LACTIC ACID IN AMPHIBIAN MUSCLE[1].

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For a generation it has been recognised that there are means available within the body by which the acid products of muscular activity may be disposed of, and there is already a large body of well-known evidence which indicates that this disposal of acid products—whatever the site of it may be—is most efficient when the conditions for oxidative processes are most favourable, and that it is incomplete when these conditions are unfavourable.

The observations to be described in this paper were undertaken in the hope of determining whether within a muscle itself means exist for an oxidative control of its own acid formation, or for the alteration or destruction of acid which has been formed, either there or by muscular activity elsewhere in the body. With this in view we examined in the first place the effect of an abundant supply of oxygen upon the development of lactic acid in a surviving excised muscle, and upon the stability of the acid within the muscle after its formation. For it has long been known that a surrounding atmosphere of oxygen has the effect of

[1] An account of the chief experimental results described in this paper was given to the Physiological Society, May 12, 1906.

preserving the irritability of a resting excised muscle[1] and of delaying the course of fatigue when contractions have been performed[2], and it has been shown that the oxygen atmosphere may indefinitely delay the onset of rigor mortis in a resting or fatigued muscle[3]. Further we know that muscle entering the state of rigor as a result of fatigue, may actually be recalled to a flaccid resting condition by immersion in oxygen gas[4].

Since an acid reaction of the muscle is, as most agree, a constant mark of the fatigued condition and a constant condition of the state of rigor, it is at once suggested that oxygen, when easily available, either restrains by some guidance of chemical events the yield of acid within the muscle, or is able to remove it after its production. And although the direct removal of lactic acid in the presence of oxygen by combustion or otherwise, does not take place under simple chemical conditions out of the body, yet an enquiry into the possibility of its occurrence within a muscle appeared very advisable in view of the facts that the resting survival yield of CO_2 by an excised muscle is at its minimum under anærobic conditions and is greatly increased in the presence of oxygen, and that the special yield of CO_2 due to contractions of the muscle is similarly increased in oxygen and may indeed be absent altogether in an oxygen-free atmosphere[5].

Our earliest experiments gave decisive evidence that lactic acid within an excised surviving muscle is actually diminished in amount, or even wholly eliminated, after exposure of the muscle to abundant free oxygen.

A study of this removal of acid can only be based upon a knowledge of the rate of survival acid-production both in resting and in contracting muscle, under anærobic and ærobic conditions; and before dealing with the question of oxidative removal it will be necessary in the first place to give an account of the main facts of this acid production. For it is notorious that, quite apart from the question of oxidative removal of lactic acid—which has not previously, we think, been examined—there is hardly any important fact concerning the lactic acid formation in muscle which, advanced by one observer, has not been contradicted by some other. Abundant lactic acid formation is said to accompany the

[1] Liebig. *Arch. f. Anat. Phys. u. Wiss. Med.* p. 393. 1850. (Humboldt's experiments in 1797 are given here.)

[2] Ludwig and Schmidt. *Ludwig's Arbeiten.* Leipzig, 1869.

[3] Fletcher. This *Journal*, XXVIII. p. 474. 1902.

[4] *Ibid.* p. 480.

[5] Fletcher. This *Journal*, XXVIII. pp. 354 and 488. 1902.

process of natural rigor in a surviving muscle (du Bois Reymond, Ranke, Boehm, Osborne), but this is denied (Blome, Heffter): it is said to accompany contraction, and to mark the advance of fatigue (Heidenhain, Ranke, Werther, Marcuse), but this is also denied (Astaschewsky, Warren, Monari, Heffter). Indeed, it may be said that since Ranke wrote in 1865, no description of the elementary facts of lactic formation in muscle, despite the fundamental importance of the subject, has been generally accepted.

We believe that the present confusion is not in chief, if at all, a result of the technical difficulties of lactic acid estimation, but that it is due to the difficulties inherent in the extractive treatment of an irritable muscle. For it is clear that in such a case no treatment for the extraction from muscle can be accepted which, acting itself as a stimulus, has among its effects an increase of the acid to be estimated.

Detailed criticisms of many previous observations will be given later in their place, but it will be convenient to refer briefly here to the main fallacies which underlie the methods of extraction hitherto described.

As solvents of lactic acid, water and alcohol have been used for extraction, and in both cases the muscle must be reduced to small pieces, by cutting or grinding, in order to ensure complete extraction. Now it will be shown that chopping an irritable muscle is, as might be expected, an acid-producing stimulus; and it is obviously fallacious to consider that the extract of a muscle after such a treatment represents its previous condition. This applies in all cases where water is used for extraction ; for water, whose virtue in this connection is that it is not itself a stimulant, by tolerating the maintenance of muscle irritability, allows time to pass after the chopping, during which, as we shall show, there occurs great augmentation of the acid yield.

For the purpose of avoiding rapid survival changes, some observers have used boiling water for the quick destruction of irritability before mincing has begun; but in these cases no analysis has been made of the effects of heating as such, and it has been left open to doubt whether sudden heating accounts for a lactic acid yield greater or less than that due to cutting injuries.

Others, again, have employed alcohol—an excellent extractive of lactic acid—and they have hoped that the alcohol in rapidly killing the muscle might not only maintain the *status in quo ante*, but allow subsequently a non-stimulating cutting up or mincing of the muscle for the completion of extraction,—and this mincing, incidentally, is aided

by the coagulative effect of alcohol. But a special dilemma is presented in the use of alcohol: the muscle must be chopped either before or after the immersion. If chopped before, in order to bring the alcohol at once to every small part, then the fallacy due to the chopping stimulation is introduced and no advantage gained by the use of alcohol. If, on the other hand, the intact muscle is bodily immersed in alcohol, without injury, then it does not escape a special stimulant action of alcohol which has not previously been recognised. We shall show that an irritable muscle taken whole, and simply immersed in strong alcohol, may in a short time suffer an eight-fold increase of its acid contents. This special effect is increased with an increase of temperature, and the use of hot alcohol, which only results in the evils of alcohol action being condensed to a shorter time-interval, must be altogether disallowed. It is a surprising fact that the dangers belonging to the use of water for extraction are not half so great as those arising from an uninformed reliance upon the use of alcohol. In view of the fallacies attending the use either of water or of alcohol for the extraction which must precede lactic acid estimation, we think, on a review of the literature, that it is not too much to say that we have as yet no trustworthy comparisons of the lactic acid content of resting and active muscle respectively, and that perhaps in no recorded observations has a genuinely resting muscle been available for examination.

We hope to show that in our experiments we have avoided these dangers of alcohol stimulation,—the magnitude of whose effects we never suspected before trial,—by using ice-cold alcohol, which has no appreciable stimulant action, while it retains its killing and coagulative influence. Immersion in alcohol has been followed by immediate rapid and thorough grinding (with sand) of the muscle ice-cold, in ice-cold alcohol. Of this and of the methods of experiment and estimation a detailed account will now be given.

I. Methods of experiment and estimation.

Changes in acidity form a feature of muscle metabolism so long familiar, and so prominent, that it is a matter for some surprise that the literature of the subject contains no satisfactory account of observations made with such continuity as would bring out the time relationships of the phenomena involved; made, that is, upon lines usually followed with profit in the study of other problems in chemical dynamics. There exist a great number of comparisons between the acidity of resting and

that of fatigued muscle, and also between fresh muscle (or muscle so described) and muscle in rigor. But except the very imperfect observations of Ranke, and those of Landsberger, which are certainly not of a kind able to yield conclusive results, there is no evidence concerning the actual course of acid production. The experiments of the two authors just mentioned are referred to later.

Nor can it be said that any satisfactory study has been made of the various conditions which control changes of acidity.

Any such investigations would be the more valuable, it would seem, if they were concerned, in the first instance, with a specific organic metabolite such as lactic acid itself. Determinations of total acidity are doubtless of value, but chiefly when special effects, due to acidity, as such, are being studied. Total acidity may, possibly at least, express the combined results of more than one specific line of change in metabolism, and the study of chemical dynamics in muscle is better served if we obtain information, first of all, with regard to lactic acid itself.

What has doubtless stood in the way of the pursuit of this study is the circumstance that accepted methods of lactic acid estimation are both lengthy and tedious. It is difficult therefore to take in hand, at any one time, a sufficient number of estimations to complete a velocity curve or other extended investigation.

Realising this difficulty we ourselves spent much effort before beginning our research in the endeavour to contrive a method of determination which should be simple and yet accurate. In particular we tried to apply oxidation methods in which the lactic acid is converted into aldehyde, and the latter distilled off for estimation. The application of this principle was at one time suggested by Boas[1], and we tried at first the method as described by that author, only to find it unreliable. Later we tried various modifications, especially taking advantage of more recent methods for estimating minute quantities of aldehyde. We attained to no great success, however, and found that, in any case, it is necessary, before applying oxidation methods, to carry out exhaustion of the muscle extracts with ether. This step proving necessary, little gain in time accrues from the final substitution of aldehyde estimations for the much more reliable process of weighing a lactic acid salt. Osborne used a method in which the acid was converted into a barium salt, the equivalent of barium being finally determined as sulphate. This method has not yet been fully described,

[1] Boas. *Deutsch. med. Wochenschr.* No. 39, p. 940. 1893.

and we ourselves decided to return to the practice of weighing zinc lactate.

At various stages in the processes of extraction and estimation we have made some slight modifications in detail, and it is desirable that we should discuss the reasons for these before summarising our routine procedure.

From remarks already made and from evidence offered in a succeeding section, it will be understood that we have found the nature of the preliminary treatment of yet irritable muscle to be a matter of the very greatest importance. Our method has been to crush under ice-cold alcohol (96 %) each individual muscle the moment it has been removed, with as little injury as possible, from the bones. In the case of frog's limb-muscles it is of course easy to do this by the simple use of pestle and mortar. The muscles contributing to any one estimation can be, finally, reduced to a thin "brei," and if this be allowed to stand for some time at room temperature under a sufficiency of alcohol, extraction becomes very complete. Before finally filtering the alcoholic extract through filter paper, we have always poured the fluid through muslin, very thoroughly squeezed the residue, and repeated its extraction with alcohol several times.

The residue left on evaporation of the clear, filtered, alcoholic extract, when rubbed up with hot water, yields a thickish magma, containing fats, lipoids, and other substances in suspension, which is very troublesome to filter. Its treatment seems always to have offered difficulties to those who have proceeded on the lines hitherto indicated. It is, of course, possible to extract this aqueous fluid very thoroughly with ether while the reaction is alkaline, and then to extract the ether-soluble acids after subsequent acidification. This method costs much time, and it is very difficult to make the removal of fat in the preliminary alkaline extraction quite complete.

We found that the use of animal charcoal offered quite unexpected help at this stage. Previous experience had shown us that charcoal absorbs lactic acid scarcely at all, and the amount removed from solutions in contact with it is negligible. If the above mentioned watery extract containing fats, etc., in suspension be heated for some time on the water bath after the addition of a reasonable amount of charcoal, and be finally boiled up before filtering, it passes, in almost all cases (*vide infra*), rapidly through a thin filter paper, yielding a filtrate which is perfectly clear and colourless. If the charcoal on the filter be well washed with hot water there is no fear of loss of lactic acid.

The clear filtrate obtained in this manner we have always evaporated completely to dryness before finally redissolving for ether extraction. Small quantities of volatile acids are thus got rid of, while any loss of lactic acid seems to be infinitesimal. Pigmentation occurs during such evaporation to a degree which is almost proportionate to the amount of breakdown products present in the original muscle; but the pigmented products do not in the smallest degree leave water for ether. The residue is taken up in a convenient quantity of water and strongly acidified with phosphoric acid in preparation for ether extraction.

In view of the work before us a decision as to the method of ether extraction to be adopted gave us, at first, some cause for anxiety. The coefficient of partage of lactic acid in ether and water is such that complete extraction is by no means easy. The use of a delicate qualitative test for the acid will indicate incomplete exhaustion at a stage when (to judge from descriptions) many observers have ceased to continue the process. A really efficient continuous liquid extraction apparatus would appear to offer the best solution of the difficulty; but such as we have tried have always brought about astonishingly slow removal of the last traces detectable by a colour reaction. In carrying out a series of simultaneous estimations (such as is necessary for constructing a velocity curve) it is almost essential to employ several individual sets of extraction apparatus, and the difficulty then arises that the rate at which they work varies greatly, and it is impossible to say (this being the chief objection to their use) that, at any moment, extraction is complete, or has proceeded to an equal degree, in all. Experience led us back to the simpler procedure of extraction by shaking, and, as we have carried it out, it is, without doubt, the most reliable method for such experiments as ours, in which a slight fall from absolute values is of less importance than rigid comparability among individual estimations.

If all the conditions of extraction are maintained strictly uniform the distribution of the lactic acid between ether and water remains, of course, at a strictly constant ratio. We have therefore always taken great care to make the volume of the aqueous solution before extraction exactly the same in every determination, to add an exactly constant amount of phosphoric acid, and finally to extract with a uniform number of exactly measured quantities of ether. We used enough ether, and sufficient renewals of the solvent, during extraction to secure nearly if not quite complete removal of the acid, and abundant experience has shown that, in any case, we have removed exactly

constant ratios in every determination. We have gone into this point at length, not because our extractions have been less complete than those of our predecessors; but because it has not always been recognised that the removal of the last traces of lactic acid from water by shaking with ether is a very lengthy process.

In almost every case the procedure so far described works smoothly, and the method is comparatively rapid. Certain exceptions occur under interesting conditions. In the case of resting muscles of well fed frogs, which have been carefully treated with ice-cold alcohol as above (a process which, as shown elsewhere, rapidly arrests change), the aqueous solution of the alcohol residue retains, even after treatment with charcoal, a "colloid" character, which makes it slow to filter. In such cases, too, the charcoal tends to pass through the filter, the colloid material (which is certainly not glycogen) having the usual effect of keeping fine particles in suspension. The results of this special condition are felt also at the stage of extraction by ether, and lead to a slow separation of the water and ether layers after shaking. Whatever the cause of these characters in the extracts from strictly resting muscle, the phenomena disappear completely when the muscles to be dealt with have undergone slight fatigue, or quite brief survival changes. As soon as breakdown products have accumulated, even to a slight degree, the extracts filter rapidly and clear, while behaviour at the ether extraction stage is normal. Only in very rare instances have we found the characters of resting muscle extracts, as described, make the final estimation in any way unsatisfactory. Their treatment calls however for a somewhat greater expenditure of time and care.

Our method of carrying on the process, after ether extraction, has been that usually adopted by others. The ether is distilled off, the residue taken up with hot water, and the solution boiled with zinc carbonate and filtered. The solution of zinc lactate is finally evaporated for the purpose of weighing. Something remains to be said however with regard to our procedure at the final stage. We have preferred always to weigh our final product as anhydrous lactate, for reasons which the following will explain. We have in the first place not been able to confirm the almost universal assumption, explicitly or implicitly made by most writers, that it is easy to prepare zinc lactate from muscle in such a condition that it shall contain exactly the normal two molecules of water of crystallisation. Although the data given by many authors indicate decided irregularities in the water determinations, even when there has been no good reason to doubt the purity of the

material, no special comment upon this fact has usually been made. Osborne[1] however has been more outspoken. He writes of the attempt to dry zinc lactate to constant weight as "a task which is well nigh impossible." For our own part we have found that, under average conditions of humidity in the laboratory, preparations, when being air dried, may stand almost indefinitely before their weight falls to that corresponding with the content of two molecules of water; their weight, moreover, may fluctuate considerably. The salt undoubtedly, as all observers agree, loses its water completely in a vacuum over sulphuric acid; on the other hand we have been unable to confirm the statement that two molecules are completely stable over sulphuric acid without vacuum; nor have we found complete stability even over calcium chloride.

As regards the influence of the sulphuric acid dessicator, without vacuum, there already exists a contradiction in the literature. Schwienig states that zinc sarcolactate, unlike the salt of the fermentation acid, scarcely loses water; Werther, on the other hand, found that the two molecules are reduced to one. An empirical use of the calcium chloride dessicator, choosing, for instance, a definite predetermined time of exposure, may lead to results which are close to theory. Such a method is clearly unsatisfactory, however, and we came to the conclusion that, at any rate in the case of small quantities of zinc sarcolactate such as are to be weighed in experiments like ours, water determinations are unavailable as a proof of purity; for this proof we have relied upon estimations of zinc oxide.

It is perfectly easy however to weigh the salt, with strict accuracy, in the anhydrous form, and it is of great advantage to evaporate the solution, after filtering from excess of zinc carbonate, completely to dryness on the water bath, and to transfer, at once, to the drying oven. The lactate separates on the water bath wholly anhydrous, and very brief drying at 110° afterwards yields constant weights. The hydrated salt loses the last traces of water more slowly. It has been our practice, after weighing, to dissolve each residue in water, to allow it to crystallise spontaneously, at room temperature, and, having carefully examined the crystals microscopically to proceed, in a large number of cases, to a determination of zinc oxide. All the products as weighed have been entirely free from pigment, have yielded uniform crystals having the exact characters of those of pure zinc lactate, and have given percentages of zinc oxide very close to theory.

[1] Osborne, *Proc. Phys. Soc.* p. xlix. 1901. (This *Journal*, vol. xxvi.)

We may now give a brief account of our treatment of the frogs employed. In all cases we used the muscles of the hind limbs only. It is abundantly shown elsewhere that it was of the utmost importance for most of our observations that absolutely intact and undamaged muscles should be used. A considerable number of animals was usually required, sometimes as many as one hundred for an individual experiment. A good deal of time was therefore occupied in the preliminary treatment during which changes in the muscles were, so far as possible, avoided by the free use of ice throughout. The frogs were pithed and the hind limbs removed by a cut across the pelvic girdle higher than the limb muscle attachments, so the musculature of the thighs remained intact.

If the muscles were to be fatigued for the purposes of the experiments in Part V. the limbs were left unskinned until after stimulation by the method described on page 280. Otherwise the limbs were skinned, the whole number well shuffled and divided into sets of ten limb-pairs (each pair still attached at the pelvis). Such a set of ten served always for individual estimations. It was, of course, desirable from every point of view, to use as few frogs for individual estimations as is consistent with accuracy, and experience led us to this choice of ten limb-pairs. It is true that in the case of strictly resting muscles it involved the weighing of as little as 20 mgms. of zinc lactate. But duplicate analyses proved the possibility of close agreement even in this case, and we have controlled the (remarkably constant) value of the resting minima by the use of larger quantities. Otherwise the amounts of lactate weighed ranged up to some 300 mgms. Weighing the final product was carried out in light, flat-bottomed, glass evaporating dishes, choice being made of such as showed satisfactory stability in weight during use. It should be understood that in the construction of (say) an anærobic survival curve, the condition of an individual set of ten limb-pairs was taken as at any moment representing the condition of the rest of the series. Full proof of the sufficient accuracy of this assumption was offered by duplicate analyses made from time to time, and by other evidence. Doubtless this desideratum was the better attained because of our practice of always using, for any one experiment, frogs caught immediately before its commencement under similar natural conditions, and also because, in shuffling (say) 100 limb-pairs into sets of ten each, we always made the weight of the sets as nearly as possible equal, thus securing average conditions of surface, etc.

Throughout the experiments care was taken to use sterilised vessels, and to work as aseptically as possible. In none of the series did putrefaction occur.

We may now summarise the processes of extraction and estimation as carried out in practice. The exact details given apply to every estimation of which the results appear in this paper.

The set of limb-pairs having undergone the treatment necessary to the experiment in hand, were placed in a weighed beaker, and their final weight observed. The beaker was next placed in ice. One operator now carefully cut the muscles from the limbs by means of sharp scissors. Each limb-pair while under treatment lay on a glass plate in contact with crushed ice. As each individual muscle was removed it was dropped into ice-cold alcohol contained in a cooled mortar. A second operator immediately crushed the muscle. As the disintegrated muscle mass accumulated in the mortar it was transferred from time to time to another vessel, and the alcohol renewed in the mortar. Finally the supernatant alcohol was poured off temporarily, the mass of crushed muscle returned to the mortar and ground to a paste. The original alcohol, the muscle, and the washings of the mortar, were now mixed in a beaker and the whole allowed to stand over night. About 400 c.c. of alcohol were used in all. Meantime the bones, with the attached debris, of all the limb-pairs were collected in the tared beaker and carefully weighed. The net weight of the muscles actually used was thus obtained and noted. Occasionally sets of limbs lost weight to some degree during the course of a long experiment, and this loss was of course greater in the sets contributing to the later estimations. By a convention, which we found justified in practice, one-fourth of this loss was attributed to the bones and the residual tissue left upon them, and three-fourths to the muscle cut off and employed for analysis. After standing, the alcohol was poured through muslin, the muscle mass squeezed thoroughly dry, and again covered with spirit and allowed to stand. This process was repeated twice more. The united alcohol extracts were filtered through paper, and the clear yellow filtrate evaporated on the water bath. The residue was carefully rubbed up with successive small quantities of hot water, and the thick fluid (about 100 c.c. in all) transferred to a small flask. Two and a half grammes of finely powdered pure blood charcoal were added, the flask placed on the water bath for half-an-hour, the contents finally heated to boiling and filtered through a small filter. The charcoal was washed once on the paper with boiling water, and the filter with its contents then transferred to a beaker, boiled with fresh quantities of water, filtered through a second paper and the whole mass washed again with boiling water. The clear colourless filtrate was evaporated just to dryness on the bath, the residue rubbed up with minute successive quantities of hot water and each successive extract poured into an accurately graduated 100 c.c. stoppered cylinder, until, after cooling, the whole volume of the aqueous solution measured exactly 15 c.c. 5 c.c. of a saturated solution of phosphoric acid, made from the pure glacial acid, were added, and on to the 20 c.c. of fluid then present 60 c.c. of ether, exactly measured, were poured. After thorough shaking the ether was removed with a pipette, renewed, and the process repeated till the aqueous fluid had been extracted five times; on each occasion with thrice its bulk of ether. The ether extracts were united and filtered through a dry filter. The ether being distilled off from a small distilling flask, the residue was carefully taken up in hot water, the aqueous fluid digested with ·5 grm. of thoroughly washed zinc carbonate, finally briskly boiled and filtered into a tared glass dish. Such filtrates were crystal-clear and colourless. The

solution was now evaporated to complete dryness on the bath, transferred at once to a drying oven and weighed till constant in weight. The residue was taken up in water, allowed to crystallise out spontaneously, the crystals examined microscopically as a test of purity, and finally a determination of zinc oxide was made, this being usually done, however, upon the united residues of three or four estimations belonging to the same series.

All the figures quoted in the paper are for convenience given as percentages of anhydrous zinc lactate.

II. THE LACTIC ACID YIELD OF RESTING MUSCLE, AND THE PART PLAYED BY TECHNICAL METHODS IN DETERMINING THE RESULTS OF ITS ESTIMATION.

During the half century which has passed since du Bois Reymond[1], followed by Ranke[2], showed that resting muscle is alkaline and becomes acid during survival, the balance of opinion on the whole has favoured their view. This opinion however has not been based either by them, or by their followers, upon the results of successive estimations of muscle extracts made at several successive periods during survival, but simply upon a comparison made between "fresh" muscle and stiff dead muscle. And while Ranke's results are supported by those of Takács (1878), of Boehm (1880), and others, who find that a stiff muscle contains more acid than one freshly excised, von Fürth, as late as 1903, finds only a slight increase of acid on rigor, and Blome (1891), followed by Heffter (1893), deny that any increase at all takes place.

This conflict of opinion is not so serious as it seems, and does not call for very elaborate analysis; the whole difficulty lies in the judgment to be passed on the condition of "fresh" muscle. All observers alike agree in finding a substantial amount of lactic acid present in stiff dead muscle, but the figures given for fresh muscle vary very widely. Those observers who find a low acid figure for fresh muscle in comparison with dead muscle, give their assent to Ranke's view of a survival production of acid. Those who find a high acid figure for fresh muscle, deny this survival production, since they tend to find, as Blome did, that "a fresh muscle has exactly the same mass of acid as the clotted muscle[3]."

The discrepancies between the various results of estimation of acid in "fresh" muscle again, while they account for the widely different

[1] *loc. cit.* [2] *Tetanus.* Leipzig, 1865.

[3] Blome's results are in part explained by the considerations brought forward by Röhmann (*Pflüger's Archiv.* L. p. 97); but the high figure obtained for resting muscle seems to call for further explanation.

views held of the survival process, significantly depend upon the technical methods of manipulation and extraction. In all cases alike the fresh resting muscle is minced or cut, to some extent at least, before extraction begins, and the question whether the resting condition outlasts this process is one which has not received notice. Independently of this, it is to be seen that those observers who kill and extract the fresh muscle by immersion in boiling water, obtain relatively low figures for resting muscle (Takács ·4%, Boehm ·2—·3%, von Fürth ·27—·33%) as compared with clotted, while those who use alcohol for fixation and extraction uniformly obtain high figures for lactic acid in resting muscle (Blome ·9%, Heffter ·59%). Hence it follows that by the use of boiling water the survival development of lactic acid is upheld as true, by the use of alcohol it is denounced.

We shall give reasons for thinking that a rapid use of boiling water, though offering a technique which is by no means ideal, happens to lead to a fairer estimate of the acid in fresh muscle, than either the use of alcohol or water after elaborate mincing, or the use of alcohol for killing and hardening before mincing. Little critical attention has been paid to this question of extraction method, but we may note that Heffter, using alcohol himself and denying, therefore, the survival production of acid, devotes a special paper[1] to a criticism of Boehm's opposing results[2] gained by the use of boiling water. Using freshly excised cat's muscle, Heffter found, after alcohol extraction, ·42% of lactic acid, after boiling water ·26%,—the latter being a low figure such as Boehm and others had obtained. Heffter, struck by this grave discrepancy, which as we have shown lies at the root of the whole long controversy, is not led to suspect his high alcohol figure as fallacious, but assumes that the lowness of the water figure, and the conclusions Boehm and others have drawn from it, are due to the faultiness of water extraction in the case of heat-coagulated muscle.

Again, Heffter shows that the lactic acid yield of muscle put straight into hot alcohol is ·37%, while the yield when mincing precedes alcohol extraction is only ·32%. He uses this comparison to decide that mincing as such is not responsible for a large yield of lactic acid, but he does not examine the possibility that while mincing accounts for a large yield of acid, alcohol itself might account for more. That he was unconscious of this possibility, in itself deprives his criticisms of his own and of Boehm's results of much of their value.

[1] *Arch. f. exp. Path. u. Pharm.* XXXVIII. p. 447. 1897.

[2] *Pflüger's Archiv.* XXIII. p. 44. 1880.

Before dealing then with the course of the spontaneous survival development of lactic acid within muscle, we shall first give our own estimation of the acid yield from fresh resting muscle, and examine the effects of cutting and mincing injuries, the special effect of alcohol and the effects of other destructively stimulating agencies, in increasing the yield of lactic acid. All the results given are those obtained with frog's muscle. We have evidence that the events in mammalian muscle are not different in kind, though they are widely different in their time-relations. It has seemed better to elucidate the simpler case of cold-blooded muscle before attempting in detail the far harder task of following the lactic acid development in the quickly changing mammalian muscle.

Resting muscle: the resting "minimum."

From the first we have obtained very low figures for the percentage of lactic acid in resting muscle, and in this we differ from other observers who have used alcohol for extraction. By the process already described (p. 252) in which the muscle, with a minimum of manipulation or cutting, was ground with sand immediately upon its admission to strong (96 %) alcohol, we have uniformly found the subsequent estimations of zinc lactate to lie between the limits of ·03—·045 %. To minimise the effect of the stimulation of crushing, as well as for another reason soon to be explained, the alcohol was always used cold, and the average figure for the lactic acid yield of resting muscle, obtained after this was adopted as the routine, lies between ·02 % and ·035 %. But with cooled muscle and ice-cold alcohol, and after scrupulous care in the avoidance of unnecessary cutting and handling during the dissection we have never obtained a negative estimation—our lowest figures have not been below ·02 % for resting muscle.

We think that, of this low resting minimum ·02 % (·015 % lactic acid),—greatly lower as it is than the result of any previous estimations—part at least is due to the unavoidable minimum of mechanical manipulation. We may at all events regard the lactic acid yield of a freshly excised muscle, which has been under normal oxidative conditions up to the time of excision, as lying close to zero.

From this point onwards, as time passes from excision, we shall show that the lactic acid yield spontaneously increases. But before proceeding to this, it is necessary to examine the effects upon resting muscle of the various manipulative methods which have been used in

the processes of experiment or estimation by other observers and by ourselves. A claim to an accurate estimation of the condition of resting and fresh muscle cannot fairly be advanced unless the special actions of the necessary technical methods of destruction are taken fully into account.

Traumatic injury: effects of cutting and mincing.

We have uniformly found that traumatic injury to an irritable muscle produces a rapid development of acid. A marked feature of the development of acid, and the rise of lactic acid yield, is that it does not attain its maximum immediately, though it reaches it rapidly. A given cutting injury to the muscle does not produce instantaneously an acid yield, maximal for that injury, but rather sets up a rapid acid-yielding breakdown which at first quickly, and then more slowly, approaches a maximum.

This is shown in the two following experiments, which may be taken as typical.

Exp. I. (Fig. 1, A). October. Ninety pairs of hind limbs were taken at 10.30 a.m.: these were kept during the intervals of all manipulations upon thin glass plates over ice. The muscles of 80 pairs were "snipped" to fine pieces with sharp scissors, upon ice-

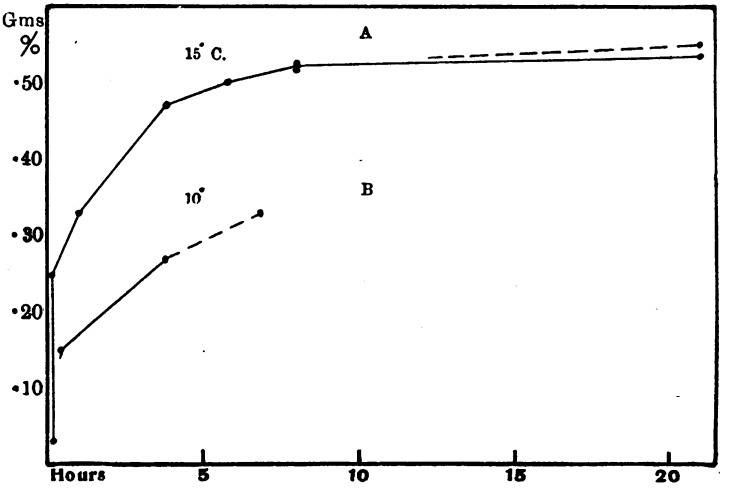

Fig. 1. The course of lactic acid production after severe cutting injury, in an atmosphere of hydrogen.

Dotted line indicates an atmosphere of oxygen. Each point shown on the curves represents a separate estimation of lactic acid in an individual set of limb muscles. Ordinates are proportionate to grammes of zinc lactate per cent. of net muscle weight.

cooled plates. Ten pairs were kept uninjured and cool. The whole chopping occupied 70 minutes, and as it proceeded the muscle fragments were collected in an ice-packed beaker. The chopped muscle was rapidly weighed out into eight lots of 26 grammes weight each, and, of these, seven were arranged upon long glass slides. The slides conveying each batch were slid into a glass tube, the tube was closed and immersed horizontally in a water bath which was maintained throughout the experiment at 15° C. All the tubes were now connected in series and a current of hydrogen was passed through all, in series. The current began at 1.45 p.m. The current after leaving the tubes was passed through a test bottle containing unmixed pyrogallol and soda. After 70 minutes these were mixed by shaking and no colouration occurred. This test of the anærobic condition of the experiment was repeated at intervals and was negative throughout the passage of the hydrogen current.

At 1.45 p.m. ten uninjured pairs of limbs were extracted for estimation, and also the batch of cut muscle which was not placed in the tubes. At the times indicated below, one tube after another was removed from the series without disturbing (except momentarily) the hydrogen current, and the muscle fragments contained in it were treated for estimation. To one tube in the series, an oxygen current was supplied at a later time in place of hydrogen. The estimation gave the following results, plotted graphically in Fig. 1, A, and expressed as weight of zinc sarcolactate per cent. of muscle weight taken.

		Zinc lactate per cent.
Undamaged resting limb muscles (weight 30·2 gms.)	...	·038
Batches of cut muscle:		
(*a*) Estimated directly	...	·246
(*b*) ,, after 1 hour in hydrogen current	...	·331*
(*c*) ,, ,, 3¾ hours in hydrogen current	...	·47*
(*d*) ,, ,, 5¾ ,, ,, ,,	...	·50*
(*e*) ,, ,, 8 ,, ,, ,,	...	·515
(*f*) ,, ,, 8 ,, ,, ,,	...	·510
(*g*) ,, ,, 20¾ ,, ,, ,,	...	·540
(*h*) ,, ,, 8 ,, ,, ,, followed by 12¾ hours in oxygen current		·546

* Zinc oxide determined on products from (*b*), (*c*), and (*d*) combined 33·43 %. Theory requires 33·42.

Exp. II. (Fig. 1, B.) March. Forty pairs of hind limbs taken. General methods as in the last experiment. The batches of cut muscle (weighing 24·6 gms. each) were placed upon glass shelves within closed chambers for exposure to hydrogen (or oxygen). The chambers were maintained throughout at 10° C. The results (see Fig. 1, B) were:

Undamaged resting limb muscles (weight 45·5 gms.)		
After 3¾ hours in hydrogen		·049
Batches of cut muscle:		
(*a*) Estimated directly	...	·150
(*b*) ,, after 3¾ hours in hydrogen	...	·266
(*c*) ,, ,, 3¾ ,, ,, followed by 3 hours in oxygen	...	·335

In Fig. 1, in both figures, A and B, it has been convenient to take the zero abscissa as representing the time of completion of the cutting,

and of the beginning of the anærobic condition: the curves—apart from the accounts just given—indicate, wrongly, that the first and largest rise of acid yield which occurred during the process of cutting, is an immediate rise. It is not an immediate, though it is a rapid rise: in Exp. I. it extended over 70 minutes, in Exp. II. it occupied 50 minutes. It would be difficult to express it otherwise in the figure because the amount of rise effected during the whole time of cutting is widely unequal in different batches of muscle, being greater in those chopped early than in those chopped late. To attain similarity of condition in the batches as finally made up and enclosed in the tubes, the chopped material was in all cases "shuffled." The success of this shuffling is indicated, we think, by the general smoothness of the results and more pointedly by the close equivalence of the duplicate observations quoted in Exp. I. The remarkable effect of chopping *per se* upon the normal survival production of lactic acid will be fully realised when the curves of Part III. are compared with those of this section.

The practical bearing of these results upon the technical methods of extraction is clear. The acid increase due to injury is rapid but not instantaneous; it does not reach an immediate maximum. In treating muscle then for the extraction and estimation of lactic acid there must be no delay between injury and the quick killing of the muscle, and the temperature should be low. Each piece as it is injured should be devitalised. In our routine method (see p. 252) we have immediately crushed and ground with sand under ice-cold alcohol each fragment of muscle as it has been removed from the limb, and the limb itself has always been kept below 2—6° C. wherever that has been possible. It is possible—we think probable—that the complete disintegration due to grinding with sand is a condition in which the acid rise which follows severe but incomplete injury, does not occur. In any case the simultaneous devitalising by alcohol does not allow a rise of acid yield to proceed. In not a few published estimations, where the muscle has waited for some time at room temperature before killing, it is clear that the resultant figures for "fresh" muscle by no means represent that condition.

It is shown in the two experiments just given that the maintenance of anærobic condition does not affect the changes set up by injury. The high lactic acid yield reached in A is not reduced by nearly 13 hours in oxygen, and the earlier stage of increase in B is not checked during 3 hours in oxygen. The significance of this will be discussed and more fully illustrated in Part V. (see p. 288).

The special alcohol stimulation effect.

Our analysis of this had its beginning in an attempt, by the avoidance of all injury during the irritable periods, to obtain a still lower resting minimum of lactic acid than that given already. One set of frog's legs after having been skinned and handled with scrupulous care, was immersed in strong alcohol and left for $2\frac{1}{2}$ hours for the completion of fixation and the loss of irritability. It was expected that these would subsequently show a smaller acid percentage than another control set which followed the usual routine and suffered the inevitable mechanical stimulation before the alcohol had killed. But the muscles which were immersed whole, showed, on the contrary, not less lactic acid but actually seven or eight times more than the cooled set treated as usual.

Exp. III. March. Two sets of ten limb-pairs each. Room temperature 15° C. Set (*a*) estimated immediately by routine method (but with alcohol used at 15° C.): yielded ·054 gms. %.

Set (*b*). Limbs trimmed from all tissues *except* the muscles to be used, as in ordinary cases, for estimation. All damage to these was scrupulously avoided. The limbs were then immersed and left undisturbed in a dish of 95 % alcohol at 15° C. After $1\frac{3}{4}$ hours the firmly hardened muscles were cut and ground as usual. The alcohol of immersion was added to the extracting alcohol for evaporation. Set (*b*) yielded ·402 gms. %.

If a comparison be made between this astonishing effect of simple alcohol immersion and the effects of other destructive and stimulating agencies, it will be seen that the alcohol effect, after an hour, may outstrip the results of the severest electrical stimulation, and in $2\frac{1}{2}$ hours it may reach a maximum as high as that attained by heat rigor (see later sections).

This special alcohol effect is reduced or abolished as the temperature sinks. Details are given in Appendix I., p. 303, of an experiment of which the results show that:

	gms.
Resting muscle, extracted as usual (but without ice) yields	·046 %
Muscle resting in alcohol 90 minutes at 15° C. „	·258 %
„ „ „ at 1—3° C. „	·059 %

Since the alcohol effect is abolished at low temperature, we have used ice-cold alcohol always in our routine method. Nor is the effect found to any important extent if the muscle be crushed immediately upon its admission to alcohol: the increased yield of acid is found only

when moderately large pieces of muscle and especially intact muscles are allowed to remain in the alcohol undisturbed.

It is quite certain that when even moderately small pieces of a muscle are immersed in strong alcohol they are not immediately killed, or even killed with a rapidity approaching that of the killing by boiling water. If the gastrocnemius of a frog be immersed in 96% alcohol for 20 minutes—a time at the end of which the muscle appears superficially to be firmly coagulated—it is found on cutting open the muscle that its inner core is still irritable, and that it responds to an induced shock by a characteristic slow spastic contraction.

It is possible that the superficial layers of muscle, rapidly coagulated, prevent the access of strong alcohol to the inner layers. The alcohol reaching the interior must be more dilute, for the diffusion both of alcohol into, and of water out of, the muscle will be delayed by the superficial film of coagulation—and this dilute alcohol may exercise a stimulative effect on the inner core as this slowly loses its irritability. That is to say, this slow action of alcohol upon an uninjured muscle may be supposed to bear the same relation to its quick action upon muscle crushed within it, as the slow process of heat rigor and their results, bear to the quick killing of boiling water and its fixing effect.

The slow spastic contraction of the inner core of muscle is similar to that seen in frog's muscle partially dried by exposure to the air, and it is possible that the stimulant action of alcohol is due to dehydration. The stimulation of the interior of the muscle, however caused, goes on, it should be noted, under anærobic conditions, and, as will appear later, those conditions would notably encourage the resulting yield of acid.

The nature of this alcohol stimulation is being further investigated. Meanwhile it must be clear that its existence, and the magnitude of its effects, are enough to throw grave doubts upon all results of lactic acid estimation obtained by the use of alcohol, where this phenomenon has not been taken into account.

Chloroform rigor.

Incidentally, the effects of another destructively stimulating agent, chloroform, may be introduced here. It has been shown that chloroform vapour, even in small doses, accelerates those survival processes in a muscle which lead to CO_2 formation. The yield of CO_2 from an excised frog's muscle is largely increased by the action of chloroform [1], and the onset of rigor is hastened.

[1] Fletcher, This *Journal*, XXIII. p. 50. 1898–9.

We find that chloroform vapour similarly accelerates the formation of lactic acid. By the use of it, a maximal yield of lactic acid may quickly and conveniently be obtained. One experiment may be quoted (for details see Appendix p. 306):

At room temperature 20° C., resting muscle extracted direct ...	·02 %
,, ,, ,, ,, muscle after 4 hours' exposure to chloroform vapour	·434 %*
,, ,, ,, ,, muscle after 11 hours' exposure to chloroform vapour	·445 %

* Zinc oxide determined on lactate weighed = 33·41 %.

Heat rigor.

Ranke[1] showed that heat rigor, which is produced rapidly in frog's muscle at temperatures just below 40° C., and almost instantaneously at 40° and for a wide temperature range above, is associated with pronounced lactic acid development. Ranke speaks of a "saürebildungsmaximum" to be attained in this way.

Our results confirm this: we find that an "acid maximum" is reached on heat rigor effected at or near 40° C., and this maximum is approximately on the same level with that produced in chloroform rigor, or in the "slow death" by alcohol. The infliction of heat rigor is a convenient method for determining the maximum potentiality of a muscle for acid production at any time and we have so used it in several experiments to be described later. Selecting observations made in other connections and at different times of the year, we find that they fall into two groups according to the season, thus giving only one for each of six months,

	Percentage of zinc lactate after heating muscles for 1 hour at 40° C.	
March	·383	
April	·315	
May	·420	·36
October	·54	
November	·51	
December	·52	

whilst we have always found a surprising constancy in the value for the acidity of heat rigor when duplicate determinations have been made upon frogs caught under similar conditions (see, for instance, p. 292), we have constantly found a higher acid maximum for the muscles of autumn frogs than for those of frogs caught in the spring.

[1] *Tetanus.* Leipzig, 1865.

Previous fatigue, when the fatigue has been produced after excision in the absence of circulation, does not appear to affect the "acid maximum" of muscle, as determined after heat rigor. This is illustrated in an experiment quoted in another connection on page 292.

Heat coagulation at 100° *C.*

Apart from other considerations, the result of the treatment of muscle by boiling water is of special interest because of the abundant use in the past of boiling water as a means of extraction from muscle for lactic acid estimation. We have already referred to this in noticing (p. 259) the strictures passed by Heffter upon Boehm's results and upon his use of boiling water, and we have seen that those who have used boiling water have in general obtained lower, and therefore more accurate figures for the acid yield of resting muscle, than those who have unguardedly used alcohol.

We find that a muscle killed and coagulated by immersion in boiling water yields always less lactic acid than a corresponding muscle which is brought less rapidly through the stages ending in loss of irritability.

Thus in the experiment of which details are given at page 303, where the resting muscle yields ·046% zinc lactate, a similar set of muscles killed quickly in boiling water yield ·081%.

In the experiment given at page 293, five sets of muscles, after various treatments are brought to heat rigor at 45° C., give respectively values of ·513, ·502, ·522, ·516, ·511%. Two similar sets, plunged into boiling water yield only ·111 and ·135% respectively.

It has been said above that the use of boiling water for killing and extraction is likely to give more accurate values in lactic acid estimation than the incautious use of alcohol. It will be obvious however that the use of boiling water is not likely to give constant values, and it must always give values which are too high. The acid yield of heat rigor must be considered as beginning at many points within a muscle-mass after the immersion of the whole in boiling water, before the higher temperature has reached every part: and the stage at which heat rigor is exchanged for heat coagulation cannot be expected to be constant even on the average except within wide limits.

III. The spontaneous survival production of lactic acid.

It is remarkable that in support of his concise statement that "acid formation in muscle entering death rigor is a function of the time: in equal times, under otherwise the same conditions, in equivalent muscles of the same animal, are formed equal quantities of acid," Ranke[1] gives little or no evidence. He does not quote the results of successive estimations made at intervals during survival from excision onwards; he establishes the "law" just quoted, upon the results of only one relevant experiment, and this is one in which a comparison is made only between already clotted rabbit's muscle yielding ·15% acid 25 hours after death, with that yielding ·2% at the end of 40 hours.

A generation later Landsberger[2], in almost identical words, again announced Ranke's conclusion as to the time relations of survival acid production, but it is very doubtful whether his methods can be admitted to justify his opinion. Taking equal (small) weights of muscle, immersed in salt solution, he found that nearly equal amounts of alkali must be added in equal time intervals after excision, on titration of the immersing salt solution, to give neutrality to phenolphthalein. Using 3 grammes of muscle at a time, only ·2—·5 c.c., or even less of the alkaline solution, was required for each titration, so that the highest significant digit of his results is a tenth of a cubic centimetre of alkali. The titration, again, of the immersing solution only indirectly, through an undetermined factor of diffusion, represented the condition of the muscle. Lastly the muscle fragments which were studied had suffered injury, and, as we have shown, an injured muscle mass does not behave like uninjured muscle: the development of acid within it is an exponential and not a linear function of the time, and Landsberger's method should properly, we think, have appeared to give evidence against Ranke.

We have seen already that the views taken by subsequent observers as to the occurrence of a survival production of lactic acid have depended in the main upon the view taken by each of the lactic acid yield for "fresh" muscle,—for all are agreed that clotted muscle gives a substantial yield.

[1] *loc. cit.* p. 148. A comparison between the many accurate pronouncements of Ranke in this chapter, and the experiments which are quoted as their basis, will provide the student with one of the greatest curiosities of physiological literature.

[2] Landsberger, *Pflüger's Archiv.* L. p. 339. 1891.

In the excised muscle of the frog, kept at room temperature in the air, we find that the survival production of lactic acid is extremely slow: in the example given in Fig. 2 (see Appendix, Exp. VIII. page 302), the yield at the end of 24 hours (13° C.) is little above that of fresh resting muscle. This is in accordance with the familiar fact that the physiological condition of excised resting frog's muscle, kept in air, hardly varies throughout the first day at low room temperature and under proper conditions of moisture.

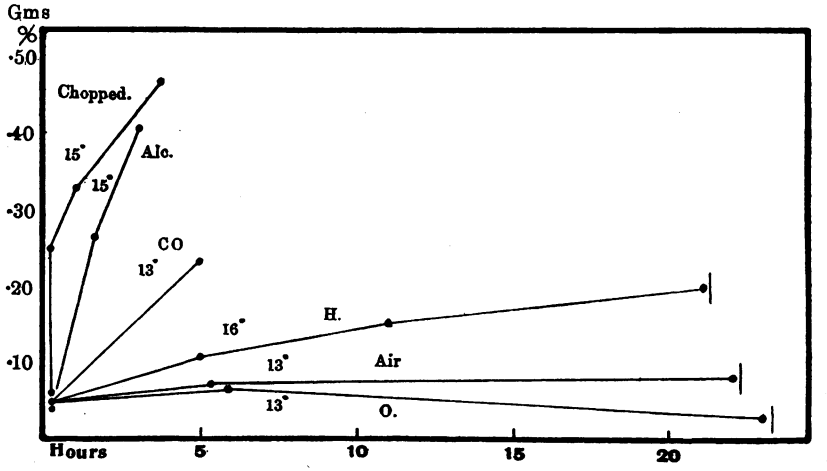

Fig. 2. The course of lactic acid production occurring during survival periods in oxygen, air, hydrogen, and coal gas (CO) respectively.

The two uppermost curves are introduced for comparison. That for chopped muscle is repeated from Fig. 1; that for alcohol immersion is based on the results given at p. 264.

That the maintenance of the "fresh" condition is due to a compromise effected between two opposing influences, one of them being the presence of available oxygen, is suggested at once by the results of parallel estimation of a control set of muscles (Fig. 2) observed at the same time intervals and the same temperature, but in an atmosphere of oxygen. In this case the acid yield is not only not increased, but is reduced, until, at 24 hours from excision, the yield is hardly one third of that for the equivalent muscles in air.

In Fig. 2 are also shown two other estimations made in the same experiment, using equivalent sets of muscle (see Exp. VIII.). One set, for comparison with the sets in air and oxygen respectively and at the same temperature, was kept in an atmosphere of coal gas: after 5 hours

this set gave a yield four times as great as those under ærobic conditions, and showed at the same time well advanced stiffness and loss of irritability. The remaining set, in this particular experiment, was forced to heat rigor at 42° C., and gave a yield of ·4%—illustrating the acid "maximum" of acid yield, potential in the other sets.

The rapid increase of acid yield induced by the atmosphere of coal gas in this experiment suggests a direct toxic effect, over and above the effects of the anærobic condition by itself. This complication need not now be discussed; in illustration of the simpler case where anærobic conditions are otherwise neutral, estimations are also shown at Fig. 2 (see also Exp. VIII.) of the survival increase of acid yield at room temperature (16° C.) in an atmosphere of hydrogen; here it is seen that the rise of yield begins in the first period after excision of the muscle and proceeds steadily, the spontaneous production of lactic acid appearing here to be a simple linear function of the time, as Ranke originally suggested.

It is apparent then that in a muscle exposed during survival to oxygen, the yield of acid which would be spontaneous and progressive in the absence of oxygen, is not exhibited—either because the spontaneous development is checked or diverted, or because the acid is removed as fast, or indeed faster, than it is found. In Part V. we shall attempt to analyse this oxidative control of the acid yield, and here deal only with the spontaneous survival yield of lactic acid under strictly anærobic conditions. Atmospheric air, it will be enough now to say, is shown in all our experiments to behave as a weak neutral solution of oxygen.

The spontaneous anœrobic production of lactic acid.

We have chiefly used for the study of the anærobic production, atmospheres of hydrogen. For variation in experimental condition we have also used nitrogen; both gases give identical results and we cannot find in the use of either, any evidence of direct action upon the muscle. Each in this relation appears to be strictly a neutral gas and to owe its effects to the negative cause only, the absence of oxygen. The results of all the experiments show, in sum, that under strictly anærobic conditions there occurs in Amphibian muscle from excision onwards a steady spontaneous increase of lactic acid yield; in this respect the muscle differs widely for many hours after excision from a muscle kept in air, and still more widely from one kept in oxygen.

It appeared to be of special interest not only to establish the fact of this spontaneous increase but to follow its course in detail as closely as possible by means of successive estimations, and so to determine the nature of the curve representing the course of increase. Limits other than those of time and patience are set to the numbers of successive estimations which can be undertaken. The limbs of ten frogs are needed for each estimation, and 100 frogs will therefore be necessary if ten points upon the survival curve are to be determined. For equivalence of individual estimations careful shuffling and weighing of the several batches, each of ten, are necessary as a preliminary. We have not attempted to determine by estimations more than ten points on the curve on account of disturbances which would be introduced by any increase of the manipulations at the beginning and their attendant delay. Estimations of from 7—10 points during the survival history are enough however to give, we hope to show, a clear indication of the course of events.

For each set of estimations it is of prime importance that the temperature with all other factors, should remain constant throughout. In obtaining the survival curves given in Fig. 3, we have used special methods, very simple in kind.

For a given series, the limbs were weighed and shuffled into nearly equivalent sets of ten each (as described on page 256), being kept cold throughout the manipulations. The skinned limbs of each set were looped at intervals along a thread, like the papers in a kite's tail—the loop passing round the metacarpal bones without any injury to the muscles subsequently to be used in estimation. Each such chain of ten limbs was drawn into a glass tube, three inches in diameter, and all the glass tubes were fixed vertically in a special frame something like an umbrella-stand. The tubes were closed top and bottom with corks and the whole series connected up so that a current of gas could be passed along from one tube to another, and so through all. The frame, with the tubes in position, was lowered into a large wooden cask filled with water at a desired temperature, so that every tube was wholly immersed. The arrangements for fixing the tubes to the frame were such that while each tube was held firmly in place, and its buoyancy in the water successfully resisted, any one tube could be removed from the series at any time without disturbing the rest, and without interrupting the gas current passing through all. To each tube moistening arrangements were introduced, and in each the chain of limbs hung freely, exposed on all sides to the current. At the beginning the current of hydrogen or nitrogen was supplied in abundance for washing out atmospheric oxygen from the tubes and from the muscles. It was found convenient to supply a special preliminary anærobic current to each four or five tubes, to give more rapid clearance. The progress of oxygen removal was tested by pyrogallate bottles in circuit. When it was complete, or very nearly so, the hydrogen or nitrogen current was economised, and maintained at a slow rate through the whole period of observation, night and day. At intervals the maintenance of the anærobic condition was tested by fresh pyrogallate bottles.

In view of the high degree of susceptibility shown by the acid-yielding functions of the muscle, to injury of any kind and to temperature changes, we think that our method has been as successful as we could hope. The consistency of the results illustrated in Fig. 3, gives us some confidence that they approach the truth, and since each point shown there upon the curves is the result of a gravimetric estimation of zinc lactate derived by lengthy processes of extraction and preparation, the irregularities which appear must be put down at least as much to our faults in estimation as to failure of experimental treatment.

Apart from general convenience there is a necessity for the use of a separate tube or chamber for each set of muscles, which we overlooked at first. It is essential to the uninterrupted course of the lactic acid production that not even a momentary break should be made in the anærobic conditions. The admission of atmospheric oxygen when

a chamber or tube common to two sets of muscles is opened for the extraction of one, even though the anærobic state is rapidly reestablished, is fatal to smooth results—as we found to our heavy cost in several long experiments. We have evidence that, after a period of deprivation, a supply of oxygen, small in amount and lasting perhaps only a few minutes, markedly and disproportionately diminishes the lactic acid yield; this seems to us to have special significance, and we propose to analyse it further.

Accidental oxygen contamination occurring at every other period of estimation (when, *e.g.*, two muscle sets are in each chamber, and one is left when the other is taken) is marked upon the graphic record of lactate values as a series of steps.

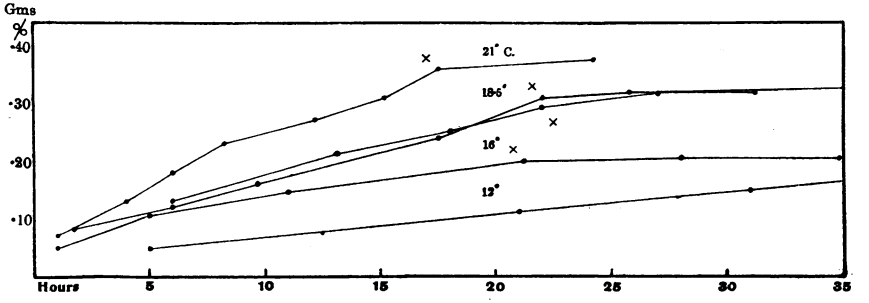

Fig. 3. The course of lactic acid production under anærobic conditions at different temperatures.

× marks approximately the points in time at which excitability was finally lost. One of the curves at 18·5° C. was obtained using an atmosphere of nitrogen; hydrogen was used for the rest. The temperature relationships here shown must not be considered apart from the question of nutritive conditions discussed on p. 276.

The results of five typical experiments, whose details are given in full on p. 306 *et seq.*, are plotted graphically in Figs. 3 and 4. All alike represent the course of survival change in anærobic conditions, at various temperatures. Of the curves in Fig. 3 which were both obtained at the temperature 18·5°C. in one hydrogen, and in the other nitrogen, was used to replace the air. The approach to identity is striking and is especially so in the cases actually given here, since the former was obtained by one of us working alone throughout, and the latter at another time by the other of us, again independently.

Taking any one of these curves, it will be seen that for the first 15 hours or more at ordinary room temperatures, and for periods which become longer with lowered temperature, the rising line representing the survival increase in acid yield is nearly a straight line: within these earlier periods after excision the production of acid is a simple linear function of the time. In each case however the increase of acid yield ceases at a certain level and ceases abruptly. Thenceforward the yield shown by subsequent estimations remains nearly constant, or shows only a very slight gain with time.

On reference to the figures, and the protocols on which they are based, it will be seen at once that the period of acid development corresponds with the period after excision during which irritability persists. Irritability and the power of spontaneous acid formation are coincident functions of the surviving muscle. If the final loss of irritability be the true mark of molecular death, then it is only living muscle, or muscle in the act of dying, which has the power of spontaneous acid production: the lactic acid content of dead muscle, *i.e.* muscle which has finally lost irritability, remains nearly constant.

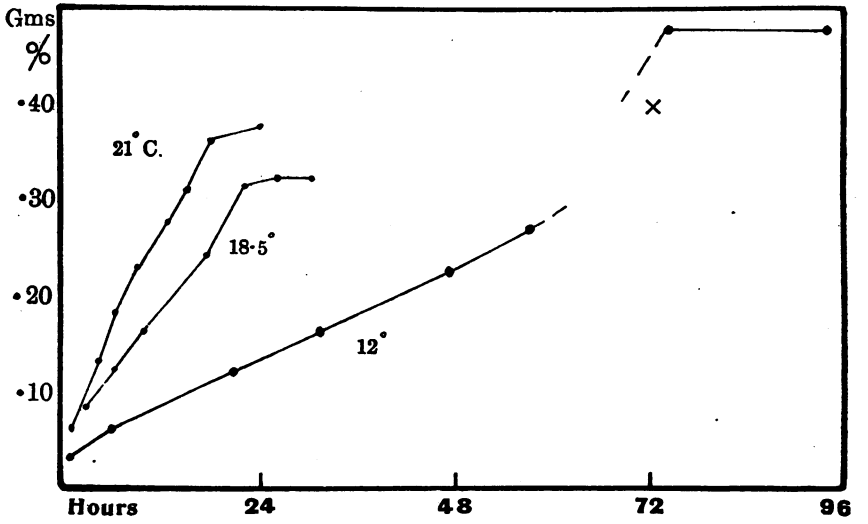

Fig. 4. The course of lactic acid production in an atmosphere of hydrogen at 12° C. This shows the whole curve of which the beginning is given in the lower part of Fig. 3. It is drawn here upon a very much shortened abscissal scale. The two upper curves are introduced (from Fig. 3) for comparison. Protocols at Exp. XII, p. 304.

This is in accord with the dictum of Salkowski that "the muscle produces lactic acid not because it is dying but because it is living, and it produces it only so long as it lives[1]." This statement was based upon experimental results obtained on lines quite different from our own. Salkowski found, as is well known, that ground up muscle placed under chloroform-water produced little or no ether-soluble acid during the progress of its *post mortem* autolytic changes. That the rapid effect of chloroform upon the disintegrated fibres should arrest all further lactic acid production is quite in accordance with our own results, but we

[1] Salkowski, *Zeitsch. f. Klin. Med.* XVII. (Suppl. Band), 97. (1890).

find it difficult to understand why no lactic acid at all was found after the muscles were chopped up. The author speaks of his material as being "möglichst schnell zerhackt" and it was either at once sterilised for control experiments, or immediately transferred to the chloroform-water for the study of autolysis. The quantity of acid formed might therefore well be small, but that none should be present is a circumstance contrary to our own experience. Magnus-Levy[1], who found (in the case of liver tissue) that lactic acid increased during aseptic, and to a less degree during antiseptic autolysis, suggested that Salkowski's negative results were due to the shortness of the period for autolysis allowed by the latter. We doubt if this explanation could hold in the case of muscle, and believe that, even though the liver was concerned, the production probably occurred in the earliest stages of Magnus-Levy's experiments and was a survival product rather than a *post mortem* autolytic product. Acid production in muscle is not confined to the period immediately related to the onset of rigor, nor to any other critical period in the survival history. It begins from the moment of excision and does not cease till the muscle fibre has lost its physiological characteristic of contracting in response to stimulation.

It cannot be claimed that the significance of all the details of the curves obtained by us is yet clear; but, as we have said, two fundamental characters seem to point to definite conclusions. These are the absence of exponential characters in the main course of the curve, and the establishment of a plateau on the disappearance of irritability.

The acid production of declining irritability.

Our experimental results lead us to agree with Ranke, that during the earlier, and, by far, the longer, period of survival life, equal amounts of acid are produced in equal times. We have pointed out however, that Ranke did not estimate the acid yield of irritable muscle, but reached his conclusion intuitively. Most of the curves plotted in Figs. 3 and 4 only approximate, we are conscious, to actual straight lines. That at 12° exhibits this character with remarkable exactness however; those at 18° offer conclusive evidence of a production essentially linear; that at 21° is rather less definite, but is clearly not an exponential curve. The greater instability at higher temperatures and the consequent greater effect of manipulations without doubt militate against accuracy. Some disturbances were to be expected as the

[1] Magnus-Levy, *Hofmeister's Beiträge* II. p. 283. 1902.

result of the idiosyncrasy of individual frogs. For each estimation ten pairs of limbs were used (as in all the experiments described in this paper) and this number is certainly not large enough to give complete smoothing of results by averages. But, as was pointed out in the discussion on methods (p. 256), our use, in any one experiment, of frogs caught immediately before under exactly similar natural conditions, and our method of shuffling the limbs into sets of nearly equal weight, serve practically to eliminate the variations of idiosyncrasy. The use of ten limb-pairs for individual estimations was sufficient for necessary accuracy, as all the work described in this paper seems to indicate. To employ more than an efficient minimum was undesirable, as leading to undue delay at the preliminary stages of an experiment. The data obtained seem to us to offer evidence which is convincing.

The fact that the curve of acid formation is approximately a straight line and is possessed, apparently, of no exponential character is certainly of no small significance. We may recall, in connection with it, the steady production of carbon dioxide—proportionate to the time—which has already been shown to occur in surviving muscle subsequently to the fifth or sixth hour after excision[1]. Linear rates of change have been found—as is well known—under quite special conditions, in reactions catalysed by enzymes; and explanations, more or less adequate, have been supplied for these cases. It would be premature to decide whether explanations of a similar kind have any bearing upon phenomena in an intact tissue. Conceivably the store of precursor in the muscle is partly in an insoluble form, and partly in solution. If change occurs only in the latter, the concentration might be kept constant by replacement from the insoluble store, and, for a period, conditions would exist for a linear rate of change. The explanation is probably less simple than this, and it is interesting, in any case, to observe how in the quiescent unstimulated muscle the survival processes which lead to lactic acid production are so controlled as to lose the exponential character of an isolated chemical reaction.

It is, moreover, especially interesting to compare the rate of change as it occurs in the intact unstimulated structure with that induced by partial disintegration. In this connection the curve of Fig. 1 should be compared with those of Figs. 3 and 4.

The velocity of formation, at first greatly accelerated, and later rapidly diminishing, which, after disintegration, takes the place of the slower linear velocity in the intact muscle, might be expected as the

[1] Fletcher, This *Journal*, XXIII. p. 10. 1898—9.

effect of a strong stimulus acting temporarily. If instead of explaining the matter in terms of a stimulation effect, we conceive of the change in the steepness and form of the curve, which results from chopping, as due to the liberation of ferment activities, which in the intact architecture of a muscle fibre are in some way controlled—perhaps by localisation—we may be altering terms only, without modifying their real significance.

Attention may here be directed to a point with some practical bearings. Autolysis of tissues is markedly accelerated by increased acidity (Schryver, Hedin *et al.*). Since chopping an organ, to judge from the case of muscle, is followed by greatly accelerated acid production, it is clear that a preliminary disintegration will, from this circumstance alone, materially affect the initial rate of autolysis.

Remembering the effect of acidity, the facts of a later section (V.) will be found to have also a bearing upon the legitimacy of applying the results of autolytic studies to the behaviour of intact tissues, duly supplied with oxygen.

The spontaneous acid maximum: the acidity of "dead" muscle.

The second characteristic of the anærobic survival curves which appears to give indications of a definite kind is the establishment of a plateau, pointing to a cessation of production, whenever loss of irritability occurs. Although such loss of irritability does not usually—under anærobic conditions—long precede the onset of obvious rigor, it is not necessarily coincident with it. Our results indicate that the spontaneous production of lactic acid in a muscle left undisturbed at constant temperature may cease while the fibres are still soft and translucent, if such a muscle has ceased to be irritable. Rigor may then be established later, without an increase in lactic acid which can be estimated.

While, then, it seems entirely clear that the survival production of lactic acid, after proceeding for long periods at a rate proportional to the time, comes abruptly to an end under the conditions just discussed, there is, on the other hand, much obscurity regarding the factors which control the concluding steps of the whole process, and determine (under given conditions) the final quantity of acid formed.

This final maximum of acidity, spontaneously reached, is probably chiefly dependent upon the initial nutritive condition of the muscles, and strict comparisons can only be made with frogs caught at the same time of year, and obtained under conditions as nearly as possible similar.

As regards the curves of Fig. 3 we have no doubt that those at 18° (obtained during August), that at 21° (late July), and, perhaps to a less degree, that at 16° (May) are really comparable. These were among the first curves obtained by us, and from them it seemed clear that the height of the final plateau, no less than the velocity of production, was some simple function of the temperature. But the curve at 12° (Figs. 3 and 4), given by frogs caught in the autumn (October), while exhibiting in a most striking manner a linear velocity of production for the first 60 hours, seemed to introduce two new features—the final plateau being higher than those attained at the higher temperatures studied, and this high maximum being reached by a sharp acceleration during the final 12 hours of irritability.

In an attempt to obtain the data for plotting a curve at any given temperature it is not easy to choose in advance right intervals for estimations which shall bear upon a critical part of the curve, such as that which immediately precedes rigor, and is associated with the period comprising the final loss of irritability. Our apparatus did not permit of a test of irritability being made within the tubes, and a group of irritable muscles once removed from the anærobic chambers, are so affected by exposure to air that even when returned to anærobic conditions their subsequent production of lactic acid is no longer parallel to the anærobic curve. The choice of intervals has therefore to be largely a matter of chance. The only other experiments we have made at low temperatures do not include estimations so numerous as to avoid this difficulty, and they do not satisfactorily express that part of the curve which more immediately precedes rigor, though they further illustrate the linear production of earlier stages.

Exp. XXV. Muscles in sets of ten limb-pairs; each set in separate chambers. Hydrogen passed continuously. Temp. 8·5° throughout.

	Zinc lactate per cent.	Increase per 24 hours
Immediately after excision	0·031	0·110
After 20 hours	0·123	0·107
,, 47 ,, ...	0·243	0·123
,, 71 ,, (muscles still just irritable)	0·366	0·137
,, 105 ,, Full rigor	0·560	
Separate set kept for 24 hours at 18°. Full rigor ...	0·550	0·550

The production during the first and second 24 hour periods is once more seen to be the same; on the next, and on the subsequent day, there was evidence of acceleration. The intervals between the estimations were, however, too long to indicate when, exactly, this acceleration began, and the final determination was made at an unknown interval after the establishment of rigor.

An incomplete study of lactic acid production was made at 0°, on frogs similar to the last mentioned. The rise was apparently slow, and fairly steady, for 96 hours after excision, while what was probably a more rapid subsequent increase led to a high final maximum.

The occurrence of a positive acceleration before the attainment of the maximum was however in this experiment uncertain, because the series was too short. Rigor was fully

established when the final estimation was made, but it was not observed at what actual period during the final 48 hours rigor first occurred. If acid production was in progress till near the hour of the last estimation, the velocity was approximately linear throughout.

Exp. XXVI. Limbs in Erlenmeyer's flasks standing in ice. Hydrogen passed continuously. Estimations made, as usual, on sets of ten limb-pairs.

	Zinc lactate per cent.	Increase per 48 hours
Immediately after excision	0·031	0·146
After 48 hours (muscles apparently non-irritable) ...	0·177	
,, 96 ,, (muscles soft and translucent, but did not respond to faradic stimulation) ...	0·310	0·133
,, 144 ,, muscles in full rigor	0·450	0·140

Apart from the high maximum of lactic acid attained and the fact that full rigor was shown to be ultimately established in spite of the low temperature maintained throughout, the results of this last experiment are interesting, because, while the muscles did not respond to stimulation after 48 hours exposure to the combined conditions of low temperature and lack of oxygen, the formation of lactic acid nevertheless continued. This is so exceptional (cf. *supra*) that it would appear as though the depression of irritability induced by freezing an anærobic muscle is something different from that due to fatigue or to the accumulation of acid and other products.

It seems clear that the high maxima found at 12°, at 8·5° and at 0° are, in part at least, due to the fact that the experiments were made upon autumn frogs, while the survival curves at higher temperatures were plotted from experiments done in spring and summer. It is evident from Exp. XXV. that the same ultimate maximum may be reached at very different temperatures when the muscles concerned are in similar nutritive condition. If the maximum yield of lactic acid obtained on exposure to 45° is a measure of the potential capacity for acid production in a muscle, we have already seen that our experiments give an average maximum for spring frogs of 0·36 %, and, for autumn frogs, an average of 0·52 % zinc lactate. There yet remains for explanation the marked positive acceleration seen at the close of the curve at 12°. Some slight indication of a positive acceleration before the plateau is seen in the curve for 21°, and in one of those for 18°, while the two experiments at lower temperatures give also indications of hastened production at the later stages. It is clear moreover that the exact slope after the last estimation made before the plateau in any curve will depend upon the interval next chosen. If production of acid has really ceased before the moment of the first estimation situated on the plateau, the correct slope would be in some degree steeper than the apparent slope; it cannot in any case be less steep. Nevertheless, the positive acceleration which appeared to precede the cessation of production in our experiment at 12°, is undoubtedly greater than any seen at higher temperatures. If the experimental result be not accidental it lacks explanation.

Such evidence as is already available indicates that the survival history of mammalian muscle is strictly comparable with that of amphibian muscle, though the time-relations are very different. Osborne[1] has said that "the *post mortem* formation of lactic acid in mammalian muscle is not confined to the period of rigor but starts

[1] *Proc. Phys. Soc.* p. xlix. 1901. (This *Journal*, vol. xxvi.)

immediately on cessation of the circulation" while von Fürth[1] shows that "the *post mortem* acid increase in mammalian muscle only changes within narrow limits." Osborne is dealing here with the period of declining irritability and rising acid yield (a survival and not strictly a *post mortem* period); von Fürth is speaking of the later periods in which death has come and rigor is established. The constancy of acid yield in a muscle which has attained to this later stage is accountable for many of the observations already referred to, in which no difference of acid yield was found in "fresh" mammalian muscle and stiff,—both in reality, owing to experimental method or delay, having alike reached the high plateau of acid yield.

IV. The lactic acid of fatigue.

The early observations of du Bois Reymond and later of Heidenhain which showed an acid reaction to be the accompaniment of contraction in excised muscle, have been opposed by several later observers—but many of the discrepancies in the results allow of simple explanations.

Ranke[2] argued that tetanus of a muscle, previous to excision, drew upon the stores of the acid yielding substance, since, as he showed, the yield of acid due to heat rigor following fatigue within the body was diminished; and there is abundant evidence that in the living animal the acid products of contraction are rapidly removed from the muscle. Astaschewsky[3], who is often quoted as opposing Heidenhain and Helmholtz, studied tetanus with intact circulation and himself admits that the diminished acid yield he obtained from the tetanised muscle is accounted for by removal by way of the blood. The similar contemporary results of Warren[4] can in part be explained in the same way.

Apart from this simple misunderstanding about the circulation, other results in which fresh resting muscle has yielded nearly as much acid as the fatigued can be explained by faults of method already noticed, which lead to unduly high estimates of the acid yield of "resting" muscle.

We have not yet made a special study of the phenomena of fatigue but we have had occasion in the course of the work to be described in

[1] Hofmeister. *Beitrage.* III. p. 543. 1903.
[2] *loc cit.* p. 151.
[3] *Ztschr. f. physiol. Chem.* IV. p. 397. 1880.
[4] *Pflüger's Archiv.* XXIV. p. 391. 1881.

the next part, to make determinations of the lactic acid content of fatigued muscles, and the results we may briefly notice here. They are in complete accordance with those of Marcuse[1] who finds, on the average, a three-fold increase of lactic acid percentage as a result of stimulating to fatigue the excised muscles of the frog. The figures he gives for the zinc lactate of resting muscle is, on an average, ·076%: that for the tetanised muscle ·229%. These approximate closely to the relations we have constantly found, though our resting figure is lower. Marcuse, we may note, employed the boiling water method.

In our experiments, the muscles have been fatigued in general by direct faradisation. For this purpose the hind limbs of a number of frogs were removed by high section across the pelvic girdle, and connected in series by zinc hooks which linked the metatarsals of one pair with those of another. Each set of 10—15 pairs of limbs hanging freely, as a chain, from a bar, was put in a secondary circuit and stimulated by the induced interrupted current, (two Daniells, secondary coil at 0—5 cm.). In most cases the limbs were left unskinned until the end of stimulation, for protection against drying. After stimulation—generally maintained with short intervals for rest during 1½—2 hours—the limbs were separated, skinned, thoroughly shuffled with the object of eliminating as far as possible accidental inequalities of fatigue, and finally made up into sets of ten each, of nearly equal weight, as in the routine described earlier.

Of 16 sets of determinations made in different experiments at different times the average percentage yield of zinc lactate for fatigued muscle is ·216. Of these the highest yield was ·28%, the lowest ·147%, and 13 of the determinations lay between the limits ·18—·25%.

It has been our object in all these experiments simply to produce a well-marked condition of fatigue, and so to obtain an intra-muscular supply of lactic acid for the purpose of examining its changes under subsequent anærobic or ærobic conditions. The results, as they stand, abundantly confirm those of Marcuse. Incidentally also they present two striking features; first, the low level of the fatigue maximum of acid yield, and second, the narrowness of the limits within which the different estimations stand.

In many cases strong stimulation was continued for 2 hours, until no further contraction could be elicited from the muscles in the chains as connected in series, and only very slight responses on the application of strong interrupted shocks to the individual muscles. In several cases the middle third of both femur and tibiofibula was removed (with minimal damage to the muscles) in each leg of every pair, so that not only was the induced current transmitted along muscle substance only, but the muscle contractions performed greatly more

[1] *Pflüger's Archiv.* xxxix. p. 425. 1886.

work in shortening the whole weighted chain. But in no case did the acid yield exceed ·28% and it exceeded ·25% in only one. These figures are roughly half those obtainable by chemical irritants, or by heat, or by mechanical violence (see p. 261 *et seq.*): and we must either suppose that the sources of lactic acid available for the contraction process are distinct from and less than those available for the break-down due to violence, chemical or mechanical, or, on the other hand that the state of fatigue is inimical to the processes by which, on contraction, lactic acid is liberated, and that the lactic acid of fatigue stops short, self-impeded, at a relatively low level.

The second point to be noticed is the very close grouping of our results within limits. Though the stimulating current used in all the cases was the same, yet the details of linkage was different, the conditions—season, temperature, the previous exercise and rest of frogs, moisture, distribution of stimulus among muscle groups, mode of dissection and so on—all varied widely, and the periods of stimulation ranged from one hour only to full two, with various combinations of rest intervals. We neither expected nor desired, for our special purposes, equal conditions of fatigue in every case: yet the lactic acid yield, whether that be a true indicator of fatigue or not, remained strikingly constant. It became almost a matter of course to assume that the acid yield would be close to ·21%. This close grouping tends to confirm the supposition that our average represents something like a fatigue maximum—probably easily reached—and that the fatigue maximum is not one which is relative simply to our peculiar methods of stimulation, but one which is clearly far below the maximum yields obtainable by the extra-physiological methods of heating, injury, and chemical damage.

In one experiment (p. 305) the hind limbs were indirectly stimulated by shocks sent to the sciatic plexuses, the muscles being unloaded. After 45 minutes only no further response was obtained, though of course the muscles remained irritable by direct excitation. Estimation of the muscles gave in this case a yield of ·203%,—very little below the average for direct and prolonged stimulation.

V. THE OXIDATIVE INTRA-MUSCULAR REMOVAL OF LACTIC ACID.

It was in the hopes of obtaining evidence either for or against the existence of an intra-muscular disposal of lactic acid that in the first place we undertook the present work. We need not refer here in detail to the evidence which points to the reality of an oxidative[1] removal of lactic acid in the body subsequent to its passage from the muscles by way of the circulation. Confining ourselves for the present to the case only of intra-muscular removal, we may point out that there is already much indirect evidence that such a removal can be effected in the presence of available oxygen. The coagulative stiffness of *rigor mortis*, and the condition of fatigue, are diminished or even removed from an excised frog's muscle in an atmosphere of oxygen[2]; and both stiffness and fatigue are held by many to be marks of the acid products of metabolism or contraction within the muscle. Resting and fatigued muscles have respectively very different osmotic properties, which must probably depend upon the presence or absence of metabolites; here again simple exposure to an oxygen atmosphere will restore to a fatigued muscle the osmotic properties of the resting[3]. It seemed in view of these facts to be highly probable then that exposure to an oxygen atmosphere would diminish the lactic acid yield of a previously fatigued and acid muscle.

At the outset we made a series of preliminary experiments, using only a delicate qualitative test for lactic acid which will be described separately (see Appendix II.). It was possible by this method to obtain results with a small bulk of muscle—a single pair of limbs, or a set of four or six gastrocnemii. The outcome showed clearly that the yield of lactic acid by a fatigued muscle was diminished after a few hours' exposure to an oxygen atmosphere, when it was compared with a control preparation similarly treated but exposed only to air, or to coal gas. In some cases indeed—to be noticed again later—a completely negative result was obtained when the extremely delicate test for lactic acid was applied after the immersion for a few hours in oxygen of a chain of fatigued gastrocnemius muscles.

We passed at once to the quantitative measurement of this oxidative removal of lactic acid, and for this we eventually adopted the standard

[1] The word oxidative, as it is used throughout this paper, does not stand for any specific chemical process, but is equivalent simply to "through the agency of oxygen."

[2] Fletcher, This *Journal*, xxvIII. p. 474. 1902.

[3] Fletcher, This *Journal*, xxx. p. 414. 1904.

process of zinc lactate estimation, modified and used as we have indicated already. In all the examples to be given, the hind limbs were used in sets of ten each, and these were fatigued in series (see p. 280) immediately after excision, then rapidly shuffled and rearranged into fresh combinations of ten, each set having a nearly similar weight and containing therefore a nearly similar constituency of large or small limbs. Each set, after the shuffling, contained also, upon the average, an equal representation of states of fatigue, accidentally different in the individual limbs. The degree to which we reached equality of condition in corresponding sets may be judged by the double control estimations to be given below. Here we give the first group of results in demonstration of the fact that an actual loss of lactic acid yield is a result of exposure to an oxygen atmosphere.

I. Fatigued muscle-sets, exposed for 18 hours to different atmospheres, under equal conditions of temperature and moisture. The figures given are, in all cases, percentage amounts of zinc lactate.

(1)	11° C.	A.	after nitrogen	·158 %		B.	after oxygen	·072 %
(2)	15° C.	A.	,, ,,	·371 %		B.	,, ,,	·205 %
(3)	11° C.	A.	,, air	·180 %		B.	,, ,,	·094 %.

II. Fatigued muscle-sets, some (A) estimated immediately after fatigue in air, the others (B) after exposure to oxygen. A and A′ indicate duplicate estimations.

(1)	13° C.	A.	·252 %	B.	oxygen 7¼ hours	·163 %
		A′.	·246 %			
(2)	13·5° C.	A.	·225 %	B.	,, 5 ,,	·114 %
		A′.	·213 %	C.	,, 20 ,,	·099 %
(3)	22° C.	A.	·242 %	B.	,, 18 ,,	·106 %
				C.	hydrogen 18 ,,	·416 %.

III. Two similar muscle-sets were arranged in vertical tubes, maintained at 13·5° C. one in hydrogen throughout, the other in oxygen, with suitable moistening arrangements. They were stimulated 5′ at a time, with alternating 5′ periods of rest for 4 hours, and then remained at rest for 1½ hours, one still anærobic, the other still in oxygen. The results of estimation were:

Hydrogen set ·240 % Oxygen set ·142 %.

For further analysis of these effects we have attempted to follow by successive estimations the velocity of change at different periods. The results at ordinary room temperatures (15°—18° C.), are shown in the lower part of Fig. 5 (for details see Exps. XVIII. *et seq.*). In the three cases given graphically in the figure the degree of acidity due to fatigue is not the same: the three initial yields are respectively ·147 %, ·193 %, and ·227 %. In all alike however the changes which accompany

exposure to oxygen belong to the same type. The diminution in acid yield is most rapid at first, and increasingly slow thereafter. It must be noticed that in all three cases given, starting from different initial amounts of acid, there is a close approximation at the tenth hour of their respectively diminished yields. Whenever we have used the whole hind limbs of the frog we have noticed that the diminution of yield due to oxygen does not proceed far below the level of ·10 %, and it is towards approximation to this level that the three curves of diminution tend.

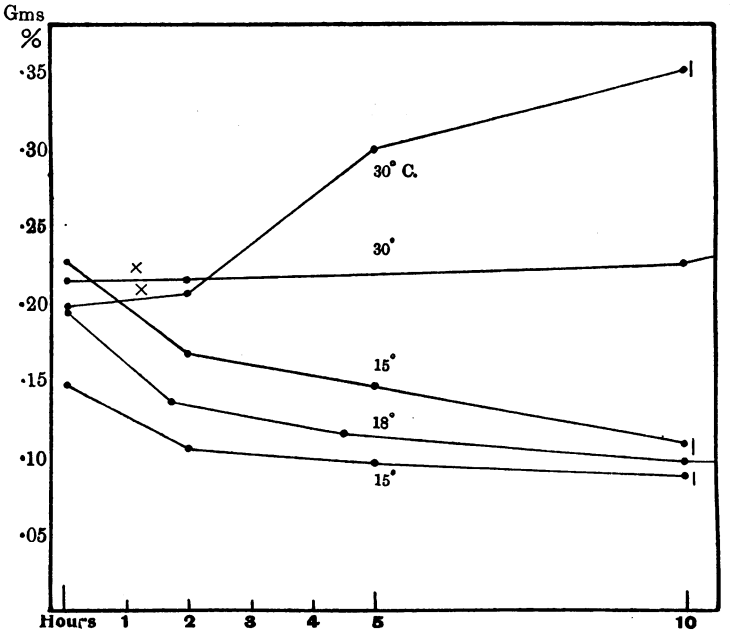

Fig. 5. Lactic acid production and loss in atmospheres of oxygen at different temperatures.

Fatigued muscles were used for all. At 30° C. gain in lactic acid is shown: at 15°—18° C. the course of loss is followed.
× loss of excitability.

It will be seen at once that these curves, obtained at room temperatures, have an exponential character, such as might be expected if the amount of acid lost in unit time, bore a simple relation to the amount at that time present in the muscle. But until a more detailed analysis of the processes underlying the described results has been made it would be unsafe to express them at all confidently in this way.

We know that in the absence of oxygen a steady spontaneous increase of acid yield takes place, but it is uncertain at present whether we have to imagine this spontaneous formation as still subsisting in the presence of oxygen notwithstanding a contemporary destruction or removal of acid, the resultant being a balance between formation and loss; or whether, on the other hand, the spontaneous formation is inhibited in the presence of oxygen, leaving exhibited the unbalanced loss alone.

Two other considerations add also to the difficulty of present interpretation.

We shall show that the loss of acid yield effected in the presence of oxygen is only exhibited by irritable muscles, and we have seen that the spontaneous survival increase of yield in the absence of oxygen is also a constant mark of remaining irritability. But whereas the spontaneous anærobic increase occurs at constant rate during the periods of declining irritability, it is possible that the ærobic loss may diminish as the irritability declines, and that the lower curves given in Fig. 5 represent not so much the rate of chemical change as affected by the materials available for it, but as affected by the physiological state which allows it; in short that the curve is essentially a curve of declining irritability.

It is to be remembered also that by immersion in oxygen gas the muscle substance is not, from the first, equally supplied in all its parts with oxygen. We have little guide as to the rate of penetration of muscle substance by oxygen. It has been shown that the diffusion of CO_2 outwards, from muscle substance artificially charged with it, follows the simple logarithmic course to be expected; its solution in muscle behaves like a solution of it in water[1]. If the diffusion of oxygen through the muscle resembles that of CO_2, then the curve of lactic acid loss may owe its exponential character in part at least to the varying rate of effective oxygen supply.

We are now engaged in a closer experimental analysis of the changing rate of loss; but we have thought it worth while to describe the actual course which the loss follows and briefly to mention some of the difficulties of interpretation. The question of diffusive penetration by oxygen, it may be further noted, has a bearing upon the circumstance that whereas we have not found a reduction of acid yield notably below ·10 % when we have used the whole musculature of the limbs, we

[1] Fletcher, This *Journal*, xxiii. p. 30. 1898–99.

have several times obtained a completely negative result (by qualitative methods) when a chain of fatigued *gastrocnemii* has been immersed in oxygen, and it is not giving these undue weight if we take them to indicate a value in any case decidedly below ·10%. This discrepancy may, we suggest, be assigned to the more efficient penetration of the smaller muscles. For the purposes of quantitative estimation we have not yet ventured to use *gastrocnemii* alone, and where whole limbs are used the larger muscle-masses of the thigh may allow the penetration difficulty seriously to affect the result of oxygen immersion.

Effects of oxygen at extra-physiological temperatures.

Some results already obtained under this head in the analysis of the ærobic loss of lactic acid may be given here. We find that both above and below the physiological limits (for these we suggest at present 10° as a lower and 25° as an upper limit) the effects of exposure to oxygen are widely different from those within the limits.

In the upper part of Fig. 5 are graphically shown the results of two series of successive estimations (see also Exps. XV. and XVI.) of fatigued muscles immersed in oxygen and maintained throughout at a temperature of 30°C. This temperature is extra-physiological for the muscle though it is not above the usual limits for enzymic action.

It will be seen that in both cases, starting from the fatigued state, in which the zinc lactate yield in each case is near to ·20%, the yield in both cases remained nearly constant for 2 hours and in one case nearly constant for 10 hours, then slowly rising. In the other case after 2 hours it rose by 50% in the next 3 hours and by half as much again in the next 5 hours.

We must defer until further experiments are completed an attempt to interpret these results in detail. It is clear that for 2 hours or more the effect of oxygen has been to hold in check an otherwise inevitable spontaneous rise of acid yield. One of the given cases provides an example of this check being maintained, or at least the acid *status in quo* being maintained, even for 20 hours, long past the cessation of irritability: the other case shows a relatively sudden release, either from check or from a counteraction, of the acid yielding process. The only conclusion we draw at present (and we here assume that increase of temperature does not impede the penetration by oxygen of the muscle substance), is that a rise of temperature to the highest physiological limits does not increase the oxidative removal of acid; or,

at the least, that it favours the acid production more than it favours the opposing oxidative process.

At the lower limits of temperature, the oxidative removal would seem to be in complete abeyance, and not merely very slow as in the case of the survival increase. It will be enough to quote one experiment, in which the fatigued muscle sets were maintained at 2—3° C. in an oxygen atmosphere.

12° C.	Fatigued muscles, estimated directly	·169 %
2–3° C.	Similar set. 2 hours in oxygen	·154 %
,,	,, ,, 5 ,, ,, ,,	·151 %
,,	,, ,, 10 ,, ,, ,,	·153 %.

The slight fall in yield after the first 2 hours is accounted for by the oxygen effect to be expected during the time in which the muscles, fatigued at room temperature of 12° C., were cooling to the temperature 2—3° C. of their chambers.

Effects of oxygen after traumatic injury.

It has been suggested already that the oxidative process, as we have studied it, may be complicated by an imperfect diffusive penetration of oxygen through the relatively large muscle-masses we have been obliged to use. It was in the course of an attempt to examine this complication by cutting the muscle to smaller pieces, and thereby to increase greatly the available surface, that we came to the effects of traumatic injury already described (p. 261). The amount of actual loss in lactic acid yield which does occur in undamaged muscles at suitable temperatures (10°—25° C.) in oxygen, had moreover suggested to us that the loss might be due to an oxidative process of a relatively direct and simple kind, not bound up with the irritable life, or with the structure of the muscle; and that the process might continue effectively when the muscle was reduced to small pieces or even to the "brei" condition, whether or not the necessary injury gave a high preliminary lactic acid percentage.

But it was at once obvious, on trial, that damage, and even relatively slight damage, if inflicted so as to leave no muscles intact, not only disallowed any process of oxidative loss of acid yield, but removed at once all the characteristic influence of oxygen. To a damaged muscle, in fact, oxygen is a neutral gas.

A comparison is made in the following experiment between the oxidative effects in the case of undamaged limbs and those when

corresponding sets of muscles have been cut each into pieces with sharp scissors (experimental details are given on p. 308).

Temperature 22° C. throughout, limbs fatigued and shuffled to sets of ten each (*a*, *b*, etc.). (Exp. XXIV.)

a	estimated directly,				·242 gms. % zinc lactate.	
c	undamaged,	18¼	hours in	hydrogen	·416 %	(gain 72 %)
d	„	„	„	oxygen	·106 %	(loss 56 %)
b	cut up, estimated directly				·334 %	
i	„	5½	hours in	hydrogen	·450 %	
k	„	18	„	„	·491 %	
f	„	5½	„	oxygen	·402 %	
g	„	18	„	„	·464 %	

Here, in marked contrast to the large oxidative loss in the undamaged limbs, the damaged muscles show little difference of yield whether in hydrogen or oxygen. A small difference which is established at the end of 5½ hours, perhaps due to slight oxidative loss, at the beginning, in pieces not at once affected by "traumatism," remains to the end of 18 hours. In the last 13 hours the cut muscles in oxygen gain actually just more in lactic acid yield than those in hydrogen.

The point may be further illustrated by reference to Exps. I (p. 261) and II (p. 262), and to Fig. 1, in which their results are plotted. In that figure the dotted lines represent periods of exposure to oxygen, the unbroken lines indicate anærobic conditions. At the end of curve A the two estimations of acid yield in the two sets of chopped muscle which had been from the 8th to the 21st hours exposed to hydrogen and oxygen respectively, are practically identical, that for oxygen being, as it happens, just the higher. In curve B, the rapidly increasing acid yield due to the cutting injuries which is seen in the first period (3¾ hours) in hydrogen, is not appreciably checked (having regard to the general time-relations of the curve) when an oxygen current is substituted for the hydrogen current during the following 3 hours.

How is this complete cessation of the oxidative control of intramuscular lactic acid, after damage to the muscles, to be accounted for? It might be supposed that the direct effect of mechanical injury, acting as a stimulus, so encourages the breakdown production of acid as to nullify the opposing influence of oxygen,—in much the same manner as the exposure to higher temperatures was seen to nullify it. But with the damaged muscle there is little sign of any compromise or balance

between stimulative production and oxidative loss. Only production is evident, and it is as evident in the presence of oxygen as it is under strict anærobic conditions.

To minimise the direct influence of the cutting stimulus, the results already given were repeated, with the only change that the muscles were cut to pieces at freezing temperature, the subsequent acid production of similar cut samples in hydrogen and oxygen respectively being tested, at intervals up to the end of 9 hours, after a low room temperature (12° C.) had been slowly reached and the muscles thawed. The results of this procedure were in all essentials the same as those already quoted.

Again, it would be difficult to explain in terms only of the potency of the initial stimulation, why, after severe injury to the muscle, the oxygen effect in controlling acid production is abolished. This initial injury produces in the cases given in Fig. 5, an initial acid yield expressed as ·150% (10° C.), and ·246% (15° C.). These are figures well within the common limits for the percentage yield of muscle stimulated to advanced fatigue by strong induced shocks. The oxidative process can effectively cope with the lactic acid production in the fatigued muscle, as we have seen; it not only prevents further increase of yield but causes the disappearance of 50%, more or less, of the initial amount of acid due to fatigue. Yet, in the case of the damaged muscle, with no higher initial content of acid, the oxidative process appears to be entirely absent or ineffective; apparently it neither leads to disappearance of pre-formed acid, nor exercises the slightest check upon continued production.

The facts indicate that we must go beyond the mere stimulative injury of cutting for the absence of the oxidative process, although if we look further, we can only summarise events by saying that, for the effective continuance of the process, the life and irritability of the muscle (both of which are almost immediately lost upon extensive cutting injuries) are essential conditions. This expression of the results can be put in a different form, but perhaps without any alteration of its significance, if we say that an essential condition for the continuance of the oxidative process is the undisturbed maintenance of the normal architecture of the muscle substance.

It is clear at all events that we have to deal not with an oxidative destruction or alteration of the lactic acid molecule carried out in the muscle tissue by simple chemical reaction or under the influence of oxydases, but with a process inextricably wrapped up with the metabolic events underlying the normal life of the muscle.

At many points in the course of current investigations into the chemistry of tissues, the suggestion is made, or implied, that a close study of changes going on in an excised and minced tissue, in a tissue "brei," or even in a tissue juice, is equivalent to a study of the chemical processes of the living tissue. We do not criticise this suggestion when it is properly safeguarded, but we think it well to point out that in the case of muscle at least we have a notable instance to the contrary effect. Here is a relatively simple chemical process—the oxidative transformation of lactic acid—which is to a high degree effective in an undamaged muscle after excision and which, we can hardly doubt, plays a very large part in the normal life of the muscle. Yet the process cannot be detached from the cycle of metabolism associated with continued irritability and appears to have at most a very brief existence or counterpart even in small pieces of muscle, damaged only by the necessary cutting.

The fate of lactic acid in the intra-muscular oxidative process.

The close association of the oxidative removal of lactic acid with the normal structure and irritability of the muscle puts special difficulties in the way of detecting the chemical nature of the process and its results. The process cannot be studied, we have shown, in minced or manipulated muscle *in vitro*, because it no longer exists.

Indirect methods only are available for ascertaining the fate of the lactic acid which disappears. It can hardly be doubted that the lactic acid, whether built up or broken down, is exchanged for molecules which have less toxic properties, if any, than itself. An obvious suggestion is that it is oxidised to yield CO_2, and in support of this we not only have the whole group of phenomena, already quoted, which indicate the removal of the toxic acid from action upon the muscle, but also the specific facts that "the normal rate of CO_2 discharge during the rigor periods for muscle in air is always largely increased in an atmosphere of oxygen" while it is "diminished by about 30% in an atmosphere of nitrogen[1]" and that the rate of survival discharge of CO_2 is increased during muscle contraction in the presence of available oxygen but is not increased under anærobic conditions[2]. Taking these considerations together, the conclusion that lactic acid is an immediate precursor of CO_2, undergoing complete conversion into CO_2

[1] Fletcher, This *Journal*, XXVIII. p. 359. 1902.
[2] *Ibid.* p. 497.

only when oxygen is available, would seem to be almost irresistible; though it should be pointed out that if such a conclusion held good, it would be difficult to account for the abolition of so simple a chemical process by injury to the muscle. If the change were simply an oxidation of lactic acid to CO_2, it would be expected to occur even in a muscle "brei," if supplied with oxygen.

We have tested the question by making use of the fact that CO_2 alone, with water, and no other substance, actually passes away from an excised muscle. If it yields CO_2 directly, then lactic acid in effect leaves the muscle. Otherwise in one form or another it remains behind.

A well-known observation of Ranke[1] suggests a means of following this up. He found that a muscle "after its removal from the blood circulation possesses an unchangeable maximum of acid yield" (Saüre bildungsmaximum) though he demonstrated this only for the case of mammalian muscles; and his results were confirmed, though unconsciously, by Astaschewsky and by Warren (see p. 279). The full establishment of rigor mortis was associated with just the same acid yield as the quickly produced heat rigor, when similar excised muscles were used. But "through tetanus in the living animal the acid maximum of the muscles becomes less. The tetanised muscle shows less acid than the resting. Tetanus of muscle uses up acid-forming material[2]." If then the passage of lactic acid out of the muscle by way of the circulation, as in Ranke's frogs during strychnine tetanus, reduces the acid-maximum attainable subsequently upon heat-rigor, similarly a removal of lactic acid in the shape of CO_2 and water from an excised muscle should reduce the ultimate heat-rigor yield. Such a reduction of the heat-rigor maximum would not show necessarily that the lactic acid has left the muscle as CO_2; the reduction might occur if the lactic acid became deconstituted within the muscle. But the absence of any reduction after oxidative disappearance of lactic acid, would negative the view that the products of the acid had escaped from the muscle.

We have consistently found that the maximum lactic acid yield reached in heat-rigor by a given excised muscle is constant, not only for resting muscle and for fatigued, but is also constant whether, to a less or greater extent, the lactic acid yield has been diminished by exposure to oxygen. The disappearance of lactic acid, as such, from the

[1] *loc. cit.* p. 146. [2] *loc. cit.* p. 151.

muscle substance under the influence of oxygen, does not cause any reduction of the lactic acid maximum subsequently reached in heat-rigor.

The constancy of the heat-rigor maximum (see also p. 266) can be shown without any elaborate precautions. But it is important that heat-rigor should be fully reached in all parts of the muscles under investigation and that, so far as possible, the heat-rigor should be induced at the same temperature—preferably a temperature not far above 40°—for all muscles which are to be put in comparison. There should be no danger (1) of incompleted rigor in any case, or (2) of any interference by quick killing at higher temperatures with the slower spontaneous heat-rigor at 40° C. and near it.

As a routine throughout our experiments we have obtained heat-rigor in each case by arranging the shuffled set of limbs at the bottom of a thin-walled Erlenmeyer flask, which has then been immersed in water maintained at 45° C. for one hour. The results obtained with control sets have exhibited constancy with almost surprising precision. In our own minds we have attached great value to the close approximations to identity of estimation which we have obtained in this set of observations, on the ground that they afford evidence of the efficiency of the routine methods of extraction and estimation.

The following experiment is typical.

Exp. V. April. Temperature 11·5° C. Limbs in sets of ten each as before.

Resting set, estimated directly	·030 %
„ „ „ after heat-rigor, induced just after excision	·312 %
Set fatigued within hydrogen tube for 8 hours, up to complete loss of irritability and marked stiffness; estimated after heat-rigor	·317 %
„ „ within oxygen tube for 48 hours, still irritable and supple; estimated after heat-rigor	·315 %

Here the muscle set fatigued to stiffness in hydrogen shows, as we expect, the same heat rigor maximum as the resting limbs, which have the same potentiality for acid production. But exactly the same maximum is also reached by the muscles in oxygen, which were stimulated to active contraction for long intervals extending over 48 hours. It cannot be doubted in view of the experimental results already given, that throughout this period the loss of lactic acid by oxidative disappearance was very considerable; and during the whole time abundant CO_2 was presumably leaving the muscle. But the store available for the lactic acid formation in heat rigor,—the same store as that called upon during contraction, the store yielding, that is to say, the lactic acid which we have shown to be subject to oxidative loss —remains undiminished at the end.

The results of another experiment exactly the same in effect, but carried out with autumn frogs, may be added here.

Exp. VI. November. Temperature 15° C. Limbs in sets of ten pairs each.

Resting set, estimated after heat-rigor at 45° C.	·513 % zinc lactate
,, ,, ,, ,, ,, (duplicate)	·502 % ,, ,,

Three other sets were all fatigued by 2 hours' severe stimulation.

Fatigued set, estimated directly, after heat-rigor	·511 % ,, ,,
,, ,, exposed 2 hours to oxygen, then estimated after heat-rigor	·522 % ,, ,,
,, ,, similarly treated in duplicate	·516 % ,, ,,

To put beyond a doubt the reality of the continued loss of lactic acid throughout long periods of activity in the presence of oxygen, and the constancy of the ultimate heat-rigor maximum, notwithstanding the successive losses, the process has been followed by successive estimations, during alternate intervals of production and loss. The results are expressed graphically in Fig. 6.

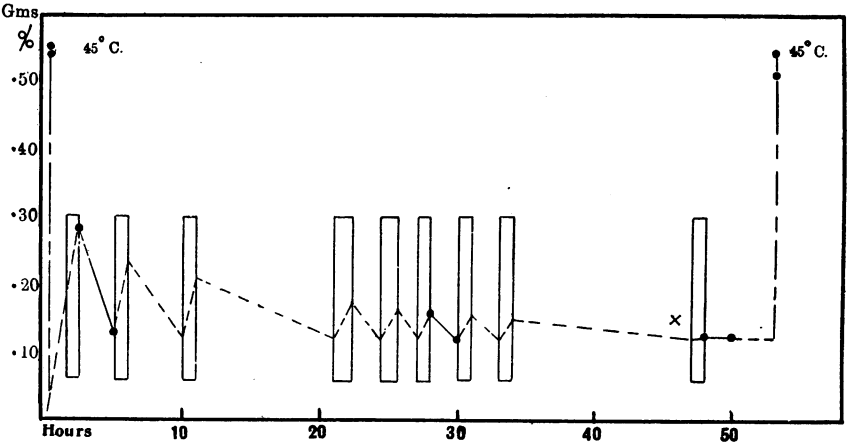

Fig. 6. The relation of the heat-rigor lactic acid "maximum," to the survival history of muscle.

Four estimations of lactic acid due to heat-rigor are shown, two at the beginning, in the case of resting muscles, two at the 53rd hour, in the case of inexcitable muscles, which had gone through nine periods of severe stimulation alternated with periods of rest in an oxygen atmosphere. The enclosed areas represent time periods (drawn proportionate to abscissæ) of stimulation by strong interrupted shocks. × loss of excitability. Temperature 15° C. Continuous line shows course of acid loss as actually determined by estimation. Dotted line shows the presumed course of acid loss and gain during other alternate periods.

Exp. VII. (Fig. 6.) Temperature 15° C. throughout. Ten shuffled sets of ten limbs each.

Two resting sets, heat-rigor (45°)	·540 %
	·545 %

The eight other sets were placed in tubes supplied with oxygen and connected so that all could be stimulated. In the periods marked on the diagram as enclosed areas, the chains of limbs were thrown into active contraction. In the third day the irritability was gone. During contraction air filled all the tubes. Immediately after each period of activity, oxygen was supplied to all and the supply maintained throughout the period of rest up to the next stimulation.

In the first day	
Set estimated directly after active period in air	·280 %
,, ,, at end of following rest period in oxygen	·125 %
In the second day	
Set estimated directly after activity in air	·16 %
,, ,, ,, following rest in oxygen	·12 %
In the third day	
Set estimated directly after stimulation period (without contraction)	·12 %
,, ,, ,, ,, rest period in oxygen	·12 %

Finally the two remaining sets, which had gone through the whole procedure described, were estimated after heat-rigor.

Set estimated after heat-rigor at end of whole procedure	·54 %
Companion set	·50 %.

Each of these last two sets of muscles had undergone nine periods of stimulation with rests following. The total amount of lactic acid caused to disappear during these three days may be judged by a rough integration based on the comparative estimates already given for each of the three days after activity and rest respectively. The figures show clearly that in spite of this large total disappearance of lactic acid, the final potentiality of the muscles for lactic acid production had not appreciably diminished.

In this particular experiment the total amount of lactic acid which was, in sum, disposed of by exposure to oxygen before the induction of heat rigor, would not be less than that afterwards produced as a direct result of the rigor. We are convinced that carefully chosen conditions as to temperature, and well selected periods for stimulation and rest, would allow this phenomenon to be demonstrated in a still more striking way. There is no question but that an excised muscle can produce, and, with access to oxygen, again dispose of, relatively large amounts of lactic acid without final diminution in that store of material which is drawn upon by the processes yielding the acid of heat rigor.

These facts apply, at present, only, of course, to excised muscle; but it seems clear that they have significance, and we believe that when they are explained some new light will be thrown upon the chemical

dynamics of living tissues. The simplest suggestion towards an explanation would be that the lactic acid of fatigue arises from sources other than those which supply the acid of heat rigor; but this seems to be against all the weight of the evidence.

We might, as an alternative, express the events in the following way: certain material, serving as a source of lactic acid, is stored at—so to speak—a certain definite chemical potential, in a condition available for immediate use, and this store alone is directly drawn upon by the processes of fatigue or rigor. But when the excised muscle is recovering from fatigue, under the favourable influence of efficient oxygenation, not only is the lactic acid produced by fatigue removed, but the store of available precursor is renewed, from reserve material present, originally, in some more stable form.

But the facts may be viewed from the standpoint of quite other possibilities. It is conceivable that the disappearance of lactic acid during recovery from fatigue does not involve its oxidative removal in the form of carbonic acid and water, but may be due to the occurrence of reconstructive processes. It is, of course, a simple and attractive view, based, as was urged above, upon available and suggestive facts, that lactic acid is a representative of a class of penultimate metabolites, formed during the breaking down processes of activity, the fate of which is to undergo subsequent oxidation under the influence of extra-molecular oxygen. This view, however, is associated with one difficulty which is not to be ignored.

Lactic acid is a substance still possessed of high potential energy, and it is not easy to see (confining our attention to the economy of active muscle) how the energy of a substance, oxidised after it has appeared as a product of the '*spaltung*' processes associated with contraction, can contribute to the sources of contractile activity. The difficulty is the greater if we adopt the usual supposition that the acid arises from carbohydrate, since its potential energy remains so nearly that of the assumed precursor. It is, of course, still more intrusive, in any consideration of muscle economy, if we suppose that, under normal circumstances, the lactic acid produced in muscle is oxidised elsewhere in the body.

The chemical relationships of lactic acid, and the results of recent research, indicate that it is a substance which, in some sort, links proteid products with carbohydrates, and carbohydrates with fats. It is at least very possible that it is an intermediary substance in the

processes of transformation of storage material in the body[1]. If such processes occur in metabolism, there is no reason why their progress should not come to light in the study of individual organs, and it is by no means fanciful to suppose that the appearance of lactic acid in muscle indicates a stage in the conversion of less readily available sources of energy into others which are more immediately available. Such processes of change would involve not only a breakdown which might continue anærobically, but also a subsequent synthesis, for which normal physiological conditions would probably be necessary. Without, however, venturing to insist upon any such details in the suggestion, we may at least indicate that, if during its disappearance in the oxygenative recovery of an excised muscle from fatigue, lactic acid does not undergo final oxidation, but shares rather in some constructive or reconstructive process, the circumstance would offer a ready explanation of the restoration of power to yield the normal acid maximum of the originally unfatigued muscle. It would explain, also, the fact that the oxygenative disappearance is no longer found when the tissue is disintegrated. This would be only in accordance with the familiar experience that in the majority of cases the integrity of the tissue unit, no less than an efficient oxygen supply, is necessary for, or greatly favours, such constructive processes. The point of view which has been indicated, whatever its present justification may be, should be susceptible of an experimental test, probably on the lines of a purely analytical study, and this we have begun.

VI. Concluding remarks and summary.

The proof obtained early in the course of this research that the lactic acid content of muscle is profoundly affected by the nature of the treatment received before or during extraction, has enabled us, we believe, to explain some of the contradictions in the statements of others about the fundamental relations of acid production.

Our experiments leave no doubt that in the survival processes which precede the disappearance of irritability there is a steady increase not only of total acidity in the muscle but of lactic acid itself. Equally certain is it that in acid production during fatigue lactic acid takes a large and probably predominant share. The necessity for reinvesti-

[1] In connection with the subject matter of this and the preceding paragraph, compare J. B. Leathes, *Some Problems of Metabolism*, 1906, Chapter III. p. 50.

gating such fundamental questions as these, before proceeding to the closer study of the phenomena which was our intended task, has given this paper essentially the nature of a preliminary communication. We propose to postpone therefore a full discussion of the bearing of our present results.

It may not be out of place however to consider briefly whether our data are to be reconciled with some current views as to lactic acid production in muscle.

We have given proof that the survival processes in excised unstimulated muscle lead from the moment of excision onwards to a steady accumulation of lactic acid, which, under most conditions, ceases entirely with loss of irritability. The increase of acid in the intact muscle is most rapid under anærobic conditions, is slower in air, and is not to be observed (at any rate for long periods after excision) in an atmosphere of pure oxygen. In the unstimulated muscle the production is, for the greater part of the survival period, very nearly proportional to the lapse of time; but stimulation produces an acceleration which may convert the curve of production velocity from a linear type into one showing exponential characters. Partial disintegration of the muscle represents a strong stimulus, inducing this acceleration to a marked degree, and a want of recognition of the rapidity of the change so induced has led many observers to ascribe much too high values to the lactic acid content of "resting" muscle. This is an error which has necessarily prevented the ascription of right values to the changes occurring during survival processes or fatigue. Exposure of the intact fibres to poisons, such as chloroform or coal gas, also accelerates the velocity of production, and, as has been shown in Part II of this paper, the action of alcohol in this respect is so marked that its uninformed use as a solvent has introduced large errors into many published estimations of lactic acid.

Our experiments make it clear that the excised but undamaged muscle when exposed to a sufficient tension of oxygen has in itself the power of dealing in some way with the lactic acid which has accumulated during fatigue. While the fibres are recovering from fatigue and regaining irritability in an atmosphere of pure oxygen, their content of lactic acid is greatly reduced. As already stated, exposure to pure oxygen also inhibits the production of the acid in fresh resting muscle. In air a slowing of the rate of production is seen, but exposure to an atmospheric tension of oxygen does not inhibit the process of formation.

There is no reason to suppose at present that an increase of oxygen tension has any influence more special than that of accelerating the

penetration of the gas into the muscle mass. If, as can hardly be doubted, there occur, in the surviving tissue, processes (encouraged by anærobic conditions) which lead to acid production, opposed to others (demanding oxygen) which make, possibly, for actual inhibition of production, and, certainly, for removal after production, then it is clear that whether we shall observe an accumulation of the product or a balance, or, as a third alternative, removal of the product when formed, will depend upon the rate at which oxygen is supplied. There is every reason to believe that a sufficient tension of oxygen (not reached in air) partially restores to an excised muscle one normal asset otherwise lost on excision, a supply, namely, of the gas sufficiently rapid to turn the balance, from an accumulation to a removal, of the particular breakdown product under consideration.

Hoppe-Seyler and his co-workers were, as is well known, the first to emphasize the importance of deficient oxidation as a factor in inducing the appearance of lactic acid in the intact animal. The facts in this connection are compatible with the view that the substance is a true intermediate product of metabolism, but one which undergoes further change with such rapidity, when oxygen supply is normal, that deficiency in oxygen supply is a necessary condition for its appearance in appreciable amount. The facts may however be taken to indicate that lactic acid is not a normal metabolite at all, but an alternative, asphyxial, product. To judge from references made to Hoppe-Seyler's view we are not sure that all writers have been quite clear as to exactly what it implied, though the author himself seems to have indicated a belief in the latter of the above alternatives[1].

The proof that muscle possesses in itself the requisite chemical mechanism for the removal of lactic acid when once formed, and that it is not in this respect wholly dependent upon the circulation, indicates, we think, that the substance is a product in its normal metabolism.

Our experiments show that a disappearance of some thirty per cent. of the lactic acid of fatigue occurs during the first two hours of exposure to pure oxygen; for the removal of fifty per cent. ten hours may be necessary. It must of course be realised that the completely fatigued excised muscle, depending upon the surrounding atmosphere instead of a circulation, is so far from being in a physiological condition that these results give no indication of the rate by which lactic acid might be dealt with under normal conditions in the body. It is clear, from

[1] Hoppe-Seyler. *Berichte* xxv., Ref. 685.

familiar evidence, that even in the body large and abnormal amounts, produced under exceptional circumstances, are removed from the muscles by the circulation; but we think that our experiments establish the existence of a power to deal with the metabolite locally, which cannot but serve the muscle under normal physiological conditions. Considerations of energy supply point strongly, as has been urged in previous paragraphs, to the value of such local utilisation. One very striking circumstance has been brought to light by our experiments. We have shown that the removal of lactic acid under the influence of oxygen only occurs when the fibres are irritable and intact. Partial disintegration of the tissue entirely prevents its occurrence.

Our experiments have not so far been concerned with the nature of the precursors of lactic acid; but the results may be held to have some indirect bearing on this matter. Numerous studies have been made by others upon excised muscle, in the endeavour to relate the disappearance of glycogen to the appearance of lactic acid in fatigue and in rigor. The results have been contradictory, but in the well-known experiments of Boehm there was no indication that, in rigor, the acid arises from glycogen. Most of the existing work upon this point (including Boehm's) was done before the importance of the pancreatic function in carbohydrate metabolism had been recognised. The possibility must now be reckoned with that the excised organ, if it does exhibit carbohydrate metabolism, may be doing so in the absence of a normal factor.

The influence of pancreatic extracts upon irritable surviving muscles might, conceivably, be studied by placing them in Ringer's fluid containing such extracts; and in view of Otto Cohnheim's results with ground up muscles, it is desirable that this possibility should be tested. Immersion of course tends to complicate the phenomena somewhat, because of the diffusion of products into the fluid. But if a proportionately small amount of fluid be used, sufficient just to cover the muscles, equilibrium inside and outside the fibres (in the case of substances for which the muscle is permeable) seems to be rapidly attained. At any rate we have found that on bubbling oxygen through Ringer's fluid containing fatigued muscles, there is (after, maybe, a preliminary increase) a final disappearance of a large proportion of the lactic acid, coincident with return of irritability to the muscles. In such experiments the lactic acid was estimated in the muscles and the surrounding fluid taken together. If the pancreatic co-agent can enter the muscle, it may prove more profitable to study the relation between carbohydrate and lactic acid when extracts of pancreas are added to Ringer's fluid as suggested, than to experiment in this connection with the muscle alone.

Since (to judge from the best available data) the average quantity of glycogen in frogs' muscle is, at most, not much more than commensurate with the amount of lactic acid which can be formed during survival

processes, it may perhaps be thought that a velocity of formation which shows no *minus* acceleration is incompatible with an exclusive derivation of the latter from the former substance. The results given in the last section, moreover, may be taken to indicate that an excised muscle is capable of producing, under special conditions, a quantity of lactic acid quite out of proportion to its glycogen content. We do not wish, however, before further data are obtained, to insist too much on considerations of this sort, and we do not claim that our present results bear with any special force upon the question of the nature of the precursors of lactic acid.

We feel however that they stand in the way of any direct application of Stoklasa's conceptions concerning lactic acid production to the case of surviving irritable muscle. As is well known this author ascribes to every living cell an anærobic mechanism, the action of which precedes oxidation processes. It brings about a change of carbohydrate into lactic acid, followed by removal of CO_2 and the formation of alcohol. The process is identical with the fermentation of sugar by the yeast-cell and the two stages of change are induced by two specific enzymes, consecutive in action, corresponding with those described in yeast by Buchner and Meisenheimer. Whenever therefore we estimate the gradual increase of lactic acid in surviving muscle, we are following, on this view, the accumulation of a product intermediate between sugar and alcohol. If our evidence for the linear relation to time exhibited by this accumulation be accepted, mathematical consideration will show that it cannot concern a product intermediate between two reactions of an exponential character. The chemical nature of the supposed reactions makes it unlikely that they would proceed on other than exponential lines; but it is, of course, possible that, in the muscle, each is so conditioned as to proceed without change of velocity. The rate of accumulation of the intermediate product will then be linear, and will depend on their relative velocity. Granting this, however, it is still not easy to see why the disappearance of lactic acid from muscle should be encouraged by a free supply of oxygen, if the enzymic change from lactic acid to alcohol is, as on Stoklasa's assumption, essentially an anærobic process. Finally, if the normal fate of lactic acid is to form alcohol by simple cleavage under the influence of an enzyme, it seems even more difficult to understand why the process which leads to its disappearance from muscle should cease so completely upon partial disintegration of the tissue.

Stoklasa's experimental evidence for the occurrence of alcoholic fermentation in the expressed juice of plant-cells, especially as

presented in his most recent paper[1], is convincing. But in the case of surviving muscle fibres, even if we assume the presence of agencies necessary to induce alcoholic fermentation, these would not, we think, suffice for a full explanation of the phenomena.

1. Estimations have been made of the lactic acid yielded by the leg muscles of frogs under various conditions. Special precautions have been taken to avoid errors due to the manipulative treatment of irritable muscle. The estimations have been made by gravimetric determinations of zinc sarcolactate according to the well-known method, used with certain modifications.

2. Freshly excised resting muscle is found to yield very small quantities of lactic acid, and these small amounts are possibly not more than can be accounted for by the unavoidable minimum of manipulation prior to extraction.

3. A large increase of the yield of lactic acid is found as the result of mechanical injury, of heating, and of chemical irritation. A large increase of acid is found, in particular, to accompany the immersion in alcohol of resting muscle in bulk, and this, with the other effects due to destructive treatment, is shown to have important bearings upon the choice of methods for extraction.

4. Lactic acid is spontaneously developed, under anærobic conditions, in excised muscles. The course of this survival development of acid has been followed by series of successive estimations. During the survival periods of subsisting irritability, and not after, equal increments of acid arise in equal times. After complete loss of irritability the lactic acid yield remains stationary.

5. Fatigue due to contractions of excised muscle is accompanied by an increase of lactic acid. The amount of acid attainable by severe direct stimulation is found, with notable constancy, to be not more than about one half of that reached in the production of full heat-rigor, or by the action of other destructive agencies than heat.

[1] *Zeitsch. f. physiol. Chem.* Bd. l. p. 303. 1907.

6. In an atmosphere of oxygen there is no survival development of lactic acid, for long periods after excision.

From a fatigued muscle, placed in oxygen, there is a disappearance of lactic acid already formed. The course of this disappearance has been followed by successive estimations in similar groups of muscles exposed to oxygen for different time intervals: it proceeds at first rapidly, then more slowly, and in general reaches a level about one half of the original yield of the fatigued muscle.

This disappearance of lactic acid due to oxygen does not occur, or is masked, at supra-physiological temperatures (*e.g.* at 30° C.). It is not found in muscle which has suffered mechanical injury: one essential condition for this effect of oxygen appears to be the maintenance of the normal architecture of the muscle.

7. The amount of lactic acid produced in full heat-rigor (at 40°—45°C.) is constant for similar muscles. This "acid-maximum" of heat-rigor is not affected by a previous appearance within the excised muscle of lactic acid due to fatigue, nor by a previous disappearance of acid in the presence of oxygen, nor by alternate appearances and disappearances several times repeated.

8. In an appendix a new colour test for lactic acid is described.

APPENDIX I.

Protocols of Experiments.

In this detailed account of experiments not given in the text such points of procedure as are common to all are omitted for the sake of brevity. The account of the treatment of the muscles given in Part I, page 256, need not be here repeated.

The weights given for the muscles are the *net* weights in all cases. For these the weight of the bones, debris, and unused muscles of the pelvic girdle and those below the ankle joints was deducted from the initial weight of the whole limbs (cf. Part I).

The calendar date of each quoted experiment is given so that seasonal allowance may be made in considering the results.

Exp. VIII. March. (See also p. 269, and Fig. 2.) Ten sets of ten pairs each, taken 10.15—10.30. Resting in skins to 11.15. Weighing, 11.15—11.45. Room temp. 12·8° C. Gas and air chambers 13° C. throughout.

Muscle weight				Gms. Zinc lactate
42·4 gms.	estimated immediately	...	...	·045 %
42·2 ,,	,, after 4½ hours, coal gas		...	·224
42·4 ,,	,, ,, 5¼ ,, in air ...		...	·066
43·7 ,,	,, ,, 6 ,, in oxygen		...	·065
42·1 ,,	,, ,, 22 ,, in air ...		...	·079
41·0 ,,	,, ,, 23 ,, in oxygen		...	·038
38·1 ,,	,, heat rigor, 1 hour in flask at 45° C.			·383

EXP. IX. March. (See page 264, and Fig. 2.) Four sets of 10 pairs each. Room temp. 15° C.

	Limb weights	Nett muscle weight		ZnL_2
a.	67·8 gms.	40·0 gms.	estimated directly	·046%
b.	67·8	(say) 40	estimated after simple immersion (as in IV) in alcohol (95 %) at 15° C. for 1½ hours ...	·258
c.	68·5	(say) 40·4	estimated after simple immersion in alcohol (95 %) at 1—3° C. for 1½ hours ...	·059
d.	68·3	(say) 40·4	muscles cut off and dropped direct into boiling water. Water evaporated: residue extracted with alcohol and this added to extract of muscle masses	·081

Zinc oxide determined on the combined residues = 33·36 % (Theory 33·42 %).

EXP. X. May. (See p. 272, and Fig. 3.) Six sets of 10 pairs each, taken 10.30 a.m. Room temp. 12·5° C. Shuffled and weighed sets arranged each to hang upon a thread freely within a vertical tube. The tubes connected in series and a current of hydrogen passed from 12 m. onwards. Tubes maintained at 16° C. throughout.

	Muscle weights	Time in hydrogen	ZnL_2
a.	37·6 gms.	0 hours (estimated directly)	·045 %
b.	37·6	5 ,, ...	·114
c.	39·2	11⅓ ,, ...	·141
d.	39·9	21½ ,, ...	·20
e.	36·5	28 ,, ...	·210
f.	39·3	35½ ,, ...	·20

The condition of the muscles at the times of estimation respectively was, as tested by induced shocks from coil with two bichromate cells in primary:

b. (5 hours), muscles contract with secondary coil at 5 cm. (at 5 cm. shock is just not painful to tongue).

c. (11⅓ hours), muscles contract well, sec. coil at 3 cm. (painful to tongue), a few thigh muscles unexcitable.

d. (21½ hours), none irritable, sec. coil at 0 cm., muscles natural in appearance, but limbs beginning to be stiff.

e. (28 hours), stiffness more obvious: some muscles still flaccid, no response to strongest stimuli.

f. (35½ hours), same, faint putrefactive smell.

Exp. XII. December. (See p. 273, Fig. 4.) 90 frogs pithed. All material kept on ice during manipulation. Limbs removed, shuffled, and divided into sets of 10 each. One set ground immediately. Each of remaining sets placed in separate tube and hydrogen passed through series. Temp. 12·5° C. throughout.

	Hours	Muscle weight	ZnL_2	Increase per 10 hours
a.	estimated directly	44·5 gms.	·031 %	
b.	5	47·1	·061	·036
c.	21¼	47·7	·120	·043
d.	31	46·1	·163	·043
e.	47	45·5	·232	·040
f.	57	46·3	·272	·104
g.	67	46·8	·481	0 (irritability lost)
h.	77	47·2	·476	
i.	77 (then 1 hr. at 45° C.)	44·7	·520	

For 50 hours (*i.e.* from the fifth hour onwards) the rate of production is seen to be almost exactly linear. Then follows the acceleration discussed on pp. 277, 278. In this experiment the spontaneous production at 12° did quite reach the level of heat rigor production.

Exp. XIII. January. Three sets of 12 pairs each, unskinned. Stimulated in series, in six chains of six each, by strong interrupted current, 11.20 a.m.—1.15 p.m. Room temp. 13° C. Skinned, weighed and shuffled. One set placed in water-jacketed chamber maintained at 12° C.

		Muscle weight	ZnL_2
a.	Fatigued limbs estimated directly ...	49·7 gms.	·252 %
b.	,, ,, ,, ,, (for duplicate)	55·7	·246
c.	,, ,, ,, after 7¼ hours exposure to oxygen atmosphere	28·5	·16

Exp. XIV. February. Four sets of 12 pairs each. Stimulated as in last Exp. 11.15—1.15 p.m. Room temp. 13·5° C. Skinned, weighed, shuffled. Two sets to oxygen chamber at 13·5° C.

		Muscle weight	ZnL_2
a.	Fatigued limbs estimated directly	45·7 gms.	·225 %
b.	,, ,, ,, ,, (for duplicate)	43·7	·213
c.	,, ,, ,, after 5 hours in oxygen	47·9	·114
d.	,, ,, ,, ,, 20 ,, ,,	44·8	·099

Zinc oxide determined on products from (*a*) and (*b*) combined = 33·20 per cent. (Theory requires 33·42.)

Exp. XV. February. 60 limb pairs taken. Stimulated as in last Exp. 10.35 a.m.—12.40 p.m. Room temp. 15·5° C. Skinned, weighed, shuffled. Four sets of 12 pairs each placed in chambers at 30° C., supplied with oxygen. Oxygen current started 2 p.m.

		Muscle weight	ZnL_2
a.	Fatigued limbs, estimated directly	40·3 gms.	·218 %
b.	,, ,, ,, after 2 hours 10 mins. in oxygen	39·9	·215
c.	,, ,, ,, ,, 5 ,, 5 ,, ,,	[lost]	
d.	,, ,, ,, ,, 10 ,, 5 ,, ,,	40·0	·223
e.	,, ,, ,, ,, 20 ,, 45 ,, ,,	39·3	·269

Exp. XVI. February. 48 pairs taken. Stimulated as in last, 10.40 a.m.—12.10 p.m. Room temp. 14° C. Three sets of 12 pairs each in chambers at 30° C., supplied with oxygen.

		Muscle weight	ZnL_2
a.	Fatigued limbs, estimated directly	44·8 gms.	·197 %
b.	,, ,, ,, after $2\frac{1}{3}$ hours in oxygen	45·2	·205
c.	,, ,, ,, ,, $5\frac{1}{3}$,, ,,	46	·317
d.	,, ,, ,, ,, $9\frac{1}{2}$,, ,,	45·4	·350

Exp. XVII. March. 48 pairs taken. Stimulated as in last Exp. 10.30—11.45 a.m. Room temp. 12·5° C. Three sets of 12 each in chambers supplied with oxygen, maintained at 2—3° C. throughout.

		Muscle weight	ZnL_2
a.	Fatigued limbs, estimated directly	31·9 gms.	·169 %
b.	,, ,, ,, after 2 hours in oxygen	35·8	·154
c.	,, ,, ,, ,, 5 ,, ,,	35·1	·151
d.	,, ,, ,, ,, 10 ,, ,,	35·9	·153

c and *d*, perfectly fresh in appearance and very flaccid and normally translucent. But no response to strong shocks directly applied. (1 Dan. coil[2] at 0 cm.)

Exp. XVIII. March. 60 pairs. Stimulated 11.10 a.m.—1.10 p.m. Room temp. 17·5° C. Five sets of 12 each. Three sets in chambers with oxygen supply, maintained at 15—16° C. Oxygen started 2.10 p.m.

		Muscle weight	ZnL_2
a.	Fatigued limbs, estimated directly	37·6 gms.	·147 %
b.	,, ,, ,, after $2\frac{1}{2}$ hours in oxygen	38·6	·107
c.	,, ,, ,, ,, 5 ,, ,,	35·4	·097
d.	,, ,, ,, ,, 9 ,, ,,	36·4	·088

Exp. XIX. March. (See p. 282, and Fig. 5.) 60 limb pairs taken. One set of 12 estimated as resting muscle. Remainder stimulated, unskinned, in chains, as in last Exp. 10.45 a.m.—12.5 p.m. Room temp. 13·5° C. Shuffled and weighed to four sets of 12 each. Three sets in oxygen chambers, maintained at 15° C. Oxygen started 1.5 p.m.

		Muscle weight	ZnL_2
a.	Resting limbs, estimated at 10.25 a.m. ...	53·1 gms.	·039 %
b.	Fatigued limbs, estimated directly (1.10 p.m.) ...	54·5	·227
c.	,, ,, ,, after 2 hours in oxygen	54·5	·169
d.	,, ,, ,, ,, 5 ,, ,,	54·3	·147
e.	,, ,, ,, ,, 10 ,, ,,	54·6	·104

Exp. XXVII. April. 40 frogs pithed. All hind limbs tetanised by stimulation with induced current of spinal cords for 45 minutes. Tetanus periods of five minutes alternate with rests of 3 minutes. Shuffled and weighed. Set of 20 limbs to oxygen chamber at 12° C.

		Muscle weight	ZnL_2
a.	20 limbs, fatigued by *indirect* stimulation	69·6 gms.	·203 %
b.	20 ,, ,, and estimated after 3 hours in oxygen	66·9	·149

Exp. XX. July. (See p. 265.) Five sets of 12 each taken at 11.45 a.m. Room temp. 20° C. At 12.30 p.m. one set estimated directly. 12.50 p.m. two sets in chamber traversed by current of CO_2, two sets in chamber supplied with chloroform vapour: chambers maintained throughout at 20° C.

	Muscle weight	ZnL_2
a.	37·2 gms., estimated directly	·05 %
b.	39·1 ,, ,, after 4 hours CO_2 ...	·054
c.	38·7 ,, ,, ,, 11 ,, ,, ...	·179
d.	42·0 ,, ,, ,, 4 ,, $CHCl_3$ ...	·434*
e.	40·0 ,, ,, ,, 11 ,, ,, ...	·445

Coil, 1 Daniell in prim. circuit.

b. All muscles give good response, sec. coil at 5 cm.

c. None give response, sec. coil at 0 cm., no stiffness: the thigh muscles are slightly spastic.

d. Very stiff: not opaque.

e. Maximum rigidity.

* Zinc oxide determined on (*d*), 33·41 per cent. Theory requires 33·42.

Exp. XXI. July. (See p. 272, and Fig. 3.) 100 limb pairs taken, 9.30. Shuffling and weighing over ice. Instruments, plates and vessels previously sterilised. Each of 10 threaded chains of 10 limbs each, secured in a glass tube, three inches diam., in which it hung freely exposed on all sides. Each tube was three feet long: and all were clamped vertically upon a stand which was bodily immersed in a very large cask of water. Hydrogen was passed through all tubes (see p. 271) from 12.30 p.m. Pyrogallate test negative at 1.20. Water temperature 21° C. throughout.

	Muscle weight	ZnL_2
1.30 p.m., chain *a* taken and estimated after 1 hour	34·9 gms.	·07 %
4.35 ,, ,, *b* ,, ,, ,, ,, 4 hours	35·2	·136
6.35 ,, ,, *c* ,, ,, ,, ,, 6 ,,	35·3	·18
8.45 ,, ,, *d* ,, ,, ,, ,, 8¼ ,,	35·8	·223
11.0 ,, ,, *e* ,, ,, ,, ,, 10½ ,,	35·8	[lost]
12.50 a.m., ,, *f* ,, ,, ,, ,, 12⅙ ,,	35·2	·27
3.45 ,, ,, *g* ,, ,, ,, ,, 15¼ ,,	35·1	·31
6.0 ,, ,, *h* ,, ,, ,, ,, 17½ ,,	36·7	·36
12.50 p.m., ,, *i* ,, ,, ,, ,, 24⅓ ,,	33·2	·376

Zinc oxide determined on first four products, 32·85 per cent., on last three, 33·01 per cent.

Pyrogallate test was applied at every hour named, and gave negative results throughout.

6.35 p.m. 1 Daniell in coil[1], coil[2] at 5 cm. (painful to tongue). All muscles respond well, B and M.

8.45 p.m. Same.

11.0 p.m. Coil[2] at 5 cm. Gastrocnemii slight response, thighs 0. At 0 cm. most thighs slight response.

12.50 a.m. $Coil_2$ at 0 cm. Thighs all 0. Most gastrocnemii respond. Thighs spastic.

3.45 a.m. Some muscles stiff, rest spastic. Responses as in last.

6.0 a.m. All but two limbs stiff and clotted. No response to stimuli, except that two gastrocnemii give surface flicker. $Coil_2$ at 0 cm.

10 a.m. All stiff and clotted, no trace of response. No signs of putrefaction.

Zinc oxide determined on products of (*b*), (*c*), and (*d*) combined = 33·50 %; on (*e*), (*f*), and (*g*) combined = 33·24; on (*h*) and (*i*) combined = 33·91.

Exp. XXII. August. (See p. 272 and Fig. 3.) 90 pairs taken 10 a.m. Room temp. 22° C. All operations over ice, and with sterilised apparatus. Nine chains of 10 pairs each in vertical tubes fixed to stand, plunged in cask under water maintained throughout at 18·5° C. Nitrogen current through whole series at 12 noon. Pyrogallate test at 1.15 negative.

	Muscle weight	ZnL_2
1.15 p.m., *a* taken after 1¼ hours	36·6 gms.	·088 %
3.30 ,, *b* ,, ,, 3½ ,,	36·8	—
6.0 ,, *c* ,, ,, 6 ,,	36·3	·12
9.35 ,, *d* ,, ,, 9½ ,,	35·8	·16
12.30 a.m. *e* ,, ,, 12½ ,,	36·7	[lost]
6.0 ,, *f* ,, ,, 18 ,,	35·8	·24
10.0 ,, *g* ,, ,, 22 ,,	35·9	·316
2.15 p.m. *h* ,, ,, 26¼ ,,	36·4	·32
9.15 ,, *i* ,, ,, 33¼ ,,	37·0	·32

Pyrogallate test negative throughout.

12.30 a.m. *e*. Slightly spastic. Coil[2] at 10 cm. (just felt by tongue) gastrocnemii fair B. response. Thighs slight B. response.

6.0 a.m. *f*. Distinctly spastic. Coil[2] at 5 cm., same responses as coil[2] at 10 cm. in last. $Coil_2$ at 10 cm., 0.

10.0 a.m. *g*. Fairly stiff, knees straight. Coil[2] at 5 cm., gastrocnemii just respond, Coil[2] at 0 cm., thighs just respond.

2.15 p.m. *h*. As in last, but most thighs give no response at all.

9.15 p.m. Stiff and clotted. Coil[2] at 0 cm. all unexcitable (two gastrocnemii showed faintest surface flicker).

Zinc oxide determined on (*f*), (*g*), (*h*), and (*i*) combined = 33·36 %.

Exp. XXIII. August. (See p. 272, and Fig. 3.) Eight pairs taken. Last Exp. repeated in every detail save that hydrogen current was used to establish the anærobic condition instead of nitrogen, soon after the beginning. Nitrogen current started at 8.15 p.m.: at midnight it failed accidentally and was replaced by hydrogen current. Pyrogallate test was negative throughout. Temp. of water cask maintained at 18·5° C.

	Muscle weight	ZnL_2
10.15 p.m., *a* taken after 2 hours	38·9 gms.	·107 %
2.10 a.m., *b* ,, ,, 6 ,,	40·6	·125
9.30 ,, *c* ,, ,, 13¼ ,,	40·3	·215
2.15 p.m., *d* ,, ,, 18 ,,	42·3	·249
6.30 ,, *e* ,, ,, 22¼ ,,	40·7	·294
11.40 ,, *f* ,, ,, 27⅓ ,,	41·6	·320
11.0 a.m. *g* ,, ,, 39 ,,	42·1	·330
5.0 p.m. *h* ,, ,, 45 ,,	41·5	·344

2.15 p.m. 1 Dan. in coil[1]. Coil[2] at 5 cm. All but few thigh muscles give fair B. and M. response.

6.30 p.m. Coil[2] at 0 cm.: most gastrocnemii give good response. All thighs give slight flickering response.

11.40 p.m. Coil[2] at 0 cm. All unexcitable, but one gastrocnemius which gave flicker.

11.0 a.m. Stiff and quite unexcitable.

Zinc oxide determined on (*a*), (*b*), and (*c*) combined = 33·95 per cent.; on (*f*), (*g*), and (*h*) combined = 33·55 per cent.

Exp. XXIV. August. (See p. 288.) 100 limb pairs taken 10.30 a.m.—limbs left in skins. All stimulated in series to active connection by strong interrupted current. Separate coil (2 Dan.) and circuits to each 12—15 limbs. Stimulation 11.12 a.m.—12.12 p.m. (including four periods of five minutes rest). Limbs skinned, shuffled (on iced plates) and weighed, to give 10 sets of 10 pairs each. Room temp. 22° C. One set estimated directly. Seven sets were severally chopped up with scissors and the chopped muscle of each set placed on slides within tubes, at 4.45. Oxygen or hydrogen supplied to the tubes as shown below.

		Muscle weight	ZnL_2
a.	Fatigued limbs, left whole, estimated directly ...	36·9 gms.	·242 %
b.	,, ,, chopped, and ,, ,, ...	32·6	·334
c.	,, ,, left whole, in hydrogen tube 18½ hours	37·5	·416
d.	,, ,, ,, ,, oxygen ,, 18½ ,,	37·5	·106
e.	,, ,, chopped, in oxygen tube 3 hours ...	32·6	·421
f.	,, ,, ,, ,, ,, 5½ ,, ...	32·6	·402
g.	,, ,, ,, ,, ,, 18 ,, ...	32·6	·464
h.	,, ,, ,, in hydrogen tube 3 hours ...	32·6	·483
i.	,, ,, ,, ,, ,, 5½ ,, ...	32·6	·457
k.	,, ,, ,, ,, ,, 18 ,, ...	32·6	·491

APPENDIX II.

A colour reaction for lactic acid.

Description may be given here of a colour test for lactic acid which was of some use to us in the preliminary stages of this research. It was introduced by one of us (F. G. H.) two years ago, but no account of it has yet appeared in any Journal[1].

The reaction yields a colour change much more definite and under most conditions more easily observed than the yellowing of Ueffelmann's reagent, but it has the disadvantage of requiring the use of strong sulphuric acid and cannot therefore be applied directly to material which chars with that acid.

The reagents required are: (1) a very dilute alcoholic solution of thiophene (10 to 20 drops in 100 c.c.); (2) a saturated solution of copper sulphate, and (3) ordinary strong sulphuric acid. The test is applied as follows:—

A glass beaker containing water briskly boiling should be ready to hand. About 5 c.c. of strong sulphuric acid are placed in a test-tube, together with one drop of the strong copper sulphate solution which serves to catalyse the oxidation that follows. To this mixture a few drops (the reaction being very delicate) of the solution to be tested are added, and the whole well shaken. The test-tube is now placed in the beaker water bath for from one to two minutes. The tube is then well cooled under a water tap, two to three drops of the thiophene solution are added from a pipette; the tube is replaced in the boiling water and the contents immediately observed. If lactic acid were present the fluid, rapidly and in a highly characteristic manner, assumes a bright cherry-red colour which is only permanent if the test-tube be cooled immediately after its appearance.

Cooling, as described, should always be carried out before the addition of the thiophene, as the gradual appearance of the colour, which then occurs on re-warming, makes the test much more delicate.

It has been described, however, in S. W. Cole's *Exercises in practical physiological Chemistry* (Cambridge and London), 1904. See page 7.

The colour change is undoubtedly due to an aldehyde reaction with thiophene, but the production of aldehyde during the preliminary oxidation appears to be associated with some special condensation or other change, as the test seems to be proportionately much more delicate for lactic acid than for acetic aldehyde.

Alcohol gives no trace of aldehyde, or any colour reaction, under the conditions of the test; but if ether is to be used for a preliminary extraction of the lactic acid it must first be well washed with water to remove aldehyde-yielding products.

The reaction is not given by any carbohydrate examined, at any rate not in concentrations such as will, by absence of notable charring, allow the colour change to be observed. From any mixture which chars with strong sulphuric, the lactic acid must, of course, be first extracted by some suitable process.

The test is not wholly specific, though much more so than is Ueffelmann's reaction. It is given by malic acid and probably by other α-oxy acids. Acetic aldehyde and glyoxylic acid give a colour with thiophene and sulphuric acid direct, but it is somewhat remarkable that though lactic acid may be supposed to yield the test because of conversion into either one of those substances during the oxidation, yet both are so rapidly destroyed by heating on the water bath with sulphuric acid and copper that they no longer react with thiophene after the briefest exposure to this treatment. The exact nature of the reaction needs therefore further elucidation.

A great number of physiological products have been submitted to the test but always with negative results, and at present it appears that for physiological material it may be considered as specific.

The oxidation goes best when little or no water is present in the mixture, and before applying the test it is well to obtain the lactic acid in alcoholic solution, or a solid residue suspected of containing it may be directly dissolved in the sulphuric acid. It may, however, be immediately applied to filtered washings from a stomach, or to any aqueous extract which contains lactic acid in such quantity that only a few drops are required. A milligramme of acid can be easily detected.

The test will supply qualitative indications as to the condition of frogs' muscles, and the description of an experiment which makes an excellent class demonstration will serve to illustrate its use. A frog is pithed and afterwards allowed to remain quiescent for half an hour or so, care being taken that the circulation in the hind limbs is not impeded by pressure or flexion. Both hind limbs are then removed and the muscles of one, immediately after cutting from the bones, are rapidly ground with sand, under 20 or 30 c.c. of ice cold alcohol. The other limb muscles may then be stimulated or caused to go into heat-rigor, after which they are ground under alcohol. The alcoholic extracts from each limb are evaporated to dryness and the residues rubbed up with a few cubic centimetres of hot water. To each aqueous extract a decigramme or so of finely powdered charcoal is added; each is heated to boiling in a test-tube and filtered. The filtrates are evaporated in small glass basins and each dry residue is dissolved in 5 c.c. of strong sulphuric acid, and the acid transferred to a test-tube for the application of the test as described. Not more than two to three drops of the thiophene should be added after oxidation. The resting limb usually gives a negative reaction, the extract of the other yields a brilliant colour.

Excess of thiophene produces a deep yellow or brown colour with sulphuric acid, and some experience is of advantage in choosing the optimum amount to add in any case. Before using the test in practice it is well to experiment with a one per cent. alcoholic solution of lactic acid and the weak thiophene solution as described above.