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The Effect of pH and Buffer Ions on the Degradation of Carbohydrates by Fenton's Reagent

BJØRN LARSEN and OLAV SMIDSRØD

Norwegian Institute of Seaweed Research, NTH, Trondheim, Norway

Hydrogen peroxide used in conjunction with ferrous ions (Fenton's reagent) is a source of free radicals and leads to a non-specific, oxidative degradation of carbohydrates, accompanied by glycosidic hydrolysis in oligo- and polymers. The results presented demonstrate that an aldonic acid, either free or as a terminal unit, will under suitable conditions undergo an oxidative decarboxylation by the action of Fenton's reagent. This reaction will occur only when conditions are favourable for the formation of a complex between ferric ions, the aldonic acid, and the hydrogen peroxide. With gluconate, this triple complex is stable only above pH 4 and is the absolutely dominating reactant above pH 7. Lowering the pH changes the reaction from the specific, oxidative decarboxylation towards a non-specific degradation. The same effect is obtained by addition of components competing with gluconate for the ferric ions. Experiments with radical scavengers, *e.g.* propanol, indicate that the oxidative decarboxylation does not involve the formation of free radicals.

The action of hydrogen peroxide on carbohydrates was recently reviewed by Moody,[1] and readers are referred to his paper for a comprehensive treatise on previous work in this field. Hydrogen peroxide used in conjunction with ferrous ions (Fenton's reagent) is a source of free radials and leads to oxidative degradation of carbohydrates and related substances. Ferric ions as a catalyst (the Ruff reagent) will, in many cases, lead to the same reactions, and, as pointed out by Moody, the postulated specificity of the Fenton reagent needs further qualification.

Provided hydrogen peroxide is in excess, the same radical entities will be formed during the stationary state, whether ferric or ferrous ions are used as catalyst (*cf.* Ref. 2). During the short induction period ferrous ions will be oxidised to ferric with the concomitant formation of hydroxyl radicals, thus leading to a higher rate of reaction in this period. The degradation of sodium alginate with hydrogen peroxide in the presence of ferrous and ferric ions, respectively, (shown in Fig. 1), may serve to illustrate this point. It, therefore, seems improbable that the radical-producing reaction *per se* should introduce

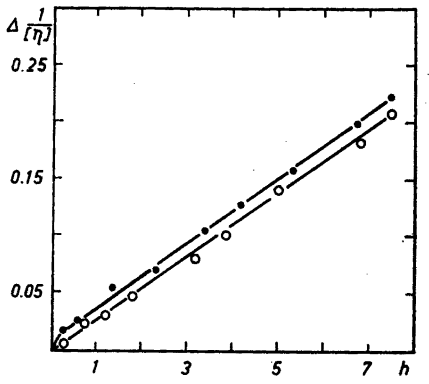

Fig. 1. Degradation of sodium alginate with hydrogen peroxide at pH 6.0 in the presence of ferrous or ferric ions. ● = Fe^{2+}; ○ = Fe^{3+}.

any specific reactions. Rather the site of an eventual specificity should be looked for either as an influence of the substrate, or of other components present in the reaction system, *e.g.* buffer components. In a previous publication [2] we demonstrated that in 0.01 N nitric acid the addition of glucose changed the reaction order of the decomposition of hydrogen peroxide from a 1st to approximately a zero order reaction with respect to the concentration of hydrogen peroxide at a glucose concentration of 0.01 M. This change may be explained by assuming a complex to be formed between the glucose, the ferrous or ferric ions, and the hydrogen peroxide, and that the degradation of this complex is the rate-determining step. It seemed reasonable to assume that such complex formation, especially if involving the substrate, might also influence the course of the reaction, *e.g.* by selectively increasing the rate of reaction of one possible pathway on the expense of others, or by completely changing the reaction mechanism. This report deals with some observations on the effect of the substrate and of external factors, like buffer ions, pH, *etc.*, on the course of degradation.

RESULTS AND DISCUSSION

In a series of preliminary experiments linkage scission was demonstrated to occur with amylose, dextran, maltose, lactose, cellobiose, and gentiobiose as a result of the action of Fenton's reagent. This conclusion mainly rests on the increase in reducing power observed for dextran and amylose and on the chromatographic demonstration of glucose for the disaccharides. Lactose also gave rise to galactose, in keeping with the observations of Adachi.[3] With melibiose (6-*O*-α-D-galactopyranosyl-D-glucose) the participation of oxidative linkage scission was also demonstrated. After 48 h degradation in a phosphate-citrate buffer at pH 6.0, (with a ferrous ion concentration of 0.001 M and two additions every day of hydrogen peroxide to 0.02 M), excess hydrogen peroxide was removed and the reaction mixture subjected to column chromatography on Dowex 1 × 8, according to the method described previously.[4] A fraction containing uronic acid was obtained, and this acid was shown to be glucuronic acid by paper electrophoresis and by paper chromatography of

the product obtained upon lactonization with formic acid. Paper chromatography was carried out in solvent A, and the single spot obtained coincided perfectly with that of an authentic specimen of glucurone. In a parallel experiment with glucose, no glucuronic acid could be detected.

Feinstein and Nejelski [5] and Wolfrom *et al.*[6] have pointed out the radiomimetic nature of Fenton's reagent. With oxygen present, irradiation of water, or aqueous solutions, leads to the formation of hydroxyl and hydroperoxyl radicals, the same entities as formed by the reaction between hydrogen peroxide and ferrous and ferric ions, respectively. Similar degradation patterns are therefore to be expected with both systems. The effect of ionizing radiation on carbohydrates has been reviewed by Phillips[7], and a comparison with Moody's review on Fenton's reagent [1] suggestively emphasizes the similarity of reaction patterns. In a paper on the irradiation of sucrose in oxygenated solution Phillips and Moody [8] demonstrated that glycosidic hydrolysis is the main primary reaction occurring, to a lesser extent accompanied by oxidative linkage scission. An analogous hydrolysis reaction may be the explanation for the rapid linkage scission occurring by the action of Fenton's reagent on oligo- and polysaccharides observed by many authors (*cf.* Ref. 1). Our preliminary results on the degradation of dextran, maltose, lactose, cellobiose, gentiobiose, and melibiose are thus in close accordance with previous experience in this field.

Modification of the reducing end of lactose, either by reduction with sodium borohydride to form lactitol or by oxidation with bromine-water to lactobionic acid, allowed the detection of other reactions taking place. Among other reaction products, lactose and glucose were detected by the action of the H_2O_2-Fe system on lactitol at pH 6. The identity of the glucose was ascertained by its disappearance from the chromatogram after treatment with glucose oxidase. Neither of these products could be demonstrated to arise from calcium lactobionate. In this case, the main products were galactose and compounds moving behind lactose in the chromatogram. After some time, a disaccharide was formed having an R_{Gal}-value of 0.75 in solvent B (glucose = 1.12, lactose = 0.61). The same disaccharide spot was detected in the reaction mixture from lactitol and also in trace amounts from lactose.

All the experiments discussed above were carried out in phosphate-citrate buffer of pH 6.0. We have previously demonstrated that the addition of carbohydrates, *e.g.* glucose, methylcellulose, and alginate, to the H_2O_2-Fe system decreased the reaction order of the hydrogen peroxide decomposition towards a zero-order reaction.[2] This effect was believed to be caused by a complex formation between the carbohydrate, the ferrous (or ferric) ions, and the hydrogen peroxide. The same effect was obtained with the phosphate-citrate buffer used in the above experiments. Substances not capable of such complex formation, (*e.g.* methanol, ethanol, and propanol), will not change the order of reaction, but may, through a radical scavenger action, lower the rate of degradation. Citrate is a well-known complexing agent for ferric ions,[9] and we considered it of importance to further investigate its influence on rate and course of degradation under various conditions.

It was noticed that when the degradation of calcium lactobionate with Fenton's reagent was carried out in the absence of buffer ions, but at the same

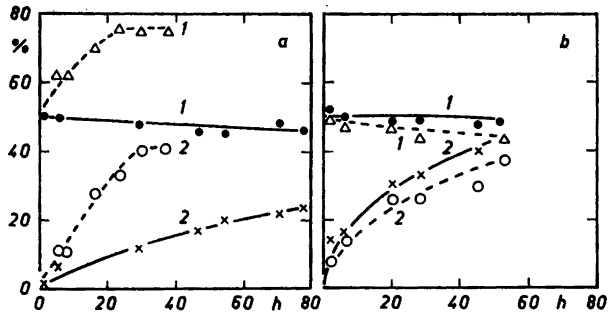

Fig. 2 a and b. Degradation of lactobionate at two different pH values. – – – = without buffer; ——— = in citrate buffer. 1 = Total carbohydrate, 2 = Reducing power. a. pH 6.0. b. pH 1.7.

pH, the disaccharide was the clearly dominating compound. The degradation was therefore followed quantitatively at two different pH values, both in the presence and absence of buffer ions. The results are shown in Figs. 2a and 2b.

Total carbohydrate was determined by the phenol-sulphuric acid reaction. As only the galactose moiety of the lactobionate molecule will respond to this reaction, the starting point was considered to be 50 % of the theoretical value. As different sugars have different extinctions in this reaction, the results can only be regarded as an estimate to show the general trend occurring during the degradation. At pH 1.7 the influence of buffer ions is hardly significant (Fig. 2b), while at pH 6.0 the initial rate of formation of reducing carbohydrate is much higher without buffer present (Fig. 2a). In the phosphate-citrate buffer the total amount of carbohydrate decreases slightly with time; in the unbuffered system there is a marked increase in reducing carbohydrates. It is difficult to see any rational explanation of this behaviour without assuming a change in mechanism at the higher pH value due to the presence of buffer ions.

In a buffer system of the same pH consisting only of phosphate, the result was the same as in experiments without buffer. The change in mechanism must, therefore, be attributed to the citric acid-citrate system. Two properties of citrate may be considered of importance in this connection: 1) its ability to form complexes with ferrous and ferric ions, and 2) its possible interaction with the radicals formed, thus lowering the concentration of free radicals available for carbohydrate degradation. To distinguish between these two alternatives experiments were carried out in the presence of propanol and EDTA in the unbuffered reaction system. EDTA forms an extremely stable complex with ferric ions at the pH of the experiment (pM $>$ 16); while the action of propanol, according to previous results,[2] must be due to a lowering of the radical concentration. The results given in Fig. 3 demonstrate that the presence of EDTA brings the rate of degradation down to the same level as was obtained with citrate. The action of citrate is thus probably a consequence of its complex formation with ferric (or ferrous) ions. It is of equal impor-

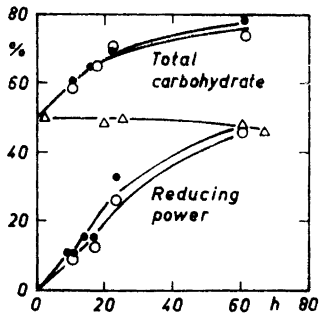

Fig. 3. The influence of EDTA and propanol on the degradation of lactobionate in unbuffered solution. ○ = Without buffer; ● = without buffer + 0.4 M propanol; △ = without buffer + 0.05 M EDTA.

tance to notice that the presence of propanol did not significantly influence the rate of degradation, thus suggesting that, in the absence of a complexing agent, the degradation is not a radical reaction.

It should now be pointed out that the curves given in this paper may not represent the total extent of reaction. Reactions with non-carbohydrate materials, for instance citrate or propanol, will not show up in any of the analytical methods used. The same also applies to reactions not involving the functional reducing group of a carbohydrate. The reaction paths investigated are thus only those leading to destruction of reducing carbohydrates or formation of new reducing functions in non-reducing reactants.

The change in mechanism suggested by the reaction curves at pH 6.0 was verified by paper chromatography. By degradation of lactobionate at pH 6 in the absence of citrate a disaccharide component was dominating, while, with citrate present, it was only formed in small amounts. The disaccharide was also the clearly dominating product in the experiments with propanol — an observation consistent with the results given in Fig. 3. The structure of the disaccharide component might provide a clue to the mechanism responsible for its formation and a tentative identification was therefore attempted. Calcium lactobionate was degraded for 48 h with Fenton's reagent in the absence of buffer ions, and excess hydrogen peroxide was removed. Paper chromatography showed the disaccharide to be the dominating reaction product. Unreacted lactobionate was removed by passing the solution through a column of Dowex 1 × 8 anion exchange resin, and the disaccharide isolated by chromatography on a carbon-Celite column. The compound obtained by this method was homogeneous as judged by paper chromatography in all solvent systems used. Acid hydrolysis with 0.5 N oxalic acid for 20 h at 100°C, followed by paper chromatography in solvent B, gave two distinct spots coinciding with galactose and arabinose, R_{Gal} 1.00 and 1.24, respectively. Reduction with potassium borohydride prior to hydrolysis gave only one spot corresponding to galactose. After periodate oxidation, no spot was present in the chromatogram in the position originally occupied by the disaccharide. After 5 h hydrolysis with 0.5 N oxalic acid at 100°C, substantial amounts of arabinose were detected. Provided the arabinose ring is in the pyranosidic configuration, this demonstrates the presence of a 3-linked arabinose as the reducing end of the disaccharide. These results strongly suggest that the

substance formed with Fenton's reagent in the unbuffered system at pH 6 was a galactose-arabinose disaccharide identical to the one isolated by Ruff and Ollendorff [10] in 1900 by Ruff degradation of lactobionate. According to the mechanism of this reaction the structure of the lactobionic acid, (4-*O*-*β*-D-galactopyranosyl-D-gluconic acid), requires the disaccharide to be 3-*O*-*β*-D-galactopyranosyl-D-arabinose. It is thus reasonable to conclude that the reaction responsible for the rapid formation of reducing power in the unbuffered system, and which is supressed by the presence of citrate, is a Ruff degradation of the aldonic acid. The aldonic acid will not register in either of the analytical methods, while, after transformation into arabinose, the new end group will be analysed for by both methods. The result will thus be a net increase both in total carbohydrate and in reducing power (Fig. 2). The detection of the same disaccharide component with lactose and lactobitol as substrates demonstrates that this change in mechanism may be important also for compounds not originally possessing the aldonic acid end group, but capable of forming such a group. All aldoses may thus be considered as potential reactants.

All the results obtained with lactobionate suggest that the action of citrate relies on its ability to form complexes with ferrous and ferric ions. The gluconate moiety of the lactobionate molecule is also capable of forming such complexes. In the absence of citrate, these complexes will, at least at higher pH values be degraded by hydrogen peroxide by oxidative decarboxylation to give the galactosyl-arabinose disaccharide. When citrate is present in the system, there will be a competition for the ferric, or ferrous, ions leading to a distribution of the ions between the two complexing agents according to the pM values of the complexes. In our case citrate is obviously the stronger complexing agent, as the Ruff degradation is greatly suppressed.

To avoid complications due to the hydrolysis and secondary reactions occurring with the hydrolysis products, sodium D-gluconate was used as a substrate for investigating further the effect of pH. The Ruff degradation will in this case give D-arabinose, and the number of products of carbohydrate nature arising from other reactions should presumably be much lower than with lactobionate. Plots of total carbohydrate and reducing power *versus* reaction time will start at zero, as the non-reducing gluconic acid will not register in either of the analytical methods. Such plots are shown in Fig. 4 for experiments carried out in the presence of citrate buffers of different pH. As in previous experiments, the total carbohydrate refers to the colour measured in the phenol-sulphuric acid method and calculated as glucose — this standard also being used for the reducing power. The initial rate of formation of reducing carbohydrate increases with increasing pH from pH 1.5 to 4.0 and, then decreases in the pH-region 4.0—7.3. The maximum yield obtained during the reaction seems, on the other hand, to be fairly independent of the pH. The decrease in rate observed above pH 4 is inconsistent with the results obtained previously for the decomposition of hydrogen peroxide and the degradation of alginate [2] — both of which increase markedly with pH. As to the radical production in the system used for D-gluconate, the same conclusion was reached through control experiments with methylcellulose at pH 4.0 and 7.3. The decrease in rate of reaction above pH 4 can therefore not be

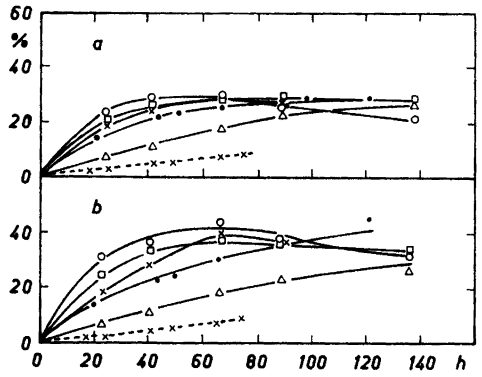

Fig. 4. Formation of reducing power and carbohydrate material during degradation of sodium gluconate in citrate buffers at different pH. ● pH = 1.5; × pH = 3.0; ○ pH = 4.0; □ pH = 6.0; △ pH = 7.3; – – – pH = 6.0, 0.4 M propanol. a. Total carbohydrate. b. Reducing power.

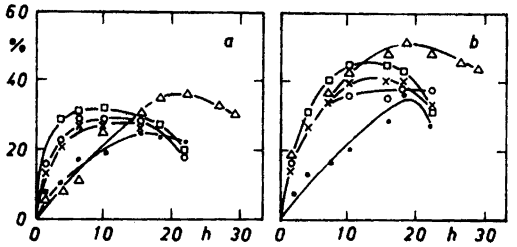

Fig. 5. Formation of reducing power and carbohydrate material during degradation of sodium gluconate in unbuffered solutions of different pH. ● pH = 1.0; × pH = 2.0; ○ pH = 2.25; □ pH = 4.0; △ pH = 6.8. a. Total carbohydrate. b. Reducing power.

due to a decrease in radical activity. The most probable explanation of this apparent anomaly seems to be a rate increase for reactions not leading to reducing carbohydrates.

The results in Fig. 4 further demonstrate that the addition of propanol to 0.4 M significantly lowers the rate of formation of reducing carbohydrates with citrate present, demonstrating that free radicals are involved in this reaction. It is therefore to be expected that the reactions occurring in this case will be of the type already described as characteristic of Fenton's reagent. In keeping with this is the detection of a variety of products in paper chromatograms.

Paper chromatography demonstrated that arabinose is formed only in trace amounts at all pH values up to 6 and only as a minor constituent of the mixture even at pH 7.3. At low pH values the dominating product was a uronic acid-like component, which, upon paper electrophoresis, could not be separated from glucuronic acid. No attempts were made to further establish the structure of this compound. As judged from the spot size, a relatively higher proportion of the products formed remains at the starting position in the chromatogram as the pH increases.

The results from corresponding experiments in unbuffered systems are shown in Fig. 5. At pH 6.8 the pH had to be adjusted at intervals, in the other experiments the pH remained constant throughout. Again the maximum initial rate of formation of reducing carbohydrate was found at pH 4, while the maximum yield obtained during the reaction seemed to increase with pH over the entire pH range. The most conspicuous results of this series of experiments are demonstrated by paper chromatography. By visually judging the spot size the results shown in Table 1 were obtained. Contrary to the

Table 1. Paper chromatography (solvent A) of products formed by degradation of gluconate with Fenton's reagent in the absence of citrate.

pH	R_{Gal}	0	0.28	0.37	0.83	1.24
1.0		+++	+	+	(+)	+
2.0		++	+	+	+	(+)
2.25		+	+	+	+	(+)
4.0		+	+	+	−	(+)
6.8		(+)	+	(+)	−	++++

results obtained in the presence of citrate, the amount of products remaining at the starting position now seemed to decrease with increasing pH. The product with R_{Gal} 0.28 may be identical to the one formed with citrate present, while the two other components of intermediate mobility did not arise in the previous experiment. The most pronounced difference between the two experiments exists in the formation of arabinose (R_{Gal} 1.24). At pH 1 arabinose is easily detected; at intermediate pH values it is only formed in trace amounts, while at pH 6.8 it is the clearly dominating product. Consequently, the curves given in Fig. 4 for total carbohydrate and reducing power at the latter pH, are calculated by using arabinose as standard.

D-Arabinose is the product formed by oxidative decarboxylation, and the contribution of this reaction to the total carbohydrate production may be estimated if arabinose is separated from other reaction products. Experiments showed that only arabinose was eluted when the reaction mixture was applied to a column of Dowex 1 anion exchange resin in the acetate form and the column then eluted with distilled water. The amount of arabinose was then determined in the eluate by the phenol-sulphuric acid method and calculated as percentage of the total amount of carbohydrate applied to the column analysed for by the same method. In the latter case the results were

Table 2. Contribution of oxidative decarboxylation given as arabinose and calculated as percentage of total carbohydrate formed.

pH	1	2.25	4	5		6		6.8	7		8	8.3
Total carbohydrate	26	29	31	2.5	8.5	1	3.5	11	13.5	22.5	13	1.5
% arabinose	19.6	7.1	9	44	54.5	76	63	92	70	103	73	72

calculated as glucose, as the correct calibration factor was not known. The results given in Table 2 must therefore be regarded only as approximate values. The total amount of carbohydrate formed is also listed in the table and given as percentage of an amount of glucose equivalent to the gluconate used in the experiment.

The trend is clearly the same as that shown by paper chromatography. At pH 1 oxidative decarboxylation is responsible for about 20 % of the total reaction. With increasing pH the contribution first drops down to a very low level, but then increases sharply between pH 4 and 7. There is a considerable variation among experiments, but we consider the rating of the oxidative decarboxylation as the dominating reaction around pH 7 to be a valid conclusion from these experiments. It then follows that, in the absence of citrate, the pH is a factor capable of controlling the reaction path almost to the complete exclusion of reactions other than the oxidative decarboxylation.

The amount of arabinose formed with ferric ions as catalyst, instead of the ferrous ions used in the preceding experiment, was 27 and 91 % at pH 4 and 7, respectively. The figures are slightly higher than those of Table 2, but indicate the same type of pH-dependence. There is thus no indication of any difference in mode of action between these catalysts.

The influence of propanol on the reactions taking place in the presence and absence of citrate was checked with gluconate by determination of the amount of arabinose formed. In citrate buffer of pH 6.0, 16 and 9 % arabinose were found with and without propanol, respectively. In unbuffered systems at pH 7 the corresponding contributions from the oxidative decarboxylation were 84 and 70—103 %, respectively. The influence of propanol is thus selectively on the non-specific oxidation occurring in the presence of citrate.

The extremely rapid increase in oxidative decarboxylation between pH 4 and 7 indicated that formation of a complex between iron and gluconate as an intermediate might be the chemical reaction responsible for this change in mechanism. The formation of this complex would have to involve the dissociation of a proton with a pK_a-value around pH 6, and the decomposition of the complex to give arabinose would have to be the rate determining step of the reaction. Disregarding for the moment the chemical structure of the complexes, two different types will have to be considered whether the hydrogen peroxide participates in the complex formation or not: {gluconate-Fe} and {gluconate-Fe-H_2O_2}. In these complexes the iron may be either in the ferrous or ferric state, thus increasing the number of possibilities to four. Experiments were then performed to distinguish between the types of complexes by means of potentiometric titration. Fig. 6 shows the results obtained by titrating equimolecular amounts of gluconate and ferrous ions with 0.2 N sodium hydroxide. The colour of the solution increased markedly with pH during the titration and was therefore measured in a spectrophotometer at 345 mμ as a function of the pH. The results are given as optical density of a 1:100 dilution of the titration mixture to allow presentation within the scale of Fig. 6. Obviously, a complex is formed between gluconate and ferrous ions, but the pK_a-value is too high to explain the change in reaction investigated here.

The fully drawn line in Fig. 7 represents the result of a parallel experiment with 0.1 M gluconate and 0.05 M ferric ions. In this case there is no discrete

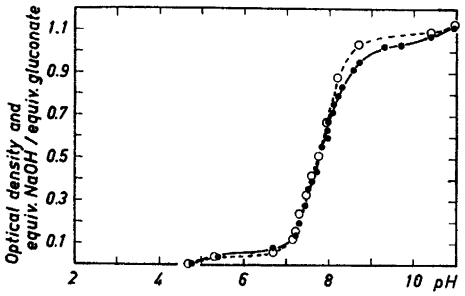

Fig. 6. Potentiometric titration and colour formation of gluconate in the presence of ferrous ions. O = Optical density, *D*; ● = Alkali consumption.

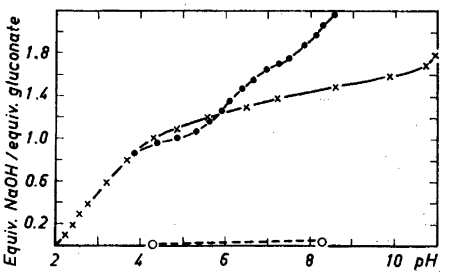

Fig. 7. Potentiometric titration of gluconate in the presence of ferric ions and hydrogen peroxide. × = without H_2O_2; ● = with 1 M H_2O_2; O = titration of 1 M H_2O_2.

dissociation step to be observed between pH 4 and 10. Thus a complex of the type {gluconate-Fe} cannot be responsible for the change in mechanism.

Addition of hydrogen peroxide to the gluconate-Fe^{3+} mixture, previously titrated to pH 4, gave a considerably higher alkali consumption than before (Fig. 7). In conjunction with the insignificant alkali titre of hydrogen peroxide alone, this result clearly demonstrates the formation of a complex between gluconate, iron salt and hydrogen peroxide. The first increase in alkali consumption after the addition of hydrogen peroxide (upper curve in Fig. 7) occurs in the same range where the yield of arabinose increases sharply.

Owing to the equilibrium set up between ferrous and ferric ions in the presence of an excess of hydrogen peroxide the above experiments do not allow the conclusion that iron is present in the complex as ferric ions. During the first part of the reaction it should, however, be possible to distinguish between the reactions occurring with ferrous and ferric ions, respectively, by adding an amount of hydrogen peroxide in moles smaller than the amount of iron present. Under these conditions ferrous ions gave a pattern of products indicative of a non-specific degradation, while ferric ions again gave arabinose as the absolutely dominating product.

The free radicals produced during the oxidation of ferrous to ferric ions must be responsible for the non-specific oxidation to a variety of acidic products. As only very small amounts of ferrous ions may have been formed from the ferric ions used at the start of the other experiment (*cf.* reactions given in Ref. 2), the high yield of arabinose obtained in this case strongly suggests the presence of ferric ions in the triple complex.

The results discussed above demonstrate that an aldonic acid will, under favourable conditions, undergo an oxidative decarboxylation (Ruff degradation) by the action of Fenton's reagent. This reaction will occur only when conditions are favourable for the formation of a complex between ferric ions, the aldonic acid, and the hydrogen peroxide. The degradation of this complex is the rate determining step, and the results indicate that this process does

not involve the formation of free radicals. Reactions occurring in a strongly acid medium may be an exception to this rule. The results indicate that the triple complex is stable only at pH values above 4 (for gluconate) and is the clearly dominating reactant above pH 7. Lowering the pH will cause a change in reaction mechanism towards a non-specific degradation involving free radicals. The same effect will also be obtained by addition to the reaction system of components, *e.g.* buffer ions capable of competing with the aldonic acid for the ferric ions. The ferric ions are then distributed between the complexes according to their pM-values.

Strong complexing agents, *e.g.* EDTA or citrate, may thus completely suppress the Ruff degradation and give a reaction pattern typical for the non-specific degradation. It should be noted here that, unless a complexing agent is present, iron salts cannot be used as a catalyst except at low pH values, owing to the low solubility of ferric hydroxide. Substances not forming complexes with ferric or ferrous ions maybe influence the rate, but not the course of reaction by acting as radical scavengers.

The conclusions above concerning the change in mechanism are, of course, only valid for compounds containing an aldonic acid, either free or as a terminal unit. As pointed out previously, the oxidation of an aldose to aldonic acid is a reaction apt to occur with Fenton's reagent, and the quantitative distribution of products may then, under suitable conditions, be influenced by the factors discussed above. The decrease in rate of formation of reducing carbohydrates above pH 4, occurring in spite of the increase in radical concentration, indicates that reactions leading to non-carbohydrate products may also be influenced by external factors. Such influence may possibly be the explanation of the specificity occasionally postulated for Fenton's reagent.

EXPERIMENTAL

Samples. All reducing mono- and disaccharides, as well as amylose and dextran, were commercial preparations. Calcium lactobionate was a synthetic specimen kindly supplied by the Institute of Organic Chemistry, the Technical University of Norway. Lactitol was prepared by reducing lactose in aqueous solution with sodium borohydride overnight. The solution was then evaporated to dryness in vacuum and borate removed by repeated evaporation with methanol. Enzymes used were commercial preparations (Boehringer).

Degradation with Fenton's reagent. For pH values above 2 the sample was dissolved in McIlvain buffer of ionic strength 0.25 to make a 2 % solution. Below pH 2, 0.1 M citrate-HCl was used as buffer. Unless otherwise stated, ferrous sulphate dihydrate, or ferric chloride, was then added to a concentration of 0.001 M. When the ferrous salt was dissolved the reaction was started by adding hydrogen peroxide until a 0.02 M solution was obtained, the same amount of hydrogen peroxide later being added twice every day. This procedure secured the presence of an excess of hydrogen peroxide during the reaction. The reaction was allowed to proceed at room temperature and samples of 3 ml were removed at suitable intervals. In these samples the degradation was stopped by adding 3 drops of a 1:5 dilution of the commercial catalase suspension.

When degrading calcium lactobionate in the absence of buffer pH was adjusted with sodium hydroxide containing a little sodium carbonate. Hydrogen peroxide was in this case added three times every day.

Sodium alginate was dissolved in distilled water to give a 2 % solution and the amount of ferrous sulphate required to give a concentration of 0.002 M was added. This solution was then diluted with an equal volume of McIlvain buffer pH 6.0 and the pH adjusted

after the dilution. Ionic strength was adjusted to 0.2. During the first 24 h, when viscosity was measured, a hydrogen peroxide concentration of 0.1 M was used. Later on three additions of 0.02 M each were used.

Analysis. After proper dilution, the samples were analysed for reducing power by the Hagedorn-Jensen method, modified to a semi-micro method by Fujita and Iwatake (*cf.* Ref. 11), and for total carbohydrate by the phenol-sulphuric acid method.[12]

Paper chromatography and electrophoresis. Solvent system A consisted of the upper phase of the mixture pyridine, ethyl acetate, and water in the proportions 11:40:6. Ascending chromatography was used with this system. The same system, but in the proportions 2:5:7, was used for descending chromatograms to allow separation of galactose and glucose. Ascending chromatography overnight was used with solvent system B, consisting of butanol, benzene, pyridine, and water in proportions 5:1:3:3. Paper electrophoresis has been described previously.[13]

Aniline-trichloroacetic acid (2.5 % solution in glacial acetic acid) was used as developing agent.

Oxidation with glucose oxidase. 1 mg of glucose oxidase was added to a 1 ml sample of the degraded solution and left open in the shaking machine overnight to secure a sufficient supply of oxygen. The treated and untreated samples were then chromatographed together in solvents A and B.

Isolation of disaccharide from degraded calcium lactobionate. The disaccharide accumulated during degradation of calcium lactobionate in the absence of buffer salts was isolated by column chromatography on a carbon-Celite column, 21 × 200 mm. 5 ml of the degraded lactobionate solution were passed through a small column of Dowex 1 × 8, 20—50 mesh, to remove acidic components. After neutralization, the solution was evaporated to about 2 ml and then put onto the carbon column. Elution was effected with a linear gradient from 0 to 25 % ethanol in distilled water and fractions of approximately 5 ml collected. Every fifth tube was evaporated in vacuum to a small volume and chromatographed in solvent B. A small amount of neutral monosaccharides was first eluted, followed by the disaccharide to be isolated. The disaccharide was spred over a rather large number of tubes, but fortunately no other compound was eluted in this region. All tubes containing the disaccharide were combined and the solution was evaporated in vacuum. No attempt was made to crystallize the isolated product. It behaved as a homogeneous substance in all solvent systems tried.

Isolation of glucuronic acid from melibiose. The degradation of melibiose (200 mg) and the destruction of hydrogen peroxide was carried out as described above. The solution was then adjusted to pH 8 and allowed to stand at this pH for 30 min to transform any lactone present into acid form. The solution was then washed onto a column of Dowex 1 × 8 (200—400 mesh, acetate form) and eluted as described previously.[4]

Periodate oxidation and acid hydrolysis. Periodate oxidation was performed with 0.05 M solution of sodium metaperiodate in distilled water for 20 h at 2—4°C. The reaction was stopped by adding ethylene glycol to a concentration of 0.05 M.

0.5 N oxalic acid was used for acid hydrolysis. Reaction conditions were 20 h at 100°C. After cooling to room temperature, the solution was neutralized, and the oxalic acid removed, by adding calcium carbonate. Calcium oxalate was removed by filtration and washed on the filter. The combined filtrates were evaporated in vacuum.

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