NONFERMENTABLE, GLUCOSE-CONTAINING PRODUCTS FORMED FROM GLUCOSE UNDER CELLULOSE ACID HYDROLYSIS CONDITIONS*

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SYNOPSIS

Solutions of D-glucose in dilute sulfuric acid were allowed to react under time and temperature conditions which simulated the production of glucose from cellulose. Under these conditions, glucose undergoes a number of reactions including isomerization, dehydration, transglycosidation, polymerization, and anhydride formation. The specific interest in this report was to investigate the reversible reactions which form "reversion products." These products, which include di- and trisaccharides, anhydrides, and glucosides, provide a protection for glucose from decomposition, but the products are not fermentable. The reactions were studied at 180 and 230°C for various times and concentrations. The products were categorized as reducing or nonreducing, and the total glucose produced by secondary hydrolysis from the nonfermentable products was determined. Depending on conditions, these products may constitute more than 20% of the potentially available glucose. A preliminary study was made on D-xylose, which will form reversion products at xylose concentrations above 20%.

INTRODUCTION

There are natural restrictions on the utilization of wood or other cellulosic biomass for the production of sugars and derived chemicals or fuels, such as ethanol. The crystalline nature and inaccessibility of cellulose in the lignocellulosic matrix restrict its enzyme digestibility and dictate the conditions required for acid hydrolysis. A preliminary purification from lignin, hemicellulose, and other material may also be desirable or necessary for subsequent processing

*Presented at Ninth Cellulose Conference, Syracuse, New York, May 24–27, 1982. This article was written and prepared by U.S. Government employees on official time, and it is therefore in the public domain.

†The Laboratory is maintained at Madison, Wisconsin in cooperation with the University of Wisconsin, Madison, Wisconsin.

Journal of Applied Polymer Science: Applied Polymer Symposium 37, 617–629(1983)
 CCC 0570-4898/83/010617-13$04.00

steps. The liquid-to-solid ratio is another variable which is constrained by processing considerations. To minimize the evaporation energy required to concentrate the sugar solution prior to fermentation or other treatment, it is desirable to conduct the hydrolysis at as low a liquid-to-solid ratio as possible.

When glucose is produced from cellulose by acid hydrolysis, it is concurrently degraded to 5-(hydroxymethyl)-2-furaldehyde (HMF) and other products. The ratio of the rate of formation to the rate of degradation determines the maximum yield of glucose that can theoretically be obtained by this method [1]. The rate ratio and the absolute rates increase with acid concentrations and temperature. Since the maximum yield of glucose continues to increase until reaction times of only a few seconds are necessary, the practical limit is imposed by the minimum time necessary for physical handling and heat transfer.

The data on lignocellulose hydrolysis available at the U.S. Forest Products Laboratory are being reviewed and reevaluated [2]. When the effects discussed above are considered in light of process requirements, economics, product purity, and other factors, the optimum conditions for the mineral acid-catalyzed production of glucose from cellulose are in the range of 230°C, 0.4% sulfuric acid, liquid-to-solid ratio of 3, and a reaction time of ca. 1 min.

Concentrated solutions of sugars containing acid are known to polymerize to form what are known as reversion products. This phenomenon was studied extensively when starch was being commercially hydrolyzed with mineral acids [3]. Most of the data, however, were collected for temperatures around 100°C. At low liquor-to-solid ratios, consideration of reversion reactions becomes important and the existing data and theories are inadequate to extrapolate to temperatures above 200°C. The present report is a study of reversion products and other nonfermentable structures such as 1,6-anhydro-ß-D-glucose (levoglucosan) which are formed from concentrated glucose solutions under the defined conditions. Although the classical use of the term "reversion" means repolymerization, it is used here to refer to combined, nonfermentable glucose regardless of its actual form. Glucose may be recovered from these products because the reactions are reversible.

EXPERIMENTAL

Reactions of Glucose

Solutions of D-glucose were prepared by dissolving the required weight of sugar in the dilute sulfuric acid solution of stated concentration and diluting to the required volume with the acid solution. The reactions were run in sealed glass ampules of 3 mm O.D., 0.3 mL (230°C) and 5 mm O.D., 1.0 mL (180°C). A silicone oil bath was used at 180°C and a molten salt bath at 230°C. In both cases, timing of the reaction was started at the moment of immersion in the preheated bath, but a 4-s heat transfer time was subtracted from the total time at 230°C. At the end of the reaction, the 5-mm tubes were cooled, opened, and

the contents quantitatively recovered. The contents of four 3-mm tubes identically treated were combined and 1-mL aliquots were taken for analysis.

In the 180°C experiments, the solutions were subjected to a dilute secondary hydrolysis: 3% sugar, 3% H_2SO_4, 120°C for 1 h. The solutions were reanalyzed for glucose with corrections for hydrolysis losses and volume changes being applied. In the experiments at 230°C, the remaining glucose was removed by fermentation before the secondary hydrolysis.

A preparative run was made in a steam-heated digester with 1200 g glucose in 4 L 0.16% H_2SO_4. The reactor was heated to 194°C, then cooled. The heating time was 5 min, and the cooling time was also 5 min. This reaction mixture was used for product identification and for the methylation analysis.

Fermentations

For the 230°C runs, 1.0-mL aliquots of the reaction mixtures were diluted to 10 mL and fermented for 72 h at ambient temperature with cultures of *Saccharomyces cerevisiae* or commercial Baker's yeast. The solutions were filtered to remove the yeast cells and concentrated under reduced pressure to remove the alcohol and to attain a suitable concentration for analysis. A control fermentation of a pure glucose solution showed glycerol to be the only soluble product detected by the analytical procedures used.

Analytical Methods

Glucose was specifically determined with a Beckman II glucose analyzer (a glucose oxidase procedure). Total reducing power was measured by the Nelson–Somogi procedure [4]. Glucose and mannose were also determined by HPLC on a Bio-Rad HPX87P heavy metal cation exchange resin column with water as eluent at 85°C.

Size exclusion chromatography was performed under gravity flow on a 110 cm × 1.5 cm column of Bio-Gel P-2, 200-400 mesh with water as the eluent. Retention times were measured relative to the exclusion peak of Dextran T-40. A preparative group separation was performed on a 2.5 × 30 cm column of AG 50WX-4 (H^+ form) 63–73 μm.

Linkage analyses by methylation were performed on the preparatively separated disaccharides. The methylated products were subsequently hydrolyzed and converted to the corresponding partially methylated alditol acetates which were analyzed by glass capillary gas chromatography on SP-1000, 10 m, 0.25 mm ID [5].

Separation and Identification of Acetylated Products

The reaction mixture after neutralization and fermentation was concentrated to dryness and acetylated with acetic anhydride and pyridine. The acetylated

products were dissolved in methylene chloride and eluted from a short (10 x 2 cm) column of silica gel with ethyl acetate cyclohexane 60/40 (v/v) to remove strongly absorbing HMF polymers and other degradation products. The acetates were chromatographed on a μ-Porasil HPLC column using a Waters liquid chromatograph with a WISP 710B processor and a 730 data module. The glycosides and anhydrides were analyzed by gas chromatography/mass spectrometry using a 182 cm x 2 mm I.D. glass column packed with 3% SP-2250 on 80-100 mesh Supelcoport. GC/MS analyses were performed by Raltech Scientific.

RESULTS AND DISCUSSION

When sugars are heated in acid, a large number of reactions are known to occur [6]. It has long been known that the acid-catalyzed decomposition of glucose from 10 to 80% decomposition can be described kinetically as a pseudo-first-order reaction whose absolute rate is dependent on the effective acid concentration [1]. The extrapolated intercepts, however, are always less than 100%, indicating a more rapid loss of glucose during the first 10% of degradation. This has been explained as the period of time in which reversion products are being formed. Because reversion products are also hydrolyzed to glucose under acid catalysis, an equilibrium between glucose and the reversion products should be established. The equilibrium is extremely complex, however, involving various di- and trisaccharide linkages, glycosidations, rearrangements, and formations of anhydrides and dehydration products and combinations of these.

The overall effect of the various reactions can be monitored by subjecting the reaction mixtures to a "secondary" acid hydrolysis which is performed at a sufficiently dilute sugar solution (3%) and low enough temperature (120°C) to shift the equilibria strongly toward glucose. The additional glucose produced by the secondary hydrolysis is a measure of the total amount of glucose "combined" or reversibly converted to some other form. The results of such monitoring of the glucose reaction at 180°C are given in Table I.

In the analysis for free glucose before hydrolysis, the copper reducing values are always higher than the values obtained by glucose oxidase. The difference is attributable to the reducing power of the disaccharides formed by reversion. The reducing powers of cellobiose and lactose, for example, have been independently measured as 0.553 and 0.528, respectively, relative to D-glucose.* The difference between the reducing values and oxidase values passes through a maximum in each concentration series and then declines. This implies that reducing disaccharides are formed and subsequently degraded.

After the secondary hydrolysis, the values obtained by glucose oxidase and copper reduction are the same within experimental error. The increase in these values over the glucose oxidase values before the secondary hydrolysis gives the total amount of glucose which is "combined." This includes disaccharides, anhydrides, and glucosides. The difference in glucose oxidase values also passes

*M. J. Effland, USDA, Forest Products Laboratory, unpublished.

TABLE I
Free and Combined Glucose After Reaction with 0.2% Sulfuric Acid, 180°C

Initial concentration of glucose	Time of reaction	Free glucose before secondary hydrolysis		Difference	Total glucose after secondary hydrolysis	Amount of glucose combined
		Glucose oxidase	Copper reduction			
mg/ml	min	mg/ml	mg/ml	mg/ml	mg/ml	mg/ml
50	10	42	43	1	46	4
	30	36	39	3	42	6
	60	32	32	0	33	1
100	10	83	86	3	94	11
	30	70	76	6	82	12
	60	63	64	1	66	3
200	10	157	165	8	189	32
	30	132	146	14	162	30
	60	120	122	2	134	14
300	10	220	238	18	279	59
	30	189	214	25	246	57
	60	163	178	15	202	37

through a maximum and declines with extended reaction. The total combined glucose products are nearly at their maximum concentration after 10 min, as contrasted with the disaccharides, which are formed more slowly. This observation is supported qualitatively by liquid chromatography of the acetylated reaction products.

If the data are plotted as a first-order reaction (Fig. 1), it is seen that the total glucose (measured after the secondary hydrolysis) does indeed follow a straight-line degradation which extrapolates to 100% at time zero. The determinations of free glucose, before secondary hydrolysis, by the oxidase method show a rapid loss during the first 15% of the degradation due to formation of reversion products. This loss increases with concentration as would be expected because the formation of disaccharides is a second-order reaction.

A similar experiment was performed at 230°C, except that the excess undegraded glucose was removed by fermentation. The results are presented in Table II. A lower concentration of acid was used to slow the reaction sufficiently so that the nonequilibrium early stage could be conveniently studied. It is evident that the major portion of the reversion reaction has occurred within the first 10 s. The absolute quantity of nonfermentable glucose does not change within experimental error over the time span 10–90s, but the absolute amount of free glucose is decreasing, so that the relative amount of glucose combined is still increasing.

Theoretically, the corrected copper reducing value before secondary hydrolysis should be a measure of the amount of the reducing di- and trisaccharides, in the reaction mixture. Assuming a minimal formation of trisaccharides, the copper reducing value before secondary hydrolysis would be half the value for glucose after the secondary hydrolysis. In Table II, the reducing values before hydrolysis range from 10 to 22% of the values after. This indicates that a major portion of the nonfermentable glucose is present as anhydrides, glucosides, the nonreducing 1,1-disaccharides, and higher oligomers.

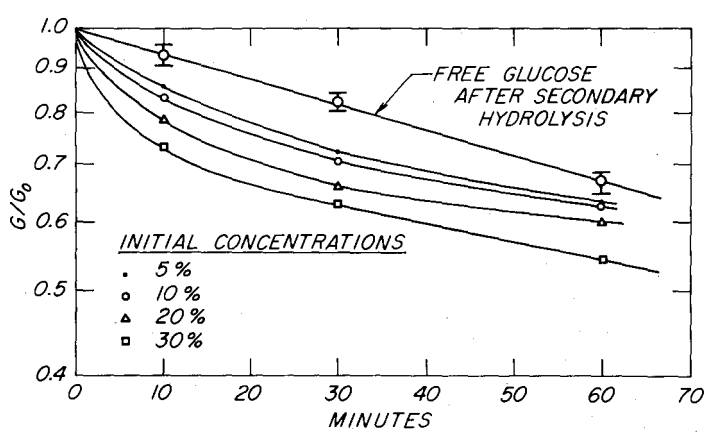

FIG. 1. Rate of disappearance pf free glucose.

TABLE II

Free and Combined Glucose After Reaction with 0.1% Sulfuric Acid, 230°C

Initial concentration of glucose	Time of reaction	Free glucose remaining [b]	Copper reduction before secondary hydrolysis [c]	After secondary hydrolysis			
				Glucose oxidase	Copper reduction	Liquid chromatography	
						Glucose	Mannose
mg/ml	sec	mg/ml	mg/ml	mg/ml	mg/ml	mg/ml	mg/ml
150	10	124	2	20	22	20	0.5
	20	121	3	20	21	--	--
	32	117	4	21	23	--	--
200	10	158	5	30	31	28	0.8
	20	148	6	29	31	--	--
	32	147	7	30	33	32	1.2
		148	6	33	31	--	--
	46	144	7	33	31	--	--
	90	131	8	30	29	--	0.5

[a]Does not include 4-s heat transfer correction.

[b]Before fermentation, determined by glucose oxidase.

[c]After fermentation, corrected for residual glucose as determined by glucose oxidase

Liquid chromatography was used to spotcheck the quantitative analyses and to test for other reducing products. The Lobry de Bruyn-Alberda Van Ekenstein rearrangement [7] of sugars occurs in aqueous acid solutions, and mannose and fructose would be expected to be formed by that mechanism although their existence may be transient [8]. The presence of these sugars has recently been reported under similar experimental conditions [9]. However, in the present experiment, sugars other than glucose would have survived the fermentation or been involved in reversion if they were observed. Mannose was detected in small amounts in the liquid chromatographic runs, and a very small peak was observed with a retention time equivalent to that of fructose.

Product Identification and Distribution

Ten of the 11 possible pyranose dimers and one pyranose–furanose dimer have been reported from the reversion reaction to glucose, with the $1 \rightarrow 6$ linked dimers gentiobiose and isomaltose predominating [3]. Attempts to chromatographically resolve the disaccharides either in the free form or as their acetates were unsuccessful. Methods investigated were paper chromatography, high-performance liquid chromatography using reverse phase or heavy metal chelation for the free sugars, and silica chromatography by HPLC, or thin-layer chromatography for the acetates. Literature reports of systems which separate disaccharides or their derivatives (alkyl bonded or amine modified silica) did not indicate adequate resolution of the dimers in the reversion mixture [10, 11]. A group separation based on size was therefore performed on cation exchange resin in the acid form. The linkage distribution of the disaccharides was determined by methylation analysis for one separation, and the results are given in Table III. The $1 \rightarrow 6$ linkage predominates under the present reaction conditions as well.

Levoglucosan was isolated by size exclusion chromatography and identified by proton NMR. Both the pyranose and furanose forms of levoglucosan were

TABLE III
Methylation Analysis of Disaccharide Fraction[a]

Methylated positions	Mole % of total	Position of linkage	% of disaccharide mixture
3, 4, 6	6	2	12
2, 4, 6	5	3	10
2, 3, 4	26	6	52
2, 3, 6	6	4 & 5	12
2, 3, 4, 6	57	1 & nonreducing end	14 (by difference)

[a]Preparative run, separated from other products on AG5OW; see experimental section for details.

identified by mass spectrometry after acetylation of the reaction mixture, preparative separation from disaccharides on silica gel, and gas chromatography. Two other products were identified as glycosides by their mass spectra but could not be further identified. Glycosides give characteristic fragmentation patterns because the cleavage of the glucosidic bond is very facile even by chemical ionization mass spectrometry and the spectrum observed is primarily that of the glucose moiety. The glucoside of HMF is the only such product to be reported in the literature thus far [12] from glucose reversion reaction mixtures. In addition, the usual dehydration products of glucose, HMF and levulinic acid, were observed.

The best overall separation of products was obtained by liquid chromatography on a "carbohydrate" heavy-metal ion exchange column of the neutralized reaction mixture after fermentation. A typical chromatogram of products obtained at 230°C is shown in Figure 2. The early peaks in the region a are the disaccharides. A small preliminary peak may be trisaccharides. Levoglucosan is peak *f*. The results of a quantitative estimation by response factors of products from three of the 230°C experiments are given in Table IV. Again, the absolute values are relatively constant in the studied time range, but because the glucose is being continuously degraded, the percent of glucose combined is increasing.

The products were also investigated by size exclusion chromatography on Bio-Gel P-2 to check for the presence of trisaccharides or higher oligomers. Two well-resolved peaks were obtained in the monomeric and dimeric regions as well as a late peak for HMF. Unfortunately, glycerol (an artifact of fermentation) is eluted as part of the early (dimeric) peak and residual glucose elutes in the trailing edge of the same peak. In longer runs, a shoulder was evident on the forward

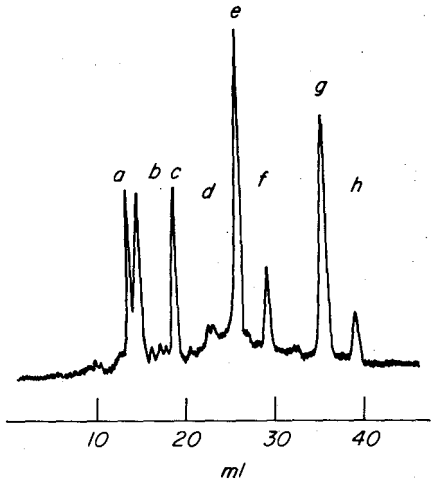

FIG. 2. Liquid chromatography of nonfermentable products. (a) disaccharide peaks; (b) glucose region; (c) 1,6-anhydro-ß-D-glucofuranose; (d) mannose and fructose region; (e) glycerol; (f) 2,5-furandimethanol; (g) levoglucosan; (h) HMF.

TABLE IV
Chromatographic Estimation of Products[a]

Reaction time	Analysis by HPLC		Analysis by size exclusion	
	Anhydrides	Dimers	Dimer peak	Monomer peak
sec	mg/ml	mg/ml	mg/ml	mg/ml
32	16	11	10	18
46	14	9	10	19
90	14	10	[b]16	22

[a] 200 mg/mL reaction, Table II.
[b] Includes significant forward shoulder due to trimer.

side of the dimeric peak, indicating trisaccharide formation. No other peaks were observed. If the amount of glycerol and glucose, as independently determined, are subtracted from the first peak, the data shown in Table IV are obtained. These total values are larger than those obtained by HPLC because the HPLC determination was more selective.

Tests of Possible Equilibria

There have been attempts to provide kinetic and equilibrium expressions for reversion reactions at lower temperatures [13–15], but extrapolation to the present range was unsuccessful. The situation is much too complex to provide a complete description with the presently available data, but some simplified expressions which consider the major effects can be tested.

The formations of glucosides, anhydrides, and disaccharides from glucose are all reversible reactions in aqueous acidic solution. It is known that the homogeneous hydrolysis of disaccharides is considerably faster than the rate of decomposition (about 35 × for isomaltose and 50 × for gentiobiose) [16]. Therefore, it would be expected that equilibrium would be established early in the decomposition reaction and be maintained throughout the reaction. The copper reducing value of Table II after fermentation has been corrected for the small amount of unfermented glucose. It may be assumed that the reported value is a measure of reducing disaccharides. Assuming further that the reducing power of 1 mol disaccharide is equivalent to 1 mol glucose, the total combined glucose may be divided into a disaccharide fraction and an anhydride fraction (which also contains nonreducing disaccharides and glucosides).

The formation of disaccharides is a second-order reaction involving two molecules of glucose. The equilibrium may thus be written as

$$K_1 = \frac{[\text{disaccharide}]}{[\text{glucose}]^2} \quad (1)$$

The formation of levoglucosan is a first-order reaction, so the equilibrium may be described as

$$K_2 = \frac{[\text{levoglucosan}]}{[\text{glucose}]} \quad (2)$$

The calculated values for such equilibria are given in Table V. It is seen that the value of K_2 for the nonreducing equilibrium is fairly constant and is established very early in the reaction. The equilibrium involving the disaccharides does not appear to be established even after 90 s at over 30% loss of free glucose. Interestingly, the formation of trisaccharides, which takes place later in the reaction, would contribute to a larger nonreducing value. It is possible that substances other than glucose dimers are contributing to the reducing value (the reducing power of HMF is 4% that of glucose) (see footnote p. 620). However, even with the constant concentration as determined chromatographically, the second-order equilibrium constant does not seem to accurately reflect the true situation. By comparison, the absolute quantity of reducing substances, the total amount of reversion products, and the ratio of each to the free glucose present were all decreasing after 60 min at 180°C. On the basis of free glucose, there was 40% reaction after 60 min at 180°C and 34% reaction after 90 s at 230°C. The total maximum concentration of "combined" glucose in the two comparable 200 mg/mL experiments was about the same, 33–34 mg/mL. It is therefore likely that the absolute and relative amounts of combined glucose and reducing substances will decrease as the reaction at 230°C is extended even further. It is possible that the system never reaches equilibrium, but more data are needed before a complete description can be obtained.

TABLE V
Calculated Product Concentrations and Equilibrium Constants[a]

Time of reaction	G[b]	L[c]	D[d]	K_2	K_1
				L/G	D/G^2
sec	mg/ml	mg/ml	mg glucose equivalent/ml		liters/mole
10	159	20	5	0.13	0.036
20	148	18	6	0.12	0.049
46	144	19	7	0.13	0.061
90	131	14	8	0.11	0.084

[a] From data in Table II.
[b] Free glucose remaining in reaction mixture as determined by glucose oxidase.
[c] Total glucose after fermentation and secondary hydrolysis minus 2 × (reducing power).
[d] Reducing power after fermentation, corrected for residual glucose.

In the presence of cellulose, the situation will be even more complex because The solution will contain oligomers produced by hydrolysis, and both the cellulose and the oligomers will provide additional substrates for the reversion reaction.

Possible Uses of Reversion Products

The nonfermentable reversion products will concentrate in the fermentation "slops" in a commercial conversion of glucose to ethanol. The additional glucose contained in these products may be recovered by an acid treatment, e.g., recycle through a prehydrolysis stage, recycle through the cellulose hydrolysis stage, or recovery through a separate hydrolysis. The $1 \rightarrow 6$ linkage is hydrolyzable by enzymes [17]. Thus, the glucose could be recovered by enzymolysis, or the reversion products could be utilized in molasses.

Xylose Reversion

The possible reversion reactions of xylose, which does not contain the primary hydroxyl group except in the furanose or acyclic form, are of practical and theoretical interest. If high-xylan-content biomass such as hardwoods is used as a substrate, a prehydrolysis removal of the xylan may be employed. If maximum xylose production is desired for fermentation or other utilization, awareness of the reversion reactions will be important. From a theoretical point of view, it would be of interest to know the extent of reversion when glycosidic linkage with a primary hydroxyl would occur only in the furanose form.

Some preliminary studies on xylose were performed using the techniques described for glucose, except that a specific xylose analyzer was not available. Xylose was determined quantitatively by HPLC. The data for 10 min at 170°C, 1% H_2SO_4 are presented in Table VI. It is seen that some reversion does take place at the higher concentrations but is only significant at 300 mg/mL. The extent is much less than that observed with glucose. These reactions are being studied further.

TABLE VI
Reaction of Xylose in 1.0% H_2SO_4, 170°C, 10 Min

	Xylose after reaction	
Initial concentration	**Before secondary hydrolysis**	**After secondary hydrolysis**
mg/ml	**mg/ml**	**mg/ml**
100	*70*	*70*
200	*139*	*142*
300	*207*	*224*

CONCLUSION

When glucose is heated in aqueous acid solution to the temperature useful for cellulose hydrolysis, disaccharides, glucosides, and anhydrides are rapidly formed. These products, which are not directly fermentable by *Saccharomyces cerevisiae*, can be a source of 20% or more of the potentially available glucose. The additional glucose can be obtained by relatively mild acid hydrolysis of dilute solutions, or by enzymatic hydrolysis (e.g., ruminant digestion). In a sense, these "reversion" products protect glucose from the degradative dehydration reactions which are initiated at the reducing end of the glucose molecule. There may be overall glucose yield advantages by promoting their formation.

The author is grateful to J. D. McSweeney for technical assistance, J. F. Harris for many helpful discussions and computer simulations, M. J. Effland for reducing sugar analyses, and V. H. Schwandt for liquid chromatography.

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