CXXV. OBSERVATIONS ON THE CHEMISTRY OF LIVER GLYCOGEN.

BY DAVID JAMES BELL¹ AND FRANK GEORGE YOUNG².

*From the Physiological Laboratory, Marischal College, the
University of Aberdeen.*

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THE means of identification of glycogen at the investigator's disposal are of necessity limited by the physical nature of the material; indeed the identity of glycogens prepared from different sources has never been satisfactorily established. Cremer [1894] and Clautriau [1895] found values for the specific rotation of yeast glycogen of $+198.9$ and $+184.5^\circ$ respectively. Harden and Young [1902; 1912] found values between $+191$ and $+203^\circ$ for glycogens prepared from oysters, rabbit-liver and yeast; in addition, the colour developed with iodine varied in tint according to the source. Norris [1913] followed the course of hydrolysis of a number of specimens of glycogen by pig pancreatic amylase and observed that his materials were hydrolysed at different rates when the optimum p_H of the reaction was taken into consideration.

The recent work of Donhoffer and Macleod [1932] has suggested that the glycogen in the livers of fasted rabbits may differ in its physiological behaviour from that in the livers of fed animals, and it is of interest to know whether this physiological differentiation is associated with a difference in chemical properties. We have therefore endeavoured by carefully controlled methods to determine satisfactory criteria for a standard type of glycogen derived from the livers of fed rabbits and then to apply our data to specimens obtained under some different physiological conditions and from various sources. This paper is concerned solely with glycogens obtained from livers.

The criteria which we finally adopted were:

- (1) Specific rotation in water.
- (2) Reduction of the Hagedorn-Jensen ferricyanide reagent.
- (3) Colour developed with dilute iodine solution, and opalescence of solution.
- (4) Ash content.
- (5) Polarimetric observations during the course of hydrolysis by dilute mineral acid.

GLYCOGEN FROM FED RABBITS.

Isolation.

The methods available fall into two types, *viz.* those in which the glycogen is extracted from the tissues by water and those in which the tissues are completely dissolved by strong alkali. Pflüger [1904] and the earlier workers concluded, with the methods then available, that glycogen was not affected by treatment with hot 30 % KOH solution, but this has been recently questioned; for example Haworth and Percival [1932] find that glycogen has a lower reducing power towards hypiodite than they would infer from the properties of their

¹ Carnegie Teaching Fellow.

² Beit Memorial Fellow.

postulated structural formula. They suggest that the reducing groups originally present in the glycogen molecules have been partly oxidised during treatment with strong alkali. We have therefore prepared, by methods of both types, *i.e.* aqueous extraction and alkali-extraction, a number of specimens of glycogen from the livers of fed rabbits, these specimens being subsequently subjected to examination under parallel conditions.

"Aqueous extracted glycogen" was obtained by the following method, which was essentially similar to that described by Carruthers [1931]. The livers, removed whole from three to five decapitated animals, were immediately plunged into about twice their volume of boiling water and the whole was heated to boiling for 5 minutes during which time the tissue was chopped into small pieces. The solid was then ground with sand and boiled with the original liquors for 30 minutes; it was then strained off and twice re-extracted with boiling water for 30 minutes. The united extracts were cooled, 40 % trichloroacetic acid solution was added until the final concentration was 4 %, and the whole was allowed to stand at 0° for 15–18 hours. The precipitated protein was centrifuged off and the glycogen precipitated by alcohol (2.5 vol.). The crude material was twice reprecipitated from water by alcohol in the presence of a little ammonium acetate as recommended by MacDowell [1927], when the glycogen separated as a pure white powder.

The "alkali-extracted glycogen" was obtained by direct alcoholic precipitation of the alkaline tissue hydrolysate from the action of 30 % KOH solution for 3 hours at 100°, using 3 to 5 livers for each specimen. As before, the crude product was twice reprecipitated from water by alcohol in the presence of ammonium acetate, but here the material invariably showed a faint brownish tinge.

As a final process of purification all the specimens were dialysed for 100 hours in running water in the presence of thymol. Dialysis was effected in collodion tubes prepared according to the directions of Wright *et al.* [1928]. The foregoing procedure yielded products which were nitrogen-free and did not reduce Fehling's solution.

In order to investigate the specific rotation, reducing power, *etc.*, the material was dried over calcium chloride at 100°/0.1 mm. until constant weight was attained. This required about 2 hours. No "denaturation" such as was observed by Sayhun and Alsberg [1930] took place during the drying of our specimens.

Table I.

Specimen	$[\alpha]_D^{18}$ $c=0.5$ $l=1.0$	Reduction (glucose = 100)	Iodine coloration (deep brownish red) and opacities were identical
Alkali-extracted			
1	+ 191.5°	3.30	
2	199.0	0.24	
3	191.0	1.94	
4	192.7	0.08	
5	193.5	0.15	
	Mean	1.14	
Water-extracted			
6	192.7	0.21	
7	191.0	0.40	
8	191.0	0.37	
9	194.1	0.60	
10	199.0	0.27	
11	195.8	0.09	
	Mean	0.32	

Solutions in water, of concentration approximately 1.3 %, were prepared and portions diluted to 0.5 % for observations on rotation and reduction.

The results are given in Table I.

From the foregoing results it will be seen that the observed specific rotations of our specimens varied between the limits $+191.0$ and $+199.0^\circ$, in both types of material. The alkali-extracted specimens showed considerable irregularity in reducing power, the average value being considerably greater than that of those prepared by aqueous extraction.

Hydrolysis of liver glycogen by aqueous acid.

The polarimetric behaviour on hydrolysis by aqueous acid was then investigated. To suitable amounts of the 1.3 % solutions small quantities of concentrated hydrochloric acid were added, sufficient to give a final concentration of 2.4 % HCl. The initial rotation was observed in a 1 dm. tube, and then the solution was heated at 100° . Observations were made at frequent intervals, using a 2 dm. tube; it was noticed that with both types of preparation the solution became clear after about 15 minutes, but while the solutions from the aqueous extracted material, when clear, were absolutely colourless, those of the alkali-extracted preparations showed a distinctly yellow tinge. On plotting rotation-time curves, a striking difference emerged between the curves derived from the two groups of specimens. Whereas the water-extracted material showed a perfectly regular and smooth curve in each case (Fig. 1), the alkali-extracted specimens, without exception, displayed varying, yet considerable, inflections during the first hour of hydrolysis (*e.g.* Fig. 2).

Fig. 1.

Fig. 2.

Fig. 1. Hydrolysis curve of specimen 8 (prepared by water process).
Concentration of glycogen, 1.3 %.

Fig. 2. Hydrolysis curve of specimen 2 (prepared by KOH process).
Concentration of glycogen, 1.3 %.

The effect of further treatment with KOH.

Specimens 6 to 10 inclusive (see Table I) were united and reprecipitated from water by alcohol to form specimen A. Amounts of this material, and also quantities of specimens 2 and 3, were heated as 5 % solutions in 30 % KOH for 2 hours, precipitated and dialysed as before.

No change in iodine coloration or opacity could be detected and no loss in material occurred. The results are given in Table II.

Table II.

Specimen	$[\alpha]_D^{18^\circ}, c=0.5, l=1$		Reduction	
	Before KOH	After KOH	Before KOH	After KOH
2	+199.0	+200.0	0.24	0.29
3	191.0	198.4	1.90	0.46
A	195.8	198.1	0.09	0.10

No significant change had occurred in the specific rotation, while the reducing power was considerably diminished where it had been initially large (specimen 3).

As before on hydrolysis by aqueous acid (2) and (3) showed marked inflection of the rotation-time curves, while (A), which had previously given a smooth hydrolysis curve, now exhibited an inflected one (Fig. 2).

The effect of precipitation from water by acetic acid.

Claude Bernard [1877] stated that glycogen could be precipitated from water by the addition of acetic acid, an observation which does not seem to have attracted the attention of later workers.

We have found that glycogen is precipitated quantitatively in 80 % acetic acid solution and have applied this to the estimation of glycogen in tissue, without, however, finding any particular advantage associated with its use in this connection. Nevertheless, precipitation with 80 % acetic acid provides an excellent means of freeing glycogen from the inorganic material with which it is normally associated. Thus we have found that, employing this process, it is possible after four precipitations to obtain glycogen with an exceedingly small ash-content, and in some instances we have prepared ash-free specimens, without having recourse to elaborate methods of electro-dialysis, *etc.* Table III gives some typical results:

Table III.

Specimen	$[\alpha]_D^{18^\circ}$		Reduction		Ash content	
	Initial	After pptn. by acetic acid	Initial	After pptn.	Initial	After pptn.
(KOH)						
2	+199.0°	+196.7°	0.24	0.11	—	0.195
3	191.0	190.7	1.90	0.06	—	0.224
P	—	196.9	—	0.09	—	0.090
(Water)						
A	195.8	197.0	0.09	0.09	0.036	Nil
11	—	196.5	—	0.09	—	Nil

It will be apparent that this method of purification afforded a series of products displaying closely similar properties; indeed, bearing in mind the nature of glycogen and the difficulties attendant on the determination of its constants, we feel justified in regarding our purified specimens as consisting of a chemical individual. The small residual ash of the alkali-extracted specimens appeared to consist chiefly of silica, presumably introduced during the treatment with strong alkali in a glass vessel.

The effect of ions on the specific rotation.

Neither H nor OH ions, nor the presence of boric acid or borate, were found to affect the rotation of any of our specimens.

The presence of iron in glycogen.

After wet-ashing with pure nitric acid minute traces of iron were detected in most of the purified specimens. In two instances, specimens 8 and 9, the thiocyanate test, which was employed throughout, gave negative results even before the materials had been purified by acetic acid. From this evidence we conclude that iron does not form an essential part of the glycogen complex.

Aqueous acid hydrolysis of glycogen precipitated by acetic acid.

Carrying out polarimetric observations as before, we found that, whether water- or alkali-extracted material was under investigation, in every case the rotation-time curves were identical and uninflected, of the type of Fig. 1. This suggested that the inflections observed in the specimens which had been, at one time or another, treated with strong alkali, originated from the action of the latter substance on the ash soluble in acetic acid. To investigate this view, specimens purified by precipitation with acetic acid were subjected to the action of 30 % KOH as described earlier. On hydrolysis of these materials by aqueous acid no inflection could be detected in the rotation-time curves, thus confirming the above suggestion.

The evidence thus afforded shows that, when free from acid-soluble ash, the rotation-time curves of glycogen obtained by both methods of extraction are identical. We are thus finally able to state that, in so far as we are able to determine, strong alkali does not produce any chemical alteration in the carbohydrate.

GLYCOGEN FROM FASTED RABBITS.

The material was prepared by the aqueous extraction method and was obtained in a yield of 1.83 % of the initial liver weight. In Table IV details are given of the usual constants determined before and after precipitation from acetic acid solution.

Table IV.

	Before reprecipitation	After reprecipitation
$[\alpha]_D^{17}$	+189.0°	+196.0°
Reducing power	0.26	0.19
Ash content %	0.66	0.13
Iodine coloration	Normal	Normal

On hydrolysis by aqueous acid the material yielded a smooth curve of the type of Fig. 1. These results are thus in no way significantly different from those obtained with the glycogen from fed animals.

RAT-LIVER GLYCOGEN.

The livers of fed rats were extracted with water by the method described in the section on rabbit-liver glycogen. The crude material was not examined but was purified by three precipitations from acetic acid (Table V).

Table V.

$[\alpha]_D^{17}$	+196.0°
Reducing power	0.08
Ash content %	0.067
Iodine coloration: identical with that given by rabbit-liver glycogen	

This specimen of glycogen was indistinguishable from the polysaccharide obtained from rabbit-liver, with the exception of a possible slightly greater opacity in aqueous solution.

FISH-LIVER GLYCOGEN.

Our material was obtained from fish of the genus *Gadus*, *G. morrhua* (cod), *G. aeglefinus* (haddock) and *G. merlangus* (whiting). The authors are indebted to Mr James Sharp, of the Torry Research Station of the Dept. of Scientific and Industrial Research, for a generous supply of crude material prepared from livers freshly removed from live fish in the following manner. The livers were digested with water at 70° and acetic acid added till the p_H was 4.5. After standing for some time the aqueous layer was run off from the supernatant oil and concentrated to small bulk, and the glycogen was precipitated by the addition of two volumes of 96 % alcohol.

The crude material, as we obtained it, contained much oil and protein. It was therefore exhausted first with boiling spirit, then with carbon tetrachloride. The residue was extracted four times with boiling water, the extract cooled and saturated with picric acid [Pertree and Alsberg, 1929]. The precipitated protein was centrifuged off and the yellow opalescent solution made up to 70 % alcohol by the addition of spirit. The precipitate was collected on a filter and washed with spirit till free from picric acid. It was then dissolved in water and treated with trichloroacetic acid as described above for rabbit-liver glycogen.

Carrying out experiments under conditions parallel with those on the material from rabbit liver we obtained the results given in Table VI.

Table VI.

	Initial	After KOH	After acetic acid
$[\alpha]_D^{17}$	+196.0°	+194.2°	+196.6°
Reducing power	0.35	0.14	0.16
Ash content %	0.127	0.147	0.028

On hydrolysis by aqueous acid the material behaved exactly as the water-extracted material from rabbit-liver. The reducing power appeared to be slightly higher but we cannot yet say whether this is significant. The coloration produced by iodine was, however, distinctly different, being a deep yellow-brown.

Examination of the products of hydrolysis by aqueous acid.

In order to determine the nature of the units composing the complex of fish-liver glycogen, we decided to employ the process of direct hydrolysis of the polysaccharide by aqueous acid, rather than the now customary procedure of treating the acetate with methyl alcoholic hydrogen chloride and examination of the resulting glucosides [cf. Irvine and Hirst, 1924]. The reason for this choice lay in the fact that the yield of acetate obtainable from our material under the mildest conditions [Haworth and Percival, 1932] did not exceed 90 %, thus leaving a not inconsiderable portion of the material unaccounted for. As will be seen our final yield of hydrolysis product consisted of pure glucose amounting to 93.6 % of the starting material.

7.517 g. of purified glycogen were subjected to hydrolysis by 2.5 % aqueous hydrochloric acid. The rotation reached a constant value of +61.3° for the Hg green line after 5 hours. The acid was neutralised with silver carbonate, the solid removed by filtration and colloidal silver eliminated from the filtrate by treatment with hydrogen sulphide and norite. On evaporating the clear solution to

dryness *in vacuo* and drying to constant weight over P_2O_5 , 7.818 g. of a light yellow glass were obtained, corresponding with a yield of hexose of 93.6 % of theory.

The crude material was dissolved in spirit and concentrated to a thick syrup under reduced pressure at 50° ; the material then crystallised spontaneously. The whole was taken to dryness and recrystallised from 92 % alcohol. Several crops of crystals, amounting to nearly 92 % of the crude material, were collected, all showing identical microscopic appearance (small prisms), M.P. $144-145^\circ$. Mixed melting-points with authentic α -glucose (M.P. $144-146^\circ$) showed no depression. The united crystals showed in water the typical mutarotation curve of α -glucose.

The mother-liquors were taken to dryness under reduced pressure; the residue again crystallised spontaneously, as before, in small prisms, contaminated by a slight trace of a yellowish syrup. The M.P. was 127° . The rotation in water was considerably lower than that of the pure crystals, but the course of the downward mutarotation was identical with that of glucose. This portion of the investigation is detailed in Table VII.

Table VII.

Material	M.P.	Specific rotation	
		Initial (extrapolated)	Final
α -Glucose	$144-146^\circ$	$+110^\circ$	$+52.70^\circ$
Pure crystals (92 %)	$144-146^\circ$	$+110^\circ$	$+52.59^\circ$
Residual material (8 %)	127°	$+77^\circ$	$+52.78^\circ$

There is thus no evidence of any units other than anhydroglucose being concerned in the structure of glycogen from this source.

DISCUSSION.

From the results of our experiments on the action of strong KOH on highly purified glycogen, prepared in the absence of alkali, we are of the opinion that the carbohydrate is not chemically affected by the treatment. Thus, the suggestion of Haworth and Percival [1932] that alkaline treatment might oxidise a proportion of the terminal reducing groups present in the glycogen chains is in no way substantiated. Indeed, it appears to us that if the suggestions of these workers were correct one would expect to find gluconic acid derivatives among the fission products of their trimethylglycogen.

The observations of previous workers have shown considerable variation in the properties of their preparations of glycogen. We believe that our method of purification, involving precipitation by acetic acid, affords specimens displaying properties as constant as might be expected to be shown by a substance of the physical nature of glycogen. The specific rotations agree closely with one another, the ash contents are low, or zero, when precautions are taken to exclude contamination by silica *etc.*, and, in those specimens examined, the total phosphorus contents were found to be of a low order. The results obtained by previous observers are compared with those of the present authors in Table VIII.

The polarimetric behaviour of our materials on hydrolysis by aqueous acid shows that this process proceeds evenly throughout, there being no evidence of "breaks," which might be expected to occur if the reaction proceeded in different stages. The very closely agreeing figures which we obtained for these observations tend to support the view that our specimens consisted of the same individual

Table VIII. *Liver glycogens.*

Authors	Source	$[\alpha]_D$	Method of purification	% Ash	% P_2O_5
Gatin-Gruzewska [1904]	Dog	+197°	16–18 pptns. from alkali	Nil	—
Harden and Young [1902]	Rabbit	+191°	—	—	—
Norris [1913]	Rabbit	—	Dialysis	0.51	—
”	Dog	—	”	0.38	—
Sayhun and Alsberg [1930]	Rabbit	—	Precipitation	0.20	0.032
Haworth <i>et al.</i> [1929]	?? (Kahlbaum)	+191°	Electrodialysis	0.37	0.14
Present authors	Rabbit (fed)	+196°	Pptn. by acetic acid	Nil	0.107
”	Rabbit (fasted)	+196°	”	0.130	—
”	Rat	+196°	”	0.067	—
”	Fish	+196°	”	0.028	—

substance. The dissimilarity in iodine colorations developed by mammalian and fish glycogens, although distinct, may not be associated with profound differences in chemical structure.

Further work in this field is at present in progress.

SUMMARY.

1. Criteria for liver glycogen have been determined, *viz.* specific rotation, reducing power, ash content, coloration with iodine and polarimetric behaviour on hydrolysis by aqueous acid.

2. Judged by these criteria, the liver glycogens from the following sources are not significantly different: fasted rabbits, fed rabbits, fed rats and fish of the genus *Gadus*.

3. Glycogen may be obtained in a highly purified, ash-free state by precipitation from water by acetic acid.

4. The chemical properties of highly purified glycogen are not altered by treatment with 30 % KOH at 100°.

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