Technical methods

A systematic error in pH-stat assays of enzymes in haemolysates

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Enzymes whose action results in a net change in hydrogen-ion concentration can be assayed in a pH-stat apparatus, in which alkali or acid is added at a rate equivalent to that at which the hydrogen ions are generated or consumed.¹ A good example is acetylcholinesterase, which produces acetic acid from acetylcholine.²

In a typical pH-stat system the reaction mixture is continuously gassed with nitrogen, to prevent its absorbing atmospheric CO₂. We wish to point out that there is an objection to this practice when the reaction mixture includes oxyhaemoglobin, as when erythrocyte acetylcholinesterase is determined.³ The objection is that when the haemoglobin-containing solution is gassed, the oxyhaemoglobin slowly loses its oxygen and becomes more basic, thus reducing the amount of alkali needed to neutralise the acid liberated in the reaction being assayed. One answer to it is to gas the reaction mixture with oxygen instead of nitrogen.

Material and methods

The reaction mixture (20 ml, containing about 0.5 μ mol of haemoglobin) was prepared by lysing 0.1 ml of washed human erythrocytes in CO₂-free distilled water, and then adjusting the pH to 7.45 with NaOH and the concentrations of NaCl and MgCl₂ to 165 mmol/l and 3.3 mmol/l respectively.

The rate of formation of hydrogen ions was measured at 30°C in a pH-stat apparatus (Radiometer, Copenhagen: a combined pH meter and titrator Type TTT 1 was coupled with a syringe burette Type SBU 1 and linked to a recorder Type SBR 2).

Results and discussion

When the reaction mixture was gassed with nitrogen

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(0.2 l/min) its pH tended to rise. This was countered by adding HCl (Figure). The amount of acid required rose significantly with time, at a maximum rate of about 0.1 μ mol/min, and after 20 minutes about 1 μ mol had been added. When the experiment was repeated using oxygen instead of nitrogen much less acid was needed (Figure), and a similar result was observed when a solution of NaCl (165 mmol/l) plus MgCl₂ (3.3 mmol/l) was gassed with nitrogen (not shown).

pH-Stat titration of haemolysate gassed with nitrogen or oxygen. Haemolysate (20 ml; approx 0.5 μ mol haemoglobin; 30°C) was gassed (0.2 l/min) with (a) nitrogen or (b) oxygen and its pH maintained at 7.45 by the addition of HCl (10 mmol/l).

Two other factors implicate the deoxygenation of haemoglobin in the spontaneous rise in pH of a haemolysate gassed with nitrogen. First, the absorption spectrum of the reaction mixture was initially characteristic of oxyhaemoglobin, but after the mixture had been gassed with nitrogen it tended to that of deoxyhaemoglobin; this change was abolished by replacing the nitrogen with oxygen. Secondly, complete deoxygenation of 0.5 μ mol of haemoglobin at pH 7.45 should be accompanied by the disappearance of about 1.0 μ mol of hydrogen ions,⁴ which is consistent with what was actually observed (Figure).

The practical significance of the spontaneous rise in pH described above depends on the experiment. For example, when acetylcholinesterase is assayed in haemolysates, the initial rate of the reaction is about

0.6 $\mu\text{mol/min}$ and will consequently be underestimated by about 10%.³ More seriously, if the integrated form of the Michaelis-Menten equation is fitted to the progress curve of the reaction, the values of the Michaelis-Menten parameters estimated from the fit will be grossly wrong.^{3,5}

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Fixation and immunohistochemistry of lymphoid tissue

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The morphological distinction between reactive and malignant processes in lymph nodes may sometimes be extremely difficult, but the introduction of immunohistochemical methods may help. While a polyclonal cell proliferation may be neoplastic,¹ it is more generally reactive, but monoclonality is very suggestive of malignancy.

The identification of monoclonality is presently limited to cells of the B lymphocyte series, most simply using antisera to kappa, lambda, and heavy chains. The antigenicity of cellular immunoglobulin appears least impaired in frozen sections or imprints, but in most cases lymphoid material arriving for histopathological diagnosis has been fixed. Routine formalin fixation may not be the most suitable for many antigens, and efforts using proteolytic enzymes to 'unmask' antigenic sites² presumed concealed by formalin fixation have gone some way to overcoming this problem. When formalin fixation is unavoidable the demonstration of cellular immunoglobulin may require such manipulations, but a more logical approach might be to use a fixative that does not denature or conceal the antigenic sites to the extent whereby reaction between antibody and antigen is impaired. We decided to examine a range of common fixatives to seek any giving adequate morphological preservation of lymphoid tissue together with preservation of immunoglobulin antigenicity.

Material and methods

TISSUES

Three to four millimetre slices of both human lymph nodes and tonsils and rabbit lymph node, spleen, and gut were fixed for periods up to two weeks in the fixatives given below. The composition of the fixatives was taken from Disbrey and Rack.³ The tissues were dehydrated in ethanol, cleared in xylene, and embedded in 56°C MP paraffin wax. Four micrometre sections were dewaxed in xylene and brought to water. Staining was performed by immunoperoxidase methods.

FIXATIVES

Simple: Formalin and glutaraldehyde 1-10%.
Compound: Susa, Bouin, Zenker, Carnoy, and formol-mercury.

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