LXXXI. A REDETERMINATION OF THE HEAT OF COMBUSTION OF GLYCOGEN, WITH SPECIAL REFERENCE TO ITS PHYSIOLOGICAL IMPORTANCE.

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Introduction.

Although the heat of combustion of glycogen has already been determined by Stohmann and Schmidt [1894] and by Emery and Benedict [1911], it has now become desirable that a more accurate determination be made in order that this constant may be satisfactorily applied in the correlation of the work of Hill and Meyerhof on the physical and chemical phenomena accompanying muscular activity.

The previous determinations were both made with care and apparent accuracy so far as the actual combustion was concerned, but in neither case was the product used submitted to a satisfactory examination, in particular with respect to the state of hydration. Stohmann and Schmidt prepared their glycogen from the liver of a single rabbit, the extraction being made in the usual manner and the protein precipitated by the potassium mercury iodide method. The resulting product did not agree with the formula $(C_6H_{10}O_5)_n$ the discrepancy being attributed by the authors to the probable presence of fat. The sample was therefore extracted with ether and dried in an air oven at 110° to constant weight. The quantity of material was so greatly reduced by this process that there remained only sufficient for the determination of the heat of combustion, and in consequence no tests could be made to prove the purity of the sample. Harden and Young [1902] have shown that glycogen can only be rendered anhydrous by drying at 100° *in vacuo* over phosphorus pentoxide. Owing to the less efficient drying method used by Stohmann and Schmidt it is probable that the material employed by them for the final determination was not anhydrous glycogen as they assumed, and that in consequence their result of 4190·6 cals. per gram is almost certainly too low.

The belief that the above determination is too low is strengthened by the results of the determinations made by Emery and Benedict, who using samples of glycogen obtained from other workers calculated the heat of combustion

[1] The major portion of this work was carried out whilst working for the Medical Research Council.

per g. on the carbon content determined by ultimate analysis. Their figures 4214 and 4241 cals. are higher than the Stohmann and Schmidt figure, but owing to the fact that these workers did not prepare and purify the glycogen themselves, the results cannot be looked upon as reliable.

In order to obtain the value for the heat of combustion in dilute aqueous solution (the constant required for physiological purposes), it is necessary to correct both the above results for the heats of wetting, hydration and solution of anhydrous glycogen. The magnitude of these corrections is influenced by the state of hydration of the glycogen burnt, and in consequence cannot be applied in the results under consideration.

The present determinations have been carried out on samples of glycogen prepared from *Mytilus edulis* and *Ascaris lumbricoides*, the state of hydration of which had been definitely determined by drying and ultimate analysis. The heats of wetting and solution have also been found for the fully hydrated substance which was used in the combustion, and in this way a value has been obtained which represents the heat of combustion in dilute aqueous solution.

The result so obtained (3836 cals. per g. of hydrated glycogen $(C_6H_{10}O_5, H_2O)_n$ in dilute solution) is relatively higher than those given above and the values calculated from it and from the heat of combustion of lactic acid for the heat evolved during isometric contraction of muscle are in excellent agreement with the direct measurements of Hartree and Hill [1923]. A paper dealing with these relationships has already been published by the author [1923]. Since the publication of this paper it has come to his notice that the value for the heat of combustion of cane sugar assumed in the calibration of the bomb differed from that employed by Meyerhof [1922] in the corresponding determination on lactic acid. The value used by Meyerhof being the better authenticated [see Henning, 1921], and moreover for physiological purposes it being essential that the two determinations should be strictly comparable, a recalculation of the results was made using the same figure as that employed by Meyerhof, viz. 3952 cals. per g. of cane sugar.

The resulting correction in the value for the heat of combustion of glycogen from 3874 to 3836 does not materially affect the argument previously advanced in order to explain the source of the "delayed anaerobic heat" described by Hartree and Hill [1923]. The corrected figures are as follows:

Heat of combustion of 1 g. of glycogen monohydrate in dilute solution	3836 cals.
Heat of combustion of 1 g. of lactic acid in dilute solution [Meyerhof, 1922]...	3601 ,,
Heat available due to the formation of 1 g. of lactic acid from 1 g. of glycogen monohydrate...	235 ,,

If it is assumed that the whole of the lactic acid is immediately neutralised by alkaline salts, the total heat liberated can be calculated by using the

figure determined by Meyerhof [1922] for the neutralisation, viz. 19 cals. per g. of lactic acid, and is found to be 254 cals. per g. This is not in very close agreement with the figure given by Hartree and Hill of 296 cals. per g. of lactic acid formed. If it is assumed however that a portion of the lactic acid is neutralised in the first place by alkali protein, which Meyerhof has shown to have a heat of neutralisation of 138 cals. per g. of lactic acid, this difference is easily explained. When the whole of the lactic acid has been neutralised by alkali protein the heat available will be 235 + 138, *i.e.* 373 cals., which is in close agreement with the value, 370 cals., which is assumed (from Meyerhof) by Hartree and Hill for the total anaerobic heat.

It is only necessary therefore to alter the previous conception to the extent of assuming (*a*) a mixed neutralisation by salts and alkali protein immediately on the formation of the lactic acid, in the ratio of 74 % of salts and 26 % of alkali protein, instead of the complete initial neutralisation by salts, and (*b*) that the subsequent change from salt neutralisation to alkali protein neutralisation goes to completion instead of only to the extent of 60 % as was supposed in the previous paper.

The figure given on p. 629 for glycogen from *Ascaris lumbricoides* is the result of only one determination, no more available material remaining after the purity had been established.

Preparation and Purification of the Glycogen.

From Mytilus edulis. Two hundred mussels which had been sent alive from the Lancashire coast were used for each preparation. The shells were opened and the mussels thrown into a litre of boiling water. After it had been ascertained that the reaction of the liquid was slightly alkaline, the boiling was continued for half an hour. The mussels were then filtered off by means of a piece of muslin and the extraction repeated. The joint extracts were rendered faintly acid, and the boiling continued for two to three minutes to precipitate protein. The coagulated protein was filtered off and alcohol added until the solution contained 60 %. The glycogen settled out rapidly and the greater part of the supernatant liquid could be decanted off. Strong caustic potash solution was now added to the glycogen sludge remaining, until the whole solution contained 50 to 60 % of potash. The solution at this stage amounted in a normal experiment to 300 to 400 cc., a volume in which the glycogen readily dissolved. The solution was heated over a wire gauze for six hours, cooled and almost neutralised by strong acetic acid; the potassium acetate which separated out was filtered off, and finally the glycogen was reprecipitated by alcohol and filtered from the solution.

The rest of the purification consisted of the repeated solution of the glycogen in water and its reprecipitation by alcohol. As the electrolytes were gradually removed the precipitation became more difficult, and it was found sometimes to be advisable to add small traces of potassium acetate in order to facilitate the formation of a curd; this salt being appreciably soluble in the

alcohol-water mixtures used for precipitation remains in solution. It is of interest to note in connection with the solution of glycogen in water, that the rate of solution is not materially increased by heating but that continuous agitation is very effective in rendering the solution rapidly complete. A yield of 23 g. of dry glycogen was obtained from 200 mussels.

From Ascaris lumbricoides. The worms freshly taken from the pig's gut were cut into small pieces and thrown into ten times their weight of boiling water. The chopped worms were filtered off and the extraction repeated; the subsequent purification process was carried out exactly as in the case of the mussels. The yield was only 2 to 3 g. from 200 g. of worms.

Method used for drying the purified glycogen.

When the glycogen was judged to be sufficiently pure, as much as possible of the alcoholic mother liquor was decanted off, and the glycogen washed twice with 70 % alcohol. The wet glycogen so obtained was transferred to a flask and an excess of dry benzene added. After the mixture had been allowed to stand over night the alcohol-water-benzene fraction and the alcohol-benzene or water-benzene fraction as the case might be, were distilled off through a fractionating column, and the excess benzene remaining poured from the glycogen. (For further description of this method of drying see Atkins and Wilson [1915].) At the end of the distillation the glycogen had formed a hard pale brown vitreous cake, which, as the adhering benzene was removed in a gentle stream of air, broke up with a concoidal fracture into a coarse glistening powder. This powder was ground in a mortar to a flour-like consistency when it became almost pure white. The final product was quite free from benzene and in appearance dry, showing no tendency to form lumps or to adhere to glass.

Examination of the glycogen.

The samples obtained by the above process were examined, in each case where the quantity of material allowed, for ash, fat, nitrogenous impurities, and an ultimate analysis was made. The examination for water content proved so interesting that it is dealt with under a separate section.

The samples were all found to be free from fat. The nitrogenous impurities were estimated by Folin's micro-Kjeldahl method. The results obtained are given in Table I.

Table I.

Sample	Ash %	Nitrogen %	Ultimate analysis C %	H %
1st sample *Mytilus*	Nil	Trace	39·90	6·64
2nd sample *Mytilus*	0·02	Trace	39·83	6·53
Sample *Ascaris*	0·10	Trace		

Calculated for $(C_6H_{10}O_5)_n$, C = 44·44 % and H = 6·17 %.
Calculated for $(C_6H_{10}O_5, H_2O)_n$, C = 40·00 % and H = 6·66 %.

There was not sufficient *Ascaris* glycogen available for ultimate analysis.

STATES OF HYDRATION OF GLYCOGEN.

From the analytical results given above it was concluded that the sample of glycogen was in a hydrated form corresponding to the formula

$$(C_6H_{10}O_5, H_2O)_n.$$

This hydrate is called for convenience in the subsequent discussion the monohydrate.

A specimen of the monohydrate was dried over calcium chloride *in vacuo*. Water was lost rapidly at first, as will be seen from the accompanying curve, but later the velocity of dehydration fell off roughly exponentially until a steady state was reached with a total loss of 5·02 %.

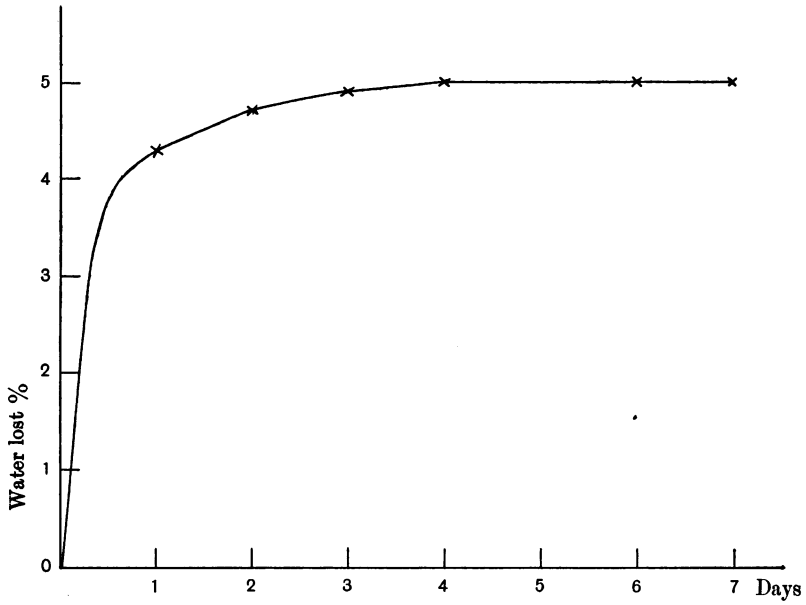

Fig. 1.

The curve in Fig. 1 shows the result of a typical experiment.

Approximately the same loss was found in all the experiments tried. The equation

$$2\,(C_6H_{10}O_5, H_2O)_n = 2\,(C_6H_{10}O_5, 1/2\,H_2O)_n + H_2O$$

requires a loss of 5·00 % of the total weight. It was therefore concluded that the drying over calcium chloride *in vacuo* results in the formation of a half hydrate. Bizio [1867] has already described this substance as the product obtained when wet glycogen is dried to constant weight over calcium chloride *in vacuo*.

When the half-hydrate was placed in an air oven at 110°, it commenced to lose weight immediately and very rapidly. After about one hour a sudden change in the rate of dehydration took place, and from this point the velocity

was extremely slow. The total loss gradually approached 10 % of the initial weight of the glycogen monohydrate.

These results are plotted in Fig. 2.

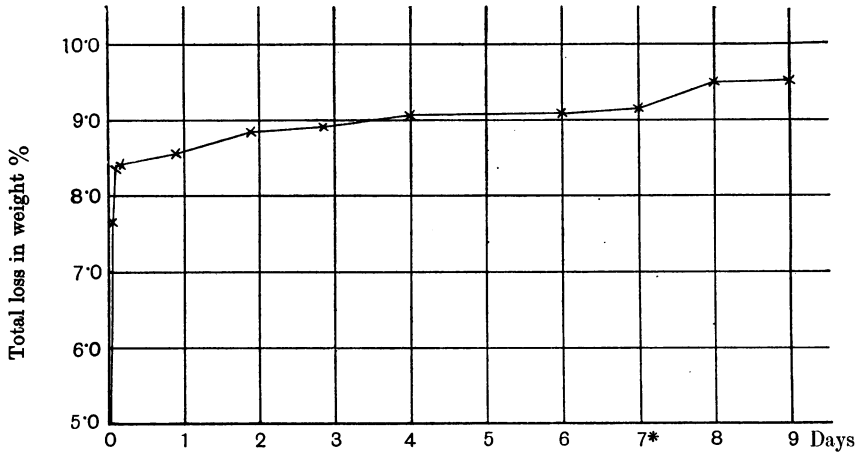

Fig. 2.

* The temperature of the oven was, at this point, raised to 120° C.

From these experiments it was concluded that sufficiently prolonged heating would produce anhydrous glycogen, but that the time necessary to do so would be approximately 15 days. Moreover, although in the present instance no decomposition was observed at 120°, it is probably unwise to use temperatures higher than 110°. It is clear from this experiment that the flat portion of the curve may be easily mistaken for the steady anhydrous state, whilst the glycogen still contains from 1·5 to 2·0 % of water.

Glycogen which is nearly anhydrous is definitely hygroscopic. A sample containing 0·5 % of water, on standing exposed under ordinary laboratory conditions for 35 hours was found to contain 4·2 % of water.

Determination of the heats of wetting and solution of the monohydrate.

Method. The apparatus used for the determination consisted essentially of an ordinary Dewar vessel from a "thermos" flask, immersed up to the neck in a thermostat which was kept at 25°.

The flask was closed by a cork carrying a thermometer graduated to 0·01°, a heating coil which could also be used for calibration purposes, and an arrangement for containing and releasing the glycogen. The latter consisted of a small specimen tube in which the glycogen was placed. The tube was supported by a rubber band resting on a copper wire ring, through which the tube could just move with ease. A piece of stout cotton thread was attached to the bottom of the tube so that when a steady temperature state had been obtained, the tube could be lifted and upset by pulling the cotton thread. This method

insured the immediate wetting of the whole of the glycogen. It was found necessary to attach another piece of cotton thread to the upper rubber ring in order to free the tube if it stuck whilst it was being raised through the copper holder. The copper ring was held in the cork by means of a short length of glass tubing, so that the heat leak through the cork might be reduced to a minimum.

In order to mix the contents, the flask was gently shaken from time to time. A control experiment was made in order to ascertain whether this procedure produced any material change in the temperature gradient. The results showed that within reasonable limits the shaking had no effect.

100 cc. of water were used in the calorimeter in two of the experiments whilst in the third 200 cc. were used, the equivalent of the whole calorimeter being redetermined. The difference in the amount of water used caused no difference in the results.

Calibration. The water was weighed into the flask and the cork placed in position. A current was then passed through the heating coil until the temperature inside the flask was within 0·2° of the temperature of the thermostat. The apparatus was then left to stand for an hour at the end of which time the temperature had become nearly steady. The change in temperature, if any, was observed over a period of 15 minutes. At the end of the initial temperature readings the heating circuit was closed for an accurately measured period, and temperature readings taken at intervals of one minute until a steady fall was observed. The necessary corrections were made for the cooling during the experiment and the actual rise in temperature calculated. The heating current was measured with an ammeter, and the heat calculated in the usual way. Table II gives the results of the calibration.

Table II.

Number of experiment	Rise in temperature (° C.)	Weight of water (g.)	Total heat equivalent (cals.)	Heat equivalent of calorimeter (cals.)
1	1·139	100·1	114·0	13·9
2	1·219	100·2	117·9	17·8
3	1·258	100·0	116·1	16·1
4	0·592	200·1	214·2	14·1

Average value for the equivalent of the calorimeter = 15·5.

Results of determination. The procedure given above was followed in each case, and the results are given in Table III.

Table III.

Number of experiment	Rise in temperature (° C.)	Weight of water (g.)	Total heat equivalent * (cals.)	Weight of glycogen (g.)	Heats of wetting and solution (cals. per g.)
1	0·082	99·2	114·7	1·1294	8·3
2	0·116	99·6	115·1	1·3880	9·6
3	0·026	198·7	214·2	0·6444	8·6

Average value = 8·8 cals. per g.

* Neglecting the dissolved glycogen.

Determination of the heats of hydration, wetting, and solution of the half hydrate.

Two measurements were made of these values using the same technique as for the monohydrate. The results obtained were as follows:

Table IV.

Number of experiment	Rise in temperature (° C.)	Weight of water (g.)	Total heat equivalent * (cals.)	Weight of glycogen (g.)	Heats of hydration, wetting and solution (cals. per g.)
1	0·143	97·5	113·0	1·3240	12·2
2	0·063	97·6	113·1	0·6180	11·6

* Neglecting the dissolved glycogen.

The weights of glycogen given are the calculated weights of the monohydrate formed from the half-hydrate taken, so that the results represent the heat given out in the formation of 1 g. of monohydrate and its subsequent solution.

Determination of the heat of combustion of the monohydrate.

Method. A Mahler-Cook bomb calorimeter was used for the determination of the heat of combustion. The established technique was employed with very slight variations to suit the particular case.

The most important variation introduced was in the arrangement of the material and the method of firing. The author has found from past experience that it is much more satisfactory to place the substance to be burnt directly in the crucible without briquetting or wrapping in a collodion envelope. The combustion under these conditions takes place evenly and rapidly, and without spurting. There is, however, a little more difficulty in the ignition, but this can be overcome by stretching the platinum ignition wire tightly between the leads, and allowing a small piece of cotton thread, tied at one end to the wire, to dip into the substance in the crucible. Such an arrangement rarely gives a misfire, and the heat given out by the thread can be corrected for, if a definite weight of cotton is used for each experiment. A good cotton yarn is sufficiently uniform to permit of a measured length being used for each experiment.

The following possible sources of error were considered and it was concluded that they could be neglected.

(1) The heat evolved by the passage of the current. A number of test experiments were made with the platinum ignition wire exposed to view and it was found that the making of the current and the fusion of the wire were, as far as could be observed, simultaneous. The needle of a delicate ammeter in the circuit was not disturbed. Under these circumstances the heat evolved must be negligible.

(2) The heat changes due to the burning of any nitrogen present in the oxygen used. The nitrogen present in the quantity of oxygen used for the combustion was not sufficient to require an appreciable amount of heat if it

were completely burned. As the reaction is endothermic any small error would be opposite in effect to a similar error due to the passage of the current as described above.

(3) Inaccuracies in the thermometer. The thermometer was a standard instrument supplied with the bomb, and was found to show no noteworthy irregularities.

(4) The change in the specific heat of water with change of temperature is not sufficient to affect the results obtained at ordinary temperatures.

Calibration of the bomb. The bomb was calibrated by burning saccharose. The saccharose contained no moisture or ash and gave on ultimate analysis C = 41·9 % and H = 6·40 %. (Calculated C = 42·10 % and H = 6·43 %.) The heat of combustion of saccharose was taken as 3952 cals. per g. The calibration determinations gave the following figures:

Table V.

Number of experiment:	1	2	3	4
Weight of saccharose (g.)	1·9550	1·9394	1·8616	1·2020
Heat available from saccharose (cals.) ...	7726·2	7664·5	7357·0	4750·3
Heat available from cotton (cals.) ...	8·2	8·2	8·2	8·2
Total heat available (cals.)	7734·4	7672·7	7365·2	4758·5
Rise in temperature (° C.)	2·370	2·355	2·257	1·462
Heat required per ° C. rise (cals.) ...	3263·5	3258·1	3263·3	3254·8
Weight of water used (g.)	2500·9	2499·9	2500·0	2497·0
Heat equivalent of calorimeter (cals.) ...	762·6	758·2	763·3	757·8

Average value = 760·5 cals. per ° C. rise.

Determination of heat of combustion of monohydrate. Six determinations were made on two samples, *M* 1 and *M* 2, of *Mytilus* glycogen, and one on the sample (*A*) of *Ascaris* glycogen. The following table gives a summary of results:

Table VI.

Number of experiment:	1	2	3	4	5	6	7
Source of glycogen	*M* 1	*M* 1	*M* 1	*M* 2	*M* 1	*M* 2	*A*
Weight of glycogen (g.)	1·2510	1·1111	1·0377	1·1161	0·8322	1·0302	0·5036*
Rise in temperature (° C.)	1·478	1·310	1·229	1·319	0·982	1·217	0·595
Weight of water in calorimeter (g.)	2500	2501	2500	2503	2501	2500	2498
Heat equivalent of calorimeter (cals.)	760·5	760·5	760·5	760·5	760·5	760·5	760·5
Total heat equivalent (cals.)	3260·5	3261·5	3260·5	3263·5	3261·5	3260·5	3258·5
Heat from cotton (cals.)	8·2	8·2	8·2	8·2	8·2	8·2	8·2
Heat evolved from glycogen (cals.)	4810·8	4264·4	3999·0	4296·4	3194·6	3959·8	1930·6
Heat of combustion per g. (cals.)	3845·6	3837·7	3853·7	3849·5	3838·7	3843·7	3833·6

* After allowing for ash.

Result and conclusions. Considering only the determinations made on the *Mytilus* glycogen the average value for the heat of combustion of the monohydrate is 3844·8 cals. per g. The *Ascaris* value is omitted as the purity of the specimen was not so definitely established, the result being regarded merely as an indication that glycogen from a second source gives a value of the same order as that from *Mytilus*.

If the value for the heat of wetting and solution of the monohydrate (8·8 cals. per g.) is subtracted, the heat of combustion of glycogen in dilute solution is found to be 3836 cals. per g. This value is lower than that previously given by the author, for reasons stated in the introduction, but is considerably higher than the corresponding values of Stohmann and Schmidt, and of Emery and Benedict. Such a deviation from the previous results is to be expected in view of the difficulty experienced in obtaining anhydrous glycogen, and suggests in the case of Stohmann and Schmidt that the glycogen used contained 1·5 to 2·0 % of water.

Summary.

(1) The previous determinations of the heat of combustion of glycogen are criticised on the grounds of the doubtful state of hydration of the samples of glycogen used, and the fact that they do not give the heat of combustion of dissolved glycogen (which is required for physiological purposes), but only of glycogen in the anhydrous state.

(2) A monohydrate of glycogen of the formula $(C_6H_{10}O_5, H_2O)_n$ can be prepared by fractionally distilling off with benzene the water and alcohol used in its preparation. This hydrate on drying over calcium chloride *in vacuo* yields a half-hydrate $(C_6H_{10}O_5, 1/2\ H_2O)_n$. The anhydrous material is very difficult to obtain, and is produced only by drying at 110° for about 15 days.

(3) The total heat of wetting and solution of the monohydrate is found to be 8·8 cals. per g., and the total heat of hydration, wetting and solution of the half-hydrate to be 11·9 cals. per g.

(4) The heat of combustion of the dry monohydrate is found to be 3844·8 cals. per g., for the average of six determinations on two samples of *Mytilus* glycogen, and 3833·6 cals. per g. in one determination on *Ascaris* glycogen.

(5) The heat of combustion therefore of glycogen monohydrate in dilute solution, taking only the *Mytilus* figure, is 3836 cals. per g.

(6) The value now given is less than that used by the author in his paper dealing with the application of this constant to the theory of muscular contraction [1923], but the change does not materially affect the argument given there. It is required only to assume that the initial neutralisation of lactic acid is carried out partly by alkali protein instead of entirely by alkaline salts, and that in the final state the neutralisation is entirely due to alkali protein, instead of only to the extent of 60 per cent.

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