Both glycogen and amylopectin are branched chain polyglucosides. A differentiation has most often been made on the basis of differences in the degree of branching of the two polysaccharides. For large molecular weight, highly ramified structures like glycogen and amylopectin, the number of branch points may be considered to be equal to the number of terminal, non-reducing glucose residues (end-groups). End-group analysis has thus provided a means for determining the average frequency or degree of branching for such polysaccharides.

The end-group assay value (EAV), here defined as the average number of glucose units per end-group, approximates 11 to 12 for glycogen (2-5). The corresponding value for amylopectin ranges from 20 to 30 (2, 3, 5), depending on the source. From these figures it is seen that glycogen is more highly branched than amylopectin since, as defined, the EAV is inversely related to the degree of branching. Indeed, glycogen has been described in this sense (6) as resembling the limit dextrin obtained from amylopectin by the action of \( \beta \)-amylase.

In this light the isolation of an "abnormal" glycogen from rabbit liver by Haworth et al. (3) is of considerable interest. The abnormal glycogen yielded an EAV of 18, a value close to the range commonly associated with the amylopectins. A study of the conditions which give rise to the "abnormal" glycogen led Bell and coworkers (7, 8) to conclude that rabbit liver glycogen may occur in either of two forms, the "normal" 12 unit structure or the "abnormal" 18 unit structure. Of the several carbohydrates tested as glycogen precursors only sucrose and galactose were found to give rise to the "abnormal" glycogen.

Glycogen synthesis is generally assumed to be the resultant of the action of at least two enzymes, phosphorylase and the "branching" enzyme. It is not unreasonable to believe that these enzymes differ in their Michaelis-
Menten constants, enzyme-substrate complex decomposition constants, and in their absolute amounts. From this it would appear that a change in concentration of the specific substrate utilized by phosphorylase and the branching enzyme would serve to favor the action of one or the other of these enzymes. Experimentally, such a condition can be induced by administering different carbohydrates to serve as the precursors for this substrate. Unless in every case the utilization of the substrate is the rate-limiting step in glycogen synthesis from carbohydrate, the change in substrate concentration, which is a function of both the type and level of carbohydrate administered, would reflect itself in the production of glycogens with different branching characteristics. Furthermore, by a proper selection of such conditions, the production of not one or two discrete glycogens but a spectrum of glycogens would be expected, some perhaps with EAV values comparable to those of the amylopectins. This would be true whether glycogen synthesis is visualized as occurring through a coupling of straight chains onto a main structure to form the branches or whether branches arise from the main stem by budding (1,6 linkage) and subsequent addition (1,4 linkage) of glucose units one at a time.

Experiments were therefore designed to verify the work of Bell and to test the above hypotheses. The extent to which each has been accomplished may be seen from the results.

EXPERIMENTAL

Glucose, galactose, and fructose were administered to fasted rabbits by stomach tube and by continuous intravenous infusion. Analysis for end-groups was then carried out on the purified, liver glycogen isolated from these animals and from a group of normal controls. The characteristics of glycogen deposited in the livers of rabbits maintained on a diet of sucrose and carrots were also studied.

The experimental procedures employed in connection with the treatment of the animals, the levels of carbohydrate administered, and the isolation and purification of the glycogens are essentially those used by Bell et al. (7, 8). However, the results obtained are at gross variance with those of Bell et al., and, since the explanation for these differences is not entirely clear and may perhaps reside in some deviation from Bell’s procedures and method for end-group analyses, a description of the methods used in the present study is presented.

Experimental Animals—Studies were carried out with eight groups of
rabbits, forty-six rabbits in all. All the animals were maintained in the colony on a stock diet for at least 1 week before use to establish their normalcy (maintenance or gain in weight, freedom from infection). So far as was possible, the animals were distributed equally, in terms of weight and sex, among the groups. The animals were fasted for 48 hours to deplete existing glycogen stores² before the test diets were administered. A series of unfasted control animals represented the normal group.

Administration of Carbohydrates—Glucose, galactose, and fructose were tested for their influence on glycogen structure. The carbohydrates were administered as 25 per cent solutions (weight of anhydrous sugar per volume of solution) to the fasted animals either by stomach tube at a level of 2.5 gm. per kilo or by continuous intravenous infusion of 4 hours duration at body temperature at a level of 4.6 gm. per kilo per hour.

The control animals were mock stomach-fed with water to simulate any nervous response due to handling. However, experiments in which the mock feeding was omitted revealed no difference in the type of glycogen deposited in these controls.

Under the infusion conditions employed, the animals exhibited no signs of nervousness or distress. It was thus possible to approximate the normal state of the animal and avoid the uncertain influence of anesthetics on carbohydrate metabolism.

An experiment was also carried out in which feeding ad libitum for 1 week on a diet of sucrose and carrots was permitted. A diet of this type was reported by Bell (10) to give rise to the 12 unit type of glycogen.

Isolation of Glycogen—4 hours after stomach feeding or initiation of infusion the animals were anesthetized (nembutal), and the livers excised, rinsed, and blended with 2.5 volumes of cold 5 per cent trichloroacetic acid. The time from anesthetization to blending was 5 to 10 minutes. After blending for 3 to 5 minutes, the preparations were centrifuged for 30 minutes, filtered, and the glycogen precipitated by the addition of 1.1 volumes of 95 per cent ethanol. The mixture was centrifuged for 30 minutes and the glycogen washed twice with 95 per cent ethanol by centrifugation, collected on a Buchner funnel, and subsequently dried in air. All steps were carried out in the cold.

Purification of Glycogen—Preliminary studies indicated that a combination of the acetic acid purification procedure of Bell and Young (11) and alcohol precipitation was most satisfactory for the preparation of glycogen "free" from phosphorus. This method was subsequently used in all the experiments.

The crude glycogen preparations from a group were pooled and dissolved in water to give a 3 per cent solution. Precipitation of the glycogen was carried out as above, but no ultracentrifugation was employed. The glycogen was washed twice with 95 per cent ethanol by centrifugation, collected on a Buchner funnel, and subsequently dried in air.

² The fasting level was approximately 0.2 gm. of glycogen per 100 gm. of liver.
LIVER GLYCOGEN. I

gen was effected by addition of glacial acetic acid to a final concentration of 80 per cent. The precipitate was separated by centrifugation. Three such precipitations from aqueous solution by acetic acid were carried out, followed by precipitation with 1.5 volumes of 95 per cent ethanol and two washings each with 95 per cent and absolute ethanol in the presence of about 0.5 mg. of ammonium acetate. The glycogen was then dried over CaCl₂ or H₂SO₄ in a vacuum desiccator and finally in air.

The recovery of glycogen purified by this process was 94 to 97 per cent by weight.

Estimation of Purity of Glycogen—Triplicate samples of each glycogen were analyzed for reducing sugar after hydrolysis in 1 N HCl. Preliminary study showed that hydrolysis was complete in 2 hours and that destruction of glucose by this treatment did not occur.

The “moisture” content of the glycogens, determined by noting the loss in weight after heating at 73° in vacuo for 18 hours, provided the basis for interpreting the reducing value data in terms of purity. The significance of this point will be treated in the discussion.

Phosphorus analyses by the method of Sumner (12) were carried out in duplicate on wet-ashed samples of glycogen from glucose-fed and galactose-fed rabbits. The value of 0.006 per cent phosphorus obtained for each of these glycogens indicates the uniform relative freedom from phosphorus which is attainable by this purification procedure.

Evidence for the absence of significant quantities of reducing substances of low molecular weight in the glycogen preparations will be presented in a later communication.

End-Group Assay—The procedure of Potter and Hassid (13), with slight modification, was used to determine the EAV of the purified glycogens. 100 mg. samples (uncorrected for “moisture” content) were analyzed. All analyses were carried out in duplicate; the reproducibility in every case was within 1 per cent.

RESULTS AND DISCUSSION

The EAV values for the glycogens prepared in this study are summarized in Table I in descending order. EAV data found for samples of glycogen and amylopectin obtained from other laboratories are likewise presented. Values from the literature for the comparable glycogens and amylopectins are included for comparative purposes.

It may be seen that arranging the EAV values in descending order of

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Treatment of the reaction mixture with ethylene glycol for 5 minutes instead of 1 hour was found to be adequate for destruction of the excess periodate. Methyl red-methylene blue indicator was used and the titration with NaOH was carried to approximately pH 6 to insure complete titration of formic acid.
magnitude shows the values for glycogen obtained after infusion of carbohydrate to be greater than those obtained after administration by stomach tube. These latter in turn display an EAV greater than that for the glycogen from animals fed rabbit pellets or sucrose and carrots.

**Table I**

*Influence of Carbohydrate on Branching Properties of Rabbit Liver Glycogen*

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Carbohydrate administered</th>
<th>Route</th>
<th>No. of animals</th>
<th>End-group assay value</th>
<th>End-group assay value from literature</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Fructose</td>
<td>I.</td>
<td>2</td>
<td>23.2</td>
<td>11-12 (8)</td>
</tr>
<tr>
<td>II</td>
<td>Galactose</td>
<td>&quot;</td>
<td>3</td>
<td>22.2</td>
<td>12 (8)</td>
</tr>
<tr>
<td>III</td>
<td>Glucose</td>
<td>&quot;</td>
<td>2</td>
<td>22.2</td>
<td>11-12 (8)</td>
</tr>
<tr>
<td>IV, a</td>
<td>Fructose</td>
<td>F.</td>
<td>3</td>
<td>21.9</td>
<td>11-12 (8)</td>
</tr>
<tr>
<td>&quot; b</td>
<td></td>
<td>&quot;</td>
<td>2</td>
<td>21.9</td>
<td>11-12 (8)</td>
</tr>
<tr>
<td>V</td>
<td>Glucose</td>
<td>&quot;</td>
<td>10</td>
<td>20.5</td>
<td>12 (8)</td>
</tr>
<tr>
<td>VI</td>
<td>Galactose</td>
<td>&quot;</td>
<td>10</td>
<td>20.1</td>
<td>18 (7)</td>
</tr>
<tr>
<td>VII, a</td>
<td>Normal</td>
<td>D.</td>
<td>8</td>
<td>19.4</td>
<td>12 (3, 14)</td>
</tr>
<tr>
<td>&quot; b</td>
<td></td>
<td>&quot;</td>
<td>2</td>
<td>19.1</td>
<td>12 (3, 14)</td>
</tr>
<tr>
<td>&quot; c</td>
<td></td>
<td>&quot;</td>
<td>2</td>
<td>19.5</td>
<td>12 (3, 14)</td>
</tr>
<tr>
<td>&quot; d</td>
<td>(KOH)</td>
<td>&quot;</td>
<td>2</td>
<td>19.3</td>
<td>12 (3, 14)</td>
</tr>
<tr>
<td>VIII</td>
<td>Sucrose and carrot</td>
<td>&quot;</td>
<td>2</td>
<td>18.9</td>
<td>12 (10)</td>
</tr>
<tr>
<td>IX, a</td>
<td>Normal rabbit liver glycogen A</td>
<td></td>
<td>17.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&quot; b</td>
<td></td>
<td>&quot;</td>
<td>18.0</td>
<td>18*</td>
<td></td>
</tr>
<tr>
<td>X</td>
<td>dog liver glycogen</td>
<td></td>
<td>11.9</td>
<td>12 (17)</td>
<td></td>
</tr>
<tr>
<td>XI</td>
<td>Oyster glycogen</td>
<td></td>
<td>11.9</td>
<td>11 (16)</td>
<td></td>
</tr>
<tr>
<td>XII, a</td>
<td>Amylopectin A</td>
<td></td>
<td>22.9</td>
<td>23 (13)</td>
<td></td>
</tr>
<tr>
<td>&quot; b</td>
<td></td>
<td>&quot;</td>
<td>25.2</td>
<td>26 (13)</td>
<td></td>
</tr>
<tr>
<td>&quot; c</td>
<td></td>
<td>&quot;</td>
<td>27.3</td>
<td>27 (13)</td>
<td></td>
</tr>
</tbody>
</table>

The carbohydrates were administered at a level of 4.6 gm. per kilo per hour for the infused (I.) animals and 2.5 gm. per kilo for the stomach-fed (F.) animals. The rabbits in Experiment VII were maintained on a diet (D.) of rabbit pellets ad libitum; those in Experiment VIII, on sucrose and carrots. The EAV values in Experiments IX, b, X, and XII were obtained on samples of the original preparations for which values from the literature are cited. The glycogen in Experiment VII, d was extracted from liver with KOH. All others were extracted with trichloroacetic acid.

* Hassid, W. Z., personal communication.

Further, it may be noted that the EAV values for "normal" glycogen found in this study are considerably higher than those reported by Bell and others (2, 3, 10, 14). The previously reported normal EAV is about 11 to 12, while that found in this study is about 19, a value generally considered to represent the "abnormal" glycogen. Similarly, the high EAV for glycogen from glucose-infused, glucose-fed, fructose-infused, fructose-fed
animals, and those fed the sucrose and carrot diet contrasts markedly with values previously reported. Thus, in none of the forty-six rabbits studied under different experimental conditions, including the normal, was the more highly branched, 11 to 12 unit glycogen obtained.

A satisfactory explanation for the difference in EAV reported here and by Bell is not yet available, though study continues. However, the following arguments are put forth to suggest that the differences are not attributable to poor sampling of the rabbits, the procedure for glycogen isolation, the state of purity of the glycogen, or the method for EAV determination.

So far as representative sampling of the animals is concerned, the good agreement in EAV obtained for three separate groups of normal animals (Experiment VII a, b, c) and two of fructose-fed animals (Experiment IV a, b) tends to minimize sampling as a causitive factor. The experiments were done with different lots of animals of different ages at different times. This reproducibility of EAV, even when small numbers of animals were used, further supports the significance which is attached to EAV differences between different experimental groups. The similar high EAV obtained for "normal" glycogen samples from other laboratories (Experiment IX a, b) further minimizes poor sampling as a responsible factor.

As for the procedure used in glycogen isolation, a selective isolation by trichloroacetic acid of a high EAV fraction appears unlikely on the following grounds. Samples of purified glycogen from normal rabbits showed no changes in EAV even after prolonged heating with 30 per cent KOH, a procedure commonly used for glycogen extraction. Direct extraction of glycogen from the livers with KOH yielded preparations having an EAV in good agreement with those obtained on an aliquot of the same tissue with trichloroacetic acid extraction (Experiment VII c, d). Agreement of EAV on crude and purified glycogen differed by no more than 1 unit, indicating that purification with 80 per cent acetic acid did not selectively extract or alter a glycogen fraction. Finally, rabbit liver glycogens prepared elsewhere by different processes (Experiment IX a, b) gave EAV values in the same range as those obtained in this laboratory. Thus, neither the extraction nor purification procedure employed appears to account for the differences found.

The possibility that some gross impurity in the glycogen might explain the high EAV observed is ruled out on the basis of the agreement between data obtained on a dry weight basis and those from the reducing value after hydrolysis, presented in Table II. The "moisture" content of each glycogen, as seen from Column A, is about 11 per cent. Column B shows that the non-reducing substance in each glycogen also approximates 11 per cent. It thus appears that the non-reducing substance is practically all accounted for by the "moisture," and hence the glycogens are all on the
order of 98.5 to 100 per cent pure. For an 11 unit glycogen sample to give an EAV of 19 or 23 would necessitate that about 42 and 52 per cent respectively of sample weight must be a non-reducing contaminant. The data in Table II show the glycogens to be 98.5 to 100 per cent pure. Again, the fact that the composition and purity of all the glycogens prepared are

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Glycogen preparation</th>
<th>Dry weight (A) per cent</th>
<th>Reducing power (B) per cent of theoretical</th>
<th>Average, (A) + (B) per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Fructose-infused</td>
<td>89.0</td>
<td>89.5</td>
<td>89.3</td>
</tr>
<tr>
<td>II</td>
<td>Galactose-infused</td>
<td>89.0</td>
<td>90.4</td>
<td>89.7</td>
</tr>
<tr>
<td>III</td>
<td>Glucose-infused</td>
<td>89.3</td>
<td>90.0</td>
<td>89.6</td>
</tr>
<tr>
<td>IV, a</td>
<td>Fructose-fed</td>
<td>87.9</td>
<td>88.9</td>
<td>88.4</td>
</tr>
<tr>
<td>V</td>
<td>Glucose-fed</td>
<td>89.5</td>
<td>90.0</td>
<td>89.7</td>
</tr>
<tr>
<td>VI</td>
<td>Galactose-fed</td>
<td>89.4</td>
<td>89.3</td>
<td>89.3</td>
</tr>
<tr>
<td>VII, a</td>
<td>Normal</td>
<td>88.6</td>
<td>90.0</td>
<td>89.3</td>
</tr>
<tr>
<td>VIII</td>
<td>Sucrose and carrot</td>
<td>88.1</td>
<td>89.5</td>
<td>88.8</td>
</tr>
<tr>
<td>XI</td>
<td>Oyster</td>
<td>89.0</td>
<td>89.3</td>
<td>89.6</td>
</tr>
</tbody>
</table>

The per cent dry weight of amylopectins A, B, C was found to be 89.8, 90.4, 90.5, respectively.

![Fig. 1. Periodate reaction curve for glycogen](http://www.jbc.org/)

Lastly, the apparent validity of the periodate oxidation procedure for EAV determination of glycogen is indicated by Fig. 1, which depicts a time versus EAV plot for samples of glycogen from glucose-fed and galactose-fed rabbits. The primary reaction is completed in 20 to 25 hours, by which time the curve has leveled off. The nature of the curve is
comparable to that found for amylopectin (13). A 25 hour end-point was uniformly used in all studies. What may be a slight rise beyond the 25 hour point is attributed to "overoxidation" (2, 15), but this would influence the EAV by less than 1 unit even if extrapolated to 36 hours. The fact that the curve does not fall off with time indicates that loss of formic acid by overoxidation to CO₂ is not appreciable. Such a loss of formic acid would give erroneous, high EAV values.

The application of the method to samples of amylopectin (Experiment XII a, b, c, Table I) obtained from Potter and Hassid yielded EAV in good agreement with the values found by them (13). The ability of the method to reveal a highly branched glycogen was checked by analyzing purified oyster glycogen (Experiment XI). The EAV of 11 is in accord with the values reported for mussel glycogen (16). A final test of the periodate method was the EAV determination of a sample of dog liver glycogen prepared by Hassid and Chaikoff and analyzed by them for end-groups by the exhaustive methylation technique (17). The results obtained by the two methods were the same (Experiment X).

If the foregoing evidence correctly supports the EAV data, then certain interesting conclusions emerge from the study. 4

End-group assay becomes a highly questionable criterion for differentiating glycogen from amylopectin, inasmuch as glycogens with EAV values comparable to those of amylopectins have been prepared under a variety of conditions, including the so called normal condition.

Consideration of the EAV data also shows that the degree of branching is a function both of the type of carbohydrate administered and the level at which it is administered. This is in accord with the hypothesis set forth earlier in this paper. Presumably the higher EAV found for the glycogen from infused compared with that from the corresponding stomach-fed rabbits for every carbohydrate tested reflects the higher blood level of the carbohydrate in the infused animals. Estimations of blood sugar in the case of fructose-infused and fructose-fed animals showed the relative levels to be about 4:1. Two inferences may be drawn from the EAV data:

4 The present work (Experiment X) as well as other reports (4, 18) indicates that EAV values determined by periodate oxidation (methyl red indicator) and by the methylation procedure agree reasonably well. Reports that the use of indicators in the alkaline range yields more accurate results ((19); also personal communication from Dr. C. F. Cori) may be argued for and against. In any event, since methyl red was used for analyses of some of Bell's glycogen samples as well as for the analyses of the glycogens prepared in this study, the marked differences noted above remain unchanged, though the values themselves may be only relative ones. Similarly, the finding by Cori (personal communication) that the EAV determined by an enzyme assay procedure is about 20 per cent lower than the values obtained by periodate oxidation affects only the absolute EAV; the basic discrepancies remain.
first, that the Michaelis-Menten constant for phosphorylase is greater than that for the branching factor; and second, that the rate constant for the decomposition of the enzyme-substrate complex is greater for phosphorylase than it is for the branching enzyme, or else that there is less of the branching enzyme present.

**SUMMARY**

The influence of the type of carbohydrate administered and the mode of its administration on the branching characteristics of rabbit liver glycogen has been studied. Values found by end-group assay have been found to be consistently higher than those reported for comparable glyogen preparations. The implications of the high EAV values and the progressive, rather than discrete, nature of their change are discussed.

The author wishes to thank Dr. W. Z. Hassid, Dr. A. L. Potter, and Dr. C. F. Cori for samples of glycogen and amylopectin prepared in their laboratories, and Mr. R. W. Handler for the analysis of blood sugar in these studies.

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