Free Rad. Res. Comms., Vols. 12-13, pp. 115-123
Reprints available directly from the publisher
Photocopying permitted by license only

OXIDATIVE GLYCATION AND FREE RADICAL PRODUCTION: A CAUSAL MECHANISM OF DIABETIC COMPLICATIONS

JAMES V. HUNT and SIMON P. WOLFF

Department of Clinical Pharmacology, University College London, 5 University Street, London WC1 6JJ

Glucose may oxidise under physiological conditions and lead to the production of protein reactive ketoaldehydes, hydrogen peroxide and highly reactive oxidants. Glucose is thus able to modify proteins by the attachment of its oxidation derived aldehydes, leading to the development of novel protein fluorophores, as well as fragment protein via free radical mechanisms.

The fragmentation of protein by glucose is inhibitable by metal chelators such as diethylenetriamine pentaacetic acid (DETAPAC) and free radical scavengers such as benzoic acid, and sorbitol. The enzymic antioxidant, catalase, also inhibits protein fragmentation.

Protein glycation and protein oxidation are inextricably linked. Indeed, using boronate affinity chromatography to separate glycated from non-glycated material, we demonstrate that proteins which are glycated exhibit an enhanced tryptophan oxidation. Our observation that both glycation and oxidation occur simultaneously further supports the hypothesis that tissue damage associated with diabetes and ageing has an oxidative origin.

KEY WORDS: Glucose oxidation, protein modification, free radicals.

INTRODUCTION

The *in vitro* exposure of macromolecules to glucose, at concentrations representative of hyperglycaemia, is widely accepted as a relevant model for tissue damage occurring in diabetes mellitus and ageing.[1] Proteins, for example, undergo structural changes and develop novel fluorophores[2] reminiscent of those found to correlate with pathological tissues obtained from diabetic individuals.[3] Such alterations have long been assumed to occur via the traditional Amadori pathway.[1,2,4] However, glucose is prone to transition metal-catalysed oxidation, generating hydrogen peroxide, hydroxyl radicals and protein-reactive ketoaldehydes.[5] Oxidative chemistry of glucose could well account for macromolecular alterations associated with experimental glycation.[5,6] This has led us to propose that tissue damage occuring *in vivo* may also involve oxidative reactions.[6] Indeed, there is considerable evidence suggesting that oxidative stress plays a role in tissue damage associated with diabetes[6] and ageing.[7] For example, plasma peroxide and copper levels, the latter catalysing free radical production from hydrogen peroxide (possibly generated by increasing glucose and other monosaccharide levels), is increased in both age[8] and diabetes.[9,10] Also, a number of antioxidant defences would seem to be compromised in diabetic individuals. These include decreases in the levels of glutathione,[11] ascorbic acid,[12] vitamin E[13] and uric acid.[14] The activity of certain antioxidant enzymes are also decreased in diabetic individuals, these include superoxide dismutase[15] and catalase.[16]

In further support to our hypothesis that oxidative reactions play a significant role

in glucose-mediated protein damage we demonstrate that glucose attachment, be it via the traditional Amadori pathway[1-4] or the novel oxidative pathway,[5,6,17] is accompanied by oxidative damage which is inextricably associated with glucose attachment.

MATERIALS AND METHODS

Protein fragmentation

Bovine serum albumin (Boehringer; Fraction V) was radiomethylated and assessed for fragmentation to trichloroacetic acid-soluble peptides by glucose as described previously. Sodium dodecyl sulphate gel electrophoresis was also performed as previously described.[18,19]

Hydrogen peroxide measurement

The prodution of hydrogen peroxide on incubating glucose under physiological conditions was monitored using horse radish peroxidase coupled phenol and amino antipyrine oxidation as previously described.[20]

Tryptophan fluorescence measurement

Alterations in tryptophan fluorescence of radiomethylated bovine serum albumin after exposure to buffer with or without glucose was determined in either buffer or buffer and 4 M guanidine hydrochloride (exciting at 280 nm and emmiting at 350 nm).[17,18] In all cases tryptophan fluorescence was corrected for protein concentration, determined by the presence of radiolabel.

Novel fluorophore formation and Glucose Incorporation

Glucose attachment (incorporation) to bovine serum albumin, determined as previously described,[17] leads to the development of novel protein fluorophores which were assessed by measuring emission between 350 nm and 500 nm when exciting at 350 nm.[15]

Ketoaldehyde (Dicarbonyl) measurement

The oxidation of glucose results in the production of ketoaldehydes (dicarbonyls). The measurement of these oxidation products was performed using Girard T reagent (carboxymethyl trimethyl ammonium chloride hydrazide) as previously described.[17,18] Gloxal standards were utilised to quantify the extent of dicarbonyl formation.

Boronate affinity chromatography

Glyco Gel B (Pierce No. 20248) was used to separate glycated from non-glycated radiolabelled bovine serum albumin.[21] The column (1.5 cm × 8 cm) was first equilibriated with 100 mM glycine (adjusted to pH 9.2–10) and sample then loaded. Non-glycated protein was eluted with this buffer. Glycated protein which adhered to the resin was eluted from the column with 100 mM citric acid (adjusted to pH 5.8).

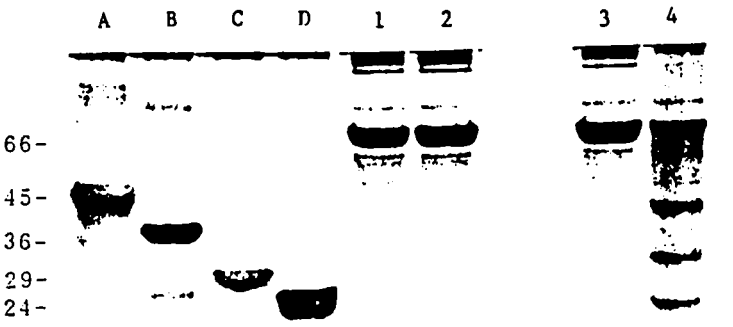


FIGURE 1 *Glucose-Mediated Protein Fragmentation.* Sodium dodecyl sulphate polyacrylamide gel electrophoresis of 1 mg/ml bovine serum albumin was exposed over 8 days to various reaction mixtures performed at 37°C in buffer (100 mM potassium phosphate pH 7.2). Lanes A-D = Molecular weight standards, lane 1 = buffer alone, lane 2 = buffer and 100 uM copper sulphate, land 3 = 25 mM glucose, 100 uM copper and 1 mM DETAPAC and lane 4 = 25 mM glucose.

RESULTS AND DISCUSSION

Glucose Fragments Protein

The incubation of radiomethylated bovine serum albumin with glucose under physiological conditions leads to frank protein scission to fragments of a finite size as

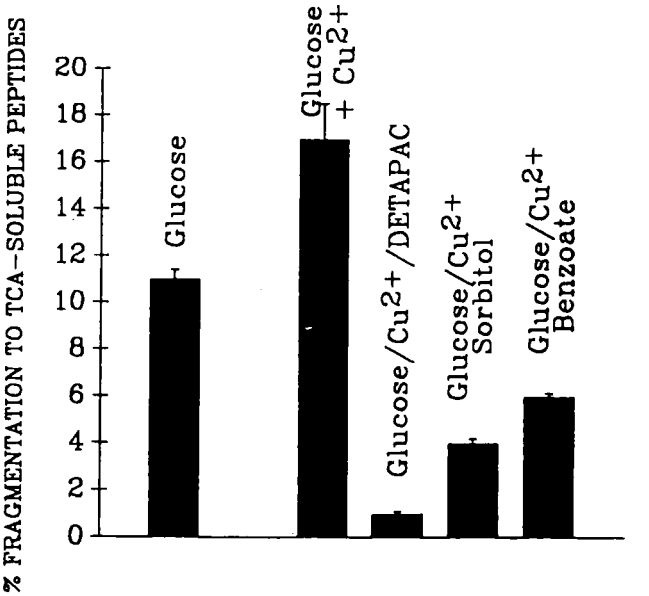


FIGURE 2 *Glucose-Mediated Protein Fragmentation: The Inhibitory Effect of Free Radical Scavengers.* Radiomethylated bovine serum albumin (1 mg/ml) was incubated with 25 mM glucose alone or in the presence of 1 mM DETAPAC, 250 mM sorbitol, 1 mM benzoic acid together with 100 uM copper in 100 mM potassium phosphate (pH 7.2). Incubations were performed at 37°C over a period of 8 days. All reaction mixtures were filter sterilised prior to incubation. Protein fragmentation was determined as the production of trichloroacetic acid-soluble radiomethylated peptides.

demonstrated by SDS-PAGE studies (Figure 1). The addition of DETAPAC inhibited fragmentation, indicating that transition metals are necessary catalysts in this proccess.[22] The production of fragments of a finite size; similar to those previously reported on exposure of bovine serum albumin to ascorbic acid[22] and hydroperoxides[19] in the presence of added copper; is due to site specificity of fragmentation resulting from a "site restricted" free radical production at copper chelation sites on this protein.[22]

Fragmentation may also be demonstrated in a quantitive fashion by the detection of changes in the trichloroacetic acid-soluble fraction of radiolabelled protein exposed to free radical generating systems.[19] Figure 2 demonstrates that fragmentation of bovine serum albumin by glucose containing reaction systems is inhibited by the metal chelator, DETAPAC, and also the free radical scavengers, sorbitol and benzoic acid. The use of benzoic acid as a free radical scavenger led to the production of fluorescent hydroxylation products, indicating the role of hydroxyl radicals[23] in fragmentation (data not shown). The oxidative origin of fragmentation has also been demonstrated by the loss in tryptophan fluorescence. The exposure of bovine serum albumin to 25 mM glucose led to 22% loss in tryptophan fluorescence, the addition of 100 uM copper increased this to 70%. Again, the addition of DETAPAC inhibited trytophan loss (5% loss).

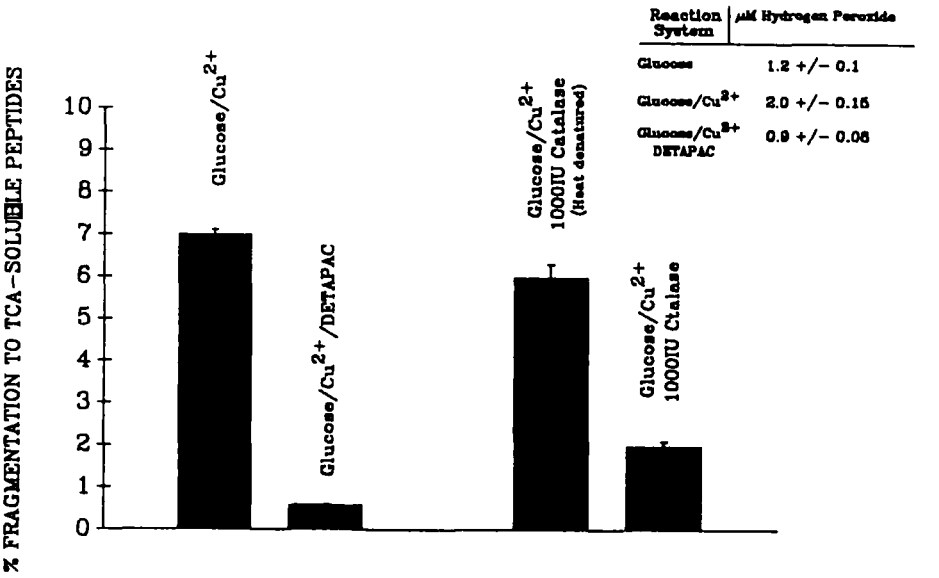


FIGURE 3 *The Role of Hydrogen Peroxide in Glucose-Mediated Protein Damage.* Radiomethylated bovine serum albium (1 mg/ml) was incubated for 3 days with 25 mM glucose and 100 uM copper in the presence on absence of 1 mM DETAPAC and 1000 IU catalase. Denatured catalase was prepared by incubation of protein at 100°C for 15 minutes prior to use in studies. Protein fragmentation was determined as the production of trichloroacetic acid-soluble radiomethylated peptides. Inset: The production of hydrogen peroxide in the absence of protein on incubating 25 mM glucose with or without 100 uM copper was determined after 3 days. A control including 1 mM DETAPAC is included. All reactions were performed in the presence of 100 mM potassium phosphate (pH 7.2) at 37°C.

TABLE I
Glucose Oxidation and Dicarbonyl Production

Ncubation	μM Methylglyoxal equivalents
Glucose	0.7 ± 0.05
Glucose/DETAPAC	0.35 ± 0.01

25 mM glucose in the presence and absence of 1 mM DETAPAC was incubated at 37 °C for 3 days in 100 mM potassium phosphate (pH 7.2). Dicarbonyl production was measured using the Girard T assay and quantified as methylglyoxal equivalent.

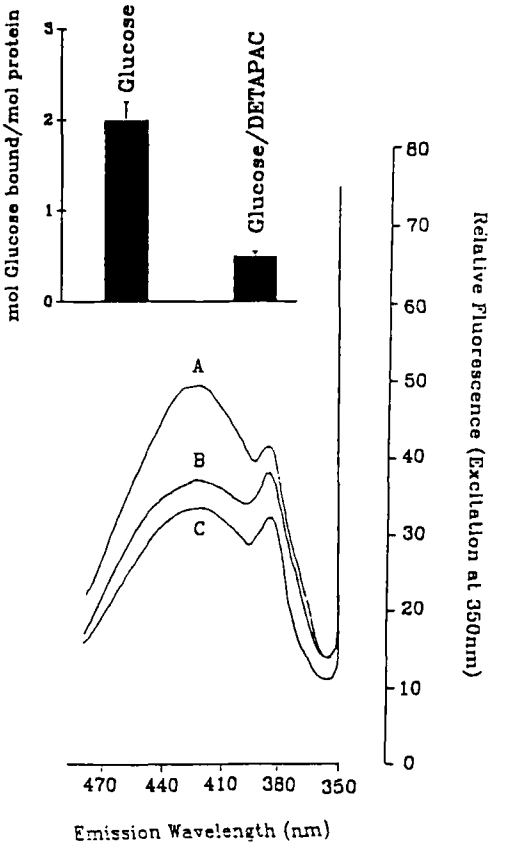


FIGURE 4 *Glucose Attachment to Protein and the Development of Novel Protein Fluorophores.* UPPER: The attachment of radiolabelled glucose to 5 mg/ml bovine serum albumin over an 8 day period was determined in the presence and absence of 1 mM DETAPAC. Incubations were carried out at 37°C under sterile conditions in the presence of 100 mM potassium phosphate. LOWER: Tfhe development of novel protein fluorophores was determined on incubating 5 mg/ml bovine serum albumin with [A] 25 mM glucose, [B] 25 mM glucose in the presence of 1 mM DETAPAC and [C] buffer alone (100 mM potassium phosphate pH 7.2). Fluorescence was determined using excitation at 350 nm and monitoring emission between 350 nm asnd 500 nm.

The Origin of Hydroxyl Radicals

The oxidation of glucose results in the production of measurable amounts of hydrogen peroxide, the extent of which is dependent on the presence of copper (Figure 3 inset). The role of hydrogen peroxide in the fragmenatation of bovine serum albumin, which is inhabitable by hydroxyl radical scavengers such as sorbitol (Figure 2), is demonstrated by the inhibitory effect of catalase (Figure 3). Also shown in Figure 3 is the effect of heat denatured catalase which led to a slight inhibition of fragmentation. The inhibitory effect of denatured catalase was due to the free radical scavenging activity of protein.[24]

Glucose Oxidation and Attachment to Protein

Glucose oxidation leads to the production of Girard T reactive material, ketoaldehydes (dicarbonyls).[17] Table I demonstrates that, in the absence of protein, glucose oxidation and the formation of dicarbonyl products is a transition metal dependent process. The attachment of radiolabelled glucose to bovine serum albumin is similarly partially dependent on the presence of transition metals (upper half of Figure 4). This

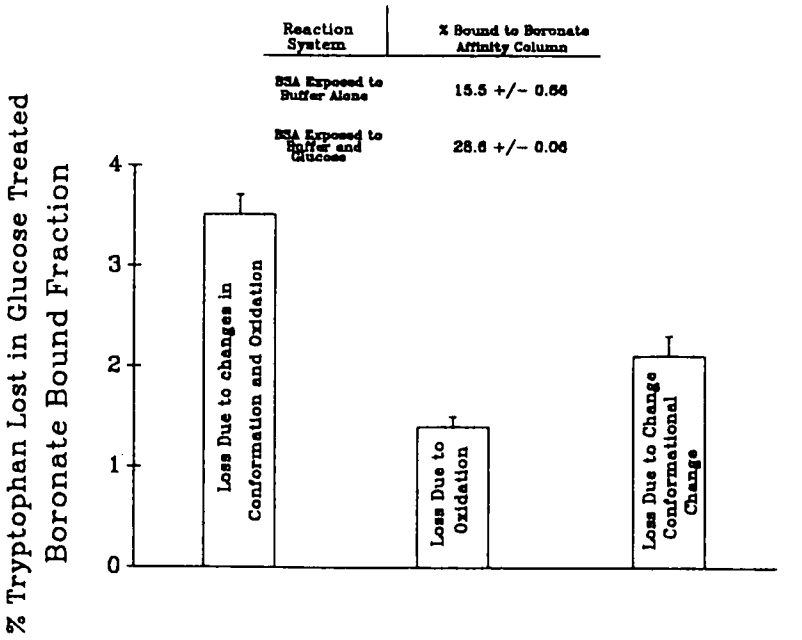
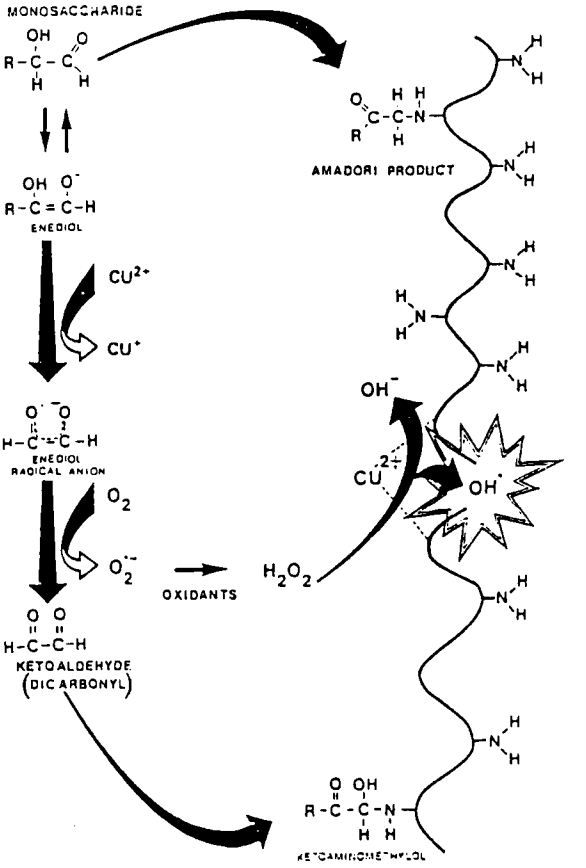


FIGURE 5 *Glucose Attachment and Protein Oxidation are Inextricably Associated.* Radiomethylated bovine serum albumin (25 mg/ml) was incubated for 3 weeks at 37°C in the presence of 100 mM potassium phosphate (pH 7.2) with or without 25 mM glucose. Incubations were prepared and carried out under sterile conditions. The reaction mixtures were then subjected to boronate affinity chromatography. The extent of attachment to the column, assessed by the presence of radioactivity in the adhering fraction, is shown in the inset. Glycated material separated by boronate affinity chromatography was then assessed for trytophan content in the presence or absence of 4 M guanidine hydrochloride. The extent of fluorescence was corrected for protein concentration (determined by radioactivity content). The percentage loss of tryptophan fluorescence occurring due to exposure to glucose, when compared to incubating in buffer alone, is shown.

is also the case for the generation of novel protein fluorophores (lower half of Figure 4). Metal-catalysed glucose oxidation may thus lead to *in vitro* protein glycation and the generation of fluorophores similar to that found *in vivo*.[1-3]

Glucose Attachment and Protein Oxidation are Inextricably Associated

So far, our studies have demonstrated that the oxidation of glucose leads to the production of oxidants capable of damaging protein (measured as either fragmentation or alterations in tryptophan fluorescence) and the production of protein reactive ketoaldehydes. We therefore considered the possibility that glycated protein is also oxidatively modified. Boronate affinity chromatography enables the separation of glycated protein.[21] The exposure of bovine serum albumin to glucose under physiological conditions leads to the generation of fluorophores (not shown) and also in an



SCHEME I *Substrate Oxidation-Derived Damage.* Autoxidisable substrates such as glucose lead to the production of dicarbonyls and oxidants. This is depicted in the scheme. Glucose oxidation is a transition metal catalysed reaction, metals such as copper may be in free solution or attached to protein. Copper attached to protein may participate in glucose oxidation, and is involved in oxidative protein alterations involving hydrogen peroxide. The attachment of glucose to protein via the Amadori pathway is also shown.

increase in boronate affinity (ca. 29%, Figure 5 inset). Albumin incubated in buffer alone also exhibited a relatively high level of boronate affinity (15%). This can be ascribed to *in vivo* glycation in the commercial product.

The separation of glycated bovine serum albumin by boronate affinity chromatography enabled the study of alterations in tryptophan fluorescence in glycated material. Figure 5 demonstrates that the exposure of bovine serum albumin to glucose leads to significant decreases in tryptophan fluorescence when compared to albumin exposed to buffer alone. This decrease in tryptophan fluorescence was due to its oxidation as well as conformational alterations induced by glucose. Our investigations thus demonstrate that glucose attachment; be it via the oxidative pathway[5,6,17] or in combination with the Amadori pathway;[1-4] is invariably accompanied by protein oxidation. This has been pictorially represented in scheme 1 in which substrate oxidation-derived damage is depicted.

CONCLUDING REMARKS

The apparent assumption that the covalent attachment of glucose to amino groups via the Amadori pathway accounts for structural changes observed in experimental glycation studies[1-4] is disputable.[5,6,17,18] Indeed, we have proposed an oxidative pathway by which products of glucose oxidation may contribute to protein glycation, leading to products indistinguishable from those produced by the Amadori pathway.[6,17]

Glucose oxidation also results in the prodution of oxidants identical in reactivity to hydroxyl radicals.[5,18] Our investigations indicate that the attachment of glucose to protein, probably via a combination of the Amadori and oxidative pathway,[6,17] is inextricably associated with protein oxidation. The combination of both oxidation and glycation may thus account for the wide variety of structural and functional alterations associated with exposure of proteins and other macromolecules to glucose *in vitro*, and perhaps also *in vivo*.

Acknowledgements

This work was funded by the Medical Research Council. We would like to thank Miss Alison Woollard for her artistic talents exhibited in the construction of the scheme shown.

References

1. A. Cerami (1986). Ageing of proteins and nucleic acids. What is the role of glucose? *Trends in Biolical Sciences*, **11**, 311–314.
2. S. Pongor, P.C. Ulrich, F.A. Bencsath and A. Cerami (1984). Ageing of proteins isolation and identification of a fluoescent chromophore from the reaction of polypeptides with glucose. *Proceedings of the National Academy of the Science U.S.A.*, **81** 2684–2688.
3. V.M. Monnier, V. Vishwanath, K.E. Frank, C.A. Elmets, P. Dauchot and R.R. Kohn (1986). Collagen-linked fluorescence is associated with increased complications in type I diabetes mellitus. *New England Journal of Medicine*, **314**, 403–408.
4. J.J. Harding (1985). Post-translational covalent modification of protein. *Advances in Proton Chemistry*, **37**, 247–334.
5. S.P. Wolff, Z. Bascal and J.V. Hunt (1989). Autoxidative Glycosylation: Free radicals and glycation theory. In: *The Maillard Reaction in Ageing, Diabetes and Nutrition*. (ed. Kunio Yagi). Alan R. Liss, Inc. pp 259–275.

6. S.P. Wolff (1987). The potential role of oxidative stress in diabetes. In: *Diabetic Complications*. (ed. Crabbe, J. M. C.). Churchill Livingstone, Edinburgh. pp 167–220.
7. D. Harman (1981). The ageing process. *Proceedings of the National Academy of Science, U.S.A.* **78**, 7124–7128.
8. D. Harman (1965). The free radical theory of ageing: Effect of age on serum copper levels. *Journal of Gerontology*, **20**, 151–153.
9. G. Noto, R. Alicata, S. Sfogliano, S. Neri and M. Bifarella (1983). A study of cupremia in a group of elderly diabetics. *Acta Diabetol. Lat.* **20**, 81–85.
10. Y. Sato, N. Hotta, N. Sakamoto, S. Matsuoka, N. Ohishi and K. Yagi (1974). Lipid peroxide level in plasma of diabetic patients. *Biochemical Medicine*, **21**, 104–107.
11. E.K. Illing, C.H. Gray and R.D. Lawrence (1951). Blood glutathione and non-glucose substances in diabetes. *Biochemical Journal*, **48**, 637–640.
12. S. Som, D. Basu, S. Mukherjee, S. Deb, P.R. Choudary, S.N. Mukherjee and S.N. Chatterjee (1981). Scorbic acid metabolism in diabetes mellitus. *Metabolism*, **30**, 572–577.
13. C.W. Karpen, S.D. Cataland, T.M. Orisio and R. Panakamala (1984). Interrelation of platelet vitamin E and thromboxane systhesis in type I diabetes mellitus. *Diabetes*, **33**, 239–243.
14. J.B. Herman, J.H. Medalie, and U. Goldbourt (1976). Diabetes, prediabetes and uricaemia. *Diabetolagra*, **12**, 47–52.
15. K. Arai, S. Iizuka, Y. Tada, K. Oikawa and N. Taniguchi (1987). Increase in the glycosylated form of erythrocyte Cu-Zn-SOD in diabetes and close association of non-enzymic glycosylation with enzyme activity. *Biochimica et Biophysica Acta*, **924**, 292-296.
16. B. Matkovics, S. Varga, L. Seabo and Witas (1982). The effect of diabetes on the acitivity of the peroxide metabolising enzymes. *Horm. etab. es.*, **14**, 77–79.
17. S.P. Wolff and R.T. Dean (1987). Glucose autoxidation and protein modification. *Biochemcial Journal*, **245**, 243–250.
18. J.V. Hunt, R.T. Dean and S.P. Wolff (1988). Hydroxyl radical production and autoxidative glycosylation. *Biochemical Journal*, **256**, 205–212.
19. J.V. Hunt, J.A. Simpson and R.T. Dean (1988). Hydroperoxide-mediated protein fragmentation. *Biochemical Journal*, **250**, 87-93.
20. J. Frew, P. Jones, and G. Scholes (1983) Spectrophotometric determination of hydrogen peroxide and organic hydroperoxides at low concentrations in aqueous solution. *Analytica Chemica Acta*, **155**, 139–150.
21. R.L. Garlick, J.S. Mazer, P.J. Higgins and H. Franklin Bunn (1983). Characterisation of glycosylated hemoglobins. *Journal of Clinical Investigation*, **71**, 1062–1072.
22. G. Marx and M. Chevion (1986). Site-specific modification of albumin by free radicals. *Biochemical Journal*, **236**, 397–400.
23. J.M.C. Gutteridge (1987). Ferrous salt promoted damage to deoxyribose and benzoate. *Biochemical Journal*, **243**, 709–714.
24. R.T. Dean, S.M. Thomas, G. Vince and S.P. Wolff (1986). Oxidation-induced proteolysis and its restriction by some secondary protein modifications. *Biomedica et Biochimica Acta*, **45**, (11–12), 1563–1573.

Accepted by Prof. G. Czapski