STUDIES OF THE REACTION BETWEEN PROTEINS AND REDUCING SUGARS IN THE "DRY" STATE

VI. THE REACTIVITY OF THE TERMINAL AMINO GROUPS OF LYSINE IN MODEL SYSTEMS

by

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In previous papers of this series[1,2,3,4] it has been shown that when solutions containing casein and glucose are freeze-dried and held at 37° C, the free amino groups of the lysyl residues of the protein react rapidly with the glucose. Complex effects, including browning and insolubility, then develop after a distinct time lag in a manner which suggests that they are consequent upon the amino-glucose reaction and evidence in support of this relationship has been provided by experiments with modified casein-glucose systems[5]. In one experiment of this type a study was made of the decomposition on further storage of a colourless amino-glucose complex prepared by reaction of casein with glucose for a short time followed by removal of the excess glucose by dialysis. In other experiments the amino-groups of the protein were blocked by selective acetylation and the reaction of the remaining non-amino polar groups of the protein with glucose investigated.

It became clear, however, that a full understanding of the chemical nature of the changes involved called for a study of the reaction between glucose and the free terminal amino groups of polypeptide-bound lysine without the complications introduced by the presence of other reactive groupings. The present communication therefore describes experiments with α-N-acetyl-L-lysine and with a preparation of poly-L-lysine[6,7] of molecular weight comparable with those of small proteins. The former permits the isolation and identification of reaction products by standard procedures, while the latter reproduces both the reactivity of truly peptide-linked lysine and physical properties approaching those of a natural protein. A comparison of the results obtained with the two systems should reveal any disturbing factors peculiar to either, such as effects of carboxyl groups in the α-N-acetyl-L-lysine or the high concentration of adjacent amino groups in the polymer.

As far as possible the reaction conditions have simulated those used with the proteins[1,2], *i.e.* (a) initial pH of 6.3, (b) 37° C, (c) only a small excess of glucose, (d) controlled low water contents obtained by freeze-drying and then equilibrating with known relative humidities. The natural buffering-power of the proteins has not been reproduced

References p. 305.

by adding buffer solutions since it has been made clear by several workers[8,9,10,11] that the buffer salts most commonly used influence the course of reactions of this type.

EXPERIMENTS WITH α-N-ACETYL-L-LYSINE

Methods

The α-N-acetyl-L-lysine used was prepared by the method of NEUBERGER AND SANGER[12]. 2% solutions of this with D-glucose added (1½ mols per amino group) were adjusted to pH 8.5 with sodium hydroxide, divided into suitable samples (usually containing 5–10 mg α-N-acetyl-L-lysine), left to stand for a few hours to complete mutarotation and then freeze-dried in high vacuum over phosphorus pentoxide. The dried material was approximately adjusted to required moisture contents at 10° C and then stored at 37° C and various relative humidities as previously described[1].

The free amino nitrogen was followed by the VAN SLYKE method (15 minutes' reaction time), glucose by use of the specific oxidase notatin and pH by diluting to approximately 1% solution and testing with narrow range pH test-papers (accurate to about 0.1 pH units except in the brownest solutions).

Selected samples were examined by descending paper chromatography on Whatman No. 1 papers with 80% aqueous propanol as solvent. The reaction products ran slowly and the chromatograms were generally developed for 40 hours with a folded filter paper pad at the lower end to promote continued solvent flow. The papers were dried at room temperature and examined both under an ultraviolet lamp and by appropriate spraying. Milligram quantities of the intermediate products could be isolated for examination and re-storage by applying the sample in a thin streak across an 8 inch wide filter paper, developing in the normal manner and eluting the appropriate transverse strips located by their fluorescence or by spraying strips cut from the edges.

Results

At high humidities a typically complex MAILLARD reaction[13] was observed but distinct phases could be recognized by considering the effect of increasing humidity (Table 1).

At 20% relative humidity (R.H.) the reaction was relatively simple. Amino groups and glucose disappeared in equimolecular amounts, no change in pH occurred and the colour development was negligible; the samples were still white after six days and only pale cream after 24 days when well over half the amino groups had reacted. A small peak developed in the ultraviolet absorption spectrum at about 2960 A (Fig. 1). (Measurements below about 2400 A were not carried out owing to the strong absorption of the initial material in this region.)

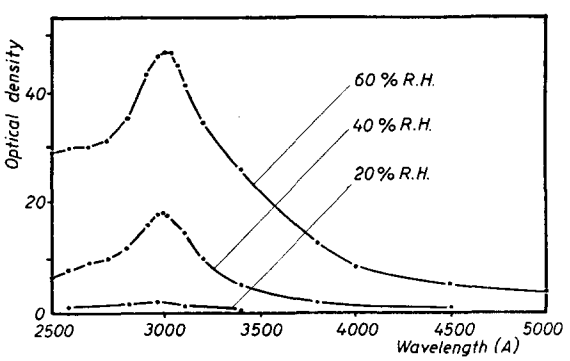

Fig. 1. Absorption spectra observed after 6 days' reaction at 37° C between α-N-acetyl-L-lysine and glucose (1½ mols/amino group) at various relative humidities. The units of optical density are the effective e values for solutions containing 1 mg original amino-N/ml.

Increasing humidities caused an increased rate of loss of amino groups until a maximum was reached at 40% R.H. but greater complexity of the reaction was now also evident. In particular, after an initial time-lag, the peak in the ultraviolet region increased considerably, and shifted slightly to 2980 A, while a small amount of undifferentiated absorption extending well into the visible region gave rise to definite browning. A small but significant loss of glucose in excess of a one-to-one ratio and a fall in pH also occurred.

References p. 305.

TABLE I

CHANGES DURING THE REACTION BETWEEN α-N-ACETYL-L-LYSINE AND GLUCOSE (1½ MOLECULES/AMINO GROUP) AT 37° C AND VARIOUS HUMIDITIES

	Reaction time (days)	*Relative humidity %*						
		20	*30*	*40*	*50*	*60*	*70*	*80*
Amino N (% of initial)	3	75	52	42	47	55	65	82
	6	66	32	23	28	40	53	72
	24	37	12	6	8	12	17	37
pH[a]	0	6.4	6.4	6.4	6.4	6.4	6.4	6.4
	6	6.4	6.3	5.6	5.0	4.9	5.0	5.0
	24	6.4	5.9	5.1	4.2	3.9[b]	3.9[b]	4.1[b]
Optical density at 3000 A[c] [d]	0	0	0	0	0	0	0	0
	3	1	2	4	9	12	8	4
	6	2	6	18	41	47	27	13
	24	5	15	70	114	157	133	65
Optical density at 4500 A[c] [e]	0	0	0	0	0	0	0	0
	3	0	0	0.2	1.0	1.2	1.0	0.4
	6	0.1	0.2	0.8	3.8	5.4	3.9	1.8
	24	0.1	0.4	2.3	7.6	16.4	13.6	7.9
Ratio mols glucose lost/NH_2 groups lost	12	—	—	0.99	—	1.22	—	1.0
	24	—	—	1.08	—	1.37	—	1.27

[a] Approx. value after dilution to 1% solution.
[b] Exact values doubtful owing to the strong colour of the solutions.
[c] Effective e value of solution containing 1 mg original amino N/ml.
[d] Measurement actually made at the peak.
[e] Measured as representative of the browning.

At 60% R.H. the rate of loss of amino groups had fallen off considerably, but the development of absorption at all wavelengths, the fall in pH and the loss of excess glucose all reached a maximum. The absorption in the visible region (browning) was now relatively much greater than at 40% R.H. (Fig. 1) and a further shift of the peak to 3020 A had occurred.

Further increase of humidity produced no other well-marked changes in the character of the reaction. The absorption spectrum remained generally similar to that at 60% R.H. and the other effects, except possibly the pH drop, developed less rapidly as the humidity rose.

It should be noted that the pH on reconstitution of these samples was about 6.5, immediately after drying, compared with the original 8.5, and this has not yet been explained. No loss of amino groups or changes in chromatographic behaviour could be demonstrated and the chemical changes involved have been assumed to be slight. A similar pH change took place even in the undried solution or in a solution of glucose alone, but not in a solution of α-N-acetyl-L-lysine alone.

Demonstration of intermediate compounds

After several days' reaction the pH had usually departed considerably from the original nearly neutral conditions and entered the acidic region where free furfurals are

References p. 305.

known to form[14]. The search for intermediate products which would account for the above effects was therefore confined to six days' time of reaction or less. Descending paper chromatography in 80% aqueous-propanol was used, and under these conditions glucose and α-N-acetyl-L-lysine ran at R_F 0.3 and 0.2 respectively.

Products at 20% *R.H.* A new well-defined spot appeared at R_F 0.1 which had all the characteristics described by GOTTSCHALK AND PARTRIDGE[15] for the corresponding lysine compound, including blue fluorescence on heating the paper and positive reactions on heating with ninhydrin or ammoniacal silver nitrate. The reaction to the ELSON AND MORGAN test as used for N-acetyl hexosamines[16] was also positive; spraying with alcoholic caustic potash followed by heating gave a deep yellow colour and subsequent spraying with the *p*-dimethylaminobenzaldehyde reagent produced a pink colour. The test of CHAPMAN AND MCFARLANE[17], as first developed for use with milk powder, was also applied by spraying with the buffered ferricyanide reagent (pH 5), heating for a short time at 70° C and then spraying with ferric chloride solution. The strong colour of prussian blue appeared only in the region of the new spot. No other new compounds of any significant intensity were observed on the papers.

Two separate samples of the compound, each of several milligrams, were eluted from unheated papers and dried over phosphorus pentoxide *in vacuo* at room temperature for three days. Their nitrogen contents, determined by the micro-Kjeldahl technique, were 6.2 and 6.9% compared with the theoretical figure for a one-to-one condensation product of 7.9%. Since part of the difference, at least, might be accounted for by contamination with inert material from the filter paper, the agreement can be considered to be fairly good and, in conjunction with the observed equimolar reaction of glucose and amino nitrogen, suggests that the unknown is in fact a simple one-to-one condensation product.

The conditions for the formation of this substance were so similar to those described by WEYGAND[18] for aromatic N-glycosides that attempts were made to prepare the same type of compound by alternative methods. The most successful was based on that of KUHN AND STROBELE[19], *i.e.* refluxing in methanol containing small amounts of water and ammonium chloride. This gave very little brown colour except on long heating and the same spot appeared on paper chromatograms.

Attempts to release free glucose by hydrolysis with hydrochloric acid, either boiling *N* acid, cold *N* acid or boiling 0.05 *N* acid, were unsuccessful; free glucose could not be detected either by paper chromatography or by the use of notatin. Although this behaviour agrees with that previously reported for the colourless complexes of glucose with casein[2], it would not be expected for a simple N-glycoside.

The compound might however be an isomer of the N-glycoside and the possibility of an *iso*glucosamine structure[20] was examined by attempted reduction with palladium black and hydrogen at room temperature in Warburg manometers with 90% ethyl alcohol as solvent[21]. Under these conditions *p*-tolyl-D-*iso*glucosamine was reduced in five hours but the unknown was, at the most, reduced much more slowly, the rate of hydrogen absorption being not significantly different from the slow but appreciable absorption of the blank.

Products at 40% *R.H.* Preliminary inspection of the results of chromatography of the 40% R.H. reaction mixtures suggested essential similarity to those at 20% R.H. except that the brown material, which was now present, remained stationary as though adsorbed on the paper.

References p. 305.

Under an ultraviolet lamp, however, a number of slow-moving compounds with a blue or purple fluorescence were revealed. Clearer separations were obtained by applying the mixture as a streak across the paper rather than as a single spot and an example of the resulting chromatograms is shown in Fig. 2. The brightest band B gave a brilliant purple fluorescence and was concentrated in a characteristic narrow band at R_F 0.09 on the rear edge of the R_F 0.1 band (A) while three other clearly separated bands with blue or pale blue fluorescence appeared with R_Fs 0.05, 0.03 and 0.01. The separate identity of these compounds was shown by their different absorption spectra after elution (Fig. 3) and by re-chromatography which gave single spots in the original positions. Although the spectra are obviously complex, one predominant feature could be recognized, *i.e.* a peak which became less sharp and moved to longer wavelengths as the R_F decreased; *i.e.* 2880, 3080, 3200, 3250 and 3280 A for B, D, E, F and G respectively A colourless non-fluorescent compound with a peak at 2800 A was also separated from the dark space C at R_F 0.07.

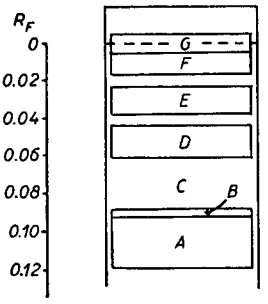

Fig. 2. Diagrammatic representation of paper chromatograms, developed in propanol/water (80:20), of a mixture of α-N-acetyl-L-lysine and glucose (1 ½ mols/amino group) after 6 days' reaction at 37° C and 40% R.H.

These results show that the apparently simple peak at about 3000 A in the intact system is in fact a complex of comparable contributions from several components. Increasing proportions of the longer wavelength fractions as the humidity rises explain both the general shape of the absorption curves and the slight movement of the peak. It should be noted that compound A did not absorb in this region and the small absorption observed at 20% R.H. must have been due to the presence of small amounts of the fluorescent compounds, particularly B.

Although these physical properties are a first guide to the nature of the new compounds, a knowledge of their chemical properties would be desirable for further identification. C–G however gave no characteristic chemical reactions while, although B appeared to give strong reduction of ammoniacal silver nitrate and ferricyanide (at pH 5), this was subject to uncertainty owing to incomplete separation from A. An observation of similar nitrogen contents for B and A is also subject to the same uncertainty. Semi-quantitative tests on small amounts of the other fluorescent and brown compounds indicated nitrogen contents of the order of 5–10%, *i.e.* of the same order as A.

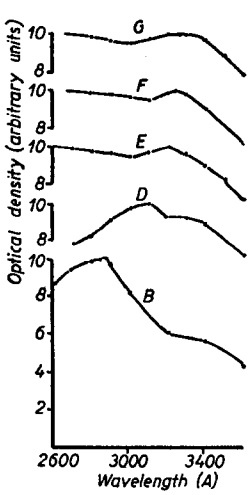

Fig. 3. Absorption spectra of the fluorescent and brown substances after elution from the chromatograms in Fig. 2. All the spectra have been adjusted to have the same effective optical density at the peak.

None of these compounds could be identified as derivatives of 5-hydroxymethyl furfural, since the main reaction product of this substance with α-N-acetyl-L-lysine at 37° C and 70% R.H. was a brilliant purple fluorescent spot at R_F 0.5 with only a faint suggestion of pale blue fluorescence at R_F 0.13. The latter would have been quite insufficient to account for any of the unknowns, while the brown products themselves

References p. 305.

behaved quite differently, streaking down the whole length of the paper rather than remaining stationary.

Products at 60% *R.H.* The chromatograms of this material were similar to those at 40% R.H. except that B was now less intense than the other fluorescent bands and there was some increase in complexity, including in particular a new pale blue fluorescent compound between B and C. This system has not yet been examined in detail.

Breakdown of the intermediate compounds

When the developed chromatograms were heated at 80° C for a few hours, pronounced browning appeared only in region A, suggesting that this is one of the main intermediate substances in the browning sequence.

Samples were therefore eluted from the chromatograms of 20% R.H. material (six days' reaction time), adjusted to pH 6.5, freeze-dried and stored at various humidities. Strong browning and a fall in pH developed without any time lag and with a maximum rate at 60% R.H. (Table II), providing a qualitative explanation of the behaviour of the intact reaction mixtures. The amount of browning however proved to be only about a third of that expected, and the discrepancy was not removed by adding excess glucose or α-N-acetyl-L-lysine before drying. Simple breakdown of A is therefore not a sufficient explanation of the browning unless some change in its stability occurs during chromatography.

TABLE II

CHANGES OBSERVED ON STORAGE OF ISOLATED SAMPLES OF COMPLEX A AT 37° C UNDER VARIOUS CONDITIONS

Relative humidity (%)	*Additions* (*per mg complex*)	*Reaction time* (*days*)	*pH**	*Optical density***	
				3000 A	*4500 A*
—	—	0	6.1	1.4	0.2
40	—	6	6.1	11.8	0.8
60	—	6	5.1	17.6	2.0
80	—	6	5.1	10.2	1.2
60	2 mg glucose	6	5.8	17.2	1.6

* Approximate value after dilution to 1 % solution.

** Effective e value for solution containing 2 mg total N/ml *i.e.* equivalent to 1 mg original amino N/ml.

Examination of the breakdown products of A, moreover, showed a marked difference from brown intact reaction mixtures. Although components D–G were all present, B was absent, implying that this itself may be a primary product of the reaction. (No search for C was made.)

Addition of B to A before drying produced no significant change in the rate of browning, but a mixture of all the fractions A to F browned about three times as rapidly as either A or B alone and one at least of these other compounds must therefore be concerned in the browning, possibly in a catalytic role. Addition of excess glucose to these mixed fractions produced no further browning but there was some indication that excess α-N-acetyl-L-lysine caused an increase.

References p. 305.

Water content of the reaction mixtures

The physical condition of the reaction mixtures at 37° C differed greatly from the protein-sugar systems previously examined[1,2,3], the mixture only appearing dry at 20% R.H. or less. At 30 and 40% R.H. it had begun to appear moist, while at 50% R.H. it had dissolved to form a viscous fluid. The water-contents estimated from the sample weights after storage and from an approximate isotherm plotted at 20° C were 5, 9, 22 and 40 g/100 g dry material at 20, 40, 60 and 80% R.H., respectively.

These water-contents are considerably higher than for the crystalline components but comparable with those of freeze-dried glucose solutions before crystallisation takes place (generally in one or two days). Since crystallisation of the reaction mixtures containing α-N-acetyl-L-lysine does not appear to occur, the water-holding properties are presumably determined by the large content of glucose (*ca* 50% by weight) in contrast with the protein-sugar systems where the large protein content (generally 80–90% by weight) is the determining factor.

EXPERIMENTS USING POLYLYSINE

Material used

Polylysine hydrochloride was prepared by a method based on that of KATCHALSKI, GROSSFIELD AND FRANKEL[6,7].

The α-ε-dicarbobenzoxy-L-lysine obtained from 10 g of L-lysine monohydrochloride[22] was boiled for 15 minutes in 50 ml of dry ether containing 10 ml of thionyl chloride. The solvent was then removed rapidly *in vacuo* and the light cream solid heated at 50° C *in vacuo* for a further 5 minutes. Two lots of 25 ml dry ethyl acetate were added and removed *in vacuo* at 50° C and the final product was taken up in 40 ml of ethyl acetate. The N-carboxy anhydride was crystallised by the slow addition of 40 ml of petroleum ether (b.p. 60–80° C) and twice recrystallised from 40 ml of ethyl acetate and 60 ml of petroleum ether. The white product was dissolved in 100 ml of ethyl acetate containing 4% of freshly-distilled pyridine and gently refluxed for 14 hours when a white copious precipitate formed. This was washed twice with ethyl acetate and on drying gave 5.5 g of pale cream powder, the ε-N-carbobenzoxy poly-L-lysine.

This was reduced with phosphonium iodide, precipitated as the picrate and re-dissolved as the hydrochloride by the methods of KATCHALSKI, GROSSFIELD AND FRANKEL and the product (3 g) was dialysed through cellulose for three days against six twelve-hourly changes of 10 volumes of distilled water. The dialysates gave approximately 1 g of yellow material on each of the first two days and 200 mg on the third. The undialysable residue gave 300 mg of a pale cream product on evaporation and only this fraction was used in the work described hereafter. All references to polylysine refer to this material.

Properties of the polylysine hydrochloride

The material was pale cream in colour with weak absorption peaks at 2700 A and 3400 A. The colour appeared to be adsorbed by the polymer since it was not removed by dialysis and was attributed to small amounts of a highly coloured impurity in negligible amounts compared with the large number of free amino groups present.

Chain-length determination with 1:2:4 fluorodinitrobenzene was unsatisfactory owing to the great difficulty of wetting the DNP-polymer with even the 50% (w/w) sulphuric acid recommended by KATCHALSKI, GROSSFIELD AND FRANKEL, but the average molecular weight of the polyionic cation determined by osmosis was 16,000 and by a fluorimetric method 10–11,000, as described below. Exact correspondence between the two methods would not be expected for poly-disperse material.

The nitrogen content was 16% and the free amino nitrogen 8% (calculated 16.9% and 8.5%).

The water absorption figures at 20° C were 4.0, 6.3, 18.3 and 31 g/100 g dry polymer at 20, 40, 60 and 80% R.H., *i.e.* very similar to those of typical proteins.

Osmotic pressure determinations

The osmotic measurement of molecular weight was carried out by Dr G. S. ADAIR. The inherent difficulties of the determination were overcome by using a small osmometer fitted with a collodion membrane of low permeability and 4 *M* sodium chloride as solvent. Nitrogen determinations on the external fluid showed no passage of polymer through the membrane although it absorbed part of the sample during the period of several days.

References p. 305.

A concentration of 0.0807 g polyionic cation/100 ml gave an osmotic pressure of 0.84 mm of mercury ± 0.2 mm at 0° C equivalent to an average molecular weight of 16,000 ± 4,000.

Fluorimetric determination of molecular volume

An independent estimate of molecular size was provided by experiments, performed by Dr G. WEBER, applying the principle that when polarized radiation falls on a small fluorescent molecule attached to a large body, the emitted light is polarized to an extent which is a measure of the molecular volume[23].

The polymer was reacted with 1 dimethylamino-naphthalene-5 sulphonyl chloride (2 molecules per polymer molecule) in 10% dioxan solution (w/w) containing approximately 1% sodium bicarbonate, and after dialysis the polarisation of fluorescence was measured over a range of temperature. The appropriate plot of the data (Fig. 4) gave a straight line at low temperatures (0–30° C) from which a molecular volume of 8,000 can be deduced, assuming the molecules to be approximately spherical. This is equivalent to a molecular weight of 10,400 assuming a density of 1.3. The corresponding curve for egg albumin is shown on the graph for comparison.

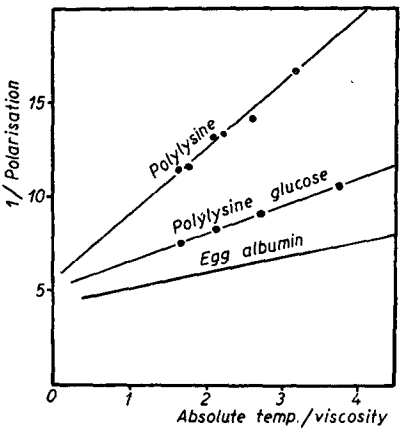

Fig. 4. The dependence on temperature of the polarisation of fluorescence of the 1-dimethylamino-naphthalene-5-sulphonyl derivatives of polylysine hydrochloride, of polylysine hydrochloride after 9 days' reaction with 1½ equivalents of glucose at 37° C and 60% R.H., and of egg albumin.

Reaction of polylysine with glucose

The polylysine hydrochloride was reacted with glucose (1½ mols/amino group under the same conditions as the α-N-acetyl-L-lysine, *i.e.* in the freeze-dried state at various humidities and with a pH before drying of 8.5.

As shown in Table III, the results were strikingly similar to those obtained with α-N-acetyl-L-lysine. Some differences are apparent, such as a slightly slower rate of loss of amino groups, a slightly higher optimum humidity for the amino-glucose reaction (45% as compared with 40% R.H.) and a slower rate of browning relative to the loss of amino nitrogen. These differences are however no greater than might be expected from the different physical and chemical form of the two model systems. In other respects there was obvious similarity. Browning developed in all cases after a distinct time-lag, the shapes of the absorption spectra at the various humidities were similar (including a movement of the peak from 2960 A to 3010 A as the humidity rose), maximum absorption at all wavelengths appeared at 60% R.H. and the browning was accompanied by a fall in pH and a disappearance of glucose in excess of a one-to-one ratio.

Although chromatographic separation of the products of the reaction was not possible, this system offered the alternative advantage that insolubility develops, similar to that observed with the proteins. The colourless amino-glucose complex formed at low humidities was freely soluble but the dark brown product after 15 days' reaction at 60% R.H. required several hours' soaking in water before it could be dispersed and complete insolubility developed after longer reaction times. Fluorimetric determination of molecular volume was applied after nine days at 60% R.H. (*i.e.* just before insolubility began to develop), using the same technique as for the initial polymer except that interference from the inherent purple fluorescence of the complex (itself polarized) was avoided by viewing the emergent light through an orange filter. When applied to the unreacted polylysine this procedure did not affect the result obtained. The resulting plot shown in Fig. 4 indicates a molecular volume of 20,000 compared with the figure

References p. 305.

TABLE III

CHANGES DURING THE REACTION BETWEEN POLYLYSINE HYDROCHLORIDE AND GLUCOSE (1½ MOLECULES/AMINO GROUP) AT 37° C AND VARIOUS HUMIDITIES

	Reaction time (days)	*Relative humidity (%)*						
		20	*30*	*40*	*50*	*60*	*70*	*80*
Amino N (% of initial)	3	83	78	74	74	80	91	97
	6	74	67	59	56	68	83	94
	15	60	45	39	42	54	64	76
pH[a]	0	6.4	6.4	6.4	6.4	6.4	6.4	6.4
	6	6.3	6.3	6.2	6.1	5.8	5.8	5.8
	15	6.3	6.3	6.1	5.8	5.2	5.2	5.3
Optical density at 3000 A[b] [c]	0	0.4	0.4	0.4	0.4	0.4	0.4	0.4
	3	0.6	0.6	0.8	1.2	1.2	1.1	0.8
	6	0.9	1.1	2.1	3.8	4.4	3.1	1.7
	15	1.8	2.5	6.3	12.1	15.5	10.9	4.8
Optical density at 4500 A[b] [d]	0	0.1	0.1	0.1	0.1	0.1	0.1	0.1
	3	0.1	0.1	0.1	0.2	0.2	0.2	0.1
	6	0.1	0.1	0.15	0.3	0.5	0.4	0.25
	15	0.1	0.2	0.4	1.2	2.2	1.9	0.9
Ratio mols glucose lost/NH_2 groups lost	15	—	—	1.08	1.2	1.29	—	1.11

[a] Approx. values after dilution to 1% solution.
[b] Effective e value of solution containing 1 mg original amino N/ml.
[c] Measurements actually made at the peak, *i.e.*, 2950 A at 20% R.H.
2980 A at 40% R.H.
3010 A at 60% R.H.
[d] Measured as representative of the browning.

of about 12,000 expected from simple addition of glucose. Even allowing for the possibility of abnormally large hydration of the complex, it is apparent that a significant increase in molecular size has occurred.

Breakdown of the intermediate complex

Material which had been dialysed free from glucose after nine days' reaction at 20% R.H. was adjusted to pH 6.4, freeze-dried and held at 40, 60 or 80% R.H. at 37° C. Even after three days the colourless compound had turned brown and insoluble with an obvious maximum rate of browning at 60% R.H. Quantitative determination of the colour of the insoluble material was not attempted.

Water content of the reaction mixtures

The reaction mixtures were similar in appearance to the α-N-acetyl-L-lysine samples, being "dry" at 20% R.H. and viscous fluids at 50% R.H. and above. The water-contents, determined on small amounts of the mixture, were 5, 10, 18 and 30 g/100 g of dry material at 20, 40, 60 and 80% R.H. respectively.

References p. 305.

DISCUSSION

The main conclusion from this work is strong confirmation for the view, previously expressed[1-5], that many of the changes in "dry" protein-glucose mixtures at 37° C can be accounted for by the reaction between glucose and the ε-amino group of lysine, without the participation of other reactive polar groupings or of surface effects peculiar to the protein. Although indications have also been obtained of the mechanism of the reaction, this aspect must at present remain incomplete, since positive identification of any of the intermediate products has not been achieved. As the work of many previous investigators shows, this is likely to be a matter of some difficulty owing to the general instability and intractability of this type of compound.

It is valuable to list the points of similarity between the three systems (casein, α-N-acetyl-L-lysine and poly-L-lysine) investigated. In all three:

1. The rates of the primary reaction between free amino groups and glucose are similar.

2. Browning develops only after a distinct time lag during which the primary reaction proceeds.

3. There is a clearly defined optimum humidity for the reaction of amino groups and an appreciably higher optimum humidity for browning.

4. Equimolecular losses of glucose and amino groups, indicating a stoichiometric reaction, are observed while the reaction mixtures are colourless.

5. Browning is accompanied by a loss of glucose in excess of the 1:1 ratio and by a fall in pH.

6. The amino group is still basic after reaction with glucose (shown by titration with the protein[3] and by the absence of pH changes in the model systems under suitable conditions).

7. Acid hydrolysis fails to regenerate glucose.

8. Reducing power towards acid ferricyanide and a positive reaction to the ELSON AND MORGAN test develop.

9. Blue fluorescence appears at the higher humidities.

10. Isolation and storage of the first-formed colourless complex produces browning and (except with α-N-acetyl-L-lysine) insolubility.

The absorption spectra observed with the model systems could not be demonstrated with the casein owing to the strong absorption of the protein in this region but the general correspondence listed strongly suggests that basically the same reaction is occurring in all three systems.

Most of these properties can be explained in terms of formation and degradation of a colourless intermediate compound; only the disappearance of excess glucose, the formation of one of the fluorescent derivatives and the origin of the unidentified catalyst for the degradation have not so far been explained on this basis.

The increased molecular volume of the polylysine (presumably indicating cross-linking), and the development of insolubility, show that the parallel effects which occur with proteins do not essentially require the presence of the arginine, histidine and other reactive side-chains in the proteins, in contrast with the action of formaldehyde. However, these other amino acids may well contribute to the reaction in its more advanced stages[5]. The most probable explanation of the cross-linking, in the early stages at least, is that there is an association between two amino-glucose groups or their

References p. 305.

degradation products; these groups would be expected to show a tendency to polymerise.

Nature of the intermediate compounds

Although tentative conclusions can be drawn about the nature of some of the intermediate compounds, two sources of possible ambiguity in the experiments should be noted, *i.e.*, the changing pH during the reaction in many cases and the possibility of isomerisation or degradation during the chromatographic procedures.

The evidence however points to A being an N-glycoside or isomer, possibly an *iso*glucosamine, R–NH–CH_2–CO–$(CHOH)_3$–CH_2OH or its ring form, produced by an Amadori rearrangement[20] as previously suggested[3,4]. The strong reducing properties, failure to regenerate glucose on hydrolysis and the positive reaction to the ELSON AND MORGAN test agree with the *iso*glucosamine structure[20] and GOTTSCHALK has recently considered similar evidence to be sufficient for assigning such a structure to a naturally occurring compound[24]. The failure to take up hydrogen in the presence of palladium black however is in contrast to the behaviour of *p*-tolyl-*iso*glucosamine. This latter compound moreover proved to be stable for 20 days under the conditions used in the present experiments, whereas N-glycosides have frequently been reported to give brown compounds under mild conditions[20,25,26,27]. MITTS AND HIXON[28] also recorded their inability to produce an Amadori rearrangement, as measured by catalytic hydrogenation, in aliphatic N-glycosides. Positive identification will only be obtained by preparation of stable derivatives under mild conditions but attempted acetylation at 0° C with acetic anhydride in the presence of pyridine has so far resulted only in extensive decomposition. HONEYMAN AND TATCHELL[27] have recently emphasized the difficulty of obtaining unequivocal results with this type of procedure even with the well-known aromatic N-glycosides. It is even possible that A is a mixture since there was some indication that the reaction to the ELSON AND MORGAN test was less intense at 20% R.H. than at higher humidities.

There is as yet no direct evidence of the identity of any of the slow-moving fluorescent or non-fluorescent compounds. The mode of formation of B and its close association with A on the papers suggest that it is also a primary product of the reaction and has a composition similar to A. The successive shift to longer wavelengths of the absorption maxima of the pale blue fluorescent compounds suggests that these are successive degradation products, possibly involving polymerisation. A similar series of compounds has recently been reported by PATTON AND CHISM[11] in boiled glucose-glycine solutions. None of these compounds has been identified as a derivative of 5-hydroxymethylfurfural, in contrast with observations made with free lysine and a large excess of glucose after a long reaction time[15].

Effect of water

For the casein system an attempt was made[1,2] to interpret the dependence of the reaction rate on the relative humidity in terms of the physical condition of the water on the protein surface. In the present work, however, such an approach cannot be justified and therefore no significance can be attached to the differences between the humidities observed for the various maxima in the three systems. The large content of hygroscopic, amorphous glucose in the model systems is probably a complicating factor but it may be significant that in both these cases the maximum amino-glucose reaction occurs in the presence of water equivalent to approximately one molecule for each

References p. 305.

molecule of the mixture and that maximum browning occurs at about twice this water content.

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The work described in this paper, a brief preliminary account of which has already appeared[29] was carried out as part of the programme of the Food Investigation Organisation of the Department of Scientific and Industrial Research.

SUMMARY

1. The reactions of α-N-acetyl-L-lysine and of polylysine hydrochloride with glucose in freeze-dried mixtures at 37° C and various humidities have been investigated and shown to be parallel in almost all respects with the behaviour of casein-glucose systems under similar conditions.

2. The pressure of aqueous vapour with which the system is in equilibrium markedly affects the nature of the reaction products. With α-N-acetyl-L-lysine a number of colourless intermediate substances have been separated and their properties described. Degradation of suitable mixtures of these substances reproduced the browning of the intact reaction mixture.

3. The reaction of the polylysine hydrochloride, under conditions where browning occurs, is accompanied by a significant increase of molecular weight, and insolubility subsequently develops.

RÉSUMÉ

1. Les auteurs ont étudié les réactions de l'α-N-acétyl-L-lysine et du chlorhydrate de polylysine avec le glucose dans des mélanges (séchés à l'état congelé) à 37° et à des degrés d'humidité divers; ils ont montré que le comportement de ces mélanges est, presque à tous les égards, parallèle au comportement de systèmes caséine-glucose dans des conditions semblables.

2. La pression de vapeur d'eau avec laquelle le système se trouve en équilibre a une influence considérable sur les produits de réaction. Dans le cas de l'α-N-acétyl-L-lysine un certain nombre de produits intermédiaires incolores ont été isolés et leur propriétés décrites. Par dégradation de mélanges convenables de ces substances les auteurs obtinrent le même brunissement quelle mélange de réaction intact.

3. La réaction du chlorhydrate de polylysine, dans des conditions où le brunissement à lieu, est accompagnée d'une augmentation considérable du poids moléculaire; puis, le mélange tend à devenir insoluble.

ZUSAMMENFASSUNG

1. Die Reaktionen von α-N-Acetyl-L-lysin und Polylysin-hydrochlorid mit Glucose in (im gefrorenen Zustand getrockneten) Gemischen bei 37° und verschiedenem Feuchtigkeitsgrad wurden untersucht. Es wurde gezeigt, dass ihr Verhalten beinahe in allen Fällen dem Verhalten von Casein-Glucose-Mischungen unter ähnlichen Bedingungen parallel ist.

2. Der Wasserdampfdruck, mit welchem das System im Gleichgewicht ist, hat einen bedeutenden Einfluss suf die Reaktionsprodukte. Im Falle von α-N-Acetyl-L-lysin wurde eine Anzahl farbloser Zwischenprodukte abgetrennt und ihre Eigenschaften wurden beschrieben. Abbau von geeigneten Mischungen dieser Substanzen ergab dieselbe Braunfärbung wie das ursprüngliche Reaktionsgemisch,

3. Die Reaktion von Polylysin-hydrochlorid, unter Bedingungen wo Braunfärbung stattfindet, ist von einer bedeutenden Zunahme des Molekulargewichtes begleitet; hierauf wird die Mischung unlöslich.

References p. 305.

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