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Note

Isoelectric focusing in chloral hydrate

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Non-ionic detergents, especially Triton X-100 and Nonidet P-40 (NP-40), have found widespread application in the disaggregation and subsequent electrophoretic analysis of membrane proteins because, in contrast with sodium dodecyl sulphate, they allow recovery of enzyme activities and of relatively intact protein structures[1]. Recently, using immunoelectrophoretic techniques[2], it has been found that Triton X-100 binds to proteins and this phenomenon has been exploited for the electrophoretic separation of normal adult haemoglobin from foetal haemoglobin[3]. The same type of binding occurs also with NP-40, and this has led to the separation, by isoelectric focusing (IEF), of β and γ human globin chains, since the binding of this ligand alters the p*K* values of neighbouring groups and thus also the p*I* values of these two chains, allowing for an increased resolution[4]. Unfortunately, these two detergents also bind to carrier ampholytes, the amphoteric substances used to generate and stabilize the pH gradient in IEF[5]. These complexes are generally insoluble in 10% trichloroacetic acid and can only be leached out from the polyacrylamide gel by long and tedious washings in alcoholic solvents at high temperatures (60°C)[4,5]. Whilst searching for a membrane solvent which would not interfere with subsequent IEF analysis of the solubilized proteins, we noticed that chloral hydrate had been successfully used for the disaggregation and electrophoretic characterization of membrane proteins[6,7], as well as for the isopycnic banding of chromatin in density gradients ranging[8] from 1.4 to 1.6 g/cm^3. As chloral hydrate is a non-ionic compound, freely soluble in water at greater than 100% concentrations, is a strong membrane-disaggregating agent, does not interfere with polyacrylamide gel polymerization and does not seem to alter or modify proteins[6–8], it seemed ideally suited also for isoelectric focusing.

We now report how, contrary to previous reports, chloral hydrate in IEF severely modifies carrier ampholytes and proteins, leading to altered or abolished pH gradients and producing a highly heterogeneous spectrum of bands from homogeneous proteins.

Fig. 1 shows the pH gradients obtained in a control gel (pH 3.5–10) and in gels containing increasing amounts of chloral hydrate, from 30 to 100%. While the control shows a regular and approximately linear pH gradient spanning the pH range 3.5–10, the same gradient is modified to a pH 2.5–6 range in the presence of 30%

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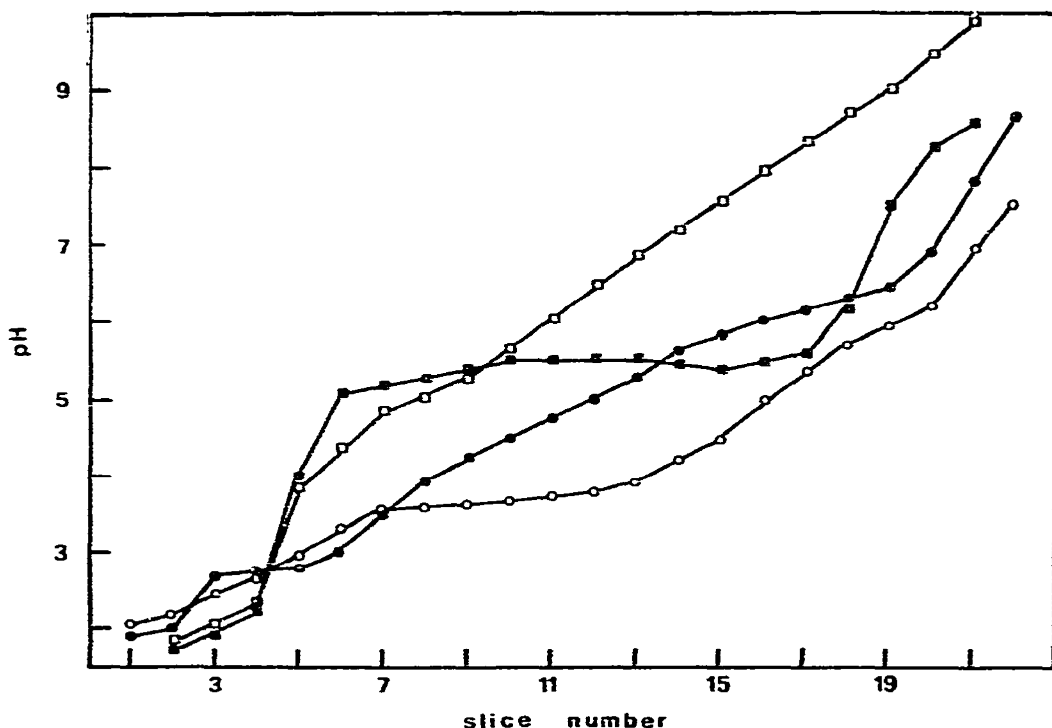

Fig. 1. IEF in the absence (□) and presence of 30 (●), 60 (○) and 100% (■) chloral hydrate. IEF was performed in a thin (0.7 mm) gel slab in an LKB Multiphor 2117 chamber with an LKB constant wattage power supply at 10°C, by applying 10 W (800 V at equilibrium) for 2 h. Gel: 7% acrylamide, 2% Ampholine pH 3.5–10. At the end, the pH gradient was measured at room temperature (22°C) by cutting 0.5-cm gel slices and eluting them with 300 μl of 10 m*M* potassium chloride.

chloral hydrate and to a pH 2.5–7 range in gels containing 60% chloral hydrate. Moreover, in the presence of 100% aldehyde, the pH gradient is completely abolished and, except for the large pH jumps at the extremes (presumably due to 1 *M* orthophosphoric acid and 1 *M* sodium hydroxide electrolyte solution diffusion into the gel), most of the gel length is covered by a wide plateau at pH *ca.* 5.5. We have interpreted this as extensive reaction of the amino groups of carrier ampholytes with the aldehyde, giving rise to Schiff bases. The carrier ampholytes thus lose positive groups and become progressively acidic. This reaction is more severe in alkaline pH ranges, which contain a greater number of deprotonated, and thus more reactive, amino groups, as compared with acidic pH ranges, which contain an average of four to five positively charged groups in an ordered sequence[9–11].

In order to see whether proteins could also be modified by reaction with chloral hydrate, we have performed IEF, in gels containing 8 *M* urea, of purified α, β and γ globin chains either untreated or incubated in 100% aldehyde. The treated samples were incubated either at pH *ca.* 2 (the pH of an aqueous solution of 100% chloral hydrate) or at pH 7 (it is impossible to titrate the aldehyde to pH 8 or above, as it is decomposed into chloroform and formic acid). As shown in Fig. 2, the pH 7 samples react to a great extent (80–90%) producing a highly heterogeneous spectrum of lower

p*I* components. This behaviour is consistent with a loss of positive charges, *i.e.* with reaction of amino groups with the aldehyde. Interestingly, even the samples incubated at pH *ca.* 2 are not completely unreactive, as each globin chain exhibits traces of lower p*I* species, clearly not present to the same extent and at the same p*I* values in the controls. By a densitometric evaluation of the IEF patterns it appears that about 5% protein has reacted, which should put a note of caution also on the use of electrophoretic techniques in lactate buffers at pH 2.5–3.0[6,7].

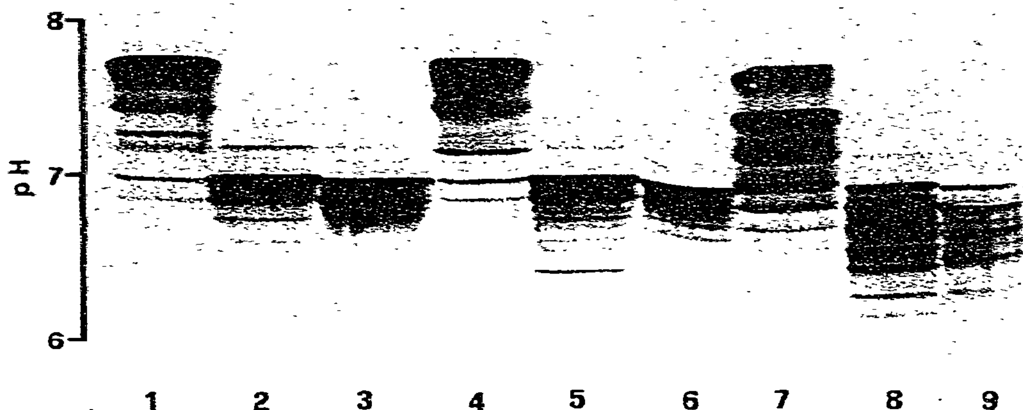

Fig. 2. IEF of α, β and γ human globin chains. The globins were chromatographically pure, haem-free chains. Slots 1–3: untreated α, β and γ chains, respectively. Slots 4–6: as for samples 1–3, except that the chains were incubated for 30 min at 25°C in 100% chloral hydrate, pH *ca.* 2. Slots: 7–9 as for samples 1–3, except that the globins were incubated for 30 min at 25°C in 100% chloral hydrate pH 7. The sample (30 μg) was applied soaked onto strips of filter paper to a pre-focused gel slab, containing 8 *M* urea and 2% Ampholine pH 3.5–10, from the anodic side. Running time: 4 h at 10 W. All other conditions as in Fig. 1. Staining: after the method of Bla' -ley and Boezi[12].

In conclusion, IEF in chloral hydrate does not appear to be a feasible technique. However, it is of i: to notice that, by treatment with 30 or 60% aldehyde, it is possible to transf a wide Ampholine pH range (pH 3.5–10) into a narrow pH range (*e.g.* pH 2.5–5) which appears to buffer particularly well in the pH region 2–4, where commercial carrier ampholytes behave rather poorly. Thus, this reaction might be exploited to produced narrow-range carrier ampholytes tailored to special separation problems.

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