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Degradation of Glycogen by Alkali

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Glycogen isolated from animal tissues by extraction methods in which all contact with alkali is avoided has been shown always to be susceptible to anaerobic degradation by hot concentrated alkali. The rapid initial decrease in molecular weight has been followed by light scattering methods. The chief products formed have been identified as a polydisperse series of relatively stable polysaccharide acids, corresponding to "glycogen" isolated by classical alkali methods and small amounts of free isosaccharinic acid. The quantities of isosaccharinic acid split off from glycogen samples of known average molecular size are in the same general order as would be predicted from calculations based upon idealized molecules of similar size having the highly branched tree-like structure indicated by enzymological studies. Borohydride reduction of the terminal aldehyde group has been found to render the glycogen molecule stable to anaerobic alkali degradation. The apparent alkali stability of glycogen after preliminary exposure to alkali is due to the replacement of the reducing end of each molecule by a saccharinic acid residue.

That glycogen is relatively stable in hot concentrated alkali has been an accepted fact since the early isolation procedures were described by Bernard[1] and by Pflüger.[2] Purified samples of glycogen isolated after alkaline digestion (KOH method) are usually found to have molecular weights between 1 and 8 $\times$ 10^6 when determined by the methods of light scattering or ultracentrifugal analysis.[3-5] On the other hand, when tissues are extracted with cold trichloroacetic acid (TCA method) the average size of the glycogen isolated is usually far higher, values up to 100 $\times$ 10^6 being obtained.[6-8] Still larger particles can be obtained following aqueous extraction.[9,10] There is considerable evidence in support of the opinion that the reported molecular weights of glycogen isolated by acid methods are not artificially high[7,10,11] and that these samples are more nearly representative of native glycogen than are those isolated by the more commonly employed hot alkali methods.

The studies reported here were undertaken in an attempt to shed some light on the nature of the changes which occur in glycogen when it is treated with hot concentrated alkali, simulating the conditions used in the isolation of glycogen from tissues.

In the past few years there has been a considerable revival of interest in the effects and mechanism of action of alkalies on carbohydrates. Excellent reviews of this subject have been written by Sowden[12] and by Whistler and BeMiller.[13] The principal steps involved in the action of an alkali on the reducing portion of a carbohydrate molecule are generally accepted to be formation and ionization of an enediol, β-elimination of a hydroxyl or alkoxyl group and rearrangement of the molecule to an α-dicarbonyl form which then undergoes an internal Cannizzaro type of reaction to yield a saccharinic acid. An O-glycosyl or O-alkyl anion is more readily extruded from the sugar enediol anion than is a hydroxyl ion. The use of alkaline degradation methods for studying the structure and branching patterns of naturally occurring polysaccharides, proposed by Corbett, Kenner and Richards[14] and by Whistler and Corbett[15] has yielded many interesting results. Detailed studies of the acidic products formed from the starch-glycogen group of polysaccharides have been restricted to the unbranched component, amylose.[16] Because of the high degree of branching of amylopectin and glycogen, changes taking place during alkali degradation are quantitatively relatively slight. These changes, however, are shown here to be qualitatively similar to those occurring with other polysaccharides.

Results

Turbidity Measurements in Alkaline Solutions.—The use of alkali resistant Teflon reaction vessels has made it possible to follow the change in turbidity which occurs when polysaccharides are

TABLE I

GLYCOGEN SAMPLES

Sample	Source	Method of isolation	Subsequent treatment	Molecular wt. $\times$ 10^{-6}
I	Rabbit liver	TCA	...	34
II	Rabbit liver	KOH	...	10.7
III	Rabbit liver	TCA	...	13[c]
IV	Sample III		10 *N* NaOH[a]	5.8
V	Sample III		0.1 *N* HCl[b]	6.9
VI	Rabbit liver	TCA	0.1 *N* HCl[b]	0.91
VII	Sample VI		10 *N* NaOH[a]	.57
VIII	Rabbit liver	TCA	0.1 *N* HCl[b]	.19
IX	Sample VIII		10 *N* KOH[a]	.1[c]
X	Sample VIII		Borohydride	.19[c]

[a] At 100° for 3 hr. [b] The decrease in turbidity of the glycogen solution with time of heating at 100° in 0.1 *N* HCl was followed until, based on previous experience, approximately the desired average molecular size was reached. The time required varied between 5 and 30 minutes for the samples used. [c] Estimated molecular weights.

(1) C. Bernard, *Compt. rend.*, **44**, 578 (1857).
(2) E. F. W. Pflüger, "Das Glycogen," Bonn, Germany, 1905, p. 53.
(3) C. T. Greenwood, *Advances in Carbohydrate Chem.*, **7**, 289 (1952).
(4) C. T. Greenwood, *ibid.*, **11**, 335 (1956).
(5) D. J. Manners, *ibid.*, **12**, 261 (1957).
(6) M. R. Stetten, H. M. Katzen and D. Stetten, Jr., *J. Biol. Chem.*, **222**, 587 (1956).
(7) M. R. Stetten, H. M. Katzen and D. Stetten, Jr., *ibid.*, **232**, 475 (1958).
(8) W. A. J. Bryce, C. T. Greenwood and I. G. Jones, *J. Chem. Soc.*, 3845 (1958).
(9) A. Lazarow, *Anat. Rec.*, **84**, 31 (1942).
(10) S. A. Orrell, Jr., and E. Bueding, *J. Am. Chem. Soc.*, **80**, 3800 (1958).
(11) S. R. Erlander and D. French, *ibid.*, **80**, 4413 (1958).
(12) J. C. Sowden, *Advances in Carbohydrate Chem.*, **12**, 35 (1957).
(13) R. L. Whistler and J. N. BeMiller, *ibid.*, **13**, 289 (1958).
(14) W. M. Corbett, J. Kenner and G. N. Richards, *Chem. & Ind. (London)*, 462 (1953).
(15) R. L. Whistler and W. M. Corbett, *J. Am. Chem. Soc.*, **78**, 1003 (1956).
(16) G. Machell and G. N. Richards, *J. Chem. Soc.*, 1199 (1958).

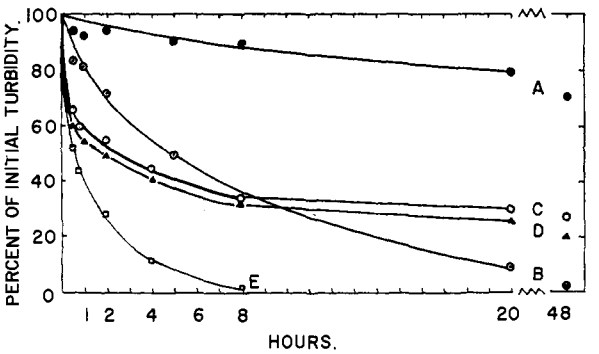

Fig. 1.—Alkaline degradation of a single sample of glycogen (TCA method, Sample I, mol. wt. 34 × 10^6) studied under various conditions: A, 10 *N* NaOH, 25°, atmosphere of air; B, 0.1 *N* NaOH, 100°, atmosphere of air; C, 10 *N* NaOH, 100°, atmosphere of nitrogen; D, 10 *N* NaOH, 100°, atmosphere of air; E, 10 *N* NaOH, 100°, oxygen bubbling through solution.

heated in alkaline solution, without resorting to isolation and purification of each sample at each point in time as was done previously.[6,7] The glycogen samples used were of various average molecular sizes, ranging from about 100,000 to 34,000,000, prepared from rabbit liver by either acidic or alkaline isolation methods as summarized in Table I.

Glycogen was heated at 100° in dust-free 10 *N* NaOH. Aliquots, removed at suitable time intervals, were added to 0.1 *M* KCl for turbidity measurements. In Fig. 1 the results obtained with a single glycogen sample (I) of average molecular weight 34 × 10^6 (TCA method) studied under different experimental conditions are plotted as per cent. of the initial turbidity of the solution against time. Each curve is the average of at least two separate runs. Curve D shows the typical rapid initial decrease, followed by a gradual continuous decline in turbidity, observed when glycogen (TCA method) was heated at 100° in concentrated alkali under normal atmospheric conditions. The solubility of oxygen in hot concentrated alkali is negligible.[17] When the same reaction was carried out in an atmosphere of N_2, using tank N_2 which had been freed of O_2 carefully, the initial decrease in turbidity observed (curve C) was almost as rapid as in air. With a stream of O_2 bubbling through the glycogen solution in hot concentrated alkali, the turbidity fell much further and after a few hours little polysaccharide remained (curve E). It thus appears that two reactions are involved. The first reaction, independent of O_2, presumably is the result of successive rapid degradative steps starting at the existent aldehyde end of the molecule. The other reaction, rapid only under O_2, is probably one of oxidation followed by splitting anywhere in the large polysaccharide molecule, producing new terminal aldehyde groups which can in turn serve as new points for rapid attack by alkali upon the molecule.[13] That heat is required for the initial rapid reaction is shown by the fact that the decrease in turbidity in concentrated alkali is very slow at room temperature (curve A).

(17) "International Critical Tables," McGraw-Hill Book Co., New York, N. Y., 1928, Vol. 3, p. 271.

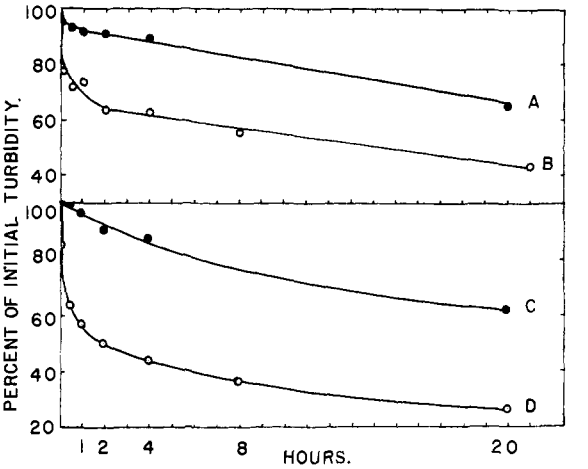

Fig. 2.—Comparison of the effect of hot 10 *N* NaOH upon glycogen samples of comparable size obtained by acidic and by alkaline isolation methods: A, rabbit liver glycogen isolated after KOH digestion, mol. wt. 5.8 × 10^6 (Sample IV); B, rabbit liver glycogen isolated after TCA extraction, mol. wt. 6.9 × 10^6 (Sample V); C, rabbit liver glycogen isolated after KOH digestion, mol. wt. 10.7 × 10^6 (Sample II); D, rabbit liver glycogen isolated after TCA extraction, mol. wt. 34 × 10^6 (Sample I). Light scattering measurements were made by removing aliquots at various time intervals and measuring turbidity after addition to dust-free 0.1 *N* KCl.

When hot dilute alkali (0.1 *N* NaOH) is employed, the initial reaction is somewhat slower than with hot concentrated alkali, but the second reaction is more prominent, persumably because of the greater solubility of O_2 in dilute alkali. The over-all breakdown of the polysaccharide thus proceeds further (curve B).

In contrast, when glycogen isolated by the KOH method was heated with concentrated alkali, a rapid initial decrease in turbidity was not observed. Figure 2 shows the results of parallel experiments with KOH glycogen (curve C) and TCA glycogen (curve D). To eliminate the possible effect of the relatively great difference in average molecular size of the samples used, two samples of glycogen with about the same molecular weight were prepared from a single rabbit liver. On treatment with hot concentrated alkali, the KOH glycogen (curve A) did not undergo the initial rapid reaction exhibited by the TCA glycogen (curve B). At later times all of the glycogen samples, regardless of the method of preparation, showed the continual slow decrease in molecular size attributed to a small amount of unavoidable oxidation.

The very rapid initial stage of the reaction was further studied by a slightly different method. Hot concentrated alkali was added to a concentrated solution of the polysaccharide in a Pyrex cylindrical cuvette and the turbidity changes measured directly without taking aliquots. Because of the compensating complications due to cooling of the solution in the cuvette and the production of light scattering material from the glass, the reaction could only be followed for a short time by this method. Figure 3 shows that

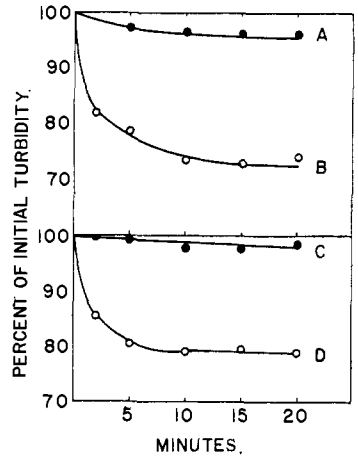

Fig. 3.—Comparison of the initial effect of hot 10 *N* NaOH on the turbidity of solutions of glycogen isolated by acidic or alkaline methods: A, 0.57 × 10^6 (alkali method) (Sample VII); B, 0.91 × 10^6 (acid method) (Sample VIII); C, 5.8 × 10^6 (alkali method) (Sample IV); D, 6.9 × 10^6 (acid method) (Sample V). Turbidity measurements were made directly upon solutions of glycogen and hot alkali reacting in a glass cuvette.

the difference in rate of alkaline degradation between glycogens of comparable size prepared by acidic and alkaline methods is immediately apparent.

Mild acid hydrolysis, splitting glucosidic bonds in a random fashion, will introduce new aldehyde groups which can serve as points of attack in subsequent alkali treatment. A sample of glycogen (TCA method), which during treatment with 10 *N* NaOH at 100° showed the degradative effect illustrated in curve D (Fig. 2), was isolated after this alkaline treatment and purified. One portion of this polysaccharide, on retreatment with concentrated alkali in exactly the same way, exhibited no rapid initial degradation. The remainder of the recovered sample was heated for a short time in 0.1 *N* HCl until the average size, measured by turbidity, was somewhat diminished. After isolation, this polysaccharide was shown to be once again subject to an appreciable rapid alkali degradation.

Characterization of Products: Isolation of Isosaccharinic Acid.—Many preliminary investigations of the products of the action of concentrated alkali on glycogen showed that an appreciable amount of the acidic compound with chromatographic properties resembling those of isosaccharinic acid $\left[\begin{matrix}\text{OH}\ \ \text{CH}_2\text{OH}\\ \diagdown\ \ \diagup\\ \text{HOOC–C–CH}_2\text{–CHOH–CH}_2\text{OH}\end{matrix}\right]$ was produced. Since the yield of saccharinic acid was relatively greater when glycogen of low average molecular weight was degraded, a sample of glycogen of molecular weight about one million was prepared from TCA rabbit liver glycogen by a mild controlled treatment with 0.1 *N* HCl. It has been observed that, as a first approximation, the α-1,4 and α-1,6 linkages of polysaccharides are hydrolyzed at a uniform rate regardless of their position in the chain or the length of the chain in which they occur.[18,19] Whereas α-1,6 linkages are somewhat less susceptible than α-1,4 linkages to acid hydrolysis, their relative scarcity in glycogen makes this difference unimportant.[19] It is therefore assumed that the random mild acid hydrolytic split to which glycogen was subjected in decreasing the average size of the molecules did not greatly change the structural pattern of the polysaccharide.

After the mild acid hydrolysis, the glycogen sample used for isolation of saccharinic acid was purified by repeated reprecipitations from aqueous solution with ethanol and was shown by chromatographic examination to be free of detectable glucose, saccharinic acids and low molecular weight oligosaccharides. It had an average molecular weight of 0.91 × 10^6 (sample VI). Four grams of this glycogen was heated at 100° in 10 *N* NaOH in a Teflon vessel for 18 hr. After removal of cations by chromatography on ion exchange resin and precipitation of the bulk of the polysaccharide by ethanol, the alcohol-soluble products were concentrated by evaporation of the solution and repeated precipitation with ethanol.

These products were separated by paper chromatography. Material eluted from the areas corresponding to the location of known isosaccharinic lactone was purified by repeating the chromatography step and by rejection from the eluate of all material insoluble in small volumes of ethanol and of ethyl acetate. The product, at first an oil, then crystalline, was recrystallized twice from ethyl acetate. Five mg., melting point 93–94°, with crystalline form identical with that of isosaccharinic lactone, was obtained. The lactone was converted to the anilide[20,21] which was found to have identical chromatographic properties with those of the anilide of isosaccharinic acid.[22,23]

Polysaccharide Acids.—Some of the material which did not migrate from the origin of the preparative chromatographic papers gave a positive test for lactones. This material, eluted with water and precipitated with ethanol, was free of monosaccharinic acids before hydrolysis, but after complete hydrolysis by dilute acid was shown chromatographically to yield, along with glucose, considerable isosaccharinic acid and some material with properties suggestive of metasaccharinic acid.

Since presumably only one monosaccharinic acid residue is liberated on hydrolysis of each polysaccharide acid molecule, the ease with which this residue can be detected varies inversely with the molecular weight of the material studied. The overwhelming amount of glucose produced on total acid hydrolysis of glycogens (KOH method) of average molecular weight larger than about a million prevented even chromatographic detection of the relatively small amount of monosaccharinic acid liberated. With samples having molecular weights in the range of a few hundred thousand, purified until completely free of the monosac-

(18) M. L. Wolfrom, E. N. Lassettre and A. N. O'Neill, *J. Am. Chem. Soc.*, **73**, 595 (1951).
(19) M. A. Swanson and C. F. Cori, *J. Biol. Chem.*, **172**, 797 (1948).
(20) B. Sorokin, *J. prakt. Chem.*, [2] **37**, 318 (1888).
(21) L. M. Utkin and G. O. Grabilina, *Doklady Akad. Nauk S.S.S.R.*, **93**, 301 (1953).
(22) J. W. Green, *J. Am. Chem. Soc.*, **76**, 5791 (1954).
(23) J. W. Green, *ibid.*, **78**, 1894 (1956).

charinic acid produced during the alkali treatment, total acid hydrolysis yielded a mixture in which, after removal of the bulk of the glucose, isosaccharinic acid readily could be demonstrated chromatographically. In control experiments with glycogen (TCA method) of comparable molecular size, acid hydrolysis liberated no saccharinic acid.

Quantitative Estimation of Isosaccharinic Acid Produced by Alkaline Degradation of Glycogens of Various Sizes.—To estimate the amount of saccharinic acid produced, 10-mg. portions of each of a series of glycogen samples (TCA method) of different average molecular size were subjected to identical conditions of alkaline degradation and separation of products. The alcohol-soluble products, after lactonization, were separated by descending paper chromatography with ethyl acetate:acetic acid:water (10:1.3:1)[24] and visualized by reaction with hydroxylamine and $FeCl_3$.[25] The quantity of isosaccharinic lactone formed was estimated by visual comparison of the lavender color with that produced from known quantities of the reference compound similarly developed and stained. The results, given in Table II, showed that the yield of isosaccharinic acid from glycogens isolated by acid methods increased as the average molecular size of the glycogen decreased.

TABLE II

QUANTITY OF FREE ISOSACCHARINIC ACID PRODUCED BY HEATING 10 MG. OF EACH GLYCOGEN SAMPLE IN 30% KOH FOR 3 HOURS

Samples prepared by acid isolation methods.

Sample	Approximate mol. wt. × 10^{-6}	Quantity of isosaccharinic acid lactone, γ	
		Produced	Calcd. from Table IV
VIII	0.19	600, 500	300
VI	0.91	100	60
V	6.9	50, 60	15
III	13	30	8
I	34	25	3

The same quantitative alkali degradation method applied to glycogens isolated by the Pflüger method or subsequently treated with concentrated alkali in the course of the preparation of the sample showed little or no free isosaccharinic acid split off. In Table III are given results obtained with glycogens of comparable size but differing in the method of preparation. Prior introduction of a terminal acidic group in the polysaccharide rendered the molecules stable to subsequent degradation by alkali. After 3 hr. of heating glycogen (KOH method) with concentrated KOH, a very small amount of free isosaccharinic acid could sometimes be detected, probably due, as in the light scattering studies, to secondary reactions resulting from the fact that the conditions were not strictly anaerobic. When hot alkali treatment was limited to 15 minutes, only the effect of the initial rapid reaction taking place from the reducing end of the molecule was evident. In the time in which appreciable isosaccharinic acid was liberated from glycogen (TCA method), none was produced from glycogen (KOH method).

(24) M. Moilanen and H. Richtzenhain, *Acta Chem. Scand.*, **8**, 704 (1954).

(25) M. Abdel-Akher and F. Smith, *J. Am. Chem. Soc.*, **73**, 5859 (1951).

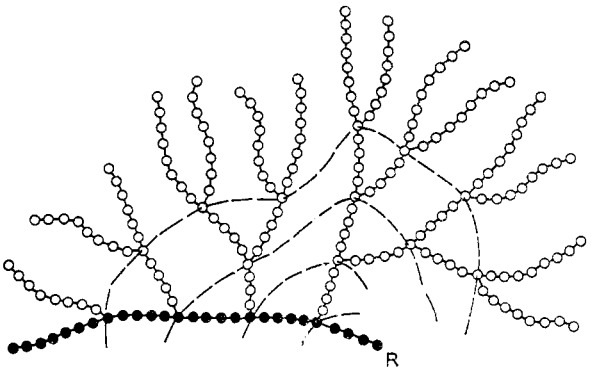

Fig. 4.—Idealized glycogen molecule having 5 tiers of glucosyl residues: ● represent those glucosyl residues which are joined in the single unbranched α-1,4 linked chain containing the free reducing end of the polysaccharide molecule (R). Dash lines mark the limits of the successive tiers.

Reduced Glycogen.—A sample of glycogen of molecular weight 0.19 × 10^6 was treated with sodium borohydride,[26] converting the terminal aldehyde group into an alcohol. The quantity of isosaccharinic acid produced by hot concentrated alkali treatment of 10 mg. of this reduced glycogen was compared with that produced in the same way from the original sample and from a sample previously treated with hot alkali. The results, samples VIII, IX and X of Table III, show that glycogen is rendered stable to anaerobic alkali degradation by prior conversion of the terminal aldehyde group into either a carboxy acid or an alcohol. In contrast, such reduced glycogen samples readily are degraded by hot dilute alkali in the presence of oxygen.[7]

TABLE III

COMPARISON OF YIELDS OF FREE ISOSACCHARINIC ACID PRODUCED FROM GLYCOGENS OF COMPARABLE AVERAGE MOLECULAR SIZE PREPARED BY DIFFERENT METHODS

Sample	Method of prepn.	Approximate mol. wt. × 10^{-6}	Quantity of isosaccharinic acid produced from 10 mg. of glycogen in 30% KOH at 100°, γ	
			In 15 min.	In 3 hr.
V	Acid	6.9	30	60
IV	Alkali	5.8	0[a]	10
VI	Acid	0.91		100
VII	Alkali	.57		0
VIII	Acid	.19	400	600
IX	Alkali	.10	0	25
X	Borohydride	.19	0	20

[a] *I.e.*, less than 5 γ, the smallest amount detectable by the methods used.

Discussion

All of the results are consistent with the picture that the principal action of concentrated alkali upon glycogen starts with an attack on the one free aldehyde group (represented by R in Fig. 4) of each molecule. The fact that the quantities of the various products formed are very different from those obtained from the α-1,4 linked polysac-

(26) M. Abdel-Akher, J. K. Hamilton and F. Smith, *ibid.*, **73**, 4691 (1951).

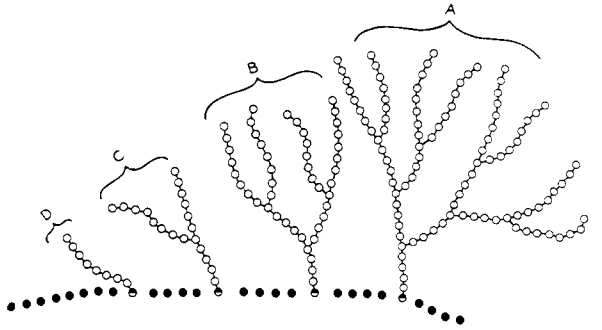

Fig. 5.—Products of the anaerobic action of concentrated alkali on an idealized glycogen molecule: ● represent free isosaccharinic acid molecules; ◒ represent the terminal isosaccharinic acid residue, to the carbon-6 of which are attached polysaccharide residues with the branching pattern of glycogen. A, B, C and D are the polydisperse series of polysaccharide acids identical with "glycogen" isolated by alkaline methods.

charide amylose[16] and the β-1,4 linked cellulose[27] can be attributed to the occurrence of appreciable 1,6 linked branching in glycogen.

The branching pattern of glycogen probably varies appreciably both between and within molecules but for purposes of calculation we shall assume that an average typical glycogen molecule has a regular multiple branched, tree-like structure[28] with an average of 4 α-1,4 linked glucosyl residues between the residues linked by α-1,6 bonds at the branch points and an average of 8 α-1,4 linked glucosyl residues per outer chain.[5,29] A relatively small molecule of this structure, having 5 tiers, is represented in Fig. 4.[30] Based upon the currently accepted mechanism of alkali degradation of carbohydrates resulting from many diverse studies,[12,13] the principal changes which such a molecule probably undergoes in the absence of oxidizing conditions and in the presence of hot concentrated alkali are as follows: 1. The glucose residue at the reducing end of the molecule (R) is converted by way of its 1,2-enediol to the 2,3-enediol of fructose. 2. The succeeding elimination of the anionic group β to the carbonyl group results in the expulsion from C-4 of the remainder of the glycogen molecule as an alkoxy anion. Kenner, *et al.*, have shown that O-glucosyl anions are more readily extruded from sugar enediol anions than are hydroxyl ions.[32] The possibility of course exists that the occasional elimination at the 1,2-enediol stage of the hydroxyl group at C-3 instead of the glycosyl anion at C-4 might occur, resulting in the production of a terminal metasaccharinic acid group which would stop further degradation, as has been observed with the longer 1,4 linked chains of cellulose[33] and amylose.[16] 3. The monosaccharide, remaining after expulsion of the bulk of the molecule, rearranges by way of an α,β-dicarbonyl intermediate to give principally D-glucoisosaccharinic acid along with acidic scission products.[16,34] 4. The polysaccharide portion, minus one glucosyl residue, now has another free reducing group exposed and can undergo a similar series of reactions. By successive reactions, called by Whistler[13] a peeling process, all of the glucosyl residues in the straight chain would be converted to isosaccharinic acid molecules, indicated by the black circles in Fig. 5. Those glucosyl residues with a side chain attached at C-6 would yield isosaccharinic acid indicated by ◒, with a polysaccharide structure still attached.

If it now be assumed that, in the absence of oxidizing conditions, no alkali sensitive linkages occur in the polysaccharide molecule other than those adjacent to the terminal aldehyde groups, estimates can be made (Table IV) for any given size of molecule, of the quantity of isosaccharinic acid which would be liberated during alkali degradation. It can be seen that the quantity of monosaccharinic acid produced varies greatly with the molecular size of the glycogen. With each halving of a polysaccharide molecule, the number of molecules of isosaccharinic acid produced per molecule is decreased by only about four. Thus (see Table IV) one gram of glycogen of molecular weight 138×10^6 would yield only 0.1 mg. while one gram with a molecular weight of 34,000 would yield 115 mg. of isosaccharinic acid. It was found that the calculated yields were in the same general order as the actual quantity of isosaccharinic acid produced from glycogen of varying average size (Table II), the deviation increasing with increasing molecular weight. This deviation is to be expected since, in the polydisperse population of molecules of

(27) G. N. Richards and H. H. Sephton, *J. Chem. Soc.*, 4492 (1957).

(28) K. H. Meyer and M. Fuld, *Helv. Chim. Acta*, **24**, 375 (1941).

(29) J. Larner, B. Illingworth, G. T. Cori and C. F. Cori, *J. Biol. Chem.*, **199**, 641 (1952).

(30) Footnote contributed by DeWitt Stetten, Jr.

Calculation of Maximum Molecular Weight of Glycogen.—If the foregoing model is rigorously adhered to and successive tiers are added peripherally, doubling the number of non-reducing termini with each additional tier, a limit is reached. The existence of such a limit, first pointed out by Pollard[31a] and subsequently considered by Madsen and Cori,[31b] results from the fact that space available for further branching and growth at the periphery of this growing spherical glycogen model will be consumed. From the model alone one may estimate the dimensions of this limiting glycogen molecule. Let:

t = number of tiers
r = radius of glucosyl residue
R = radius of glycogen molecule
n = number of non-reducing ends = 2^{t-1}
a = equatorial area of cross-section of glucosyl residue = πr^2
na = minimal peripheral surface consumed by glycosyl residues at non-reducing termini = $2^{t-1}\pi r^2$
A = available area on surface of spherical glycogen molecule.

The maximum value for $R = (6 + 10t)r$; hence the maximum value for $A = 4\pi r^2(6 + 10t)^2$.

The maximum value for t, when all the available area on the surface of the glycogen molecule shall have been consumed by glucosyl residues, therefore is given by the solution of the equation

$$4\pi r^2(6 + 10t)^2 = 2^{t-1}\pi r^2$$

or

$$(3 + 5t)^2 = 2^{t-5}$$
$$t \cong 18$$

Since the branch points at which the 18th tier originates are only 17 tiers removed from the reducing end, it follows that 17 completed tiers are the greatest number provided by this model and the above assumptions. Assuming $t = 17$, then

Maximum molecular weight = $(5 + 8) \times 2^{16} \times 162 = 138 \times 10^6$.

(31) (a) Polysaccharides in Biology (3rd Conference), Ed., G. F. Springer, Josiah Macy Jr. Foundation, New York, N. Y., 1958, p. 129-130. (b) N. B. Madsen and C. F. Cori, *J. Biol. Chem.*, **233**, 1251 (1958).

(32) J. Kenner and G. N. Richards, *J. Chem. Soc.*, 278 (1954).

(33) G. Machell and G. N. Richards, *ibid.*, 4500 (1957).

(34) G. Machell and G. N. Richards, *ibid.*, 1924 (1960).

any glycogen sample, the smaller molecules which make a minimum contribution to the weight average molecular weight make a maximum contribution to the saccharinic acid production. Simultaneously, the progressive alkali degradation would liberate from the glycogen molecule a series of stable polysaccharide acids, indicated by A, B, C and D in Fig. 5. These molecules would consist of a terminal isosaccharinic acid residue to which is attached at C-6 a side chain with the characteristic branching pattern of glycogen. For any given glycogen molecule a series of such polysaccharide acids would be produced with an average size of somewhat less than half that of the glycogen before alkali degradation. Curiously, even if the original glycogen were not polydisperse before alkali had acted on it, the resulting alkali stable mixture of polysaccharide acids, *i.e.*, Pflüger type glycogen, would be polydisperse.

TABLE IV

THEORETICAL ISOSACCHARINIC ACID YIELDS FROM THE ACTION OF CONCENTRATED ALKALI IN THE ABSENCE OF OXYGEN ON IDEALIZED GLYCOGEN MOLECULES[a] OF DIFFERENT MOLECULAR SIZES

Tiers (t)	Mol. wt. $\times 10^{-6}$	No. of glucose residues per molecule	Isosaccharinic molecules produced per glycogen molecule[b]	Theor. yield of isosaccharinic acid, %
5	0.034	208	24	11.5
6	.067	416	28	6.7
7	.13	832	32	3.9
8	.27	1,660	36	2.2
9	.54	3,330	40	1.2
10	1.1	6,650	44	0.66
11	2.2	13,300	48	.36
12	4.3	26,600	52	.20
13	8.6	53,200	56	.11
14	17.2	106,000	60	.06
15	35.5	213,000	64	.03
16	69.0	425,000	68	.02
17	138.0	852,000	72	.01

[a] For the purpose of these calculations the average glycogen molecule was assumed to be of the idealized type represented in Fig. 4, in which branching is regular, inner branches have 5 and outer branches 8 glucose residues. t = number of tiers of glucosyl residues. The total number of glucosyl residues per molecule approximately equals $2^{t-1} \times 5 + 2^{t-1} \times 8$ or $2^{t-1} \times 13$. Molecular weight = 162 × number of glucose residues. [b] Isosaccharinic acid molecules produced per glycogen molecule = average number of residues between branch points (4) × number of interior tiers + average length of exterior tiers (8). See Fig. 5 for the case where $t = 5$.

Of interest in connection with the present findings are the observations of Putzeys and Verhoeven[35] who noted that glycogen prepared by a water and picric acid extraction method behaved as an ideal non-electrolyte with apparent molecular weights determined by light scattering, independent of glycogen concentration and ionic strength. The molecular weights of samples prepared by alkaline methods were found to depend strongly upon the glycogen concentration and upon the electrolyte concentration of the solution. They suggested that the presence of acidic groups introduced into glycogen by an alkaline degradation might explain these differences in behavior.

Glycogen isolated by the common alkali methods appears, as a first approximation, to be resistant to alkali degradation. This is probably only because a point is reached, under the conditions of treatment with concentrated alkali, at which each glycogen molecule is in fact a large polysaccharide acid. The terminal acidic group, in the position usually referred to as the "reducing end," prevents further action of alkali except that which may occur as a result of random oxidation somewhere in the molecule, splitting it into several large fragments which are once again susceptible to further alkali degradation. Glycogen itself, separated from possible protein or nucleotide binding sites, and isolated by acidic or other non-alkaline methods, is probably always alkali-labile. The large molecular size and the nature of the branching pattern of glycogen makes the quantitative loss of polysaccharide material during isolation by concentrated alkali methods relatively slight.

Most enzymatic studies in the past have been on alkali-isolated "glycogen." Since the relative abundance of the acidic group in such large molecules is exceedingly small, and all the known enzymes concerned in glycogen metabolism act at the remote non-reducing end groups, this probably has had little effect on the results obtained.

Experimental

Preparation of Glycogen Samples.—Trichloroacetic Acid (TCA) Method.—Glycogen was isolated from normal, well fed rabbits by extraction with trichloroacetic acid at 0° and was purified by repeated reprecipitations from water with ethanol as previously described.[6] Contact with alkali was avoided throughout the isolation procedure.

The glycogen sample to be used for the isolation of saccharinic acid was prepared by the trichloroacetic acid method, followed by heating of the purified sample in 0.1 *N* HCl to reduce the average molecular size. About 20 minutes at 100° were required to effect the desired decrease in turbidity of the solution. This material, after extensive purification by repeated reprecipitation from water with ethanol, was examined by paper chromatography and was found to be free of detectable glucose, saccharinic acids and low molecular weight oligosaccharides. Molecular weight = 0.91×10^6 (Sample VI, Table I). The molecular weight of each sample was determined and calculated as previously described[6] by the method of light scattering.

KOH Method.—Glycogen was isolated from rabbit liver essentially by the method of Pflüger[2] but using less alcohol for precipitations[36] and KOH instead of NaOH for digestions.[7] After the tissue was digested for 3 hr. at 100° in 10 *N* KOH, glycogen was precipitated at room temperature with one volume of ethanol and purified by repeated solution in water, filtration and reprecipitation with alcohol. Contact with acid was avoided throughout the isolation. Purification was continued until the addition of an electrolyte (LiBr) was needed for final precipitation.

Reduced Glycogen.—Five hundred mg. of glycogen, sample VIII, having a molecular weight of 0.19×10^6, was allowed to stand for two days at room temperature with 200 mg. of sodium borohydride in 5 ml. of water.[26] After neutralization of the solution, the polysaccharide was recovered and purified by repeated solution in water and reprecipitation with ethanol. Four hundred and fifty-two mg. was obtained.

Turbidity Measurements of Alkaline Solutions.—Preliminary experiments revealed that, under the heating conditions used in studying the action of concentrated alkalies on glycogen, boric acid and other extraneous materials which interfered with light scattering measurements and complicated attempts to isolate the products were produced from glass

(35) P. Putzeys and L. Verhoeven, *Rec. trav. chim. Pays-Bas*, **68**, 817 (1949).

(36) M. Somogyi, *J. Biol. Chem.*, **104**, 245 (1934).

vessels. It was found that Teflon vessels, prepared by drilling holes in cylindrical blocks of Teflon, could be used for prolonged heating of solutions of NaOH without the introduction of any light scattering or soluble interfering substances. The openings of the Teflon vessels were machined to fit standard taper joints for attachment of glass accessories.

The concentrated alkaline solution used for the degradation studies was prepared by diluting 5 parts of 50% NaOH (w./w.) with 4 parts of water and centrifuging the solution at high speed until no significant amount of light-scattering material remained in the supernatant solution. About 2 hr. in a Servall centrifuge at 32,000 *g* were required. Such a clarified solution could be stored unchanged in polyethylene bottles. A suitable aliquot of the NaOH solution was heated in a Teflon vessel under a reflux condenser at 100° in a water-bath. The turbidity of the alkali solution, heated alone, did not change significantly. Glycogen was introduced in solution in one part of water so that the final concentration of NaOH was 25%. After rapid mixing aliquots were removed at various times for turbidity measurements while the remaining solution was heated continually in the Teflon vessel. Usually a 1-ml. aliquot of the alkaline solution was added in a semi-octagonal cuvette to 20 ml. of 0.1 *N* KCl which had been clarified by repeated filtrations through a millipore filter. The solution was mixed rapidly by means of a small magnetic stirrer in the cuvette. Light scattering was measured at an angle of 90° using an American Instrument Company Photomultiplier Microphotometer. Appropriate corrections were made for varying volumes and for the blank turbidity readings of the NaOH and KCl solutions. Anaerobic conditions were obtained by the use of tank N_2, carefully freed of O_2 by passing it through a tube containing Cu wire heated at 375°.

The decrease in turbidity of solutions of acid isolated glycogen samples was so rapid after contact with hot alkali that proper zero time readings could not be obtained in the time required for mixing the glycogen solutions with the alkali. Therefore, zero time values were obtained by measuring light scattered by aliquots of aqueous glycogen solutions introduced directly into 0.1 *M* KCl without the use of alkali.

The above methods were used for experiments summarized in Figs. 1 and 2 in which the conditions of alkali treatment or the nature of the glycogen were varied. Somewhat different methods were used to investigate further the very rapid initial stages of the reaction, reported in Fig. 3. An aqueous solution of the glycogen was placed in a Pyrex cylindrical cuvette. The dust-free NaOH was heated at 100° in a Teflon vessel and at zero time was poured into the glycogen solution in the cuvette. After the solution was rapidly mixed with a magnetic stirrer the turbidity changes were followed directly without the necessity of taking aliquots. The reaction could only be followed for a short time by this method because the temperature of the solution in the cuvette continued to fall from its initial value of nearly 100° to about 70° in 20 minutes, and the effect of the production of extraneous light scattering material from the glass was soon evident. Since in this type of experiment the change of turbidity was measured directly in concentrated alkali, the zero time values were obtained by the mixing of corresponding aliquots of concentrated alkali and glycogen solutions at room temperature.

Chromatographic Methods.—Whatman #1 paper was used for characterization purposes and Whatman #3 for isolations.

For separation of substances on paper the chief solvent mixtures used were ethyl acetate:acetic acid:H_2O (10:1.3:1)[24] when saccharinic acid lactones were sought and butanol:ethanol:H_2O (3:2:1) for detection of possible glucose and oligosaccharide contamination of polysaccharide samples. For location of compounds, silver nitrate–sodium hydroxide[37] was used for carbohydrate in general, benzidine[38] for reducing sugars and hydroxylamine–ferric chloride[25] for lactones.

Known compounds used for comparison included isosaccharinic lactone prepared from lactose by the method of Kiliani,[39] isosaccharinic anilide synthesized according to Sorokin[20] and Utkin and Grabilina[21] and α- and β-metasaccharinic lactones and glucosaccharinic lactone kindly supplied by Dr. Hewitt Fletcher. Each of these reference compounds was mixed with a large excess of glucose prior to chromatography to simulate the mixtures which were obtained by acid hydrolysis of the experimental polysaccharide acids.

Isolation and Characterization of Isosaccharinic Acid after Alkali Treatment of Glycogen.—Four grams of glycogen of average molecular weight 0.91×10^6 (Sample VI) was heated with 20 ml. of 10 *N* NaOH for 18 hr. in a Teflon vessel at 100°. After the resulting mixture was diluted with water, Na^+ ions were removed by means of Amberlite IR-120(H) resin. The bulk of the acidic polysaccharides, corresponding to glycogen isolated by the Pflüger method, was precipitated with ethanol. The supernatant solution was evaporated *in vacuo* to a small volume and a further crop of material insoluble in cold 95% alcohol was removed. After the removal of a third small crop of insoluble material in the same way, the alcohol soluble fraction was estimated to contain about 300 mg. of total solids. Aliquots of this solution were subjected to descending paper chromatography (ethyl acetate:acetic acid:water [10:1.3:1]) for 7 hr., and the materials were located by staining with silver and with hydroxylamine–$FeCl_3$. By comparison with samples of known compounds it was estimated that the solution contained about 45 mg. of material which migrated identically with isosaccharinic lactone and a small amount of free isosaccharinic acid. The polysaccharide material which remained stationary at the origin was also stained lavender with hydroxylamine–$FeCl_3$, suggesting the presence of a lactone.

The remaining solution was deposited on sheets of previously washed and dried Whatman #3 filter paper and developed in the same way. The segments corresponding to the location of isosaccharinic lactone were eluted with water and the extracts evaporated. This material was further purified by repeating the steps of paper chromatography, elution and evaporation. Material insoluble in water or alcohol was discarded and only that portion of the residue which was soluble in hot ethyl acetate was retained. The mixture of crystals and oil obtained after seeding and refrigeration was twice recrystallized from ethyl acetate; yield 5 mg. of crystals identical in form with those of the lactone of isosaccharinic acid; m.p. 93–94°.

The lactone was converted to the anilide[20,21] by heating with a 40-fold excess of aniline at 150–200° for 4 hr. The anilide was found to be identical with that of isosaccharinic acid when studied chromatographically by means of the solvent acetone:water:benzene (30:1:20, v./v.) containing the ultraviolet fluorescent dye Rhodamine B.[22,23]

Aqueous elution of the material which did not migrate from the origin on the paper sheets used for isolation of isosaccharinic lactone yielded a glycogen-like polysaccharide acid which was shown to be free of monosaccharinic acids. Total hydrolysis of this material yielded chiefly glucose and an appreciable amount of isosaccharinic acid lactone. In addition there were traces of material with chromatographic properties suggesting the presence of some glucometasaccharinic lactone.

Estimation of the Quantity of Isosaccharinic Acid Produced from Various Glycogen Samples under Standard Conditions of Treatment with Hot Concentrated Alkali.—Ten mg. of glycogen was dissolved in 0.5 ml. of 30% KOH and heated under reflux in a small Teflon vessel for 15 minutes or for 3 hr. at 100°. The resulting solution was diluted with water and K^+ was removed with 1.5 g. of Amberlite IR-120(H) resin. The slightly acidic filtrate was lyophilized to dryness and the fluffy white residue extracted 4 times with successive 1-ml. portions of hot ethanol. The combined alcohol extracts were evaporated to dryness on a steam-bath and the residue was taken up in a few drops of water. Water was evaporated off and the residue was heated for about 10 minutes to ensure conversion of the saccharinic acids to lactones. This residue was dissolved in water and all or a suitable aliquot was subjected to paper chromatography. The quantity of material was roughly estimated visually by comparison with known quantities of isosaccharinic lactone, deposited, developed and stained in the same way. The smallest quantity detectable by the methods used was about 5 γ.

(37) W. E. Trevelyan, D. P. Procter and J. S. Harrison, *Nature*, **166**, 444 (1950).

(38) W. J. Whelan, J. M. Bailey and P. J. P. Roberts, *J. Chem. Soc.*, 1293 (1953).

(39) H. Kiliani, *Ber.*, **42**, 3903 (1909).