three non-mammalian species (chicken, trout, frog), in which red blood cells still contain nuclei [2].

Briefly, the method to prepare insulin gold complexes consists of coupling insulin to colloidal particles with a definite size.

Figure 1 A shows insulin gold complex binding to a mature human red blood cell without any determinable internalization. In contrast, Figure 1 B shows a nucleated chicken erythrocyte which both binds and internalizes insulin gold complex. This micrograph (Fig. 1 B) is representative of similar results obtained with other nucleated erythrocytes from frog and trout, as well as with human reticulocytes still possessing nuclei.

Specific [$^{125}$I]-insulin binding (at 15 °C for cell concentrations approaching the respective blood erythrocyte values) were as follows: human red blood cells: 7.6 ± 1.6% (4 × $10^9$ cells/ml); chicken red blood cells 8.9 ± 1.6% (2.6 × $10^9$ cells/ml), trout red blood cells 1.6 ± 0.5% (5 × $10^8$ cells/ml), and frog erythrocytes 16.7 ± 4.5% (2 × $10^8$ cells/ml). When a high concentration of reticulocytes were present in the human red blood cell population (16 ± 23%), the specific binding rose to 13.65 ± 1.05%. The number of receptors per cell were 44 ± 16 for human, 309 ± 5 for chicken, 466 ± 36 for trout, 1188 ± 320 for frog erythrocytes, respectively, and 109 ± 36 for red blood cells enriched in reticulocytes. Similar differences in binding were revealed when electron microscopy studies were carried out. The human white blood cells (in particular monocytes) also showed the two capacities: binding and internalization of insulin gold complexes.

From these results the following conclusions might be offered: (1) binding is not necessarily followed by internalization of insulin; (2) only cells with nuclei internalize insulin; (3) insulin may well exert different effects on intracellular metabolism depending upon nuclei content; and (4) reticulocytes that can internalize insulin might be of importance concerning the rate of insulin degradation in circulating blood.

Yours sincerely,
X. Bihr, C. Thun and E. F. Pfeiffer

**References**

1. Gambhir KK, Archer JA, Bradley CJ (1978) Characteristics of human erythrocytes insulin receptors. Diabetes 27: 701–708
2. Repasky EA, Eckert BS (1981) A re-evaluation of the process of enucleation in mammalian erythroid cells. In: Brewer GJ (ed) Progress in clinical and biological research, vol 55. The red cell. (Fifth Ann Arbor Conference). A. R. Liss, New York, pp 679–690

Dr. X. Bihr
Centre of Internal Medicine
University of Ulm
Steinhövelstr. 9
D-7900 Ulm
FRG

## Rapid, Accurate Colorimetric Assay of Non-Enzymatically Glycosylated Serum Proteins

Dear Sir,

It is now recognised that many tissue proteins besides haemoglobin, are modified by the process of non-enzymatic glycosylation. Several recent reports have shown that measurement of non-enzymatically glycosylated serum proteins in diabetic patients provide an index of integrated glycaemia over the preceding 1–2 weeks [1–5]. The colorimetric technique used for measuring glycosylated serum proteins is similar to that originally described for glycosylated haemoglobin by Fluckiger and Winterhalter [6]; full details have been published elsewhere [1, 4]. Because of the need to free samples of glucose and to run a sodium borohydride blank with each sample, the whole assay procedure, when applied to serum proteins, is more time consuming than when applied to haemoglobin. In our hands, a coefficient of variation of 8% within and 12% between assays has not matched the reproducibility of glycosylated haemoglobin estimation. We would like to report several modifications which have resulted in a considerably shorter and more reproducibile assay.

Sodium borohydride reduces the ketoamine link in glycosylated proteins to a form which does not give the characteristic colour formation. Incubation of serum with sodium borohydride for 4.5 h has been the basis of the blank run with each sample [1, 2]. We have found that the reaction between sodium borohydride and protein is essentially complete after 15 min; there is thus no need to prolong the blank incubation further.

The central step of the assay is release of adducted glucose from the protein as 5-hydroxymethylfurfural (5-HMF) by weak acid hydrolysis, a reaction which is time- and temperature-dependent. This is not an end-stage reaction and in the conventional assay is performed for 5 h at 100 °C. This step could be shortened using a higher temperature and Parker et al. [7] showed that for haemoglobin, this could be achieved by performing the hydrolysis in an autoclave. When serum pooled from diabetic patients was hydrolysed for 1 h in an autoclave (121 °C, 1.05 kg/cm$^2$), the production of 5-HMF, measured by specific absorbance at 443 nm after reaction with thiobarbituric acid, was 45% greater than after hydrolysis for 5 h at 100 °C in a heat block.

Glycosylated serum protein levels may be expressed in units of nmol 5-HMF/mg protein by comparing the absorbance at 443 nm when the 5-HMF released by hydrolysis is reacted with thiobarbituric acid with the absorbance of known dilutions of purified 5-HMF. A disadvantage of this is that there is no allowance for possible variability of hydrolysis or evaporation of samples in different assay runs, which may therefore contribute to interassay variability. Fructose provides a suitable alternative standard which overcomes these problems as it is converted to 5-HMF by weak acid hydrolysis [8].

By introducing the three modifications outlined above (15-min sodium borohydride blank incubation, weak acid hydrolysis for 1 h in an autoclave, and fructose standards), we have been able to reduce the time taken to assay glycosylated serum proteins in dialysed serum from over 10 h to approximately 2.5 h. Glycosylated serum protein formation during incubation of non-diabetic serum in vitro with $^{14}$C-glucose was measured by this modified technique and showed an extremely close correlation ($r$ = 0.98) with the amount of radioactivity incorporated into the protein. Figure 1 shows the glycosylated serum protein levels in 17 control and 32 diabetic patients using the modified technique; these levels correlated with those measured by the longer,

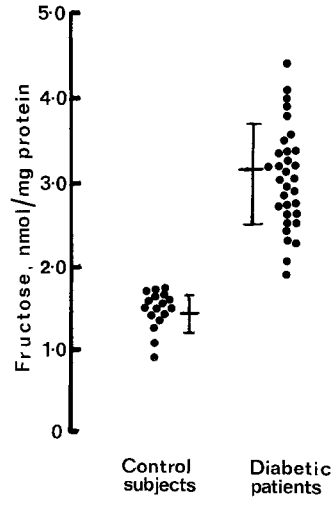


**Fig. 1.** Glycosylated serum protein levels in 17 control and 32 diabetic patients using the modified technique

conventional technique ($r = 0.68$) and the results obtained with autoclave hydrolysis were consistently higher (mean 61%) than with conventional hydrolysis.

To test the reproducibility of the modified technique, serum from a diabetic patient was assayed 13 times within one assay run. The mean ± SD was 3.3 ± 0.145 nmol fructose/mg protein. When assayed in 12 separate runs the result was 3.24 ± 0.273 nmol fructose/mg protein (mean ± SD). These results represent intra- and interassay coefficients of variation of 4.3 and 8.6% respectively, a considerable improvement over the unmodified assay.

We therefore suggest that these simple modifications could be applied to measurement of non-enzymatic glycosylation of other proteins, such as collagen [9] and basement membrane proteins [10]. These improvements in technique may be of particular value in studies aimed at answering the crucial question as to whether non-enzymatic glycosylation is of pathophysiological importance in diabetes mellitus.

Yours sincerely,
E. Elder and L. Kennedy

**References**

1. Kennedy L, Mehl TD, Riley WJ, Merimee TJ (1981) Non-enzymatically glycosylated serum protein in diabetes mellitus: an index of short-term glycaemia. Diabetologia 21: 94–98
2. McFarland KF, Catalano EW, Day JF, Thorpe SR, Baynes JW (1979) Nonenzymatic glycosylation of serum proteins in diabetes mellitus. Diabetes 28: 1011–1014
3. Yue DK, McLennan MS, Turtle JR (1980) Glycosylation of plasma protein and its relation to glycosylated hemoglobin in diabetes. Diabetes 29: 296–300
4. Kennedy L, Merimee TJ (1981) Glycosylated serum protein and hemoglobin $A_1$ levels to measure control of glycemia. Ann Intern Med 95: 56–58
5. Gragnoli G, Tangonelli I, Signorini AM, Tarli P (1982) Non-enzymatic glycosylation of serum proteins as an indicator of diabetic control. Diabetologia 22: 224 (Letter)
6. Fluckiger R, Winterhalter KH (1976) In vitro synthesis of hemoglobin $A_{1c}$. FEBS Letters 71: 356–360
7. Parker KM, England JD, DaCosta J, Hess RL, Goldstein DE (1981) Improved colorimetric assay for glycosylated hemoglobin. Clin Chem 27: 669–672
8. Pecoraro RE, Graf RJ, Halter JB, Beiter H, Porte D, Jr. (1979) Comparison of a colorometric assay for glycosylated hemoglobin with ion exchange chromatography. Diabetes 28: 1120–1125
9. Schnider SL, Kohn RB (1980) Glycosylation of human collagen in aging and diabetes mellitus. J Clin Invest 66: 1179–1181
10. Cohen MP, Urdanivia E, Surma M, Ciborowski CJ (1981) Nonenzymatic glycosylation of basement membranes. In vitro studies. Diabetes 30: 367–371

Dr. L. Kennedy
Sir George E Clark Metabolic Unit
Royal Victoria Hospital
Belfast BTIZ GBA
N. Ireland, UK

# *Book Reviews*

**H. Keen and J. Jarret. Complications of Diabetes, 2nd edn.** London: Edward Arnold 1982. Hardback, pp 331, £ 25.00

The new *Complications of Diabetes* has retained the organization and, laudibly, the length of the 1975 edition. The number of sections has increased only from 8 to 9; a section describing the skin disorders associated with diabetes has been added. The bibliography has been consolidated just ahead of the index, a useful space-saving modification for which the publisher is to be saluted. Each major field of diabetic complications is presented from the perspective of one or more of the leaders in that field. The introductory section has been subdivided in this edition to a review of the role of genetics and of immunological factors in diabetic complications and an update on the linkage between hyperglycaemia and the long-term complications of diabetes. Both are characterized by a balanced and systematic approach. The largest section, as in the first edition, is devoted to eye changes. The review of retinopathy contains a great number of useful illustrations and large areas of the text have been updated and modified. The section on management of diabetic neuropathy is less altered but new headings on autonomic neuropathy and vascular changes associated with nerve damage have been incorporated and a well-organized section on management of the diabetic foot has been included. The section on diabetic renal disease has been extensively rewritten and is well illustrated. It now focuses quite intensely on early nephropathy management. The diabetes-associated manifestations involving the heart and large arteries are treated epidemiologically, emphasizing identifiable environmental factors more than clinical assessment techniques for cardiovascular problems. The dermatology section is brief and not as well illustrated as might be hoped. The section on diabetes in pregnancy has been revised to demonstrate the importance of vigorous control for both the insulin-requiring and the gestational diabetic, but the appropriate management of the latter is not as developed as might be hoped. The section on biochemistry has added a review of control over cell myoinositol, arterial wall metabolism, and the role of haematologic (haemorheologic and coagulation) factors. The last section on emotional complications of diabetes has been extensively revised. It is aggressively critical of current educational practices and makes recommendations for effective changes. It complements the other chapters, capturing the flavour of the entire book, a compendium targeted at the experienced diabetologist. Those who are ready for this book will find it most provocative in their first reading. It will remain a current and useful reference source on their office or library shelf for the rest of this decade.

D. E. McMillan (Santa Barbara, California)