Immobilized-Enzyme Pipette

SCOPE AND LIMITATIONS OF A SIMPLE DEVICE

By P. V. SUNDARAM

Max-Planck-Institut für Experimentelle Medizin, Abteilung Chemie, D-3400 Gottingen,
Federal Republic of Germany

(Received 29 January 1979)

Disposable pipette tips made of polymeric nylon tube with enzymes bound covalently to their inside surface and fixed to the stem of an automatic, adjustable-volume pipette holder together constitutes an immobilized enzyme pipette or 'Impette'. The present paper describes the application in research laboratories and clinics of this new development, with urease as an example in the determination of blood urea.

Ever since the early experiments of immobilization of enzymes and other biologically active molecules on insoluble polymers with a view to harnessing their activity in specific reactions, there have been several developments, such as the immobilized-enzyme nylon-tube reactors and enzyme electrodes (Sundaram & Hornby, 1970; Sundaram *et al.*, 1978a; Guilbault & Lubrano, 1973; Clark, 1972; Meyerhof & Rechnitz, 1976), that show promise in routine use. A common feature of these developments is a decrease in cost during routine use by virtue of their repeated usability and high operational stability. However, many of them require to be used in conjunction with rather expensive and sophisticated equipment.

In contrast to the above, the present paper describes the development of a device that is simple to prepare and use with the minimum additional equipment.

Materials and Methods

Nylon₆ tubing (int. diam. 1 mm) was supplied by Portex Ltd., Hythe, Kent, U.K. Urease type III (28 units/mg) was obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. All other chemicals were obtained from Aldrich Chemical Co., Milwaukee, WI, U.S.A.

Disposable pipette tips containing immobilized enzyme may be prepared by coupling the enzyme directly to activated nylon tubes as described by Sundaram *et al.* (1978b). Coiled tubing was filled with 0.1 M-triethyloxonium tetrafluoroborate made up in dichloromethane and allowed to react for 4 min at room temperature. The tube was then washed well with ice-cold methanol, followed by deionized water. The tube was then filled with freshly made

urease enzyme solution at 2 mg/ml in phosphate buffer (0.1 M-KH₂PO₄/K₂HPO₄), pH 7, containing 1 mM-EDTA and left for about 16 h at 4°C. At the end of this period of time the tube was washed well with 0.1 M-NaCl and then with deionized water to remove any adsorbed protein.

The Impette tip was connected by a piece of flexible silicone tubing to the commercially supplied pipette tip of an automatic pipette. Fig. 1 shows a photograph of an Impette.

The Impette tips were stored soaking in a buffer of choice at 4°C when not in use.

Results and Discussion

Calibration and principle of operation

An Impette may be used in the determination of specific substrates in unknown solution by using either a kinetic or equilibrium method. In the kinetic method, standard curves have to be drawn from known standard solutions. For example, in the case of urea, standard solutions of concentrations ranging from 1 to 50 mM are made up in 0.1 M-phosphate buffer, pH 7, containing 1 mM-EDTA. The volume in the Impette was adjusted to, for example, 0.25 ml, and the sample was sucked in, kept exactly for 5 min, and then expelled. The amount of ammonia formed in a portion of the substrate that had been made to react was determined by the modified method of Chaney & Marbach (1962) as described in Sundaram *et al.* (1978a). A standard curve was drawn from these results.

Unknown solutions of the same volume were sucked into the Impette and allowed to react for 5 min, expelled, and ammonia in a portion was determined as for standards. The A_{630} value obtained was read off against the standard curve. This procedure is a kinetic method of determination, because during the 5 min reaction time only a certain percentage of

Abbreviations used: Impette, immobilized-enzyme pipette; Impette tips, disposable pipette tips containing the immobilized enzyme.

Fig. 1. Photograph of an Impette

Key to labelled parts: a, Stem of an automatic pipette; b, commercially supplied disposable pipette tip; c, Impette tip connected to b by flexible silicone tubing; d, Eppendorf tube that contains serum sample.

the substrate is converted into the product. Thus care is necessary to time the residence of the sample within the Impette exactly with a stop-watch.

The main factors that affect performance during calibration and operation are:

(1) The effect of time on the linear range; the longer the assay time, the greater the concentration range over which the calibration curve is linear (Fig. 2 supports this statement). This is dependent on two variables: (a) the specific activity of the Impette tip, which may be controlled either by immobilizing an enzyme of higher activity or by using a longer

Fig. 2. Calibration curves for urea determination by the Impette kinetic method

Curves (a), (b), (c), and (d) are for 2, 3, 5 and 10 min reaction times respectively.

coil as the Impette tip; (b) dilution of the samples being tested. For example a serum sample containing 98 mg% (16.33 mM) urea measured directly shows a 90.2% recovery, whereas a 10-fold dilution increases recovery to 105%. This is because the efficiency is better when the metabolite concentration falls within the linear range.

(2) It is sometimes important to exercise care in the choice of standards used in calibration. One should not be surprised if serum determinations based on aqueous standards are not satisfactory. Sometimes such a discrepancy arises because the immobilized enzyme does not display the same activity towards a substrate in the presence of other serum components, such as the salts and serum proteins. In such cases it is better to use commercial sera for calibration.

The equilibrium method depends on a total conversion of the substrate into product. Total conversion of substrate would demand Impette tips of high specific activity. With the exception of urease, which may be immobilized to make Impette tips of about 15 units/50 cm, none of a host of different enzymes that have been tried give such high specific activities. Thus a kinetic method will be a method of choice in most cases, since total conversion of substrate will be time-consuming.

Application

Table 1 contains recovery values of urea obtained from standard solutions of the substrate made up in phosphate buffer, pH7, and determined by the kinetic method, with a reaction time of 5 min. The results, which looked promising, led to some trials with determination of urea in serum.

Blood collected from patients was deproteinized with perchloric acid, and urea in the resulting serum was determined by the kinetic method. Table 2 shows results of some of these determinations. Some samples obtained from the clinic were carefully chosen so that a wide spectrum of urea concentrations (i.e. from below normal to uraemic concentrations representing kidney damage) were chosen. These

sera were analysed in the clinic with a urease-solution method by using a Technicon Autoanalyser. The performance of the Impette by the present method was indeed promising and encourages further development. Urea determination in non-deproteinized serum is also possible.

We have already tried several determinations such as those of glucose and uric acid, which are important in clinical biochemistry, amino acids that are needed in food technology, and pesticides in environmental control, by the present method. The results suggest that immobilized-enzyme pipettes will make a valuable contribution to analysis. Of course these can never replace autoanalysers, which guarantee rapid sampling and vast turnover that is needed in large hospitals.

In contrast, Impettes will be an invaluable addition to research laboratories, private physicians and small clinics, especially those that can neither afford, nor need, automation.

Impettes containing phosphatases (for cleaving terminal phosphates), proteinases (for splicing peptides at specific points), and similarly endo- and exo-nucleases (for splitting polynucleotides, which are used as catalytic probes in research), facilitate the above objectives, eliminating the tedium of separating the enzymes from small volumes of sample. When needed, the reacted samples may be applied directly to gel columns for further purification.

I acknowledge the excellent technical assistance of Mrs. R. Wassermann. The project was financed by a grant from D.F.V.L.R. (Deutsche Forschungs und Versuchsanstalt für Luft- und Raumfahrt).

References

- Chaney, A. L. & Marbach, E. P. (1962) *Clin. Chem.* **8**, 130-134
- Clark, L. C., Jr. (1972) *Biotechnol. Bioeng. Symp.* **3rd** 377-394
- Guilbault, G. G. & Lubrano, G. J. (1973) *Anal. Chim. Acta* **64**, 439-444
- Meyerhof, M. & Rechnitz, G. A. (1976) *Anal. Chim. Acta* **85**, 277-285
- Sundaram, P. V. & Hornby, W. E. (1970) *FEBS Lett.* **10**, 325-327
- Sundaram, P. V., Igloi, M. P., Wassermann, R., Hinsch, W. & Knoke, K.-J. (1978a) *Clin. Chem.* **24**, 234-239
- Sundaram, P. V., Igloi, M. P., Wassermann, R. & Hinsch, W. (1978b) *Clin. Chem.* **24**, 1813-1817

Table 1. Recovery of urea from standard solutions determined by the Impette kinetic method

Urea (mg%)		
Theoretical value	Observed value with Impette	Difference (%)
75	76.2	+1.6
100	100.4	+0.4
150	150	0
200	199.8	-0.1

Table 2. Serum urea measurement with Impette compared with the autoanalyser method

The Impette method used a 5 min kinetic method. The autoanalyser method was performed on a Technicon autoanalyser II, with urease solution. Ammonia detection was by the Berthelot method of Chaney & Marbach (1962).

Method	Urea (mg%)	
	Impette	Autoanalyser
	16	15
	20	19
	31	29
	94	90
	41	40
	142	135
	250	250
	60	60