Potentiometric pH-Stat Titration: Importance of an Inert Atmosphere in Reaction Vessels when Using Alkali Titrant

The use of electrochemical methods[1] for the assay of enzymes necessitates the elimination of any non-specific factors which could modify the rate at which titrant is added to the reaction vessel. Any such effect could lead to an erroneous interpretation of experimental results, or give apparently abnormally high enzyme levels leading to false conclusions. The latter might be of importance in clinical or forensic studies, as in the determination of acetylcholinesterase levels in cases of suspected organophosphorus poisoning[2].

During a study of the levels of cholinesterases in the avian supra-orbital gland using a pH-stat continuous potentiometric technique[3], unexpectedly high and variable results were obtained even when corrections were applied for the non-specific liberation of acid from the tissue homogenate[4]; the error was found to be due to the uptake of acidic substances from the air. The evidence for the existence of this error, and a method of eliminating it are reported below.

All experiments were carried out using a Radiometer TTT 1 titrator, an SBR 2 recorder, and an autoburette equipped with a G2222C glass electrode and a K4112 calomel reference electrode; titrations were carried out at 37 °C in thermostatted vessels equipped with magnetic stirrers.

If the air is a source of extraneous acidic substances then a slow continuous addition of alkali will occur when water alone is exposed to air in a reaction vessel, and the effect should be considerably greater with mechanical agitation of the water. This has been proved to be the case; slope A in Figure 1 shows the titration of 3 ml deionized-distilled water, agitated in an air-filled reaction vessel, with $1.10^{-3}N$ NaOH at pH 7.4. A slow continuous uptake of acidic substances from the air occurred equivalent to 0.720 µmoles NaOH/h. According to the HENDERSON-HASSELBACH relationship, the rate of such uptake should be pH-dependant; Figure 2 shows that the rate of uptake increases with a decrease in $[H^+]$; the equivalent values in µmoles NaOH/h added were 1.25 at pH 7.5, 1.70 at pH 8.0 and 2.05 at pH 8.5.

Further evidence that uptake of acidic substances from the air occurs was provided by the observation that when titrating water as above, then exclusion of air from the reaction vessel by an inert gas stopped the addition of titrant, which re-started when CO_2 was introduced into the vessel. This is also shown in Figure 1. When air in the reaction vessel was replaced by N_2, the addition of titrant ceased (B). A gas mixture of 95% O_2 + 5% CO_2 was then fed into the vessel, and resulted in a continuous addition of alkali due to H_2CO_3 formation (C). Pure N_2 was reintroduced into the vessel, and again the addition of titrant ceased (D).

The marked effect that atmospheric pollution may have on the results of enzyme assays performed by continuous electrometric titration is shown in Figure 3, taken from an experiment in which the levels of butyrylcholinesterase in a supra-orbital gland from a normal duck were being determined, using 15 mM butyrylcholine chloride as substrate together with $1.10^{-5}M$ 1,5-*bis*(4-trimethylammoniumphenyl)-pentan-3-one di-iodide (BW62C47) to inhibit any acetylcholinesterase present[5]. The reaction was carried out at pH 7.4 with $5.10^{-2}N$ NaOH as titrant. Slope A shows the rate of addition of alkali with air in the reaction vessel, and gave an apparent concentration of butyrylcholinesterase in the tissue of 129.3 µmoles/g/h; when the air was replaced by N_2 the value dropped to 85.6 µmoles/g/h (slope N). Thus, without correction for the atmospheric effect, the value obtained by titrating in air was 51% too high, even after a correction for spontaneous non-specific liberation of acid from the tissue homogenate had been made.

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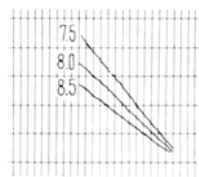

Fig. 2. Titrations of deionized-distilled water with $1 \times 10^{-3}N$ NaOH to demonstrate pH-dependancy of the uptake of acidic substances from the air. T = 37 °C, pH = 7.5, 8.0 and 8.5. Titrant, $1 \times 10^{-3}N$ NaOH. Proportional band, 0.1 pH. Chart speed, 2.5 mm/min.

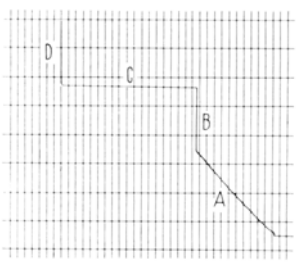

Fig. 1. Effect of the exclusion of atmospheric acidic substances from reaction vessels by N_2 on the titration of water with $1 \times 10^{-3}N$ NaOH. A, air in vessel; B, N_2 in vessel; C, 5% CO_2 + 95% O_2 in vessel; D, N_2 in vessel. T = 37 °C, pH = 7.4. Titrant $1 \times 10^{-3}N$ NaOH. Proportional band, 0.1 pH. Chart speed, 1.25 mm/min.

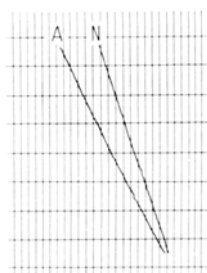

Fig. 3. Titration curves for the estimation of butyrylcholinesterase levels in a homogenate of normal duck supra-orbital gland; A, performed in air; N, performed under an atmosphere of pure N_2. T = 37 °C, pH = 7.4. Tissue concentration, 14.8 mg/ml. Titrant, $5 \times 10^{-2}N$ NaOH. Proportional band, 0.1 pH. Chart speed, 2.5 mm/min.

Regional and temporal variations in the composition of air make it impossible to obtain a constant correction factor for this effect. Thus, analysis of the composition of 3 samples of air[6] taken at different sites in the City of Leeds on 12/6/67 gave values for the SO_2 content of 66, 56 and 74 $\mu g/m^3$. The occurrence of temporal variations was demonstrated by titration; thus determinations of the rate of uptake of acidic substances in 3 ml deionized-distilled water on 7 consecutive days gave equivalent values of 0.730, 0.750, 0.690, 0.852, 0.647, 0.577 and 0.703 μmoles NaOH/h.

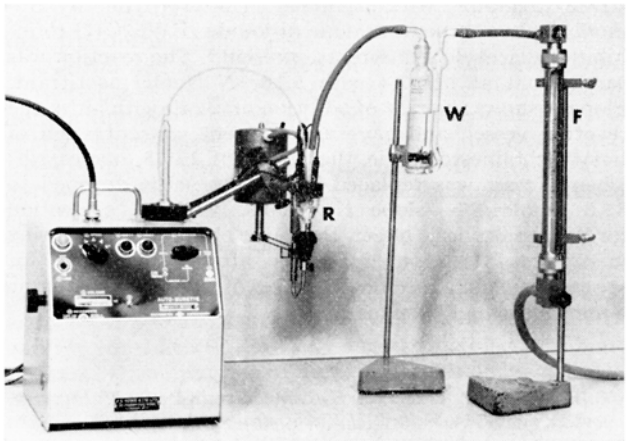

Fig. 4. Equipment suitable for producing an inert atmosphere in a reaction vessel (R) during potentiometric titrations. F, flowmeter (0–25 ml/min); W, wash bottle containing CO_2-free distilled water.

It is clear that the most convenient way to eliminate the error is to carry out titrations in an inert atmosphere, using an arrangement like that shown in Figure 4. Pure N_2 from a cylinder is led through a pressure reducing valve and then to a flowmeter (Figure 4, F) of suitable calibration (0–25 ml/min). It is advisable to pass the N_2 through CO_2-free distilled water in a wash bottle (Figure 4, B) before it reaches the reaction vessel, particularly when titrations are prolonged, to prevent undue evaporation of the vessel contents[7].

Résumé. Les estimations des enzymes dans de petits échantillons de tissu, ou dans des tissus de faible activité, par des méthodes potentiométriques pH-stat avec un alcali comme titrant sont sous la dépendance d'erreurs causées par l'absorption des substances acides de l'air. On peut éviter ces erreurs par l'usage en éprouvette d'une atmosphère pure et inactive.

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The Relationship of Phosphate and Lipids to Xanthine Dehydrogenase

Phosphate is required for the reduction of one electron acceptor by cream xanthine dehydrogenase[1] (XD) and seems to have a stabilizing action on this enzyme[2]. On the other hand, there is evidence that both the milk[3] and the mammalian enzymes[4,5] are associated with lipid components. The postulate that lipid or phospholipide material is responsible for binding together various oxidative enzyme systems was the main support for our investigation on the phosphate and lipid participation in the XD activity of the mammalian enzyme.

For the purification of the enzyme, a homogenate (1:5 w/v) obtained from rat liver was heated at 65 °C for 15 min. After centrifugation at 1000 *g* the aqueous phase was siphoned off and submitted to the following steps performed at 0 °C.

Preparation A1. To each 100 ml of the homogenate were added 47 ml of saturated ammonium sulphate. After 20 min most of the protein which had been salted out was separated by centrifugation and discarded. To each 100 ml of the clear aqueous solution were added 47 ml of saturated ammonium sulphate. After 45–60 min the brown protein was collected by centrifugation and dissolved in twice-distilled water.

Preparation A2. Obtained by dialysis of preparation A1 for 18 h against twice-distilled water at 5 °C. The solution was centrifuged (1000 *g*) for 15 min and a supernatant free of turbidity with a deep red-brown colour was obtained. The E_{280}/E_{450} for this preparation was usually 13.5–14.7.

Preparation B1. Obtained by solubilization of the enzyme by treatment with butanol as suggested by MORTON[3]. While stirring, 1 vol of aqueous butanol was slowly added to the homogenate. After centrifugation (1000 *g* for 15 min) the transparent yellowish aqueous phase was siphoned off, fractionated with ammonium sulphate, and dialysed as A2. This preparation presented an E_{280}/E_{450} equal to 11.3.

XD activity was determined by the method previously described[5]. Phosphorus was determined by the method described by GÖMÖRI[6].

The activity of the enzyme preparations measured by the production of formazan/mg dry weight are shown in Figure 1. Results on aged preparations showed that B1 and A2 were inactivated differently. When incubated at 37 °C, B1 preparation lost activity more rapidly than A2 and both preparations presented 100% inhibition after 24 h of incubation (Figure 2).

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