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The structure of hydroxyethyl starch

W. BANKS, C. T. GREENWOOD AND D. D. MUIR

Flour Milling and Baking Research Association, Chorleywood, Rickmansworth, Herts. WD3 5SH

Summary

1. It is suggested that the structure currently used to describe hydroxyethyl starch (a blood plasma volume expander) is a gross oversimplification.

2. A model having a much more complex substitution-pattern is proposed, and an accurate, rapid test has been devised for distinguishing between the two models.

3. This test shows that the simple model has no basis in reality, and that the more complex substitution pattern is correct.

4. Studies involving the enzymic hydrolysis of hydroxyethyl starch are shown to provide qualitative support for the new model, and the significance of the findings is discussed.

Introduction

In the past decade, considerable interest has been shown in the therapeutic uses of hydroxyethyl starch (HES), first as a blood plasma volume expander, and latterly as a cryoprotective agent for erythrocytes. In the search for a suitable blood plasma volume expander, the branched component of starch, amylopectin, was one of the many substances tested, in the belief that its close structural relationship to glycogen should ensure its non-toxicity. Unfortunately, the persistence of amylopectin in the blood was too short to be of any use because blood α-amylase rapidly reduced the polymer to a size where it was excreted. However, Wiedersheim (1957) suggested that the hydroxyethyl derivative of amylopectin be used rather than the polysaccharide itself, because derivatization would reduce the number of bonds available for hydrolysis by the amylase, thus decreasing the rate of hydrolysis of the material, and so extending its persistence time in the intravascular system. Wiedersheim's work showed HES to have useful properties as a blood plasma volume expander, and to be relatively non-toxic. These conclusions were subsequently confirmed and greatly extended by Walton and co-workers (Thompson, Britton & Walton, 1962; Thompson, Wayt & Walton, 1964; Thompson, Fukushima, Rutherford & Walton, 1970). Indeed, it is now widely accepted that HES produces no more deleterious effects in experimental subjects than does the widely-used bacterial polysaccharide, dextran, and that HES has superior storage properties. However, some opposition to the claim for low toxicity has been expressed (Granath, Strömberg & de Belder, 1969), although the validity of this work has been challenged (Brake, 1970; cf. Granath, Strömberg & de Belder, 1970).

Another advantage of HES over dextran is that, as a man-made polymer, its molecular architecture may be modified by altering the hydroxyethyl content, so giving a degree of control over the ultimate properties of the material, e.g. the time

it would remain in the intravascular system following injection. If full use is to be made of this facility, however, it is necessary to understand the structure of HES, and the factors governing that structure. Those concerned with the medical aspects of HES are, judging by the diagrams in their publications (Thompson *et al.*, 1962 ; Russell, Bradham & Lee, 1966 ; Cerny, Graham & James, 1967), using a model which is grossly over-simplified and may give rise to serious misconceptions.

We wish to present here a simple test which can immediately decide whether or not the commonly-accepted model for HES is correct, and to discuss the significance of our findings.

Methods

Amylose, the linear starch component, was isolated from amylomaize starch (Banks, Greenwood & Muir, 1971), and amylopectin from waxy maize starch (Banks, Greenwood & Khan, 1970).

Hydroxyethyl derivatives were prepared by dissolving the polysaccharides in aqueous alkali (1·0 M KOH) at a concentration of 1–3%, cooling the solution to 2° C, whilst sparging with nitrogen (the latter to minimize degradation), and adding ethylene oxide, also at 2° C, by pipette. (Because of side-reaction, an excess of ethylene oxide over the stoichiometric amount required for any given hydroxyethyl content was employed ; the exact amount was determined by trial and error. The use of low temperature enabled the ethylene oxide to be added as a liquid rather than a gas, which is much more convenient.) After rapid mixing, the flask was sealed, and placed in a thermostat bath for 16 h at 40° C, with constant stirring (magnetic). It was then cooled to 2° C, opened, and the contents neutralized with dilute acid. After extensive dialysis to remove salts and ethylene glycols, the solution was concentrated on the rotary evaporator and freeze-dried.

The hydroxyethyl contents of the derivated polysaccharides were determined by the method of Morgan (1946), in which the ether linkage is cleaved by means of constant-boiling hydroiodic acid at atmospheric pressure to yield a mixture of ethylene and ethyl iodide. These products were estimated volumetrically after reaction with bromine in acetic acid, and silver nitrate, respectively.

This measurement gives the *molar substitution* (MS), defined as the average number of hydroxyethyl groups per anhydroglucose residue, by applying the relation:

$$MS = \frac{W_H}{1-W_H} \times \frac{162}{44} \qquad (1)$$

where W_H is the weight-fraction of hydroxyethyl group.

The weight-fraction of unsubstituted glucose residues in each derivatized polysaccharide was obtained as follows: The material was dried *in vacuo* at 70° C overnight and dissolved in sufficient water to give a known concentration in the range 1–3 mg/ml. An equal volume of sulphuric acid (1·5 M) was added, and the polysaccharide derivative hydrolyzed for 3 h on a boiling water bath. This procedure has been shown to achieve complete hydrolysis of the glycosidic bonds in amylose and amylopectin without any concomitant acid reversion of the glucose.

(The ether linkages of the derivative are, of course, resistant to this relatively mild hydrolysis.) The samples were cooled to room temperature, neutralized by the addition of a predetermined amount of 1·0 M KOH, and diluted in a graduated flask to give a free-glucose content in the range 5–40 μg/ml. An aliquot (1·0 ml) was taken for analysis by the coupled glucose oxidase/peroxidase/chromogen system previously described (Banks, Greenwood & Muir, 1970). Control experiments with the various monosubstituted hydroxyethyl derivatives of glucose showed that only free glucose could react with the enzyme/chromogen system.

The measurement of free-glucose content, in conjunction with that of hydroxyethyl content, enables the *degree of substitution* (DS) to be measured. This parameter is defined as the fraction of glucose residues substituted, and may be obtained from the relation:

$$DS = 1 - \frac{0.9W_G\,(162 + 44\,MS)}{162W_P} \qquad (2)$$

where W_G is the weight of free glucose in a polymer sample of weight, W_P.

The resistance of hydroxyethyl amylose (HEA) to the action of α-amylase was determined by measuring the viscosity number of the polysaccharide during hydrolysis by the enzyme, with an Ubbelohde viscometer. The solution (15 ml; 0·15% HEA in 0·9% saline) was placed in a viscometer at 25° C and, after temperature equilibrium, the viscosity number measured. An aliquot (0·10 ml) of a dilute solution of pig pancreatic α-amylase (3,000 units/ml, the unit of activity being that defined by Greenwood, MacGregor & Milne (1965)), was then added, and the solution mixed thoroughly. The viscosity number was re-determined as a function of time.

Results

In the widely-accepted model of HES (which is explained in some detail in the following section), the molecule is supposed to consist of a random array of singly-substituted and unsubstituted anhydroglucose units. No consideration appears to have been given to the possibility that a significant fraction of the substituted residues may have reacted with two or more molecules of ethylene oxide. Two quite simple tests may be used to determine whether this latter complex substitution has occurred; namely, (a) the relation between the number of moles of ethylene oxide which have been incorporated into the polysaccharide and the proportion of glucose residues which are unsubstituted (in the accepted model for substitution, there is an inverse proportionality between these two parameters, i.e. the degree of substitution is equivalent to the molar substitution, whereas in the case of complex substitution no such simple relation exists), and (b) the susceptibility of the HES to α-amylolysis (as the substitution approaches the level at which one mole of ethylene oxide is incorporated per mole of anhydroglucose residue, the number of bonds available to the action of α-amylase decreases to zero if the simple model of substitution is correct, but remains finite in the case of complex substitution).

A comparison of MS and DS for various samples of hydroxyethyl amylose and hydroxyethyl starch is shown in Table 1; the susceptibility of these samples of hydroxyethyl amylose to α-amylolysis is given in Table 2.

TABLE 1. *A comparison of the molar substitution (MS) and degree of substitution (DS) for hydroxyethyl amylose (HEA) and hydroxyethyl starch (HES) of varying hydroxyethyl contents*

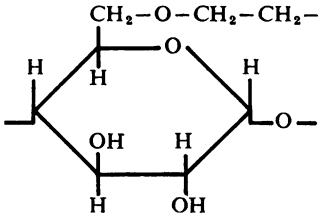
	Hydroxyethyl amylose					Hydroxyethyl starch				
MS	0·45	0·60	0·75	0·90	1·10	1·25	0·43	0·55	0·64	0·81
DS	0·45	0·56	0·64	0·69	0·74	0·77	0·42	0·52	0·59	0·69

TABLE 2. *The viscosity number (η sp./c) of hydroxyethyl amylose of different molar substitutions as a function of time of exposure to α-amylase at 25° C*

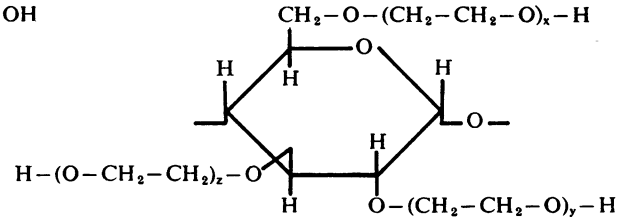
Molar substitution	Degree of substitution	η sp./c at t minutes				
		0	2	10	20	1500
0·45	0·45	76	12	9	8·5	5·5
0·60	0·56	77	13	9·5	9	6
0·75	0·64	84	31	22	19	11
0·90	0·69	88	34	25	22	13
1·10	0·74	85	47	37	33	18
1·25	0·77	85	60	51	47	29

Discussion

Formula *A* below shows the widely-accepted structure for a substituted glucose residue in HES (Thompson *et al.*, 1962 ; Russell *et al.*, 1966 ; Cerny *et al.*, 1967).



A



B

In this case, substitution has occurred only at the hydroxyl group on carbon atom 6. There are, however, two free hydroxyl groups on carbon atoms 2 and 3, which are also capable of reacting with ethylene oxide. Moreover, each hydroxyethyl group carries a hydroxyl group which may also react with ethylene oxide, building up first oligomeric- and then polymeric-chains of ethylene oxide. Formula *B* represents this structure ; the parameters, x, y and z may take on the value of any integer between zero and infinity. Formula *A* is, in fact, the simple case when $x=1$, and $y=z=0$.

Formula *A* appears to be due to Schoch (1963), who later (Schoch, 1965a) mentioned the random nature of the derivation procedure, during discussion of his work (Schoch, 1965b). However, he did add (Schoch, 1965b), that there was no evidence of complex derivatization (monomer units carrying more than a single substituent group) when, on average, each glucose residue was substituted with 0·8 moles of ethylene oxide, but the experimental basis of this statement was not given.

To differentiate between (a) monosubstituted glucose residues, and (b) a mixture of monosubstituted glucose residues plus complex derivatives, it is necessary to measure two quite different parameters, namely, molar substitution and degree of substitution. These two terms are often used interchangeably, but they are not synonymous. As noted above, MS is defined as the average number of hydroxy-

ethyl groups per anhydroglucose residue, and is an easily-accessible, experimental parameter, whereas DS is defined as the fraction of glucose residues substituted. To obtain the DS, it is necessary to measure MS and one additional parameter, namely the free-glucose content of the derivatized polysaccharide. Our technique of hydrolyzing the polymer and specifically estimating the free glucose in the presence of hydroxyethyl derivatives of glucose provides a rapid, and very accurate, method of obtaining the latter parameter. Knowing both MS and the free-glucose content of the polymer, the DS can be obtained from relation (2).

The presence of complex glucose derivatives in admixture with the simple monohydroxyethyl derivatives must obviously be reflected in the non-equivalence of MS and DS. For such a mixture, in fact, MS>DS. Table 1 shows a comparison of MS and DS values for a number of samples of HEA and HES of varying hydroxyethyl content. (The term 'HES' in the field of blood plasma volume expanders is applied to the derivatives of waxy maize and waxy sorghum starches. These genetic variants of maize and sorghum have only an amylopectin component in the starch, and hence our preparation of hydroxyethyl amylopectin is synonymous with the appellation HES.)

As the hydroxyethyl content increases, the divergence between MS and DS also increases in the case of both HEA and HES. There can be no doubt that these figures demonstrate quite unambiguously that the complex glucose derivatives reach a measurable level when MS=0·45; at MS=0·8 (the material widely used as a blood plasma volume expander has a similar value of MS), some 15% of the hydroxyethyl groups are present as complex derivatives of glucose. In the face of this evidence, the simple structure represented by formula *A* must be abandoned, and replaced by the more complex form given by formula *B*.

It must be stressed that some samples of HEA and HES were subjected to repeated extraction with 95% acetone, in case any ethylene glycol or its oligomers had survived the dialysis stage. (Such a mixture would exhibit the same properties as those displayed by the samples in Table 1.) However, in no case did this treatment lead to any significant change in the values of MS or DS, and hence it is concluded that the results shown in Table 1, and the inferences derived from them, are real.

A necessary corollary of the model of complex substitution is that some of the substituent groups are not effective in conferring resistance to α-amylolysis. For example, the introduction of a single hydroxyethyl group into a glucose residue will confer on the glycosidic bonds in the immediate vicinity immunity from α-amylolytic attack; the introduction of another hydroxyl group into that same glucose residue will not increase this immunity. Consequently, the parameter which determines the rate and extent of amylolytic attack will be DS rather than MS. In Table 2, we show the variation with time of the viscosity numbers of a series of HEA samples of different hydroxyethyl content (HEA was chosen in preference to HES because the viscosity technique is more sensitive to changes in the molecular weight of linear polymers than it is to such changes in a branched polymer).

The results in Table 2 show clearly that DS is the dominant factor in determining the resistance of the molecule to α-amylolysis. Even ignoring the values of DS, the fact that samples with MS values of greater than unity are susceptible to amylolytic attack shows, qualitatively at least, that complex derivatization has occurred. It should also be remembered that the action of α-amylase requires a

sequence of unsubstituted glucose residues. The minimum number of residues in such a sequence to allow α-amylolytic degradation is three; a more realistic estimate is probably four or five. If we accept the minimum figure, then the probability of finding three unsubstituted glucose residues in sequence when the DS= 0·75 is $(1/4)^3$, or 1 in 64; the probabilities of finding four or five residues in sequence are 1 in 256, and 1 in 1,024, respectively. The number average degree of polymerization of the initial HEA is approximately 500 monomer units, thus the number of bonds accessible to the action of α-amylase varies in the range (8–0·5) per HEA molecule, depending on the minimum size-requirement of the enzyme. With these calculations in mind, the susceptibility of samples of high MS values to α-amylolysis provides striking confirmation of our model of substitution.

We may conclude, therefore, that a fairly high proportion of the hydroxyethyl residues present in the HES used as a blood plasma volume expander are ineffective and do not decrease further the susceptibility of the macromolecule to hydrolysis by α-amylase. It seems reasonable to conclude that there is less danger in infusing solutions of the native polysaccharide, starch, into the intravascular system, than there is in infusing solutions of hydroxyethyl starch, that is to say, any potential toxicity probably arises from the presence of the substituent hydroxyethyl group. Therefore, if a method could be devised for preventing complex substitution, a suitable resistance to the action of α-amylase would be attained at a lower hydroxyethyl content, and presumably there would be a smaller probability of toxic side-effects. This might be particularly germane if the complex substitution took the form of poly-(ethylene oxide) side-chains, i.e. if the hydroxyl group on the newly-introduced substituent is more reactive than either of the two remaining functionalities within the glucose residue. The ultimate structure of HES must reflect the relative values of 4 rate constants, i.e. those governing substitution at carbon atoms 2, 3 and 6 of the glucose residues, and at the newly-introduced hydroxyethyl groups. Comparatively minor changes in the conditions under which derivatization is carried out could easily influence the relative values of these 4 rate constants. Consequently, even samples of HES having the same MS may differ quite profoundly in DS, and it is the latter parameter which is of interest in the proposed pharmacological uses of HES. In this connexion, it is of interest that the debate regarding the toxicity of HES (Granath *et al.*, 1969; Brake, 1970; Granath *et al.*, 1970) is based not on the use of a single sample of HES, but on two preparations emanating from different laboratories. Whilst these samples are supposed to have the same chemical structure, as reflected by their equivalence in the measured MS, we would suggest that the absence of a proper characterization involving the determination of DS renders the debate meaningless.

We are presently attempting to elucidate the nature of the complex substitution in HES, and to eliminate it by modifying the conditions by which HES is prepared.

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