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REACTION OF LYSINE WITH ALDOSES*

GIOVANNI CANDIANO, GIAN MARCO GHIGGERI, GEROLAMO DELFINO, CARLO QUIEROLO,
Department of Internal Medicine and Hemodialysis Service, Hospital of Lavagna, Via don Bobbio, 16033 Lavagna, Genova (Italy)

CARLA CUNIBERTI,
Institute of Industrial Chemistry, University of Genova (Italy)

ELISABETTA GIANAZZA, AND PIER GIORGIO RIGHETTI
Chair of Biochemistry and Department of Biomedical Sciences and Technology, University of Milano, Via Celoria 2, 20133 Milano (Italy)

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ABSTRACT

The reactivity of amino acids with trioses and hexoses under physiological conditions (37°, pH 7.5) has been investigated. The most reactive species were lysine, glyceraldehyde, and 2-amino-2-deoxyglucose. They generated yellow, oligomeric compounds having molecular weights in the range 500–1000 and pI values in the range pH 4–6. In the Lys–glyceraldehyde adduct, the ε-amino group is incorporated into a pyrrole-type structure, which gives a positive reaction with the Pauly reagent. The largest oligomers correspond to the condensation of 4 mol of Lys with 8–10 mol of glyceraldehyde. It is hypothesised that the primary Amadori-compound rearranges during the Maillard reaction to give browning products, since no yellow oligomers are produced if the Lys–aldehyde adduct is reduced immediately with cyanoborohydride.

INTRODUCTION

In investigating the non-enzymic reaction of human serum albumin (HSA) with sugars in cases of diabetes mellitus, we have described a procedure[1] for the separation of modified and unreacted species. Total albumin is first adsorbed on Blue Sepharose CL-6B, and the modified molecules are selectively removed by chromatography on concanavalin A (Con A)–Sepharose. The albumin peak bound to Con A–Sepharose was shown to contain glucose, galactose, and mannose in amounts considerably greater than those found in the albumin species having no affinity for the lectin–Sepharose[2]. Moreover, these very high levels of modified

*Reaction of Aldoses with Amino Acids and Proteins, Part I.

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albumins in diabetes have altered renal clearance, in that they are retained with lower efficiency by the glomerular wall and are concentrated in the urine[3].

We have demonstrated[4] by isoelectric focusing (i.e.f.) in polyacrylamide matrices that the peaks of modified albumins purified from sera and urine are heterogeneous, giving 25–30 fully resolved bands in the range pH 4–6. This finding suggested that, in addition to the two lysine residues (Lys 199 and 525) reported[5,6] to undergo non-enzymic reaction with sugars, many more lysine residues must be modified in diabetic patients. Given a total of 59 Lys/HSA molecule, it was concluded that the reacted Lys residues represented ~35% (*i.e.*, 16) of those on the surface of the HSA molecule. This view was confirmed by chemical analysis of Con A–Sepharose-purified, modified albumin[7]. Extensive modification *in vitro* of albumin by trioses and hexoses was also described[7], with glyceraldehyde being by far the most reactive molecule (after 7 days of incubation at 37°, 130 mol were covalently bound to 1 mol of albumin).

Non-enzymic reaction of sugars occurs with hemoglobin[8], red-cell membrane components[9], plasma proteins such as albumin[2], low-density lipoproteins[10], nerve proteins (particularly myelin[11]), lens crystalline[12] and extracellular matrix proteins such as collagen[13]. During the preparation and storage of foods, non-enzymic browning products are formed as a result of the Maillard reaction between reducing sugars and free amino acids and proteins[14–19]. As a consequence, the nutritional and physiological properties of foods are alteed, due to loss of availability of essential amino acids and to the presence of new molecular features. Unfortunately, the extensive literature available on foodstuffs and on model browning reactions cannot be transferred readily or applied to physiological systems since high temperatures, pH extremes, and non-aqueous solvents were used. We now present data on the reaction of lysine and other amino acids with aldoses under simulated physiological conditions.

EXPERIMENTAL

Incubations. — Amino acids (40mM) were incubated with various monosaccharides (20mM) in the dark under nitrogen in various buffers (100mM), namely, phosphate for pH 2, 7.0, and 7.5, acetate for pH 4.5, and borate for pH 9.0, 9.5, and 10.5. In some experiments, sodium cyanoborohydride (100 mmol) was added simultaneously with the sugar.

Esterification. — For esterification, a suspension of each amino acid in methanolic 0.2M hydrogen chloride was stored at 2° for 76 h[20].

Determination of molecular weight. — A column (1.7 × 135 cm) of Bio Gel P-2 at room temperature was used with elution by 0.05M NaCl at 5 mL/h. Oxidised and reduced glutathione, recrystallised Lys, and vitamin B12 were used as standards. Lys was determined by the trinitrobenzenesulfonic acid reaction[21].

Electrophoretic titration. — pH-Mobility curves were obtained[22] by using an

Ampholine mixture in the pH range 4–9.5. The titration curve of Lys was determined by staining a dried gel layer[23] with ninhydrin.

Detection of pyrrole groups. — Pyrroles were determined either by the *p*-dimethylaminobenzaldehyde reaction[24] or by the sulfanilic acid–$NaNO_2$ stain[24], using 3 scalar solutions of histidine as standards (limits of linearity 0.1–0.5mM). For the Pauly reaction[24], 200 μL of sample was mixed with 100 μL of 29mM $NaNO_2$ and 1 mL of 32mM sulfanilic acid in 0.165M HCl. After incubation at 37° for 1 h, 100 μL of 5M NaOH was added and the mixture was stored for 45 min at 37° before reading the absorbance at 492 nm. Blanks contained 20mM glyceraldehyde or 40mM Lys.

Spectra. — U.v.-visible spectra were recorded with a Beckman DU-8 spectrophotometer at 33°. Control Lys and α-Boc- and ε-Boc-modified Lys were initially incubated with sugars in H_2O and afterwards placed in buffers of various pH values (2–10). Fluorimetric spectra were recorded with a Perkin–Elmer MP44A instrument in standard 10-mm quartz cuvettes, and were uncorrected.

RESULTS AND DISCUSSION

The reactivity of amino acids with glyceraldehyde at 37° in phosphate buffer (pH 8) is shown qualitatively in Table I. Lys was the most reactive amino acid, producing quasi-yellow pigments that had a strong absorbance at 340 nm. Of the aldoses tested (Table I), glyceraldehyde was the most reactive. Two systems were investigated further, namely, Lys–glyceraldehyde, which involved the most reactive components, and Lys–2-amino-2-deoxyglucose (GlcN) since the sugar component is normally present in physiological fluids at fairly high concentrations.

Fig. 1 shows the fluorescence emission spectra of the Lys–glyceraldehyde and

TABLE I

REACTION OF AMINO ACIDS WITH ALDEHYDES

Amino acid[a]	*Absorbance (340 nm)*	*Reagents*	*Absorbance (340 nm)*
Ala	++	Lysine + D-glucose	−
Arg	+−	Lysine + 2-amino-2-deoxy-D-glucose	+++
Asp	−	Lysine + D-ribose	+−
Cys	−	Lysine + D-erythrose	+++
Gly	+++	Lysine + DL-glyceraldehyde 3-phosphate	+
Gln	+++	Lysine + DL-glyceraldehyde	++++
Glu	−	Lysine + acetaldehyde	−
His	++		
Lys	++++		
Phe	−		
Ser	−		
Tyr	−		
Trp	++		

[a]Reaction with glyceraldehyde.

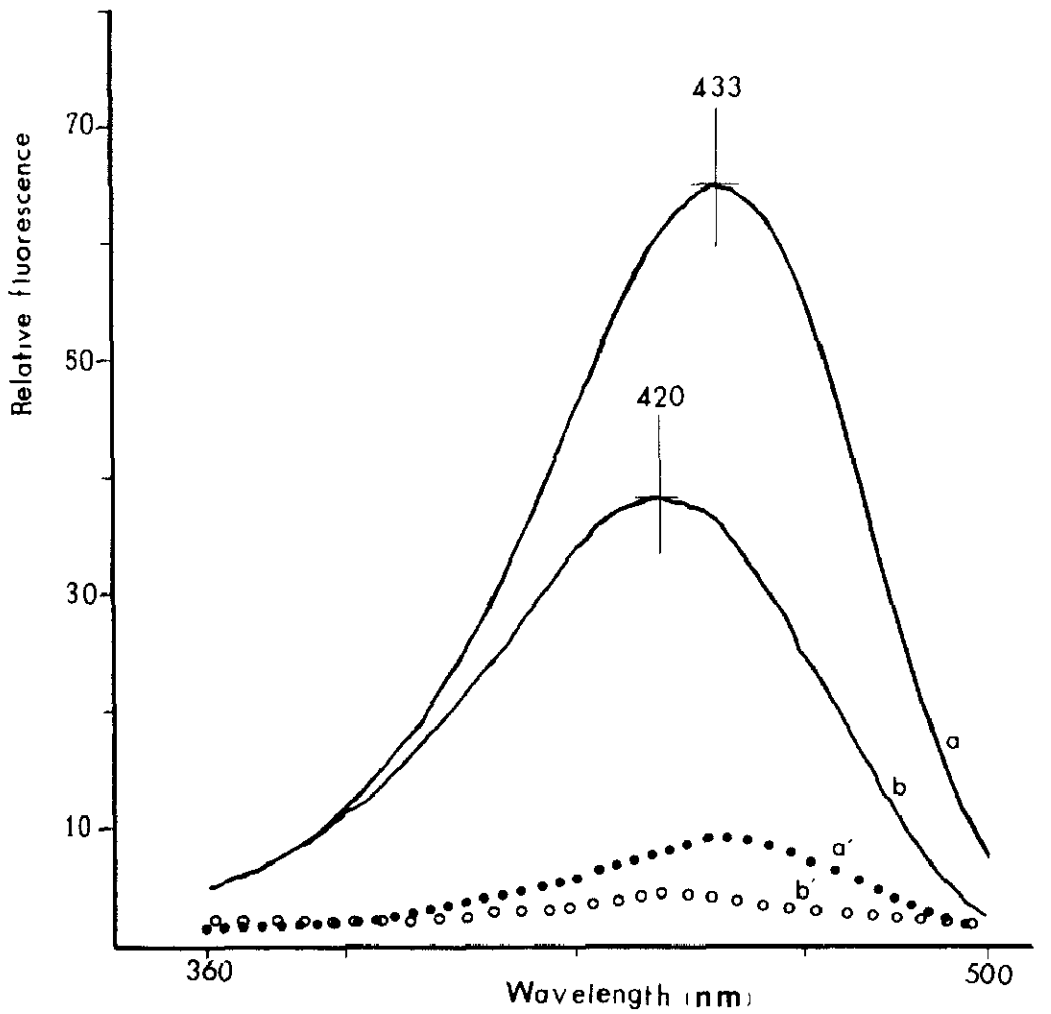
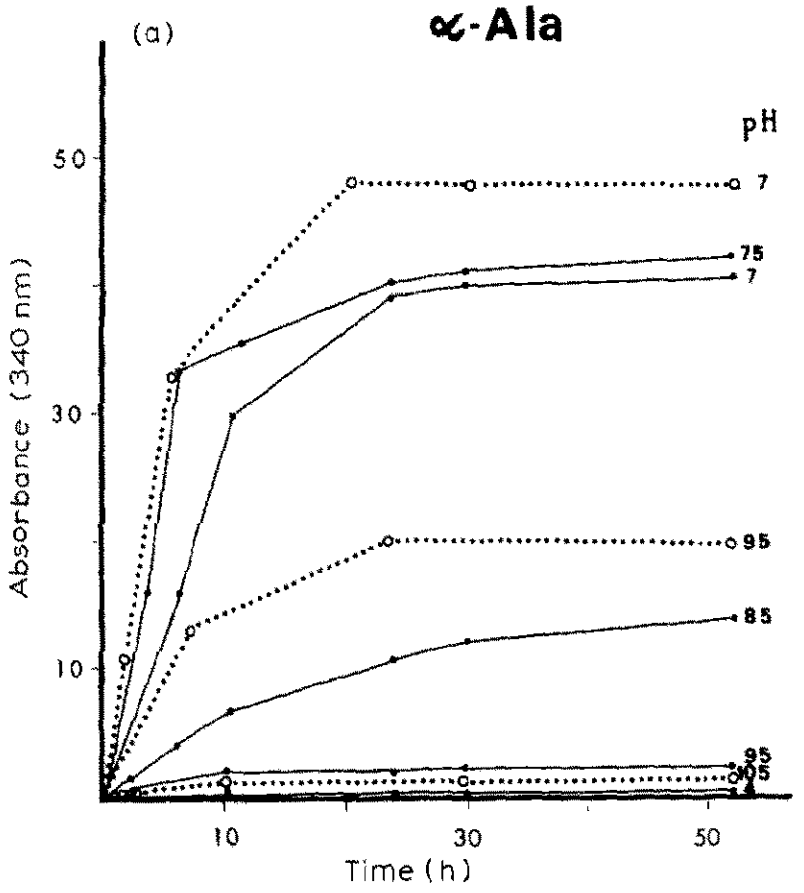


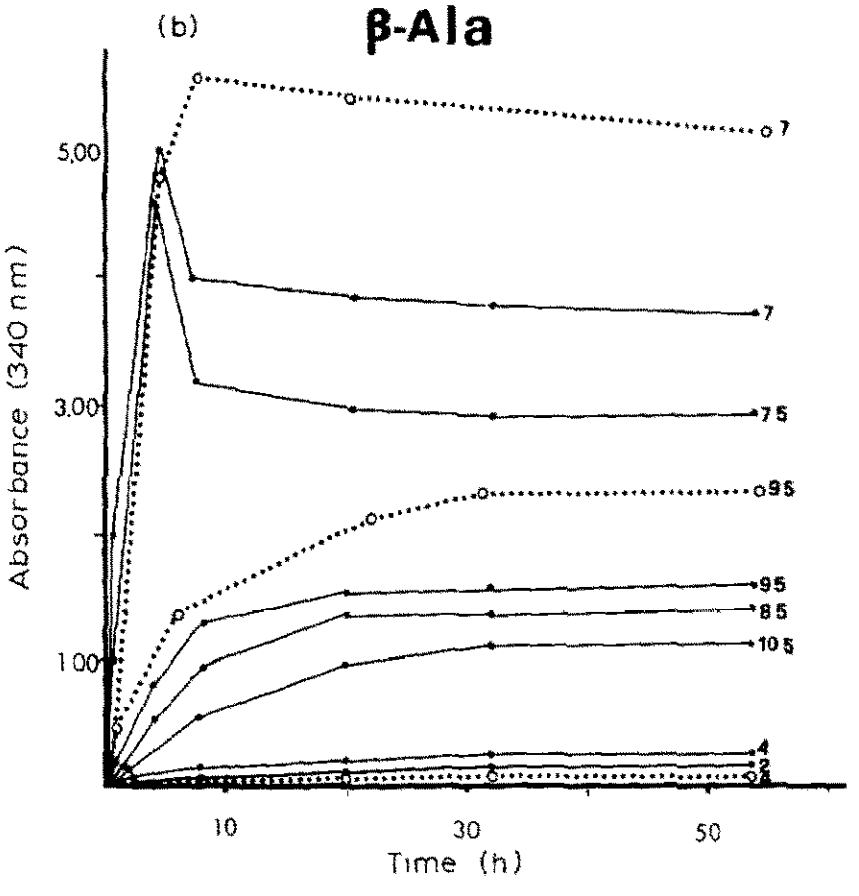
Fig 1. Fluorimetric emission spectra of lysine–glyceraldehyde and lysine–GlcN with (a',b') and without (a,b) sodium cyanoborohydride Incubation (see Experimental) for 48 h at 37° and pH 7 Excitation wavelength, 340 nm.

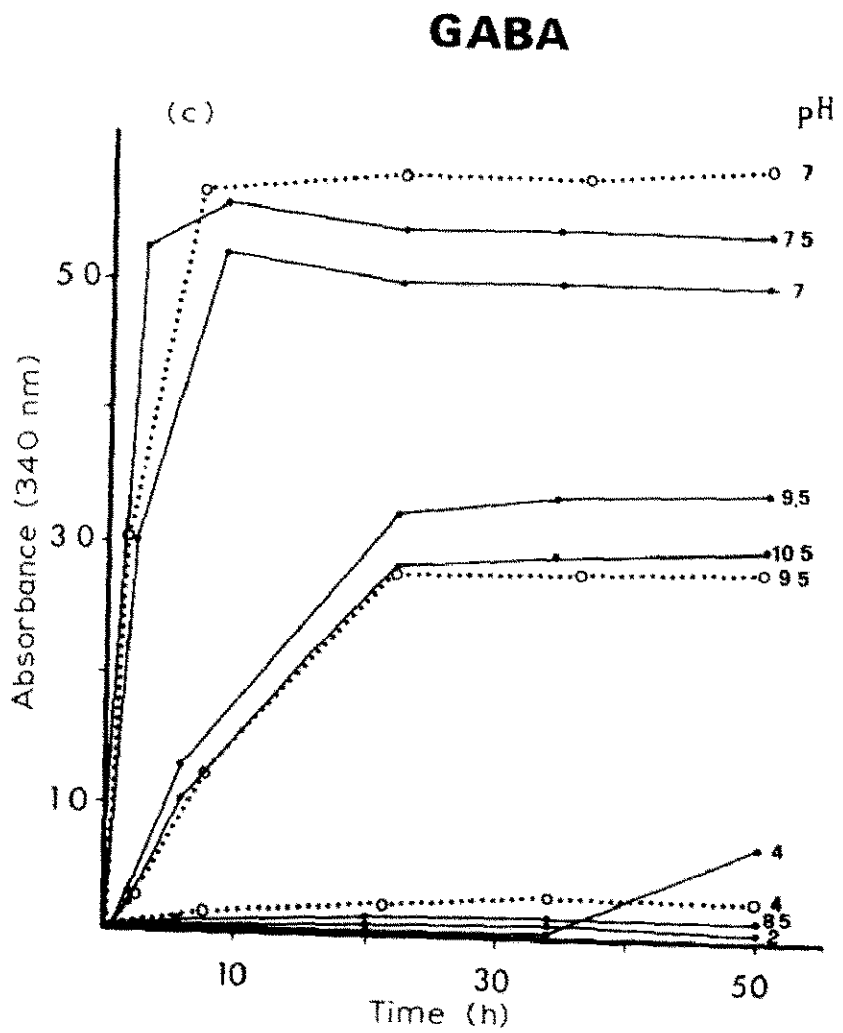
Lys–GlcN adducts on excitation at 340 nm (the wavelength of the absorbance peaks of the reacted species, see Table I), which had peaks at 433 and 420 nm, respectively. In the presence of cyanoborohydride, both peaks were almost completely quenched (Fig. 1).

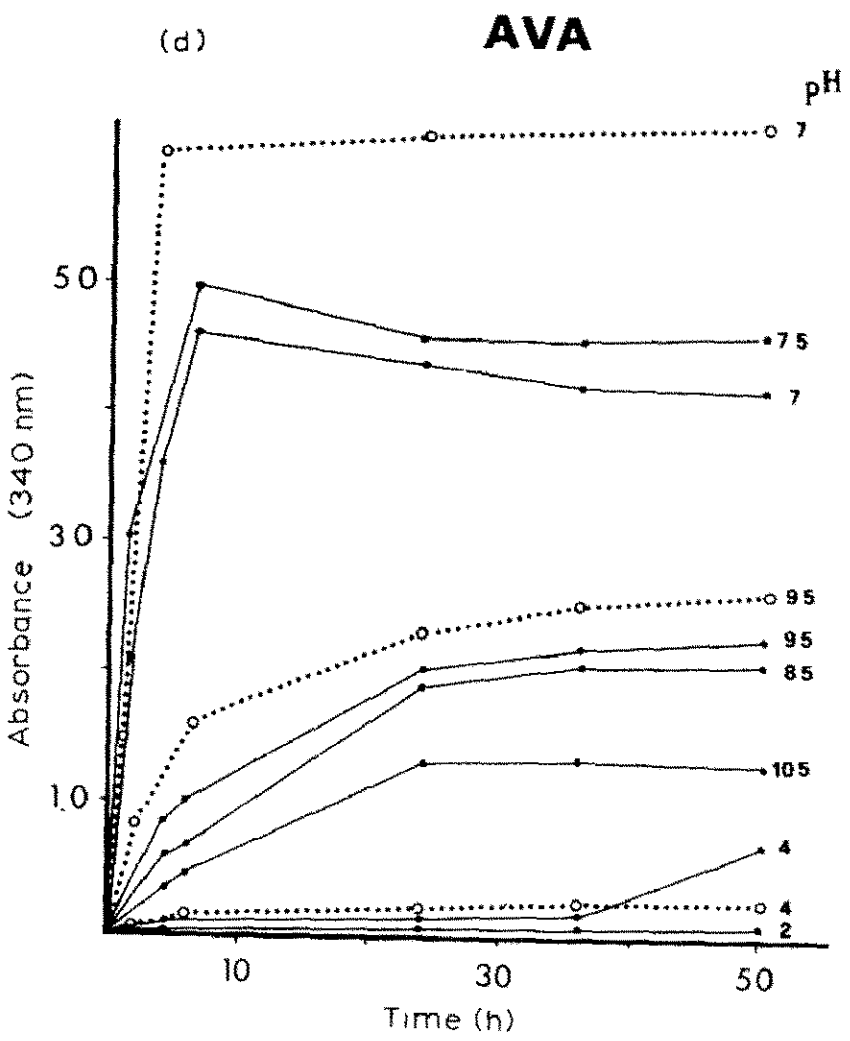
The reaction of amino acids with aldehyde groups essentially involves the amino group, and the following parameters have been investigated: (*a*) distance between amino and carboxyl group, (*b*) pH of the solution, and (*c*) esterification of the carboxyl group. The results shown in Fig. 2 indicate that (*a*) adduct formation is practically nil at pH 2–4; (*b*) maximum reactivity is found at pH 7–7.5; (*c*) reactivity decreases as the pH is increased; (*d*) at neutral pH, esterified amino acids are the more reactive, but the divergence in reactivity becomes progressively smaller as the NH_2–COOH distance increases (see Fig. 2e,f); (*e*) the same phenomenon as in (*d*) occurred at alkaline pH for α-Ala and β-Ala, but there was a reversal of this trend for longer amino acids (GABA, 6-ACA, Lys); (*f*) the rate of reaction increased at neutral pH as the NH_2–COOH distance increased (*e.g.*, 30 h for the α-Ala adduct and a few hours for the Lys-adduct). The non-reactivity at pH 2–4 reflects protonation of the NH_2 group, and the apparently much reduced reactivity at pH 9–10 reflects hydrolysis of the adduct.

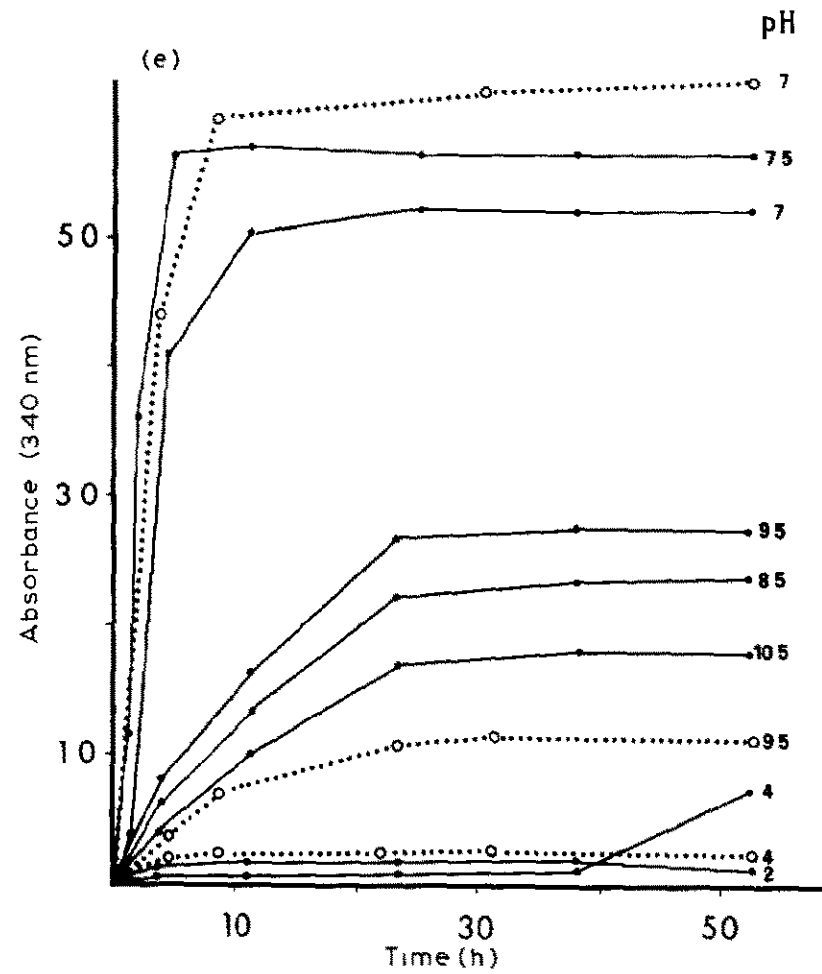
Fig. 3 shows the reactivity of α-Boc-Lys (α-*tert*-butoxycarbonyl-lysine) with


(a)
α-Ala
pH
50
30
10
Absorbance (340 nm)
7
75
7
95
85
95
95
10
30
50
Time (h)


(b)
β-Ala
5,00
3.00
1.00
Absorbance (340 nm)
7
7
75
95
95
85
105
4
2
10
30
50
Time (h)


(c)
GABA
pH
7
7 5
7
9,5
10 5
9 5
4
4
8 5
2
Absorbance (340 nm)
5 0
3 0
1 0
10
30
50
Time (h)


(d)
AVA
pH
7
7 5
7
9 5
9 5
8 5
10 5
4
4
2
Absorbance (340 nm)
5 0
3 0
1 0
10
30
50
Time (h)



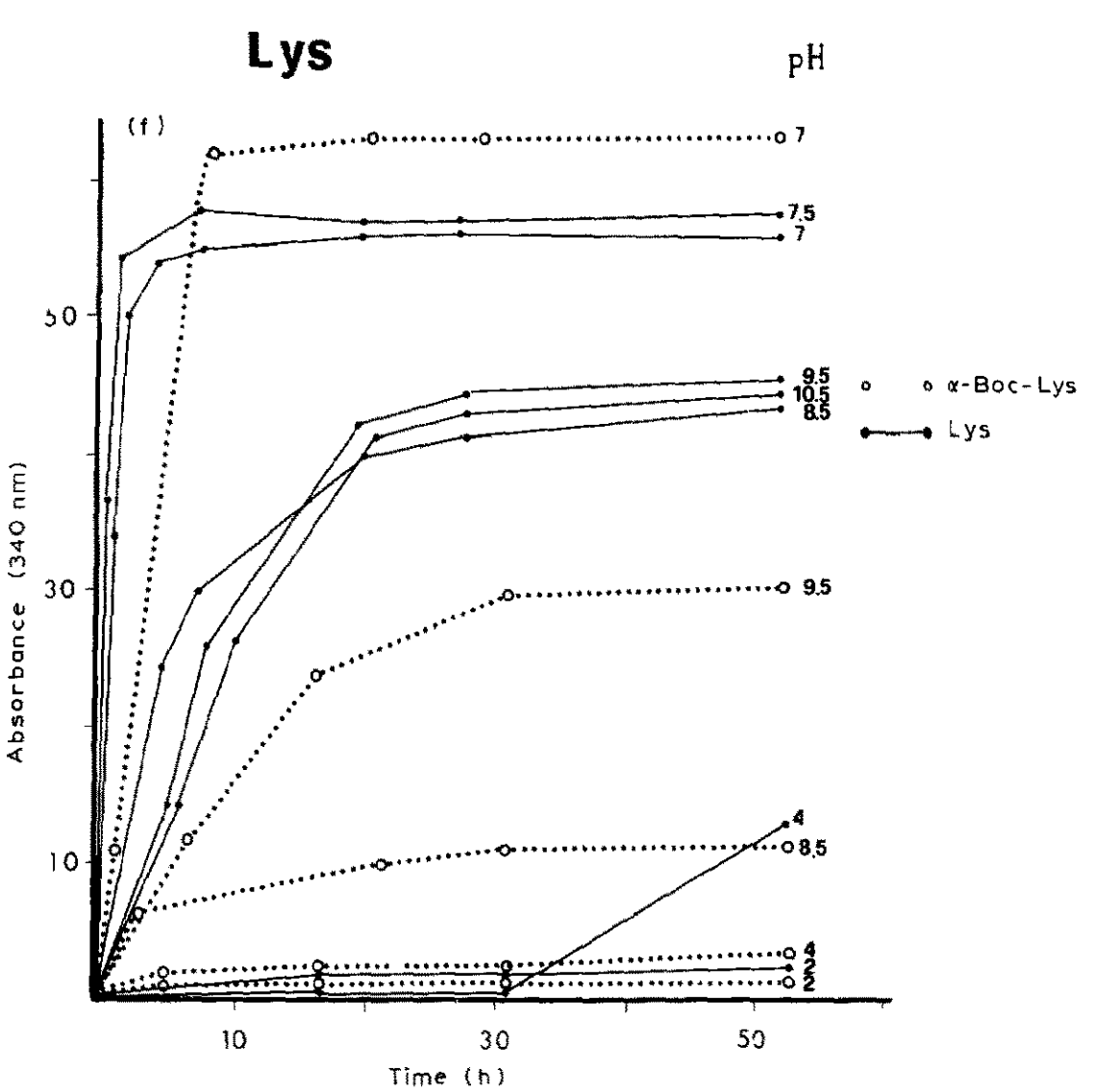


Fig. 2. Effect of pH on the rate of reaction of amino acids with glyceraldehyde (see Experimental): (a) α-alanine, (b) β-alanine, (c) γ-aminobutyric acid (GABA), (d) 5-aminovaleric acid (AVA), (e) 6-aminocaproic acid (ACA), (f) lysine. ——, Amino acids; ·····, esterified amino acids; for Lys, ····· indicates α-Boc-Lys.

2-amino-2-deoxyglucose. Only the ε-amino group is unsubstituted in α-Boc-Lys. The overall pattern is similar to that in Fig. 2 except that the divergence in reactivity at pH 7 between Lys and α-Boc-Lys is much greater and the rates are much lower. The reactivity as a function of the NH_2–COOH distance (Fig. 4) increases almost linearly and with a shallow slope up to four carbon atoms, and then with a steep slope.

The electronic spectra of the Lys–glyceraldehyde and Lys–GlcN adducts are shown in Fig. 5. For Lys–glyceraldehyde at pH 9, a complex spectrum was obtained with peaks at 257 and 296 nm and a shoulder at 332 nm. A similar spectrum was obtained for α-Boc-Lys but with a considerably increased peak at 296, suggesting that the longer wavelength chromophores are associated with reaction occurring on the ε-amino group. At pH 2, the 257-nm peak was almost abolished, but at pH 5 the situation was reversed. For the products from 2-amino-2-deoxyglucose, the spectra were quite different from those for the glyceraldehyde adducts. For Lys, a single peak at 270 nm was not substantially modified in the pH range 2–9, but a shoulder appeared at 340 nm at pH 9. An essentially identical spectrum was given by the α-Boc-Lys adduct at pH 9, but at pH 5 and 2 the λ_{max} exibited a marked ipsochromic shift and splitting (λ_{max} 245, 250, 255, and 260 nm).

When the products of the Lys–glyceraldehyde reaction were analysed on Bio-

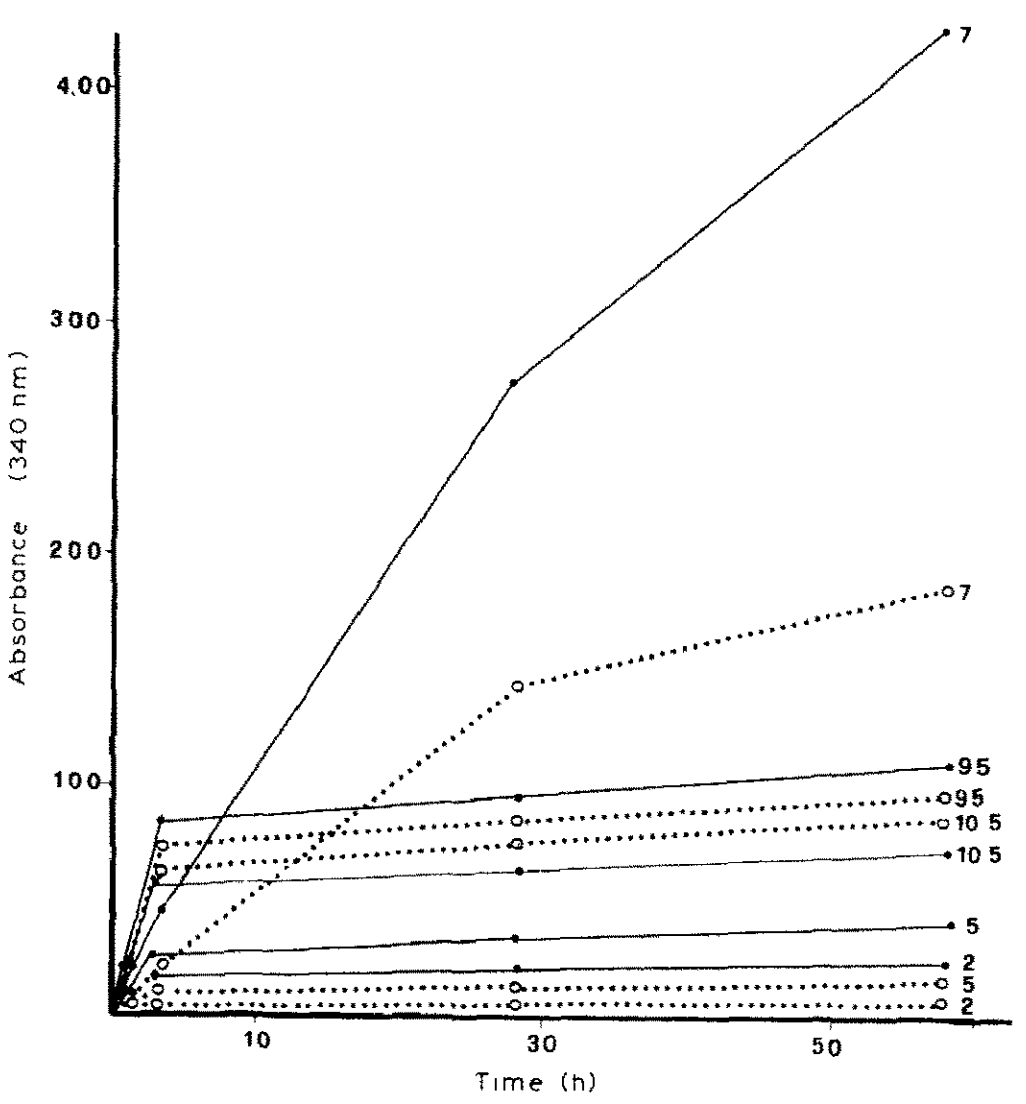


Fig. 3. Reaction of Lys (——) and α-Boc-Lys (·····) with 2-amino-2-deoxyglucose (see Experimental).

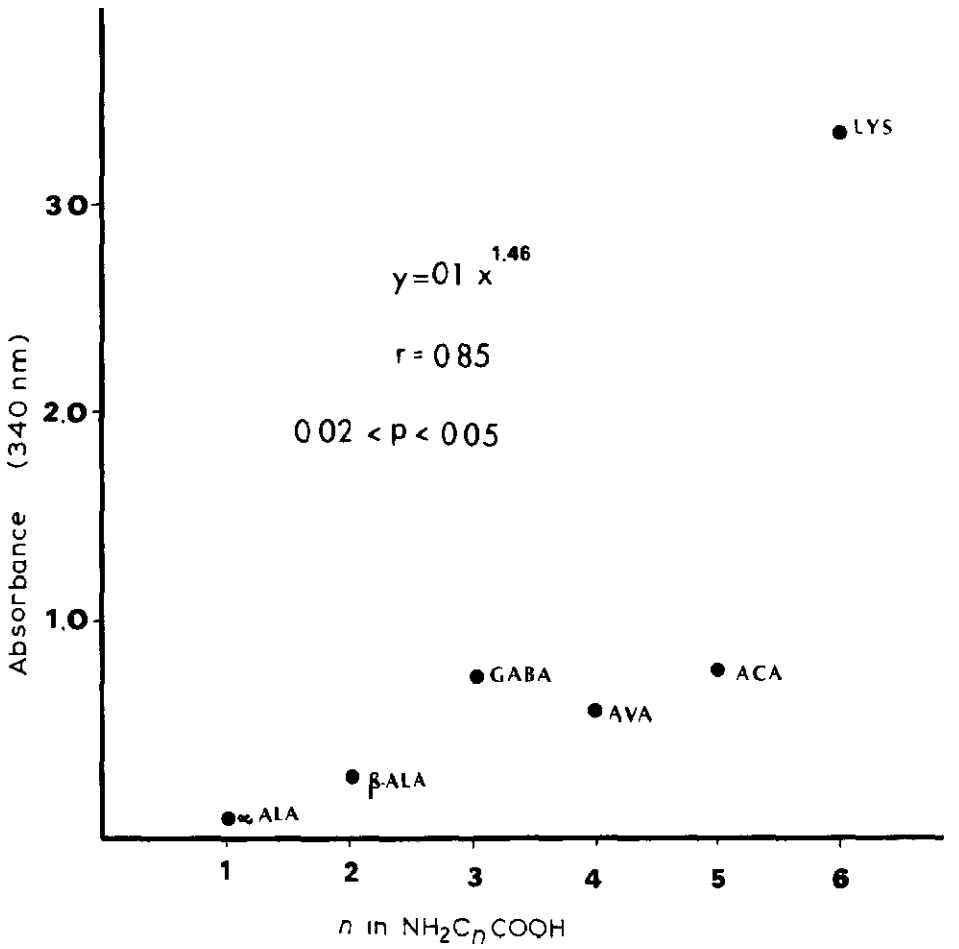
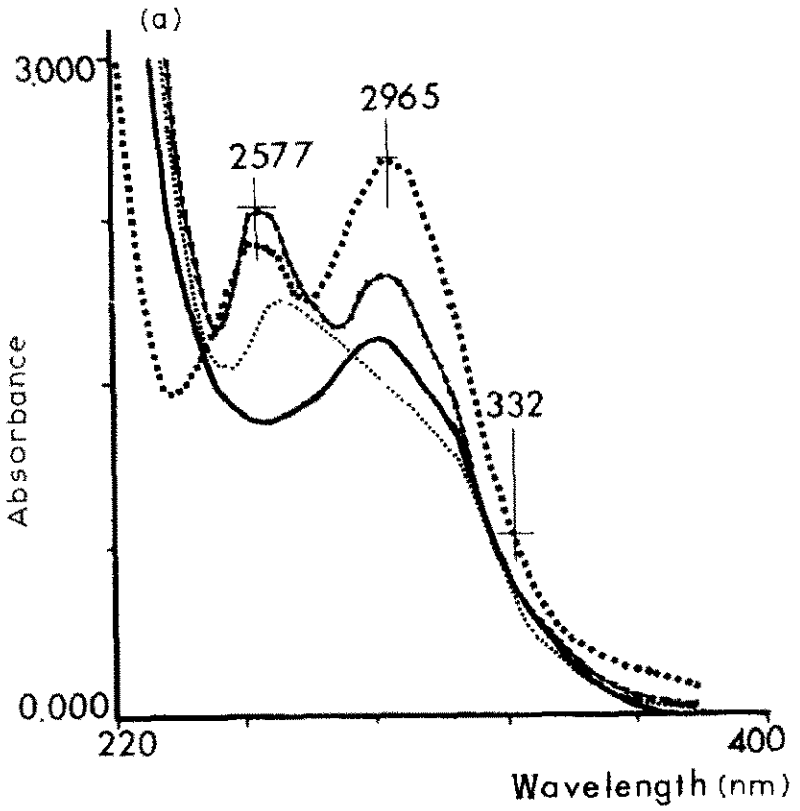


Fig. 4. Rate of "browning" of amino acids with glyceraldehyde at pH 7.5 as a function of the number of carbon atoms between the NH_2 and COOH groups. Incubation (see Experimental) for 30 min.

Gel P-2, at least three peaks were obtained with apparent mol. wts. of >1000, 670, and 565 (Fig. 6). These results indicated polycondensation products, since a 1:1 Lys–glyceraldehyde complex would have a mol. wt. of 200. The Lys–GlcN complex would have a mol. wt. of 240.

Further evidence for the formation of oligomeric products was obtained by titration of the charged groups. Two approaches were used. A pH–mobility curve was obtained by forcing Lys and reacted Lys to migrate perpendicularly to a quasi-stationary pH gradient formed by a stack of focused carrier ampholytes[22] (Fig. 7); Lys behaved as an amphoteric compound (pI 9.3), whereas the adduct exhibited at least two titration curves, with a behaviour typical of acidic ampholytes (pI values in the range pH 4–5). The carboxyl groups of these oligomers seem to have similar pK values and the titration curves merged into a single line below pH 5. In a second approach, the adducts were subjected to equilibrium isoelectric focusing (i.e.f.) over a narrow range of pH (4–7). Fig. 8 shows that the adducts were resolved into at least 8–9 isoelectric species, with pI values in the pH range 4–6. The strongly acidic character of these molecules is compatible with the presence of 4–7 carboxyl groups counterbalanced by 2–3 protonated amino groups and suggests that not only the ε- but also a substantial portion of the α-nitrogens of Lys cannot be protonated even at pH 4–5 and that a minimum of 4–7 Lys must have been condensed into a single molecule. Alternatively, if it is assumed that only the ε- and not the α-amino groups react with the aldehyde, additional acidic groups must have been generated



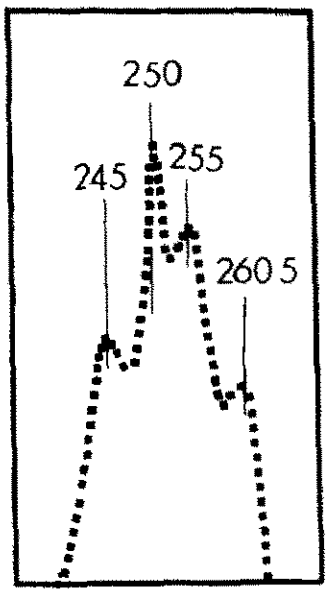


Fig. 5. U.v. spectra of (a) Lys–glyceraldehyde and α-Boc-Lys–glyceraldehyde adducts at pH 9 (◀◀◀◀◀), 5 (●●●●●), 2 (———), and (for α-Boc-Lys) 9.5 (■■■■); (b) Lys-GlcN at pH 9 (●—●—●—●—●), 5 (———), and 2.7 (▶▶▶▶), and α-Boc-Lys at pH 9 (○—○—○—○—○—○), 5 (▷▷▷▷▷), and 2.7 (■■■■). After incubation in H_2O, 100 mL of solution was added to 5 mL of the appropriate buffer (see Experimental).

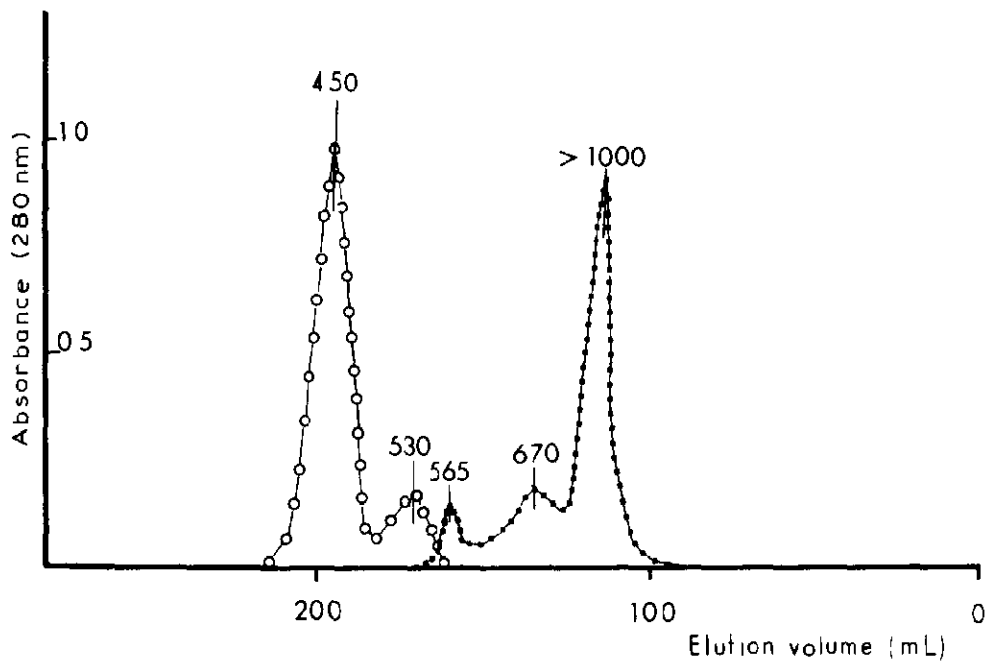


Fig. 6. Elution of the Lys–glyceraldehyde (—■—) and Lys–GlcN (—○—) adducts from a column (1.7 × 135 cm) of Bio-Gel with 50mM NaCl at 5 mL/h; standards: glutathione (mol. wt. 612), Lys (mol. wt. 146.2), and vitamin B12 (mol. wt. 1355).

in order to have species focusing in the pH 4–5 region. The bands are visible due to their intense yellow-brownish colour and fluoresced strongly when the gel was excited with u.v. light (not shown). Since, in this pH range, an oligo-anion could produce artefacts by binding to the oligo-amino backbone of the carrier ampholytes (the minimum for cooperative binding being six negative charges[25,26]), 8M urea was used to disrupt any such complexes. Since the pattern of bands was maintained in 8M urea, the species observed must be different oligomeric condensation products of Lys and glyceraldehyde.

Audisio *et al.*[27] suggested that the reaction of Lys with methylglyoxal yields a yellow polymer containing pyrrole nuclei in the backbone. We assayed our reaction products with the Ehrlich and Pauly reactions[24] and, whereas no colour was developed with *p*-dimethylaminobenzaldehyde–sulfuric acid, a strong reaction was developed with sulfanilic acid–$NaNO_2$. As shown in Table II, extensive reaction between Lys and glyceraldehyde had occurred after 1 h and with a large excess of glyceraldehyde (400mM) practically all of the Lys was incorporated into the polymer. The excess of reactants did not interfere in the Pauly reaction with the Lys–glyceraldehyde adduct, but GlcN, which could not be removed easily from the reaction products by dialysis or gel filtration, did interfere when the reaction was applied to the Lys–GlcN adducts.

The reaction of aldehydes with free amino acids and proteins has been extensively studied, mostly in food chemistry and technology, in order to reproduce and understand the "browning" reaction. Unfortunately, most of the published data cannot be applied readily to biological systems, since the reactions were performed under relatively harsh conditions (60–100°, pH 10). The "non-enzymic browning products" thus obtained (reductones, furfurals, osones, melanoidins, unidentified fluorescent compounds) are not formed in body fluids under physio-

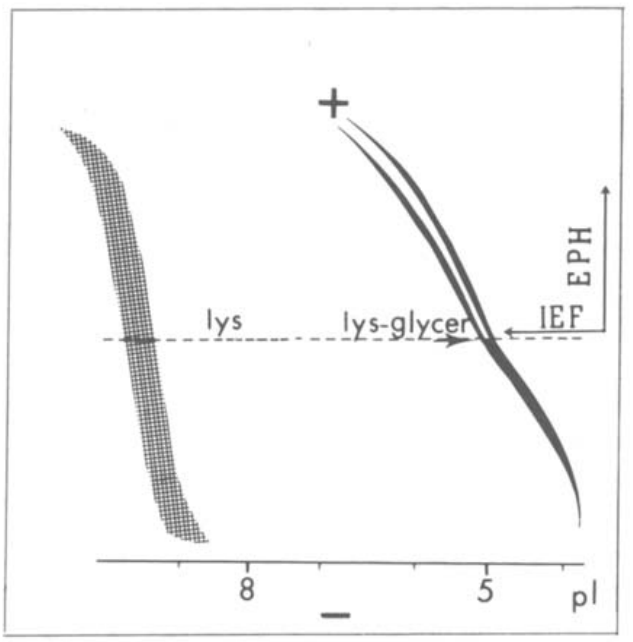


Fig. 7 Electrophoretic titration curves of Lys and the Lys–glyceraldehyde adduct; ---- represents the zero-mobility plane (application trench) and its intersection with the titration curve represents the pI value of the species The adducts were detected by their yellow colour, and Lys, on the dried gel, by the Sakaguchi reaction.

logical conditions. The present data, obtained for reactions at 37° and pH 7.5, show that (*a*) Lys is much more reactive than any other amino acid; (*b*) glyceraldehyde is more reactive than tetroses, pentoses, and ketoses; (*c*) 2-amino-2-deoxyglucose is significantly reactive (Table I). The high reactivity of Lys has long been recognised in food chemistry[16,17], and in the advanced stages of the "browning" reaction the digestibility of the protein is decreased. In the presence of an excess of glyceraldehyde, practically all Lys molecules are converted into a polymeric, yellow product. Fortunately, *in vivo*, glyceraldehyde is present in the cell cytosol, mainly as the 3-phosphate which has a much reduced reactivity (Table I), and its

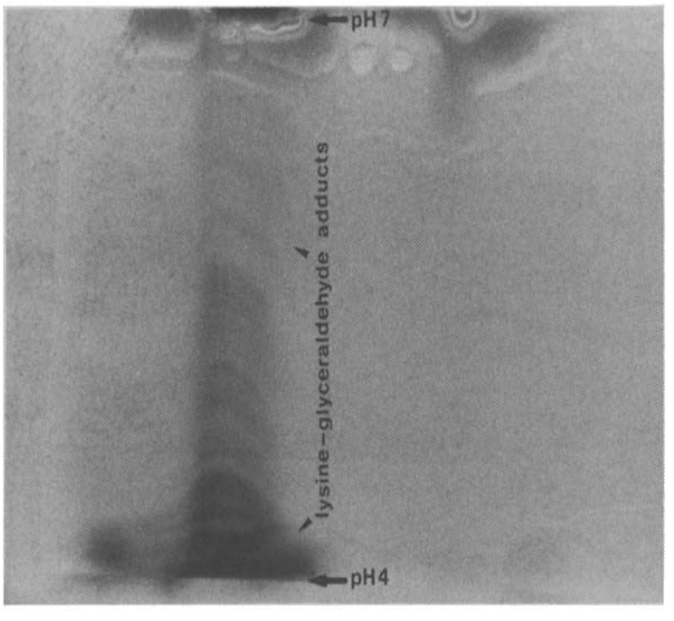


Fig. 8. Equilibrium isoelectric focusing of the Lys–glyceraldehyde adducts. The 0.5-mm thick gel contained 6% of total acrylamide monomer, 2% of carrier ampholytes (pH 4–7), and 8M urea, and was cast on a gel-Bond PAG foil. Focusing was terminated after 4000 Vh at 10° and 10 W Anolyte, 100mM acetic acid; catholyte, 100mM NaOH The yellow zones were photographed as such

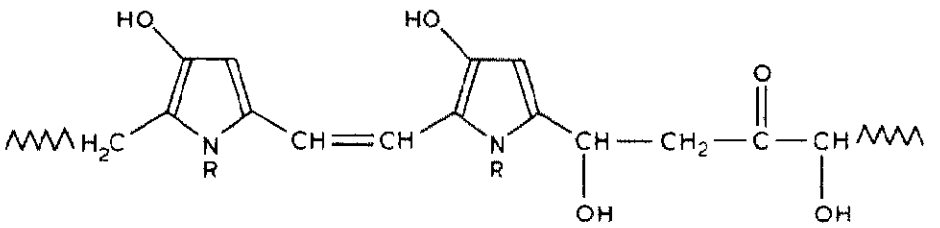
TABLE II

FORMATION OF PYRROLE GROUPS (mM) IN THE REACTION OF 40mM LYSINE AND GLYCERALDEHYDE

Glyceraldehyde concentration (mM)	*Time (h)*		
	1	*96*	*158*
20	2.7	3.1	2.2
100	14.3	11.5	10.3
200	18.7	23 3	17.8
400	20.6	45 2	41.5

equilibrium concentration is small, because it is rapidly converted into either the inert dihydroxyacetone phosphate or into 2,3-diphosphoglycerate. 2-Amino-2-deoxyglucose, however, is present at a fairly high concentration in the blood stream, and its reaction with Lys residues could be important in diabetic patients in the formation and secretion of heavily modified albumins in urine[1–4].

The Lys–glyceraldehyde and Lys–GlcN adducts were heterogeneous in size, with mol. wts. in the range 500–1000, and were heterodisperse in charge, with apparent pI values in the range pH 4–6. The Lys–glyceraldehyde adduct could be similar to the Lys–methylglyoxal adduct described by Audisio *et al.*[27]. According to these authors, there should be no difference between the two aldehydes since "Lys, normally present in all animal tissues, catalyses the transformation of glyceraldehyde and glyceraldehyde 3-phosphate into methylglyoxal". On the basis of n.m.r. data, these authors suggested that the ε-amino group of Lys is incorporated into the pyrrole structure **1**, with the yellowish colour reflecting the extended conjugation.



1 R = $(CH_2)_4-CH(^+NH_3)-COO^-$

The data reported here show partial similarities with those of Audisio *et al.*[27]: the molecular weights agree (~1000), but the data on surface charge point to further, previously undetected, reactions. According to Audisio *et al.*[27], only the ε-amino group of Lys reacts, so that the R group in **1** contains a carboxyl pK 3.0) and an α-amino group (pK 10.2) (see Table II in ref. 27). If this is correct, **1** should have a pI value of 6.6. Moreover, as the pK is 7.2 and [pI − pK(prox)] is 3.5, then, according to Rilbe's law[28], it should be classified as a "poor carrier ampholyte" over a broad range of pH and thus be unable to focus at its theoretical pI. Our findings

that these yellow oligomers focus sharply at pH 4–6 suggest that, if the α-amino group remains intact, additional acidic groups must be generated. These groups could arise from the rearrangement of the Amadori compound, the first product of the addition of the aldehydic to the amino group. If the primary adduct is left in solution, intense browning occurs which can be prevented by the addition of cyanoborohydride. With modified albumins, this inhibition of colour development also inhibits the production of more acidic species[4].

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