J. Enzyme Inhibition, 1999, Vol. 15, pp. 79–89
Reprints available directly from the publisher
Photocopying permitted by license only

NON-ENZYMATIC GLYCOSYLATION (OR GLYCATION) AND INHIBITION OF THE PIG HEART CYTOSOLIC ASPARTATE AMINOTRANSFERASE BY GLYCERALDEHYDE 3-PHOSPHATE

CATHERINE FITZGERALD, TIMOTHY A. SWEARENGIN, GEORGE YEARGANS, DAVID McWHORTER, BRAD CUCCHETTI and NORBERT W. SEIDLER*

Department of Biochemistry, University of Health Sciences, 1750 Independence Boulevard, Kansas City, MO 64106-1453, USA

(Received 26 March 1999)

Glyceraldehyde 3-phosphate (Glyc3P), a glycolytic intermediate, non-enzymatically glycosylated (or glycated) and inhibited the pig heart cytoplasmic aspartate aminotransferase (cAAT). Glyc3P (5.0 mM) decreased cAAT activity by 47% after 1 min at 23°C. cAAT activity remained unchanged after a 24 h incubation with either glucose 6-phosphate (5.0 mM) or ribose 5-phosphate (5.0 mM). Increasing the incubation pH from 6.4 to 7.8 or the incubation temperature from 23°C to 50°C enhanced Glyc3P's inhibitory effect on cAAT activity. Glyc3P (250–500 μM) decreased the thermal stability of cAAT as evidenced by lowering the T_m or temperature that caused a 50% irreversible loss of cAAT activity (69°C, control; 58.5°C, 500 μM Glyc3P). Glyc3P decreased cAAT amino group content and increased glycation products, which were measured by adduct formation, fluorescence and protein crosslinking.

Keywords: Maillard reaction; Glycation; Glyceraldehyde 3-phosphate; Asparate aminotransferase

Abbreviations: cAAT, cytosolic aspartate aminotransferase; Glyc3P, glyceraldehyde 3-phosphate; AGEs, advanced glycation endproducts; NBT, nitroblue tetrazolium; TNBS, 2,4,6-trinitrobenzene 1-sulfonic acid

* Corresponding author. Tel.: 816-283-2207. Fax: 816-283-2357.
E-mail: NSEIDLER@fac1.uhs.edu.

INTRODUCTION

Myocardial energy production depends in part on aspartate aminotransferase isozymes (EC 2.6.1.1.),[1] with fatty acids used for fuel. Carbohydrate breakdown however exceeds fatty acid utilization when circulating insulin or tissue work-load increases and during ischemic episodes.[2] In ischemia the cytosolic aspartate aminotransferase (cAAT) helps sustain glycolysis, and the mitochondrial isoform provides a residual capacity for ATP production.[3] The heart and liver, which are tissues relatively resistant to ischemic damage, contain the highest cAAT activity per tissue weight.

While cAAT's role in disease is poorly understood, liver cAAT activity decreases in diabetes[4] suggesting that cAAT is modifiable. Hypertensive hearts exhibit increased myocardial cAAT activity[5] presumably due to increased energy demands on the tissue. Interestingly, cAAT substrates, when given exogenously in the ischemic heart model, protect the myocardium against injury.[3]

Transcriptional and post-transcriptional events regulate cAAT activity, with liver but not myocardial cAAT mRNA levels under hormonal control,[6] and nutritional status is believed to play a role in regulating cAAT activity[7] involving as yet undefined mechanisms. Control may include post-translational non-enzymatic reactions such as non-enzymatic glycosylation (or glycation).

Glycation of proteins is a type of chemical modification that impairs protein function.[8] The rat liver cytosolic cAAT isoform is susceptible to *in vitro* glycation;[9] additionally liver obtained from diabetic rats contains glycated cAAT.[4] Glycating sugars react with the α- and ϵ-amino groups in proteins forming unstable Schiff bases with rearrangement products ultimately giving rise to advanced glycation endproducts (or AGEs). While all reducing sugars are capable of glycating proteins,[10] carbohydrates differ in their reactivity to proteins.[9] Glyceraldehyde 3-phosphate (Glyc3P), which is a glycolytic intermediate that becomes elevated in ischemic myocardium, was the focus of the current study. Glyceraldehyde, the dephosphorylated form of Glyc3P, reacts with hemoglobin[11] and albumin[12] and affects other proteins.[9,13–15]

The current study examined the glycating and inhibiting effects of Glyc3P on the pig heart cytosolic cAAT isoform. The dimeric cAAT protein has two identical chains each with 412 amino acids; the functional dimer contains 2α-amino groups and 38 ϵ-amino groups,[16] which are the likely target sites for glycation.[9] Pyridoxal 5-phosphate, which is an essential coenzyme, is bound to the ϵ-amino group of lysine-258 via a reversible Schiff base.[16]

MATERIALS AND METHODS

Experimental Design

Samples containing commercially prepared cAAT (0.1–1.0 mg protein/ml) (Sigma Chemical Company) were incubated with various concentrations of D,L-glyceraldehyde 3-phosphate (Glyc3P) (Sigma Chemical Company) in sodium phosphate (50–100 mM) under various conditions (i.e. incubation times, pH and temperature). Unless otherwise indicated the pH was set at 7.4. Working solutions of Glyc3P were freshly prepared for each experiment. Stock solutions of Glyc3P were kept at −70°C and potency was assessed periodically. All chemicals were of reagent grade or better.

Immediately following incubation samples were diluted or dialyzed to prevent Glyc3P from effecting the assays. Samples were either diluted 1 : 5000 in 80 mM sodium phosphate (pH 7.4) or dialyzed overnight (Spectra/Por CE Dispo-Dialyzer MWCO 10,000, Spectrum Laboratory Products) against 50 mM sodium phosphate (pH 7.4) with three changes of the dialysis medium (1000-fold). Samples were then assayed for enzyme activity and protein modification, which involved determining early glycation products (amino group content and enzyme–Glyc3P adducts) and late glycation products (fluorescence and protein crosslinking) as described below.

Enzyme Activity

Enzyme activity was measured at 37°C by a coupled-enzyme assay.[17] cAAT (0.1–0.5 μg) was added to a 1.0 ml medium containing 80 mM sodium phosphate (pH 7.4), 200 mM L-aspartate, 12 mM α-ketoglutarate, 0.2 mM NADH, and 1.1 I.U. malate dehydrogenase (Sigma Chemical Company). The reaction was followed spectrophotometrically as a decay in the 340 nm absorbance corresponding to NADH oxidation. Maximal rates were calculated using the following formula: absorbance ÷ extinction coefficient for NADH (6.22 = absorbance of one μmole NADH per ml at 340 nm in a 1 cm light path) × assay volume, ml ÷ assay time, minutes ÷ [protein], mg = μmoles/min/mg protein. The residual concentrations of Glyc3P in the assay medium were below 1.0 μM. Unless otherwise indicated pyridoxal 5-phosphate was not added to the assay medium.

Amino Group Content

Amino group content of the samples was determined spectrophotometrically using 2,4,6-trinitrobenzene 1-sulfonic acid (TNBS). TNBS reacts

rapidly with primary amino groups forming trinitrophenyl–amino acid complexes[18–20] that show characteristic absorbance increases at 420 nm. TNBS (0.17 μmol) was added to the assay medium (1.0 ml) which contained 50 mM sodium phosphate (pH 8.3) and cAAT (0.8 μg). The assay medium was placed in a 50°C water bath for 10 min, cooled to room temperature and read against TNBS controls (minus cAAT) at timed intervals. The content of amino groups was determined using β-alanine as a standard. All spectrophotometric measurements were made using either Turner (Model 350) or Bausch and Lomb (Spectronic 600) spectrophotometers.

Glycation Assay

Early glycation products, which are ketoamine adducts (or so-called Amadori products) derived from the initial unstable Schiff base adducts, were determined by a spectrophotometric procedure[21,22] involving nitroblue tetrazolium (NBT) (Sigma Chemical Company). Dialyzed samples which contained cAAT (20–40 μg) were added to the assay medium (1.0 ml) which consisted of 100 mM sodium carbonate (pH 10.8) with and without 0.25 mM NBT. Following overnight incubation at 24°C samples were read at 530 nm against minus cAAT controls. The fructosamine analogue, 1-deoxy 1-morpholinofructose (10–30 nmols) (Sigma Chemical Company), was used as a standard.

Fluorescence Measurements

Fluorescence measurements were based on the observations that prolonged incubation of sugars with proteins produces diverse products which exhibit characteristic absorbance and fluorescence spectra.[23,24] Assay media which contained 100 mM sodium phosphate (pH 7.4), cAAT (50–125 μg/ml) and Glyc3P (0–5 mM) were incubated for various times at 37°C. Fluorescence (excitation wavelength: 365 nm, band width 100 nm; emission wavelength: 460 nm, band width 10 nm) was measured using a Hoefer DQ 200 Fluorometer (Pharmacia Biotech Products). Relative fluorescence was recorded using the minus Glyc3P samples as a reference, which was set at an arbitrary value of 100.

Protein Electrophoresis

Electrophoresis was performed on pre-cast 4–20% polyacrylamide slab gels (Sigma Chemical Company) with a Tris (25 mM)–glycine (192 mM,

pH 8.3)–sodium dodecylsulfate (SDS) (0.1%, w/v) running buffer (BioRad Laboratories) using a Mini-Protean II apparatus and PowerPac 200 power supply (BioRad Laboratories). Samples were denatured by heating in sample buffer, which contained SDS (0.1%) and β-mercaptoethanol (5%, v/v) (Sigma Chemical Company), prior to electrophoresis of aliquots with 4–8 μg protein. The protein bands were stained with Coomassie Brilliant Blue Concentrate (Sigma Chemical Company), and band intensities were measured using the SigmaGel computer program (SDSS, Inc.).

RESULTS AND DISCUSSION

Glyc3P (5.0 mM) inhibited cAAT activity, which fell to 53% of control within the first minute of incubation at 23°C and to 20% of control after 50 min (Figure 1). In a previous study[9] glyceraldehyde (1.0 mM) was shown to inhibit liver cAAT activity by 50% after 50 h of incubation at 37°C. In the present study physiological concentrations of Glyc3P (500 and 50 μM, respectively) caused an 81% and 70% decline in cAAT activity after 24 and 90 h at 37°C (data not shown). These observations suggest that the presence of a phosphate may enhance Glyc3P's inhibitory effect. The Glyc3P-mediated inhibition of cAAT activity was irreversible. The loss in enzyme activity in the Glyc3P-containing samples was not recovered following dialysis or addition of 0.1 mM pyridoxal 5-phosphate (data not shown).

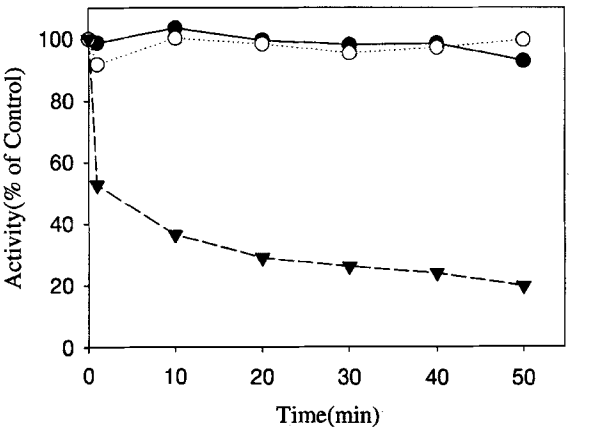

FIGURE 1 Effects of phosphorylated carbohydrates on enzyme activity. cAAT was incubated with the phosphorylated carbohydrates (5 mM) at 24°C for the indicated times. Assays were performed as described in Materials and Methods. Data represent means from 2–3 experiments. Glyc3P (▼); glucose 6-phosphate; (●) ribose 5-phosphate (○).

Contrary to the effects of Glyc3P, ribose 5-phosphate (5.0 mM) and glucose 6-phosphate (5.0 mM) did not inhibit cAAT activity when tested for 50 min (Figure 1) and after 24 h (data not shown). The differences among the sugars are consistent with previous observations.[9,12,25] Ribose 5-phosphate and glucose 6-phosphate, respectively, are 5- and 6-carbon sugars, which exist predominantly in a non-reactive cyclical form. Glyc3P is a straight chain triose. The free aldehyde group as well as the smaller size of Glyc3P may contribute to its reactivity.

Inhibition of cAAT activity by Glyc3P was concentration and temperature dependent (Figure 2). After 60 min at 20°C, 41°C and 50°C, respectively, cAAT activity was 105%, 93% and 76% of control with 1.0 mM Glyc3P and 39%, 9%, and 0% of control with 4 mM Glyc3P. These data suggest that the observed inhibition may be due to a chemical reaction like glycation rather than non-covalent binding, since temperature is known to increase glycation reaction rates.[26] It has been shown that cAAT resists denaturation as temperature is increased;[27,28] interestingly micromolar concentrations of Glyc3P decreased cAAT's thermal stability (Figure 3). In the current study untreated cAAT irreversibly lost 50% of its activity at 69°C, which was designated as I_m. The T_m for cAAT in the presence of 250 and 500 μM Glyc3P was 62.5°C and 58.5°C, respectively. The decrease in T_m with increasing concentrations of Glyc3P suggests that Glyc3P may be facilitating the denaturation of the enzyme. Increased thermal motion may further enhance glycation reactions by making cryptic sites (e.g. lysyl residues

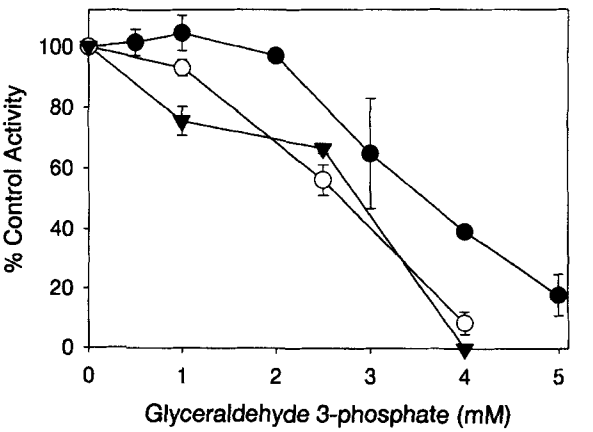

FIGURE 2 Inactivation of enzyme activity as a function of Glyc3P concentration. cAAT was incubated with various concentrations of Glyc3P for 60 min at 20°C (●), 41°C (○), and 50°C (▼). Data represent mean ± SE from 1–4 experiments.

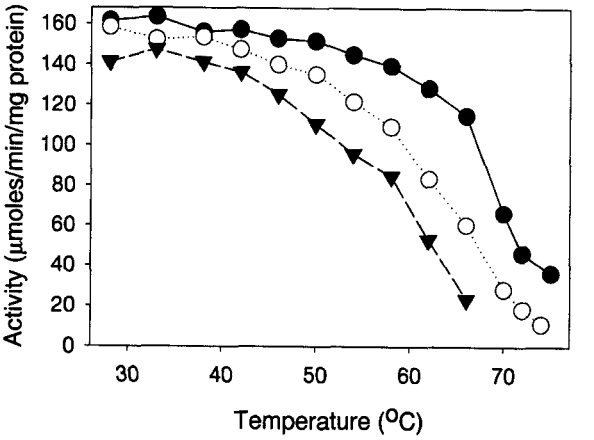

FIGURE 3 Irreversible thermal inactivation of cAAT. cAAT was incubated with various concentrations of Glyc3P for 3 min at the indicated temperatures. Samples were diluted 1 : 50, allowed to cool to room temperature, and assayed as described in Materials and Methods. Control (●); 250 μM (○); 500 μM (▼).

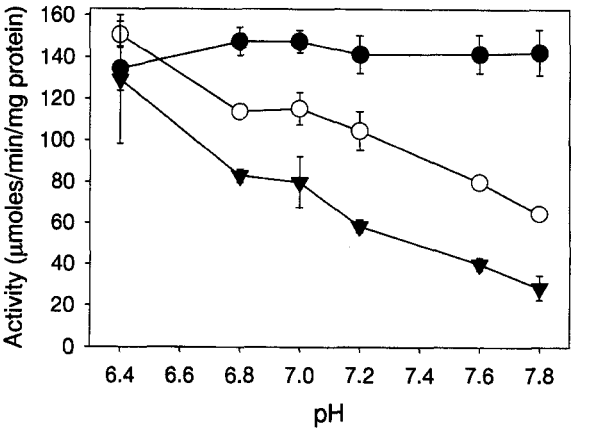

FIGURE 4 pH dependence of Glyc3P-mediated inhibition of cAAT activity. cAAT was incubated with various concentrations of Glyc3P for 93 min at 37°C in media at various hydrogen ion concentrations. Control (●); 1.25 mM (○); 2.5 mM (▼).

positioned towards the interior of the protein) more accessible. Additionally, the enzyme–Glyc3P adduct(s) may impair domain–domain interactions that may lead to loss of native conformation.

The incubation pH affected Glyc3P's inhibition of enzyme activity (Figure 4). At pH 6.4 there was no loss of activity following 90 min of incubation with Glyc3P (1.25–2.5 mM) at 37°C. However, at pH 7.2 and 7.8

activity declined by 26% and 55%, respectively, following 90 min of incubation with Glyc3P (1.25 mM) at 37°C. These data are consistent with the literature, which shows that glycation reaction rates increase in alkaline solutions.[29] A pH-induced change in protein conformation may have also contributed to Glyc3P's inhibitory effects.

Concurrent with the Glyc3P-mediated decline in cAAT activity, amino group content decreased (Figure 5, main figure) and early glycation products appeared (Figure 5, inset). Amino group content provided a measure of Glyc3P-induced chemical modification of the α and ϵ-amino groups in cAAT. Adducts between Glyc3P and protein amino groups result in the formation of secondary amino groups. TNBS only binds to primary amino groups; hence, the TNBS method monitored early glycation events. Direct measurement of glycation products by determining NBT-reactivity coincided with the loss of amino groups (Figure 5, inset). These observations suggest that the loss in cAAT activity was due to glycation of the enzyme.

We observed further evidence of glycation when we measured the fluorescent properties of cAAT samples incubated over several days with various concentrations of Glyc3P at 37°C (Figure 6). Fluorescence measurements at excitation 365 nm (band width 100 nm) and emission 460 nm (band width 10 nm) monitored the formation of AGEs. There was a time-dependent increase in fluorescence. Incubation of cAAT with 500 µM and 5 mM Glyc3P at 37°C brought about a 2-fold increase after 6 days and a 33-fold

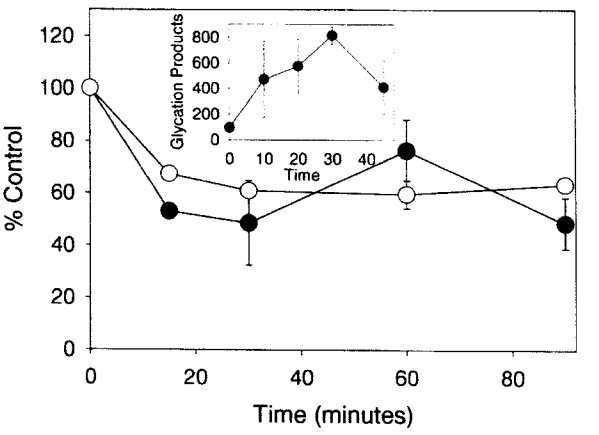

FIGURE 5 Effects of Glyc3P on enzyme activity, amino group content, and levels of glycation products. cAAT was incubated with 2.5 mM Glyc3P at 37°C for the indicated times. Samples were tested for enzyme activity (○, main figure) and amino group content (●, main figure) and levels of glycation products (●, inset) as described in Materials and Methods.

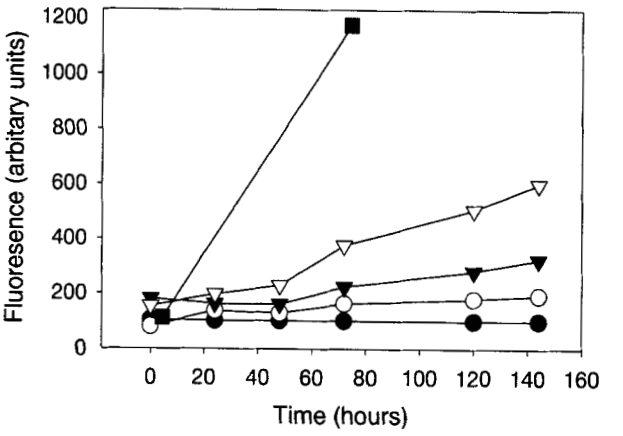

FIGURE 6 Glyc3P-mediated fluorescence of cAAT. cAAT was incubated with various concentrations of Glyc3P at 37°C for the indicated times and fluorescence measurements were made as described in Materials and Methods. Control (●); 500 μM (○); 1.0 mM (▼); 2.5 mM (▽); 5.0 mM (■).

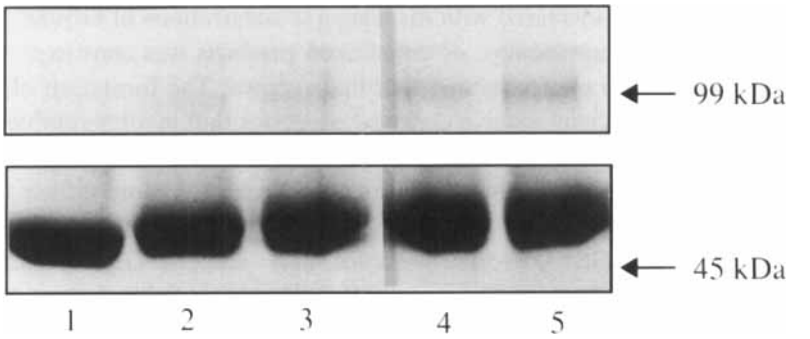

FIGURE 7 Glyc3P-mediated modification of cAAT. cAAT was incubated with various concentrations of Glyc3P (lane 1, no Glyc3P; lane 2, 0.5 mM Glyc3P; lane 3, 1.0 mM Glyc3P; lane 4, 2.5 mM Glyc3P; lane 5, 5.0 mM Glyc3P) at 37°C for 5 days and samples were electrophoresed under denaturing conditions as described in Materials and Methods.

increase after 4 days, respectively. The time scale for the appearance of fluorescence was considerably longer than the time needed to observe enzyme inhibition.

When cAAT was incubated with Glyc3P for 5 days at 37°C, in addition to the band at about 45 kDa, which represents the cAAT monomer, a 99 kDa protein band appeared in SDS-polyacrylamide gel electrophoresis (Figure 7). The apparent molecular weight of 99 kDa suggests that this protein band may be composed of a crosslinked dimer with several attached

TABLE I Quantitation of protein band intensities (in arbitrary units)[a] from SDS-polyacrylamide gel electrophoresis

	Glyc3P (mM)				
	0	0.5	1.0	2.5	5.0
99 kDa	0	20 ± 12.7	46	93 ± 23.4	127 ± 23.5
cAAT	937 ± 129.8	1016 ± 154.5	901	1126 ± 115.1	1123 ± 170.6

[a]The data, which are presented as mean ± SE, were obtained from duplicate analyses of protein bands from 2 experiments using the SigmaGel program (SDSS, Inc.).

Glyc3P molecules. Glyc3P has a molecular weight of 170 daltons suggesting that as many as 16 Glyc3P molecules per subunit may be attached to the crosslinked product. At the higher Glyc3P concentrations, an additional band appeared that migrated at 110 kDa, which may be composed of a crosslinked dimer with about 33 Glyc3P molecules attached per subunit (Figure 7). The intensities of the cAAT band at about 45 kDa and the 99 kDa protein band were quantitated (Table I). The amount of crosslinked product at 99 kDa increased with increasing concentrations of Glyc3P. The time scale for the appearance of crosslinked products was consistent with the time needed to observe changes in fluorescence. The formation of late glycation products may involve chemical processes that involve residues on different protein chains. Additionally, the cAAT band at about 45 kDa in the plus Glyc3P samples migrated more slowly and was more diffuse than the control cAAT band (Figure 7). These observations suggest that the proteins in the Glyc3P-treated samples may contain Glyc3P–enzyme adducts of varying amounts increasing the molecular weight of the protein. In summary, we observed that Glyc3P inhibited the pig heart cAAT activity and that the Glyc3P-mediated inhibition of enzyme activity increased with increasing Glyc3P concentration, incubation time, temperature and pH. Glyc3P–enzyme adducts were observed, which decreased the amino group content of cAAT, increased cAAT fluorescence, and caused protein crosslinking. These data indicate that Glyc3P glycated pig heart cAAT and that glycation of the protein correlated with loss of enzymatic activity.

Acknowledgements

The authors thank Paula Clevenger, Ruth Carrier and Bill Glosser for technical and clerical assistance. Electrophoretic protein bands were quantified by Sharagim S. Shokry.

References

[1] D.M. Cohen and R.N. Bergman (1995) *Amer. J. Physiol.*, **268**, E397.
[2] N. Sperelakis (1995) (Ed.). *Physiology and Pathophysiology of the Heart*, pp. 385–411. Boston: Kluwer Academic Publishers.
[3] O.I. Pisarenko, I.M. Studneva, V.S. Shulzhenko, O.V. Korchazhkina and V.I. Kapelko (1995) *Biochem. Mol. Med.*, **55**, 138.
[4] M. Okada, Y. Murakami and E. Miyamoto (1997) *J. Nutri. Sci. Vitamino.*, **43**, 463.
[5] A. Atlante, F. Abruzzese, T.M. Seccia, V. Vulpis, S. Doonan, A. Pirrelli and E. Marra (1995) *Biochem. Mol. Biol. Int.*, **37**, 983.
[6] M. Pavé-Preux, N. Ferry, J. Bouguet, J. Hanoune and R. Barouki (1988) *J. Biol. Chem.*, **263**, 17459.
[7] A.O. Adisa and A.A. Odutuga (1998) *Med. Sci. Res.*, **26**, 25.
[8] M.X. Fu, K.J. Knecht, S.R. Thorpe and J.W. Baynes (1992) *Diabetes*, **41**, 42.
[9] M. Okada, A. Sogo and N. Ohnishi (1994) *J. Nutri. Biochem.*, **5**, 485.
[10] C.A.L.S. Colaco (1997) (Ed.). *The Glycation Hypothesis of Atherosclerosis*, pp. 57–87. Austin, Texas: Landes Bioscience.
[11] A.S. Acharya and J.M. Manning (1980) *J. Biol. Chem.*, **255**, 7218.
[12] I. Syrovy (1994) *J. Biochem. Biophys. Meth.*, **28**, 115.
[13] A.K. Bures, H.H. Daron and J.L. Aull (1991) *Int. J. Biochem.*, **23**, 733.
[14] M. Callens and F.R. Opperdoes (1991) *Mol. Biochem. Parasitol.*, **47**, 11.
[15] M. Kohlhardt, H. Fichtner and U. Frobe (1989) *FASEB J.*, **3**, 1963.
[16] P.D. Boyer (1973) (Ed.). *The Enzymes*, pp. 379–481. New York: Academic Press.
[17] Recommendations of the German Society for Clinical Chemistry (1972) *Z. Klin. Chem. Klin. Biochem.*, **10**, 281.
[18] P. Cayot and G. Tainturier (1997) *Anal. Biochem.*, **249**, 184.
[19] Y. Endo (1983) *Meth. Enzymol.*, **94**, 42.
[20] U.P. Steinbrecher (1987) *J. Biol. Chem.*, **2**, 3603.
[21] G.M. Ghiggeri, G. Candiano, F. Ginevri, R. Olegginin, M.T. Piccardo, R. Bertelli, F. Perfumo and R. Gusmano (1988) *Analyst*, **113**, 1101.
[22] R.N. Johnson, P.A. Metcalf and J.R. Baker (1982) *Clin. Chim. Acta*, **127**, 87.
[23] S. Pongor, P.C. Ulrich, F.A. Bencsath and A. Cerami (1984) *Proc. Nat. Acad. Sci. USA*, **81**, 2684.
[24] D.R. Sell and V.M. Monnier (1989) *Connective Tissue Res.*, **19**, 77.
[25] H.F. Burn and P.T. Higgins (1981) *Science*, **213**, 222.
[26] C.H. Lea and R.S. Hannan (1949) *Biochim. Biophys. Acta*, **3**, 313.
[27] C. Twomey, S. Doonan and A. Giartosio (1995) *Biochem. Soc. Trans.*, **23**, 396S.
[28] M. Martinez-Carrion, A. Artiques, A. Berezov, M.L. Bianconi, A.M. Reyes and A. Iriarte (1995) *Meth. Enzymol.*, **259**, 590.
[29] S.H. Ashoor and J.B. Zent (1984) *J. Food Sci.*, **49**, 1206.