## 237. *The Alkaline Degradation of Polysaccharides. Part III.* Action of Sodium Hydroxide on Amylose.*

By G. Machell and G. N. Richards.

The action of 0·5N-sodium hydroxide at 100° on amylose is of two main types: "degradation" producing mainly D-gluco*iso*saccharinic, formic, and lactic acid; and a "stopping" reaction affording alkali-stable amylose probably bearing terminal D-gluco*meta*saccharinic acid units. The analogy with the similar degradation of cellulose is discussed.

Much of the earlier work on the alkaline degradation of starch and its amylose component was concerned with attempts to relate the extent of degradation to the initial molecular weight of the material. This work led to the development of the "alkali-number" determination,[1] which is a measure of the amount of acid formed when starch or amylose is degraded in alkali under standard conditions. The alkali number is related to the initial molecular weight provided the degradation commences only at reducing glucose end-units present in the original material. However, the method is particularly sensitive to traces of oxygen, which cause a random scission of the polysaccharide,[2] with possible production of new reducing end-groups. Additional interest in the alkaline degradation

* Part II, *J.*, 1957, 4500.

[1] Schoch and Jensen, *Analyt. Chem.*, 1940, **12**, 531.
[2] Bottle, Gilbert, Greenwood, and Saad, *Chem. and Ind.*, 1953, 541.

of starch arises from the possibility of modification of the polysaccharide during alkaline extraction.

Bottle and Gilbert [3] examined the effect of boiling, oxygen-free, aqueous sodium hydroxide on potato amylose of D.P. 415 and found that the degradation ceased when an average of 230 glucose residues per chain had been degraded. This cessation of attack was ascribed to a termination reaction, *i.e.*, conversion, during the degradation of a reducing glucose end-unit into an alkali-stable group. No suggestion was made as to the nature of the latter, and this interpretation was in conflict with that of Taylor and Salzmann [4] who considered that amylose contained two fractions, one completely degraded by, and the other stable to, alkali. Bottle and Gilbert's observations can be related to the results obtained from a recent study of the action of alkali on the chemically similar cellulose under similar conditions.[5] In this case also, the progressive stepwise degradation from the reducing end-group is arrested by a reaction in which a terminal reducing glucose unit is rearranged to an alkali-stable group.

The material used in the present work was cold-alkali extracted potato amylose hydrolysed in acid to D.P. *ca.* 450; the results of the degradation of this material in sodium hydroxide are shown in Table 1, and agree well with Bottle and Gilbert's finding.[3] It is evident that the degradation has virtually ceased after about 20 hr., when approximately 60% of the amylose has been degraded, yielding *ca.* 1·4 equiv. of acid per glucose residue removed. Schoch and Jensen [1] had stated that the degradation products formed under such conditions included formic, acetic, and lactic acid and pyruvaldehyde, although they gave no supporting evidence. A preliminary examination [6] of the major acidic products has now been extended, these products being examined by paper chromatography and, where possible, identified by isolation and preparation of derivatives. The proportions of the main products have also been determined, and all of these results are summarised in Table 2. For comparison, the yields of acids obtained from cellulose under similar conditions [5a] have been included.

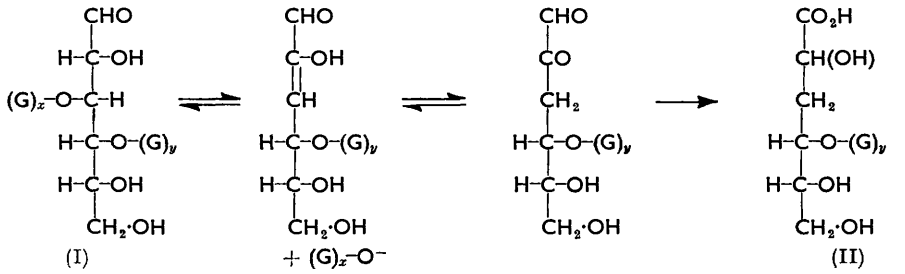
The alkali-stable polysaccharide obtained from the alkaline degradation of amylose was hydrolysed in dilute acid, and the products separated into acidic and neutral fractions by an ion-exchange procedure.[7] In the neutral products were found glucose and traces of maltose and a disaccharide reversion product. Rigorous identification of the acidic, alkali-stable end-unit was ruled out by the small amount isolated, but paper chromatography indicated the presence of approximately equal amounts of α- and β-D-gluco-*meta*saccharinic acid.

The nature and proportions of both the acidic degradation products and the alkali-stable polysaccharide obtained from the alkaline degradation of amylose are, as expected, similar to those of the analogous products from the cellulose. It follows, therefore, that in the alkaline degradation of amylose, as for cellulose, there are two main types of reaction: "degradation" giving mainly D-gluco*iso*saccharinic, formic, and lactic acid, and the "stopping" reaction in which a terminal glucose unit rearranges to D-gluco*meta*saccharinic acid. Mechanisms proposed are the same as those given [5] for cellulose.

It has been suggested, however, that the results of certain enzymatic degradations of amylose can only be explained by assuming the presence of branches linked to the main amylose chain, possibly by 1 : 3-β-glycosidic linkages.[8] On the basis of experiments with simpler related compounds,[9] the presence of 1 : 3-linked branches would lead to rearrangement in alkali, to D-gluco*meta*saccharinic acid, of the glucose units bearing such branches when these units became exposed during the course of the degradation. This situation is

[3] Bottle and Gilbert, *Chem. and Ind.*, 1954, 1201.
[4] Taylor and Salzmann, *J. Amer. Chem. Soc.*, 1933, **55**, 264.
[5] (*a*) Richards and Sephton, *J.*, 1957, 4492; (*b*) Machell and Richards, *J.*, 1957, 4500.
[6] Kenner and Richards, *Chem. and Ind.*, 1954, 1483.
[7] Machell, *J.*, 1957, 3389.
[8] Peat, Thomas, and Whelan, *J.*, 1952, 722; Peat and Whelan, *Nature*, 1953, **172**, 494; cf. Bird and Hopkins, *ibid.*, p. 493.
[9] Kenner and Richards, *J.*, 1954, 3277.

depicted in the annexed scheme. However, the analogy with cellulose, in which such 1 : 3-linked branches are very unlikely, suggests that at least part of the " stopping " reaction involves rearrangement at an unsubstituted reducing end-unit.




(I) = main amylose chain of D.P. (*y* + 1) with a 1 : 3-linked branch of D.P. *x*. (II) = resulting alkali-stable amylose.

The results obtained from the alkaline degradation of amylose and cellulose, particularly the relative yields of D-gluco*iso*saccharinic, formic, and lactic acid, should be paralleled in the alkaline degradation of 1 : 4-linked glucans in general and should also be applicable to other polyhexoses, such as mannans. It would be expected that a study of the alkaline degradation of amylopectin, in particular the effect on D.P., would help in their structural investigation.[10]

TABLE 1. *Degradation of amylose in* 0·5N-*sodium hydroxide at* 100°.

| Time (hr.) | Acid formed (milliequiv.) | Decrease in A.V. at $\lambda_{max.}$ (%) | $\lambda_{max.}$ (mμ) | Time (hr.) | Acid formed (milliequiv.) | Decrease in A.V. at $\lambda_{max.}$ (%) | $\lambda_{max.}$ (mμ) |
|---|---|---|---|---|---|---|---|
| 0 | 0 | 0 | 637 | 7 | 0·214 | 53·3 | 635 |
| 0·5 | 0·096 | 26·9 | 637 | 21 | 0·292 | 62·8 | 632 |
| 2 | 0·192 | 48·0 | 637 | 40 | 0·316 | 64·7 | 632 |

A.V. = absorption value of iodine-stained polysaccharide residue.

TABLE 2. *Acids from degradation of amylose in* 0·5N-*sodium hydroxide at* 100°.

| Acid | $R_L$ value (solvent *a*) | Yield (% total equivs.) from amylose | Yield (% total equivs.) from cellulose |
|---|---|---|---|
| α,β-D-Gluco*iso*saccharinic | 0·14 (acid), 0·55 (lactone) | *ca.* 23 | *ca.* 31 |
| Supposed dihydroxybutyric | 0·59 (acid), 1·04 (lactone) | — | — |
| Supposed αβ-dihydroxy-α-methylpropionic | 0·69 | — | — |
| Glycollic | 0·74 | — | — |
| Lactic | 1·00 | 6 | 6 |
| Lactyl-lactic | 1·23 | — | — |
| Formic | — | 35 | 33 |
| Unknown | 0·47, 0·51, 0·81 | — | — |

## EXPERIMENTAL

Amylose was kindly provided by Mrs. L. M. Gilbert; it was prepared by cold-alkali fractionation of potato starch,[11] followed by hydrolysis of the amylose fraction with 0·002N-hydrochloric acid at 100° for 20 min. under nitrogen, and isolation as the butan-1-ol complex.[12] From measurements of the hydrolysis constant,[13] the D.P. of the hydrolysed material was estimated as *ca.* 450.

The following solvents and sprays were used for paper chromatography with Whatman No. 1 paper at 25°: solvents; (*a*) [14] ethyl acetate–acetic acid–water (10 : 1·3 : 1);

[10] Cf. Greenwood, *Adv. Carbohydrate Chem.*, 1956, **11**, 335.
[11] Baum and Gilbert, *J. Colloid Sci.*, 1956, **11**, 428.
[12] Lansky, Kooi, and Schoch, *J. Amer. Chem. Soc.*, 1949, **71**, 4066.
[13] Baum and Gilbert, unpublished work.
[14] Richtzenhain and Moilanen, *Acta Chem. Scand.*, 1954, **8**, 704.

(*b*) ethanol–ammonia (*d* 0·88)–water (85 : 1 : 14); (*c*) ethyl acetate–pyridine–water (8 : 2 : 1); (*d*) butan-1-ol–ethanol–acetic acid–water (45 : 5 : 1 : 49). Sprays; (*a*) [15] B.D.H. 4·5 Indicator; (*b*) [16] hydroxylamine–ferric chloride; (*c*) [17] sodium periodate–potassium permanganate; (*d*) [18] B.D.H. 4·5 Indicator buffered with citric acid; (*e*) [19] silver nitrate–sodium hydroxide. For lactic acid, $R_L = 1·00$.

Total acids in solution were determined by adding a two-fold excess of alkali, keeping the mixture for 30 min. at room temperature to decompose lactones, and titrating it with acid to pH 9. Free acids in the same solutions were titrated directly with alkali to a transient end-point with bromothymol-blue.

*Degradation of Amylose in Sodium Hydroxide*: *Quantitative Experiments.*—Amylose–butan-1-ol complex (*ca.* 0·5 g. of amylose) was suspended in water (80 ml.), and the suspension heated at 100° in a stream of nitrogen to remove the butanol. The resulting clear solution was cooled, and diluted to 100 ml. with water, and an aliquot portion (10 ml.) evaporated to dryness as described by Bottle,[20] to give a residue of amylose (0·0544 g.). The blue value [21] measured by a Unicam spectrophotometer was 1·36 at 680 mμ.

Further 10 ml. portions of the same solution were transferred to Polythene ampoules, which were each placed inside a glass tube partly filled with water. Oxygen was removed by a stream of nitrogen, which was continued while oxygen-free N-sodium hydroxide (10 ml.) was added, and the assembly was immersed in a boiling-water bath. After varying times, the reaction was arrested by cooling with ice, and the resulting solution was run through a column of Amberlite IR-120(H) resin (25 ml.) to remove sodium ions. The total acid in the effluent was determined by titration of an aliquot portion, and the absorption value [21] (A.V.) of the iodine stain of the residual amylose on a second portion. Results are given in Table 1.

*Soluble Products from the Degradation of Amylose in Sodium Hydroxide.*—(a) *Qualitative.* A suspension of amylose–butan-1-ol complex (*ca.* 20 g. of amylose) in water was freed from butanol by steam-distillation under nitrogen. The volume of the suspension was then adjusted to 250 ml., and oxygen removed by a stream of nitrogen. Oxygen-free N-sodium hydroxide (250 ml.) was added, and the clear solution heated in a boiling-water bath for 20 hr. The resulting dark brown solution was cooled in ice, diluted to 3 l., and run through a column of Amberlite IR-120(H) resin (300 ml.) to remove sodium ions. Alkali-stable amylose in the acidic effluent was precipitated as its complex with butan-1-ol, and the complex recovered in the centrifuge, washed thoroughly with water previously saturated with butan-1-ol, and retained for later examination.

The acidic centrifugate and initial washings were combined (4 l.), distilled at 20 mm. with the bath temperature at 40—50°, and the distillate collected at 0°. Distillation was arrested when *ca.* 3 l. of distillate had collected; the latter was made 2N with respect to hydrochloric acid, and a saturated solution of 2 : 4-dinitrophenylhydrazine in 2N-hydrochloric acid (200 ml.) added. Slow deposition of a yellow solid took place and, after one month, this was collected. Crystallisation of this material from anisole afforded dark red crystals (*ca.* 40 mg.) which decomposed at *ca.* 265°, and were not further investigated. The distillation was continued to give a syrupy residue, to which were added two successive portions of water (30 ml.), each addition being followed by distillation to a syrup. An aliquot portion of the distillate (350 ml. from 1 l.) was neutralised with sodium hydroxide, and the solution of sodium salts evaporated to small bulk (5 ml.) under reduced pressure. From this concentrate was prepared *p*-bromophenacyl formate, m. p. and mixed m. p. 134—135° (from acetone–light petroleum).

The residue of non-volatile acids remaining from the above distillation was dried to constant weight (4·02 g.) over phosphoric oxide *in vacuo*. Paper chromatography of the acid mixture was carried out with solvent *a*, and for comparison a standard mixture containing D-gluco*iso*-saccharinic acid and its lactone, glycollic and lactic acid. Spray *a* developed components corresponding to the following acids: α,β-D-gluco*iso*saccharinic ($R_L$ 0·14), dihydroxybutyric ($R_L$ 0·59), αβ-dihydroxy-α-methylpropionic ($R_L$ 0·69), glycollic ($R_L$ 0·74), lactic ($R_L$ 1·00), and lactyl-lactic ($R_L$ 1·23). In addition there were traces of unknown acids having $R_L$ 0·47, 0·51,

[15] Nair and Muthe, *Naturwiss.*, 1956, **43**, 106.
[16] Abdel-Akher and Smith, *J. Amer. Chem. Soc.*, 1951, **73**, 5859.
[17] Lemieux and Bauer, *Analyt. Chem.*, 1954, **26**, 920.
[18] Cf. Kennedy and Barker, *ibid.*, 1951, **23**, 1033.
[19] Trevelyan, Procter, and Harrison, *Nature*, 1950, **166**, 444.
[20] Bottle, *Chemist-Analyst*, 1956, **45**, 82.
[21] Bourne, Haworth, Macey, and Peat, *J.*, 1948, 924.

and 0·81. Application of spray *b* revealed components corresponding to D-gluco*iso*saccharinolactone ($R_L$ 0·55) and dihydroxybutyrolactone ($R_L$ 1·04). Use of spray *c* indicated that D-gluco*iso*saccharinic acid and its lactone were the main constituents of the non-volatile acids.

An aqueous solution (250 ml.) of the residue of non-volatile acids was found by titration to contain 5·75 milliequiv. of free acids and 24·75 milliequiv. of lactones. This solution was heated to 70—80° and stirred with an excess of calcium hydroxide to decompose lactones and form the corresponding calcium salts. Excess of lime was removed by filtration, and the filtrate saturated with carbon dioxide, boiled for 5 min., and again filtered. The solution of calcium salts was decolorised with charcoal and evaporated to *ca.* 20 ml. After storage at 4° for 3 days, the concentrate deposited a white solid (0·4 g.) which recrystallised from boiling water. The purified salt was heated with an excess of Amberlite IR-120(H) resin at 70° for 1 hr. to liberate the acid and promote its lactonisation, the latter process being further facilitated by evaporating the solution and drying the residue over phosphoric oxide *in vacuo*. Crystallisation of the dried syrup (0·11 g.) from ethyl acetate gave α-D-gluco*iso*saccharinolactone, m. p. and mixed m. p. 93—94°. Paper chromatography of the lactone in solvent *a*, with sprays *b* and *c* gave a single spot, $R_L$ 0·55; in solvent *b* and with sprays *c* and *d*, the corresponding ammonium salt prepared from the lactone gave a single spot, $R_L$ 0·71.

(b) *Quantitative.* Amylose (*ca.* 1 g.) was treated with 0·5N-sodium hydroxide (50 ml.) at 100° for 30 min. in the absence of oxygen, as previously described. The cooled mixture was then freed from sodium ions by passing it through a column of Amberlite IR-120(H) resin (50 ml.), and stored at 4° overnight. Retrograded amylose was then removed by filtration, and the filtrate, which still contained amylose, was stirred with De-Acidite FF (micro-bead; 2% cross-linked) carbonate (5 g.) for 24 hr.[7] The resin was transferred to a column and washed with water (500 ml.), and the filtrate and washings containing the amylose and any other neutral products were rejected.

Sorbed acids were eluted from the resin with N-ammonium carbonate (100 ml.), and the eluate evaporated to dryness under reduced pressure to decompose the excess of eluant. The residue of ammonium salts was taken up in water (50 ml.) and passed through a column of Amberlite IR-120(H) resin (10 ml.). By titration of an aliquot portion of the effluent, the total acid present was found to be 2·16 milliequiv. Lactic acid in this solution was determined by Hullin and Noble's method [22] (the lime treatment being omitted); formic acid, other volatile acids, and D-gluco*iso*saccharinic acid were determined as described earlier.[5a] The results (as percentages of acid equivalents) were: formic acid, 35%; other volatile acids, 3%; lactic acid, 6%; and D-gluco*iso*saccharinic acid, *ca.* 23%.

*Acidic Hydrolysis of Alkali-stable Amylose and Identification of Products.*—The alkali-stable amylose–butan-1-ol complex (*ca.* 7·5 g. of amylose) obtained in an earlier experiment was freed from butan-1-ol as described, and suspended in N-hydrochloric acid (200 ml.). After 2 hr. at 100° in a stream of nitrogen, all the solid had dissolved, leaving a pale brown solution, which was decolorised with charcoal. The solution was then substantially freed from hydrochloric acid by shaking it with methyldi-*n*-octylamine [23] (51 g., 0·2 mole) in chloroform (500 ml.), and the aqueous layer was separated, washed with chloroform, and concentrated to 30 ml. at 40°/20 mm. Cations were removed from the concentrate by passage through a column of Amberlite IR-120(H) resin (10 ml.), and the acidic effluent was neutralised with silver carbonate. The small precipitate of silver chloride was removed and silver ions removed from the filtrate by treatment with Amberlite IR-120(H) resin (10 ml.) in a column. A slightly acid solution (pH 3) resulted, and this was stirred with De-Acidite FF (micro-bead; 2% cross-linked) carbonate (5 g.) for 24 hr. to sorb the acids and lactones. The resin was then transferred to a column, in which it was washed with water (500 ml.), and the filtrate and washings were evaporated to a syrup under reduced pressure. After the syrup had been dried to constant weight (7·3 g.) over phosphoric oxide *in vacuo*, it was examined by paper chromatography in solvent *c*. Applications of spray *a* indicated that the material consisted of glucose ($R_F$ 0·30) with traces of maltose ($R_F$ 0·18) and a substance of $R_F$ 0·12. The latter corresponded to isomaltose and gentiobiose, which would be expected to be formed by reversion during the acid hydrolysis.[24]

Acids were eluted from the De-Acidite resin with N-ammonium carbonate (100 ml.), and the

[22] Hullin and Noble, *Biochem. J.*, 1953, **55**, 289.
[23] Smith and Page, *J. Soc. Chem. Ind.*, 1948, **67**, 48.
[24] Thompson, Anno, Wolfrom, and Inatome, *J. Amer. Chem. Soc.*, 1954, **76**, 1309; E. E. Bacon and J. S. D. Bacon, *Biochem. J.*, 1954, **58**, 396.

eluate freed from ammonium ions as described in the previous experiment. Titration of an aliquot part of the resulting acid solution revealed the presence of 0·18 milliequiv. of total acid, but tests showed that inorganic acids including hydrochloric and sulphuric acids were present. Paper chromatography of this acid solution in solvent *a* with sprays *b* and *e* indicated that the main constituents of the mixture were probably α- and β-D-gluco*meta*saccharinolactone having $R_F$ 0·38 and 0·32 respectively. There was a small amount of a further unidentified lactone with $R_F$ 0·21. With solvent *d* and sprays as above, the α- and β-D-gluco*meta*saccharinolactone had $R_F$ 0·40 and 0·34 respectively, and the unknown lactone $R_F$ 0·19. The last could be D-glucosyl-D-gluco*meta*saccharinolactone present as a result of either incomplete hydrolysis or condensation of D-glucose with the lactone during the isolation.[25] Further investigation of these hydrolysis products was precluded by the small amount of material available.

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[25] Aspinall, Carter, and Los, *J.*, 1956, 4807.