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GLYCATION OF COLLAGEN IN HYPERTRIGLYCERIDEMIC RATS

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Summary

Nonenzymatic collagen glycation and modification with lipid derived metabolites was studied in rat skin and tail tendon collagen of control and hypertriglyceridemic (HTG) rats. Age-dependent changes typical for lipid and sugar derived adducts were evaluated by measuring fluorescence of these collagens at wavelengths typical for sugar (335/385 and 370/440 nm) and lipid derived adducts (356/460 and 390/460 nm). In addition pentosidine assay (corresponding to the fluorescence parameters 335/385 nm) was performed as well. It was found that pentosidine concentration as well as fluorescence intensities in skin collagen was the same for control and HTG rats and significantly increased with age. On the other hand, no significant age-dependent changes in fluorescence intensities were observed in tail tendon collagen. Pentosidine concentration in tail tendon collagen was much lower than that in skin and it was decreased in young HTG rats compared to cotrol ones. It increased with age, more distinctly in HTG rats than in their control counterparts, in such a way that at the age of 19 months the pentosidine levels were undistinguishible in both rat strains. Possible mechanisms underlying these results are discussed.

Key Words: collagen, glycation, hypertriglyceridemic rats

Nonenzymatic collagen glycation and reaction with lipid peroxidation products bearing an aldehydic group are spontaneous chemical modifications of proteins, caused by reactions between free oxo-groups (sugar aldehydes or ketones and products of lipid metabolism) and free aminogroups of peptide chains. These modifications are accumulated under normal physiological conditions (aging) or pathophysiological situations (diabetes). Glycation starts by condensation of oxo- and amino- groups; the resulting aldimine undergoes an Amadori rearrangement to form more stable Amadori product. The arising moieties are called "early glycation products". In a set of next steps these compounds undergo further reactions producing fluorophores and chromophores the chemical nature of which has not been fully elucidated yet. These products are known collectively

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as AGE (advanced glycosylation end products) or "browning products" (1-4). Most of the work in this respect refers to sugar-protein interactions (i.e. nonenzymatic glycation). However, analogous reactions can involve aldehydes derived from lipoperoxidation, such as malondialdehyde or hydroxynonenal. These two sources of reactive aldehydes form fluorescent end products which are accumulated in long-lived proteins during aging (5-9).

The most significant, fully identified product of this category is pentosidine described by Sell and Monnier (10). From the analytical point of view the advantage of this product follows from its acid-hydrolysis resistance and the resulting possibilities of its quantitation in proteins. Pentosidine arises in a reaction between free epsilon groups of lysyl and arginyl residues in a peptide chain with free oxo groups of pentoses or hexoses (11). The content of this product in collagen increases with aging and in diabetes and is decreased by caloric restriction, however, it constitutes only a minor proportion of glycated adducts found in long-lived proteins (12-16).

In this study we have used hereditary hypertriglyceridemic (HTG) rats which represent genetic model of hypertension with metabolic disturbances of lipid and carbohydrate metabolism (17-19). This animal model seemed to be suitable for testing the posttranslational products arising from both the reaction with reducing sugars and lipid-derived oxo compounds. Fluorescence determination at wavelengths typical for glucose adducts, lipid-derived compounds and pentosidine content were used as indicators of protein modification.

Materials and Methods

Experimental animals: Hereditary hypertriglyceridemic (HTG) rats were originally selected from Wistar rats as previously described (20). HTG rats aged 7, 14 and 19 months (bred in Institute of Physiology, Academy of Sciences of the Czech Republic, Prague) were studied. Age-matched normotensive Wistar-derived Lewis rats were used as controls. Rats were housed under controlled conditions (temperature 23±1 °C, 12 h : 12 h light-dark cycle). They were fed a standard rat chow (Velaz, ST-1) containing 0.4% sodium chloride. Water and food were available *ad libitum*.

Blood pressure was measured by a direct puncture of the carotid artery under light ether anaesthesia. Serum triglycerides and glucose were determined by commercial kits (Lachema, Brno, Czech Republic).

Preparation of collagen samples: Samples of skin collagen were prepared according Dunn *et al.* (21). Briefly, after thawing, the skin samples were scraped vigorously with a scalpel to remove adherent fat and other tissues, and then extracted sequentially for 24 h at 4°C with 1 M NaCl, chloroform-methanol (2:1), and 0.5 M acetic acid to remove lipids and soluble proteins. After the final extraction, the samples were rinsed in distilled water and dried by lyophilization.

Tendons dissected from tails were washed three times with 1 M NaCl and then dried by lyophilization.

Collagen content in individual samples were assayed by the hydroxyproline method (22).

Preparation of collagen digest: For the measurement of collagen-linked fluorescence collagen was digested by collagenase according the procedure described previously (8). Ten mg of collagen were homogenized in 1.0 ml of buffer (0.01 M $CaCl_2$/0.02 M Tris-HCl, pH 7.55) containing 0.05% toluene to prevent bacterial growth. To each sample 0.5 ml of solution containing 1 mg collagenase (from *Clostridium histolyticum*, 0.8 U/mg; Fluka, Buchs, Switzerland) dissolved in the above specified Tris-HCl buffer was added. The samples were digested with shaking at 37°C for 24 h.

Preparation of samples for pentosidine measurement: For the pentosidine assay (23), 10 mg of collagen (dry weight) was wetted by 2.5 ml of water, heated at 65°C for 2 min and then reduced by addition of 5 ml 3.3 mM $NaBH_4$ in 0.1 M NaOH. After incubation for 18 h at room temperature, residual borohydride was discharged and the sample diluted to contain 2 mg/ml of digested

collagen by the addition of an equal volume of concentrated HCl. Borohydride reduction decreased fluorescence background and was not essential for pentosidine measurement. The protein was then hydrolyzed at strictly deareated conditions under N_2 for 18 h at 95°C. HCl was removed by rotary evaporation.

HPLC assay for pentosidine: HPLC was carried out using a Waters automated gradient controller (Millipore, Milford, MA, USA) with Waters Model 510 pumps. The steel column Supelcosil LC-318 (250 x 4.6 mm I.D., 5 μm, 300 Å pores; Supelco, Bellefonte, PA, USA) was mounted in the instrument. The sample was dissolved in 300 μl 2% heptafluorobutyric acid (HFBA) and 100 μl was injected into the column. Elution was done by a linear gradient H_2O (A) – 50% acetonitrile (B) (both containing 0.1% HFBA) from 10% B to 21% B at 15 min, next 10 min isocratic washing with 21% B was followed by 10 min isocratic washing with 100% B. The flow rate was 1.0 ml/min and the column temperature was held at 30°C. The eluate was monitored by fluorescence at $328_{ex}/378_{em}$ nm using a Fluorescence monitor RF-530 (Shimadzu, Kyoto, Japan). Peak areas were integrated using software Apex v3.1 (DataApex, Prague, Czech Republic).

Preparation of pentosidine standard: The pentosidine standard was prepared by a modified procedure of Dyer *et al.* (23). Briefly, an aqueous solution (1000 ml, 100 mM N^{α}-acetylarginine, lysine and glucose in 200 mM phosphate buffer, pH 9.0) was heated at 65°C for 1 day. The solution was lyophilized and the product was extracted with 200 ml of methanol. Methanol was removed by rotary evaporation, the residue dissolved in 150 ml H_2O (with 0.1% HFBA) and 1 ml aliquots were applied to a Sep-Pak C18 cartridge (Waters), washed with H_2O (with 0.1% HFBA), crude product was eluted by 15% acetonitrile (with 0.1% HFBA). Eluates were pooled, dried and then disolved in 1% trifluoracetic acid (TFA). Crude pentosidine was purified in two subsequent separation steps by HPLC using a Supelcosil LC-318 column (250 x 4.6 mm I.D., 5 μm, 300 Å pores). In the first step separation was done with 0.1% TFA while in the second step TFA was replaced by 0.1% HFBA. After de-acetylation by heating in 6 M HCl for 1 h at 110°C, pentosidine was finally purified by the system with 0.1% HFBA. Identification of pentosidine was confirmed by measuring of its flurescence spectra.

Spectroscopical measurements: Fluorescence spectra were measured with LS 50B Luminescence Spectrometer (Perkin Elmer, Beaconsfield, UK) and fluorescence intensities at designed wavelengths were measured using LS-5 Luminescence Spectrometer (Perkin Elmer).

Statistical analysis: Statistical calculations were based on the Student's t-test and data were expressed as mean ± SD (standard deviation).

Results

Experimental animals were characterized by body weight, blood pressure, plasma triglyceride levels and fasted and non-fasted plasma glucose (see Table 1). Body weight of HTG rats was significantly lower as compared to controls. This finding suggests that these animals were not obese. HTG rats had markedly higher plasma triglycerides and elevated blood pressure.

Fluorescence intensities were measured at four wavelength combinations — 370/440 nm (excitation/emission) and 335/385 nm for glycation-related products, and at 356/460 nm and 390/460 nm for the protein adducts of hydroxynonenal and malondialdehyde (7). In the case of skin collagen (Table 2), similar significant age-related changes in fluorescence intensities at all measured wavelengths were observed. There were no differences between control and HTG rats. On the contrary, in the case of tail tendon collagen (Table 3) no significant changes depend on age or strain were observed.

Direct analysis of pentosidine level in rat skin collagen indicated a significant increase with age. There were, however, no differences between HTG and control groups (Table 4 and Fig. 1). Pentosidine showed a linear increase in both cases with the similar slope (HTG: $y = -6.27 + 5.09x$;

TABLE 1

Basal characteristics of experimental animals (BW - body weight, SBP - systolic blood pressure, TG - serum triglycerides).

Experimental group		BW g	SBP mm Hg	TG mmol/l	Serum glucose non-fasted mmol/l	Serum glucose fasted mmol/l
7 months	control	386 ± 20	126 ± 17	1.47 ± 0.62	10.22 ± 2.94	4.02 ± 0.65
	HTG	322 ± 32***	144 ± 10*	2.49 ± 0.59**	9.19 ± 2.30	6.42 ± 1.00***
14 months	control	474 ± 11	135 ± 13	1.84 ± 0.40	9.02 ± 1.48	4.48 ± 1.14
	HTG	335 ± 26***	174 ± 20**	2.38 ± 0.22*	9.71 ± 3.50	6.60 ± 1.44*
19 months	control	498 ± 54	124 ± 11	1.91 ± 0.38	10.49 ± 1.52	6.02 ± 0.85
	HTG	329 ± 44**	156 ± 18*	2.55 ± 0.32*	8.06 ± 2.16	5.04 ± 0.70

Statistical differences from controls: *P<0.05, **P<0.01, ***P<0.001.

TABLE 2

Fluorescence of glycation and lipoperoxidation products in skin collagen of hypertriglyceridemic (HTG) and control rats (mean ± SD).

Age (months)	Experimental group (n)	Fluorescence intensities (a.u.)			
		Glycation products		Lipoperoxidation products	
		370/440 nm	335/385 nm	356/460 nm	390/460 nm
7	control (8)	62 ± 19	71 ± 23	61 ± 19	35 ± 12
	HTG (6)	60 ± 14	67 ± 17	59 ± 13	33 ± 8
14	control (7)	75 ± 7	85 ± 10	74 ± 7	45 ± 5
	HTG (4)	76 ± 13	92 ± 17	75 ± 12	41 ± 8
19	control (5)	96 ± 11°° **	121 ± 15°° ***	93 ± 11°° **	57 ± 7 °° **
	HTG (4)	85 ± 4 °°	107 ± 6 °°	83 ± 4 °°	49 ± 4 °°

Statistical differences: from 7-months-old animals °°P<0.01
from 14-months-old animals **P<0.01, ***P<0.001

TABLE 3

Fluorescence of glycation and lipoperoxidation products in tail tendon collagen of hypertriglyceridemic (HTG) and control rats (mean ± SD).

Age (months)	Experimental group (*n*)	Fluorescence intensities (a.u.)			
		Glycation products		Lipoperoxidation products	
		370/440 nm	335/385 nm	356/460 nm	390/460 nm
7	control (8)	52 ± 13	69 ± 18	69 ± 13	25 ± 9
	HTG (6)	63 ± 11	79 ± 14	64 ± 12	33 ± 9
14	control (7)	63 ± 21	74 ± 20	62 ± 20	35 ± 9
	HTG (4)	64 ± 14	77 ± 18	63 ± 14	38 ± 9
19	control (5)	70 ± 20	85 ± 25	70 ± 25	39 ± 18
	HTG (4)	68 ± 14	82 ± 7	65 ± 13	34 ± 8

No significant statistical differences were observed.

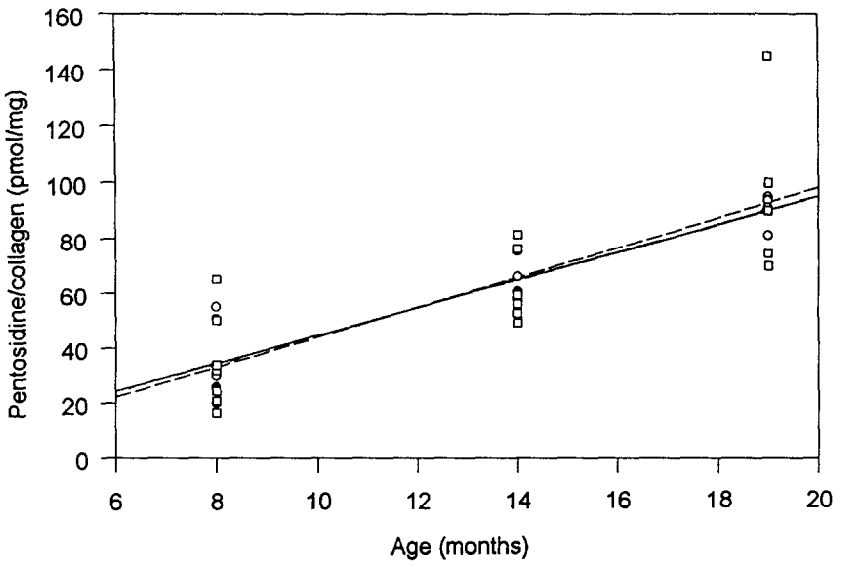

Fig. 1

Age-dependent changes in the concentration of pentosidine in rat skin collagen for control (□, dashed regression line) and HTG (○, full regression line) animals.

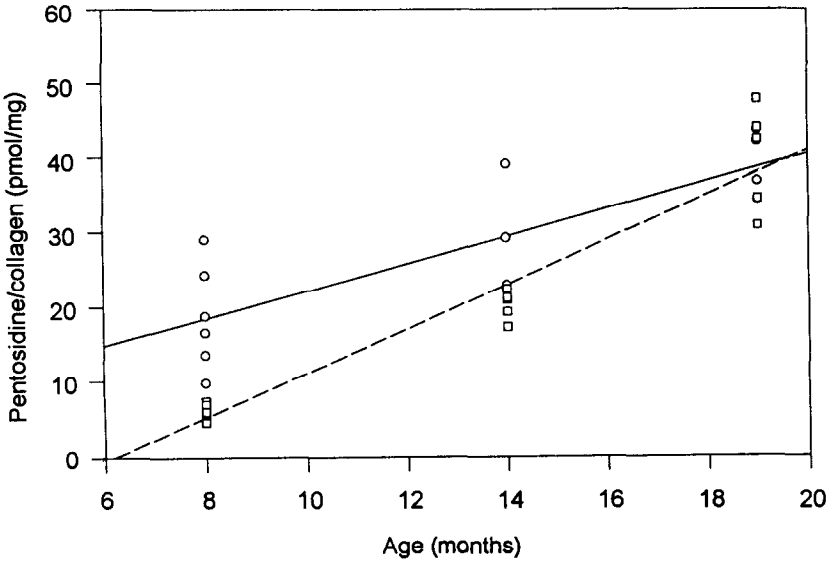

Fig. 2

Age-dependent changes in the concentration of pentosidine in rat tail tendon collagen for control (□, dashed regression line) and HTG(○, full regression line) animals.

TABLE 4

Pentosidine content in skin and tail tendon collagen of hypertriglyceridemic (HTG) and control rats (mean ± SD).

Age (months)	Experimental group (*n*)	Pentosidine/collagen (pmol/mg)	
		skin	tail tendon
7	control (8)	34.5 ± 16.0	6.4 ± 1.0
	HTG (6)	34.4 ± 14.7	18.7 ± 7.0†††
14	control (7)	62.2 ± 12.2°°	20.9 ± 1.7°°°
	HTG (4)	65.2 ± 7.7°°	28.6 ± 7.7†
19	control (5)	96.0 ± 29.9°°° *	39.4 ± 7.0°°° ***
	HTG (4)	90.4 ± 6.2°°° **	39.3 ± 4.3°°°

Statistical differences: from 7-months-old animals °°$P<0.01$, °°°$P<0.001$
from 14-months-old animals *$P<0.05$, **$P<0.01$, ***$P<0.001$
from control animals of the same age †$P<0.05$, †††$P<0.001$

R = 0.922, P<0.0001 and control: y = −10.70 + 5.46x; R = 0.802, P<0.0001). A completely different picture was seen with the tail collagen (Table 4 and Fig. 2). In younger animals (aged 7 months) there was a distinct difference between the HTG and control groups (three times higher level of pentosidine in HTG, $P < 0.001$); at the age of 14 months this difference was diminished and concentration of pentosidine was only about 40% higher in HTG ($P < 0.05$). In animals aged 19 months there was no difference of pentosidine level in tail tendon collagen between both strains. Thus the pentosidine concentration in tail tendon collagen showed an age-dependent linear increase, though with different slopes in HTG and control rats (HTG: y = 3.68 + 1.85x; $R = 0.823$, $P = 0.0003$ and controls: y = −18.3 + 2.97x; $R = 0.961$, $P < 0.0001$).

Discussion

Collagen-linked fluorescence which reflects advanced protein glycation is measured routinely at 370/440 nm (8,9,24), while the respective parameters for pentosidine are 335/385 nm (12,13). In addition Odetti *et al.* (7) determined fluorescence of lipoperoxidation-related products derived from hydroxynonenal at 356/460 nm and malondialdehyde at 390/460. It was reported (7) that all these fluorescence intensity parameters increased with aging. Our findings on control animals are in concert with published data as far as skin is concerned. However, in spite of the fact that HTG animals had a higher serum triglycerides, they did not exhibit any increase of fluorescence typical for lipoperoxidation products. This indicates that *in vivo* posttranslational modifications represent a more complex system than can be expected from *in vitro* experiments. There are several reasons for this situation. First of all not all modifying metabolites can be readily available at target place (lysine and/or arginine residues) because of different blood supply, different penetration of reactive metabolites through tissues and because of different packing of collagen protofibrils in different tissues which may make some reactive amino groups sterically not available.

As documented in Tables 2 and 3 there was a slight age-dependent increase observed at fluorescence parameters corresponding either to hydroxynonenal or malondialdehyde adducts in skin collagen but no age-dependent difference at these fluorescence parameters was seen in tail tendons. Surprising enough, no difference in the lipid-derived products fluorescence parameters was observed in skin collagen of HTG rats when compared with controls. It may be speculated that in normotriglyceridic rats all the predisposed sites in skin collagen are altered by the reactive metabolites and a further increase of serum triglycerides in HTG rats is consequently without effect.

Pentosidine fluorescence in tail tendon collagen showed slight, but not significant age-related increase, which, however did not correspond to the highly significant increase observed when pentosidine was assayed directly. It must also be taken into consideration that the lack of a statistically significant increase of fluorescence parameters in tail collagen may be just due to the limited number of animals. In case of skin, pentosidine fluorescence and directly assayed pentosidine concentration increased significantly with age. This finding is in good agreement with the published data (12-16). On the other hand, the pentosidine level was much lower in tail tendon collagen compared to skin. The lowest concentration was seen in young HTG rats. However, it increased with age much faster in HTG rats than in controls, being almost identical at the age of 19 months in both groups, though it was still lower than one half of pentosidine concentration observed in skin.

The reasons may be either in the different accessibility of the reactive metabolite (glucose) to the target amino groups (due to differences in blood supply or sterical hindrances in differently packed collagen fibers) or in the presence of additional reactive sites which the reactive metabolites may meet along their transport between the vascular supply and the target collagen fibre.

From the comparison of direct pentosidine measurement (by HPLC) and pentosidine fluorescence (335/385 nm) it may be concluded that fluorescence method cannot be easily used for pentosidine determination. In fact, complex reactions of posttranslational modifications may yield

various, probably fluorescent, products. From this view of point other results obtained by fluorescent measurement must also be considered. Fluorescence intensities at certain wavelengths need not only reflect a given product (e.g. lipid-derived) but other arising compounds may also contribute to the fluorescence observed.

It is demonstrated here that long-lived proteins (collagens) in different connective tissues are differently available for posttranslational modifications and that results obtained with tail tendons as a typical connective tissue model (e.g. refs. 25,26) cannot be automatically applied to other soft connective tissues in the body. It appears that in general pentosidine is formed at a much lower rate in tail tendon than in skin, moreover, in tendon considerable strain differences in pentosidine formation exist in young animals where in the HTG group very little of this cross-linking amino acid is formed. In addition to the biological aspects discussed, one has to keep in mind the differences in the proportion of individual collagen types in different tissues.

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References

1. K.M. REISER, Proc. Soc. Exp. Biol. Med. 196 17-29 (1991).
2. F. LEDL and E. SCHLEICHER, Angew. Chem. Int. Ed. Engl. 29 565-594 (1990).
3. J.J. HARDING, Adv. Protein Chem. 37 247-325 (1985).
4. K.M. REISER, M.A. AMIGABLE and J.A. LAST, J. Biol. Chem. 267 24207- 24216 (1992).
5. M. TSUCHIDA, T. MIURA, K. MUZITANI and K. AIBARA, Biochem. Biophys. Acta 834 196-204 (1985).
6. J.V. HUNT, C.C.T. SMITH and S.P. WOLFF, Diabetes 39 1420-1424 (1990).
7. P. ODETTI, M.A. PRONZATO, G. NOBERASCO, L. COSSO, N. TRAVERSO, D. COTTALASSO and U.M. MARINARI, Lab. Invest. 70 61-67 (1994).
8. I. MIKŠÍK and Z. DEYL, J. Gerontol. 46 B111-116 (1991).
9. I. MIKSIK, R. STRUZINSKY and Z. DEYL, Mech. Ageing Dev. 57 163-174 (1991).
10. D.H. SELL and V.M. MONNIER, J. Biol. Chem. 264 21597-21602 (1989).
11. S.K. GRANDHEE and V.M. MONNIER, J. Biol. Chem. 266 11649-11653 (1991).
12. D.G. DYER, J.A. DUNN, S.R. THORPE, K.E. BAILIE, T.J. LYONS, D.R. MCCANCE and J.W. BAYNES, J. Clin. Invest. 91 2463-2469 (1993).
13. D.R. MCCANCE, D.G. DYER, J.A. DUNN, K.E. BAILIE, S.R. THORPE, J.W. BAYNES and T.J. LYONS, J. Clin. Invest. 91 2470-2478 (1993).
14. W.T. CEFALU, A.D. BELL-FARROW, Z.Q. WANG, W.E. SONNTAG, M.-X. FU, J.W. BAYNES and S.R. THORPE, J. Gerontol. 50A B337-B341 (1995).
15. P.J. BEISSWENGER, L.L. MOORE, T. BRINCK-JOHNSEN and T.J. CURPHEY, J. Clin. Invest. 92 212-217 (1993).
16. D.R. SELL, R.H. NAGARAJ, S.K. GRANDHEE, P. ODETTI, A. LAPOLLA, J. FOGARTY and V.M. MONNIER, Diabetes/Metab. Rev. 7 239-251 (1991).
17. I. KLIMEŠ, A. VRÁNA, J. KUNEŠ, E. ŠEBÖKOVÁ, Z. DOBEŠOVÁ, P. ŠTOLBA and J. ZICHA, Blood Press. 4 137-142 (1995).
18. P. ŠTOLBA, Z. DOBEŠOVÁ, P. HUŠEK, H. OPLTOVÁ, J. ZICHA, A. VRÁNA and J. KUNEŠ, Life Sci. 51 733-740 (1992).
19. J. KUNEŠ, Z. DOBEŠOVÁ and J. ZICHA, Physiol. Res. 44 421-424 (1995).
20. A. VRÁNA and L. KAZDOVÁ, Transplant. Proc. 25 2579 (1990).
21. J.A. DUNN, D.R. McCANCE, S.R. THORPE, T.J. LYONS and J.W. BAYNES, Biochemistry 30 1205-1210 (1991).
22. H. STEGEMANN and K. STALDER, Clin. Chim. Acta 18 267-273 (1967).

23. D.G. DYER, J.A. BLACKLEDGE, S.R. THORPE and J.W. BAYNES, J. Biol. Chem. 266 11654-11660 (1991).
24. J.J. TOMASEK, S.W. MEYERS, J.B. BASINGER, D.T. GREEN and R.L. SHEW, Life Sci. 55 855-861 (1994).
25. I.A. TRONCOSO, M.M. ESTEBAN, M.A. RUIZ, L. FLOREZ and L. BARNEO, Transplant. Proc. 27 3345-3346 (1995).
26. S. TANAKA, G. AVIGAD, E.F. EIKENBERRY and B. BRODSKY, J. Biol. Chem. 263 17650-17657 (1988).