720. *Observations on the Reduction of Alkaline 3 : 5-Dinitrosalicylate by Certain Carbohydrates.*

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Reduction of alkaline solutions of 3 : 5-dinitrosalicylate can be used for determination of certain methylated derivatives of D-fructose which do not appreciably reduce alkaline copper reagents. Various partially methylated hexoses derived from D-glucose and D-fructose display differing reducing powers, calculated on a molar basis, indicating that factors other than the presence of the potential aldehydic or ketonic group are critical. Feeble or complete lack of reduction was observed with a number of fructosans (inulins, levans, and irisins); this supports the view that these polysaccharides are built up by lengthening the fructosidic chain of the non-reducing sucrose molecule. The dinitrosalicylate reagent is not suitable for determination of the molecular size of certain amylolytic degradation products of starch and glycogen.

Meyer, Noelting, and Bernfeld (*Helv. Chim. Acta*, 1948, **31**, 103) obtained good agreement between estimates of the molecular weights of amylose and of lichenin deduced from certain physical measurements and from the reduction by the polysaccharides of 3 : 5-dinitrosalicylic acid ("DNS") in alkaline solution (Sumner, *J. Biol. Chem.*, 1921, **47**, 5). Meyer *et al.* interpreted their results on the basis of the assumption that the terminal reducing end-groups of their polysaccharides were oxidised by DNS as was the terminal reducing end-group of maltose.

Chanda, Hirst, Jones, and Percival (*J.*, 1950, 1289) and Chanda, Hirst, and Percival (*J.*, 1951, 1240) found substantial agreement between molecular-size determinations on several xylans when comparing the DNS method with physical determinations. On the other hand, Percival and Ross (*J.*, 1951, 156) noted considerable discrepancies between molecular weights of laminarin assessed from the two different approaches. In an exhaustive investigation on starch, Lansky, Kooi, and Schoch (*J. Amer. Chem. Soc.*, 1949, **71**, 4066) compared the results of several reduction methods with those of physical procedures and concluded, with regard to estimations of molecular weights, that (*a*) hypoiodite methods were inaccurate because of "over-consumption," (*b*) alkaline ferricyanide, copper, and DNS reagents were somewhat more selective but were influenced by pH, reaction temperature, reactant concentration, etc., (*c*) of the (*b*) reagents DNS at 65° gave the most reliable and reproducible results while at 100° higher reductions were obtained, and (*d*) in the starch series none of the values obtained by alkaline reduction methods could be translated into terms of molecular weights.

We have made some investigations of the DNS method with three ends in view, namely, (i) the possible determination of certain methylated fructoses which, by reason of their structures, gave little or no reduction of alkaline copper, (ii) the detection of reducing terminal radicals in certain fructosans, and (iii) the possible estimation of molecular sizes of certain dextrins obtained by amylolytic degradation of glycogen.

While the first aspect of this investigation has yielded satisfactory, but somewhat unexpected, results, the experiments on the fructosans and the dextrins have led us to the same conclusion as Lansky *et al.* (*loc. cit.*).

For our experiments we used the modified reagent of Sumner and Sisler (*Arch. Biochem.*, 1944 **4**, 333; cf. Sumner, *J. Biol. Chem.*, 1924–25, **62**, 287; 1925, **65**, 393). In a few experiments we carried out parallel reductions with the original type of DNS reagent used by Meyer *et al.* (*loc. cit.*). These experiments gave results differing only in numerical values. We found that equal amounts of glucose and fructose reduced equal amounts of DNS (cf. Hostettler, Borel, and Deuel, *Helv. Chim. Acta*, 1951, **34**, 2132) but that considerable variations in "molar" reducing power were exerted by a number of methylated sugars examined. For our purposes, however, it was important to learn that various methylated fructoses, especially those substituted in positions 1 and 3, could be

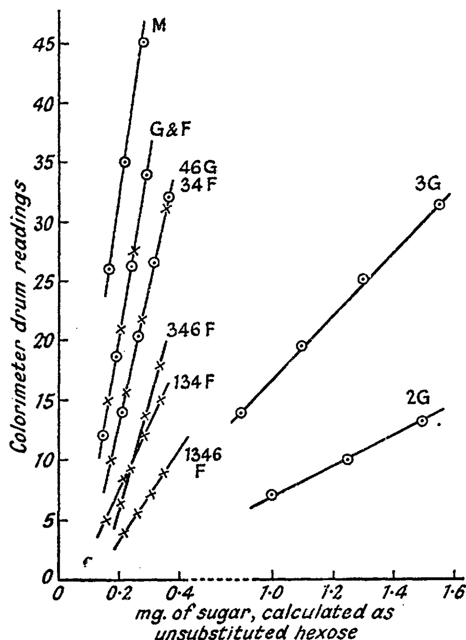
accurately determined on a micro-scale by DNS reduction. Such sugars are not oxidised by alkaline copper and, theoretically at least, are inert towards alkaline hypoiodite. Our results are set forth graphically in the Figure. We shall not comment on the various reducing powers of the sugars examined. It may be noted that the modified DNS reagent markedly decreased the amount of colour produced by *e.g.*, glucose, a fact previously observed by Lansky *et al.* (*loc. cit.*). A further remarkable fact emerged: neither 2 : 3-dimethyl nor 2 : 3 : 6-trimethyl glucoses reduce alkaline DNS, but both 2-methyl and 3-methyl do so, though to a lesser extent than the unsubstituted sugar. Yet the reducing powers of 4 : 6-dimethyl glucose and 3 : 4-dimethyl fructose are closely similar to those of the unsubstituted sugars.

Recent work on fructosans, especially on inulin, has proved that these polysaccharides contain constituent radicals of D-glucose (cf. Palmer, *Biochem. J.*, 1951, **48**, 389; Schlubach and Elsner, *Ber.*, 1929, **62**, 1493). Methylated inulin has been shown by Hirst, McGilvray,

Reducing action of various sugars on alkaline 3 : 5-dinitrosalicylate.

Key: M = Maltose
G = Glucose
F = Fructose
46G = 4 : 6-Dimethyl glucose
3G = 3-Methyl glucose
2G = 2-Methyl glucose
34F = 3 : 4-Dimethyl fructose
346F = 3 : 4 : 6-Trimethyl fructose
134F = 1 : 3 : 4-Trimethyl fructose
1346F = 1 : 3 : 4 : 6-Tetramethyl fructose

Fructose and derivatives are indicated ×
Glucose and derivatives are indicated ⊙



and Percival (*J.*, 1950, 263) to yield, on hydrolysis, approximately equal amounts of 2 : 3 : 4 : 6-tetramethyl D-glucose and 1 : 3 : 4 : 6-tetramethyl D-fructose, in addition to the major product, 3 : 4 : 6-trimethyl D-fructose. It thus seems highly probable that the inulin molecule is terminated at one end by a *non-reducing* sucrose radical from which the tetramethyl glucose is derived, while the other end consists in a fructofuranoside radical, likewise non-reducing. Strong confirmation of this chemical evidence is afforded by the simultaneous and independent enzymic experiments of Bacon and Edelman at Sheffield (*Biochem. J.*, 1949, **45**, xxvii; 1951, **48**, 114; Edelman and Bacon, *Biochem. J.*, 1950, **47**, 42) and Dedonder at Paris (references to preliminary communications are to be found in the completed papers, *Bull. Soc. Chim. biol.*, 1952, **34**, 144, 157, 171).

Grass levans (Bell and Palmer, *J.*, 1952, 3763; Laidlaw and Reid, *J.*, 1951, 1830), triticin (Arni and Percival, *J.*, 1951, 1822), and irisin (Bell, unpublished), all of which contain component radicals of D-glucose, may also be built up from sucrose acting as a "primer."

If the above hypothesis is true, all these fructosans should contain no reducing terminal radical. As regards inulin, previous reports are conflicting. Irvine and Steel (*J.*, 1920, 1474) found that repeatedly purified inulin was devoid of action on Fehling's solution (cf. Pringsheim and Ohlmeyer, *Ber.*, 1933, **66**, 1292), but Drew and Haworth (*J.*, 1928, 2690)

were unable to purify commercial inulin so that it did not reduce this reagent. Schlubach and Elsner (*loc. cit.*) found several commercial samples which definitely reduced Bertrand's copper reagent, but a sample purified by Kiliani's method (*Annalen*, 1880, **205**, 145) did not. Levan from the grass *Poa trivialis* was noted by Challinor, Haworth, and Hirst (*J.*, 1934, 1560) to be non-reducing (method not stated). Levan from barley leaves (Archbold and Barter, *Biochem. J.*, 1935, **29**, 2689) did not reduce alkaline copper. Triticin (Arni and Percival, *loc. cit.*) reduced Fehling's solution slightly after 15 minutes' heating, but these authors noted a similar reduction with pure sucrose (see below). Schlubach, Knoop, and Liu (*Annalen*, 1933, **504**, 30) found irisin to be non-reducing.

We have found that irisin is completely non-reducing to alkaline copper, but all specimens of inulin, despite repeated crystallisation from water, and all specimens of grass levans, show very slight reduction. It may be that alkaline solutions of heavy-metal ions are not generally suitable reagents for the detection of small proportions of reducing radicals in large molecules (cf. Pringsheim *et al.*, *Ber.*, 1929, **62**, 2378). There are many reports of the apparent "reducing action" of sucrose itself, *e.g.*, by Browne (*J. Amer. Chem. Soc.*, 1906, **28**, 45; *J. Assoc. Off. Agr. Chem.*, 1919, **3**, 261), Maquenne (*Compt. rend.*, 1915, **161**, 617), Quisimbing and Thomas (*J. Amer. Chem. Soc.*, 1921, **43**, 1503), and Bruhns (*Centr. Zuckerind.*, 1929, **37**, 280, 882, 875, 1268, 1467). Moreover several glycosides and other non-reducing polyhydroxy-compounds have been shown to react with alkaline silver (Hough, *Nature*, 1950, **165**, 400). In a personal communication, Dr. H. N. Munro of Glasgow states that many "non-reducing" polyhydroxy-compounds do, in fact, reduce alkaline ferricyanide in presence of nickel ions.

We therefore decided to examine the reducing action of several fructosans on the Sumner–Sisler DNS reagent, choosing this solution as it seemed to be more diagnostic than Sumner's original solution as used by Meyer *et al.* (*loc. cit.*). Samples of irisin from *Iris pseudacorus*, both wild and cultivated, were found to be definitely non-reducing. Samples of levans from Italian rye-grass (*Lolium italicum*) and leafy cocksfoot grass (*Dactylis glomerata*) showed faint reducing properties, unchanged after acetylation and deacetylation; this reduction was only a small fraction of that expected from the molecular weights estimated from sedimentation–diffusion data (Bell and Palmer, *Biochem. J.*, 1949, **45** xiv) or from the glucose radical content (Palmer, *loc. cit.*). Repeatedly recrystallised specimens of inulin behaved similarly. It therefore seems reasonable to suggest that all these fructosans are esentially composed of non-reducing molecules and that any reduction observed is not caused by the presence of reducing terminal radicals.

The DNS reagent was also used in an attempt to determine the reducing power, and hence the degree of polymerisation ("D.P."), of the dextrins "L.D.2" and "L.D.3," produced by the stepwise degradation of rabbit-liver glycogen (Bell and Manners, *Biochem. J.*, 1951, **49**, lxxvii). L.D.2 had a D.P. of 16 when measured by both a hypoiodite method (Kline and Acree, *J. Res. Nat. Bur. Stand.*, 1930, **5**, 1063, modified to a semi-micro-scale by Hanes, personal communication) and an alkaline copper method (Shaffer and Somogyi, *J. Biol. Chem.*, 1933, **100**, 695, as modified by Hanes and Cattle, *Proc. Roy. Soc.*, 1938, *B*, **125**, 387). The reduction of DNS was of an order indicating a D.P. of 5. From its other properties L.D.2 could not possibly be as small as a pentasaccharide. L.D.3 which had a D.P. of 11 (by hypoiodite and alkaline copper methods) behaved as a trisaccharide towards the DNS reagent. Furthermore a sample of a "maltodextrin" (produced by the action of malt α-amylase on starch, and provided by Professor C. S. Hanes, F.R.S.), on examination by the copper method, had a D.P. of 11; the apparent D.P. by the DNS method was 3·7.

Experimental

Standard sugar solutions were made up in saturated benzoic acid and diluted as required. Benzoic acid was found not to interfere with reduction of the DNS reagent.

General Method (Sumner and Sisler, *loc. cit.*).—Suitable amounts of the solution to be examined were measured into glass-stoppered boiling-tubes, and water was added to bring the volume up to 2 ml. The DNS reagent (1 ml., containing 0·63% of 3 : 5-dinitrosalicylic acid) was added to each tube. A blank with water (2 ml.) in place of the carbohydrate solution was prepared similarly. After mixing of the solutions, the tubes were placed in a boiling-water bath,

and heating was continued for 5 minutes. The tubes were then cooled and 5 ml. of water added to each. The intensity of the colour produced was then measured in a photometer, with a green filter, and compared with the colour of the blank. It was noted, with the carbohydrates examined, that prolongation of the heating to **30** minutes did not intensify the colour.

Examination of L.D.2 and L.D.3 and Maltodextrin.—Calibrations of each set of reagents were made at the same time as the measurements on the dextrins—the hypoiodite and copper reagents against standard maltose, and the DNS reagent against glucose and maltose. The Table shows the results.

Reagent	Analysis	L.D.2	L.D.3	"Maltodextrin"
Hypoiodite	Dextrin analysed (mg.)	87·0	77·0	—
	Maltose equiv. (mg.)	11·7	14·8	—
	D.P.	16	11	—
Alkaline Cu	Dextrin analysed (mg.)	16·38	9·96	10·34
	Maltose equiv. (mg.)	2·01	1·79	1·91
	D.P.	16	11	11
D.N.S.	Dextrin analysed (mg.)	1·67	0·58	1·29
	Maltose equiv. (mg.)	0·73	0·24	0·66
	D.P.	4·8	2·8	3·7

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