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Note

Characterisation of the phenylhydrazone derivatives of "glycated albumin" purified from diabetic sera

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Many proteins, circulating and structural, undergo non-enzymic glycosylation that has been implicated in the pathogenesis of the long-term complications[1] of diabetes mellitus. Sugars are initially bound to proteins through a Schiff's base (aldimine) linkage, which is followed by an Amadori rearrangement[2,3] (ketoamine form) and by a cascade of other rearrangements (Maillard reaction), the end products (browning compounds) of which are unstable and toxic.

There is evidence that browning compounds occur *in vivo* for such long-lived proteins as collagen[4], but their occurrence is only supposed for such short-lived proteins as albumin on the basis of *in vitro* experiments involving the exposure of the protein either to non-physiological concentrations of D-glucose[5] (up to M) or other sugars[6]. Furthermore, it is still a matter of debate whether the sugars attached to these proteins are in the ketoamine form. Indeed, based on the observation that glycated hemoglobin does not react with phenylhydrazine, Fisher and Winterhalter[7] hypothesised that, for glycated hemoglobin, the carbohydrates are not present in the ketoamine form. We applied this reaction in order to search for keto groups in glycated albumin.

Protein from diabetic sera was purified by a double affinity-chromatography technique[8,9] and the carbohydrates covalently bound to the protein, determined by g.l.c. of the alditol acetates after hydrolysis, were (mol/mol of albumin) hydroxymethylfurfural, 1.48; glucose, 24.0; mannose, 1.7; and galactose, 2.0. Glycated albumin was then treated with phenylhydrazine, producing an adduct with λ_{max} at 350 nm (Fig. 1), the formation of which was abolished by pretreatment with

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borohydride. Since phenylhydrazine, besides having a strong affinity for ketoamine groups, can also induce transaldiminidation or other amine substitution reactions which lead to the formation of phenylhydrazone derivatives of low mol. wt., the products of the reaction of glycated albumin with phenylhydrazine were fractionated on a column of Sephadex. Two peaks were obtained (Fig. 2), the first of which was eluted at the V_0 with a mol. wt. of >30,000 and corresponded to a phenylhydrazone derivative of albumin (~15% ketoamine form); the second was eluted at V_t and corresponded to products of low mol. wt. (<3,000). Isoelectric focusing in polyacrylamide gel revealed the product of high mol. wt. to be homogeneous with a pI 4.7 (pI of unreacted albumin), suggesting that the phenylhydrazine had reacted with the carbohydrate bound to the albumin without

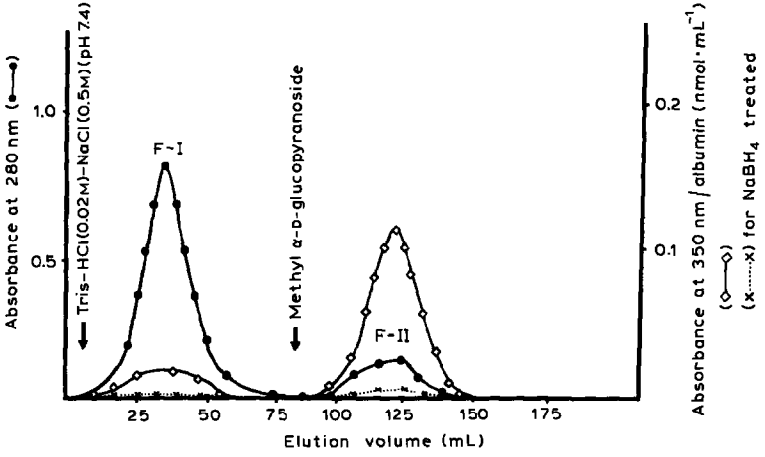

Fig. 1. Fractionation of the products of reaction of albumin with phenylhydrazine on Concanavalin A–Sepharose: F-I had a low and F-II a high content of carbohydrate; F-II is glycated albumin.

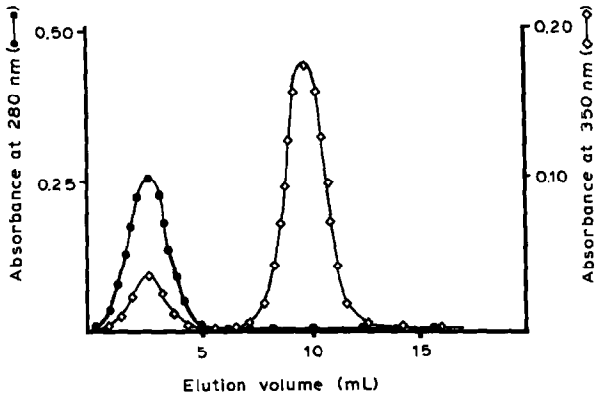

Fig. 2. Fractionation of F-II (Fig. 1) on Sephadex G-50. Albumin was eluted at V_0. The absorbance at 350 nm indicates that only a small amount of phenylhydrazine was linked *via* the ketoamine form of the rearranged carbohydrate.

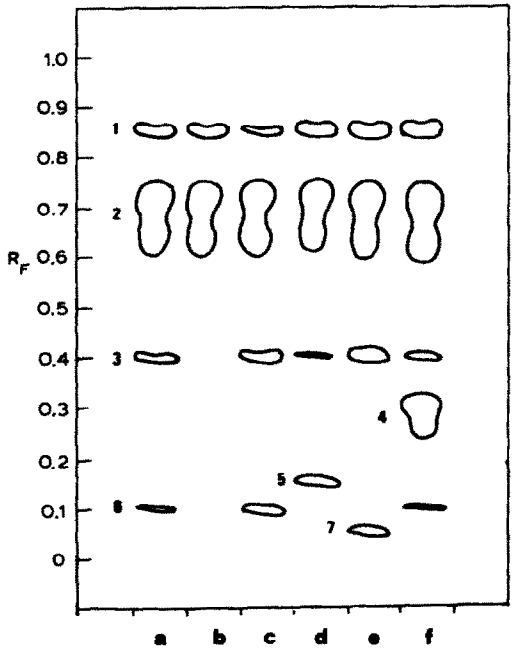

Fig. 3. T.l.c. on silice gel of (**a**) products of low mol. wt. of the reaction between glycated albumin and phenylhydrazine, (**b**) phenylhydrazine, (**c**) Schiff's base of D-glucose linked to lysine in the presence of $NaCNBH_3$, (**d**) Schiff's base of D-mannose and lysine, (**e**) Schiff's base of D-galactose and lysine, (**f**) browning compounds of D-glucose and lysine. The formation of yellow pigments of browning was monitored at 340 nm. See text for the identification of 1–7.

affecting the surface charge of the protein nor its conformation. T.l.c. of the products of low mol. wt. is shown in track **a** of Fig. 3. Spots 1 and 2 correspond to phenylhydrazine (*cf.* track **b**), spot 3 corresponds to the Schiff's base of glucose (*cf.* track **c**), and spot 6 corresponds to unreacted glucose. Furthermore, no unreacted mannose and galactose (that correspond to spots 5 and 7 of tracks **d,e**) were detected, nor any products of the advanced rearrangements (spot 4, track **f**). Thus, unlike glycated hemoglobin, ketoamine- and aldimine-linked carbohydrates are detectable by the reaction of phenylhydrazine with serum glycated albumin, but the amounts of these forms are low in comparison to the overall glycation of the protein.

EXPERIMENTAL

Albumin was purified from the sera of 7 diabetic patients using Affi-Gel Blue[8], and the more glycated fraction was absorbed on Concanavalin A–Sepharose[9]. The determination of monosaccharides (as their aldital acetates) covalently bound to the protein was carried out after treatment for 3 h in

methanolic M hydrogen chloride at 85°, by g.l.c. of the alditol acetates[10]. For reaction with phenylhydrazine, the lyophilised protein was dissolved in 10mM phosphate buffer (pH 7.25, 0.3 mL) containing 3M guanidine · HCl[11]. In some experiments, reduction was achieved with 200 mol of $NaCNBH_3$. The separation of the products of high and low mol. wt. was carried out using a column (0.7 × 20 cm) of Sephadex G-50 equilibrated with 10mM phosphate buffer. Isoelectric focusing in polyacrylamide gel was performed according to Bianchi-Bosisio *et al.*[12], with silver staining using the photochemical method of Merril *et al.*[13]. T.l.c. of the products of low mol. wt. was carried out on silice gel with chloroform–methanol–water (8:5:1).

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