GLYCOSYLATION OF SERUM ALBUMIN: ELEVATED GLYCOSYL-ALBUMIN IN DIABETIC PATIENTS

R. DOLHOFER and O. H. WIELAND

Klinisch-chemisches Institut and Forschergruppe Diabetes, Städtisches Krankenhaus München-Schwabing, Kölner Platz 1, D-8000 München 40, FRG

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1. Introduction

Non-enzymatic sugar incorporation into proteins during technical food processing, the so-called Maillard reaction has been known for a long time [1,2]. However, glucosylation of proteins occurs also under physiological conditions and might have an importance which is not yet fully recognized. Recently we could show that glucose is incorporated into insulin, in vitro, and that glucosylated insulin has biological activity different from that of non-glucosylated insulin [3]. Another example is the glycosylation of the lens crystalin proteins, which might be of significance in cataract formation [4]. Earlier work on the existence of glycosylated forms of hemoglobin ($HbA_{Ia\text{-}c}$) in normal red blood cells and the elevation of these fractions in diabetes has gained much attention, particularly with respect to the diagnostic use of this reaction for long term control of carbohydrate metabolism (reviewed in [5]). This led us to study whether the most abundant serum protein, albumin, is also subject to glucosylation, in vitro and in vivo and found that similar to hemoglobin, glucosylated albumin is a natural constituent of human blood whose concentration appears to be markedly elevated in diabetes mellitus. Our results confirm and extend a report on non-enzymatically glucosylated albumin [6], which appeared after this study was completed. Incidentally there is also a report in the older literature on the glucosylation of horse serum albumin [7].

Abbreviations: Natriumtimerfonat®, *p*-aethyl-mercuri-mercaptobenzolsulfonsaures Natrium

2. Materials and methods

2.1. *Materials*

Human serum albumin and horse anti-human-serum was from Behringwerke, Marburg. D-[U-^{14}C]glucose was from the Radiochemical Centre, Braunschweig. Enzymes and coenzymes were products of Boehringer, Mannheim. Affi-Gel Blue was from Bio-Rad, Munich. Whatman DE52 cellulose was obtained from Hormuth and Vetter, Wiesloch. NCS Tissue Solubilizer® was from Nuclear Chicago. Natriumtimerfonat® was from Asid, Munich. D(+)-Glucose, thiobarbituric acid and all other chemicals were from Merck, Darmstadt. Diaflo UM-10 ultrafiltration membranes were from Amicon (Witten/Ruhr) and collodium bags from Sartorius (Göttingen).

2.2. *Methods*

Albumin from freshly drawn human serum was purified by DEAE-cellulose and Affi-Gel blue chromatography. Prior to DEAE-cellulose chromatography the serum was mixed with an equal volume of 100% saturated $(NH_4)_2SO_4$. After centrifugation for 10 min at 35 000 × *g* the (0–50%)-pellet was dissolved in 0.9% NaCl and dialyzed against the same solution prior to sugar analysis. The supernatant was dialyzed against distilled water, centrifuged for 15 min at 40 000 × *g* and chromatographed on DEAE-cellulose according to [8]. The fractions containing albumin were pooled, dialyzed against 0.02 M Na^+-phosphate buffer, pH 7.1, and chromatographed on Affi-Gel Blue according to the supplier's instructions. The albumin containing eluate was dialyzed against distilled water and concentrated by ultrafiltration.

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For assay of 5-hydroxymethylfurfural 1 ml protein solution (corresponding to ~15 mg) was mixed with 0.15 ml glacial acetic acid, and kept for 2 h in a boiling water bath [9]. After cooling, 0.4 ml 40% (w/v) trichloroacetic acid (TCA) was added and 1 ml of the supernatant obtained after centrifugation for 15 min at 3000 × *g* was mixed with 0.5 ml 0.05 M thiobarbituric acid and incubated for 30 min at 40°C [10] before extinction at 443 nm was measured. Protein was determined by the biuret method [11].

3. Results and discussion

Purified human albumin under physiological conditions with respect to pH, ion composition and temperature incorporates [^{14}C]glucose in a dose-dependent manner. The rate of glucose uptake was also dependent on reaction time, temperature and pH (fig.1a–d). The radioactivity was recovered in the TCA precipitate and was not removed by extensive dialysis against 11 mM unlabelled glucose, suggesting covalent incorporation. Furthermore, the location of protein and radioactivity was the same on electrophoresis in SDS-polyacrylamide gels (fig.2). When glucosylated albumin was treated with 4 N CH_3COOH at 110°C, the hydrolysate contained glucose, and also mannose at a nearly constant ratio. Both sugars amounted to ~5% of the incorporated glucose (table 1). Moreover, glucosylated albumin (in contrast to the non-glucosylated protein (not shown)), kept in 2 N CH_3COOH for 120 min in a boiling water bath, yielded a supernatant after precipitation with TCA that reacted with thiobarbituric acid as shown in fig.3. Thus 5-hydroxymethylfurfural was in the hydrolysate, indicating ketoamine formation on incubation of albumin with glucose. Apparently glucosylation of albumin occurs by the same mechanism as in HbA_{Ic} synthesis. In this case the sugar forms initially a Schiff base with the N-terminal valine of the β-chain which, by Amadori rearrangement gives the ketoamine

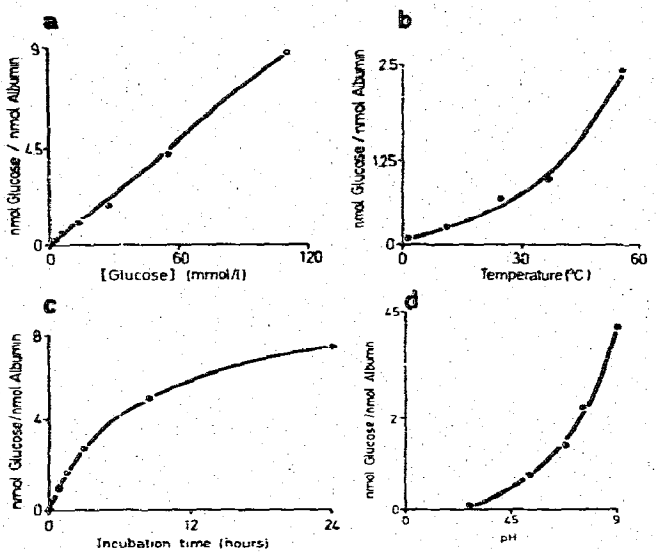

Fig.1.(a–d). Formation of [^{14}C]glucose–albumin as a function of: (a) glucose concentration; (b) temperature; (c) incubation time; (d) pH. If not stated otherwise albumin (1 mg/ml) was incubated at 37°C for 3 h in Earle's medium [14], containing 27.7 mmol/l [^{14}C]glucose and was brought to pH 7.5 with carbogen. After the incubation 0.1 ml 3 M TCA was added to 0.5 ml of the reaction mixture. The precipitate obtained by centrifugation (Eppendorf centrifuge model 5412) was washed 3 times with 1 ml 10% (w/v) TCA and dissolved by adding 1 ml NCS Tissue Solubilizer® and 0.2 ml H_2O. After addition of 0.05 ml glacial acetic acid radioactivity was determined in a Packard liquid scintillation counter.

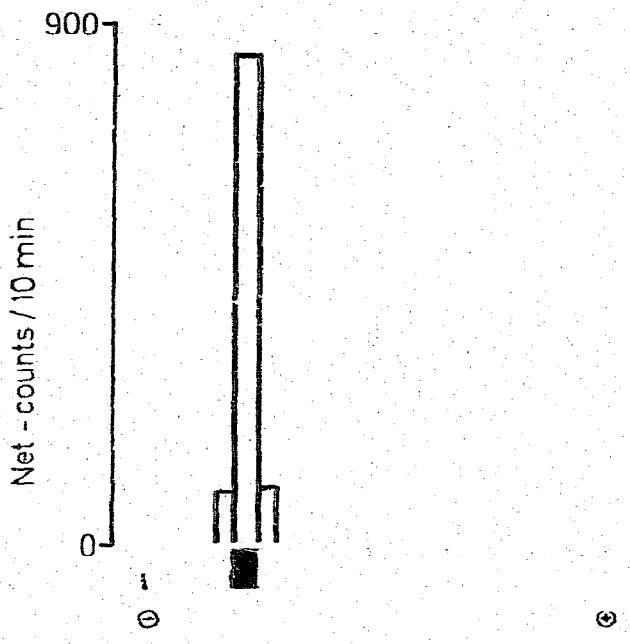

Fig.2. Analysis of [^{14}C]glucose–albumin by SDS–polyacrylamide gel electrophoresis. Glucosylation of albumin was performed as in the legend to fig.1, except that the incubation was carried out for 48 h in the presence of 0.01% Natriumtimerfonat®. The mixture was then extensively dialyzed at 4°C against 0.9% NaCl before it was subjected to SDS–gel electrophoresis according to [15].

Table 1
Analysis of carbohydrates released from [^{14}C]glucose–albumin

Exp.	Glucose incorporated (mol Glucose. mol Albumin^{-1})	Glucose and mannose released after acid hydrolysis (mol Glucose and mannose mol Albumin^{-1})	Release in % of incorporation	Glucose / Mannose
I	10.8	0.56	5.2	2.6
II	20	0.81	4.0	2.5
III	36	1.78	4.9	3.2

Human serum albumin (see section 2.1) (8 mg/ml in exp. I; 6 mg/ml in exp. II; 20 mg/ml in exp. III) in Earle's medium [14] (pH 8.0) containing [^{14}C]glucose (444 mM in exp. I; 888 mM in exp. II, III) was incubated at 37°C in the presence of 0.01% Natriumtimerfonat® for 42 h (exp. I, II), and 70 h (exp. III), respectively. The samples were dialyzed extensively against distilled water, and analyzed for protein-bound radioactivity as in the legend to fig.1. The sugar was released by incubating 221 nmol [^{14}C]glucose–albumin with 4 N acetic acid at 110°C for 4 h, as in [12]. Hydrolysis for up to 24 h did not increase the recovery. Glucose, fructose and mannose were assayed in 50 mM triethanolamine, 0.5 mM $NADP^+$, 2.5 mM ATP, 2.5 mM $MgCl_2$, buffer (pH 7.5) containing glucose-6-phosphate dehydrogenase, by sequential addition of hexokinase, phosphoglucose-isomerase and phosphomannose-isomerase [12]. There was no detectable release of fructose

[12]. Preliminary experiments suggest that in albumin ϵ-amino groups of lysine are the sites for sugar attachment.

When freshly prepared serum was incubated with radioactive glucose, a considerable portion of the label which remained constant after repeated washings, was recovered in the TCA precipitate. Electrophoresis on cellulose-acetate revealed that not only albumin but also other serum proteins were radioactive (table 2). Radioactivity was highest in the albumin and α_1-globulin fractions, followed by the γ-globulin fractions. For further differentiation the serum was

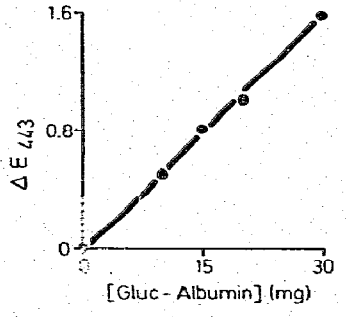

Fig.3. Formation of 5-hydroxymethylfurfural from glucosylated albumin. Albumin (20 mg/ml) was incubated with 888 mM glucose at 37°C for 70 h prior to dialysis against distilled water. The solution was concentrated with a collodium-bag to ~30 mg protein/ml.

Table 2
Distribution of radioactivity from [^{14}C]glucose among the fractions of human serum

Fraction	Protein		Radioactivity		
	μg	%	total cpm	%	cpm μg protein^{-1}
Albumin	22.5	68.2	6624	78	294
α_1-Globulin	0.76	2.3	234	2.8	308
α_2-Globulin	2.9	8.7	477	5.6	164
β- Globulin	1.98	6.0	324	3.8	164
γ- Globulin	4.72	14.3	827	9.7	175

Normal serum was incubated with 111 mM [^{14}C]glucose at 37°C in the presence of 0.01% Natriumtimerfonat® for 50 h. The serum was dialyzed extensively against 0.9% NaCl. Electrophoresis was carried out on cellulose-acetate strips at pH 8.6. The stained protein bands were quantified with a Multiphorese II apparatus (Vogel, Giessen). For radioactivity measurement the stained protein bands were dissolved in scintillator fluid of [17]

Table 3
Release of sugar by acid hydrolysis from albumin from normal and diabetic serum

Subjects	n	Glucose (pmol glucose mg protein^{-1})	Mannose (pmol mannose mg protein^{-1})	Glucose / Mannose
Normal	8	320 ± 88	149 ± 37	2.1 ± 0.4
Diabetic	8	715 ± 284 ($p < 0.0025$)	361 ± 163 ($p < 0.0025$)	2.0 ± 0.2

Chromatographically purified human serum albumin was hydrolyzed and further processed as described in the legend to table 1. Mean values ± SD are given. Albumin amounted to 99.8 ± 0.2% and 99.7 ± 0.3%, respectively, of total serum protein of normal and diabetic sera. Fructose was present only in traces

subjected to immunoelectrophoresis. As illustrated in fig.4 each immunoprecipitate was radioactive. Furthermore, the protein bands stained with Coomassie blue after electrophoresis on SDS–polyacrylamide gels coincided with those obtained after autoradiography of the gels (not shown).

In order to see whether glucosylation of serum proteins also occurs in vivo and is increased in diabetes, serum proteins from normal and diabetic persons were treated with acetic acid and the glucose, fructose and mannose released were determined enzymatically. Secondly, the acid hydrolysates were assayed for hydroxymethylfurfural with thiobarbituric acid. The results in tables 3 and 4 indicate that glucose is a natural constituent of serum proteins and that protein bound glucose is considerably increased in diabetes.

The observation shown in tables 3 and 4 that highly purified albumin from normal and diabetic serum differs in its extent of glucosylation is of interest. Until now only hemoglobin A_{Ic} has been considered as a diagnostic indicator for the long-term control of diabetes. Measurements of glucosylated albumin may be more advantageous, since the half-life of albumin is only 20 days [13] compared to 120 days for the erythrocyte. Assuming that glucosylated and non-glucosylated albumin have the same turnover, one would expect fluctuations in blood glucose to be sensed more sensitively by the changes in glucosylated albumin than in glucosylated hemoglobin.

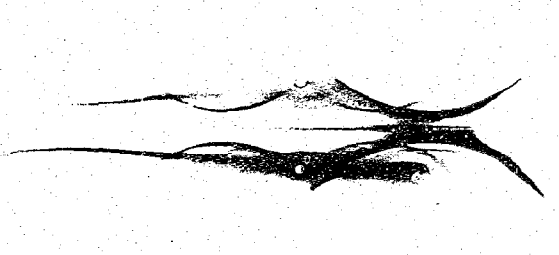

Fig.4. Analysis of [^{14}C]glucosylated serum proteins by immuno-electrophoresis and autoradiography. To 0.2 ml normal human serum 22.2 μmol D-[U-^{14}C]glucose (corresponding to 100 μCi) were added and the serum was incubated for 48 h at 37°C in the presence of 0.01% Natriumtimerfonat®. After dialysis against 0.9% NaCl at 4°C, immunoelectrophoresis was carried out according to [16]. After staining with amido black 10B and destaining autoradiography was performed with Kodak X-omat R film. (a) Protein-staining; (b) autoradiogram.

Table 4
Release of 5-hydroxymethylfurfural (HMF) from albumin and non-albumin proteins from normal and diabetic serum

Subjects	n	Albumin (pmol HMF mg protein^{-1})	n	Non-albumin proteins (pmol HMF mg protein^{-1})
Normal	8	86 ± 18	8	211 ± 24
Diabetic	8	174 ± 62 ($p < 0.0025$)	12	305 ± 82 ($p < 0.0025$)

Mean values ± SD are given. Albumin amounted to 99.8 ± 0.2% and 99.7 ± 0.3%, respectively, of total serum protein of normal and diabetic sera. Extinction of the thiobarbituric acid reaction product at 443 nm was proportional up to 30 mg protein/assay. For experimental details see section 2

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