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Quantification of Glycated Hemoglobin by Electrospray Ionization Mass Spectrometry†

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Glycated hemoglobin, considered to be the best index for the treatment of diabetes mellitus, was measured by electrospray ionization mass spectrometry (ESI/MS) according to the method proposed by Morris *et al.* at the 44th ASMS Conference on Mass Spectrometry and Allied Topics, 1996. They compared the values obtained by MS and affinity chromatography. Here, the values obtained by ESI/MS were compared with those obtained by high-performance liquid chromatography and by latex agglutination immunoassay. Whole blood samples were diluted 500 fold with 0.2% formic acid–50% acetonitrile solution and 5 µl of the diluted solution was injected with the ESI/MS system (TSQ 7000) via a sample loop. The within-run and between-run relative standard deviations of the ratio of glycated and non-glycated β-chain were less than 5%. The correlation coefficients between ESI/MS and conventional methods were higher than 0.96. However, considerable discrepancies were observed among methods. ESI/MS will allow reproducible measurements of glycated hemoglobin and will be useful in the quality control of HbA1c measurement by other principles and also in routine clinical laboratory tests. © 1997 by John Wiley & Sons, Ltd.

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INTRODUCTION

The measurement of glycated hemoglobin in blood is an excellent tool for the long-term control of the glycemic state of diabetes mellitus patients.[1] HbA1c is the major component of glycated hemoglobin and is believed to be the best index for the treatment of diabetes mellitus. HbA1c is biochemically characterized as a stable adduct of glucose to the NH_2-terminal amino group of the β-chain of hemoglobin A_0 [*N*-(1-deoxyfructosyl)hemoglobin].[2] A wide variety of analytical methods are currently used by clinical laboratories for the measurement of glycated hemoglobin in blood.[3]

The methods for the assay of glycation are based on charge difference[4] (ion-exchange chromatography, electrophoresis and isoelectrophoresis), on the affinity between the *cis*-1,2 diol groups of hemoglobin-bound glucose and immobilized boronic acid[5] and on antigenic differences between glycated- and non-glycated-hemoglobin.[6] Medical laboratories can currently choose between more than 20 different commercially available tests. However, the glycated hemoglobin value varies considerably with the method of measurement.[3] The molecular mass difference between glycated and non-glycated globin chains is 162 u, which is expected to be well separated by mass spectrometry (MS). MS is expected to give more precise glycated hemoglobin values than other conventional methods.

Lapolla *et al.*[7] suggested the usefulness of matrix-assisted laser desorption/ionization (MALDI) for the determination of glycated and non-glycated α- and β-chains in the diagnosis of diabetes mellitus. They did not present any quantitative data and did not compare the measurements of glycated hemoglobin with those of other analytical methods. Morris *et al.*[8] reported more successful measurements using electrospray ionization mass spectrometry (ESI/MS) for the determination of glycated hemoglobin. They reported that the procedure gave good precision and reproducibility between and within analyses and that the MS results compared favorably with those given by affinity chromatography. We measured glycated hemoglobin by ESI/MS according to the procedure reported by Morris *et al.*[8] after modification for calculation of the peak height ratio. We compared the data obtained by this ESI/MS

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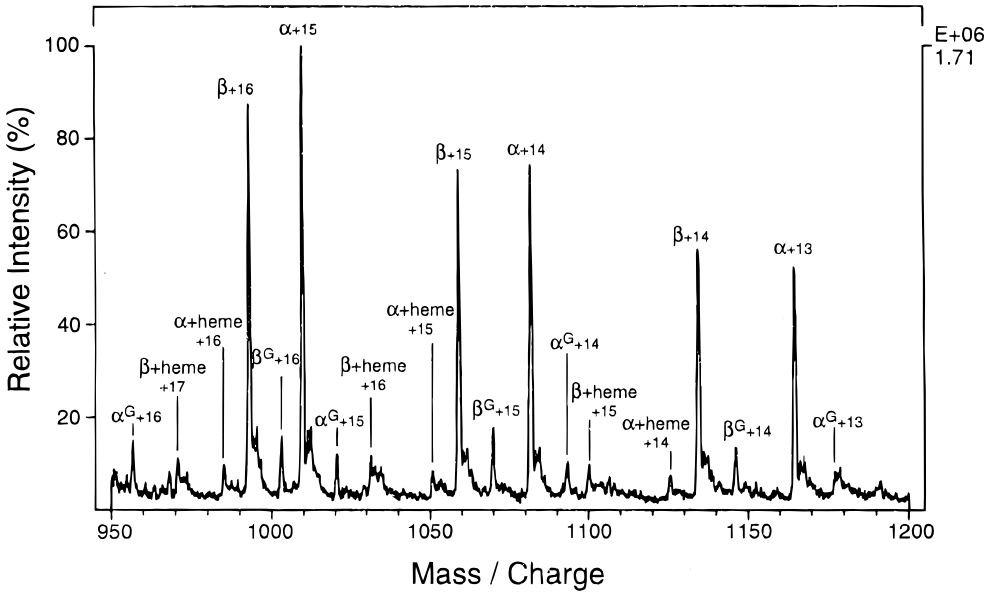

Figure 1. Raw ESI mass spectrum of hemolysate taken from a diabetic patient. Deconvoluted data are shown in Fig. 2 and the predicted assignments are given in Table 1. α^G, glycated α-chain; β^G, glycated β-chain.

method with those obtained by HPLC and the latex agglutination method. The idea of measuring glycated hemoglobin by MS occurred to the both groups through their experience of abnormal hemoglobin studies using MS, which was originally advanced by Wada *et al.*[9]

EXPERIMENTAL

Preparation of hemoglobin solution

Blood specimens were collected in EDTA-containing tubes from healthy control and diabetic patients (total number 96). Volumes of 10 μl of whole blood samples were diluted in 490 μl of deionized water and an aliquot (20 μl) of the solution was mixed with 40 μl of 1% (v/v) formic acid, 40 μl of deionized water and 100 μl of HPLC-grade acetonitrile to give the working solutions for mass spectrometric analysis. The final solution was 50% aqueous acetonitrile containing 0.2% formic acid.

Measurement of HbA1c by high-performance liquid chromatography (HPLC) and by latex agglutination methods

HbA1c was measured by fully automated ion-exchange (HPLC) using a Hi-AUTOA1c HA-8121 HbA1c analyzer (Kyoto Daiichi Kagaku, Kyoto, Japan) according to the procedures recommended by the manufacturer. The labile fraction of glycated hemoglobin, which is the reversible glycated component with a Schiff base, was converted in to non-glycated form by addition of a reagent (4-polyphosphate) dissolved in dilution buffer.[10]

HbA1c was also measured by the latex agglutination method using reagents and equipment purchased from Roche Diagnostic Systems (F. Hoffmann-La Roche, Basle, Switzerland). Hemolysate was treated with pepsin and liberated glycated peptides from the amino-terminus of the β-chain were determined using latex coated with specific mouse monoclonal antibodies against glycated peptides from the amino terminus of the β-chain. The total hemoglobin concentration was determined by measuring the absorbance at 550 nm at alkaline pH. These procedures were performed automatically by a Cobas Mira system (F. Hoffmann-La Roche). Standard hemoglobin specimens for HbA1c analysis provided by manufacturer were measured by the three methods. Specimen HbA1c Kokusai for HPLC assay, was purchased from International Reagents (Kobe, Japan), HbA1c Roche for latex agglutination was purchased from F. Hoffmann-La Roche.

Mass spectrometry

A 5 μl aliquot of the above-prepared solution was introduced via a sample loop at a flow rate of 5 μl min^{-1} into the electrospray source of a TSQ 7000 triple-stage quadrupole mass spectrometer (Finnigan MAT, San Jose, CA, USA). ESI scanning was performed from m/z 900 to 1300 in 8 s and the results were summed over 1.5 min to obtain the final spectrum. Usually the data acquisition time was 4 min as the ions may remain in the electrospray chamber after infusion has stopped. The spectra of multiply charged ions were transformed by using software provided by the manufacturer, according to Watkins *et al.*[11] Calibration was performed by using multiply charged ions from horse heart apomyoglobin.

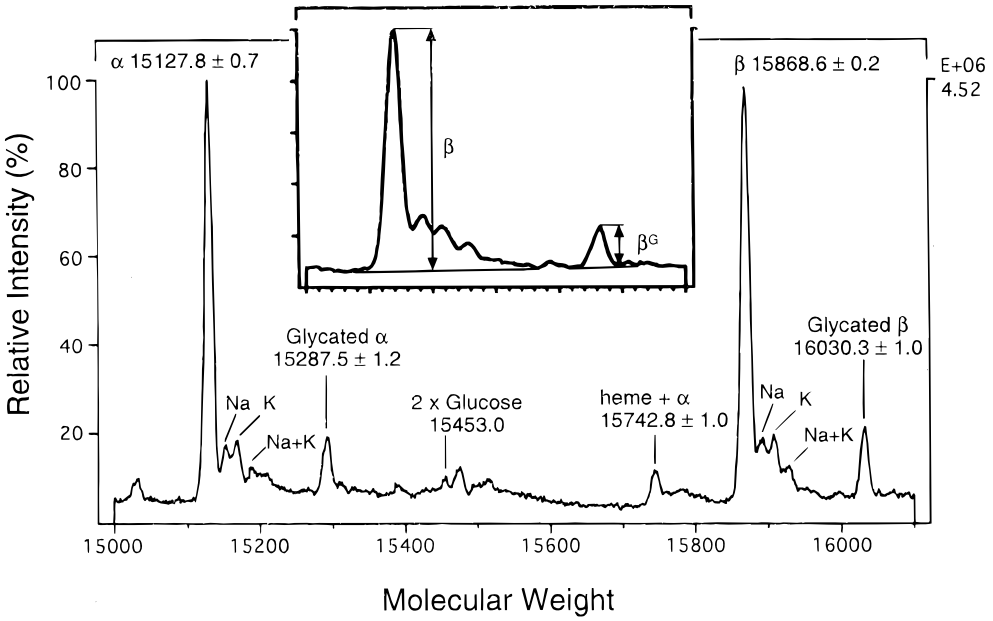

Figure 2. Deconvoluted data of the spectrum shown in Fig. 1. The predicted assignments are given in Table 1. The inset shows the manual measurement of the relative intensity of peak height.

Calculation of ratio of glycation

The ratio of glycated β-chain to total β-chain was calculated by the following equation:

glycated hemoglobin (%) = 100

[peak height of glycated β/(peak height of glycated β + peak height of non-glycated β)].

The peak of subunit glycated with 1 mol of hexose was used for the calculation. The peak height was calculated from the deconvoluted spectra as shown in the inset of Fig. 2. The baseline was drawn by connecting two points of the neighboring region with no ion peaks.

Table 1. Expected and measured molecular masses for the free and modified globin chains

Predicted adduct	Expected	Measured[a]
α-Chain:		
None	15126.4	15126.9 ± 0.6
Sodium	15149.4	15149.7 ± 1.9
Potassium	15165.5	15166.3 ± 1.7
Glucose	15288.6	15289.3 ± 0.9
2 mol glucose	15450.7	15451.6 ± 1.0
Heme	15742.6	15743.7 ± 0.8
β-Chain:		
None	15867.2	15868.6 ± 0.7
Sodium	15890.2	15891.5 ± 1.6
Potassium	15906.3	15908.5 ± 1.5
Glucose	16029.4	16031.3 ± 0.8
Heme	16483.4	16485.3 ± 1.9

[a] The measured values are average data from ten analyses of samples from different patients with high glycated hemoglobin levels.

The ratio of glycated α-chain was also calculated by the same equation. The ratio of glycated to non-glycated chain was calculated also using multiply charged ions on raw spectra. The values calculated using four pairs of multiply charged ions were averaged, viz. 12–15 charged ions for the α-chain and 13–16 for the β-chain. For

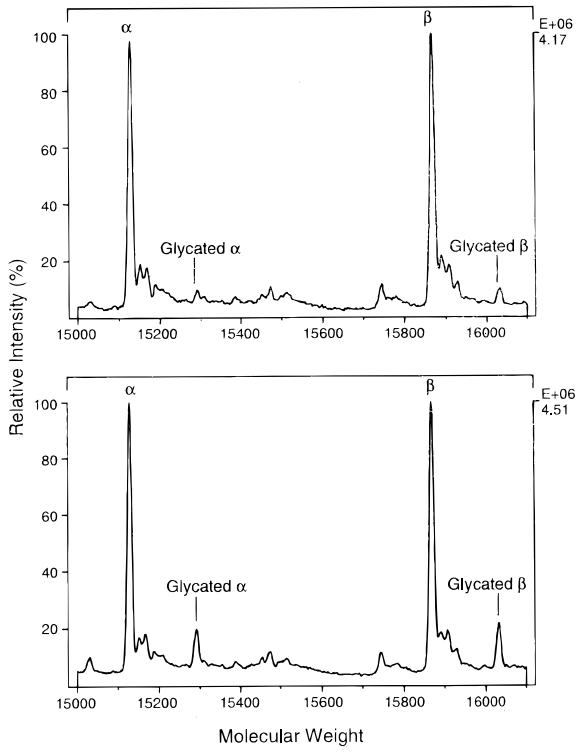

Figure 3. Deconvoluted data for a normal control (top) and a diabetic patient (bottom). HbA1c value by HPLC: 4.8% (top) and 11.0% (bottom).

comparison with other methods of measuring HbA1c, the ratio calculated with ions of the β-chain was used. The relative standard deviation (RSD) of the measurement was calculated for the ratio of β-chain glycation.

RESULTS

The electrospray mass spectrum of diluted hemolysate taken from a diabetic patient and its deconvoluted data are shown in Figs 1 and 2, respectively. The observed values of the ions assigned to glycated and non-glycated α- and β-chains coincided with the theoretical values within an average of ± 2SD of ten analyses of different specimens as shown in Table 1. The RSD was $<0.01\%$. The other ions corresponded to α- and β-chains coupled with sodium, potassium, 2 mol of hexose and heme. The probable adduct ions of doubly glycated globin chains were small but increased in parallel with singly glycated globin chains. The ion, slightly above 2 mol glycated α-chain, was not identified; it was higher in stored hemoglobin specimen than in fresh and the peaks did not change on reduction of hemoglobin with 10 mM dithiothreitol.

Figure 3 shows a comparison between deconvoluted data for a normal control (top) and a diabetic patient (bottom); the HbA1c values obtained by HPLC measurement were 4.8% and 11.0%, respectively. The ions corresponding to glycated subunits are comparable to these values. The RSDs of the measurements are given in Table 2. The ratio of glycation of the α- and β-chains was nearly unity by calculation from deconvoluted spectra but it was about 2:3 by calculation from raw data.

Figure 4 shows the values obtained by the HPLC method vs. ESI/MS and Fig. 5, those obtained by the latex agglutination method. The correlation coefficient between the ESI/MS method and the conventional HPLC method was 0.980 and that between the ESI/MS method and the latex agglutination method was 0.961. Regression equations are shown in the figures.

Four standard hemoglobin specimens from two sources, Kokusai and Roche, were measured by the three methods and the measured values are given in Table 3 in comparison with the assigned values. Large differences were observed among the methods.

Sample preparation was completed in 2 min and MS analysis in 4 min.

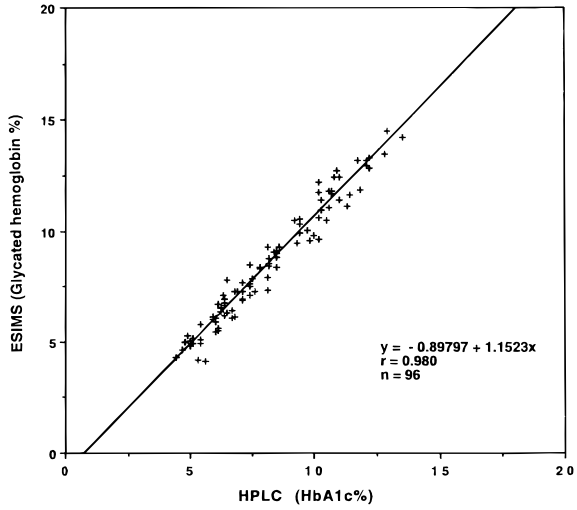

Figure 4. Correlation between the percentage obtained by the ESI/MS (β-chain glycated) and the values obtained by HPLC (HbA1c).

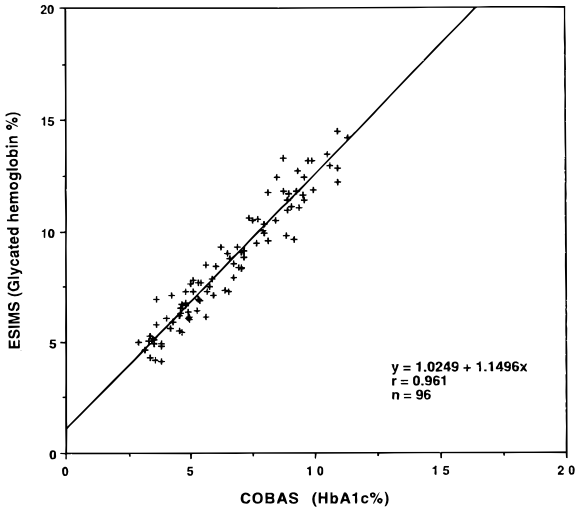

Figure 5. Correlation between the percentage obtained by the ESI/MS (β-chain glycated) and the values obtained by the latex agglutination method using Cobas Mira autoanalyzer (HbA1c).

Table 2. Relative standard deviations for the within-run and between-run results for % glycation in whole blood samples by ESI/MS

Parameter	Within-run		Between-run	
No. of measurements	10	10	6	6
Mean	5.49	13.13	5.79	13.54
SD	0.23	0.23	0.28	0.58
RSD (%)	4.11	3.94	4.86	4.27

Table 3. Comparison of the values (%) of HbA1c and glycated β-chain assigned by manufacturers and measured by the three methods using four specimens from two sources

Name of Hb control specimen	Level	Assigned value	$\beta^G/\beta^G+\beta$ By MS	HbA1c (%) By HPLC	By latex
HbA1c control Kokusai	Low	5.0 ± 0.5	5.62 ± 0.28	4.94 ± 0.08	3.08 ± 0.10
	High	10.7 ± 0.9	11.44 ± 0.45	10.02 ± 0.12	8.93 ± 0.14
HbA1c control Roche	Low	5.1 ± 0.8	4.57 ± 0.22	4.36 ± 0.08	3.08 ± 0.10
	High	11.5 ± 1.7	12.29 ± 0.49	9.96 ± 0.17	9.86 ± 0.26

DISCUSSION

Discrepancies among methods

The reproducibilities of the two non-MS methods and the MS method were fairly good (<5%). The correlation among these methods was also high (>0.96). However, the discrepancies among values measured by these methods were considerable, as there are large regression slopes and intercepts in the regression equations shown in Figs 4 and 5. Possible causes of the discrepancies are as follows. (i) The calibrator or standard hemoglobin specimens for HbA1c assay provided by the manufacturers were not adjusted among specimens. The hemoglobin molecule may be unstable during lyophilization and storage. We measured standard specimens provided by each manufacturer by the three methods and the values assigned by the manufacturer and the measured values were found to be different (see Table 3). (ii) Analysis of HbA1c by ion-exchange HPLC may give falsely high estimates because of non-glycated Hb contaminants that co-elute. These components are HbF, cabamylated hemoglobin, acetylated hemoglobin, acetoaldehyde–hemoglobin and some unknown non-glycated contaminants. (iii) The glycated hemoglobin measured by non-MS methods is HbA1c, that is, biochemically characterized as a stable adduct of glucose to the NH_2-terminal amino group of the β-chain of hemoglobin A_0. However, the glycated β-chain includes the β-chain glycated on α-NH_2-valine and/or ε-NH_2-lysine residues. The difference in the molecular derivatives detected by each method may be a cause of discrepancies among the data. In diabetics and in normal individuals, the glycated β-chain may change in parallel with HbA1c. Thus the correlation between non-MS and MS methods was high in spite of the large discrepancies.

Accuracy of peak height determination

The deconvolution method and baseline determination may affect the accuracy of the peak height measurement. Since our MS instrument has no program for baseline subtraction, measurements were made manually as shown in the inset in Fig. 1. The signal-to-noise ratio, resolution, reproducibility of peak abundance and program for deconvolution and for background subtraction may not be sufficient for the practical application of these techniques. We compared the glycated/non-glycated ratio calculated using multiply charged ions in raw mass spectra and the ratio calculated from the deconvoluted spectra. For the β-chain the ratio calculated by the two procedures was similar. However, the glycated α-chain/non-glycated α-chain ratio, calculated from the deconvoluted spectra was considerably higher than that calculated from the raw mass spectra. This may be due to the error accompanying deconvolution. We await improvement of the deconvolution program.

Ratio of glycation rate of α- and β-chains

The ratio of α- and β-chain glycation obtained by Morris *et al.*[8] by MS measurements was 2:3. In another report,[12] the ratio obtained by a conventional method was about 1:2. The ratio calculated from the deconvoluted spectra in our analyses for α-chain may not be reliable. The height of low peaks on the deconvoluted spectrum may be incorrect especially between peaks of non-glycated α- and β-chains. The ratio of α- and β-chain glycation calculated from the raw spectra, that is, the average ratios for four pairs of multiply charged peaks, was about 2:3 and resembled the ratio given previously.[8]

MS method in comparison with conventional methods

ESI/MS proved to be useful for the determination of glycated hemoglobin as proposed by Morris *et al.*[8]. The various commercially available methods for the determination of glycated hemoglobin require sophisticated materials such as a high-quality ion-exchange column and a specific antibody against glycated peptide or immobilized affinity particles. The ion-exchange column deteriorates with repeated use and requires careful maintenance to obtain a consistent result in each control assay. The quality of the reagents for immunoassay and for affinity chromatography is difficult to maintain, and differs between lots, requiring the use of reference materials for various analytes.[3] However, it is difficult to prepare stable reference materials for the assay of glycated hemoglobin. MS analysis does not require any sophisticated reagents. The relative abundance between ion peaks with similar molecular masses and similar chemical natures should bear an inherent relationship to the actual ratio of the concentration in the mixture of the material. The ratio is theoretically precise, as is the measurement of absorbance to determine the concentration of analytes which have a known molar absorptivity. With the development of bench-top mass spectrometers with an automatic sample loading system, the MS assay may become easier to use.

This measurement system may also be applied for the diagnosis of diseases other than diabetes mellitus. Carbamylated hemoglobin is increased in the patients with uremia[13] who have a high level of urea, and the urea is easily changed to cyanate, which forms a carbamylated protein. In some patients with hemoglobinopathy, the peak of the glycated chain may be superimposed on an abnormal non-glycated chain. In such cases the normal subunit without an abnormal counterpart (α-chain in the β-variant cases and vice versa) can be used for calculation.

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REFERENCES

1. R. J. Koenig, C. M. Peterson, R. L. Jones, C. Saudek, M. Lehrman and A. Cerami, *N. Engl. J. Med.* **295**, 417 (1976).
2. H. F. Bunn, D. N. Haney, K. H. Gabbay and P. M. Gallop, *Bopchem. Biophys. Res. Commun.* **67**, 103 (1975).
3. W. Hoelzel and K. Miedema, *J. Int. Fed. Clin. Chem.* **9**, 62 (1966).
4. D. E. Goldstein, R. R. Little and K. M. Parker, *Clin. Lab. Sci.* **21**, 187 (1984).
5. D. C. Klenk, G. T. Hermanson, R. I. Krohn, E. K. Fujimoto, A. K. Mallia, P. K. Smith, J. D. England, H-M. Wiedmeyer, R. R. Little and D. E. Goldstein, *Clin. Chem.* **28**, 2088 (1982).
6. J. Karl, G. Burns, W. D. Engel, A. Finke, M. Kratzer, W. Rollinger, E. Schickaneder and C. Seidel, *Klin. Lab.* **39**, 991 (1993).
7. A. Lapolla, D. Fedele, R. Aronica, M. Garbeglio, M. D'Alpaos, R. Seraglia and P. Traldi, *Rapid Commun. Mass Spectrom.* **10**, 1133 (1996).
8. M. Morris, S. Jarvis, B. Green and N. Roberts, paper presented at the 44th ASMS Conference on Mass Spectrometry and Allied Topics, 1996 May 12–16.
9. Y. Wada, A. Hayashi, T. Fujita, T. Matsuo, I. Katakuse and H. Matsuda, *Biochim. Biophys. Acta* **667**, 233 (1981).
10. K. Nakashima, Y. Hattori, K. Yamazaki, M. Takechi, Y. Andoh and T. Miyaji, *Diabetes* **39**, 17 (1990).
11. P. J. Watkins, I. Jardine and J. X. Zhou, *Biochem. Soc. Trans.* **19**, 957 (1991).
12. R. L. Garlick, J. S. Mazer, P. J. Higgins and H. F. Bunn, *J. Clin. Invest.* **71**, 1062 (1983).
13. R. Fluckiger, W. Harmon, W. Meier, S. Loo and K. H. Gabby, *N. Engl. J. Med.* **304**, 823 (1981).