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Substrate Cleavage Point of the α- and β-Amylases[1]

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Hydrolysis of glycogen by crystalline β- and α-amylases has been carried out in H_2O^{18}. Enzymatically formed saccharides contained O^{18} in the 1 position to the extent of about 90%. Maltose controls contained only traces of O^{18}. It is concluded that both β- and α-amylases rupture the C-1-O bond of the α-1,4 linkages hydrolytically. A mechanism consistent with these findings is presented and discussed.

Introduction

Amylases have been conveniently divided into two types.[3] Type I amylases (α-amylases) when acting on polysaccharides of the starch glycogen class produce a rapid decrease in relative viscosity, iodine coloration and a mixture of products which mutarotate downward. Type II amylases (β-amylases) when acting on the same substrates produce a very slow decrease in relative viscosity, iodine coloration and a single product, maltose, which mutarotates upward.

By optical rotation experiments, it has been shown that β-amylases from barley and sweet potato liberate maltose in the β-form; α-amylases from hog pancreas, malt, *Aspergillus oryzae* and *Bacillus subtilis* liberate their products with an α-configuration.[4] Thus, β-amylases invert the configuration at the C-1 position during cleavage of the α-1,4-glucosidic bond, whereas with α-amylolytic hydrolysis the configuration at the C-1 carbon is retained.

In order to account for the inversion and retention of configuration with the β- and α-amylases, respectively, it has been suggested that in the former case the C-1–O bond is cleaved, while the C-4–O bond is ruptured in the latter.[5] Alternatively, it has been proposed that the β-amylases facilitate a "single displacement" while the α-amylases catalyze a "double displacement" at the C-1 carbon, the same bond being cleaved by the two types of amylases.[6]

Using O^{18} as a tracer, evidence has been obtained which indicates that both classes of amylases cleave the same bond, namely, the C-1–O bond. In this paper the evidence will be presented and the results interpreted in terms of a mechanism in agreement with the structure and chemical behavior of the substrate.

Experimental

A. Materials.—Rabbit liver glycogen was prepared from frozen livers by the trichloroacetic acid extraction method.[7] All operations were carried out in a cold room (2–4°). Liver was cut into pieces, and ground into a thick paste with the aid of a small amount of sand. The paste was extracted with an equal volume of cold 10% trichloroacetic acid. The mixture was centrifuged, and the residue re-extracted a second time with 1.5 volumes of cold 5% trichloroacetic acid. To the combined extracts was added an equal volume of 95% ethanol and the flocculated glycogen allowed to stand overnight. Supernatant fluid was poured off and the glycogen collected by centrifugation. It was dissolved in a minimum amount of cold water and dialyzed exhaustively against cold demineralized water. The product was precipitated with 66% ethanol, washed with 95% ethanol and then with ether. After air drying, it was stored at room temperature in a desiccator.

Commercial maltose was recrystallized three times from ethanol containing 1% nitric acid by the method of Bates, *et al.*[8] The β-maltose monohydrate was stored at room temperature in a desiccator.

Sweet potato β-amylase, 4×-recrystallized, was a gift of A. K. Balls. An aliquot of the crystal suspension was diluted with H_2O^{18} (approximately 1.4 atom per cent excess O^{18}). After standing for 12 hours in a cold room, the amylase solution was frozen and stored in a deep-freeze. The enzyme was assayed by the method described by Bernfeld,[9] except that a 5% solution of glycogen was used as substrate in place of 1% soluble starch. Under these conditions, 1 ml. of the amylase solution liberated 32,000 mg. of maltose monohydrate in 3 minutes at 20°.

Hog pancreatic α-amylase, twice recrystallized, was generously provided by E. H. Fischer. A stock solution was prepared as follows.[10] Seven mg. of amylase was dissolved in 2 ml. of 0.02 *M* phosphate, 0.007 *M* NaCl, *p*H 7.1, in H_2O^{18} (1.4 atom per cent. excess O^{18}). The tube was allowed to stand in a cold room for 12 hours before being frozen. All but a very small amount of material went into solution. The enzyme was assayed by the method described by Bernfeld,[9] and was found to liberate reducing material equivalent to 700 mg. of maltose monohydrate per ml. of enzyme solution.

Bacillus subtilis α-amylase, 3×-recrystallized, also was generously provided by E. H. Fischer. A stock solution was prepared by dissolving 5 mg. of amylase in 1 ml. of 0.02 *M* $CaCl_2$ in H_2O^{18} (1.4 atom per cent. excess O^{18}). The enzyme solution was allowed to stand in a cold room for 12 hours before storing in a deep-freeze. The enzyme was assayed by the same method used for the hog pancreatic amylase,[9] except that the *p*H was set at 6.5. Under these conditions, 1 ml. of the enzyme solution liberated reducing material equivalent to 2850 mg. of maltose monohydrate.

The O^{18} water, containing 1.4 atom per cent. excess O^{18}, was purchased from the Stuart Oxygen Co., San Francisco Calif.

B. Methods.—Reducing power was measured by the 3,5-dinitrosalicylic acid method of Meyer, *et al.*[11] Measurements were made in a Klett-Summerson colorimeter with filter no. 54.

The number of mmol.–CHO/mg. in a sugar sample was determined by the iodometric method as described by Caldwell, *et al.*[12]

The enzymatic reactions were followed by ascending paper chromatography (Whatman No. 1) in order to ascertain the number and nature of components in a hydrolysate. A one-phase system composed of butanol–pyridine–water (6:4:3)

(1) Taken from a thesis submitted by F. C. Mayer to the Graduate College of the University of Illinois in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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(3) P. Bernfeld, *Advances in Enzymol.*, **12**, 379 (1951).

(4) G. G. Freeman and R. H. Hopkins, *Biochem. J.*, **30**, 451 (1936); H. Fuwa and Z. Nikuni, *Mem. Inst. Sci. and Ind. Research, Osaka Univ.*, **9**, 194 (1952).

(5) S. Schwimmer, *Brewers Dig.*, **26**, 29T (1951); *Ann. Rev. Biochem.*, **26**, 83 (1957).

(6) D. E. Koshland, Jr., *Biol. Revs.*, **28**, 416 (1953).

(7) M. R. Stetten, H. M. Katzen and D. Stetten, Jr., *J. Biol. Chem.*, **22**, 587 (1956).

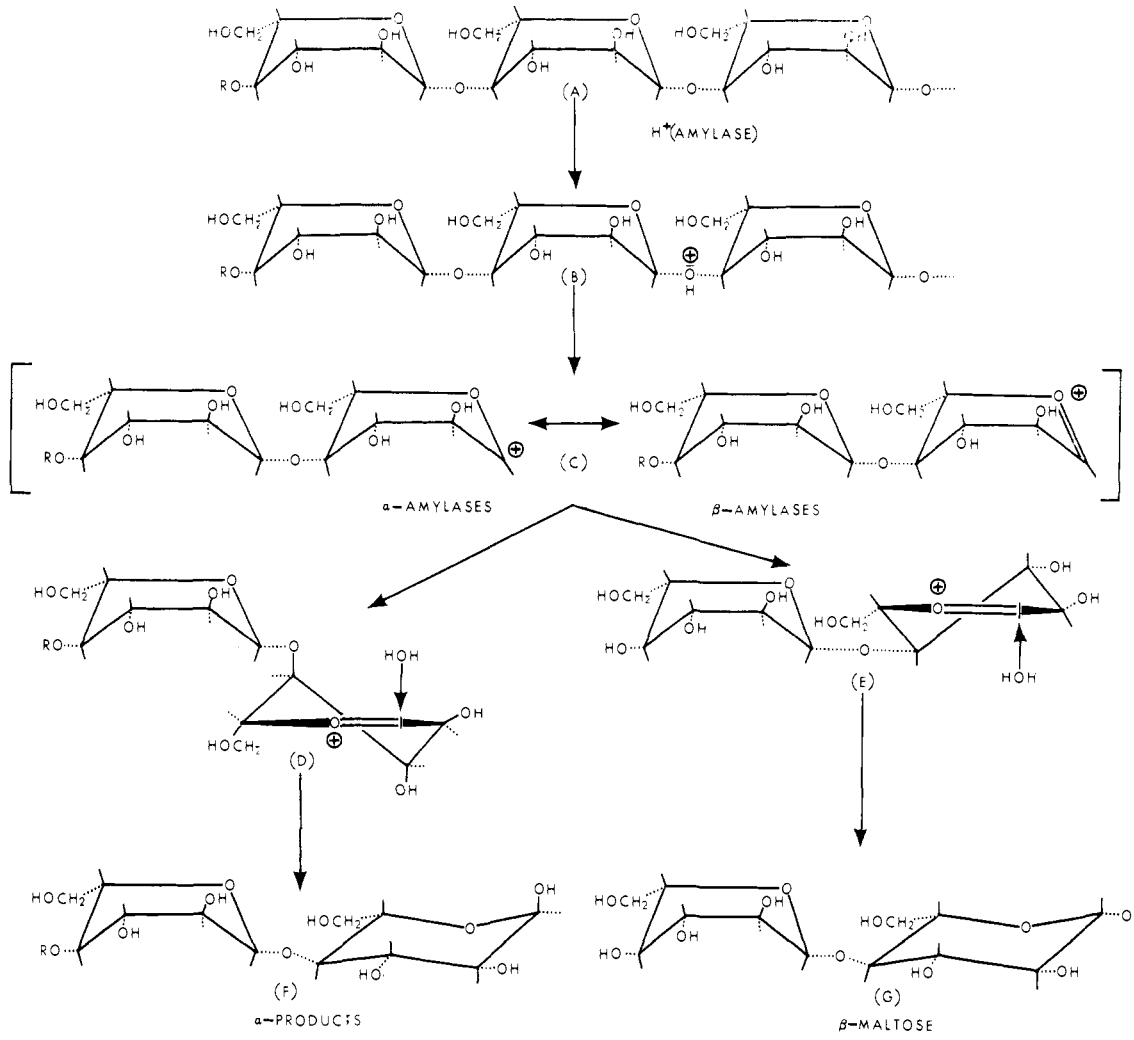
(8) F. J. Bates and Associates, "Polarimetry, Saccharimetry, and the Sugars," Circ. of the National Bureau of Standards C440, U. S. Govt. Printing Office, Washington, D. C., 1942, p. 470.

(9) P. Bernfeld, *Methods in Enzymol.*, **1**, 149 (1955).

(10) M. L. Caldwell and J. T. Kung, THIS JOURNAL, **75**, 3132 (1953).

(11) K. H. Meyer, E. H. Fischer and P. Bernfeld, *Helv. Chim. Acta*, **30**, 64 (1946).

(12) M. L. Caldwell, S. E. Doebbeling and S. H. Manian, *Ind. Eng. Chem., Anal. Ed.*, **8**, 181 (1936).



was employed for development.[13] The sugars were detected by a silver dip method[14] modified as follows[15]: solution 1 was prepared by diluting 1 ml. of saturated $AgNO_3$ to 6 ml. with water and then to 200 ml. with acetone. Solution 2 consisted of 1 volume of 10% aqueous NaOH and 5 volumes of methanol. Solution 3 was an aqueous 0.05 *M* solution of sodium thiosulfate. The solutions were poured into separate pans and the paper was dipped into solution 1 and allowed to dry. The paper was next dipped into solution 2 until the characteristic "black spots" began to appear. After washing under the tap, it was placed in solution 3 until the background coloration disappeared. A final washing under the tap yielded a chromatogram which was stable.

The identity of the sugars was ascertained by plotting the logarithm of a partition function against molecular size as described by French and Wild.[13]

All sugar samples were dried in an Abderhalden pistol at the temperature of boiling ethanol and with phosphoric anhydride in the desiccator section, at a pressure less than 1 mm. for 5 hours. The samples were allowed to cool over phosphoric anhydride in a stoppered test-tube. The O^{18} and aldehyde determinations were made on the dried samples.

All O^{18} measurements were made on carbon dioxide with a Consolidated–Nier isotope-ratio mass spectrometer, model 21-201; O^{18} content of water was determined by equilibrating H_2O^{18} and tank carbon dioxide at room temperature for 24 hours.[16] Carbon dioxide was analyzed and O^{18} content of the water was calculated with the equation derived by Dostrovsky and Klein.[17] In general, approximately 0.4 mmol. carbon dioxide was equilibrated with 10–18 mmol. of water.

The O^{18} content of sugar samples was determined by an equilibration method.[18] Weighed amounts of dried sugar and of ordinary water were placed in a break-seal tube.[19] The tube was heated in a water-bath at 83° for 5 hours. The heater was turned off and the bath was allowed to come to room temperature (approximately 12 hours). The tube was removed and the water was distilled and equilibrated with carbon dioxide. The O^{18} content of the sugar (–CHO) was calculated from the O^{18} content of the water by simple isotope dilution.

Determination of O^{18} content of water and sugar by pyrolysis[20]: A train for direct oxygen determination[21] modified essentially as described by Doering and Dorfman[22] was used for the pyrolysis. Differences between the train described by Doering and Dorfman and the train used here were as follows: The bubbler tube containing the bromine solution and the liquid nitrogen trap were omitted. Effluent gases from the carbon tube were passed directly into

(13) D. French and G. M. Wild, THIS JOURNAL, **75**, 2612 (1953).

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

(15) E. A. Moscatelli, personal communication.

(16) M. Cohn and H. C. Urey, THIS JOURNAL, **60**, 679 (1938).

(17) I. Dostrovsky and F. S. Klein, *Anal. Chem.*, **24**, 414 (1952).

(18) M. Anbar, I. Dostrovsky, F. S. Klein and D. Samuel, *J. Chem Soc.*, 155 (1955).

(19) The break-seal tubes with an outside diameter of 19 mm. were obtained from Corning Glass Co., Corning, N. Y.

(20) Pyrolyses were performed by the Clark Microanalytical Laboratory, Urbana, Ill.

(21) A. Steyermark, "Quantitative Organic Microanalysis," Blakiston Co., Philadelphia, Pa., 1951, p. 208.

(22) W. von E. Doering and E. Dorfman, THIS JOURNAL, **75**, 5595 (1953).

the I_2O_5. Iodine vapor was condensed in a straight tube cooled with Dry Ice. Carbon dioxide was collected in a U-tube immersed in liquid nitrogen.

It was found that two consecutive pyrolyses of a given sample were sufficient to remove any memory effect. For example, three consecutive pyrolyses of a water sample gave an atom per cent. excess O^{18} of 0.115, 0.124 and 0.121, respectively. In all cases the carbon dioxide from the second aliquot was collected and analyzed.

In calculating the O^{18} content of the carbon dioxide the dilution of CO going to CO_2 and the number of oxygens in the sample were taken into consideration. For water the dilution of oxygen was 1:2, for maltose the dilution was 1:22.

Enzymatic hydrolyses were performed as follows: 1 g. of liver glycogen was dissolved in 20 ml. of H_2O^{18} with the aid of gentle heating (less than 60°). After cooling to room temperature, the *p*H was appropriately adjusted. In the case of the two α-amylases the glycogen solution was made 0.007 *M* in NaCl.

The glycogen solution was brought to 0°, enzyme added and, after removal of aliquots for reducing power and isotope analysis, the reaction mixture was incubated at 29° for 30 minutes.

At the end of the incubation period, aliquots were again removed for reducing power and isotope analysis. The two aliquots for isotope analysis were combined.

The remainder of the digestion mixture was added to enough cold absolute ethanol to a final concentration 75–80%, placed in a deep-freeze for 2 hours and then centrifuged at cold room temperature. The clear supernatant was distilled *in vacuo* at room temperature, the white sirup remaining after solvent removal taken up in a small amount of water (7 ml.) at 0° and shaken with 1 ml. of chloroform. The mixture was recentrifuged in the cold; the clear top layer removed and lyophilized. The resultant white powder was dried and analyzed for O^{18} and aldehyde content.

Controls with β-maltose monohydrate were treated in an identical manner; 300 mg. maltose was dissolved in 10 ml. of H_2O^{18} in all cases.

Results

A. Preparation and Analysis of Maltose-O^{18}.—Cohn and Urey[18] have shown that acetone and acetaldehyde (carbonyl groups) completely exchanged oxygen with water; phenolic and alcoholic oxygen did not exchange. Recently, glucose has been shown to contain one exchangeable oxygen position.[23] As a preliminary step to the present investigations, it was desirable to ascertain the extent of exchange between maltose and O^{18}-water. The evidence supporting the statement that maltose also contains one exchangeable oxygen position has been recorded below.

Recrystallized maltose, dried as described previously (Methods), and H_2O^{18} were equilibrated in a sealed tube under conditions described for the equilibration method. Initial and final waters were analyzed for O^{18} by the carbon dioxide method. Results are presented in Table I.

TABLE I

MALTOSE-H_2O^{18} EQUILIBRATION

Expt.	H_2O, mmol.	-CHO, mmol.	At. % excess O^{18} initial water	At. % excess O^{18} final water Calcd.	Obsd.
1	27.78	1.06	0.688	0.662	0.661
2	27.78	0.94	0.688	0.665	0.658
3	16.67	0.81	1.38	1.32	1.33

3×-Recrystallized maltose (anhydrous) gave a value of 0.00268 mmol.-CHO/mg., calculated from hypoiodite oxidation. The theoretical value for pure maltose (anhydrous, mol. wt. 342) is 0.00293 mmol.-CHO/mg. Therefore, the maltose used in the equilibrations was about 92% pure. Examination of a multiple-ascent chromatogram showed that the recrystallized maltose was contaminated with small amounts of maltotriose and higher oligosaccharides.

From the data it may be concluded that maltose has one exchangeable oxygen. For two exchangeable O-positions (including water of hydration) the atom per cent. excess O^{18} in the final water of experiment 3 (Table I) would have been 1.26, which is well outside the range of experimental error. Contamination of the maltose in no way invalidates the above conclusion, since the mmol.-CHO was calculated from direct aldehyde determination which included higher homologs, not from the molecular weight of maltose.

Maltose-O^{18} was analyzed by pyrolysis and by the equilibration method. Comparison of the O^{18} content of maltose as determined by equilibration with the calculated value is given in Table II.

TABLE II

MALTOSE-O^{18} EQUILIBRATION

Expt.	At. % excess O^{18} in maltose Calcd.	Obsd.	Deviation, %
1	1.19	1.19	0
2	0.679	0.643	−5
3	1.31	1.36	+4

The data show that the equilibration method was satisfactory for the determination of O^{18} in the 1-position of sugars. The calculated value was obtained from a material balance.

Although the atom per cent. excess O^{18} in the water is correct to 1% or less, a small error in its atom per cent. excess O^{18} leads to a much larger error in the calculated value for the sugar. In view of this fact, the agreement between the experimental and the theoretical values are considered reasonable.

Comparison of the O^{18} content of maltose as determined by pyrolysis and by equilibration is given in Table III. A sample of O^{18} water also is included.

TABLE III

COMPARISON OF O^{18} CONTENT BY PYROLYSIS AND EQUILIBRATION

Material	Atom per cent. excess O^{18} Pyrolysis	Equilibration	Deviation, %
Water	0.123	0.140	−12
Maltose	1.09	1.19	− 9
Maltose	0.374	0.419	−11
Maltose	0.471	0.511	− 8

In all cases the O^{18} content as determined by pyrolysis was about 10% lower than the O^{18} content determined by equilibration on a portion of the same sample.

The data in Tables II and III demonstrate that the equilibration method accounts for all the isotope in the sugar.

B. Glycogen Hydrolysis by β-Amylase.—Results for this series of experiments are presented in Table IV. For experiments 1 and 2, 0.1 ml. of enzyme solution was used; in experiment 3, 0.2 ml. was added.

(23) D. E. Koshland, Jr., and S. S. Stein, *J. Biol. Chem.*, **208**, 139 (1954).

TABLE IV

ENZYMATIC HYDROLYSES

Enzyme	Expt.	Substrate	Hydrolysis, %	At. % excess O^{18}, medium	Equilibration: Mmol. H_2O	Equilibration: Mmol. -CHO	Equilibration: At. % excess O^{18} in H_2O	At. % excess O^{18} in products
β-Amylase	1	Glycogen	25	1.36	11.11	0.55	0.0561	1.20
	2	Maltose	0	0.696	16.67	1.17	.0039	0.059
	3	Glycogen	32	1.40	11.11	0.49	.0513	1.22
Hog pancreatic amylase	1	Glycogen	50	1.33	11.11	.62	.0630	1.20
	2	Maltose	0	1.43	11.11	.59	.0029	0.06
	3	Glycogen	1	51.42	11.11	.62	.0649	1.23
Bacillus subtilis amylase	1	Glycogen	38	1.42	11.11	.25	.0300	1.36
	2	Maltose	0	1.42	11.11	.49	.0052	0.12
	3	Glycogen	38	1.43	11.11	.34	.0359	1.22

The product resulting from β-amylase action on glycogen was found to be maltose by paper chromatography, the mmol.–CHO/mg. was determined as 0.00289 (the theoretical 0.00293).

A comparison of the observed atom per cent. excess O^{18} in the maltose and the atom per cent. excess O^{18} of the medium shows that approximately 90% of the theoretical incorporation was obtained. The small amount of isotope in the control will be considered further in a later section. It should be noted that the maltose resulting from glycogen hydrolysis had 20 times the isotope content of the control. It is concluded that isotope was incorporated into maltose during enzymatic hydrolysis and not after the maltose had been produced.

From the data it may be concluded that β-amylase cleaves the α-1,4-glycosidic bond between the potential aldehyde group of the maltose produced and the oxygen of the bridge. The reaction may be depicted as

$$R\text{–}O\text{–}R' + H_2O^{18} \longrightarrow R\text{–}O^{18}H + HOR'$$

where R = maltosyl radical and R′ = *n* glucose units.

C. Glycogen Hydrolysis by Hog Pancreatic Amylase.—In Table IV it may be observed that the sugars produced by the action of pancreatic α-amylase on glycogen incorporated approximately 90% of the theoretical atom per cent. excess O^{18}. The incorporation of isotope into the control is again one-twentieth of the experimental value. In experiments 1 and 3, 0.25 ml. of enzyme was used; for the control (experiment 2) 0.12 ml. was added.

The main components of the hydrolysate were glucose, maltose, maltotriose and maltotetraose, as determined by paper chromatography. There was also a very faint trace of material at the origin. Glucose was present as a minor component. By visual examination of the chromatogram, it was estimated that maltose and maltotriose composed approximately 60–70% of the mixture. The mmol. –CHO/mg. of the hydrolysate was 0.00190. This value is reasonable considering the presence of higher oligosaccharides and the very probable presence of salts.

Although hog pancreatic amylase produces a mixture of sugars when acting on glycogen, the results support the conclusion that the oxygen bridge was cleaved on the C-1 side of the sugar produced.

D. Glycogen Hydrolysis by *Bacillus subtilis* Amylase.—As in previous cases, the sugars produced by enzyme action incorporated approximately 90% of the theoretical amount of isotope. Again, the maltose control showed a small amount of incorporation of isotope. The enzymatically produced sugars contained 10 times the atom per cent. excess O^{18} of the control. For experiments 1 and 3, 0.14 ml. of enzyme was used; in the control (experiment 2) 0.07 ml. was added.

Bacillus subtilis amylase, like hog pancreatic amylase, produced a mixture of sugars. However, the mixture was quantitatively different. For the bacterial amylase these several sugars have been identified chromatographically: glucose, maltose, maltotriose, maltotetraose and probably maltopentaose. The last component moved very slowly and after 3 ascents was still close to the origin. From a plot of molecular size *versus* partition coefficient, the components appeared to be of the same series (normal α-1,4). However, it is possible that the spot closest to the origin was a mixture containing sugars with both α-1,4- and α-1,6-linkages.

It was estimated visually that maltose, maltotriose and glucose accounted for 50–70% of the sugars. The mmol.–CHO/mg. was 0.00125 suggesting a high percentage of higher oligosaccharides. Considering the presence of salts, this value is a reasonable one.

The data support the conclusion that *Bacillus subtilis* amylase cleaved the oxygen bridge of the α-1,4-linkage on the potential aldehyde side. Similar conclusions have been reached for β-amylase and pancreatic α-amylase using the same substrate in each case. A few comments on the results of the enzymatic hydrolyses and the methods used to obtain them are in order.

The atom per cent. excess O^{18} in the products represent a small difference between two large numbers. For example, from experiment 1, Table IV, pancreatic amylase hydrolysis, it is observed that the equilibration mixture was composed of 11.11 mmol. of H_2O plus 0.62 mmol.–CHO, giving a total of 11.73 mmol. of oxygen positions. The atom per cent. O^{18} in the equilibration water was 0.2639. Ordinary water on analysis contained 0.2009 atom per cent. O^{18}. Thus, the atom per cent. excess O^{18} in the equilibration water was 0.0630 and the atom per cent. excess O^{18} in the sugars was 1.20. The atom per cent. O^{18} of ordinary water as a result of 24 determinations was 0.2010 ± 0.0010. If it is assumed that the determination of O^{18} in the equilibration water was also accurate to ±0.0010 atom per cent. O^{18}, then it can be calculated that the maximum variation permitted in the atom per cent.

excess O^{18} in the water would be 0.0630 ± 0.0020 (3%). The maximum variation in the atom per cent. excess O^{18} values for the sugars are precise to 3%. Since the O^{18} contents of the controls are ten- to twentyfold lower than the O^{18} content of the experimental samples, the uncertainty in the atom per cent. O^{18} values of the controls is much greater. It is for this reason that no more definitive statements can be made concerning the possible very small incorporation of O^{18} into the controls.

The atom per cent. excess O^{18} in the hydrolytic products was always *less* than the theoretical value. This could mean that either the incorporation was incomplete (isotope effect) or the isotope was removed during the isolation procedure, or both. The equilibration method was developed for the purpose of measuring large differences in atom per cent. excess O^{18}. Since an isotope rate effect would be expected to be small and the amount of isotope lost in the isolation procedure is small, it is concluded that the method employed in these investigations is not of sufficient accuracy to detect these small differences in atom per cent. excess O^{18} (differences of less than 10%). The results show, however, that the incorporation of isotope in the hydrolytic products was a result of enzyme action and was not due to other factors.

Discussion

The hydrolysis of most simple acetals is known to be specifically acid-catalyzed.[24] Apparently the electron density around the potential aldehyde carbon is so great that a hydroxyl group cannot approach sufficiently close to displace an alkoxide group. However, the high electron density would favor the addition of a proton with subsequent removal of an alcohol molecule and formation of intermediate carbonium ions. The syntheses of dimethyl and diethyl acetal have been shown to be specifically acid-catalyzed.[25] From reversibility considerations, the synthesis of most simple acetals would be expected to be specifically acid-catalyzed.

Polysaccharides may be classed as simple acetals. It has been reported that when glucose is heated with dilute mineral acid, there is produced a mixture of oligosaccharides.[26] Thus the carbohydrates behave toward acid in a manner that could have been predicted from the chemical behavior of simple acetals.

For the hydrolyses of α- and β-methyl glucoside in H_2O^{18} by an α-glucosidase and by the β-glucosidase in emulsin, respectively, and also by acid, Bunton, *et al.*,[27] have demonstrated that the methanol liberated did not contain excess O^{18}. On the basis of these observations and the above considerations, any hydrolytic cleavage of the simple glycosidic bond would have been predicted to occur between the C-1 carbon and the oxygen of the bridge. This prediction has been verified for the amylases by the data recorded in the previous section.

According to Koshland[6,28] β-amylase facilitates a direct collision between reactants, *e.g.*, polysaccharide and water. The water molecule attacks the potential aldehyde carbon from the backside, displacing the R–O group. This inverts the configuration at the C-1 carbon, giving β-maltose as the product.

In order to account for the retention of configuration in the products of α-amylase action, Koshland[6,28] has suggested that the reaction may proceed through two independent displacements on the potential aldehyde carbon (two SN2 reactions).

We wish to propose a general mechanism for hydrolysis by both α- and β-amylases, (Fig. 1) some features of which (ionic intermediates) have been suggested by Koshland.[6,23,28] The steps are: (a) orientation of the substrate on the enzyme surface, (b) protonation of the bridge oxygen to form an oxonium ion which is cleaved on the C-1 carbon side, leaving an intermediate carbonium (oxonium) ion, and (c) solvolysis of the carbonium (oxonium) ion. The course of the transformation follows SN1 (solvolysis) mechanics.

The available evidence indicates that in cellulose,[29] sucrose[30] and many simple glucosides[31,32] the glucopyranose residue exists in the staggered or stepformed ring of Hassel[33] or the C1 chair conformation of Reeves.[31,34] With the starch-like polysaccharides objection has been raised against the plausibility of the glucopyranoside group existing in the C1 (chair) conformation on steric grounds. Thus, it has been proposed that amylose is composed of glucose units having a boat conformation which permits the substituents on both the 1- and 4-carbon atoms to assume equatorial positions.[35,36] From studies on the influence of alkali on the rotation and behavior of amylose in cuprammonium, Reeves[36] concluded that this polysaccharide contains glucopyranoside rings which exist in more than one conformation with reversible changes in ring structure upon addition of alkali (B1 → 3B conformational shift).[37] Recently, Bentley has stated that the non-reducing ring of maltose most likely exists in either the B1 or 3B conformations.[38]

Although the conformation(s) of the glucopyranoside group in the starch-like polysaccharides has not been established definitely, the current data point toward a boat form. For the sake of argument, it has been assumed that (1) in each scission

(24) P. D. Bartlett, in H. Gilman's "Organic Chemistry," John Wiley and Sons, Inc., New York, N. Y., 1953, p. 115; J. Hine, "Physical Organic Chemistry," McGraw-Hill Book Co., Inc., New York, N. Y., 1956, p. 239.

(25) A. J. Deyrup, THIS JOURNAL, **56**, 60 (1934); R. P. Bell and A. D. Norris, *J. Chem. Soc.*, 118 (1941).

(26) E. E. Bacon and J. S. D. Bacon, *Biochem. J.*, **58**, 396 (1954).

(27) C. A. Bunton, T. A. Lewis, D. R. Llewellyn, H. Tristram and C. A. Vernon, *Nature*, **174**, 560 (1954).

(28) D. E. Koshland, Jr., in W. D. McElroy and B. Glass, "The Mechanism of Enzyme Action," John Hopkins Press, Baltimore, Md., 1954, p. 608.

(29) W. T. Astbury and M. M. Davies, *Nature*, **154**, 84 (1944).

(30) C. A. Beebers and W. Cochran, *ibid.*, **157**, 872 (1946).

(31) R. E. Reeves, THIS JOURNAL, **71**, 215 (1949).

(32) R. E. Reeves and F. A. Blouin, *ibid.*, **79**, 2261 (1957).

(33) O. Hassel and B. Ottar, *Acta Chem. Scand.*, **1**, 929 (1947).

(34) R. E. Reeves, THIS JOURNAL, **72**, 1499 (1950).

(35) K. Freudenberg and F. Cramer, *Ber.*, **83**, 296 (1950).

(36) R. E. Reeves, THIS JOURNAL, **76**, 4595 (1954).

(37) In the B1 conformation the hydroxyl group at carbon atom 2 would be in an axial position and the hydroxyls at carbons 2 and 3 should not form complexes in cuprammonium. In the 3B conformation they should form complexes with cuprammonium (*cf.* R. E. Reeves, *ibid.*, **71**, 212 (1949)).

(38) R. Bentley, Abstracts of the 132nd Meeting of the American Chemical Society, New York, N. Y., September, 1957, p. 6-D.

of an α-1,4-glucosidic bond by an amylase the residue is always in the same conformation, and (2) that conformation is the B1 boat form of Reeves. The solvolysis or carbonium ion mechanism is depicted in Fig. 1.

In the figure (A) represents a linear α-1,4-linked chain with the glucose residues in the B1 conformation and with R = H for β-amylase action and R = H or n glucose units as an α-amylase substrate. After the substrate has been oriented on the protein surface, it is protonated by the enzyme to form the oxonium ion B. The bond is broken on the C-1 carbon side, leaving the carbonium (oxonium) ion C which is represented by two limiting structures. So far, the addition of the proton and cleavage of the bond are the same for both types of amylases although the orienting surfaces would be different.

Once the α-1,4-link is broken, the conformation of the reducing ring is disrupted. The first ring of the hybrid ion C can now assume a half-chair conformation. However, in the α-amylase case the ion C is permitted to turn only in the direction which leads to the half-chair D with the substituents at carbon atoms 2, 3, 4 and 5 in axial positions. For the β-amylases the first ring of ion C rotates in the opposite direction to give E in which the substituents at carbon atoms 2, 3, 4, and 5 are in equatorial positions. The direction of rotation of the potential reducing ring of the intermediate C depends upon the surface of the enzyme and the steric restrictions imposed by it.

The intermediates D and E, still under the influence of the catalytic protein, undergo solvolysis in the manner indicated. The intermediate D yields F, products with an α-configuration (α-amylases), while intermediate E yields G, β-maltose (β-amylases). The reducing ring of the maltose (or maltooligosaccharides) assumes the chair conformation with the substituents at carbon atoms 2, 3, 4 and 5 in equatorial positions, but the non-reducing ring(s) still retain their original conformation.[39]

The above mechanism is in agreement with the chemical behavior of starch-like polysaccharides (acetals) and with the cleavage point of the α- and β-amylases. It does not account for the mode of action of these enzymes in terms of products, since the sequence depicted takes place *after* the substrate becomes oriented on the enzymes' surface. The α-amylases from different sources evolve their products at different rates,[40] but it is suggested that once the bond to be cleaved has been selected, the cleavage always proceeds by the same mechanism.

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Potential Purine Antagonists. XVI. Preparation of Some 2-, 6- and 8-Methylpurines[1]

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The preparation of 2-methylpurine (VIII), 2,6-dimethylpurine (XII), 2,8-dimethylpurine (VII), 6,8-dimethylpurine (XV) and 2,6,8-trimethylpurine (XVI) has been accomplished. All the methyl derivatives of the purine nucleus possessing a methyl group attached to a carbon atom are now known. The synthesis of several new 2-substituted-6-methylpurines is described.

Recently reported anti-tumor activity and toxicity of 6-methylpurine[4] prompted us to investigate the preparation of related homologs with a methyl substituent at positions 2, 6 and 8 as candidate anti-tumor agents. Of the possible homologs of purine with a methyl group attached to a carbon atom of the purine nucleus, only 6-methylpurine[5] and 8-methylpurine[6,7] previously have been reported.

Recent studies involving a new method of synthesis of 8-methylpurine[7] from 6-chloro-8-methylpurine suggested similarly the preparation of 2-methylpurine (VIII) from 2-methyl-6-chloropurine (III).[8] This was accomplished directly by reduction of III with hydrogen and palladium-on-charcoal catalyst in the presence of ammonium hydroxide. 2-Methylpurine (VIII) also was obtained from 2-methyl-6-purinethiol[8] by removal of the thiol group with Raney nickel. Bendich, Russell

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