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NMR-invisible lactate in blood plasma

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Resonances for lactate are broadened in 500 MHz ^{1}H NMR spectra of human blood plasma and only about one-third is visible in Hahn spin-echo spectra. Similar effects are observed for some other carboxylate anions. Lactate added to the high-M_r fraction of plasma can give rise to peaks which are too broad to observe in either single-pulse or spin-echo spectra. Addition of agents such as NH_4Cl of SDS dramatically increases the intensities of lactate peaks. Some glycoproteins appear to broaden lactate resonances.

^{1}H-NMR; Lactate; Blood plasma; Glycoprotein; (Human)

1. INTRODUCTION

Proton NMR spectra of blood plasma are very complicated even at high frequency (e.g. 500 MHz). They contain many overlapping broad resonances arising largely from proteins and lipoprotein particles, and sharper resonances from small molecules and highly mobile regions of macromolecules. Despite this, some specific assignments can be made, especially with the aid of spin-echo methods which, in effect, act as 'mobility filters', allowing only peaks from protons with long T_2 values to appear in the spectrum [1–5].

There have already been suggestions that ^{1}H NMR spectra of plasma will be of diagnostic clinical value. For example, resonances for ketone bodies have been correlated with the control of diabetes mellitus by insulin, those for lipids with hyperlipidaemia [2], peaks for lactate [6], fucosylated lipids [7] and lipoproteins [8] with malignant cancer, and resonances for other small molecules with inherited metabolic disorders [9], organ damage through drug overdose [10], and the efficiency of haemodialysis [11].

However, it seems unlikely that high-resolution ^{1}H NMR spectra of plasma will be of routine clinical use until the factors which affect the intensities and linewidths of resonances are better understood. Because plasma is a heterogeneous mixture of lipoprotein particles, proteins, small molecules and ions there are a large number of possibilities for molecular interactions. The binding of small molecules to proteins, for example, can lead to severe broadening of resonances [12,13].

We report here that resonances for lactate, and some other small organic anions, are often specifically broadened in ^{1}H NMR spectra of blood plasma, and the intensities of their Hahn spin-echo peaks greatly reduced. In effect, there is a pool of 'NMR-invisible' lactate, which makes quantitation difficult. We show that such effects can be observed when lactate is added to the high-M_r fraction of plasma and that, in some circumstances lactate resonances are too broad to detect in either single-pulse or Hahn spin-echo spectra. We have discovered that a variety of agents can increase the intensity of these resonances, and the possible involvement of glycoproteins in anion binding is considered.

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Our observations not only have implications for the use of ^{1}H NMR in clinical measurements and in other in vivo ^{1}H NMR experiments [14], but also raise wider questions about the role of organic anions in controlling protein function and that of proteins in controlling anion transport and delivery.

2. EXPERIMENTAL

2.1. *Blood plasma*

Venous blood from healthy consenting volunteers was drawn and placed in standard clinical vials containing anticoagulant. It was centrifuged and the plasma was either used fresh, or stored at −20°C and thawed at room temperature immediately before use. A plasma sample from a ketoacidotic subject was kindly supplied by Dr A.F. Macleod (St Thomas' Hospital).

Low-molecular-mass plasma ultrafiltrates were obtained using Amicon centricon devices (10 kDa cut-off) washed with MeOH followed by H_2O. High-molecular-mass fractions were obtained by dialysis in washed cellulose sacks (Sigma, 10 kDa cut-off) at 4°C over ~12 h with frequent changes (3–4) of external medium (H_2O or 0.15 M NaCl, ~1 l/ml plasma). Sodium bicarbonate (26 mM) was added to some samples (e.g. that for fig.2) before dialysis to enhance lactate displacement. When freeze-dried, samples were made up to their original volume with D_2O. Proteins were also removed from whole blood and plasma by $HClO_4$ precipitation (1:2, v/v; 8% ice-cold $HClO_4$, shaken vigorously, centrifuged and neutralized with KOH).

2.2. *NMR spectroscopy*

500 MHz ^{1}H NMR spectra were recorded on a Bruker AM500 spectrometer (MRC Biomedical NMR Centre, Mill Hill). Typically, 0.45 ml blood plasma or ultrafiltrate was placed in a 5 mm tube and 0.05 ml D_2O added; either added TSP (sodium 3-trimethyl[2,2,3,3-$^2H_4$]propionate), or internal alanine (1.487 ppm) was used as a chemical shift reference.

Single-pulse spectra are the result of 32 (plasma)–128 (proteins) 45° pulses separated by a T_1 relaxation delay of 1.5–2.4 s. The probe temperature was 298 K. Water saturation was achieved either with continuous or gated (off during acquisition) secondary irradiation. Free induction decays (FIDs) were accumulated into 16384 computer points and an exponential, line-broadening function of 0.8 Hz was applied.

Hahn spin-echo spectra [15] were acquired using the sequence 90–t–180–t–collect–FID, usually with t = 60 ms. The CPMG sequence 90–(t_1–180–t_1)$_n$–FID [16] was used for T_2 studies. T_1 values were measured with the inversion-recovery sequence 180–t–90–FID [17].

Additions of sodium D- or DL-lactate, lactic acid methyl ester, sodium 3-D-hydroxybutyrate or 2-hydroxybutyrate, L-alanine (all purchased from Sigma), NH_4Cl, and $NaHCO_3$ were made as microlitre aliquots of stock solutions in D_2O. SDS was added as a solid.

Human apotransferrin, iron-saturated transferrin and α_1-antitrypsin were purchased from Sigma and dissolved in D_2O (~14 mg/ml). Very low density lipoprotein (VLDL) was the kind gift of Dr P. Turner (St Thomas' Hospital). The pH meter reading in D_2O is referred to as pH*.

2.3. *Enzyme assay*

L-Lactate was determined using a 'Sigma Diagnostics' pyruvate/lactate assay kit (lactate dehydrogenase/NAD^+). This was checked with 0.88 and 12.3 mM reference solutions of L-Lac and DL-Lac, respectively.

3. RESULTS

Typical 500 MHz ^{1}H NMR single-pulse and Hahn spin-echo spectra of heparinised human blood plasma are shown in fig.1a and c, respectively. In spin-echo spectra of the corresponding ultrafiltrates (< 10 kDa, not shown) there are large increases in the intensities of the lactate resonances

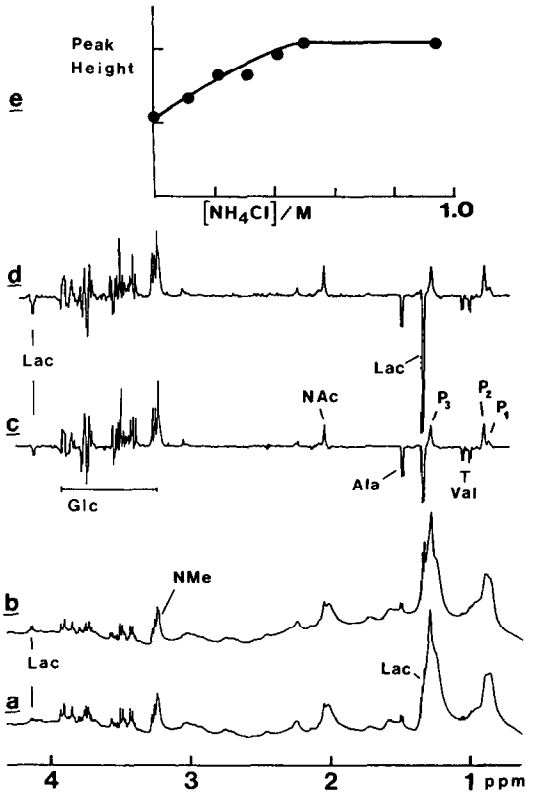

Fig.1. 500 MHz ^{1}H NMR spectra of human blood plasma: (a,b) single-pulse spectra, and (c,d) Hahn spin-echo spectra, before and after addition of NH_4Cl (0.5 M), respectively. (e) Increase in height of the lactate CH_3 spin-echo peak with increasing concentration of added NH_4Cl; for this experiment a separate sample containing added sodium DL-lactate (2 mM) was used. Assignments: Ala, alanine; Glc, glucose; Lac, lactate; NAc, *N*-acetyls of glycoproteins; NMe, choline head groups of HDL (and LDL for single-pulse); P_1,P_2, CH_3 of HDL and LDL, and chylomicrons and VLDL, respectively; P_3, CH_2 of chylomicrons and VLDL; Val, valine.

(up to 200%) relative to those of alanine, or valine. Addition of certain agents to intact plasma dramatically increases the relative intensities of both the lactate CH_3 (doublet, 1.33 ppm) and CH (quartet, 4.11 ppm) resonances. These include SDS (50–200 mM), NaCl (2 M), $HClO_4$, NH_4Cl (fig.1b,d) as well as increases in temperature (to 50°C).

As shown in fig.1e, the lactate CH_3 peak intensity increases almost linearly with added NH_4Cl concentration, plateauing at about 0.5 M. Typically, after addition of NH_4Cl (0.5 M), lactate CH_3 linewidths decrease from ~3.5 to 2.2 Hz, whilst those for alanine remain unchanged (2.3 Hz). It is possible that all the lactate contributes to the single-pulse spectrum; however, although an allowance can be made for the overlapping threonine peak, integration is hampered by the large lipid CH_2 signal, hence the need to use spin-echo spectra.

Only after addition of NH_4Cl is it possible to make a reliable estimation of the total lactate concentration in plasma from the spin-echo spectrum. For 3 typical plasma samples, the concentrations of lactate determined from spin-echo peak heights, calibrated via standard additions of alanine were 1.10, 1.20 and 0.80 mM, about one-third of those obtained by enzyme assay (2.90, 3.50 and 2.45 mM, respectively). The NMR determinations on intact plasma approached the latter values after addition of NH_4Cl (0.5 M, 2.30, 3.00 and 1.80 mM) or SDS (200 mM, 2.50, 2.90 and 2.00 mM), but those from NMR spectra of plasma ultrafiltrates were closer: 2.80, 3.20 and 2.30 mM. SDS and NH_4Cl had no effect on the relative intensities of signals in the spectra of ultrafiltrates.

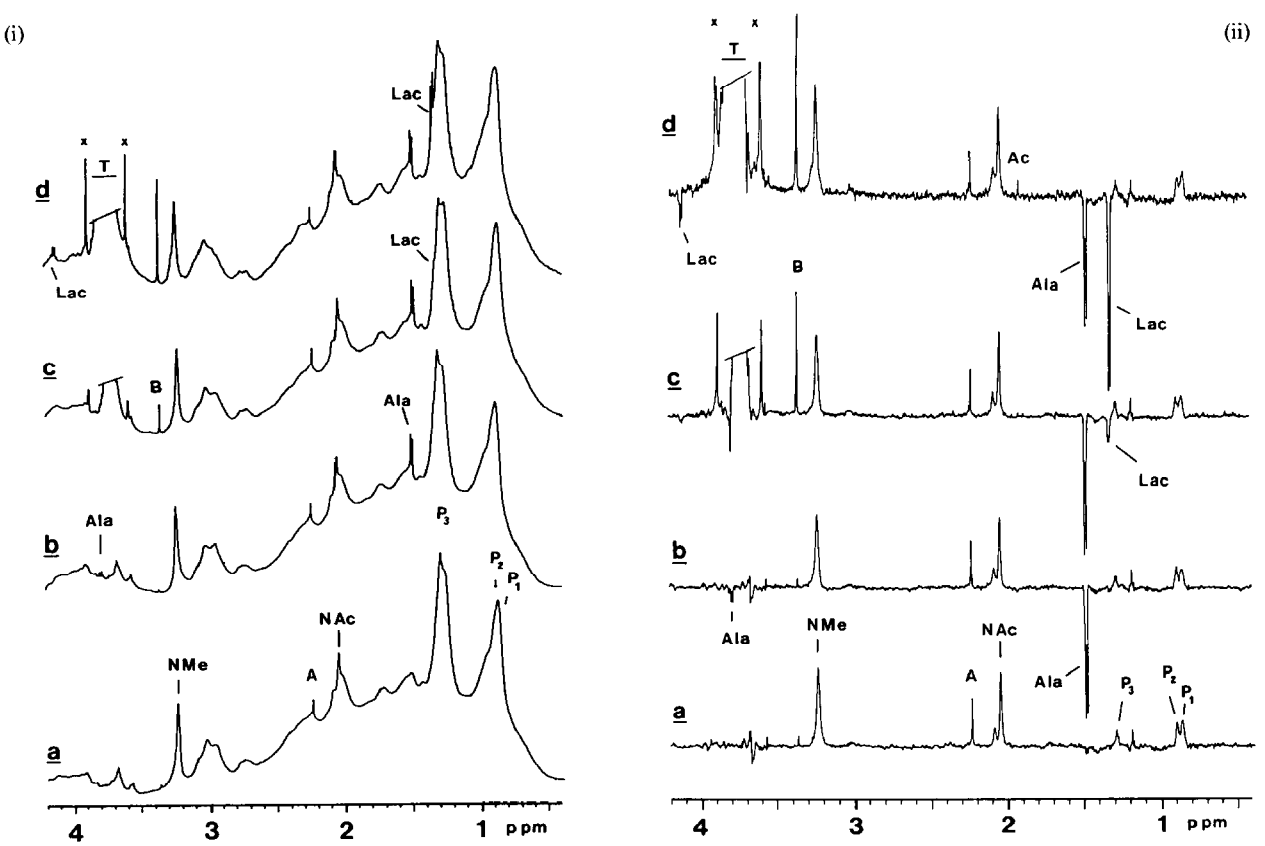

Fig.2. 500 MHz ^1H NMR spectra of the high-molecular-mass (>10 kDa) fraction of human blood plsma. (i) Single-pulse spectra, (ii) Hahn spin-echo spectra. (a) No addition, (b) addition of alanine and lactate (0.25 mM each), (c) addition of Tris-DCl buffer to raise the pH* from 5.5 to 7.4, (d) addition of NH_4Cl (0.5 M). Assignments as in fig.1, also T, Tris; A, acetone (tube contaminant); B, unassigned; x, spinning side-bands.

Experiments were carried out to investigate the origin of the effects on lactate resonances. Spectra acquired without H_2O suppression from a freeze-dried sample of plasma redissolved in D_2O showed similar increases in the intensities of lactate on addition of NH_4Cl. This suggested that cross-saturation and spin-diffusion effects arising from H_2O irradiation were not involved. The relaxation delay was varied from 2 to 10 s with little change in spectra, appearing to rule out T_1 effects. The behaviour of lactate peaks was similar in both Hahn and CPMG spectra, eliminating molecular diffusion as a cause, and was not dependent on the type of anticoagulant (EDTA, heparin or citrate).

As judged from CPMG spectra ($2nt$ = 20–180 ms), there appeared to be little difference between the decay of the slowly relaxing components of the transverse magnetization of the CH_3 groups of lactate and alanine, either before or after addition of NH_4Cl. It seems likely that there is also a fast-relaxing component for lactate. However, the interpretation of these data is complicated by the problems of spectral overlap and spin-spin couplings [18].

Dramatic attenuations of peak intensities were observed when lactate was added to the high-molecular-mass (>10 kDa) fraction of plasma. Equimolar standard solutions of lactate and alanine were added to plasma dialysed vs H_2O. Alanine CH_3 and CH resonances are well-resolved and increase linearly in intensity with added standard (0.5–1.6 mM), but no peaks for lactate are visible in either single-pulse or Hahn spin-echo spectra (fig.2(i)a,b,(ii)a,b). When the pH* was raised from 5.5 (isoionic point) to 7.4 with Tris-DCl buffer, a small shoulder for the lactate CH_3 appeared in the single-pulse spectrum (fig.2(i)c) and a small inverted doublet in the spin-echo spectrum (fig.2(ii)c). Only after addition of NH_4Cl did the intensity of lactate resonances reach those of Ala (fig.2(i)d,(ii)d). The increase in intensity of the lactate doublet beyond that of alanine suggests that even extensive dialysis did not remove all the lactate in this sample, and that some is tightly bound. When 0.15 M NaCl was used in the dialysis, similar results were obtained.

The possible involvement of specific plasma proteins in lactate interactions was investigated. As shown in fig.3, commercial samples of human diferric transferrin contain NMR-invisible lactate

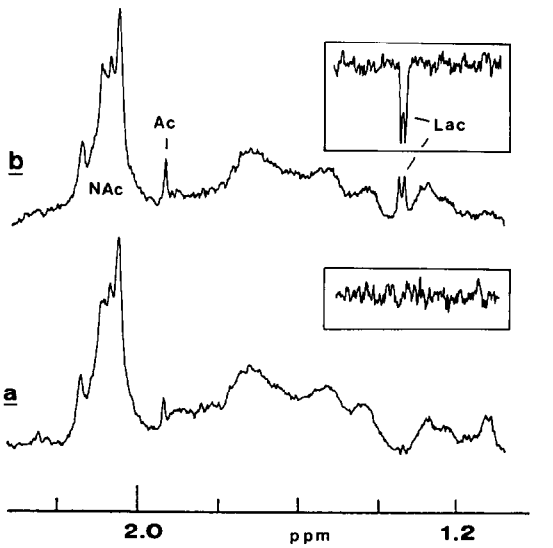

Fig.3. 500 MHz ^1H NMR spectra of human iron-saturated transferrin. (a) D_2O, pH* 5.5; (b) after addition of $NaHCO_3$ (15 mM) and 95% O_2/5% CO_2, pH* 7.5. Assignments: Lac, lactate; Ac, acetate; NAc, *N*-acetyls of carbohydrate side chains. Insets: portions of the corresponding Hahn spin-echo spectra.

which appears after addition of competing anions such as bicarbonate. Apotransferrin [19] and α_1-antitrypsin behave similarly. On the other hand, isolated VLDL had no effect on lactate peaks.

Similar effects were found for some other physiologically important carboxylic acids. Thus, although the intensities of the peaks for lactate methyl ester added to plasma were not affected by NH_4Cl addition, those for 3-D-hydroxybutyrate, acetoacetate (ketone bodies), and 2-hydroxybutyrate did increase in intensity (>100%) relative to those of alanine, valine, glucose, and *N*-acetyls of glycoproteins. Such effects were also observed with plasma from ketoacidotic diabetic subjects.

4. DISCUSSION

The detection and quantitation of lactate and other small organic molecules in blood plasma, plasma fractions and other biological materials is a subject of much current interest. The work reported here has clearly shown that specific broadening of lactate resonances can occur in normal single-pulse spectra of plasma such that the intensities of spin-echo resonances are severely

attenuated. Under some conditions, lactate added to the high-molecular-mass (>10 kDa) fraction of plasma gives resonances which are too broad to detect in either spin-echo or normal spectra.

In a previous paper [2], we noted that although NMR assays for alanine and valine in intact plasma were in reasonable agreement with conventional assays, there were discrepancies between NMR and enzyme assays of lactate. The data reported here provide insight into the reasons for these discrepancies. Recently, Grasdalen et al. [11] measured, by spin-echo NMR, the concentrations of small molecules in plasma of subjects with chronic renal failure during haemodialysis. It was notable that they had to use a correction factor of 6.1 to convert lactate peak intensities to concentrations compared to a value of 1.1 for alanine. Again the present data suggest why this is so. Unfortunately, until the molecular basis of lactate interactions is understood, it cannot be assumed that the same correction factor will apply to all samples.

Ohsaka et al. [6] have reported that lactate concentrations in serum are easily determined by NMR from single-pulse spectra and can be used for cancer diagnosis. Their comments can only be said to refer to 'NMR-visible' lactate. We have found that NMR-visible lactate levels sometimes do provide useful information, particularly where comparisons are required within a similar set of samples, for example in maternal plasma they correlate with the length of the second stage of labour [20].

Our findings also complicate the attempts by Mountford et al. [7] to link resonances with long 'T_2' values and chemical shifts similar to lactate in ^{1}H NMR spectra of plasma and serum with cancer-associated lipoproteins. The interpretation of this region of the spectrum either in single-pulse or spin-echo spectra can now no longer be regarded as straightforward. Broadened lactate CH_3 peaks could also contribute to the lipoprotein CH_2 linewidth which has been used in correlations with malignant cancer by Fossel et al. [8]. However, this test for cancer is of little general use in view of the complexities of the lipoprotein resonances themselves [4,21,22].

Although there are now many reports of the detection of lactate in vivo by ^{1}H NMR (e.g. [23–25]), few have examined the quantification problem. Because of the need to suppress the intense H_2O signal and to simplify the spectra, spin-echo pulse sequences are used. Recent measurements by Chang et al. [14] have shown that only 25% of the lactate present in hypoxic or ischaemic rat brains appears to be observable by NMR.

In our studies, the suppression of lactate signals may arise from binding to plasma proteins. The negatively charged detergent SDS, which is known to interact strongly with proteins [26], decreased lactate binding. NH_4^+ may cause local changes in protein structure through hydrogen-bonding interactions, although the Cl^- added may also play a role in competing with lactate. An awareness of these effects is important if NH_4Cl is used as a relaxation agent for H_2O suppression [27].

Further studies will be required to establish whether interactions with specific plasma proteins are sufficient to account for the effects on lactate resonances. We investigated transferrin initially because it is known to possess carboxylate-anion-binding sites [28]. Our finding that both apo- and diferric transferrin can contain NMR-invisible lactate was unexpected. Other 'acute-phase' glycoproteins present in higher concentrations may also bind lactate. We have found that α_1-antitrypsin can cause severe broadening of lactate peaks under certain conditions. The factors which influence the rates of lactate exchange processes on the NMR time scale are not yet clear.

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REFERENCES

[1] Nicholson, J.K., Buckingham, M.J. and Sadler, P.J. (1983) Biochem. J. 211, 605–615.
[2] Nicholson, J.K., O'Flynn, M.P., Sadler, P.J., Macleod, A.F., Juul, S.M. and Sonksen, P.H. (1984) Biochem. J. 217, 365–375.
[3] Bell, J.D., Brown, J.C.C., Nicholson, J.K. and Sadler, P.J. (1987) FEBS Lett. 215, 311–315.
[4] Bell, J.D., Sadler, P.J., Macleod, A.F., Turner, P.R. and La Ville, A. (1987) FEBS Lett. 219, 239–243.
[5] Bock, J.L. (1982) Clin. Chem. 28, 1873–1877.
[6] Ohsaka, A., Yoshikawa, K. and Matuhasi, T. (1979) Jap. J. Med. Sci. Biol. 32, 305–309.

[7] Mountford, C.E., May, G.L., Wright, L.C., Mackinnon, W.B., Dyne, M., Holmes, K.T., Van Haaften-Day, C. and Tattersall, M.H.N. (1987) The Lancet, April 11th, 829–833.
[8] Fossel, E.T., Carr, J.M. and McDonagh, J. (1986) N. Engl. J. Med. 315, 1369–1376.
[9] Iles, R.A., Buckingham, M.J. and Hawkes, G.E. (1983) Biochem. Soc. Trans. 11, 374–375.
[10] Bales, J.R., Bell, J.D., Nicholson, J.K., Sadler, P.J., Timbrell, J.A., Hughes, R.D., Bennett, P.N. and Williams, R. (1988) Magn. Res. Med. 6, 300–306.
[11] Grasdalen, H., Belton, P.S., Pryor, J.S. and Rich, G.T. (1987) Magn. Res. Chem. 25, 811–816.
[12] Fischer, J.J. and Jardetzky, O. (1965) J. Am. Chem. Soc. 87, 3237–3243.
[13] Jardetsky, O. and Roberts, G.C.K. (1982) NMR in Molecular Biology, Academic Press, New York.
[14] Chang, C.-H., Pereira, B.M., Weinstein, P.R., Keniry, M.A., Murphy-Boesch, J., Litt, L. and James, T.L.L. (1987) Magn. Res. Med. 4, 575–581.
[15] Hahn, E.L. (1950) Phys. Rev. 80, 580.
[16] Meiboom, S. and Gill, D. (1958) Rev. Sci. Instrum. 29, 688.
[17] Vold, R.L., Waugh, J.S., Klein, M.P. and Phelps, D.E. (1968) J. Chem. Phys. 48, 3831.
[18] Martin, M.L., Delpuech, J.-J. and Martin, G.J. (1980) Practical NMR Spectroscopy, Heyden, London.
[19] Bell, J.D., Brown, J.C.C., Kubal, G. and Sadler, P.J. (1988) Biochem. Soc. Trans., in press.
[20] Bell, J.D., Brown, J.C.C., Sadler, P.J., Garvie, D., Macleod, A.F. and Lowry, C. (1988) submitted.
[21] Bell, J.D., Brown, J.C.C., Newell, D.R., Norman, R.E. and Sadler, P.J. (1988) NMR Biomed. 1, in press.
[22] Small, D.M. and Hamilton, J.A. (1987) N. Engl. J. Med. 316, 1412–1413.
[23] Rothman, D.L., Behar, K.L., Hetherington, H.P. and Shulman, R.G. (1984) Proc. Natl. Acad. Sci. USA 81, 6330–6334.
[24] Arus, C., Barany, M., Westler, W.M. and Markley, J.L. (1984) FEBS Lett. 165, 233–238.
[25] Williams, S.R., Gadian, D.G., Proctor, E., Sprague, D.B., Talbot, D.F., Young, I.R. and Brown, F.F. (1985) J. Magn. Res. 63, 406–412.
[26] Nozaki, Y., Reynolds, J.A. and Tanford, C. (1974) J. Biol. Chem. 249, 4452–4459.
[27] Rabenstein, D.L. and Fan, S. (1986) Anal. Chem. 58, 3178–3184.
[28] Schlabach, M. and Bates, W.B. (1975) J. Biol. Chem. 250, 2182–2188.