XXXIII. CHEMICAL CHANGES IN MUSCLE.

PART I. METHODS OF ANALYSIS.

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SOME of the chemical changes responsible for activity in vertebrate skeletal muscle are fairly well known. On the other hand, in plain muscle, cardiac muscle and certain muscles of invertebrate animals, these changes are not clearly understood, possibly because the analytical methods which have been used for vertebrate skeletal muscle do not always give good results on application to other types of muscle. For this reason it was thought advisable to examine and if possible to improve the methods available for the estimation of carbohydrates and lactic acid in muscle.

Treatment of muscle preparatory to analysis.

In order to follow the changes involved in muscular activity, the tissue must be fixed in a known state and lactic acid, glycogen and other carbohydrates estimated. Fletcher and Hopkins [1907] found that special treatment was necessary to avoid formation of lactic acid before analysis. Alcohol was used at a temperature below 5° to precipitate protein and extract lactic acid. They minced the muscle in the cold alcohol and then ground it with sand until extraction was complete. Laquer [1914] and Schenck [1924] used dilute hydrochloric acid at $-10°$ and subsequently precipitated interfering substances with mercuric chloride. Eggleton and Eggleton [1927] used cold trichloroacetic acid solution to extract soluble phosphate from muscle; this seems to be a good method for use when acid soluble substances alone are to be estimated. It would, however, be unsuitable if glycogen were to be estimated on the same sample of muscle. Freezing of the tissue with liquid air or carbon dioxide snow, followed by grinding, should give even sampling and prevent post-mortem lactic acid production, but Meyerhof [1926] found that resting values for lactic acid obtained by this method were not lower than those obtained by the use of cold alcohol. Inositol can be estimated, after the method of Needham [1923], in the residue, after extracting the muscle with alcohol. The inositol is extracted with acetone and the residue from this extraction can be used for glycogen estimation. When lactic acid and glycogen are to be estimated in

the same sample of muscle, alcohol must be used to extract the lactic acid and precipitate the glycogen and protein. For complete precipitation of the glycogen the alcohol should be stronger than 60 %, while the protein is better precipitated with 80 % alcohol.

Method. The muscle is minced with alcohol cooled in a freezing mixture to about $-5°$ and then made up to a known definite volume, five times the volume of the muscle. After standing overnight, the alcohol is filtered off and a measured volume of the filtrate evaporated for the estimation of lactic acid and lower carbohydrates. When the alcohol has been removed by evaporation, the extract must be cleared of lipins and protein. If present in large amounts, hexoses must also be removed. The extract is therefore treated with ammonium sulphate solution or phosphotungstic acid. Lactic acid can be separated from hexoses in an ammonium sulphate solution by extracting the acid with ether as did Fletcher and Hopkins, or with amyl alcohol as described by Ohlson [1917]. If the lactic acid is to be estimated by oxidation, however, it is advisable to avoid extraction with ether as this usually contains aldehydes which would probably affect the estimation of lactic acid. Hexoses may be removed from a phosphotungstic acid solution by precipitation with copper sulphate and calcium hydroxide, as used by Van Slyke [1917]. In many cases the amount of hexoses present is too small to have any marked effect on the lactic acid estimation. The error due to hexoses is small, as is shown in Table I, but it is too irregular for the application of a correction.

Table I. *The effect of glucose on the estimation of lactic acid, by the oxidative method, using steam distillation.*

5·0 mg. glucose required	3·7	0·5	1·2	1·7	0·9 cc. *N*/1000 iodine
equivalent to	0·17	0·02	0·05	0·08	0·04 mg. lactic acid
10·0 mg. glucose required	4·3	5·0	1·1	3·0	2·1 cc. *N*/1000 iodine
equivalent to	0·19	0·22	0·05	0·13	0·09 mg. lactic acid

The evaporated alcoholic extract is therefore heated with 10–20 cc. of saturated ammonium sulphate solution for half-an-hour on the water-bath. The last traces of alcohol are then removed by heating with 2 cc. of benzene, when the low boiling alcohol-benzene-water tertiary mixture quickly boils off. The precipitated fat and protein are filtered off through asbestos and washed with saturated ammonium sulphate solution. The filtrate and washings are made up to a known volume with saturated ammonium sulphate solution and aliquot parts taken for estimation of lower carbohydrates and lactic acid. The solution will not keep indefinitely, so that estimations should be made within two days.

Estimation of total carbohydrates.

In examining the changes occurring in muscle it would be convenient to estimate the total carbohydrates present, if possible, in one estimation. This would provide an excellent check on the separate estimation of glycogen and lower carbohydrates, but no simple method of determination of the total carbohydrates could be found. By hydrolysis of the whole tissue with acid,

all carbohydrates would form simple hexoses, while the protein would largely form amino-acids. The amount of protein degradation products, however, is too great to allow of estimation of the hexoses even by a ferricyanide method. Such products of hydrolysis give the biuret reaction; they also give large and irregular glucose titration values. After ultra-filtration through collodion, which should remove the protein compounds, the result is very little better. Attempts to separate the glucose with alkaline copper sulphate are useless, owing to the combination of the copper with the amino-compounds present. The carbohydrates must be estimated therefore in two parts—soluble and insoluble in alcohol—or lower carbohydrates and glycogen respectively. This has the disadvantage that some dextrins may not be estimated.

Glycogen estimation.

Glycogen is estimated by methods which are variations on the procedure described by Pflüger [1905]. Improvements in the method for estimation of small amounts have been made by Evans [1926].

Method. The residue of tissue insoluble in alcohol is heated for 3 hours on a water-bath with 0·25 cc. of 60 % potash for each gram of original tissue. When cool, the hydrolysed material is diluted with water to three times the volume of potash taken. Alcohol is added till the total volume is seven times the original volume of potash. After standing overnight, a precipitate of glycogen forms and is filtered off on asbestos on a Gooch filter. It is well washed with 60 % alcohol and the washed precipitate is transferred with the asbestos into a beaker. 50 to 100 cc. of 2·2 % hydrochloric acid are added and the beaker and contents heated for 5 hours on a boiling water-bath. The solution is then filtered and the filtrate and washings made up to a known volume. Aliquot parts of this are taken for estimation of glucose, which is effected by oxidation with excess of alkaline ferricyanide and determination of the excess iodometrically [Hagedorn and Jensen, 1923; Issekutz and Both, 1927]. For the estimation of an amount below 5 mg., the glucose is heated for 15 minutes in a flask in a boiling water-bath with 20 cc. of a solution containing 0·33 % potassium ferricyanide and 2·12 % sodium carbonate. When cooled, 10 cc. of a solution containing 7·5 % zinc sulphate, 25 % sodium chloride and 5 % potassium iodide, followed by 10 cc. of 3 % acetic acid, are added. The liberated iodine is titrated with $N/100$ sodium thiosulphate. A blank is carried out on 20·0 cc. of the ferricyanide solution without glucose. The difference in titre between blank and determination is almost proportional to the amount of glucose present, as shown in Table II.

Table II. *Estimation of pure glucose.*

mg. glucose	Equivalent $Na_2S_2O_3$ in cc.			mg. glucose per cc. $Na_2S_2O_3$
1·25	3·90	3·95	3·90	0·315
2·50	8·00	7·90	7·95	0·32
3·75	12·0	12·1	12·0	0·31
5·00	16·1	16·1	16·0	0·31

The error of assuming the titre to be proportional to the glucose present; is thus negligible. The sodium thiosulphate is standardised by carrying out the estimation with a standard glucose solution preserved with benzoic acid. Holden [1926] showed this method of glucose estimation to be less sensitive to error from amino-acids than the copper reduction methods. It is also unaffected by ammonium sulphate solution, which makes it useful for estimating lower carbohydrates in such a solution. The estimation by this method, however, is impossible in the presence of moderate amounts of protein degradation products. After treating as in the glycogen estimation, muscle protein previously freed from glycogen reduces ferricyanide to some extent, as shown in Table III. Another error arises from the incomplete hydrolysis of glycogen by acid. Nerking [1901] obtained a 97 % yield of glucose after boiling for 3 to 5 hours. Harden and Young [1902] found that the rate of hydrolysis slowed down after 4 hours; they obtained maximum conversions of 96 % with rabbit glycogen after 8 hours, 97·6 % with yeast glycogen after 7 hours and 98·3 % with oyster glycogen after 7 hours at 100°. A sample of purified *Mytilus* glycogen, kindly supplied by Dr Slater, gave yields of about 93 % after 5 hours' hydrolysis, as shown in Table III. The average conversion of glycogen into glucose in presence of lobster or frog muscle is 92 %. It is necessary to allow for this in the calculation of results, which should be accurate to less than 5 %, using 10 g. of tissue containing at least 5 mg. of glycogen. It is convenient to state the results in terms of the hydrate $(C_6H_{12}O_6)_n$ which Slater [1924] has shown to exist.

Table III. *Glycogen estimations with purified* Mytilus *glycogen containing* 96·2 % $(C_6H_{12}O_6)_n$.

Amount taken	mg. glucose	% recovery and conversion	
5·00 mg. glycogen	4·43	92·7	
5·00 mg. glycogen	4·48	93·8	
2·0 g. dried lobster muscle	0·95	—	
2·0 g. dried lobster muscle	0·74	—	
2·0 g. dried lobster muscle and 5·00 mg. glycogen	5·28	92·3	Allowing for muscle blank
2·0 g. dried lobster muscle and 5·00 mg. glycogen	5·13	88·0	Allowing for muscle blank
4·72 g. frog muscle	16·6	—	
4·72 g. frog muscle and 37·0 mg. glycogen	50·8	92·6	
6·43 g. frog muscle	25·4	—	
6·43 g. frog muscle and 37·0 mg. glycogen	60·3	94·0	

Estimation of lower carbohydrates.

The so-called lower or soluble carbohydrates of muscle are probably intermediates in the conversion of glycogen into lactic acid. Although they do not change apparently to any great extent in muscular work, yet they have some significance. Usually quick moving muscles contain more than slow muscles; the muscles of summer frogs contain more than those of hibernating frogs.

Parnas and Wagner [1914] estimated the carbohydrate extracted with 60 % alcohol and boiling water. These two extracts were combined; they contained all the soluble carbohydrate and a little of the glycogen. Mercuric acetate and caustic soda were added to precipitate protein. Excess mercury was removed with hydrogen sulphide, which in turn was removed by blowing air through the solution. Hydrochloric acid was added to make it up to 2·0 % hydrochloric acid and it was heated for 2 hours on the water-bath, after which the glucose was estimated by the Bertrand method. Meyerhof [1920, 2] simplified this by extracting the muscle with 60 % alcohol only and estimating glycogen in the residue. The same methods of protein precipitation and hydrolysis were used. By use of the modified Hagedorn and Jensen method of glucose estimation, which is not influenced by ammonium salts, it is possible to estimate the lower carbohydrates in the same extract as is used for estimation of lactic acid.

A measured volume of the muscle extract which has been treated with ammonium sulphate and benzene, as previously described, is made up to 2 % hydrochloric acid. This is heated for 2 hours on the water-bath, neutralised, and the glucose present is estimated by heating with excess ferricyanide solution. In some instances, such as lobster muscle the heating with acid can be omitted without affecting the results; this is not so with frog muscle.

Estimation of lactic acid.

Although lactic acid can be estimated gravimetrically, colorimetrically, volumetrically and by purely physical methods, no method has the accuracy and reliability that is desired. Long [1924] and others have indicated that the only method suitable for the estimation of small amounts of lactic acid is the oxidative process. In this process the acid is oxidised to acetaldehyde which is determined iodometrically. If more than 50 mg. is to be estimated, isolation as zinc lactate [Fletcher and Hopkins, 1907] is usually adopted. Phelps and Palmer [1917] isolated and weighed quinine and guanidine lactates, which are insoluble in carbon tetrachloride. This method is rather tedious and appears to be little used. The main objection to the oxidative method is that it is not very specific and should only be used on pure solutions of lactic acid. On the other hand, the method of Fletcher and Hopkins is not free from this defect; it is, for example, interfered with by succinic acid and by urea, while the oxidative method is not. Moyle [1924] has shown that rabbit muscle may contain 0·1 % of succinic acid and this would cause lactic acid values determined by the zinc lactate method to be too high. Succinic acid is soluble in ether and zinc succinate is soluble in water, though it contains 44·1 % zinc oxide, as compared with 33·4 % for zinc lactate. It is difficult to distinguish between crystals of pure zinc lactate and of zinc lactate contaminated with zinc succinate, from the appearance of the crystals. The resting value of lactic acid, 0·02 % obtained by Fletcher and Hopkins is probably too high for this reason. Meyerhof [1920, 1] using the oxidative method of estimation obtained a lower resting value of 0·01 %. Fletcher and Hopkins do not give any analyses of

zinc content of zinc lactate obtained from resting muscle, probably on account of the small amount of zinc lactate obtained in those experiments. The lower figure obtained by the oxidative method is almost certainly nearer to the true resting value than that originally obtained. The determinations of lactic acid in fatigued muscle and in muscle in rigor will be less affected than the determinations of the small amount of lactic acid found in resting muscle. On account of the unspecific nature of the methods of estimation, lactic acid should be identified qualitatively before any quantitative method is applied. It is then advisable to estimate the acid by at least two methods and to compare the results obtained.

Long [1924] has examined the early work on the oxidative method. He adopted with slight modification the method published by Clausen [1922]. The oxidation of the lactic acid, by sulphuric acid or potassium permanganate, gave acetaldehyde which was collected in sodium bisulphite solution. After oxidising the excess bisulphite with iodine, the aldehyde-bisulphite compound was decomposed with sodium bicarbonate and the liberated bisulphite titrated with standard iodine. This accurate method of estimating acetaldehyde, due to Clausen, made the estimation of lactic acid much more reliable than it had ever been before. Since Clausen's paper, the only improvements have resulted in the increase of the yield from 85 to 98 % and reduction of the time necessary to make the estimation. Long obtained better oxidation with potassium permanganate than with sulphuric acid. Brehme and Brahdy [1926] used sulphuric acid. They heated the lactic acid with sulphuric acid, for 1 to 1½ hours, at 140° in an all glass apparatus in a metal-bath. They obtained yields of 92 to 95 %, but their process was very lengthy. Friedeman, Cotonio and Shaffer [1927] describe a modification of the Clausen process, by means of which estimations take only 15 minutes and a 96 % yield is obtained. The improvements lie in the apparatus for carrying out the oxidation and in the use of manganese sulphate as catalyst. This addition of manganese sulphate may be necessary with pure lactic acid, as without this there is often a considerable induction period before oxidation begins; manganese sulphate is then formed and the action proceeds rapidly. In estimating lactic acid from any biological source there is practically no induction period, as traces of organic impurity are first oxidised, with simultaneous production of manganese sulphate.

In the above methods with the exception of those of Meyerhof [1920, 1] and Long [1924], an air current is used to carry the acetaldehyde from the oxidising vessel to the absorption vessels. Meyerhof and Long used direct distillation. The air current, which is obtained by sucking air through the apparatus, will contain the variable impurities of the laboratory air. Alcohol or acetone vapour in the air would materially affect the results. In order to avoid this source of error, Brehme and Brahdy found it necessary to use outside air, led by a tube from outside, to the apparatus; this however is cumbersome. In addition to this, the Consortium für elektrochemische Industrie [1913] state

that manganese sulphate is an excellent catalyst for the aerobic oxidation of acetaldehyde to acetic acid. It would therefore seem advisable to avoid the presence of air, if possible, in the estimation.

Some experiments were made using direct distillation in the determination, after the method of Meyerhof, but this did not give good results, as can be seen from Table IV. This is probably due to over-oxidation of the acetaldehyde to acetic acid, owing to insufficient agitation allowing acetaldehyde to remain too long in the presence of the permanganate. Steam distillation should avoid this and on trial gave better results. At first this was performed in a Claisen flask fitted with rubber stoppers. Later the apparatus[1] (Fig. 1) was designed for

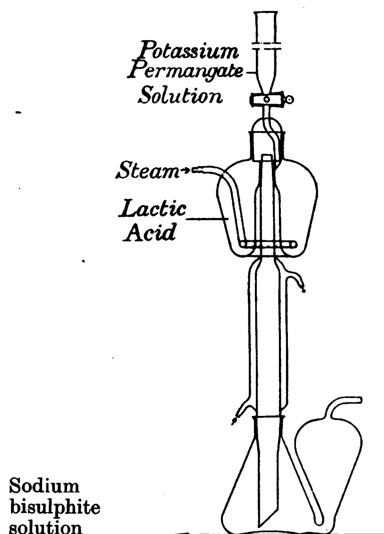

Fig. 1. Apparatus for the estimation of lactic acid.

the process; it gives slightly better results and is very convenient in working. This method gives the same results as that of Friedeman, Cotonio and Shaffer in a laboratory free from organic vapours. The method of steam distillation is independent of the air and generally gives more regular conditions for oxidation. Results obtained by various methods and the recovery of lactic acid from muscle are given in Table IV. Except for the use of steam and the standardisation of the iodine solution with a lactic acid solution, the procedure in the estimation is similar to that of Clausen.

Material containing between 0·5 and 5 mg. of lactic acid is placed in the upper vessel, with 10 cc. of 10 % sulphuric acid. 20 cc. of $N/20$ sodium bisulphite are placed in the Volhard flask. The condenser is well cooled with a fast stream of water and a vigorous current of steam is led through the solution of lactic acid. $N/200$ potassium permanganate is slowly dropped in from the funnel, so as to maintain a pink colour. The acetaldehyde produced by oxidation is immediately carried over by the steam, the distillate collects in the

[1] Made by the Scientific Glass Blowing Co., Manchester.

Table IV. *Estimation of* 1·00 *mg. lactic acid—from* 1·07 *mg. pure lithium lactate.*

Process	cc. $N/1000$ iodine	mg. lactic acid	% yield average
Clausen	20·8	0·94	
	18·2	0·83	
	15·9	0·72	85·2
	21·8	0·98	
	20·1	0·90	
	17·3	0·78	
Friedeman, Cotonio and Shaffer	21·2	0·95	
	20·9	0·94	
	20·1	0·90	93·6
	19·6	0·88	
	19·7	0·89	
	22·8	1·04	
Direct distillation	15·0	0·68	
	17·3	0·78	
	19·6	0·89	81·7
	18·5	0·83	
	19·1	0·88	
	18·4	0·84	
Steam distillation	20·9	0·94	
	19·8	0·89	
	20·6	0·93	92·1
	21·5	0·97	
	19·7	0·89	
	20·4	0·92	

Recovery of lactic acid from muscle.

Muscle taken	mg. lactic acid	Recovery %
4·73 g. frog muscle	6·0	
4·73 g. frog muscle and 5·0 mg. lactic acid	10·8	96
6·43 g. frog muscle	20·2	
6·43 g. frog muscle and 5·0 mg. lactic acid	25·4	104
3·97 g. frog muscle	6·9	
3·97 g. frog muscle and 5·0 mg. lactic acid	11·5	92
Average	—	97

bisulphite solution. In a few minutes the pink colour becomes permanent without further addition of permanganate and after another 5 minutes' distillation the steam is shut off and the apparatus dismantled. The condenser is washed down into the bisulphite in the Volhard flask. Starch is added to the bisulphite and the excess is titrated with $N/20$ iodine, to a pale blue colour. Saturated sodium bicarbonate solution is added to decompose the aldehyde-bisulphite compound and the bisulphite so liberated is titrated with $N/1000$ iodine solution. Each cc. of iodine should represent 0·045 mg. of lactic acid. The iodine is best standardised by carrying out the determination on a solution of pure lithium lactate. At least one standardisation should be made with each batch of estimations.

SUMMARY.

The methods of estimation of glycogen, lower carbohydrates and lactic acid have been examined. An improved method for the determination of lactic acid using steam distillation combined with oxidation is described.

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