LXXVI. THE TOTAL CARBOHYDRATE CONTENT OF ISOLATED FROG MUSCLE.

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Introduction.

There are two methods in general use for the determination of the total carbohydrate content of muscle. In one [Parnas and Wagner, 1914; Parnas, 1921] alcoholic and aqueous extracts of the tissue are made, glycogen being estimated in the residue and soluble carbohydrate in the extracts. In the other [Loewi, 1918; Lesser, 1920; Meyerhof, Lohmann and Meier, 1925] the whole muscle is hydrolysed in dilute acid and the glucose formed is estimated by its reducing power. Both are liable to error since it is difficult to remove non-carbohydrate reducing substances completely. The second method gives higher values than the first. Lesser considers that this is due to incomplete extraction of a dextrin. Parnas and Meyerhof *et al.* attributed it rather to the inclusion of non-carbohydrate reducing substances split off from protein during hydrolysis and not precipitated by mercuric acetate. Bissinger [1926] introduced an adaptation of the Van Slyke [1917] method in which sugar is recovered quantitatively from a copper sulphate-lime precipitate. He and Lesser [1926] found that when this technique was used for the estimation of total carbohydrate in the mouse the extraction and hydrolysis methods gave the same results. The same procedure has been applied to the estimation of alcohol-soluble carbohydrate in nerve by Holmes and Gerard [1929] and in muscle by Anderson and Macleod [1930]. Ochoa [1930] in estimating the total carbohydrate content of frog muscle later dispensed with the separation of sugar by copper-lime precipitation and relied on the complete removal of protein and non-sugar reducing substances by West, Scharles and Peterson's [1929] method. The results of the present work show that although neutral mercuric sulphate does give lower results than mercuric acetate or chloride, copper-lime precipitation will still further diminish the reducing value of the filtrates.

The present investigation is an attempt to apply a modification of Bissinger's method to the estimation of the total carbohydrate of frog muscle and to compare the results with the values found for the glycogen and alcohol-soluble carbohydrate in resting muscles, in muscles incubated in phosphate and bicarbonate buffers and in muscles poisoned with sodium monoiodoacetate.

There is some evidence that a carbohydrate intermediate in complexity between glycogen and glucose may exist in muscle. Laquer [1914, 1921] found that in a phosphate buffer solution the total yield of lactic acid exceeds the initial content of glycogen and can usually be accounted for by the lactacidogen content. Simpson and Macleod [1927] found that the breakdown of glycogen in a muscle frozen in liquid air and allowed to thaw was much more rapid than the formation of lactic acid. Anderson and Macleod [1930] have shown that on keeping after death the glycogen content of mammalian muscle remains steady, or may increase, whilst the lactic acid content increases. Lohmann [1926] has found a trisaccharide among the products of action of muscle extract on glycogen. This trisaccharide was not fermentable by yeast and did not give rise to lactic acid. More recently Barbour [1930] has isolated a trisaccharide formed as the sole product of the hydrolysis of glycogen by muscle extract at p_H 6·3. If such a trisaccharide does exist in muscle it might well escape estimation as an alcohol-soluble carbohydrate and would almost certainly be destroyed, in part at least, by the drastic alkaline hydrolysis required for the glycogen estimation.

The method employed in this work should include such a compound, whilst non-carbohydrate reducing substances should be largely, if not entirely, excluded. It was hoped that the solutions made from the copper-lime precipitates would be free from nitrogen, but this was not found to be the case. It is however unlikely that the nitrogen-containing substances are responsible for more than a small part of the reducing value, since after incubation in a phosphate buffer the carbohydrate content of a muscle falls to about 10 % of the initial value, whilst the nitrogen content of the copper-lime precipitates does not alter appreciably, whence it may be assumed that non-carbohydrate reducing substances have not altered in amount. The error from this cause, if the whole of the final reducing value is due to nitrogen-containing substances, will not be above 10 %. Another source of error, acting in the opposite sense, is the incomplete hydrolysis of carbohydrate. Glycogen is hydrolysed under optimum conditions to the extent of 94 % and other carbohydrate, should any be present, is unlikely to give values as high as 100 %; all values in this paper are given as glucose found, without arbitrary correction. In the time allowed all the hexosephosphate will not be hydrolysed, but as the amount present in muscle is small compared with the total carbohydrate, the error from this cause will be insignificant [Meyerhof, 1930]. In some cases, such as in muscles poisoned with monoiodoacetate, where exceptionally large amounts of hexosephosphate are known to be present, special allowance must be made.

The chief difference between the results found in this work and those of previous workers is the much greater difference between the total carbohydrate content and the sum of the glycogen and alcohol-soluble carbohydrate. Glycogen values vary considerably and it is difficult to compare results found by different workers, but the alcohol-soluble carbohydrate values are much lower.

Meyerhof [1920] found as an average value for resting frog muscle, 194 mg./100 g., whereas the average resting value for the present results is 40 mg./100 g. This may be due both to the use of copper-lime precipitation and to the use of absolute alcohol as an extractant in place of 60 % alcohol, which must extract some glycogen as well as other carbohydrate and so make the results for alcohol-soluble carbohydrate too high. The values for total carbohydrate are decidedly higher than those found by other workers, although lower values would have been expected since sugar was separated by copper-lime precipitation and protein was removed after 30 minutes' hydrolysis, the total period of hydrolysis (1·5 hours) being much shorter than that usually allowed. In one experiment the values for total carbohydrate estimated by several variations of the method were compared. When hydrolysis was discontinued at the end of 30 minutes, protein removed by Schenk's method, and the hydrolysis continued for another hour, the total carbohydrate content of the muscle was 0·973 %, if sugar were separated by copper-lime precipitation, but 1·219 % if calculated from the reducing value of the solution before copper-lime precipitation. If protein were removed by West, Scharles and Peterson's method the values were 0·840 % and 1·022 % respectively, the discrepancy being less after copper-lime precipitation. When hydrolysis was continued for 3 hours before removal of protein by Schenk's method the value was 1·071 % after copper-lime precipitation and 1·347 % before; if protein were removed by West, Scharles and Peterson's method the values were 1·202 % and 1·044 % respectively, higher in all cases when protein was not removed before the completion of hydrolysis. The Schenk method was used throughout this work, as when most of the experiments were carried out the superiority of the West, Scharles and Peterson method was not appreciated; the results are therefore probably too high by 10 % to 15 %, a figure comparable with that estimated for the error due to non-carbohydrate reducing substances.

Methods.

Preparation of material. In all the experiments recorded in this paper single isolated frog muscles were used. When working with single muscles it is essential that all comparisons should be made either in simultaneous determinations on the same muscle, or on pairs of corresponding muscles from the two legs of the same frog. The glycogen and total carbohydrate contents of resting muscles from the same frog vary very much, and a totally wrong idea of the changes taking place may result from comparing different muscles or even by taking average figures. The glycogen and total carbohydrate contents of corresponding muscles of a pair from opposite legs of the same frog agree reasonably well (Table VI), and a much truer picture of changes taking place results from confining all the estimations to one pair of muscles.

The frogs were in all cases the large Hungarian variety. They were killed by a cut across the base of the skull, followed immediately by section of the

spine just above the pelvic girdle. The muscles were dissected, weighed on a torsion balance and dropped into Ringer solution at p_H 7·3, containing 0·012 % sodium dihydrogen phosphate and 0·057 % disodium hydrogen phosphate, but no bicarbonate. Oxygen was slowly bubbled through the solution. The muscles were left in the solution for at least 1 hour and usually longer before further treatment. Dulière and Horton [1929] have shown that muscles treated in this way are not subject to the onset of inexcitability, and that their resting phosphagen content is high and inorganic phosphate low. The lactic acid content is also low, on an average 20 mg./100 g., as is shown by the results in this paper. These figures indicate that the muscles are in a resting condition and have recovered from any stimulation or injury inflicted during killing and dissection. This method is suitable only for thin muscles, such as sartorii or semi-membranosi, and cannot be used for thick muscles such as gastrocnemii, as the oxygenated solution will not diffuse into them; where such muscles were used they were treated immediately after dissection without any soaking in Ringer solution.

Estimation of total carbohydrate: extraction and hydrolysis. 2·2 % hydrochloric acid has been found most suitable for the hydrolysis of glycogen and, since glycogen forms the greater part of the carbohydrate of muscle, this strength of acid was used for the hydrolysis of the whole muscle. In the first experiments the muscles were thrown into boiling acid, but later they were ground in ice-cold acid, and extract and residue were hydrolysed together in a boiling water-bath, as changes due to killing are better avoided in this way. The completeness of the extraction of carbohydrate was tested by determining the amount of glycogen in the residue after varying the times of hydrolysis. Protein was precipitated by Schenk's method, the solid residue removed by centrifuging, and glycogen estimated in it. It was found that after 15 minutes most of the glycogen was removed, and that heating for longer than 30 minutes did not remove appreciably greater quantities, so the latter period was adopted as standard. There was always some glycogen left in the residue, and it did not change in amount during the various treatments applied to the muscle.

Hydrolysis for 30 minutes will not hydrolyse all the carbohydrate present in the muscle completely, but it is inadvisable to heat longer, owing to the danger of further protein breakdown. The time necessary for maximum hydrolysis, after removal of protein, was investigated by hydrolysing the protein-free extract, obtained from a number of muscles, in a boiling water-bath and at intervals removing portions for sugar estimation, after precipitation with copper-lime. The results are plotted in Fig. 1. In one experiment (curve b_2), the nitrogen values of the copper-lime precipitates were determined, and it will be seen that they fall off slowly, whilst the sugar values rise sharply to a peak and then fall away. This may be explained by supposing that a part of the reducing value is due to some nitrogen-containing substance which is slowly being destroyed, as is indicated by the decreasing amounts of nitrogen associated with the copper-lime precipitates. In this experiment (curve b_1), protein was

removed by trichloroacetic acid, which may make the sugar values low; although most of the acid is destroyed during the hydrolysis, sufficient is left to interfere with the sugar estimation, and for this reason the method was not used in later work. One hour was adopted as the standard period for the second hydrolysis. The curves plotted in Fig. 1, which are typical of several obtained, show that hydrolysis was complete in this time.

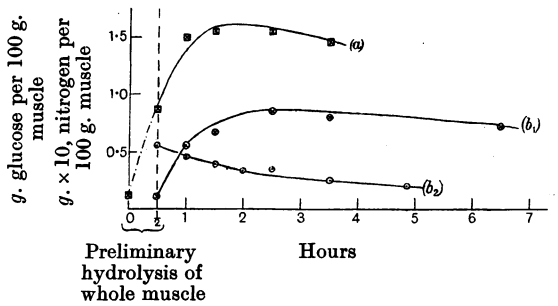

Fig. 1. Sugar formed on hydrolysis of hydrochloric acid extract of muscle.
Exp. (*a*). Sugar formed: protein removed by Schenk's method.
Exp. (*b*) 1. „ „ „ „ „ trichloroacetic acid.
(*b*) 2. Nitrogen content of copper-lime precipitate.

Estimation of sugar in hydrolysed solutions. Sugar was estimated in the solutions after hydrolysis by an adaptation of Bissinger's [1926] method. A further investigation of the conditions of precipitation and recovery of sugar was undertaken, using pure glucose solutions.

Freedom of precipitate from non-carbohydrate reducing substances. Holmes and Gerard [1929] state that creatinine is not precipitated by the copper-lime reagent. In order to test this the effect of treating a solution containing 25 mg./100 cc. of creatinine with copper sulphate and lime was determined. After removing the precipitate the creatinine content was reduced to 80 % of the original. That this was partly due to adsorption or occlusion on the precipitate was shown by dissolving the latter in normal hydrochloric acid and reprecipitating the copper compound with an equal volume of normal sodium hydroxide. By using two precipitations in this way it was possible to recover 95 % of creatinine in the mother-liquor.

In an experiment using a solution formed by the hydrolysis of muscle the nitrogen content of the first copper precipitate was 150 mg./100 g. of muscle. This solution gave a biuret reaction. Solution of the precipitate in normal hydrochloric acid and reprecipitation with normal sodium hydroxide reduced the value to 40 mg./100 g. muscle. This second solution gave no biuret reaction. From these results it was decided to use this method of two precipitations throughout.

Optimum conditions for precipitation of sugar. The solutions were allowed to stand for 30 minutes [Van Slyke, 1917] after addition of copper sulphate and lime, and again for 30 minutes after the second precipitation with sodium

hydroxide. If the alkaline solutions are allowed to stand too long some of the sugar will be destroyed (Table I), in fact some is destroyed in any case, for higher values are obtained when hydrochloric acid is added before copper sulphate and lime, and precipitation is not allowed to take place, the rest of the determination being carried out in the same way. The loss is a constant percentage of the whole and can be corrected.

Table I.

Effect of time of standing on recovery of sugar from copper-lime precipitates. An equal time was allowed after each precipitation.

Time in minutes	Sugar recovered, mg.
25	5·88
30	6·16
35	6·06
40	5·94
45	5·78

The proportion of sugar recovered was constant at any one temperature, and did not vary much with temperature so long as the other conditions remained unaltered (Table II). The solutions were allowed to stand at laboratory temperature, and the factor required was redetermined from time to time, especially if there was known to be any large variation in temperature.

Table II.

Effect of variation of temperature during standing on recovery of sugar from copper-lime precipitates.

Temperature	Sugar recovered, mg.
0°	8·9
18°	8·5
24°	7·9

Proportions of copper sulphate and lime required for complete precipitation of sugar. The amounts both of copper sulphate and of lime used by different workers vary very much. The minimum amounts required were determined for concentrations of sugar up to 0·1 % in a volume of 25 cc. The sugar remaining in solution after addition of increasing amounts of a 10 % solution of crystalline copper sulphate and a 10 % suspension of calcium hydroxide was determined, the precipitated copper compound and excess of lime being removed by centrifuging. The amount of calcium hydroxide was adjusted so that there should always be an excess present after allowing for the formation of calcium sulphate and saturation of the solution with calcium hydroxide. The amount of sugar remaining in solution decreased with increasing amounts of copper sulphate up to 2 cc., but beyond this there was no improvement, the solution after centrifuging had always about 1 % of the original reducing value. Using 2 cc. of 10 % copper sulphate, 0·94 cc. of a 10 % suspension of calcium hydroxide is required for formation of calcium sulphate and for saturation of 25 cc. of solution with calcium hydroxide; in order that there should always be a considerable excess present, 2 cc. were added.

Recovery and estimation of sugar. If a method could be found in which the copper used in precipitating the sugar could be used as the oxidising agent it would simplify the procedure very much. Folin's method [1926] seemed the most likely to be satisfactory, especially as Eggleton (private communication) has shown that it is possible to estimate the molybdenum blue, formed on addition of the acid reagent, by titration with potassium permanganate, instead of colorimetrically. Eggleton found that 1 mg. of glucose required 8·20 cc. of 0·004 *N* permanganate. This method was found very satisfactory when a modification of the alkaline tartrate reagent was made. The large excess of copper did not affect the reaction, and the final titration was sharp enough to make the accuracy of the method sufficient for work on isolated muscles.

The copper precipitate did not dissolve in the tartrate reagent, made up without copper sulphate, owing to the calcium hydroxide still present, but dissolved easily in a slight excess of normal hydrochloric acid, and an aliquot part of this solution was used for sugar estimation. It was not possible to use more than 2 cc. per estimation as Folin's special tubes were used, and as the volume of the solution after dissolving the copper precipitate in *N* hydrochloric acid was not far short of 10 cc., only about $\frac{1}{5}$th could be taken for each determination. The tartrate reagent was made up without copper sulphate and with four times the amount of tartrate used by Folin, since $\frac{1}{5}$th of the dissolved copper precipitate contained four times as much copper as 2 cc. of Folin's reagent. The amount of sodium carbonate was increased from 0·7 % to 7·5 % to allow for neutralisation of hydrochloric acid, the formation of calcium carbonate and for the absorption of the additional carbon dioxide liberated. The amount of sodium bicarbonate was not altered from the 2·0 % in Folin's solution. The ratio of carbonate and bicarbonate formed in this solution was found adequate for buffering changes in p_H due to variation in excess of hydrochloric acid. The accuracy of the estimation was not affected by small excess either of copper or of tartrate, nor did the calcium carbonate precipitated when the two solutions are mixed interfere with the reaction as long as the mixing was efficient.

The final composition of the alkaline tartrate solution using $\frac{1}{5}$th of the dissolved copper precipitate for each determination was as follows:

Sodium potassium tartrate	6 %
Sodium carbonate	7·5 %
Sodium bicarbonate	2 %.

The acid solution was that recommended by Folin:

Sodium molybdate	150 g.	$Na_2MoO_4.2H_2O$ per litre
Phosphoric acid	225 cc.	85 % acid ,,
Sulphuric acid	150 cc.	25 % ,, ,,
Acetic acid	75 cc.	99 % ,, ,,

Summary of procedure for estimation of total carbohydrate. The muscle, weighing usually 0·15–0·35 g., was ground with sand and 10–15 cc. of ice-

cold 2·2 % hydrochloric acid, and the mixture was hydrolysed for 30 minutes in a boiling water-bath. After cooling an excess of solid mercuric chloride was added, the solution was made up to 25 cc. and allowed to stand. It was then filtered, moist hydrogen sulphide was passed through the filtrate, the mercuric sulphide was filtered off, and the filtrate thoroughly aerated to remove hydrogen sulphide. 15 cc. were hydrolysed for 30 minutes, and after cooling the solution was washed into a centrifuge tube graduated at 25 cc. and at 10 cc.; 2 cc. of a 10 % solution of crystalline copper sulphate were added, and the solution was treated with 40 % sodium hydroxide until the copper was just precipitated. 2 cc. of a 10 % suspension of calcium hydroxide were then added, the solution was well stirred, and water was added to 25 cc. The tube was left to stand for 30 minutes and centrifuged for 3 minutes. The supernatant liquid was poured off and used for lactic acid estimation. The precipitate was dissolved in 5 cc. of *N* hydrochloric acid, 5 cc. of *N* sodium hydroxide were added, the solution was well stirred, and the volume brought back to 25 cc. After 30 minutes the precipitate was separated by centrifuging and dissolved in 3 cc. of hydrochloric acid. Less acid was needed for the second solution as the second precipitate contained less calcium hydroxide. The volume was made up to 10 cc., and 2 cc. of this solution were added to 2 cc. of the tartrate solution in a Folin tube. The sugar solution should be run into the bottom of the tartrate solution, otherwise the calcium carbonate formed greatly impedes the mixing which is absolutely essential for satisfactory results. The tube was placed in a boiling water-bath for 8 minutes, cooled for at least 1 minute in cold water, and the acid reagent was added. When all the cuprous oxide and the calcium carbonate were dissolved, the solution was washed into a large boiling-tube and titrated with 0·004 *N* potassium permanganate. The solution may safely be left for about 30 minutes after addition of the acid reagent, but should be titrated immediately after it has been washed into the tubes for titration. The end-point is not difficult to see after some practice and in a good light, but the titration must be rapid as the blue colour reappears on standing.

When the estimation was carried out in this way, the relationship between the amount of potassium permanganate required and the amount of glucose in solution was linear. The results of estimating pure glucose by this method are plotted in Fig. 2. Curve (*a*) was obtained using only the precipitation and sugar recovery part of the method, curve (*b*) by including the Schenk protein removal technique, starting with addition of hydrochloric acid and mercuric chloride. The mean value for the factor for converting cc. of potassium permanganate into mg. of glucose is 0·1 when the full method is used, but 0·094 when the Schenk procedure is omitted (temperature 16°). Possibly some glucose is lost during aeration of the solution in removing hydrogen sulphide. The recovery of added glucose in a determination of total carbohydrate in muscle was measured and the results calculated, using curve (*b*) Fig. 2, are shown in Table III. The accuracy of the sugar recovery is not nearly as great as in the determinations on pure glucose solutions. The inaccuracies are

probably due to conditions of extraction and hydrolysis of carbohydrate and not to errors in the sugar method.

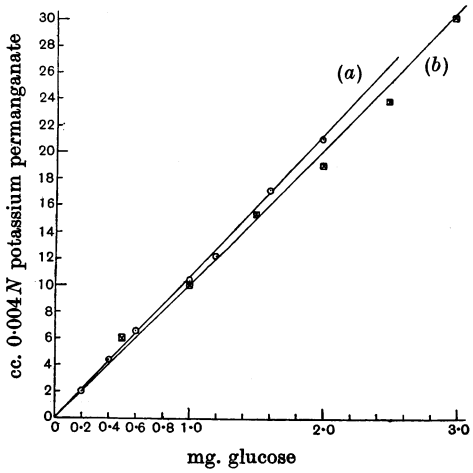

Fig. 2. Titration of glucose recovered from copper-lime precipitate against 0·004*N* potassium permanganate.

(*a*) Recovery from copper precipitates. (*b*) Including Schenk protein precipitation.

Table III.

3 g. muscle extracted with 100 cc. 2·2 % hydrochloric acid, after 30 minutes' hydrolysis extract filtered and a solution containing 1, 2 and 3 mg. sugar added to portions of 10 cc. Total carbohydrate estimated in each portion.

Sugar added mg.	Carbohydrate found mg. glucose	Glucose recovered mg.	% recovery
0	1·41		
	1·45 Mean 1·43	—	—
1	2·62		
	2·39 „ 2·51	1·08	108
2	3·40		
	3·26 „ 3·33	1·90	95
3	4·53		
	4·47 „ 4·50	3·07	102

If the reducing value finally measured is due only to glucose, it should be the same when measured by different methods. In order to check this, in an experiment on muscle the reducing value of the second copper precipitate, dissolved in hydrochloric acid, was estimated by Hagedorn and Jensen's method, after removal of copper and calcium [Holmes and Gerard, 1929], as well as by this adaptation of Folin's method. When the results were corrected by a factor found by carrying out the estimations on pure glucose solutions, and necessitated by losses occurring during the copper precipitation, they agreed very well with those found by the Folin method.

Estimation of glycogen. It was necessary to estimate both glycogen and lactic acid in the same muscle, so the muscles were crushed in 10–15 cc. of ice-cold absolute alcohol in well-cooled mortars and washed into cooled centri-

fuge tubes. The tubes were left overnight in the refrigerator to allow complete extraction of lactic acid. The alcoholic extract was separated by centrifuging, the residue was washed twice with absolute alcohol, and glycogen was estimated in it as described in a previous paper [Kerly 1930], the correction for solubility in aqueous alcohol being applied. Sugar was estimated after hydrolysis by the original Hagedorn and Jensen method, by Hanes's [1929] modification or by the Shaffer-Hartmann method. Loss of glycogen is insignificant except perhaps for muscles containing very little glycogen, since the volume of alcohol is very large in relation to the volume of the muscle, and the water content of the muscle has an inappreciable effect on the concentration of the alcohol. Anderson and Macleod [1930] find that for mammalian muscle this procedure is not satisfactory for glycogen estimation, but the method is not quite comparable for the two types of muscle. In the case of mammalian muscle the muscles were cut off the bones and finely minced under alcohol, a process that must take an appreciable time, whilst the frog muscles were allowed to recover after dissection and then dropped into the ice-cold alcohol; within a few seconds at most they were completely disintegrated.

Estimation of soluble carbohydrate. Alcohol-soluble carbohydrate was estimated in the alcoholic extracts of those muscles used for estimation of glycogen and lactic acid. In some cases determinations were made of the initial reducing value only after copper-lime precipitation, in others of the reducing values both initially and after hydrolysis. For convenience the former is described arbitrarily as the alcohol-soluble sugar, the latter as alcohol-soluble carbohydrate. The alcoholic extract and washings were evaporated nearly to dryness, water was added and a few drops of benzene, and heating was continued to remove the last traces of alcohol [Boyland, 1928]; the solution was then made up to 25 cc. 15 cc. were used for determination of sugar and 10 cc. for carbohydrate. Copper sulphate and lime were added and sugar was estimated as described above. For alcohol-soluble carbohydrate hydrochloric acid was added to make 2·2 % and the solution was hydrolysed for 3 hours in a boiling water-bath, sugar being estimated in the hydrolysate. The insignificant amounts of fat and protein extracted by the absolute alcohol were not removed. The conditions chosen were those most suitable for hydrolysis of glycogen, but the time is quite inadequate for hydrolysis of hexosemonophosphate which should be included both in the determination of sugar and of carbohydrate, since it is carried down by the copper-lime precipitate [Embden and Zimmermann, 1924].

The values found for both alcohol-soluble sugar and carbohydrate were much lower than those usually quoted, probably owing to the failure of most workers to remove reducing substances other than sugar. The average values found for resting muscle were 20 mg./100 g. for sugar (of the same order as those found by Anderson and Macleod for mammalian muscle, using a copper precipitation method), and 40 mg./100 g. for carbohydrate, the variation from the mean being large in both cases. The value for alcohol-soluble sugar may be compared with that found for hexosemonophosphate by Embden and Jost

[1928] and by Eggleton and Eggleton [1929], who found a value of 5–8 mg./100 g. P corresponding with 30–40 mg./100 g. hexose. If it be assumed that hexosemonophosphate gives a reducing value corresponding to 50 % of its hexose content [Meyerhof, 1930], then 20 mg. of alcohol-soluble sugar represents 40 mg. of sugar as hexosemonophosphate. The agreement suggests that the alcohol-soluble sugar of muscle is hexosemonophosphate, but as absolute alcohol may not extract all the lower carbohydrate [Holmes, 1929] there may be other reducing sugars present. Neither Embden and Jost nor Eggleton and Eggleton used absolute alcohol as an extractant, so their results are not low for this reason. The reducing value of the alcoholic extracts after hydrolysis is approximately doubled, but hexosemonophosphate is only hydrolysed to the extent of 20 % during 3 hours in dilute acid [Meyerhof, 1930] so that there must be other carbohydrate constituents present in the alcoholic extract of muscle.

The accuracy of individual values for both alcohol-soluble sugar and carbohydrate is not high, but the values taken as a whole indicate approximately the amount present, and whether any gross changes are taking place during activity of the muscle.

Estimation of lactic acid. The method used depended on whether the muscles were used for determination of total carbohydrate or for glycogen and alcohol-soluble carbohydrate.

In a hydrochloric acid extract of muscle. When estimating total carbohydrate the lactic acid was extracted during the preliminary 30 minutes' hydrolysis. Experiments with a solution of zinc lactate in hydrochloric acid showed that none of the lactic acid was destroyed either during prolonged boiling, as long as efficient condensers were used, or during aeration to remove hydrogen sulphide. A measured volume of the supernatant fluid poured off after removal of the first copper-lime precipitate was taken for estimation of lactic acid. A modification of Friedemann, Cotonio and Shaffer's [1927] method was used, in which manganese sulphate was used to catalyse the reaction, but the aldehyde formed was distilled over in a current of steam instead of air. This method gave a mean recovery of 98 % of lactic acid from zinc lactate solutions. The recovery of lactate added to a muscle extract after hydrolysis is shown in Table IV, the results being calculated by means of the recovery factor found for pure zinc lactate solution.

Table IV.

Recovery of lactic acid from zinc lactate solution added to a hydrochloric acid extract of muscle after the first hydrolysis and before removal of protein.

Zinc lactate added mg. lactic acid	Lactic acid found, mg.	Lactic acid recovered mg.	% recovery
0	0·098	—	—
0·422	0·525	0·427	101
0·633	0·741	0·643	101
0	0·114	—	—
0·379	0·504	0·390	103
1·140	1·314	1·200	106

In an alcoholic extract of muscle. When estimating glycogen lactic acid was extracted by the alcohol, the pulverised muscles being left overnight in the refrigerator to ensure complete extraction. Experiment showed that washing with two portions of 5 cc. of absolute alcohol was sufficient to remove all the lactic acid. Lactic acid was estimated, as described above, in the mother-liquor of the first copper-lime precipitate in the portion of the alcoholic extract used for alcohol-soluble sugar estimation. The temperature at which the alcohol is removed must not rise much above 60° as if the solution becomes too hot or if it is evaporated absolutely to dryness, loss is liable to occur in the lactic acid content. In any case the recovery of added lactic acid is not so good as from a hydrochloric acid extract of muscle. The mean of a number of experiments (Table V) was 85 % recovery, and the amounts of lactic acid found were corrected by this figure. The small quantities of fat and protein, which were not removed, were found not to interfere with the estimation.

Table V.

Recovery of lactic acid from an alcoholic extract of muscle, extract divided into two equal portions and 1·03 mg. lactic acid (as zinc lactate) added to one portion, (B), lactic acid estimated as described in text in both portions.

Portion A lactic acid mg.	Portion B lactic acid mg.	Lactic acid recovered mg.	Recovery %
0·247	1·095	0·848	82
0·254	1·187	0·933	91
0·259	1·170	0·911	88
0·229	1·106	0·877	85
0·265	1·173	0·908	88
0·209	1·030	0·821	80
0·129	0·992	0·863	84
0·101	0·925	0·824	80
0·441	1·326	0·885	86
0·305	1·153	0·849	82
			Mean 85

A COMPARISON OF THE CHANGES TAKING PLACE IN THE TOTAL CARBOHYDRATE CONTENT AND IN THE DIFFERENT CARBOHYDRATE FRACTIONS.

The total carbohydrate, glycogen and alcohol-soluble carbohydrate of resting muscles. The total carbohydrate content and the glycogen content of pairs of muscles from opposite legs of frogs are shown in Table VI. The muscles were all in a resting condition, having recovered from dissection in oxygenated Ringer solution. The agreement between the two muscles is not very exact, but can be said generally to fall within 10 %. The variation from one muscle to another in the same frog is considerable and is greater for total carbohydrate than for glycogen.

In order to compare the total carbohydrate content with the glycogen content one muscle of a pair was analysed for total carbohydrate, the other for glycogen. In some cases the alcohol-soluble sugar, and in two cases the alcohol-soluble carbohydrate, was also determined. In those cases where the alcohol-

Table VI.

Total carbohydrate and glycogen content of pairs of muscles from opposite legs of a frog, muscles allowed to recover in oxygenated Ringer solution.

Frog	Muscle	Weight mg.	Total carbohydrate mg. per 100 g.	Glycogen mg. per 100 g.	Difference %
I	S	319	969	—	3
		311	938	—	
I	Sm	413	1110	—	16
		408	1320	—	
I	B	276	868	—	7
		280	929	—	
II	Sm	377	607	—	2
		397	595	—	
II	B	250	460	—	10
		268	513	—	
III	B	175	920	—	9
		174	834	—	
IV	S	310	1310	—	7
		335	1220	—	
V	S	317	—	523	10
		342	—	463	
VI	B	266	—	568	1
		270	—	563	
VI	S	324	—	648	3
		325	—	668	
VII	S	335	—	758	9
		369	—	694	
VIII	B	217	—	571	6
		241	—	539	
VIII	S	305	—	495	8
		317	—	457	
*IX	G	1131	—	675	9
		1169	—	742	
*X	G	1584	—	558	4
		1588	—	578	

Note. In this and subsequent tables S = Sartorius, Sm = Semi-membranosus, B = Biceps, G = Gastrocnemius, Gr = Gracilis.

* Immediately after dissection, no recovery in Ringer solution.

Table VII.

Comparison of total carbohydrate, glycogen and alcohol-soluble sugar and carbohydrate of pairs of muscles, resting, mg. per 100 g. muscle.

Frog	Muscle	Weight mg.	Total carbohydrate	Glycogen	Alcohol-soluble sugar	Alcohol-soluble carbohydrate	Excess total carbohydrate over glycogen
I	S	315	810	591	30	(60)	219
I	Sm	371	1375	915	14	(28)	460
I	B	224	1305	735	34	(68)	570
II	S	239	965	636	—	—	329
II	B	192	995	721	—	—	274
III	S	203	758	656	49	47	102
IV	Sm	352	1775	931	52	92	844

soluble carbohydrate was not determined, an estimated value for this equal to twice the alcohol-soluble sugar content is included in Table VII for comparison, the values so calculated are enclosed in brackets.

The glycogen content is in all cases much less than the total carbohydrate content, the greatest variation is shown where the total carbohydrate is highest. In no case does the alcohol-soluble carbohydrate account for more than a fraction of the excess of total carbohydrate over glycogen. The values for alcohol-soluble carbohydrate may be low, owing to inefficient extraction from the muscle and to incomplete hydrolysis of the carbohydrates present, *e.g.* hexosephosphate, but when every allowance is made for these corrections, it is impossible that the alcohol-soluble carbohydrate can account for the difference between the glycogen and total carbohydrate. The difference also is too great and too regular to be accounted for by the variation in total carbohydrate and glycogen content of two muscles of a pair, and it is much greater than the maximum amount of non-carbohydrate reducing substance likely to be associated with the total carbohydrate fraction. It may in part be accounted for by ribose derived from adenylic acid. Ochoa [1930] states that as much as 100 mg./100 g. muscle may arise from this source. But even taking into consideration both ribose and non-carbohydrate reducing substances the difference is so great that there must almost certainly be some other substance present.

Changes taking place during incubation in phosphate and bicarbonate buffer solutions. The muscles used in these experiments were allowed to recover from dissection in Ringer solution. One muscle was then analysed and used as the resting control, the other was cut finely with a pair of scissors and placed in a test-tube under 1–2 cc. of the buffer solution. The tube was placed in an incubator at 37° for 3 hours, then the cut muscle was analysed in the same way as the control. The buffer solution was added to the extract. So that estimations of total carbohydrate should be comparable with estimations of glycogen and soluble carbohydrate both experiments were carried out on frogs from the same batch.

Phosphate buffer. A mixture containing 95 % $M/5$ disodium hydrogen phosphate and 5 % $M/5$ sodium dihydrogen phosphate was used, p_H 8. The results are shown in Tables VIII and IX. The total carbohydrate and lactic acid changes and some glycogen and lactic acid changes were estimated using autumn frogs. A more extensive set of experiments on the glycogen and lactic acid changes was carried out on two further batches, one of autumn and one of summer frogs.

In all the cases examined nearly the whole of the carbohydrate of the muscle has disappeared, a mean value of 92 mg./100 g. muscle remaining, or 5–14 % of the initial content, with an average value of 9 %. In no case is the increase of lactic acid equal to the decrease of carbohydrate, in all cases except the first a large amount of carbohydrate has disappeared that has not been converted into lactic acid. It is possible that some or all of it has been changed into hexosephosphate, but from the large difference between the carbohydrate unconverted to lactic acid and the residual value, it is not likely that the final

Table VIII.

Change in glycogen, lactic acid, alcohol-soluble sugar and carbohydrate of muscle incubated for 3 hours at 37°, mg. per 100 g.

Frog	Muscle	Weight mg.	Glycogen initial final change	Alc.-sol. sugar initial final change	Alc.-sol. carbohydrate initial final change	Lactic acid initial final change
		(*a*) Phosphate buffer, autumn frogs, same batch as Table IX (*a*).				
I	Sm	210	749	2		10
		212	67 – 682	9 + 7	—	615 + 605
I	S	172	400	0		4
		162	72 – 338	19 + 19	—	341 + 337
II	Sm	164	916	10		10
		161	350 – 566	46 + 36	—	592 + 582
		(*b*) Phosphate buffer, autumn frogs, another batch.				
III	B	246	710			74
		206	102 – 608	—	—	707 + 633
IV	Sm	509	1090			14
		478	60 – 1030	—	—	989 + 975
		(*c*) Phosphate buffer, summer frogs.				
V	S	244	598			17
		—	76 – 522	—	—	700 + 683
V	B	235	569			63
		—	72 – 497	—	—	603 + 540
VI	S	274	555			18
		—	84 – 471	—	—	622 + 604
VI	B	261	605			15
		—	92 – 513	—	—	632 + 617
VII	S	293	588			14
		—	89 – 499	—	—	564 + 550
VIII	S	259	802			5
		—	97 – 705	—	—	703 + 698
IX	B	175	696			9
		—	137 – 559	—	—	559 + 550
		(*d*) Bicarbonate buffer, autumn frogs, same batch as Table IX (*b*).				
X	S	198	915	24	35	44
		186	469 – 446	23 – 1	43 + 8	241 + 197
XI	Sm	181	1150	0	36	26
		192	400 – 750	42 + 42	57 + 21	695 + 669
XI	B	112	951	24	27	21
		112	594 – 357	12 – 12	72 + 45	337 + 316
XII	B	190	754	47	16	56
		189	473 – 281	58 + 11	79 + 63	444 + 388
XIII	Sm	199	1855	20	0	79
		200	799 – 1056	65 + 45	85 + 85	445 + 366

reducing value is all due to hexosephosphate. It is also possible that this final reducing value is due partly or entirely to non-carbohydrate reducing substances, since the copper-lime precipitates were not free from nitrogen. If this excess of reducing substance is converted into a compound allied to lactic acid it should be estimated, in part at least, with lactic acid, though it might possibly be destroyed during the acid hydrolysis.

The amount of carbohydrate which is changed into lactic acid is of the

Table IX.

Change in total carbohydrate, lactic acid and "residual glycogen in muscles after incubation for 3 hours at 37°, mg./100 g.

Frog	Muscle	Weight mg.	Total carbohydrate initial / final / change	Lactic acid initial / final / change	Residual glycogen (i) initial / final / change	Residual glycogen (ii) initial / final / change
	(*a*) Phosphate buffer, autumn frogs, same batch as Table VIII (*a*).					
I	Sm	215	720	24	64	87
		215	98 – 622	640 + 616	80 + 16	103 + 16
I	B	144	582	34		
		114	60 – 522	283 + 249	—	—
II	Sm	473	1610	20	28	39
		490	84 – 1526	1030 + 1010	16 – 12	32 – 7
II	B	322	1092	8	45	64
		304	119 – 973	608 + 600	79 + 34	101 + 37
III	Sm	243	1250	42	*34	*53
		256	85 – 1165	487 + 445	*35 + 1	*55 + 2
IV	B	231	1695	20	*25	*49
		231	108 – 1587	970 + 950	*32 + 7	*65 + 16
	(*b*) Bicarbonate buffer, autumn frogs, same batch as Table VIII (*d*).					
V	Sm	264	1335	38		
		234	831 – 504	486 + 448	—	—
V	B	162	1055	34		
		157	658 – 397	604 + 570	—	—
VI	S	152	826	50		
		154	309 – 517	462 + 412	—	—
VII	Sm	210	1530	55		
		182	527 – 1003	793 + 738	—	—
VIII	S	161	1298	33		
		173	564 – 734	745 + 712	—	—

(i) Residual glycogen calculated without solubility correction.
(ii) Residual glycogen calculated including solubility correction.
* Shaffer-Hartmann sugar estimation.

same order as the amount which can be accounted for as glycogen when the total carbohydrate and glycogen are determined on the same muscle (Table VII). This is in agreement with the evidence of Table VIII, that the greater part of the lactic acid is formed from glycogen. These experiments do not give sufficient evidence to determine the fate of the carbohydrate not converted into lactic acid.

Laquer [1914, 1921] found that during incubation in phosphate buffer the increase in lactic acid is sometimes greater than the initial carbohydrate content, measured by adding together the glycogen and the lactacidogen. In only two of the results in Table VIII is the initial glycogen content less than the increase in lactic acid, but in several the increase of lactic acid is greater than the decrease in glycogen. This is more often the case with summer frogs than with autumn frogs, and may be a seasonal difference, but both types of result are found in both groups of frogs. The change in alcohol-soluble sugar was only measured in three cases, in all of which it increased, probably owing to the formation of hexosemonophosphate. This increase in alcohol-soluble sugar makes the difference between the decrease of the sum of glycogen and

soluble sugar, and the increase of lactic acid greater where the lactic acid change is already greater than the glycogen change, and more nearly equal, or greater, in the remaining cases, and brings the figures generally more into agreement with those of Laquer. But when the total carbohydrate is measured by acid hydrolysis, as already discussed, the position is quite different (Table IX *a*).

Determinations of residual glycogen, *i.e.* glycogen left in the solid residue after hydrolysis with hydrochloric acid and removal of protein, were carried out on those muscles used for total carbohydrate analysis. The mercuric chloride in the residue does not upset the results, which are of the same order when trichloroacetic acid is used for precipitating protein[1]. In all the muscles where the change in glycogen during incubation was determined a small amount of glycogen was found after incubation. This was approximately the same as the residual glycogen found in the total carbohydrate determinations. It seems possible that this residual glycogen is of a different nature from the main bulk of that present in muscle, since it is not extracted during hydrolysis with hydrochloric acid and is not removed during incubation with phosphate buffer. This raises the question whether or not the correction for solubility in the alcohol used to precipitate the glycogen should be applied in calculating the amount of residual glycogen present, since, if the latter is of a different nature from the rest of the glycogen, it possibly has a different solubility in alcohol. In Table IX the results are shown both with and without the correction; in Table VIII the correction is used in the calculation of all the results.

Bicarbonate buffer. The results of estimations on muscles from autumn frogs of the same batch incubated in bicarbonate buffer are shown in Tables VIII (*d*) and IX (*b*). Although the decrease in total carbohydrate is in all cases except one greater than the increase in lactic acid, the discrepancy is not nearly so great as in the case of incubation in phosphate buffer, and moreover a large amount of carbohydrate is left in the muscle. An increase in hexosephosphate during incubation would tend to make the final figures for total carbohydrate low in comparison with the initial, and possibly this accounts for the greater decrease of total carbohydrate compared with increase of lactic acid. The increase in soluble carbohydrate in Table VIII (*d*) supports the idea of increase in hexosephosphate.

These results and the determination of glycogen and lactic acid agree with those of Meyerhof, of Laquer and of Embden, that lactic acid formation ceases whilst there is still carbohydrate left in the muscle. In two cases in Table VIII (*d*) the lactic acid increase exceeds the carbohydrate decrease calculated from the change in glycogen and alcohol-soluble carbohydrate, though in one it is less than the glycogen change taken alone. If, as is probable, the figures for alcohol-soluble carbohydrate are low, then the change in this fraction should be greater than appears in Table VIII (*d*). This change is always an increase and it is possible that were its full value known it would account for the difference between glycogen increase and lactic acid decrease.

[1] The reducing value after hydrolysis was estimated by the Hagedorn and Jensen method in all cases except two, in which the Shaffer-Hartmann method was used.

As is generally accepted, this difference between the action of the two buffer mixtures suggests that phosphate is required before all the carbohydrate can be converted into lactic acid. When the difference in the behaviour of total carbohydrate is considered it seems probable that in either case the chief source of lactic acid is glycogen, and that the large excess of carbohydrate found when the muscle is extracted with hydrochloric acid, as in this method of determining total carbohydrate, over that found using an alcoholic extraction, cannot form lactic acid at all. It may be noted in this connection that the trisaccharide of Lohmann does not give rise to lactic acid. From a comparison of the action of the two buffer mixtures it seems further very probable that this excess of carbohydrate does not react at all in a bicarbonate buffer, but is converted into a phosphate during incubation in a phosphate buffer. This phosphate may of course only be of a transitory nature.

Changes taking place in muscles poisoned with sodium monoiodoacetate. Lundsgaard [1930] has shown that for muscles poisoned with sodium monoiodoacetate although there is no increase in lactic acid content during anaerobiosis or when the muscle is fatigued, the glycogen content falls and the hexosephosphate content rises proportionately. Lundsgaard has shown that the hexosephosphate is in large part a diphosphate, and from the hydrolysis curve he concludes that it is probably identical with the hexosediphosphate of Harden and Young. This phosphate is much more easily hydrolysed than the monophosphate so that total carbohydrate should show little or no change during anaerobiosis or after fatigue. This was confirmed by experiment, the results of which are shown in Table X. The frogs were injected in the dorsal lymph sac with a 1 % solution of monoiodoacetic acid neutralised to p_H 7 with sodium carbonate. 4 cc. per 100 g. body weight were given. After injection the frogs were placed in the refrigerator for 40 minutes so that the muscles should be in a resting condition when the frog was killed. The muscles were dissected on a board which was well cooled and surrounded by ice so that the changes taking place after death and not due to the treatment given should be a minimum. The control muscles were ground in a mortar with ice-cold alcohol or hydrochloric acid immediately after dissection and weighing. For these experiments it was considered better not to soak the muscles in oxygenated Ringer solution as the changes taking place are not yet fully worked out, and a continuous breakdown might be taking place in the Ringer solution instead of recovery. The muscles were fatigued by single induction shocks at the rate of about 2 per second in an atmosphere of hydrogen. The record of a lever attached to the muscles was taken and in all cases a curve typical of a poisoned muscle was obtained, that is the muscle became inexcitable after 100–200 shocks and remained in a contracted state. For anaerobiosis the muscle was hung in a tube containing hydrogen and either left at 16° for 24 hours, or placed in an incubator at 37° for 2 hours. Determinations were also made of the change in glycogen and alcohol-soluble carbohydrate and sugar in poisoned muscles. The glycogen change is of the same order as that found by Lundsgaard, but the increase in alcohol-soluble carbohydrate does not correspond

Table X.

Change in total carbohydrate, glycogen, alcohol-soluble carbohydrate and sugar, and lactic acid, in muscles poisoned with monoiodoacetate, mg. per 100 g.

Frog	Muscle	Weight	Total carbohydrate initial / final / change	Glycogen initial / final / change	Alc.-sol. carbohydrate initial / final / change	Alcohol-soluble sugar initial / final / change	Lactic acid initial / final / change
			(*a*) Fatigue in hydrogen.				
I	G	1·25	—	1200 964 – 236	17 31 + 14	6 20 + 14	20 22 + 2
II	G	1·10	1260 1237 – 23	— —	— —	— —	51 50 – 1
III	G	0·77	1115 1115	— —	— —	— —	29 48 + 19
IV	G	0·90	1082 1085 + 3	— —	— —	— —	35 3 – 32
V	G	0·44	—	768 547 – 221	27 57 + 30	12 20 + 8	19 28 + 9
			(*b*) Anaerobiosis in hydrogen.				
I	Gr	0·80	—	1325 795 – 530	42 92 + 50	46 48 + 2	16 30 + 14
I	Sm	0·30	—	738 653 – 85	43 103 + 60	— —	43 28 – 15
II	S	0·20	720 709 – 11	— —	— —	— —	11 30 + 19
II	Gr	1·10	1270 1040 – 230	— —	— —	— —	51 50 – 1
III	Gr	0·85	—	704 281 – 423	12 73 + 61	11 84 + 73	8 7 – 1
V	Gr	0·30	878 905 + 20	— —	— —	— —	20 62 + 42
			(*c*) 2 hours in air.				
I	S	0·20	—	670 590 – 80	104 273 + 169	4 63 + 59	37 78 + 41

with the decrease in glycogen. The values obtained for the former may be low, owing to incomplete extraction of the carbohydrate present by alcohol. In this case the low values cannot be explained by supposing that there is hexosephosphate present which is incompletely hydrolysed, since there is no change in total carbohydrate content, where the time allowed for hydrolysis is less than that allowed in the case of the alcohol-soluble carbohydrate.

Summary.

1. A modification of the method of estimating total carbohydrate of muscle by hydrolysing the whole muscle is described.

2. A modification of Bissinger's method of separating sugar from solution and estimating it quantitatively by precipitation with copper-lime and using the copper as the oxidising agent for sugar estimation is described.

3. The resting value of the total carbohydrate of isolated frog muscles estimated by this method is found to be much greater than the sum of the glycogen and alcohol-soluble carbohydrate.

4. During incubation in a phosphate buffer at p_H 8 this excess carbohydrate is removed from the muscle but does not appear as lactic acid. The lactic acid increase is in general somewhat greater than the decrease of the sum of glycogen and alcohol-soluble carbohydrate.

5. The excess carbohydrate is not removed during incubation in 2 % bicarbonate buffer.

6. In muscles poisoned with monoiodoacetic acid, during anaerobiosis and fatigue the total carbohydrate content does not change significantly whilst the glycogen content falls. The lactic acid content rises very slightly on the average. The alcohol-soluble carbohydrate rises but not sufficiently to account for the fall in glycogen.

7. When the total carbohydrate of muscle is estimated by this method there is left in the muscle a small residual amount of glycogen, on the average 50 mg. per 100 g. muscle. This glycogen does not change significantly in amount during incubation in phosphate buffer. Approximately the same amount of glycogen is found remaining after incubation when the whole glycogen content of the muscle is estimated by Pflüger's method. It is suggested that this glycogen may be of a different nature from the main bulk of that present in muscle.

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