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Spectrophotometric Determination of Aldoses by an Iodometric Procedure

GAIL LORENZ MILLER and ANNE L. BURTON

Pioneering Research Division, Quartermaster Research and Engineering Center, Natick, Mass.

► An iodometric procedure for determination of aldoses is described in which the excess of iodine is measured spectrophotometrically and a glucose solution is used as the reference standard. In the development of the procedure, consideration was given to pH, potassium iodide concentration, reaction time, and temperature. Tests were made with pentoses, hexoses, oligosaccharides, and enzymic digests of carboxymethylcellulose. Consideration was also given to complex formation of iodine with carboxymethylcellulose.

THE usual iodometric procedure for determination of aldoses consists of treating test samples at an alkaline pH

with an excess of standard iodine-potassium iodide solution, followed, after a suitable period of standing, by acidification and back-titration with sodium thiosulfate solution. This procedure has been reported to be applicable to the determination of glucose, galactose, maltose, lactose, arabinose, xylose, ribose, and methylated sugars (2, 6), but not to rhamnose, lyxose, talose, and mannose (6), which exhibit low uptakes of iodine, or to higher oligosaccharides (1), which give erratic results. Certain work (16) has indicated, however, that mannose can be determined by lengthening the time of action of the iodine, and it may be supposed that other difficultly determined aldoses would respond similarly.

The iodometric procedure has also been used with variable success for determinations of molecular weights of starch fractions (11, 13).

In the present paper a modified procedure is described in which the excess of iodine is measured spectrophotometrically instead of by titration with sodium thiosulfate, glucose solution being used as a reference standard. The amount of aldose present in an unknown test sample is determined by comparing the difference between the absorbance of the color of iodine in a blank and a test sample with the difference between the blank and the glucose standard. As spectrophotometric procedures are generally more convenient than titrimetric procedures and as the

preparation of standard glucose solutions is more easily carried out than the preparation and standardization of iodine solutions, the modification represents a simplification. The modified procedure is tested with pentoses, hexoses, oligosaccharides, and also with enzymic digests of carboxymethylcellulose.

REAGENTS

IODINE-POTASSIUM IODIDE, 0.02*N*. Iodine (analytical grade, approximately 2.6 grams) and potassium iodide (analytical grade, 100 grams) are diluted to 1 liter with distilled water. Standardization is not required.

IODINE-POTASSIUM IODIDE, 0.002*N*. Same as 0.02*N* iodine-potassium iodide except that only 0.26 gram of iodine is used.

SODIUM CARBONATE, 0.75*M*. Anhydrous sodium carbonate (analytical grade, 40 grams) is diluted to 500 ml. with distilled water.

PHOSPHORIC ACID, 1.2*N*. Sixty per cent phosphoric acid (85%, 55 ml.) is made up to 2 liters with distilled water.

GLUCOSE STANDARD. Glucose (100 mg., weighed to ± 0.1 mg.) is made up to 100 ml. with distilled water.

TEST MATERIALS

Aldoses. Glucose was obtained from the Matheson Co.; galactose from General Biochemicals, Inc.; cellobiose from Eastman Kodak Co.; mannose and xylose from Fisher Scientific Co.; arabinose from Pfanstiehl Chemical Co.; and lyxose from Bios Laboratories. Cellotriose, cellotetraose, and cellopentaose were supplied through the courtesy of M. L. Wolfrom, The Ohio State University, and were also prepared in this laboratory by gradient elution chromatography of a mixture of oligosaccharides obtained by saponification of acetolyzed cellulose (14). The higher oligosaccharides were found to contain 3 to 6% moisture and allowance was made for moisture in the calculation of results. The different sugars were each dissolved in distilled water to give final concentrations equivalent to 1 mg. of glucose per ml. on a mole basis.

Enzymic Digests of Carboxymethylcellulose. Mixtures of 1 ml. of buffered carboxymethylcellulose (15) and 1 ml. of cellulase containing 4 units of enzyme activity (5) were incubated at 50° C. for 80 minutes and cooled briefly in a cold tap water bath before application of the iodometric procedure.

PROCEDURE

A 2-ml. aliquot of test solution, containing the equivalent of not more than 2 mg. of glucose on a mole basis is transferred to a 14 × 125 mm. tube. A 2-ml. aliquot of glucose solution, containing 2 mg. of glucose to serve as a standard, and a 2-ml. aliquot of water to serve as a blank also are transferred to tubes. Next, 1 ml. of 0.75*M* sodium

carbonate and 2 ml. of 0.02*N* iodine-potassium iodide are added to each tube. The aliquots of test sample, standard, and blank are measured out with the aid of volumetric transfer pipets; the various reagents are measured with Krogh-Keyes type pipets. The contents of the tubes are mixed by twirling and the tubes are allowed to stand for a period of time at a temperature which depends upon the nature of the sugar being tested. Next, the mixtures are each acidified with 5 ml. of 1.2*N* phosphoric acid and mixed by pumping motion with a 3-mm. glass rod having a 7-mm. flattened end. Mixing is carried out carefully to avoid losses resulting from too rapid an evolution of carbon dioxide, and thoroughly to remove the excess carbon dioxide and avoid error due to formation of gas bubbles in the spectrophotometric cuvettes. The absorbances of the final mixtures thus obtained are measured at room temperature in a Beckman Model DU spectrophotometer at a wave length of 480 $m\mu$ and a slit width of 0.2 $m\mu$. The wave length of 480 $m\mu$ is used instead of that of 352 $m\mu$ where maximum absorbance is obtained (4), as the higher wave length permits a more practical range of spectrophotometer readings, 0 to 0.5, for the measurement of the quantities of aldose used in the proposed procedure. Use of the longer wave length does not affect linearity detectably.

For determinations of enzymic digests of carboxymethylcellulose, the blanks are prepared to contain carboxymethylcellulose in the absence of the enzyme.

For determinations of very small samples of aldose—e.g., the mole equivalent of 0.2 mg. of glucose—0.002*N* iodine-potassium iodide is used and the reaction time is doubled. To increase the numerical values of the spectrophotometric readings obtained under these conditions, measurements of absorbance are made at 404 $m\mu$ rather than 480 $m\mu$.

VARIABLES

In the development of the procedure, consideration was given to pH, potassium iodide concentration, reaction time, temperature, and also a problem of interaction of iodine with carboxymethylcellulose.

pH. To provide the optimum pH of 11.3 (7), sodium carbonate was selected as the source of alkali. To cope better with the loss of alkali which takes place during the formation of the active agent—hypoiodous acid—and the oxidation of aldehyde groupings to carboxyl groupings, the proportion of sodium carbonate relative to iodine and aldose was made 4 to 11 times greater in the present procedure than in previously reported procedures (2, 12). Under the conditions used, the pH drops a maximum of 0.2 unit.

Potassium Iodide Concentration.

The effects of concentration of potassium iodide in the iodine reagent upon the rate and degree of uptake of 0.02*N* iodine in tests with 2 mg. of glucose at 25° C. are shown in Figure 1. The maximum absorption of iodine by glucose observed in these tests was taken as 100% reaction, as it was found to correspond to that required by theory (2, 7). At low iodide concentrations the uptake of iodine is incomplete. At higher iodide concentrations, the uptake approaches a maximum although the rate tends to become slower. In comparable tests with mannose and galactose, at low iodide concentrations the reaction of iodine with mannose is even less complete than with glucose, whereas that with galactose is more complete. The use of iodine reagent containing 10% potassium iodide is best for assuring the theoretical uptake of iodine at a reasonable rate.

The incomplete reaction of aldoses with iodine at low iodide concentrations is attributable to the rapid decomposition of the active agent, hypoiodous acid, which takes place under these conditions (3). Complete reaction, however, apparently can be obtained even at low iodide concentration, provided a sufficient excess of the iodine is available (3). In the present spectrophotometric modification of the iodometric procedure, the use of a large excess of iodine would adversely affect the accuracy of measurement and cannot be resorted to.

Reaction Time and Temperature.

The time required for complete reaction of aldose with iodine was found to depend upon the structure of the aldose, as was to be expected from previous work (7, 8, 16). The rates observed at 25° C. for galactose, glucose, cellobiose, cellopentaose, a digest of carboxymethylcellulose (CMC), and mannose illustrate the range encountered (Figure 2). To facilitate comparison of rates of the different sugars in this figure, the quantities of iodine absorbed at the end of 60 minutes were taken to correspond to 100% reaction. Rates were also measured at 17° C. and were found to be approximately half those at 25° C. In separate tests, arabinose exhibited a rate similar to galactose; xylose, similar to glucose; and lyxose, similar to mannose. The monosaccharides with hydroxyl groups on C₂ and C₃ which are configurationally trans are oxidized more rapidly than those which are configurationally cis.

To determine the degree to which secondary reactions might take place with the different aldoses, further tests were made with the use of much longer reaction times. This might be particularly important in the case of the higher oligosaccharides in view of the anomalous shape of the rate curve for cellopentaose (Figure 2). The results are

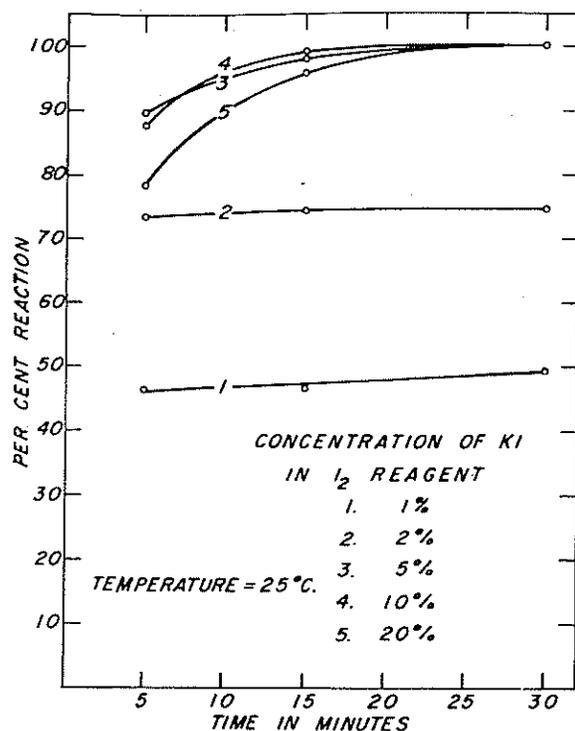


Figure 1. Effect of potassium iodide concentration in iodine reagent on rate and degree of uptake of iodine by glucose

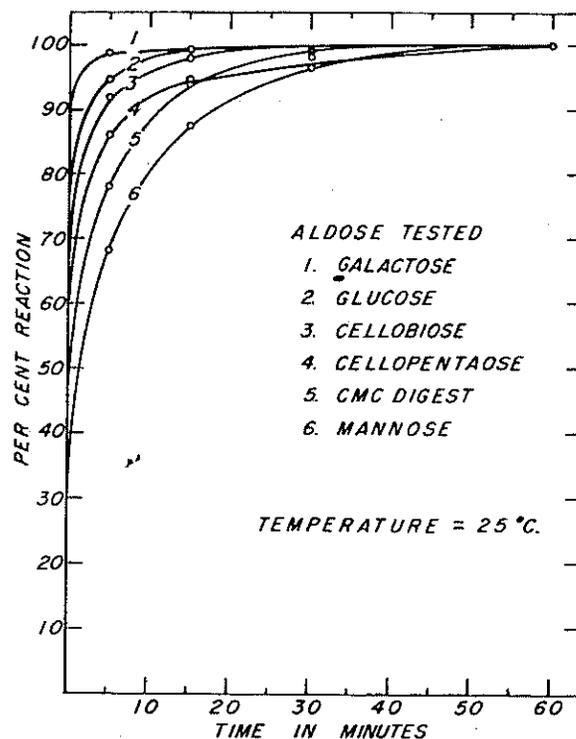


Figure 2. Rates of reactions of aldoses with iodine at 25° C.

shown by the solid lines in Figure 3 in which the quantities of iodine absorbed at the end of 1 hour are again taken to correspond to 100% reaction. The findings revealed slight secondary reactions with monosaccharides, but increasingly significant reactions with higher and higher oligosaccharides. In separate tests other monosaccharides, specifically galactose and mannose, like glucose, revealed slight secondary reactions while the secondary reaction shown by cellobiose was similar to that of cellopentaose.

The secondary reactions involve alcoholic groupings (13), probably both primary (3), and, by analogy with the action of hypobromite and periodate on sugars, also secondary (9). The observed differences between the behaviors of oligosaccharides and those of monosaccharides are attributable to the fact that oligosaccharides contain higher proportions of alcoholic groupings relative to aldehyde groupings. The finding that carboxymethylcellulose digests exhibit only slight secondary reactions may be the result of one or more of several possible circumstances: the presence of substituent carboxymethyl groups on hydroxyl groups, steric interference by carboxymethyl groups, or catalytic effect of carboxymethylcellulose on liberation of iodine from potassium iodide, counterbalancing absorption of iodine in secondary reactions.

To determine whether the secondary reactions of iodine with aldoses might be reduced by the use of a lower temperature, experiments were carried out with

glucose and cellopentaose at 13° C. The results observed were favorable, as shown by the dotted lines in Figure 3, and were confirmed when the tests were repeated.

When rates were measured for the reaction of 0.2-mg. levels of glucose with 0.002*N* iodine-potassium iodide at 25° C., the time required for complete reaction was increased to 2 hours, and that for mannose to 4 hours.

Interaction of Iodine with Carboxymethylcellulose. Iodine forms a strongly colored complex with carboxymethylcellulose (10). This is demonstrated by the results of an experiment, presented graphically in the left-hand portion of Figure 4, in which 2-ml. aliquots of 0.5% carboxymethylcellulose were treated with 1-ml. aliquots of 0.75*M* sodium carbonate, 2-ml. aliquots of different concentrations of iodine in 10% potassium iodide, and 1-ml. aliquots of 3*N* sulfuric acid. Sulfuric acid is the acid usually employed in iodometric procedures. In control tests the carboxymethylcellulose was replaced with water. The tests shown were carried out at 17° C. with a reaction time of 2 hours, although similar results were obtained at 25° C. with a reaction time of 1 hour. Absorbances were measured at a wave length of 590 $m\mu$ where the results were brought out more strikingly. The formation of the complex was most marked when iodine concentrations above 0.01*N* were added.

To determine whether the formation of the colored complex might be reduced

by the use of a large volume of less concentrated acid, tests were made in which 5-ml. aliquots of 0.6*N* sulfuric acid were substituted for the 1-ml. aliquots of 3*N* acid used before. To determine whether the use of phosphoric acid in place of sulfuric acid might further reduce the formation of the colored complex, tests were also made in which 5-ml. aliquots of 1.2*N* phosphoric acid were used. The phosphoric acid brought the final pH of the mixture to 2.2, compared to a pH of 1.4 effected by the sulfuric acid. The quantities of either acid used were shown to be adequate to ensure complete liberation of the iodine in the mixtures. Absorbances of the colors which resulted under these conditions are presented graphically in the right-hand portion of Figure 4. The findings show clearly that the greatest advantage is realized through the use of the 5-ml. aliquots of the 1.2*N* phosphoric acid. The phosphoric acid reagent was selected for use in the final form of the aldose test.

ACCURACY

For glucose in the range of 0 to 2 mg. (iodine concentration 0.02*N*, time of reaction 1 hour, temperature 25° C., and wave length for absorbance measurements 480 $m\mu$), the relation between amount of sample and absorbance was found to be precisely linear; this also was obtained for the range 0.0 to 0.2 mg., where iodine concentration used was 0.002*N*, time and temperature were the same as above, and the wave length for measurements was 404 $m\mu$. The rela-

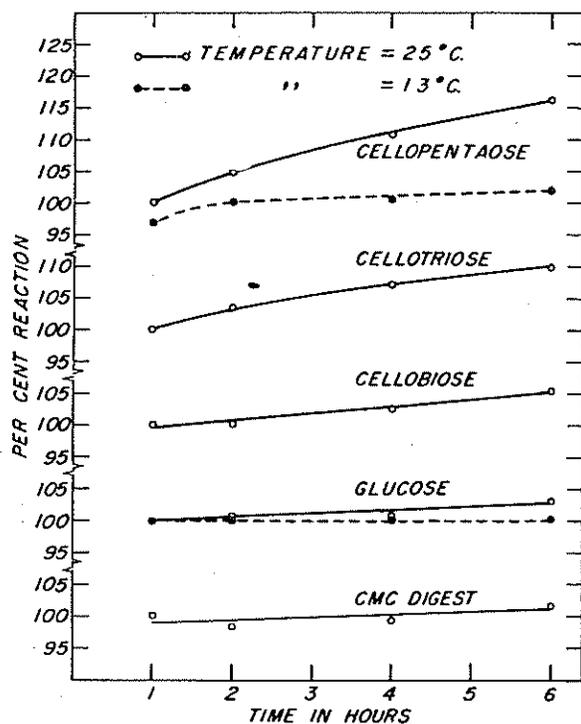


Figure 3. Rates of secondary reactions of aldoses with iodine at 25° and 13° C.

tion failed to be linear above the ranges indicated because of incomplete uptake of iodine when its concentration was reduced too greatly by reaction with aldose. Carboxymethylcellulose, when present, had a slight depressing effect on the readings of absorbance obtained with glucose and 0.02*N* iodine; the effect was more marked with glucose and 0.002*N* iodine.

Where the structure of the aldose to be tested is known, the conditions of time and temperature for carrying out its measurement can be adjusted accordingly. For mono- and disaccharides of the types tested in the present study, or for carboxymethylcellulose digests, a temperature of 25° C. is used advantageously because at this temperature the secondary reactions appear of minor importance and the times required for complete reaction are shorter than they would be at lower temperatures. The times required can then be judged on the basis of the data shown for representative aldoses in Figure 2. When small amounts of mono- or disaccharides are being measured with the aid of 0.002*N* instead of 0.02*N* iodine, the times indicated by data of Figure 2 should, by analogy with the results described for glucose under these conditions, be doubled. Where the structure of a mono- or disaccharide to be tested is unknown, a time of 2 hours at 25° C. should probably be used to allow a reasonable safety factor which will assure complete reaction.

For oligosaccharides, a temperature of 13° C. instead of 25° C. should be used

to obviate uncertainties occasioned by secondary reactions at the higher temperature. At the lower temperature a reaction time of 2 hours assures complete reaction of the type of oligosaccharides tested.

When compared with glucose under the appropriate conditions outlined above, spectrophotometric values for uptakes of iodine by samples of galactose, cellobiose, mannose, arabinose, xylose, lyxose, cellotriose, cellotetraose, and cellopentaose were found to be 99, 101, 99, 99, 100, 98, 98, 96, and 97%, respectively. The slightly low values for the higher oligosaccharides are probably due to the presence of difficultly removed inert impurities; the rate of response of these materials to the action of iodine would not seem to suggest any other explanation.

To determine the reproducibility of the method, twenty 2-ml. aliquots of a mannose solution serving as an unknown were carried through the final form of the analytical procedure. Each solution contained 1 mg. of aldose per ml. For determination of the blank, three 2-ml. aliquots of water were substituted for the sugar solutions. The reagents were added alternately to the samples of glucose and mannose in order that the results obtained with each pair might be treated as an individual analysis. The differences between the average reading of the blanks and the readings of each of the sugar samples were calculated first. Next, the ratios of the differences for the mannose samples to those for the glucose samples were calculated

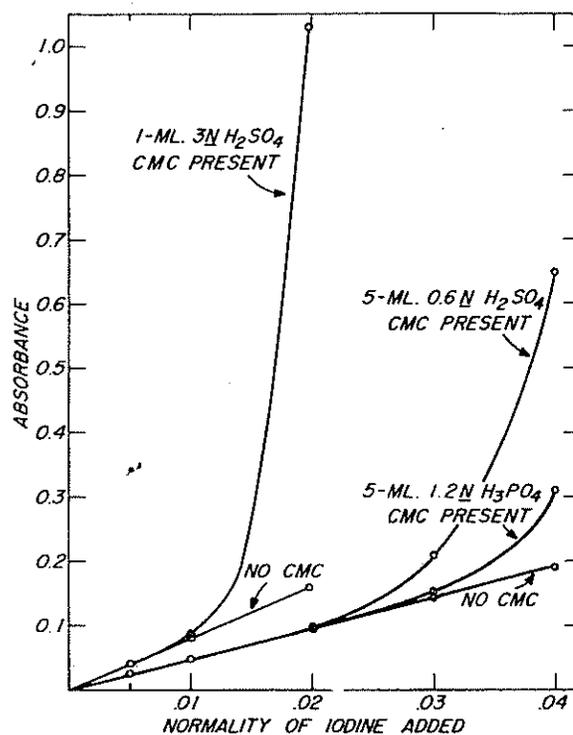


Figure 4. Effect of carboxymethylcellulose on absorbance of iodine

for each pair. Finally, the standard deviation of the different values of the ratios from the value of the mean ratio was calculated, which amounted to only 0.005.

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