Biochem. J. (1983) **211**, 605–615
Printed in Great Britain

High resolution ^1H n.m.r. studies of vertebrate blood and plasma

Jeremy K. NICHOLSON,* Malcolm J. BUCKINGHAM† and Peter J. SADLER*
**Department of Chemistry, Birkbeck College, University of London, Malet Street, London WC1E 7HX, U.K., and †U.L.I.R.S., Department of Chemistry, Queen Mary College, University of London, Mile End Road, London E1 4NS, U.K.*

(*Received 29 November 1982/Accepted 15 February 1983*)

Spin echo Fourier transform proton n.m.r. spectra of whole blood contain resonances from both erythrocytes and plasma. A large number of well-resolved signals from mobile protons of low-molecular-weight metabolites in plasma and serum have been identified. Spectra from the plasmas of eight animal species and commercial, quality control sera are compared. $CaEDTA^{2-}$ and $MgEDTA^{2-}$ resonances can be used for the simultaneous determination of EDTA-chelatable calcium and magnesium concentrations in intact plasma and other biological fluids. Cholesterol is too immobile to contribute to the spectra of intact plasma, but is readily estimated by n.m.r. in both its free and esterified forms after extraction into methanol.

Our interest in the metabolism of metal compounds led us to investigate the application of ^1H n.m.r. methods to a study of intact human blood. Such studies are hampered by the large number of overlapping resonances and by the large signal from water. Previous workers have shown that both of these can be largely overcome by using the SEFT (Brown *et al.*, 1977; Rabenstein & Nakashima, 1979) or magnetization transfer (Rabenstein *et al.*, 1980) techniques combined with high frequency observation (e.g. 400 MHz). In the SEFT technique broad resonances from macromolecules and other relatively immobile groups disappear via spin–spin (T_2) relaxation during the waiting periods (τ) of the two pulse sequence. In magnetization transfer, selected protein resonances are irradiated and the remainder of the protein envelope is effectively saturated via spin diffusion during a small time delay before the observation pulse is applied.

^1H n.m.r. spectra from blood appeared to consist of resonances from red cells observed previously (Brown *et al.*, 1977; Brindle *et al.*, 1979; Brown & Campbell, 1980) together with resonances from plasma. However, spectra from plasma alone were more highly resolved and reproducible. In this paper we discuss the assignment of resonances including free amino acids, creatine, glucose, lactate, D-3-hydroxybutyrate, choline, citrate and fatty acids. We show that there are significant variations in the proton n.m.r. profiles of plasma observable from a range of animal species and suggest that these will be valuable for comparative biochemical studies.

When EDTA is used as an anti-coagulant for blood instead of heparin, we show that plasma contains well-resolved resonances from $CaEDTA^{2-}$ and $MgEDTA^{2-}$. These can provide a rapid, simultaneous estimation of free (EDTA-chelatable) calcium and magnesium concentrations: a topic of considerable physiological interest. Cholesterol, which is too immobile to contribute to plasma spectra, is readily estimated by n.m.r. following extraction into methanol. Free and esterified cholesterol are readily distinguished by this method.

Given the rapid, direct and simultaneous estimation of low-molecular-weight metabolites as well as Ca and Mg by ^1H n.m.r., it seems likely that the technique will be of value in routine clinical chemical and biochemical analysis. Since the technique provides information about molecular mobility and molecular interactions it will complement currently-used, purely quantitative assays.

Abbreviation used: SEFT, spin-echo Fourier transform.

Experimental

Blood samples

Fresh venous blood was drawn from consenting, healthy, male volunteers and placed into sterile plastic vials of the type routinely used in clinical laboratories, containing either K_2EDTA (Sterilin) or lithium heparin (ML Ltd.) as anticoagulants, or into glass vials prior to defibrination. The blood was centrifuged and the plasma separated. ^2H_2O (50 μl) was added to 0.45 ml aliquots of plasma giving 10%

(v/v) ^2H_2O as a field-frequency lock. The resulting 0.5 ml samples were placed into 5 mm outside diameter n.m.r. tubes. For measurements at 200 MHz in 10 mm tubes, at least twice this volume was used. Red cells were washed twice in saline (0.9% NaCl) made with ^2H_2O, being centrifuged after each washing. For the estimation of intracellular Ca and Mg concentrations, known volumes of red cells were haemolysed by three cycles of freezing and thawing in the presence of 5 mM-K_2EDTA. Some EDTA- and heparin-treated whole blood samples were placed directly into n.m.r. tubes with no further treatment other than sometimes with the addition of 10% saline in ^2H_2O.

Blood containing no preservatives was prevented from clotting by defibrination using a long, sealed, stainless steel syringe needle to which had been attached short (1 cm) pieces of stainless steel wire at 1 cm intervals down its length in a helical pattern (designed by Professor A. Grimes, St. Thomas' Hospital, London). The gentle rotation of this device in the blood allowed the fibrin fibres formed during clotting to adhere to the tool over a period of 3–7 min. Then the tool was removed leaving a suspension of red cells in serum (i.e. plasma without fibrinogen) which could be manipulated without danger of clotting.

Venous blood samples were also obtained from several species of wild animals undergoing routine examination at the London Zoo (numbers in parentheses indicate the number of animals in each species studied): common zebra (*Equus burchelli*) (2); Bennett's wallaby (*Macrapus rufogriscus rufogriscus*) (1); mouflon (*Ovis musimon*) (1); capybara (*Hydrochoerus hydrochaeris*) (1); common marmoset (*Callithrin jacchus jacchus*) (6).

Blood samples from the European starling (*Sturnus vulgaris*) (6) were obtained from Monks Wood Experimental Station, Huntingdon, U.K. Blood was also drawn from laboratory mice (*Mus musculus*, Swiss White strain A_2G) (10). Samples were preserved and plasma separated as described above.

L-α-Phosphatidylcholine and other standards were purchased from Sigma. Quality control animal sera, Welcomtrols 1, 2 and 3, were purchased from Wellcome Reagents, Beckenham, Kent, U.K. These lyophilized powders were redissolved in the appropriate volume of ^2H_2O for n.m.r. measurements. Welcomtrols 1 and 3 are of bovine origin and Welcomtrol 2 is of equine origin.

For the preparation of methanol extracts, 1 ml of serum was lyophilized and 5 ml of methanol was added to the powder. After shaking well, the clear yellow supernatant was separated by decantation and filtration, and then evaporated to dryness (rotary evaporator). The residue was dissolved in 1 ml of (^{2}H)methanol ($C^2H_3O^2H$) for n.m.r. measurement. This procedure allowed extraction of most of the lipid-soluble components of plasma, including phosphatidylcholine and cholesterol.

N.m.r. measurements

Bruker WH 400 and JEOL FX200 spectrometers operating at 400 and 200 MHz respectively in quadrature detection mode and ambient temperature (approx. 298 K) were used. Spectra were accumulated either by 300 repetitions of the Hahn spin-echo sequence ($90°-\tau_2-180°-\tau_2$–collect), $\tau_2 = 60$ ms, with additional continuous proton irradiation at the water frequency in the case of unlyophilized plasma samples, or by a magnetization-transfer method involving inverse gated-decoupling at the H_2O resonance frequency (4.8 p.p.m.) with 1 s of proton decoupling in the interval between successive (30°) pulse and acquisition cycles.

Results and discussion

SEFT ^{1}H n.m.r. spectra of red cells resuspended in saline made with ^2H_2O are compared with spectra from EDTA- and heparin-treated whole blood in Fig. 1.

In the Hahn spin-echo experiment, the amplitude of the observed signal (echo) at time $2\tau_2$ after the 90° pulse is dependent on the intrinsic T_2 of the observed nuclear resonance, homonuclear spin–spin coupling (which can also produce phase modulation), and a term arising if the molecule diffuses to a region of different applied magnetic field during the refocusing period (Brown & Campbell, 1980). At a τ_2 value of 60 ms ($1/2J$) doublets with J = approx. 8.2 Hz are inverted whereas singlets and triplets are upright (Rabenstein & Nakashima, 1979). Sufficient time (120 ms) then elapses for the net magnetization associated with most broad resonances (short T_2) to decay to zero and so they do not contribute to the spectrum. As previously reported (Brown *et al.*, 1977; Brindle *et al.*, 1979; Brown & Campbell, 1980; Chapman *et al.*, 1982) the spectrum of red cells contains many well-resolved resonances for small intracellular metabolites such as ergothioneine, choline, acetylcholine, carnitine, creatine, lactate, alanine, glycine and glutathione. It can be seen that the spectra of whole blood contain additional peaks attributable to metabolites present in plasma. Prominent amongst these are resonances from mobile fatty acids (P_2 and P_3) and $CaEDTA^{2-}$ and $MgEDTA^{2-}$; their assignment is discussed below.

Two marked differences in the spectra of whole blood compared with red cells arises from the high ^2H_2O content of the latter (approx. 100%) compared with the former (approx. 10%). Thus in whole blood the glycine-CH_2- resonance (g_1) of glutathione is inverted since it is coupled to the ex-

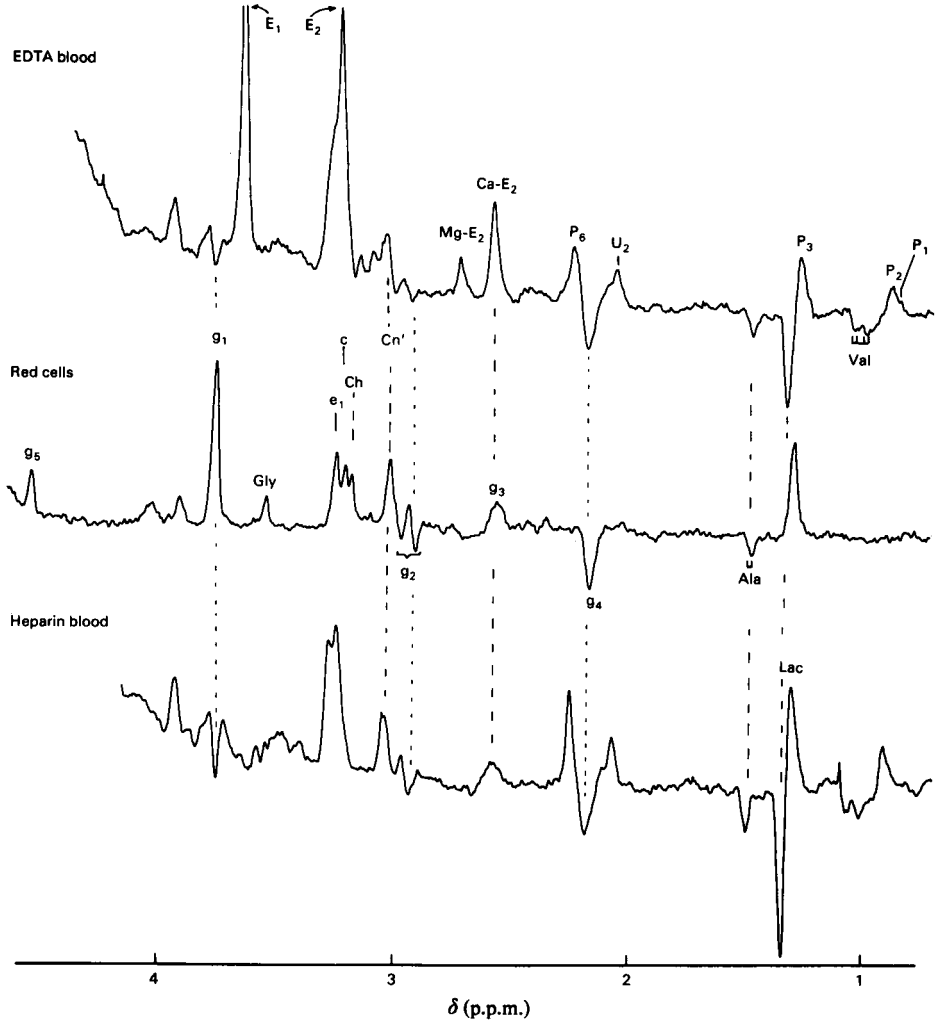

Fig. 1. *400 MHz ^1H n.m.r. SEFT spectra ($\tau_2 = 60$ ms) of EDTA- and heparin-treated whole blood (top and bottom respectively) containing 10% ^2H_2O, and red cells resuspended in saline made with ^2H_2O (centre)*

For conditions see Fig. 2. Additional assignments not listed in Fig. 2: Gly, glycine; g_1, glycine CH_2, g_2, cysteine β-CH_2, g_3, glutamate γ-CH_2, g_4, glutamate β-CH_2; g_5, cysteine α-CH, all of glutathione; e_1, ergothioneine CH_3; c, carnitine CH_3; Ch, choline CH_3; Cn', creatine CH_3. The phase of the lactate -CH_3 and g_1 signals is discussed in the text.

changeable -NH proton of the neighbouring peptide bond. This was confirmed by irradiation at the -NH resonance frequency. Similarly the lactate-CH_3 resonance is inverted since it is a doublet through coupling to the adjacent -CH-proton. In ^2H_2O, red cell lactate is deuterated via NAD^{2}H and a series of enzymic reactions (Brindle *et al.*, 1979; Brown & Campbell, 1980).

Well-resolved spectra were obtained routinely from resuspended red cells (haematocrit approx. 80%), but not from whole blood. Various factors were investigated, e.g. oxygenation, mixing prior to measurement, addition of ^2H_2O, heparin versus EDTA addition, and variation of τ_2, but the problem was not fully solved. Spectra sometimes contained no signals at all, except for H_2O, or only broad unresolved resonances. The most likely cause was thought to be small amounts of paramagnetic deoxyhaemoglobin in the venous blood, but repeated treatment with O_2, or even CO, did not always improve spectra. We suggest that a contributing factor is erythrocyte sedimentation and movement, perhaps in association with plasma components, during the course of spectral accumulation. On some occasions when a 1 ml sample of whole blood did not give rise to resolved resonances, the erythrocytes

were allowed to sediment completely over a period of a few hours, without opening the tube. The packed cells were then placed into the receiver coil and subsequently gave rise to well-resolved spectra. We also found that resuspension of well-oxygenated red cells in an equal volume of plasma gave rise to problems of broadening.

In the remainder of this paper we therefore concentrate on studies of plasma and serum.

Human plasma and serum

SEFT ^1H n.m.r. spectra of human serum and plasma are compared in Fig. 2. The aromatic region rarely contained any resonances with comparable signal-to-noise after the same accumulation time (a few min) and will not be discussed further here. The plasma was obtained simply by separating the blood cells by centrifugation from a sample of whole blood which had been preserved in a standard clinical vial containing K_2EDTA. No preservatives were added to the serum sample; the fibrinogen responsible for clotting was removed and the cells again separated by centrifugation.

The spectra are well-resolved, and have a high information content. Those for normal healthy subjects were highly reproducible. Assignments were based on a knowledge of expected low-molecular-weight metabolites present at >0.1 mM concentration, chemical shifts and spin–spin coupling patterns, and were confirmed by direct additions of metabolites to the n.m.r. samples. Metabolites within the n.m.r. concentration range include particularly those with several equivalent protons, especially methyl groups [values in parentheses are approximate expected upper limits (White *et al.*, 1973)]: alanine (0.9 mM), glycine (0.7 mM), isoleucine (0.3 mM), leucine (0.4 mM), valine (0.4 mM) threonine (0.3 mM), creatine (0.07 mM), creatinine

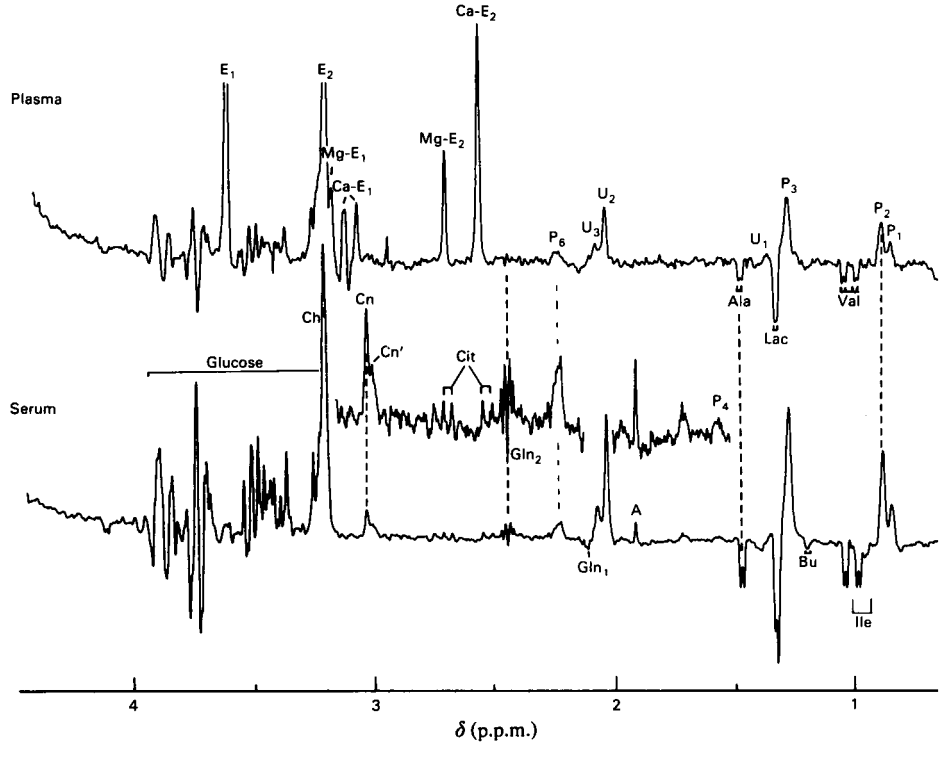

Fig. 2. *400 MHz ^1H n.m.r. SEFT spectra of 12 h fasting human serum (bottom) and plasma (top)*
Spectra are the result of 300 accumulations at 297 K of the Hahn spin-echo sequence with $\tau_2 = 60$ ms, 90° pulse 9.5 μs, 180° pulse 19 μs, relaxation delay 0.1 s, acquisition time 0.80 s (8 K data points, zero-filled to 16 K data points). Exponential multipliers corresponding to a 1 Hz line-broadening have been applied. Assignment of peaks: E_1, Ca-E_1, Mg-E_1 and E_2, Ca-E_2, Mg-E_2, the acetate and ethylenic protons respectively of EDTA and its metal complexes; P_2 and P_3, terminal-CH_3 and -$(CH_2)_n$ groups respectively of triacylglycerols; Ala, alanine -CH_3; Val, valine CH_3s; Lac, lactate -CH_3; A, acetate CH_3; Cn, creatinine N-CH_3; Cn′, creatine N-CH_3; Gln_1 and Gln_2, β-CH_2 and γ-CH_2 groups of glutamine respectively; Bu, D-3-hydroxybutyrate CH_3; Ch, choline N-$(CH_3)_3^+$; Ile, isoleucine -CH_3. High levels of citrate (Cit) can interfere with the integration of peaks Mg-E_2 and Ca-E_2 but in such instances at least one of the four citrate peaks is clearly resolved and can be subtracted. Some peaks (U) have not yet been assigned.

(0.17 mM), and lactate (2 mM). Peaks from most of these are observed (Fig. 2) and those from glutamine (0.7 mM) are usually prominent even though the protons are highly coupled. The high multiplicity of peaks from other amino acids which may be present at levels up to 0.4 mM, e.g. lysine and proline, makes them difficult to detect. However, binding to proteins or paramagnetic metal ions could also cause their disappearance from SEFT spectra.

The concentrations of the α- and β-anomers of glucose can be determined from the intensities of their anomeric proton resonances at 5.25 p.p.m. and 4.65 p.p.m. respectively.

The most variable metabolite was lactate, being dependent on the amount of exercise by the subject prior to venepuncture. The typical 'finger-prints' of small molecules and mobile groups shown in Fig. 2 are substantially altered in disease states, e.g. diabetes mellitus (J. K. Nicholson, M. P. O'Flynn, P. J. Sadler, A. F. Macleod, S. M. Juul & P. H. Sönksen, unpublished work).

The intensities of resonances have to be interpreted with caution at this stage since they may be influenced by both T_1 and T_2 relaxation. All the spectra discussed in this paper were accumulated with similar pulse repetition rates so that T_1 effects are likely to be similar in each case. Recent experiments have shown that intervals of approx. 4 s are necessary to eliminate them. The factors affecting T_2 include the binding of small molecules to macromolecules, to paramagnetic ions (e.g. Cu^{2+}) and viscosity differences between plasma samples. Several methods are useful for investigating T_2 differences, including the measurement of SEFT spectra with a range of τ values (peaks from most of the metabolites present in human plasma are still observable with $\tau_2 = 300$ ms, i.e. a total delay of 600 ms in the Hahn sequence), comparison of SEFT spectra with magnetization-transfer spectra, linearities of peak intensity with concentration after standard additions, and comparison with independent, e.g. enzymic, assays of metabolites. We have found a good comparison between the levels of lactate, alanine, and valine in human plasma samples determined from SEFT ^{1}H n.m.r. spectra by standard additions with conventional assays for these metabolites (J. K. Nicholson, M. P. O'Flynn, P. J. Sadler, A. F. Macleod, S. M. Juul & P. H. Sönksen, unpublished work).

Peaks P_2 and P_3 appear to be associated predominantly with the $-CH_3$ and $-CH_2$-(chain) groups respectively of mobile fatty acids. We have noted that these are most intense in lipaemic plasma samples from obese human subjects and the capybara (see below), and decrease in intensity during fasting (J. K. Nicholson, M. P. O'Flynn, P. J. Sadler, A. F. Macleod, S. M. Juul & P. H. Sönksen, unpublished work). These appear to arise from fatty acids which are not only mobile on the n.m.r. timescale but also readily metabolized. These may be part of high and low density lipoproteins which contain both triacylglycerols and lipids, or chylomicrons which contain largely triacylglycerols. Chapman *et al.* (1969) have previously observed ^{1}H n.m.r. signals from mobile lipids during studies of isolated lipoproteins. Fatty acids bound to albumin would be expected to be highly immobile and to give rise to very broad resonances (Steim *et al.*, 1968) not seen in these SEFT spectra. In lipaemic samples resonances from other parts of fatty acid chains are also present (see Fig. 5).

Although peak P_1 may be due to $-CH_3$ groups of fatty acid pools, it is sometimes seen without an associated P_3 ($-CH_2$-) peak (see wallaby in Fig. 5 and Welcomtrol 1 in Fig. 6). It may be due to terminal-CH_3s of amino acids in proteins, but isolated albumin and immunoglobulin G do not give such a peak.

Peaks U_2 and U_3 are of special interest since their intensities appeared to be highly conserved in most of the human plasma and sera samples we have examined (normal, fasting, diabetic, rheumatoid). They may be due to *N*-acetyls from sugars of glycoproteins. They do not penetrate 1000 mol.wt. cut-off ultrafiltration membranes.

The appearance of separate peaks for $CaEDTA^{2-}$ and $MgEDTA^{2-}$ in plasma from EDTA-preserved blood offers a novel method for studying Ca and Mg levels which will now be discussed further.

Calcium and magnesium estimations: EDTA resonances

A SEFT spectrum of plasma for EDTA-treated blood in the region 2.5–3.7 p.p.m. is shown in Fig. 3 and compared with both SEFT and single pulse spectra of a model solution containing 2 mM-Ca^{2+} and 1 mM-Mg^{2+} and excess K_2EDTA. It can be seen that $MgEDTA^{2-}$ and $CaEDTA^{2-}$ complexes give resonances for the acetate ($-CH_2CO_2^-$) protons at 3.23 and 3.13 p.p.m. (AB multiplets) and resonances for the ethylenic protons ($N-CH_2-CH_2-N$) as singlets at 2.76 and 2.57 p.p.m. respectively.

In an earlier study, Kula *et al.* (1963) noted that peaks from free and bound EDTA in solutions containing Ca^{2+} and Mg^{2+} were observable in the pH range 6–10 even at 60 MHz, i.e. there was slow exchange on the n.m.r. timescale. At physiological pH 7.4, free EDTA is predominantly in the form $HEDTA^{3-}$ and the rate constant for exchange with $CaEDTA^{2-}$ has been estimated to be $<1\,s^{-1}$, whereas at high pH, where $EDTA^{4-}$ predominates, the rate increases to approx. $10^9\,s^{-1}$ (Bryson & Fletcher, 1970).

The association constants of $EDTA^{4-}$ with Ca^{2+} and Mg^{2+} are high: $\log(K) = 11.0$ and 8.7 respectively at 20°C in 0.1 M-KNO_3 (Sillen, 1971). The conditional stability constants at pH 7.4 are approx.

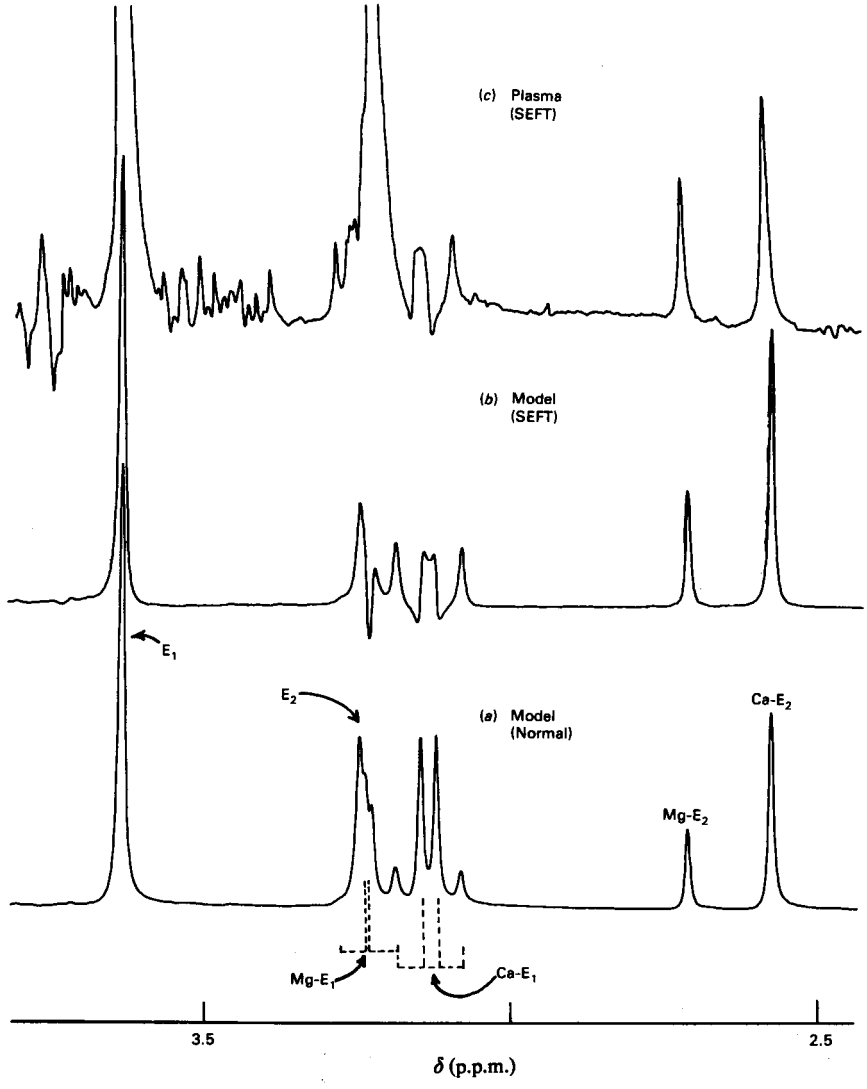

Fig. 3. *400 MHz ^1H n.m.r. spectra in the region 2.5–3.7 p.p.m. of (a) single-pulse spectrum of a model solution containing 2 mM-Ca^{2+}, 1 mM-Mg^{2+}, pH 7.4, in the presence of excess EDTA (300 scans), (b) SEFT spectrum of the above solution (300 scans), and (c) SEFT spectrum of human plasma (300 scans)*

10^3-fold smaller: $\log(K_c) = 7.8$ and 5.8 for Ca^{2+} and Mg^{2+} respectively (Ringbom, 1959). Hence it can be calculated that $\ll 1\%$ of the total Ca^{2+} and Mg^{2+} will remain uncomplexed in our experiments at physiological pH, if EDTA is maintained at mM excess levels. Under these conditions, we find by the method of standard additions and intensity measurements of the ethylenic resonances of CaEDTA and MgEDTA that the values obtained for Ca^{2+} and Mg^{2+} concentrations in human serum are generally within 5% of the values obtained by atomic absorption spectrophotometry. Thus the total amounts of these metal ions in serum are being detected by n.m.r. Typical n.m.r. determinations for our subjects were: 1.01 ± 0.05 (1.1 ± 0.05 mM by atomic absorption spectrophotometry) for Mg^{2+}, and 2.24 ± 0.08 mM (2.25 ± 0.08 mM by atomic absorption spectrophotometry) for Ca^{2+}.

The accuracy of the n.m.r. method was assessed by the determination of Ca^{2+} and Mg^{2+} concentrations in five samples of standard lyophilized plasma, Welcomtrol 1. The results were: Ca^{2+}, 2.50 ± 0.13 mM (2.55 ± 0.08 mM) and Mg^{2+}, 0.77 ± 0.04 mM (0.74 ± 0.02 mM) where the values in parentheses are based on peak areas obtained manually by peak weight and the others by

computer integration. The coefficient of variation of the measurements was 5%, most of this variability being due to inaccuracies in the peak area measurements. The manufacturers independent determinations were reported to be: Ca^{2+}, 2.48mM (cresolphthalein complexone), 2.44mM (Ca–EGTA complexiometric titration), 2.46mM (atomic absorption spectrophotometry) and for Mg^{2+} 0.78mM (atomic absorption spectrophotometry). It is estimated that concentrations down to 10μM can be detected with present ^{1}H n.m.r. equipment, the four magnetically equivalent ethylenic protons effectively amplifying the metal concentration by a factor of 4.

We have shown that Ca^{2+} and Mg^{2+} EDTA complexes are readily detectable at lower field, (200MHz) using the magnetization-transfer technique. Irradiation at the resonance frequency of water (present at approx. 50M) had the effect of reducing the intensity of this resonance as well as saturating the protein (largely albumin and immunoglobulin) envelope. There was a good correspondence of peaks observed in SEFT and magnetization transfer spectra.

It is important to note that this method of simultaneous metal estimation can be readily applied to other biological fluids or cell extracts. As an example, we applied it to the estimation of the intracellular Mg^{2+} concentration in erythrocytes. The cells were lysed in the presence of 5mM-EDTA (which does not really penetrate unlysed cells), and the resultant ^{1}H SEFT n.m.r. spectrum gave clearly resolved $MgEDTA^{2-}$ signals.

We have calculated the intracellular Mg^{2+} concentration to be 2.25 ± 0.1mM, a typical literature figure being 2.35mM (Bowen, 1979). The intracellular Ca^{2+} concentration is much lower, approx. 0.08mM and the ethylenic peak of $CaEDTA^{2-}$ overlaps with the much larger αCH_2 resonance of the glutamate of glutathione.

A number of other metal–EDTA complexes can be studied in the same way. For example, in a solution at pH*7 (meter reading in ^2H_2O) we resolved six peaks for the ethylenic protons of seven

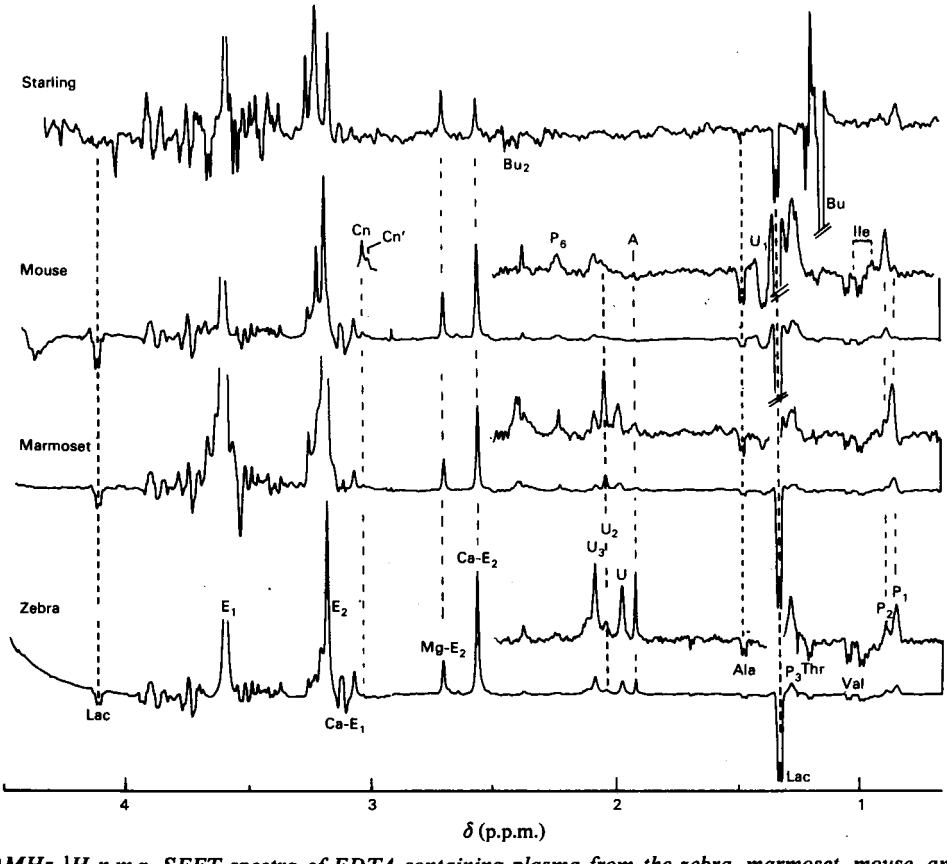

Fig. 4. *400MHz ^{1}H n.m.r. SEFT spectra of EDTA-containing plasma from the zebra, marmoset, mouse, and starling*
Conditions and assignments are as described in the legends to Figs. 1 and 2. Also, Bu_2, D-3-hydroxybutyrate CH_2; Thr, threonine CH_3.

metal–EDTA complexes (Mg^{2+}, Cd^{2+}, Ca^{2+}, Ba^{2+}, Sr^{2+}, Zn^{2+} and Pb^{2+}). The only overlap problem was for Mg^{2+} and Cd^{2+}. Kula *et al.* (1963) have discussed the n.m.r. behaviour of a range of metal–EDTA complexes.

The concentration of Zn^{2+} in human erythrocytes is approx. 0.22mM (Bowen, 1979). A small peak at 2.86p.p.m. adjacent to the cysteine βCH_2 resonance of glutathione was observed in the red cell lysate spectrum. This was tentatively assigned to $ZnEDTA^{2-}$. This Zn^{2+} was probably derived mainly from carbonic anhydrase and other proteins. SEFT n.m.r. experiments on the pure carbonic anhydrase (J. K. Nicholson & P. J. Sadler, unpublished work) have shown that some Zn^{2+} is EDTA-chelatable and that removal from the enzyme takes place over several hours at neutral pH with a two-fold excess of EDTA present. It follows that the interpretation of zinc concentration data from ^1H n.m.r. of cell extracts should be treated with caution as the binding of metal to ligand is likely to be kinetically limited. However such kinetic information is itself very useful.

Comparison of vertebrate plasmas

We studied ^1H SEFT spectra of a range of animal species in order to explore the range of metabolites which might be observable by n.m.r. and to provide

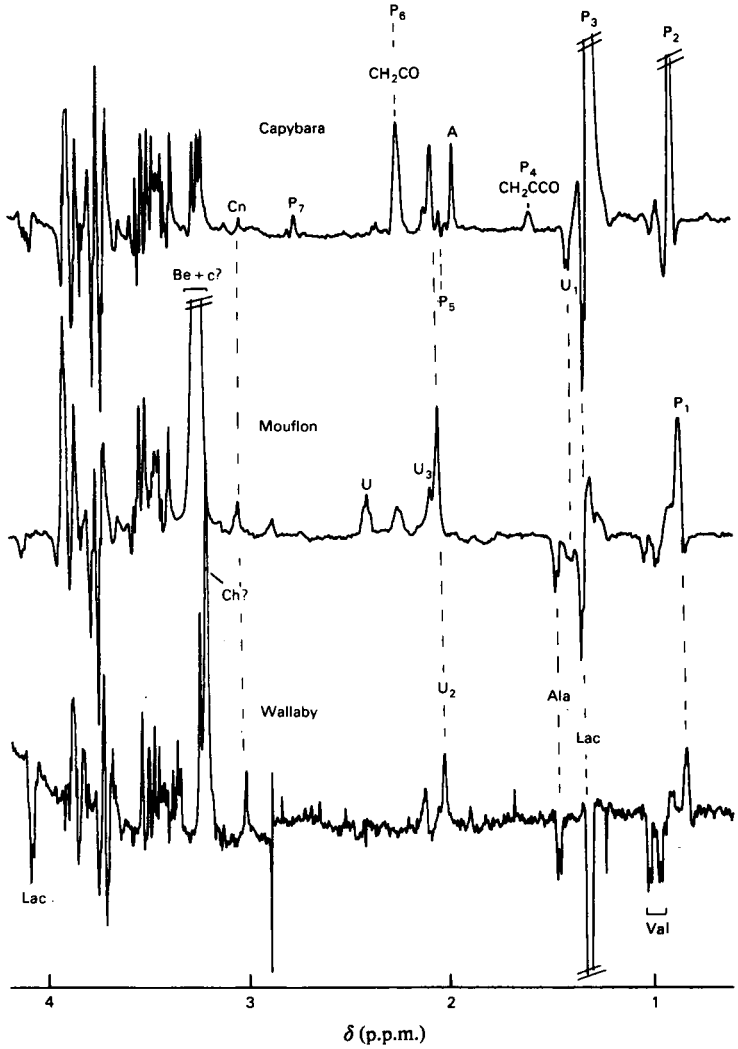

Fig. 5. *400MHz ^1H n.m.r. SEFT spectra of heparin-containing plasma from the wallaby, mouflon and capybara*
The capybara sample was lyophilized and redissolved in ^2H_2O before measurement. The peak positions for the N-$(CH_3)_3^+$ groups of betaine (Be), carnitine (c) and choline (Ch) are indicated, although they overlap with three peaks of glucose.

an additional assignment aid for resonances. Fig. 4 shows spectra of plasma from EDTA-treated blood of starling, mouse, marmoset and zebra and Fig. 5 plasma of heparin-treated blood of the capybara, mouflon and wallaby. Each species appears to have a characteristic pattern of metabolites.

Apart from the sharp methyl doublet of lactate at 1.34 p.p.m. the clearest features are the well-resolved doublets from the CH_3s of alanine (1.48 p.p.m.) and valine (1.0 and 1.05 p.p.m.) present at around 0.3 mM in all species except the starling. Birds rely heavily on efficient metabolism of triacylglycerols for fuel (Hodges, 1974). This is reflected in the large peaks for (>1 mM) D-3-hydroxybutyrate (see -CH_3 at 1.21 p.p.m., Fig. 4), one of the ketone bodies. It is only formed in large quantities in mammals when fats, rather than glycogen, are used as the main source of energy, e.g. on fasting, or when abnormal lipolysis occurs as in diabetic keto-acidosis. Indeed our observations on starling plasma have prompted us to study metabolic changes in insulin-dependent diabetes mellitus (J. K. Nicholson, M. P. O'Flynn, P. J. Sadler, A. F. Macleod, S. M. Juul & P. H. Sönksen, unpublished work). In the latter, peaks for acetone and acetoacetate were seen which have not been present in our starling plasmas.

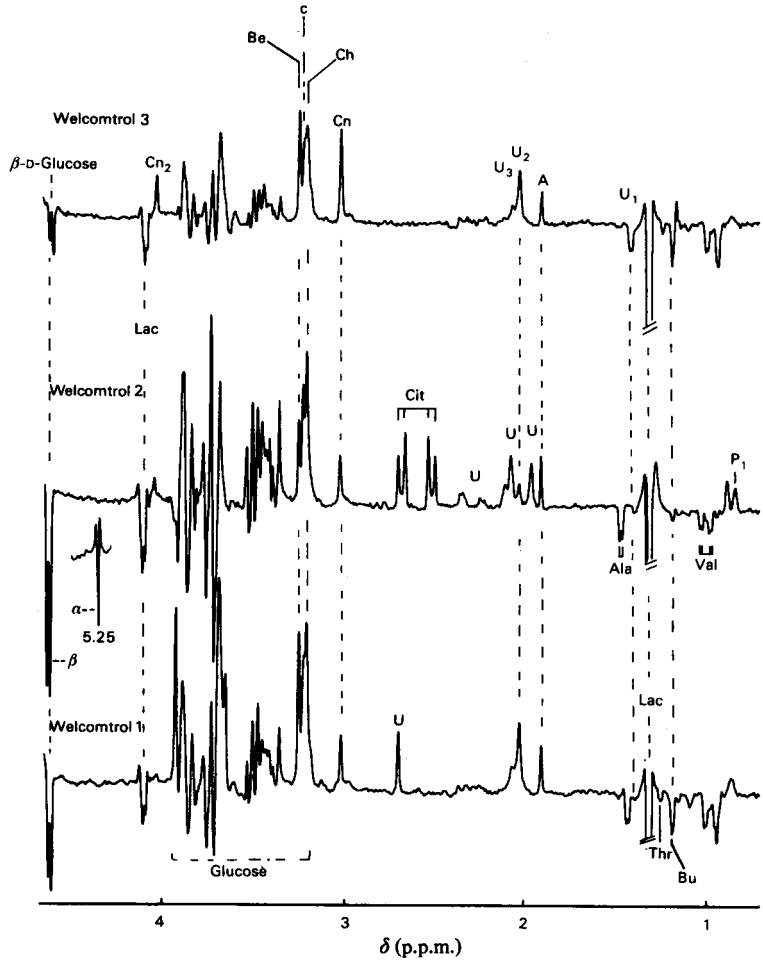

Fig. 6. *400 MHz ^{1}H n.m.r. SEFT spectra of Welcomtrols 1 (bottom), 2 (middle) and 3 (top), prepared by dissolving lyophilized powder in ^2H_2O*

The two resonances at 1.955 and 2.02 p.p.m. for Welcomtrol 2 are also found in the plasma spectrum of the zebra (Fig. 6). Welcomtrols 1 and 3 are of bovine origin. The intensities of the glucose peaks parallel the manufacturers assay values of 5.8, 12.1, and 3.0 mM for Welcomtrols 1, 2 and 3 respectively. The creatinine values (0.16, 0.35 and 0.63 mM) parallel the intensities of the CH_2 peak (Cn_2) but not, for Welcomtrols 1 and 2, the N-CH_3 peak (Cn), suggesting an overlap problem.

The relative heights of the $CaEDTA^{2-}$ and $MgEDTA^{2-}$ ethylenic proton peaks in Fig. 4 show that there are significant variations in Ca^{2+} and Mg^{2+} levels, man being similar to marmosets and mice, Ca^{2+} being slightly higher in the zebra and lower in the starling plasma shown. Atomic absorption spectrophotometry measurements on the latter indicates that only 70% of the total Ca^{2+} was being seen by n.m.r. It is possible that avian plasma contains proteins which bind Ca^{2+} more strongly than $EDTA^{4-}$; however, four other lyophilized starling plasma samples redissolved in ^2H_2O did not show this effect. Birds are known to undergo many more diurnal and seasonal physiological changes than mammals.

The intense peaks P_2 and P_3 in the spectrum of capybara plasma, Fig. 5, are attributable to high levels of triacylglycerols. These appear to contain unsaturated fatty acids (e.g. palmitoleate, oleate, linoleate) since peaks for $CH_2{-}CH{=}CH{-}CH_2$ (CH=CH at approx. 5.4 p.p.m., results not shown) and $HC{=}HC{-}CH_2{-}CH{=}CH$ protons also appear in the spectrum (compare also dipalmitoylphosphatidylcholine, Fig. 7). The capybara, the world's largest rodent, has a bulky body containing plenty of fatty tissue which counterbalances skeletal weight especially during swimming. The spectrum closely resembles that from the plasma of an obese non-insulin-dependent diabetic subject (J. K. Nicholson, M. P. O'Flynn, P. J. Sadler, A. F. Macleod, S. M. Juul & P. H. Sönksen, unpublished work). Both samples had a milky appearance suggestive of a high chylomicron content. It is clear that such studies are potentially very useful in work on fatty acid metabolism.

Clear differences between humans and other mammals are seen for $-CH_3$ resonances near 0.9 p.p.m. (ratio P_1/P_2) and other unassigned peaks

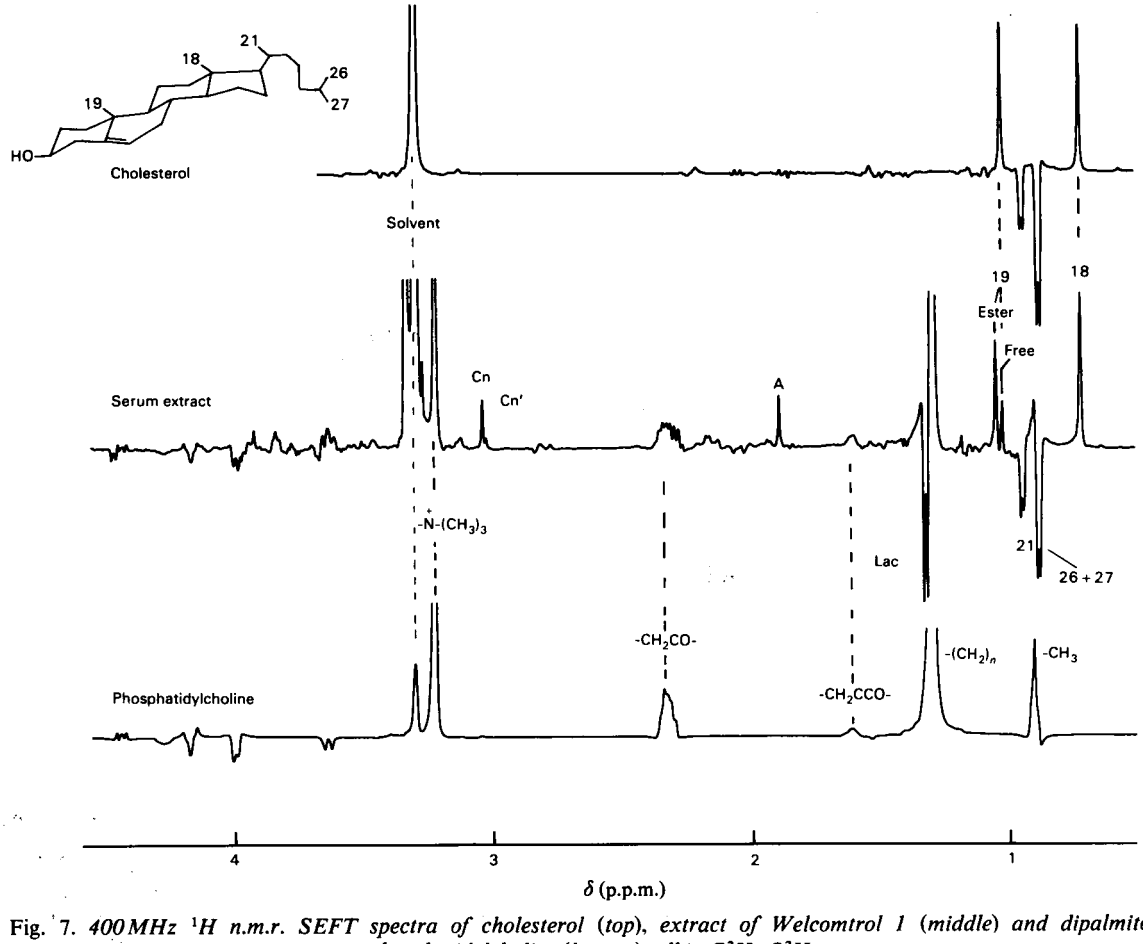

Fig. 7. *400 MHz ^1H n.m.r. SEFT spectra of cholesterol (top), extract of Welcomtrol 1 (middle) and dipalmitoyl phosphatidylcholine (bottom), all in C^2H_3-O^2H*

Assignments for phosphatidylcholine are based on those of Birdsall *et al.* (1972). Note the two peaks for the C-19 CH_3 of cholesterol corresponding to free (1.023 p.p.m.) and esterified (1.050 p.p.m.) forms.

between 2.2 and 2.5 p.p.m. (Figs. 4 and 5). Significant variations in the intensities of the -$N(CH_3)_3^+$ resonances from choline and its derivatives around 3.2 p.p.m. also occur, although these can only be resolved in the absence of EDTA. Notable is the high level in the (1-day-old) mouflon (Fig. 5).

Quality control sera

The results described so far suggested that plasma and sera n.m.r. studies might be routinely useful in clinical chemistry laboratories. We therefore examined widely used quality control sera, Welcomtrols 1, 2 and 3. Their spectra are shown in Fig. 6. A wide variety of substances in these sera are assayed by the suppliers and supplemented to bring them within certain specifications. For example, the creatinine concentration is much greater in Welcomtrol 3 than in Welcomtrols 1 and 2 as reflected in the greater intensity of the N-CH_3^+ peak at 3.02 p.p.m. Similarly the glucose content is highest in Welcomtrol 2 (see Fig. 6). N.m.r. also provides information about metabolites not routinely measured by the suppliers, e.g. Welcomtrol 2 contains a high citrate concentration whereas Welcomtrols 1 and 3 do not.

It is also of interest to note that Welcomtrols 1 and 3 are bovine sera whereas Welcomtrol 2 is equine. The latter contains a number of metabolites which are not normally present in bovine serum but which are found in zebra plasma (compare Figs. 4 and 6). Zebras and horses are phylogenetically close relatives, being members of the *Equidae* family.

We have noted that lyophilization of plasma and serum samples often leads to a small low-frequency (high-field) shift of the -CH_3 resonances of alanine and valine. The alanine doublet appears to shift to the positions of unassigned resonance U_1 (see Fig. 1 also). In Fig. 5 the spectra of Welcomtrols 1 and 3 show this effect but Welcomtrol 2 does not. Similarly in Fig. 5 the capybara plasma was lyophilized but the other two were not. The origin of this effect is being investigated further.

Methanol extracts of plasma

We have noted that resonances for molecules present in relatively immobile micellar structures such as cholesterol and phosphatidylcholine are not seen in the spectra of aqueous plasma. However, we find that they are readily extracted from lyophilized samples into methanol. In Fig. 7, a methanol extract of Welcomtrol 1 is compared to SEFT ^{1}H n.m.r. spectra of cholesterol and dipalmitoylphosphatidylcholine.

After extraction, single resonances are obtained for the C-18, C-21 (doublet), C-26 and C-27 -CH_3 groups of cholesterol but two resonances in approx. ratio 2:1 for the -CH_3 at C-19. These are attributed to cholesterol esterified to linoleic acid and free cholesterol respectively, known to be present in plasma in approximately this ratio (Bell *et al.*, 1976). C-19 is the closest -CH_3 to the esterification site, C-3. The C-19 -CH_3 resonance assigned to free cholesterol increased in intensity on addition of further cholesterol to the methanol extract. Thus n.m.r. provides a novel method of simply estimating the proportion of free and esterified cholesterol in plasma. The total cholesterol level, 4.15 mM, calculated from n.m.r. measurements by a method of standard additions, was close to the total as determined by the manufacturer's assay.

Fig. 7 also shows that dipalmitoylphosphatidylcholine is extracted from plasma into methanol along with acetate, lactate, creatine, creatinine and a few other unidentified substances.

We are very grateful to Rio Tinto Zinc Services, the S.E.R.C., M.R.C. and U.L.I.R.S. for support, to Lynette Wilson (U.C.L.) and Dr. D. Osborn (Monks Wood Experimental Station) for their generous supply of animal blood and plasma, to Professor A. Grimes (St. Thomas' Hospital) for advice on defibrination, and to Professor D. L. Rabenstein, Professor B. Kratochvil, Dr. A. A. Isab (University of Alberta) and Dr. N. J. Birch (Wolverhampton Polytechnic) for useful discussion. P. J. S. was a Nuffield Foundation Research Fellow (1981–2).

References

Bell, G. H., Emslie-Smith, D. & Peterson, C. R. (1976) *Textbook of Physiology and Biochemistry*, 9th edn., Churchill Livingstone, Edinburgh

Birdsall, N. J. M., Feeney, J. Lee, A. G., Levine, Y. K. & Metcalfe, J. C. (1972) *J. Chem. Soc. Perkin 2* 1441–1445

Bowen, H. J. M. (1979) *Environmental Chemistry of the Elements*, Academic Press, London

Brindle, K. M., Brown, F. F., Campbell, I. D., Grathwohl, C. & Kuchel, P. W. (1979) *Biochem. J.* **180**, 37–44

Brown, F. F. & Campbell, I. D. (1980) *Proc. R. Soc. London Ser. B* **289**, 395–406

Brown, F. F., Campbell, I. D., Kuchel, P. W. & Rabenstein, D. L. (1977) *FEBS Lett.* **82**, 12–16

Bryson, A. & Fletcher, I. S. (1970) *Aust. J. Chem.* **23**, 1095–1100

Chapman, B. E., Beilharz, G. R., York, M. J. & Kuchel, P. W. (1982) *Biochem. Biophys. Res. Commun.* **105**, 1280–1287

Chapman, D., Leslie, R. B., Hirz, R. & Scanu, A. M. (1969) *Biochim. Biophys. Acta* **176**, 524–536

Hodges, R. D. (1974) *The Histology of the Fowl*, Academic Press, New York

Kula, R. J., Sawyer, D. T., Chain, S. I. & Finley, C. M. (1963) *J. Am. Chem. Soc.* **85**, 2930–2936

Rabenstein, D. L. & Nakashima, T. T. (1979) *Anal. Chem.* **51**, 1465A–1474

Rabenstein, D. L., Isab, A. A. & Brown, D. W. (1980) *J. Magn. Reson.* **41**, 361–365

Ringbom, A. (1959) *Treat. Anal. Chem.* **1**, 543–628

Sillen, L. G. (ed.) (1971) *Stability Constants of Metal–Ion Complexes*, **25**, Suppl. **1**, The Chemical Society, London

Steim, J. M., Edner, O. J. & Bargoot, F. G. (1968) *Science* **169**, 909–911