XXXI. THE BREAKDOWN OF GLYCOGEN BY THE GLYCOGENASE OF HEART MUSCLE

By LEWIS BLAND WINTER

From the Department of Physiology, University of Sheffield

(Received 16 December 1936)

It has been shown by Evans *et al.* [1933] that under certain conditions the heart utilizes lactic acid supplied by the blood circulating through it in preference to glucose; on the other hand, it is believed that the skeletal muscles normally draw upon the blood glucose for their supply of carbohydrate. Both heart and skeletal muscle contain reserve carbohydrate in the form of glycogen, and it seemed possible that the glycogenase in the two situations might form different products when acting on this polysaccharide. Barbour [1929] investigated the action on glycogen *in vitro* of the glycogenase of the skeletal muscles of the rabbit and found that the sole product of hydrolysis is a reducing trisaccharide. Carruthers & Lee [1935] and Carruthers [1935] do not agree that a trisaccharide is the sole product; these authors obtained evidence that a disaccharide may be formed, and complete breakdown to glucose may also occur. The products isolated were amorphous, and the identity of any one could not be established with certainty. The action of the glycogenase from heart muscle does not appear to have been investigated. Dogs were used in the present work in order to have available from a single heart a sufficient quantity of enzyme for several experiments. The glycogen used was prepared from rabbit liver and purified according to Barbour's description. The investigation has been purely qualitative; after a series of trials had shown that a glycerol extract of the dog's heart muscle was capable of hydrolysing glycogen *in vitro*, no attempt was made to determine the optimum concentration of glycogen, enzyme or hydrogen ions; it was assumed that they are not widely different in the dog and the rabbit. Identification of the main product of hydrolysis proved to be difficult; not only had the carbohydrate to be separated from glycerol and protein, but the hydrolysis of glycogen *in vitro* is likely to give rise to traces of sugars other than the main product, and these tend to prevent the latter from crystallizing.

EXPERIMENTAL

The animal was stunned electrically, the throat was cut, and the heart was removed, minced in an ice-cold mincer and weighed. A glycerol extract was then made by the method of Hunter & Dauphinee [1924]. Since no details are given in Barbour's paper of the precise conditions under which hydrolysis of glycogen was effected in the experiments in which the trisaccharide was isolated, his Table V was relied upon: the figures in this table show that with a 2% glycogen solution in the presence of phosphate buffer pH 6.3, the disappearance of glycogen is almost complete in 36 hours. In the present work 2% glycogen was also used, and the action of the enzyme was allowed to proceed for 40 hours. For the isolation of the product formed it was found advantageous to work on a comparatively small scale, using 20 ml. 2% glycogen, 10 ml. phosphate buffer, pH 6.3, 10 ml. glycerol extract of muscle and 2 ml. toluene. The mixture, in a

small flask closed with cotton wool, is maintained at 37° for 40 hours, with occasional shaking; 50 ml. water are then added and the precipitate may be readily removed by filtration. The carbohydrate is then separated by the lead-barium method [Winter, 1930] with the following modifications. 5 ml. of a saturated solution of lead acetate are used; the precipitate is invariably colloidal at first, and no way of avoiding this has been found. After 3 or 4 days at a low temperature the precipitate aggregates sufficiently to be removed in a high-speed centrifuge. The alkaline lead precipitate is washed once only with 75 % alcohol and dried. After treatment of the dry precipitate with sulphuric acid, alcohol is added to 70 %. The precipitate which forms is often colloidal, and much delay may be caused before it can be centrifuged clear. In addition to traces of lead, any unchanged glycogen is removed at this stage. The barium compound is decomposed by passing CO₂ into its suspension in 100 ml. 70 % alcohol at 40°. After the precipitate has settled the fluid is evaporated to dryness by distillation at low pressure: 15 ml. methyl alcohol are placed in the flask, and it is allowed to stand at room temperature for a day. The extraction is repeated and the united alcohol extracts are evaporated in a basin over sulphuric acid. The residue in the flask is taken up in water, any insoluble matter is removed at the centrifuge, and the solution is evaporated to dryness. These two fractions were investigated separately.

Alcohol-soluble fraction. The syrup obtained in the first experiment began to crystallize after some months and after another month became a white, almost solid mass. The maximum yield in such an experiment has been 70 mg. Purification has proved unexpectedly troublesome: the crystals drain slowly on a porous tile, and it has not been found possible to recrystallize the sugar from methyl alcohol. For the analytical work the purification was carried out as follows. After draining on a tile as completely as possible the solid was extracted with warm methyl alcohol, and some insoluble matter was removed. On evaporation of the solvent the syrup was induced to crystallize by seeding with a crystal of the crude product. After crystallization was complete, the solid was taken up in cold methyl alcohol, in which it was completely soluble. The solvent was removed, and the syrup was allowed to crystallize spontaneously in a damp atmosphere.

As is usual with sugar hydrates, the melting-point was indefinite; softening took place at about 95°, with a complete melt at 115–120°.

Micro-analyses (Schoeller). Found: C, 37.96, 37.91; H, 6.94, 7.00 %. C₆H₁₂O₆, H₂O requires: C, 36.36; H, 7.07 %.

The reducing power of the substance was estimated by the Hagedorn-Jensen method and was found to be that of a monosaccharide. 2.530 mg. in 0.9958 g. water: $c=0.230\%$ (all values for c are calculated for the anhydrous sugar); found: 0.231 %. Glucose, 3.117 mg. in 1.2340 g. water: $c=0.255\%$; found: 0.248 %.

Molecular weight. This was determined by Barger's method. In his paper Barger states that good results were obtained with as little as 30 mg. of solid. In the present work it was necessary to economize material and tests were carried out with smaller amounts, with excellent results. Solutions of sucrose in water were used as standard, and 18–24 hours were allowed for equilibrium to be attained at room temperature. In each estimation six capillaries were used, containing the unknown and 0.10, 0.12, . . . , 0.20 *M* sucrose. Inspection of the aggregate differences in the drop lengths showed quite clearly which solution was isotonic with the unknown. Using a solution of 2.759 mg. glucose in 0.0998 g. water, the mol. wt. was found to be 197; with a solution of maltose,

5.299 mg. in 0.1009 g. water 354; with the sugar derived from glycogen, 2.610 mg. in 0.1015 g. water, a mol. wt. of 179 was found: a second experiment gave 167.

Optical activity. The substance is optically active, giving a positive rotation with mutarotation in aqueous solution. The following figures were obtained with 22.61 mg. in 1.593 g. water. $c = 1.288$, $l = 1$.

Time (hours) ...	0-16	0-36	0-68	2-41	3-23	24-0
α	+1.69°	1.59	1.43	1.06	0.99	0.87
$[\alpha]_{5461}^{16^\circ}$	130.4°	123.4	111.0	82.2	76.8	67.6

Phenylosazone. This was insoluble in hot water, with the typical crystalline form of glucosazone. After recrystallization from hot aqueous pyridine the substance melted at 208–210° (corr.): the same melting-point was given by a specimen of glucosazone prepared and recrystallized under the same conditions.

Alcohol-insoluble fraction. No preparation has crystallized, even after 2 years. There appears to be little reducing sugar of any kind in this fraction: heated with phenylhydrazine and acetic acid it forms no osazone insoluble in the hot mixture; on cooling there may be deposited in the course of a day a trace of amorphous precipitate, insufficient for identification.

DISCUSSION

In the hydrolysis of sugars by enzymes *in vitro* there is always some doubt as to what part bacteria may have played in bringing about the final result, even when toluene was present; this is specially the case when the reaction is allowed to proceed for more than a few hours. It is probable, however, that bacteria cannot have materially influenced the result recorded above, for their action would tend to remove the product formed, glucose being fermented readily by many micro-organisms. That the product isolated was glucose cannot seriously be doubted, bearing in mind that glycogen was the substrate: the reducing power and molecular weight were those of a reducing hexose sugar. As is always the case with glucose, even when impure, the confirmatory evidence of the phenylosazone was easy to obtain. Some reasons however must be sought for the difficulty in purification, the refusal to crystallize from methyl alcohol and the high percentage of carbon. The sample submitted for combustion may have suffered some loss of water, but an additional explanation may be found in the values for the specific rotation, which are higher than those of glucose. The initial figure need not be considered, for it is very dependent on the interval which elapses between solution and the first reading: the equilibrium figure however differs from the theoretical value 62 by at least twice the probable experimental error of the observer. It is possible that traces of another sugar, or sugars, were responsible for these results: the only ones which might have been present are maltose and Barbour's trisaccharide; both have a greater rotation than glucose; both are richer in carbon than is glucose hydrate; $C_{12}H_{22}O_{11}$, H_2O requires C, 40.00, H, 6.66%; $C_{18}H_{32}O_{16}$ requires C, 42.86, H, 6.35%. To obtain further information on this point, three porous tiles used for draining the crude glucose crystals were extracted several times with water: the united extracts were then evaporated as completely as possible over sulphuric acid. In the absence of any sign of crystallization the syrup, which weighed 0.2 g., was taken up in a small quantity of water and heated with excess of phenylhydrazine and acetic acid for 40 min. The phenylosazone which precipitated from the hot mixture was separated and recrystallized; yield 44 mg., m.p. 208–210°. The filtrate on cooling yielded a crystalline deposit which appeared to

consist largely of glucosazone. This was separated, digested at 100° with a small quantity of water and filtered. A small amount of precipitate was obtained, with none of the microscopic appearances of maltosazone, but consisting entirely of star-shaped aggregates of needles, as described by Barbour. The product was dried, and the whole yield (2.8 mg.) was submitted to analysis (Schoeller). Found: N, 8.05 %, which agrees closely with the theoretical figure for the phenylsazone of a trisaccharide; $C_{30}H_{42}O_{14}N_4$ requires N, 8.21 %. The failure to obtain any evidence for the presence of maltose is interesting, in view of the results of Carruthers & Lee [1935] with skeletal muscle, and suggests that the glycogenase of heart muscle is capable of forming glucose from glycogen without the aid of maltase. In the absence of definite information whether an alkaline lead salt is an efficient precipitant for Barbour's trisaccharide, it is impossible to decide whether the glycogen was almost entirely converted into glucose: probably the small yield of glucose was due more to the losses attendant on the method and to the fact that glucose is a sugar isolated and purified with difficulty, than to the formation of a considerable amount of trisaccharide which escaped precipitation. Experiments have shown that the trisaccharide raffinose may be readily removed from solution by the method adopted.

SUMMARY

The end-product of the action of a glycerol extract of heart muscle on glycogen is glucose. Evidence was obtained which indicated that the formation of a trisaccharide may be an intermediate stage in the hydrolysis, but no disaccharide was detected. It is suggested that the complete conversion of glycogen into glucose may be effected by the glycogenase of heart muscle.

It is a pleasure to express my indebtedness to Profs. G. A. Clark and H. S. Raper at different stages of the work. The polarimeter readings were taken in the Department of Physiology, University of Manchester.

REFERENCES

- Barbour (1929). *J. biol. Chem.* **85**, 29.
 Carruthers (1935). *J. biol. Chem.* **108**, 535.
 — & Lee (1935). *J. biol. Chem.* **108**, 525.
 Evans, de Graff, Kosaka, Mackenzie, Murphy, Vacek, Williams & Young (1933). *J. Physiol.* **80**, 21.
 Hunter & Dauphinee (1924). *Proc. roy. Soc. B*, **97**, 209.
 Winter (1930). *Biochem. J.* **24**, 851.