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Changes in Apparent pH on Freezing Aqueous Buffer Solutions and their Relevance to Biochemical Electron-Paramagnetic-Resonance Spectroscopy

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Changes in apparent pH occurring during fast freezing of aqueous buffer solutions and cooling to −196°C were studied by various semiquantitative methods, including simple visual measurements of colour changes with pH indicators, as well as measurements of pH-dependent changes in the e.p.r. (electron paramagnetic resonance) spectra of solutions of three different metalloenzymes. It is concluded that apparent pH changes of up to about 3 pH units may occur under particular conditions. Such changes were independent of the time taken to freeze the samples, when this was varied from about 3 ms to 20 s, but were affected by the presence of some proteins in solution. Recommendations on the buffers that should be used to avoid such apparent pH changes in e.p.r. spectroscopy and other low-temperature biochemical work are made. Phosphate and pyrophosphate buffers, which gave large decreases (2–3 pH units), and Tris, which under some conditions gave increases of about the same magnitude, are to be avoided. Certain zwitterionic buffers such as Bicine [*NN*-bis-(2-hydroxyethyl)glycine] are satisfactory. Apparent pH effects were found to depend on buffer and protein concentration. It is therefore recommended that as a prelude to future detailed low-temperature biochemical work, appropriate tests with an indicator system should be performed.

Investigations of biological molecules by physical methods are frequently carried out at low temperatures in frozen aqueous solutions (for early studies see, e.g., Keilin & Hartree, 1949). Such work, which may be performed at temperatures ≤4.2 K, includes e.p.r.* spectroscopy, Mössbauer spectroscopy, optical spectroscopy and measurements of magnetic susceptibility and magnetic circular dichroism. It is well known that freezing is likely to modify systems under study in some way, and that therefore direct correlation between results obtained at ambient temperatures in solution and results obtained in the frozen state may not always be possible. Nevertheless, it is surprising how few systematic studies on the effects of freezing have been carried out. pH changes occurring in buffers dissolved in unfrozen mixtures of water and organic solvents ('antifreeze' solutions), on cooling to temperatures down to −50°C, have been reported (Hui Bon Hoa & Douzou, 1973; Maurel *et al.*, 1975), but are not directly relevant to the situation in frozen solutions. In connexion with the frozen-foodstuffs and pharmaceuticals industries, studies on pH changes in the residual liquid phase resulting from phase separation during very slow freezing of aqueous buffers and cooling to temperatures in the region of −20°C have been reported (see, e.g., Van den Berg, 1966; Larsen, 1973). Such results are not necessarily relevant to biochemical e.p.r. studies, for example, where fast freezing is usually used, so that supersaturation rather than phase separation would be expected. Here, freezing times are generally in the range of 20 s to approx. 3 ms (Bray, 1961; Bray *et al.*, 1973; Ballou & Palmer, 1974).

In the present work, apparent pH changes occurring on fast freezing of aqueous buffer solutions have been studied in several ways as part of an e.p.r. study. We present semiquantitative observations of apparent pH changes during the freezing of buffer solutions containing pH-sensitive dyes or pH-sensitive paramagnets ('e.p.r. chromophores'). Because of the complexity of the systems studied, it is not yet

* Abbreviation: e.p.r., electron paramagnetic resonance.

possible to make precise measurements or to determine the exact mechanism of the observed effects. We have therefore used as an operational term 'apparent pH', which is defined below (see the Results section). Though our findings are presented as empirical observations only, nevertheless they should be of considerable practical importance to other workers.

Materials and Methods

Buffers

Unless otherwise stated, all buffers were adjusted to the appropriate pH with NaOH or HCl. Potassium phosphate buffer was KH_2PO_4 adjusted with KOH. Pyrophosphate buffer was made from $Na_4P_2O_7,10H_2O$. Other buffers were morpholine, Tris, Taps (3-{[2-hydroxy-1,1-bis(hydroxymethyl)-ethyl]amino}propane-1-sulphonic acid), Hepps [4-(2-hydroxyethyl)piperazine-1-propanesulphonic acid], Bicine [*NN*-bis-(2-hydroxyethyl)glycine], Tricine {*N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]-glycine}, Hepes [4-(2-hydroxyethyl)piperazine-1-ethanesulphonic acid], Mes (4-morpholine-ethanesulphonic acid), Ches [2-(cyclohexylamino)ethanesulphonic acid], Mops (4-morpholinepropanesulphonic acid) and Pipes [piperazine-1,4-bis(ethanesulphonic acid)].

Method of freezing

For most of the work, samples were frozen in one of two ways, both of which are widely used in biochemical e.p.r. investigations. The first involved immersing the samples, contained in thin-walled silica tubes (internal diam. 3mm), cautiously into liquid N_2. The total time taken with this procedure was about 20s, corresponding to an initial cooling rate of approx. 20°C/s. The second procedure was the 'rapid freezing' method of Bray (1961) (see also Bray *et al.*, 1973) that involves squirting the solution into cold isopentane. Freezing times with this method (as measured by the time required to stop a particular test reaction) can be as short as approx. 3ms (Bray *et al.*, 1973; Barber, 1976; see also Ballou & Palmer, 1974). This is equivalent to an initial cooling rate of 5000–20000°C/s. Slow freezing at −20°C was achieved by placing the samples in a refrigerated cabinet.

pH measurements with indicators

A commercial mixed indicator solution was used ('Universal Indicator Solution' from British Drug Houses, Poole, Dorset, U.K.). An appropriate concentration of the indicator was added to the buffer solutions before final pH adjustment. Colours of the frozen solutions were matched by eye with the standard colours printed on the label of the bottle by the makers. To obtain an estimate of the reproducibility of such measurements, each sample was examined by four to six different observers. The average of their estimates of the pH was recorded together with the standard deviation.

Enzymes

Nitrate reductase was prepared from *Escherichia coli* K12 by the method of Clegg (1976). Reduction was not required to elicit the e.p.r. signals from Mo(V) (Bray *et al.*, 1976). Bovine liver catalase was from Sigma Chemical Co., Kingston-upon-Thames, Surrey, U.K. (grade C-40); human erythrocyte catalase was isolated as described by Saha *et al.* (1964) with final purification on DEAE-cellulose (Whatman; DE-52) and Sephadex G-150. Xanthine oxidase was prepared and reduced with salicylaldehyde as described previously (Cammack *et al.*, 1976; Barber, 1976). In calculating pH values from $FADH^{\bullet}$ e.p.r. signal intensities of xanthine oxidase, a pK of 8.2 was assumed for an ionization controlling conversion of the enzyme between forms assumed to give (under the reduction conditions used) 1.95% of the flavin as $FADH^{\bullet}$ at low pH and 0.35% at high pH. Errors with this procedure (Fig. 1) correspond to the individual values for duplicates. In the experiment reported by Cammack *et al.* (1976), the $FADH^{\bullet}$ intensity in phosphate buffer was wrongly plotted.

Computation of species present in solutions containing Cu^{2+} ions, EDTA and buffer

A digital computer program was written in ALGOL 60 and run on the ICL 1906A computer at the University of Leeds (in collaboration with Dr. D. G. Herries). The Newton–Raphson iteration method was used to calculate concentrations of each of the five or more copper-containing species, including mixed complexes, which might be present.

E.p.r. measurements

A Varian E9 spectrometer was used. The techniques were as described by Lowe *et al.* (1972).

Results

Definition of 'apparent pH'

The point of interest to us is whether and to what extent the state of ionization of groups affecting a chromophore in a particular frozen system differs from that in solution at, say, 20°C. Conversely, if the state of ionization of a chromophore can be measured in a frozen sample, then obviously, provided that a pK value is available (for example, we might arbitrarily assume that the pK value in solution at 20°C applies), then this measurement will furnish an apparent local pH value. Although the precise meaning of the value so obtained may be obscure, its relevance to the study of chromophore is obvious. To facilitate further consideration of ionizations in frozen aqueous systems, we make an operational

definition of the term 'apparent pH' (denoted by the symbol $pH_{app.}$), to be used throughout this paper, as meaning the pH value deduced from the state of ionization of a particular chromophore in a particular frozen system together with its pK value in solution at 20°C. The 'apparent pH change' ($\Delta pH_{app.}$) on cooling is then given by: $\Delta pH_{app.} = pH_{app.} - pH$ (20°C).

Studies with mixed indicators

We first describe some simple experiments in which pH-indicator molecules served as the chromophores whose ionizations were monitored in a series of frozen samples. We found, on freezing a series of buffer solutions containing mixed indicators and cooling them to the temperature of liquid N_2, that quite large variations in the apparent pH as indicated by the colour of the frozen solution, took place, with the sign and magnitude of the apparent pH change depending markedly on the nature of the buffer (see Table 1). Although with some buffers $\Delta pH_{app.}$ was small, with others it was quite large, ranging from about +2 to −2 or −3. Samples showing large increases were mainly cationic buffers and those showing large decreases were anionic buffers, whereas the zwitterionic buffers, apart from Taps, gave smaller changes.

In general, on cooling buffers in liquid solution below 0°C, only increases of pH are expected (Hui Bon Hoa & Douzou, 1973). These increases are relatively small for anionic buffers (e.g. phosphate buffer in aqueous 50% methanol has $\Delta pH/°C = -0.003$ in the temperature range +20°C to −50°C), but larger for cationic ones. Temperature coefficients for the pK values of common indicators in solution are comparable in magnitude with those of common buffers (Benkenkamp & Rieman, 1975; Hui Bon Hoa *et al.*, 1970). These considerations do not, however, affect comparison of one buffer with another or our definition of $pH_{app.}$.

In our experiments, the apparent pH change for a given buffer was found (Table 1) not to depend in any obvious way on the initial pH of the solution nor on the time taken to freeze it, when this was varied from about 20s to about 3ms. (Freezing procedures were modelled on ones used widely in biochemical e.p.r. investigations; see the Materials and Methods section for details.) Also, we found that varying the observation temperature from −196°C to −142°C did not affect $\Delta pH_{app.}$ (results not shown). In phosphate buffer, which we studied in some detail, the marked colour changes were reversible when the cycle of manual freezing and thawing was repeated several times. However, if samples were frozen much more slowly, at −20°C, or if they were allowed to warm slowly to this temperature from −196°C, then with some buffers there were signs of separation of solute. Apparent pH changes for slow freezing at −20°C are listed in the footnote to Table 1. For some, e.g. pyrophosphate, most of the overall apparent pH change occurring between +20°C and −196°C took place in the narrow range between +2°C and −20°C. Thus it would seem that in these cases at least, it is the freezing process itself, rather than the actual

Table 1. *$\Delta pH_{app.}$ measured with indicators on cooling various buffer solutions from 20°C to −196°C*

Values of $\Delta pH_{app.}$ are given. Details of the buffers (0.1M) and of the measurement procedure are given in the Materials and Methods section. Two sets of buffer solutions were used, with different pH values at 20°C. Alternative freezing procedures (see the Materials and Methods section) involved immersing the tubes in liquid N_2 ('manual' freezing), or rapid freezing by squirting into cold isopentane. In one experiment bovine serum albumin (40mg/ml) was added.

Buffer	$\Delta pH_{app.}$ for pH 8.1–8.2 at 20°C	$\Delta pH_{app.}$ for pH 6.9–7.4 at 20°C			Average pH change (without albumin)
		Rapid freeze	Manual freeze		
	Manual freeze		Alone	With albumin	
Tris	+1.6±0.5	+2.1±0.3	+3.3±0.4*	+0.1±0.2	+2.3
Taps	+1.8±0.6	+2.0±0.5	+0.9±0.7	—	+1.6
Morpholine	+1.3±0.2	—	—	—	+1.3
Hepps	+0.6±0.2	—	—	—	+0.6
Bicine	+0.4±0.2	−0.4±0.2	−0.4±0.1*	0 ±0.1	−0.1
Tricine	—	0 ±0.2	−0.4±0.1	+0.1±0.2	−0.2
Hepes	—	—	−0.4±0.2	+0.4±0.1	−0.4
Potassium phosphate	−1.2±0.1	−1.4±0.7	−1.1±0.3*	+0.1±0.1	−1.2
Sodium phosphate	—	−1.8±0.2	−1.9±0.5*	−0.3±0.1	−1.8
Sodium pyrophosphate	−2.5±0.2	−2.9±0.3	−2.9±0.7*	−1.2±0.5	−2.8

* Corresponding values in samples frozen slowly at −20°C and observed at this temperature in Tris, Bicine, potassium phosphate, sodium phosphate and sodium pyrophosphate were respectively +0.9±0.4, +0.2±0.4, −0.2±0.3, −3.4±0.1 and −2.5±0.7.

low temperatures, that give rise to these apparent pH changes.

To test the effects of a protein on the system, we added bovine serum albumin to some samples. Apparent pH changes, measured at low temperatures by the indicator procedure, were largely suppressed (Table 1) in all buffers except pyrophosphate, when albumin was present at 40mg/ml. At 10mg of albumin/ml, the suppression was less complete (results not shown).

In further experiments (Table 2) we studied the effect of varying the concentration of the buffer. Quite large effects of concentration on $\Delta pH_{app.}$ were found with Mops (whether sodium or potassium salts were used) and there was also a significant effect with Pipes. On the other hand, with the phosphate buffers, concentration effects were small.

Though we have not established the theoretical basis of the results in Tables 1 and 2, they do seem to be relevant to biochemical e.p.r. investigations. Indeed they appear to provide a ready explanation of a considerable number of experimental observations. The following are some systems that are familiar to us.

Xanthine oxidase

Cammack *et al.* (1976) found that e.p.r signals from the FADH• semiquinone obtained on partial reduction of xanthine oxidase changed some fivefold in intensity when the nature of the buffer was varied, under particular conditions of reduction. They worked at a fixed pH of 8.2, measured at 20°C before freezing. Although different buffer ions might influence molybdenum e.p.r. signals from the enzyme by binding directly to the metal, such an explanation seems unlikely for the FAD moiety. We therefore speculated that apparent pH changes that occurred on freezing the samples might have been the cause of the FADH• signal changes. It is known from experiments not involving freezing that the yield of FADH• from the enzyme is pH-dependent. Thus from stopped-flow studies at 25°C, Olson *et al.* (1974) concluded that, though little if any semiquinone is formed at high pH values, it is markedly stabilized at low pH. In the absence of detailed studies of effects of pH, a pK of about 8.2 at 25°C for the transformation of a high-FADH• into a low-FADH• reduced form of the enzyme would appear consistent with the data of Olson *et al.* (1974). By using this pK and assuming that there are no specific effects, either direct or indirect, of buffers on FADH• signal intensity, it is then possible to use the FADH• e.p.r. signal intensity [as reported by Cammack *et al.*

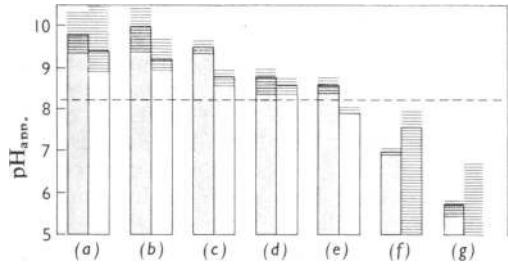

Fig. 1. *Histogram comparing apparent pH values estimated with indicators, and from FADH• e.p.r. signals from xanthine oxidase, for various frozen buffer solutions*

$pH_{app.}$ values obtained (at −196°C) with mixed indicators (stippled areas) are taken from Table 1. The alternative method (unshaded areas) involved measurement of the intensity of the FADH• e.p.r. signal (at about −150°C) given by xanthine oxidase samples in the various buffers (0.1M, also containing 0.1M-NaCl), after they had been reduced under standard conditions (see the text for details). All samples were frozen in liquid N_2. Areas with horizontal shading correspond to estimated errors. The dashed line corresponds to the initial pH of all the buffers at 20°C (8.2). (*a*) Tris; (*b*) Taps; (*c*) morpholine; (*d*) Hepps; (*e*) Bicine; (*f*) potassium phosphate; (*g*) sodium pyrophosphate.

Table 2. *Effect of buffer concentration on $\Delta pH_{app.}$ observed on cooling from 20°C to −196°C*

Values of $\Delta pH_{app.}$ (measured with indicators) were obtained by the procedure given in the Materials and Methods section. Freezing was by immersing in liquid N_2.

Buffer (pH at 20°C) Concn. ...	$\Delta pH_{app.}$ 10mM	100mM	0.5M	1.0M
Pipes (6.8)	−0.4±0.1	−0.3±0.1	+0.5±0.2	—
Mops (7.2)	−0.3±0.1	+0.1±0.1	—	+1.2±0.4
Mops (7.3)*	−0.2±0.3	+0.5±0.5	—	+1.3±0.6
Sodium phosphate (7.4)	−2.9±0.4	−3.1±0.3†	—	−3.3±0.2
Potassium phosphate (7.4)	−1.7±0.2	−1.4±0.5	—	−1.2±0.7

* Adjusted with KOH instead of NaOH.

† Agreement between this value and the corresponding one in Table 1 is poor. This may be due to small differences in the exact freezing procedure, which could be crucial with this buffer; the samples were frozen by different workers.

(1976) and Barber (1976)] to measure the apparent pH change resulting from freezing each of the buffers (see the Materials and Methods section for further details). In Fig. 1 apparent pH values calculated in this way are compared with those obtained for the same buffers by using the mixed-indicator procedure. Despite the rather arbitrary numerical assumptions, the generally good agreement strongly supports the proposal that the buffer effects of Cammack *et al.* (1976) on the flavin of xanthine oxidase were in reality the result of apparent pH changes, of about the magnitudes revealed by the indicator studies, which occurred on freezing and cooling the samples.

Catalase

Catalase binds a number of small molecules, such as formate, nitrite, acetate and fluoride, to produce characteristic changes in its optical and e.p.r. spectra. The pH-dependence of binding indicates that the free acids are bound (Chance, 1952). In a study of the e.p.r. spectra of catalase complexes at 9.0K (Williams-Smith & Patel, 1975) the dissociation constant for catalase–formate was 1.0± 0.5mM for bovine liver and human erythrocyte catalases, when the pH before freezing was 7.0–7.4, at 2–20°C in 0.25M-Hepes buffer. In 0.01M-sodium phosphate, and at similar pH values before freezing, the dissociation constant was two orders of magnitude lower. This is consistent with $\Delta pH_{app.}$ being lower by 2pH units in sodium phosphate buffer than it is in Hepes buffer, in agreement with the indicator results. This effect of phosphate on the dissociation constant was abolished by high concentrations of albumin (170mg/ml). Comparable effects of phosphate were obtained when studying nitrite binding to catalase.

Nitrate reductase

E.p.r. has been widely used in investigations of quinquivalent molybdenum in enzymes (see Bray, 1975). Recent studies on nitrate reductase from *E. coli* K12 (S. P. Vincent & R. C. Bray, unpublished work) show that the major form of this enzyme gives a simple rhombic molybdenum signal with $g_{av.}$ 1.976 at high pH values, that changes to a proton-split rhombic signal with $g_{av.}$ 1.983 (Bray *et al.*, 1976) when the pH is lowered. Typical spectra at different pH values (measured at 20°C) in a number of buffers are shown in Fig. 2, arranged in order of increasing proportions of the high-pH form, as judged particularly from the features at the high-field end of the spectrum. This sequence is also, in general, that of increasing initial pH, and the data are consistent with an apparent p*K* for the transformation between the two species of about 8.2 (S. P. Vincent & R. C. Bray, unpublished work). However, as shown in Fig. 2(*a*), the spectrum of the enzyme in sodium phosphate buffer is seriously out of line and indicates

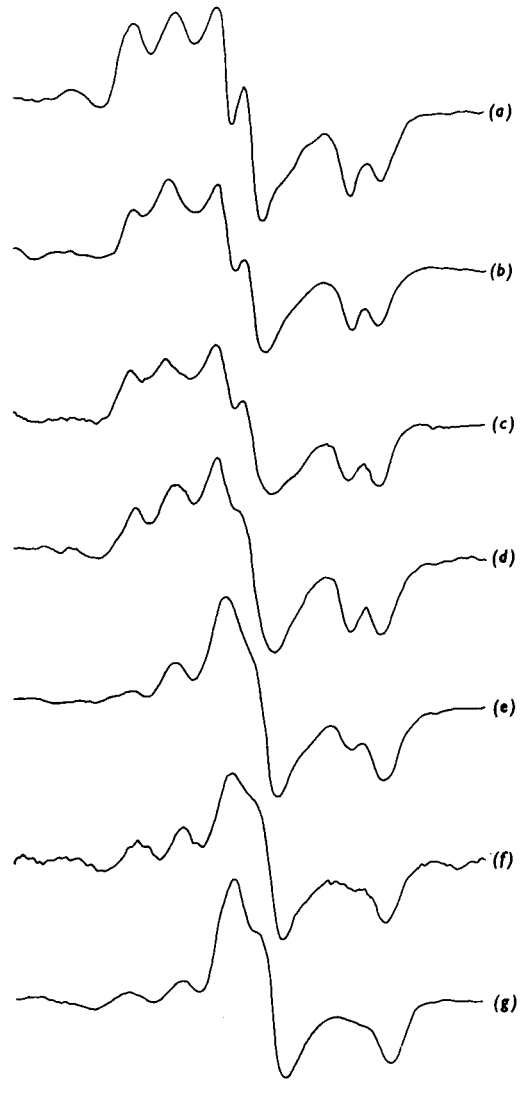

Fig. 2. *E.p.r. spectra of molybdenum(V) in nitrate reductase from E. coli K12*

Spectra were recorded at about −150°C. Buffers and pH values (at 20°C) were as indicated. Buffer concentrations were 0.05M, except (*f*) (0.25M) with the addition of 0.4M-NaCl in all cases. Samples were frozen in liquid N_2. (*a*) Sodium phosphate, pH7.7; (*b*) Mes, pH6.1; (*c*) Tris, pH7.0; (*d*) Mops, pH7.2; (*e*) Hepps, pH8.2; (*f*) Mops, pH7.2; (*g*) Ches, pH9.3.

that a substantial lowering of apparent pH (perhaps of 2pH units) relative to the other samples has taken place. Conversely, use of a high concentration of Mops (Fig. 2*f*) results in an increase in apparent pH relative to the other samples of at least 1pH unit. Thus although a p*K* for the enzyme has not been measured at 20°C, so that all our results for

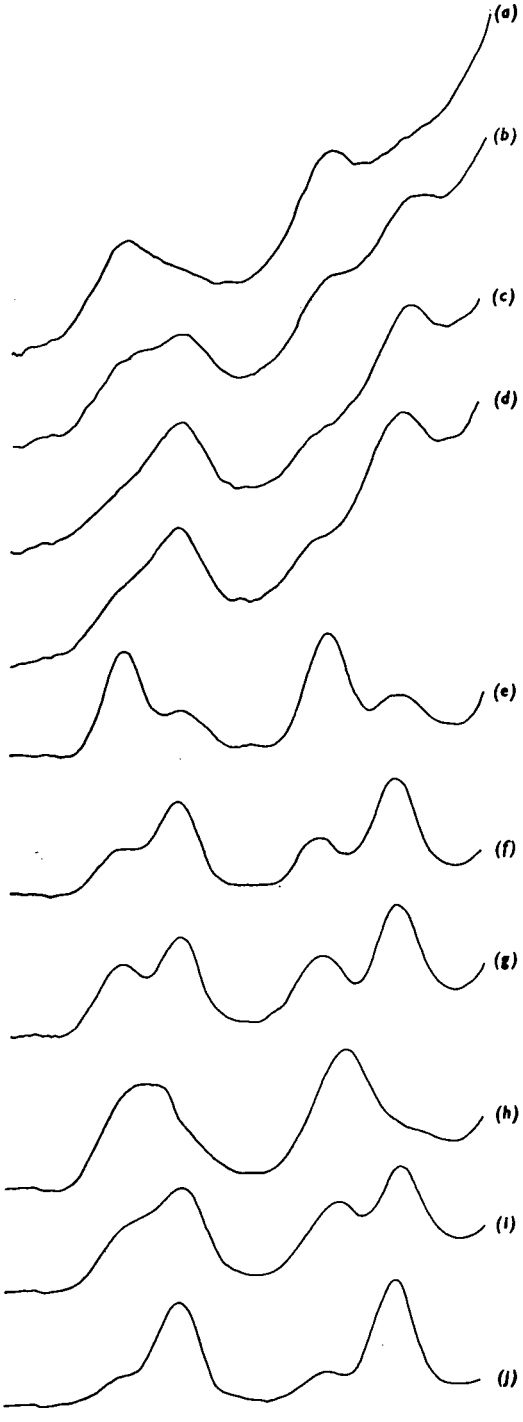

Fig. 3. *E.p.r. spectra of Cu^{2+}–EDTA at various pH values in different media*

Only the low-field part of the spectrum (first two $g_{\parallel}$ hyperfine lines) is shown. Spectra were recorded at about −150°C. In all cases the Cu^{2+} concentration was 0.4mM. pH values (at 20°C) were adjusted to those indicated. In (*a*)–(*f*), EDTA served as the buffer, whereas in (*g*)–(*j*) additional buffers were present. The rapid-freezing procedure of Bray *et al.* (1973) was used for (*g*) with sodium phosphate buffer; in other samples freezing in liquid N_2 was used. (*a*)–(*d*) 0.4mM-EDTA at pH3.5, 4.0, 5.0 and 6.0 respectively; (*e*) and (*f*) 50mM-EDTA at pH3.5 and 6.0respectively; (*g*) 0.4mM-EDTA and 50mM-sodium phosphate at pH6.0; (*h*) and (*i*) 0.4mM-EDTA and 50mM-sodium pyrophosphate at pH5.0 and 6.0 respectively; (*j*) 0.4mM-EDTA and 40mM-Mes at pH5.5.

the enzyme are only relative, nevertheless there is good parallelism between the indicator data and that on nitrate reductase.

Cu^{2+}–EDTA complexes

In search of a non-enzymic system for measuring changes of apparent pH in frozen solution by e.p.r., we carried out some studies on Cu^{2+}–EDTA complexes. Though these have so far been only partly successful, some results seem relevant. The spectrum of Cu^{2+}–EDTA in frozen solution under most conditions is rather complex and consists of at least two species. One species which predominates at low pH values has $g_{\parallel}$ about 2.34 (Hathaway *et al.*, 1969). As shown in Fig. 3, when the pH (measured at 20°C) is raised within the range pH3.5–6.0, another species with a lower value of $g_{\parallel}$ increases in intensity. Computer studies based on the known values of the dissociation constants (Sillén & Martell, 1971; Johansson & Wänninen, 1975) show that only two species are present in substantial amounts in this pH range, these being Cu–H–EDTA and Cu–EDTA. These species are predicted to be present in equal amounts at a pH value of 3.0–3.1. Our data in Figs. 3(*a*)–3(*d*) obtained at low ionic strengths in EDTA as buffer indicate an apparent p*K* for this transformation, under our conditions and measured in the frozen state, of about 4, corresponding to a $\Delta pH_{app.}$ of about +1. Spectra from Cu^{2+}–EDTA were sharper but otherwise similar if the ionic strength was raised, either by increasing the EDTA concentration (Figs. 3*e* and 3*f*) or by addition of NaCl. Addition of ethylene glycol, up to a concentration of 50%, also sharpened the signals, but, as may be predicted from the work of Douzou (1974), the solvent had little effect on the apparent p*K*, as indicated by the relative intensities of the two $g_{\parallel}$ signals.

Introducing buffers into the system had effects that depended on the pH and the nature of the buffer. Figs. 3(*i*) and 3(*j*) show that pyrophosphate, pH6.0, and Mes, pH5.5, gave spectra not differing very greatly from those expected at high ionic

strengths. However, other spectra in phosphate, pH6 (Fig. 3*g*), or still more noticeably, in pyrophosphate, pH5 (Fig. 3*h*), are out of line and suggest decreases in apparent pH in both these samples relative to the others of possibly 2pH units. (Computer calculations indicated that pyrophosphate would not compete effectively with EDTA for copper under the conditions of our experiments.)

The spectra illustrated in Fig. 3 were all integrated and there was no loss of signal intensity, despite the relatively high concentrations of metal (0.4mM) used. Thus it is unlikely that dipolar broadening due to solute aggregation occurred (Ross, 1965). This will also be the case for the metalloenzymes, where, even if aggregation did occur, the bulk of the protein should keep the paramagnetic centres in a magnetically dilute state.

Discussion

Apparent pH values in frozen samples

Our results will be discussed primarily in relation to physical studies on biological molecules in frozen aqueous solution. It is noteworthy that with several buffer systems (particularly zwitterionic ones), the state of ionization of a chromophore of a macromolecule (such as FADH• in xanthine oxidase) can remain about the same in a frozen aqueous sample at the temperature of liquid N_2 as it is when in liquid solution at 20°C. However, effects on the macromolecule of varying the medium must clearly be interpreted with great caution. Thus there are usually substantial decreases in apparent pH when phosphate or pyrophosphate buffers are frozen, whereas there are some increases in apparent pH on freezing Tris and at least one other cationic buffer. Another interesting practical point is that there was little difference in the magnitude of the apparent pH changes, whichever of the two freezing procedures customarily used in biochemical e.p.r. investigations was applied.

Cause of apparent pH variations

In a series of investigations Van den Berg (1966) and Larsen (1973) have made measurements of pH changes on cooling common buffers to about −20°C. This they did by monitoring (e.g. with a pH electrode containing 'antifreeze') the pH of the liquid phase during phase separation at extremely slow cooling rates (about 0.002°C/s), to avoid supersaturation. In several buffer systems, large changes in pH were observed during phase separation. For phosphate this could be attributed to the lower solubility of Na_2HPO_4 compared with NaH_2PO_4. As the temperature is lowered, Na_2HPO_4 preferentially precipitates, leaving a higher NaH_2PO_4 concentration in the residual liquid phase and a lower pH. Since in our experiments we would expect that the high solubility of our enzymes would ensure that they remain in the liquid phase, such a mechanism could account for our observations of lowered $pH_{app.}$ in sodium phosphate buffers. However, the rapid rates of freezing that we have used (manual freeze, about 20°C/s; rapid freeze, 5000–20000°C/s) will in general lead to incomplete phase separation (supersaturation). Our results for slow freezing to −20°C for sodium phosphate and potassium phosphate, as well as for Tris, are in agreement with those of Larsen (1973). However, with fast freezing the parallelism sometimes breaks down. Thus, in particular, from the results of Larsen (1973) it can be predicted that potassium phosphate, unlike sodium phosphate, should show only small positive pH shifts, since the solubilities of the relevant potassium salts are similar. As shown in Tables 1 and 2, an apparent fall in pH of up to 1.7pH units was nevertheless observed in our system for potassium phosphate. Conversely, we found that apparent pH decreases with sodium phosphates were rather smaller with fast freezing than they were with slow freezing.

Further work will be required, particularly studies with single species (pH indicators or chromophores in macromolecules) having well-established p*K* values to put our findings on a firmer quantitative and theoretical basis.

Conclusion

There is good parallelism between $\Delta pH_{app.}$ values obtained by e.p.r. spectroscopy and those obtained with indicators. Thus it would appear that the very simple expedient of using indicator solutions can be an approximate guide to apparent pH changes. We therefore recommend that when weakly coloured enzymes or other samples are used for low-temperature studies, preliminary tests should be carried out for such effects of freezing on apparent pH. In protein solutions the changes in apparent pH may be somewhat different from those when only buffer and indicator are frozen, as is shown particularly by our experiments with albumin. Also, the effects of buffer concentration, and no doubt that of other salts as well (cf. Larsen, 1973), are obviously important. In general it seems that zwitterionic buffers of the type introduced by Good *et al.* (1966) (e.g. Bicine) may be less likely to generate pH-dependent artifacts on freezing than are other buffers, so that their use in future work seems desirable. We conclude that phosphate and pyrophosphate buffers and, if possible, also Tris buffers should not be used for low-temperature work, though their use has been widespread.

No doubt there are other examples in the literature of buffer effects on apparent pH changes on freezing. Specifically, we suggest that effects on oxidase and dehydrogenase properties of rat liver xanthine oxidase occurring on freezing homogenates in Tris, but not in

phosphate or pyrophosphate buffers (Stirpe & Della Corte, 1969), may have been due to apparent pH changes in the frozen state.

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