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Non-Enzymic Browning: the Reaction between D-Glucose and Glycine in the 'Dry' State

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The interaction between amino acids or protein and reducing sugars has been shown to be the cause of much of the browning that occurs during the manufacture and storage of dried foods (cf. reviews by Stadtman, 1948; Danehy & Pigman, 1951) and consequently much research has been done on the reaction. Many of the investigators, however, have conducted the experiments in aqueous solution at elevated temperatures, that is, they have used experimental conditions similar to those of Maillard (1912). Under these conditions a large number of compounds are formed (Chichester, Stadtman & Mackinney, 1952) and it becomes difficult to decide which are intermediates in the browning and which are products of side reactions. Also these conditions for the reaction are very different from those found in dried foods. It has been shown, however, (Gottschalk & Partridge, 1950) that mixtures of amino acids and simple sugars under 'dry' conditions, i.e. at low moisture content and at lower temperatures, turn brown and yield chromatographically relatively simple mixtures. These mixtures contain new compounds with the colour reactions of both an amino compound and, more weakly, of a sugar. On their evidence Gottschalk & Partridge (1950) suggested structure (III) for the compounds. Other workers (cf. Hodge, 1953) consider such intermediates to be 1-amino-1-deoxy-2-ketoses formed by an Amadori rearrangement of an initially formed *N*-substituted glycosylamine.

The present investigation is concerned with the interaction between a simple sugar (D-glucose) and an amino acid (glycine) at pH 6.7 in an atmosphere of 70% relative humidity at 37°, conditions which approximate to those which might be found during the tropical storage of foodstuffs.

EXPERIMENTAL

Chromatography. In the following experiments, sheet-paper partition chromatography was done by the descending method (Partridge, 1948) on Whatman no. 1 filter paper. R_F values were given with *n*-butanol-ethanol-water (40:11:19, by vol.) as the mobile phase. The chromatograms were run for about 15 hr., dried and then sprayed with one of the following reagents: (a) ammoniacal silver nitrate (Partridge, 1948); (b) aniline hydrogen phthalate (Partridge, 1949); (c) resorcinol-HCl in butanol (Forsyth, 1948) and modifications; (d) 0.2% ninhydrin in wet butanol; (e) the Elson & Morgan reagent for glycosylamines as modified for use as a spray (Partridge, 1948); (f) alkaline ferricyanide solution followed 5 min. later by ferric reagent (Borsook, Abrams & Lowy, 1955). If sprays (a)-(d) were used the papers were then heated.

5-Hydroxymethylfurfuraldehyde. This compound was prepared according to Haworth & Jones (1944).

Examination of the reaction between D-glucose and glycine

Equal volumes of M D-glucose and 0.25M glycine were mixed and brought to pH 6.7 with HCl and NaOH. Portions (2 ml.) were transferred to specimen tubes, dried at 20° in a vacuum desiccator, equilibrated to 70% relative humidity, and then, still in the same atmosphere, held at 37°. At intervals two samples were removed, one for chromatographic and one for spectroscopic examination.

Chromatographic examination. The first sample, which was taken after 2 days of heating, had an amber colour when dissolved in water (2 ml.). In samples taken at longer time intervals the colour was progressively darker until at 8 weeks the solution was almost black. Chromatography revealed the presence of only one additional compound (R_F 0.11) apart from a brown material which did not move from the base-line. The quantity of the compound having R_F 0.11 was so small after 2 days that it was barely detectable on paper chromatograms, but by 6 days it was present

in sufficient quantity to be detected as a strong spot. It gave only a weak reaction with the ammoniacal silver nitrate and aniline hydrogen phthalate sprays but with the ninhydrin spray gave a very strong yellow spot which after a few minutes changed to the usual violet colour as given by glycine. With the Elson-Morgan spray the compound gave a weak purple colour but the Seliwanoff test and modifications (see later) were negative. Spray (f) gave a strong blue spot in the cold.

Spectroscopic examination. The spectrum of the second sample in 1000-fold aqueous dilution was determined in a Beckman u.v. spectrophotometer in a 1 cm. cell (see Fig. 1).

Separation of the glucose-glycine reaction mixture. D-Glucose (45 g.) and glycine (5 g.) were dissolved in water and brought to pH 6.7 with HCl and NaOH and evaporated to dryness in a vacuum desiccator. The mixture was then equilibrated to 70% relative humidity and held at that humidity at 37°. After 14 days the reaction mixture was dark brown. It was dissolved in water (1 l.) and slowly added to a column (3.5 cm. $\times$ 30 cm.) of Amberlite resin IR 100 (72-100 mesh B.S.S.). The column was then exhaustively washed with water. The eluate and washings contained only glucose and a brown material with zero R_F value. Ammonia solution (0.1 N) was then slowly added to the column and the eluate collected on an automatic fraction cutter. The fractions containing only the compound having R_F 0.11 were combined, concentrated under reduced pressure, shaken with activated charcoal, filtered and finally taken to dryness. The compound (0.45 g.) was further purified by chromatographic separation of bands on sheets of Whatman no. 3 MM filter paper. The strips containing the compound were cut out, eluted with water and the eluate was concentrated. In this way the compound having R_F 0.11 was obtained chromatographically pure as judged by its migration in three solvents. It could be slowly crystallized as the sodium salt, which was submitted for infrared examination.

A mixed fraction with glycine followed the above compound off the resin column and then a fraction which on chromatographic examination contained only glycine, characterized as 2:4-dinitrophenylglycine, m.p. and mixed m.p. 201°.

Properties of the compound having R_F 0.11

Infrared examination. A Nujol (liquid paraffin) mull of the sodium salt of the compound having R_F 0.11 was prepared, and the infrared spectrum of this mull was recorded in the frequency range 4000-650 cm^{-1} (see Table 3) with a Perkin-Elmer Model 21 infrared spectrophotometer equipped with sodium chloride optics.

Products formed on treatment with acid. The compound (30 mg.) and $\text{N-H}_2\text{SO}_4$ (2 ml.) in a sealed hydrolysis tube were heated in a boiling-water bath for 1 hr. The mixture was then neutralized with barium carbonate and the precipitate removed by centrifuging. Paper-chromatographic analysis showed the presence of unchanged material (R_F 0.11), a compound with the same R_F (0.15) and giving the same colour reaction with ninhydrin as glycine, and also a compound of R_F 0.8, with the same colour reactions with aniline phthalate and resorcinol as 5-hydroxymethylfurfuraldehyde.

To ensure more complete acid breakdown the experiment was repeated with a reaction time of 8 hr. The neutralized mixture, which contained the same products as those obtained with a shorter reaction time, was concentrated and

then separated by sheet-paper chromatography on Whatman no. 3 MM paper. The strips containing the compounds were cut out and the compounds were eluted with water.

To the fraction (2.5 ml.) containing the compound of R_F 0.15 was added sodium bicarbonate (0.2 g.) and a solution of 2:4-dinitrofluorobenzene (0.14 ml.) in ethanol (5 ml.). The mixture was stirred for 1.5 hr., concentrated and then extracted with ether. When the aqueous solution was acidified an oil separated which did not crystallize. The oil was extracted with ether, dissolved in ethanol-ether and streaked on to Whatman no. 3 MM filter paper and developed with the organic phase of butanol-ethanol-water (4:1:5, by vol.). The band corresponding to 2:4-dinitrophenylglycine was cut out and extracted with ether. The extract crystallized from water in fine needles, having m.p. 197°, undepressed on admixture with genuine 2:4-dinitrophenylglycine.

The compound having R_F 0.8, in addition to having identical R_F and colour reactions, had the same u.v. absorption spectrum (principal maximum at 285 $\text{m}\mu$. in water) as genuine 5-hydroxymethylfurfuraldehyde.

Preparation of D-glucosazone from the compound. The compound (75 mg.) was treated in the usual way to form an osazone (35 mg.) which had the characteristic crystalline

Fig. 1. Changes with time of absorption curves of brown mixture obtained by the interaction of D-glucose with glycine at pH 6.7, 37° and 70% relative humidity.

Table 1. Periodate oxidation of the compound having R_F 0.11 formed by reaction between glucose and glycine

Time (hr.)	Periodate uptake (mol.prop.)	Formic acid released (mol.prop.)
4.5	4.42	2.78
22	4.55	2.92
72	4.72	2.96
168	4.79	3.01

form of glucosazone, and m.p. 203°, not depressed on admixture with genuine D-glucosazone.

Periodate oxidation. The compound (52.8 mg.) was dissolved in water (50 ml.) and 5 ml. portions were taken. To each was added 0.2M sodium metaperiodate (1 ml.) and the reaction mixtures were left in the dark at room temperature. At intervals samples were removed and neutral 0.1N sodium arsenite (5 ml.) was added, followed by back titration with 0.01N iodine to determine the amount of periodate consumed. A control determination on 1 ml. of 0.2M periodate and 5 ml. of 0.1N arsenite solution gave a titration of 12.5 ml. of 0.01N iodine. Similar oxidation of another portion with sodium metaperiodate (0.2M, 1 ml.) gave formic acid, which was determined by titration with 0.02N-NaOH (see Table 1). Similar oxidation of another portion gave formaldehyde, identified as the dimedone derivative, m.p. 186° (Bell, 1948).

Tests for hexuloses. The Seliwanoff test was carried out (1) as is usually done in 12% final concn. of HCl; (2) as modified for chromatography (Bryson & Mitchell, 1951); (3) in ethanol saturated with HCl gas; and finally (4) as modified by Roe (Dische, 1955). Tests (2) and (3) were conducted on paper chromatograms. In all the modifications the reagent gave a negative test with the compound but a strong positive reaction with fructose. The test with α -naphthol and phosphoric acid was carried out by spraying the compound with a 10:1 (v/v) mixture of 2% ethanolic α -naphthol and phosphoric acid. This test also was negative for the compound but gave a strong positive reaction with fructose. The test with stannous chloride, H_2SO_4 and urea (Feigl, 1947) was also negative. The compound gave a negative test when sprayed on filter paper with a saturated solution of 2:4-dinitrophenylhydrazine in ethanol-HCl (100:1, v/v). Under the same conditions fructose gave an orange spot at room temperature.

Tests for reducing power. To a dilute solution of the compound in 0.1N-NaOH (1 ml.) at room temperature were

Table 2. *Effect of pH on the decomposition of the compound having R_F 0.11*

pH	Optical density ($\times 10^3$) at 350 m μ . after days at 37°				
	2	4	8	16	32
3	22	28	31	53	85
5	11	14	16	28	60
6.7	17	21	45	60	80
8	75	85	105	146	170
10	117	120	115	121	122

Fig. 3. *Effect of relative humidity on the decomposition at pH 6.7 and 37° of the compound having R_F 0.11. Measurements of optical density (350 m μ .) of aqueous solution.*

Fig. 2. *Effect of temperature on decomposition at pH 6.7 and 70% relative humidity of the compound having R_F 0.11 formed in the reaction between glucose and glycine under 'dry' conditions. Measurements of optical density (350 m μ .) of aqueous solution.*

Fig. 4. *Changes with time of absorption curves of brown mixture obtained by decomposition at 37°, pH 6.7 and 70% relative humidity of the compound having R_F 0.11.*

added a few crystals of *o*-dinitrobenzene. The solution turned purple in about 1 min. The compound decolorized Fehling's solution at room temperature. The test involving the reduction of ferrieyanide and the conversion of the ferrocyanide into Prussian blue at room temperature (Borsook *et al.* 1955) gave a strong positive reaction.

Effects of temperature, pH, and relative humidity. The compound (0.2 g.) was dissolved in water (50 ml.) and portions of this solution were brought to pH 3, 5, 6.7, 8 and 10 respectively with HCl and NaOH. The volumes of these solutions were adjusted with water and portions containing 1 mg. of the compound were transferred to small tubes and freeze-dried.

Samples adjusted to pH 6.7 were stored in air-tight jars over a H_2SO_4 -water mixture producing a relative humidity of 70% at 0°, 22°, 37° and 55° respectively. At intervals tubes were removed, the contents were dissolved in water (10 ml.) and their absorption spectra were measured in a 1 cm. cell (see Fig. 2).

Samples adjusted to pH 3, 5, 6.7, 8 and 10 were stored in an air-tight jar over H_2SO_4 -water mixture producing a relative humidity of 70% at 37°. The colour developed was measured as above (see Table 2).

Samples adjusted to pH 6.7 were stored at 37° over H_2SO_4 -water mixtures producing relative humidities of 0, 20, 50, 70 and 100%. The colour developed was measured as above (see Fig. 3).

Isolation of brown material

The eluate from the Amberlite IR 100 column containing only glucose and a brown material with zero R_F value (see above) was concentrated and then dialysed against running tap water until paper chromatography revealed only the brown material which did not move from the base-line. The solution was dialysed against distilled water and then concentrated under vacuum below 40°. The concentrate was

dried to a black glass in a vacuum desiccator. This was completely soluble in water, in which in ultraviolet light it gave a strong bluish green fluorescence (see Fig. 5).

The polymer (0.1 g.) was dissolved in $\text{N-H}_2\text{SO}_4$ (5 ml.) and heated in a boiling-water bath for 15 hr., neutralized with barium carbonate, centrifuged, concentrated, and then examined by paper chromatography. The ninhydrin spray revealed two faint blue spots having values R_F 0.18 and 0.04, and also a strong purple spot, migrating with glycine (R_F 0.14). The aniline phthalate spray revealed a faint brown spot identical with glucose (R_F 0.24).

DISCUSSION

The reaction between D-glucose and glycine under 'dry' conditions has been followed both by spectroscopic and by chromatographic analysis. As the reaction mixture slowly turned brown in colour a strong absorption developed in the near ultraviolet (λ_{max} 2900 Å) with decreasing absorption continuing into the visible. The maximum is close to that of 5-hydroxymethylfurfuraldehyde (λ_{max} 2850 Å) but that compound could not be detected by chromatography. The continuing absorption into the visible can be explained by the absorption of the brown polymer (see Fig. 5). It is probable then that the absorption band at 2900 Å is that of the Schiff's base (II) of 5-hydroxymethylfurfuraldehyde, postulated by Gottschalk & Partridge (1950), which then can form brown polymers.

The chromatographic analysis revealed in addition to the reactants only the compound having R_F 0.11, which was isolated, and the brown polymer, which did not move from the base-line. The compound of R_F 0.11 was isolated by displacement chromatography on an ion-exchange resin. It was chromatographically pure and could be very slowly crystallized, but because of its instability it was not obtained in a form suitable for elementary analysis. In its general properties the compound seems very similar to the intermediate isolated from the browning of dehydrated carrot (Wager, 1955) and that isolated from liver (Borsook *et al.* 1955). It has properties such as very strong reducing power, acid breakdown to glycine and 5-hydroxymethylfurfuraldehyde rather than acid hydrolysis, and the colour given with *p*-dimethylaminobenzaldehyde after pretreatment with acetylacetone, all of which have been used as indications of 1-deoxy-1-amino-hexuloses (Gottschalk, 1952; Hodge, 1953) formed by Amadori rearrangement. However, Amadori rearrangement compounds give positive colour reactions for ketoses and form hydroxylamine derivatives (Kuhn & Weygand, 1937; Gottschalk, 1952) and the compound of R_F 0.11 did not give any ketose reactions although many tests were tried.

Certain structural evidence emerges from the experimental work. The glycine moiety is bound into the new compound virtually unchanged, for it

Fig. 5. Absorption curve in aqueous solution of brown polymer formed in the reaction between glucose and glycine in the 'dry' state.

is readily released from it with acid treatment, and under some conditions, even on standing. The yellow colour at first given with ninhydrin is consistent with an $-\text{NH}$ group (cf. proline). The preparation of D-glucosazone from the compound shows that its reducing and amino functions are at the first two carbon atoms and that the configurations of the lower four carbon atoms are identical with those of glucose. The release of three molecular proportions of formic acid and the release of formaldehyde on oxidation with sodium metaperiodate confirm the presence of this tetrahydroxybutyl group but do not differentiate between a carbonyl and an hydroxyl group at C-2. The periodate oxidation does, however, demonstrate that the compound is in a straight chain and not in a ring form.

These findings are best explained by the scheme illustrated, which is similar to that proposed by Gottschalk & Partridge (1950). This scheme envisages the reaction of glucose (I) with glycine to pass probably by way of the *N*-glucosylamine (II), although that compound was not detected, to the compound (III) which is the enolic form of *N*-(carboxymethyl)amino-1-deoxyfructose. The tautomeric equilibrium must be almost completely in favour of the enol as no ketose reactions were observed under a variety of conditions. The intermediate (III) can then pass under suitable conditions through the Schiff's base of 5-hydroxymethylfurfuraldehyde (IV) either to brown polymers or to 5-hydroxymethylfurfuraldehyde and glycine.

The allocation of structure (III) is further substantiated by a study of the infrared spectrum of the compound (see Table 3). The peaks found can be compared with those expected in the 1500 cm^{-1} – 1700 cm^{-1} region for structure (III) and for the alternative structures (VI) and (VII) (Bellamy, 1954).

Table 3. Infrared spectrum of the compound having R_f 0.11

Abbreviations: *br*, broad; *m*, moderate strength; *s*, strong; *sh*, sharp; *v*, very; *w*, weak.

Peak frequencies (cm^{-1} , corrected)		
3530 <i>m, sh</i>	1192 <i>m</i>	862 <i>m</i>
3160 <i>vs, br</i>	1127 <i>s</i>	829 <i>m</i>
1626 <i>s</i>	1100 <i>s</i>	780 <i>m</i>
1549 <i>s</i>	1075 <i>vs</i>	713 <i>w</i>
1327 <i>s</i>	1043 <i>s</i>	675 <i>w</i>
1298 <i>w</i>	985 <i>vs</i>	660 <i>w</i>
1260 <i>m</i>	929 <i>w</i>	
1213 <i>m</i>	909 <i>m</i>

Structure (III)	$-\text{CO}_2\text{Na}$	$1610\text{--}1550\text{ cm}^{-1}$
	$-\text{NH}-\text{CH}=\text{C}(\text{OH})-$	$1680\text{--}1620\text{ cm}^{-1}$
Structure (VI)	$-\text{CO}_2\text{Na}$	$1610\text{--}1550\text{ cm}^{-1}$
	>C=N-	$1690\text{--}1640\text{ cm}^{-1}$
Structure (VII)	$-\text{CO}_2\text{Na}$	$1610\text{--}1550\text{ cm}^{-1}$
	>C=O	$1725\text{--}1705\text{ cm}^{-1}$

Although the presence of a peak does not prove that a particular grouping is present in a compound, the absence of a peak conclusively proves the absence of that grouping. Thus the peaks at 1549

and 1626 cm^{-1} in the spectrum of the compound rule out structure (VI), because the C:N stretching at 1670 cm^{-1} is absent, and rule out structure (VII), because the simple ketone peak at 1724 cm^{-1} is not present. The peak at 1626 cm^{-1} would not be given by a substance in the pyranose form. The presence of this peak in the spectrum confirms chemical evidence that the compound does not occur in the pyranose form.

Although the peak at 1645 cm^{-1} expected in structure (III) is not present the group adjacent to the double bond could decrease the frequency, and as there is no definite information upon the C=C

frequency of the double bond in $\text{C}(\text{OH})=\text{CH}\cdot\text{N}$ this could be 1626 cm^{-1} . The -NH stretching peak expected from structure (III) or (VII) is present.

Of the three structures the spectrum excludes structures (VI) and (VII) and is compatible with structure (III).

That the compound isolated in these experiments is indeed an intermediate in the 'browning' reaction can be seen from the study of its decomposition under varied conditions. It is self-browning and the rate of browning increases rapidly with temperature (see Fig. 2) and is strongly influenced by water content (see Fig. 3), showing a maximum at water contents corresponding to a relative humidity of about 70%, and falling away to low values at very low and very high water contents. The influence of pH is not so clear, perhaps because the pH recorded is that of the solutions from which were prepared the samples for browning (in equilibrium with 70% relative humidity), and the significance of pH in the presence of so little water is doubtful. However, the results do show a slower initial rate of browning under acid conditions and increases with increasing pH. The self-browning of this compound is thus influenced in the same way as the browning reaction (Lea & Hannan, 1949). Also the spectrum of its decomposition products is very similar to that of the browning reaction (compare Figs. 1 and 4).

SUMMARY

1. The reaction between D-glucose and glycine in the 'dry' state at 37° , pH 6.7 and 70% relative humidity has been studied chromatographically and spectroscopically. An intermediate appeared which gave the colour reactions of both a sugar and an amino acid.

2. The intermediate has been isolated chromato-

graphically pure from the reaction mixture by displacement chromatography on an ion-exchange resin. It is very strongly reducing but it is not a ketose. Glucosazone can be formed from it. On oxidation with sodium metaperiodate it released three molecular proportions of formic acid and also formaldehyde. On treatment with acid it gave 5-hydroxymethylfurfuraldehyde and glycine.

3. The infrared spectrum of the sodium salt of the intermediate is consistent with that expected for the sodium salt of the enolic form of *N*-(carboxymethyl)amino-1-deoxyfructose.

4. On this evidence the intermediate has been formulated as the enolic form of *N*-(carboxymethyl)-amino-1-deoxyfructose, with the tautomeric equilibrium completely in favour of the enol.

5. The compound has been shown to be a true intermediate in the 'browning' reaction.

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