

## SHORT COMMUNICATION

# A Micromethod for Urease

J. De Bersaques

Department of Dermatology, University of Ghent, Belgium

The determination of urease activity by measurement of the  $^{14}\text{CO}_2$  set free from Carbon-14-labelled urea appears to be a simple, sensitive and specific technique. The  $^{14}\text{CO}_2$  has been measured after precipitation as Barium-Carbon-14-carbonate. Recently,<sup>2</sup> a method was described in which the reaction was stopped by the addition of sulphuric acid and the escaping  $^{14}\text{CO}_2$  was trapped in Hyamine 10-X and counted by liquid scintillation counting. The whole reaction took place in a 10 or 25 ml Erlenmeyer flask fitted with a well for the trapping agent.

By using the microtechniques developed by Linderstrøm-Lang and Holter,<sup>3</sup> viz. by putting a liquid alkaline 'seal' in the neck of the reaction tube to trap the  $^{14}\text{CO}_2$ , the reaction volumes can be reduced considerably and the sensitivity is very much increased. Such techniques were used for the study of enzymatic reactions in which  $^{14}\text{CO}_2$  is liberated, viz. for glutamic decarboxylase<sup>4</sup> and glycolysis.<sup>5</sup>

In our experiments, small throw-away tubes were made from 5 mm i.d. borosilicate glass tubing and siliconised (Silikon Lösung Serva, Heidelberg). The reaction mixture, 10  $\mu\text{l}$  with 20 mM urea, 40 mM pH 7.0 Tris-HCl buffer, 70,000 c.p.m. Carbon-14-urea (55 mCi/mM, CEA France) and urease (crystalline, type C-3, Sigma Chem. Co., St. Louis), was present at the bottom of the tube. A 5  $\mu\text{l}$  drop of 1 N sulphuric acid was placed on the side wall. The 30  $\mu\text{l}$  liquid seal was put in a separate piece of glass tubing, linked to the reaction tube by a short length of latex tubing. This two-piece construction was adopted in order to avoid contamination of the seal by the radioactive solution. A glass stopper was placed at the other end of the second tube (Fig. 1). The tubes could be incubated horizontally on the bottom of a water bath. After the chosen incubation period, the acid was tipped into the reaction mixture to destroy the enzyme and liberate  $^{14}\text{CO}_2$ . After a suitable volatilisation period, the latex tubes were removed and the glass tube with the alkaline seal was dropped into a counting vial with scintillator and counted (Tri-Carb liquid scintillation spectrometer, model 2111, Packard Instrument Co.) at room temperature.

Numerous agents and methods have been advocated for the trapping and liquid scintillation counting of  $^{14}\text{CO}_2$ : KOH, NaOH, hydroxide of Hyamine 10-X, ethanolamine, Primene, phenylethylamine, etc. in various homogeneous and inhomogeneous scintillating mixtures. Some of these alkaline solutions were thought to be less suitable, either because of their viscosity, making it difficult to use micropipettes, because of streaking due to low surface tension, or because

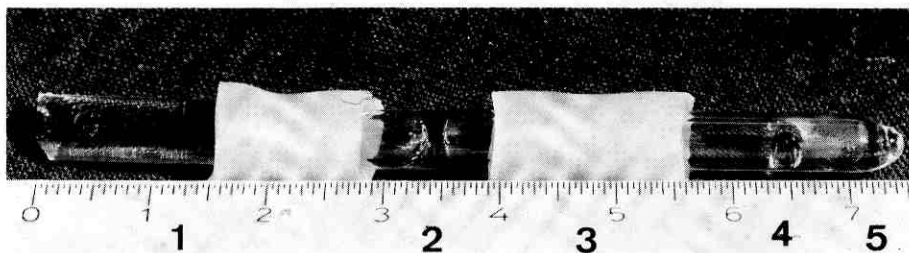


Fig.1. Assay device (scale in cm). 1. Glass stopper. 2. Tube with alkaline seal. 3. Latex tubing. 4. Drop of 1 N sulphuric acid. 5. Reaction mixture at the bottom of the reaction tube.

of their content in organic solvents, which might inhibit the enzyme; for these reasons, Hyamine 10-X in methanol was not used.

Most of the papers published on  $^{14}\text{CO}_2$  absorption are concerned with the maximal capacity of the trapping solution, while in our experiments the total quantity of  $\text{CO}_2$  was very small:  $0.2 \mu\text{mole}$  at 100% hydrolysis. It appeared that with some combinations of alkali and scintillator severe losses of  $\text{CO}_2$  and radioactivity occurred, although the amount of alkali was theoretically more than sufficient:

- with phenylethylamine and a toluene scintillator: 50% loss after 40 min;
- with phenylethylamine and a dioxane scintillator: 15% loss after 40 min;
- with 0.3 N KOH in methanol and a dioxane scintillator: 20% loss after 20 h.

Such a decrease in counts has been mentioned only once,<sup>6</sup> when  $^{14}\text{CO}_2$  trapped in Primene was diluted with toluene scintillator and kept at room temperature.

Loss of counts was only minimal with 1 N KOH or NaOH in water as the trapping agent and 5 ml of a scintillating solution made up from 4 volumes toluene, 2 volumes Triton X-100 and 1 volume 0.1 N NaOH, with 6 g/litre PPO (a formula derived from Ref. 7). The efficiency, measured with Carbon-14-benzoic acid, was 75%. The blank was about 15 c.p.m. above background after 1 h incubation.

The time needed to capture quantitatively the  $^{14}\text{CO}_2$  released by the acid was less than 10 min. The radioactivity measured was proportional with time and amount of enzyme, up to about 20% hydrolysis of the substrate. The sensitivity of the method was such that  $2 \times 10^{-6}$  Sumner units gave 1000 c.p.m. after 1 h incubation at  $37^\circ\text{C}$ .

Recently, Wilson and Kronick<sup>8</sup> described a device made from scintillator plastic, enabling continuous measurement by scintillation counting of the  $^{14}\text{CO}_2$  released from an enzymatic reaction. This means that  $\text{CO}_2$  already escapes from the reaction mixture kept at pH 7 and that the addition of sulphuric acid is not essential. However, when our system was used without acid the radioactivity measured in the NaOH seal was always 10 to 30% lower than when sulphuric acid had been added.

A direct method of continuous measurement was also tried: the reaction tube was put into the scintillation vial, slightly inclined downwards to avoid penetration of scintillator into the tube (Fig. 2), and covered with 5 ml scintillator. The vial was then counted continuously at room temperature, or at chosen intervals, in which case the incubation can be done at  $37^\circ\text{C}$ . In the latter case, there is a very real danger of splashing some of the radioactive mixture into the scintillator during the manipulations to and from the counter or because of the movements in the counter itself.

A lag period of about 15 min when incubating at  $37^\circ\text{C}$  and of 30 min when



Fig.2. Scintillation vial with reaction tube (without scintillator) for direct measurements.

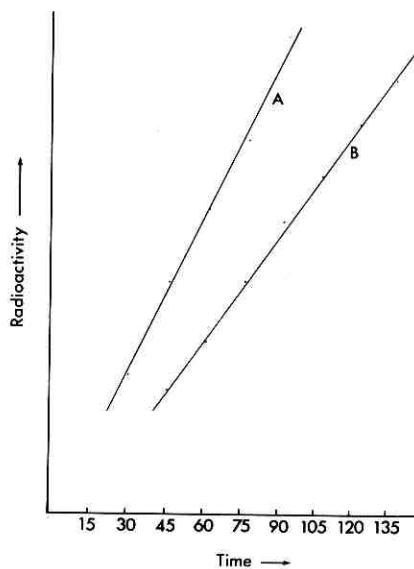


Fig.3. Increase of radioactivity in function of time (in min), measured with a device as shown in Fig.2 (with scintillator). A Incubation at 37°C  
B Incubation at 26°C (room temperature).

incubating at room temperature was apparent before the increase in radioactivity proceeded in a linear fashion and at maximum speed (Fig. 3). This maximum speed was equal to that measured with the first, indirect method. The delay in equilibration in our experiments was much longer than that of 2 min mentioned by Wilson and Kronick.<sup>8</sup>

#### REFERENCES

- 1 R.J. Roon and B. Levenberg, J. Biol. Chem. 243, 5213 (1968).
- 2 J.A. McDonald, K.V. Speeg, J.W. Campbell, Enzymologia 42, 1 (1972).
- 3 K. Linderstrøm-Lang and H.C.R. Holter, Trav. Lab. Carlsberg 19, 14, 1 (1933).
- 4 R.W. Albers and R.O. Brady, J. Biol. Chem. 234, 926 (1959).
- 5 K. Adachi and H. Uno, Adv. Biol. Skin IX, 511 (1969).
- 6 R.A. Opperman, R.F. Nystrom, W.O. Nelson and R.E. Brown, Intern. J. Applied Radiation and Isotopes 7, 38 (1959).
- 7 J. Murray, Intern. J. Applied Radiation and Isotopes 22, 209 (1971).
- 8 S.H. Wilson and M.N. Kronick, Anal. Biochem. 43, 460 (1971).