Nonenzymatic glucosylation of proteins: a new and rapid solution for in vitro investigation

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The rates of nonenzymatic glucosylation of albumin, high density lipoprotein (HDL) and low density lipoprotein (LDL) were determined in vitro using [^{14}C]glucose repurified by a new and rapid HPLC method. All commercial preparations were found to contain contaminants reacting 15–20-times faster with protein than the repurified [^{14}C]glucose. Removal of contaminants was critical to the rate determinations and constitutes a substantial improvement over the widely used existing method. The initial rates of nonenzymatic glucosylation determined in vitro for albumin, HDL and LDL were used to predict normal in vivo levels of 0.40, 0.65 and 0.08 mol glucose per mol protein, respectively. This is within the range of values found in vivo for albumin and LDL, but low for HDL. These values would be expected to increase 2–4-fold in diabetes.

Glucosylation *Glycation* *Diabetes* *High density lipoprotein* *Low density lipoprotein* *Albumin*

1. INTRODUCTION

Attention in recent years has focused on one potentially deleterious consequence of the high glucose levels found in diabetes, the nonenzymatic glucosylation of proteins [1,2]. Proteins whose function is affected by glucosylated amino-terminal or lysine ϵ-amino groups would be the most vulnerable in diabetes mellitus. There is a growing list of proteins which fit into this category including haemoglobin, collagen, lens crystallins, albumin, peripheral nerve protein (review [1]), LDL [3,4] and HDL [5].

The methods currently used to measure nonenzymatic glucosylation were mainly devised for haemoglobin and with the exception of chemical analyses [6–8] not directly applicable to other proteins. Therefore, an attractive alternative is to isolate the protein of interest and to explore glucosylation and the subsequent functional consequences in vitro. Unfortunately, monitoring incorporation of ^{3}H- or ^{14}C-labelled glucose has been flawed by the presence of radioactive contaminants in all commercially available preparations [7]. These contaminants, albeit representing only a few percent of the total radioactivity, react much more rapidly with protein than the glucose itself [7]. This has led to inadvertant overestimation of the rates of glucosylation [9,10] and possibly galactosylation [11]. To circumvent this problem the radioactive glucose has been incubated with albumin for 2–3 days, followed by gel filtration to separate the cleansed sugar [4]. However, this method is time-consuming and gives relatively poor yields. It also relies on the probably erroneous assumption that all the contaminants are consumed during the incubation.

We report here a rapid and convenient solution

Note: nonenzymatic glucosylation is now also referred to as glycation

Abbreviations: PBS, phosphate-buffered saline (pH 7.4); HPLC, high-performance liquid chromatography; HDL, high density lipoprotein; LDL, low density lipoprotein

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to this problem. [^{14}C]Glucose was purified by HPLC and was then used to follow the course of glucosylation of 3 plasma proteins (albumin, LDL and HDL) for which nonenzymatic glucosylation may have significant functional consequences [5,13,16]. The HPLC method presented here should also be directly applicable to the repurification of other radiolabelled monosaccharides.

2. MATERIALS AND METHODS

D-[U-^{14}C]Glucose was purchased from the Radiochemical Centre (Amersham) and from the Commissariat à l'Energie Atomique (CEA, Gif-sur-Yvette). Human serum albumin and glucose (puriss) were supplied by Fluka (Buchs). Bovine serum albumin was from Sigma (St. Louis). Lumagel SB and Rialuma were purchased from Lumac (Schaesburg). LDL and HDL were prepared as in [14].

The separation of [^{14}C]glucose from ^{14}C-labelled contaminants was achieved by HPLC on a 4 × 250 mm Lichrosorb NH_2 column (Merck, Darmstadt). Injection of the radioactive glucose, dissolved in either 20 or 100 μl water, was followed by isocratic elution with 1.5 ml/min acetonitrile: water (80:20, v/v). Forty fractions of 20 drops each were collected, aliquots of each fraction dissolved in 4 ml Rialuma and ^{14}C measured in a Nuclear Chicago Isocap 300 scintillation counter.

Albumin (0.92 mg/ml), LDL (0.93 mg/ml) and HDL (0.63 mg/ml) were incubated in sterile capped tubes (Corning, Avon Cedex) at 37°C with 100 mM glucose buffered to pH 7.4 with PBS. All solutions were sterile filtered and 1 mM NaN_3 was added as preservative. Samples were removed at timed intervals and dialysed at 4°C against several changes of PBS over 2–3 days. Aliquots from these samples were measured for ^{14}C as in [7]. Bovine serum albumin was used for all experiments presented here, however human serum albumin was found to incorporate glucose at an identical rate (not shown).

After incubation with untreated or purified glucose, protein samples were prepared as in [7] for amino acid analysis [15]. Albumin concentrations were measured as in [16] relative to an albumin standard. The apolipoproteins were quantitated from their amino acid analyses with reference to published compositions for apo B [17] and apo A-1 and A-2 [18].

3. RESULTS

[^{14}C]Glucose preparations from either Amersham or CEA were resolved by HPLC into similar patterns, as illustrated in fig.1 for batch 116 from Amersham. The [^{14}C]glucose peak eluted at 11 min and was preceded by two other peaks at 4 and 9 min. The proportions of these contaminant peaks to that of glucose ranged from 1.5 to 15% between individual commercial preparations of [^{14}C]glucose but were consistent for a given batch.

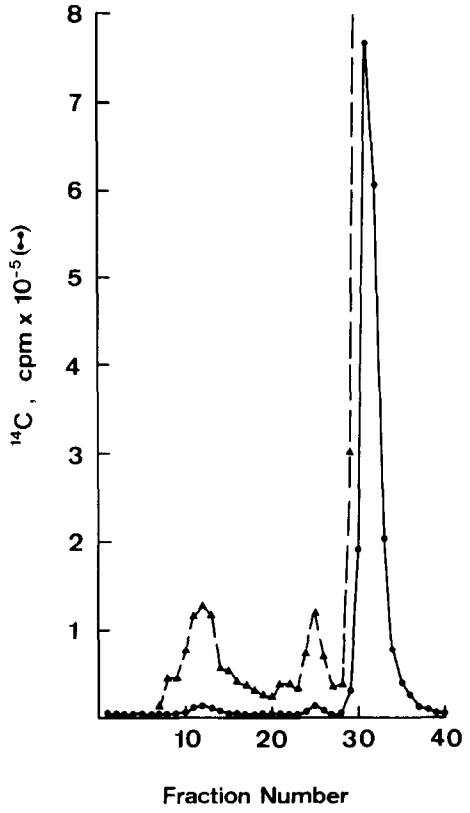

Fig.1. HPLC of [^{14}C]glucose on a 4 × 250 mm column of Lichrosorb NH_2. 200 μCi [^{14}C]glucose (batch 116, Amersham) was dissolved in 20 μl water, loaded and eluted with acetonitrile: water (80:20, v/v) at a flow rate of 1.5 ml/min. Fractions of 20 drops each were collected and 5-μl aliquots taken for ^{14}C analysis. An expanded scale is included (cpm × 10^{-4}, ▲---▲) to show more clearly the elution of the two contaminant peaks centred on fractions 12 and 25 relative to glucose on fraction 31.

These differences between batches were not necessarily related directly to the age of the samples. This was illustrated by the analyses of batches 105, 111, 116, 163 produced in March 1978, November 1978, February 1979 and June 1983, respectively, by Amersham. Although listed as 99, 98, 99 and 99% radiochemically pure at time of production, respectively, they contained 15, 2, 4 and 1.5%, respectively, of ^{14}C-labelled contaminants, when measured in December 1983.

However, time-dependent accumulation of radiolysis products is to be expected [19]. When repurified [^{14}C]glucose (1 mCi/ml) was stored either at 20°C in 3% (v/v) ethanol in water, or at 4°C in the same solution plus gentamycin (0.01%, w/v), it was found that although the samples were stable for up to 1 month in either of the above conditions, longer storage resulted in reappearance of fast reacting ^{14}C-labelled compounds (not shown). Thus, for the glucosylation experiments below, [^{14}C]glucose was freshly purified before each incubation with protein.

The courses of nonenzymatic glucosylation of albumin, LDL and HDL were followed using either the untreated or purified [^{14}C]glucose tracer in a 100 mM glucose solution buffered to pH 7.4 with PBS. The incorporation of trichloroacetic acid-precipitable glucose is plotted as a function of time in fig.2. The initial reaction rates of the untreated glucose were consistently 16–20-times higher than that of the HPLC-purified sugar. The result shown here was obtained with batch 116 from Amersham which had 4% ^{14}C-labelled contaminants. Similar results were obtained with batch 91-1279 (CEA) with 6% ^{14}C-labelled contaminants.

Albumin bound purified glucose linearly for 16 days. Both HDL and LDL showed a more rapid incorporation of purified glucose for the first 3 days. This was followed by declining incorporation over the following 13 days in the case of HDL and by a slower but linear rate than initially for LDL.

The initial rates of glucosylation by either untreated or purified [^{14}C]glucose were calculated as mol glucose incorporated after 24 h per mol protein and/or per mol lysine (table 1). HDL was modified by purified glucose at twice the rate of LDL or albumin when expressed per mol lysine. However, because of its much greater size (550 kDa) apo B or LDL bound 6-times as much

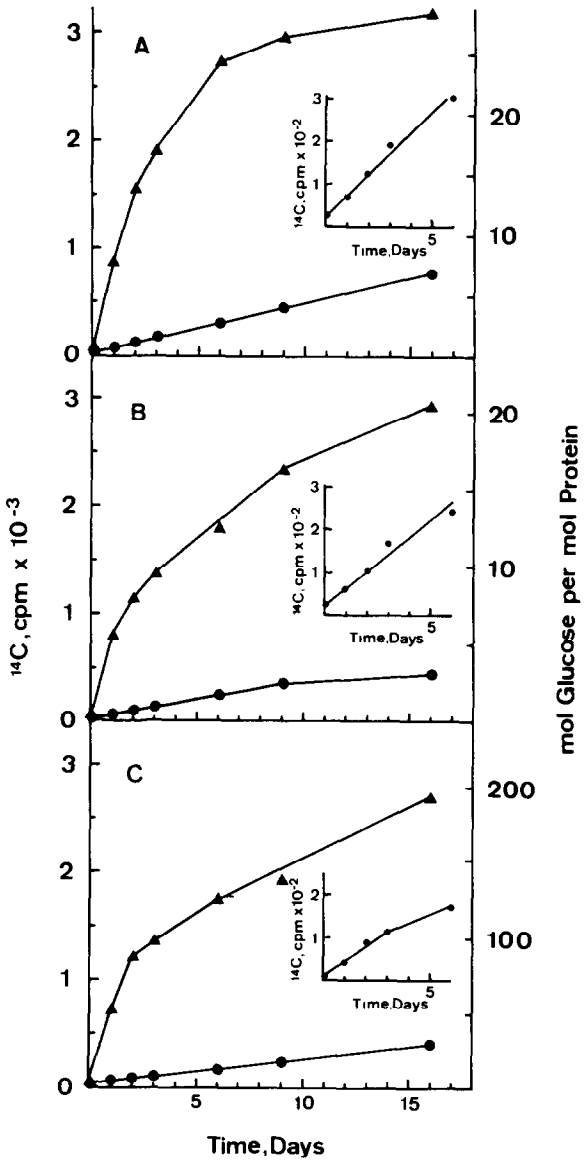

Fig.2. Incorporation of ^{14}C into protein from untreated (▲—▲) and purified (●—●) [^{14}C]glucose as a function of time. (A) Albumin (13.5 μM), (B) HDL (17.6 μM) and (C) LDL (1.7 μM) were incubated in 100 mM [^{14}C]glucose (spec. act. 240 dpm/nmol) at 37°C. Data points show trichloroacetic acid-precipitable ^{14}C in 50-μl aliquots of incubation solutions. Insets have expanded scale on ordinate axis to show more clearly incorporation of purified [^{14}C]glucose into protein over the first 5 days.

glucose on a molar basis relative to albumin (68 kDa) and 4-times more glucose relative to apo A1 (27 kDa) and A2 (7.8 kDa) of HDL. These dif-

Table 1

	Initial rates of [^{14}C]glucose incorporation			
	Untreated [^{14}C]glucose		Purified [^{14}C]glucose	
	A	B	A	B
Albumin	71.2	1.19	4.32	72.0
HDL	50.3	19.6	3.27	125.0
LDL	932.0	2389	25.83	66.2

(A) 10^{-3} mol Glc·mol^{-1} protein·day^{-1}·mM^{-1} Glc, (B) 10^{-6} mol Glc·mol^{-1} Lys·day^{-1}·mM^{-1} Glc

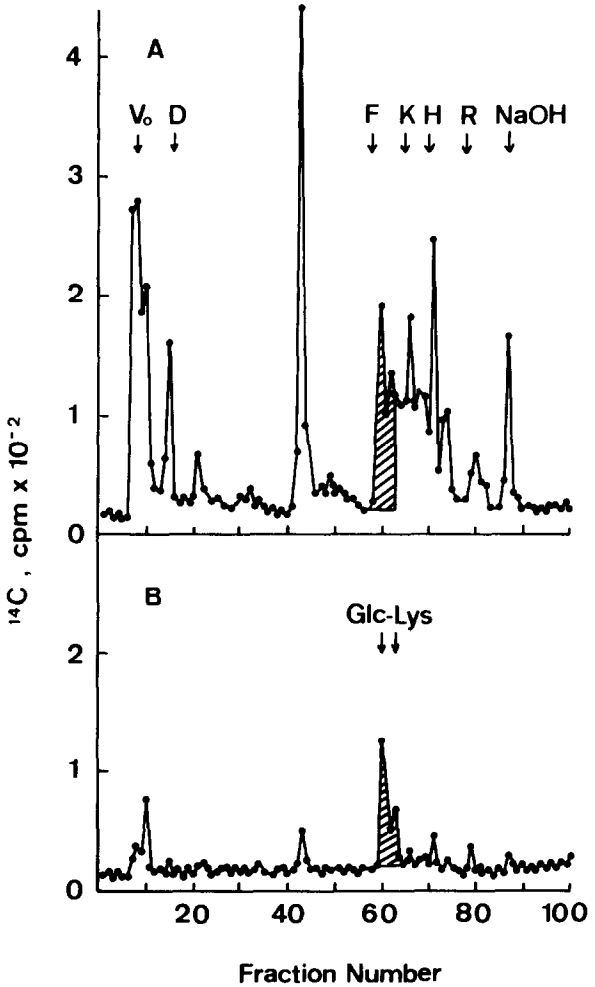

Fig.3. Amino acid analysis of albumin glucosylated with untreated (A) and purified (B) [^{14}C]glucose. Fractions of 0.7 ml were collected. Elution positions of the glucitollysine peaks (GlcLys, shaded) are shown relative to a number of amino acids (single letter code). The void volume (V_o) and 0.2 M NaOH regeneration step are also indicated.

ferences were not discernible with untreated glucose (table 1).

To compare the specificity of the reaction between either pure or impure [^{14}C]glucose and protein, aliquots of the dialysed albumin solution from day 9 of the incubations were reduced to convert fructosyllysine into the stable glucitollysine, hydrolysed and then applied to an amino acid analyser. The normal elution program was adapted to allow the characteristic [1] double peaks of the glucitollysine standard to elute clearly between phenylalanine and lysine (fig.3). The hydrolysate of albumin incubated with the impure [^{14}C]glucose showed a broad distribution of the incorporated radioactivity (fig.3a). In contrast, the major ^{14}C peaks derived from the reaction between purified [^{14}C]glucose and albumin coincided with those of the glucitollysine standard (fig.3b). The remainder of the ^{14}C-labelled material eluted primarily in the void volume and probably represents acid-degraded glucitollysine.

4. DISCUSSION

The presence of minor radioactive impurities, either from manufacture or radiolysis products of storage, would appear to be inevitable in most radiochemical preparations. Although often not of concern, these minor contaminants can assume major importance when the reaction being traced consumes only a few percent of the substrate. Nonenzymatic glucosylation is such a reaction.

Thus, a method which enables radiolabelled glucose preparations to be easily and promptly repurified is necessary. The HPLC method presented fulfills these requirements. The rates of reaction of [^{14}C]glucose with protein, from batches

repurified at different times, was found to be reproducible. The specificity of the reaction between the repurified [^{14}C]glucose and the lysine ϵ-amino groups of albumin is clearly demonstrated in fig.3. However, only about half of the [^{14}C]glucose precipitable with trichloroacetic acid could be recovered as glucitollysine, indicating that as much as 50% of the bound [^{14}C]glucose was degraded under the protein hydrolysis conditions employed.

Even after 3 days radioactivity from the untreated [^{14}C]glucose was still incorporated 6-fold more rapidly into protein than the purified glucose. This is the maximum time normally used for adsorptive removal of contaminants by protein, which in turn illustrates that glucose purified by preincubation with albumin is probably unsatisfactory. The reactions of the untreated and the purified [^{14}C]glucose approached similar rates only after 10 days incubation with albumin. With the lipoproteins the rate of ^{14}C incorporation was still 4-times higher for the nonpurified glucose after 16 days (fig.2).

The in vitro rates of glucosylation obtained with the purified [^{14}C]glucose were used to estimate glucosylation in vivo. Thus, plasma half-lives of 18 days for albumin [12] and 5 days each for LDL and HDL [20,21] would lead to 0.40, 0.65 and 0.08 mol glucose per mol protein at normal plasma levels of glucose and protein. The predicted values are within the range of the very preliminary data reported for albumin [1,8] and LDL [4] from euglycemic individuals of 0.1–0.7 and 0.5 mol glucose per mol protein, respectively. The range of 1.1–3.8% glucosylated lysines reported for HDL [5] for 6 euglycemic subjects is above the values of 0.3% calculable from table 1. Although these results were presented as preliminary [5], thus precluding a detailed comparison, the 4–12-fold difference would require the lysines of HDL to be 8–24-times more reactive than those of albumin or LDL (table 1).

In addition, repurified [^{14}C]glucose can now serve as a specific label to identify susceptible glucosylation sites or to synthesise protein standards for which the absolute level of glucosylation can be measured. Exploration of potential Maillard reactions of the non-enzymatically glucosylated protein, suggested to be involved in the complications of diabetes [1] will also become possible.

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