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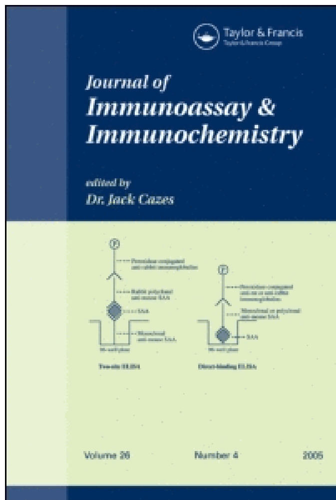

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THE USE OF 3, 3', 5, 5'-TETRAMETHYLBENZIDINE AS A PEROXIDASE SUBSTRATE IN MICROPLATE ENZYME-LINKED IMMUNOSORBENT ASSAY

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ABSTRACT

The assay conditions for the use of 3,3',5,5' tetramethylbenzidine (TMB) in microplate enzyme-linked immunosorbent assay are described. TMB is a safe (non-mutagenic) chromogen that is more sensitive than OPD as a substrate for horseradish peroxidase. We describe the optimum storage and assay conditions for this chromogen.

Key words: (Tetramethylbenzidine, Peroxidase, Giardia lamblia, dimethyl sulfoxide, ELISA).

INTRODUCTION

Horseradish peroxidase (HRP) is a commonly used enzyme for enzyme immunoassay (EIA). The traditional substrates for this enzyme are 0-phenyldiamine (OPD), 2,2'-azino-di-(3-ethyl-benzthiazole-sulphate) (ABTS), and 5-aminosalicylic acid (5-ASA). ABTS and OPD however are mutagenic in the Ames test (1,2). 5-ASA is non-mutagenic (1) but has relatively low sensitivity due to poor colour yield (3).

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Recently 3,3',5,5'-tetramethylbenzidine (TMB) has been introduced as a more precise and sensitive chromogen for use with HRP (4,5). Major additional advantages are that TMB is non-mutagenic and non-carcinogenic (6,7). During the development of an enzyme-linked immunosorbent assay (ELISA) for the detection of Giardia lamblia antigen, we experienced some difficulty with the stability of this substrate and therefore performed experiments to establish:

1. The stability of TMB under various storage conditions
2. The optimal incubation time with HRP
3. The stability of the reaction products.

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MATERIALS AND METHODS

TMB, Dimethyl sulfoxide (DMSO), HRP, sodium acetate, citric acid, and hydrogen peroxide were obtained from Sigma Chemical Company, Poole, Dorset. Antigiardia serum was raised in rabbits using Portland 1 strain of Giardia lamblia trophozoites (~10^7 organisms in complete Freund's adjuvant) from axenic culture as previously described (8). Anti-rabbit-IgG-HRP conjugate was purchased from Miles Laboratories Limited and Sigma Chemical Company. ELISA was performed in U-bottomed polyvinyl microtitre plates (Dynatech Microlisa). Giardia antigen for the antigen capture ELISA was obtained either from whole or sonicates of

axenically cultured G. lamblia trophozoites (8,9) or from human faecal samples containing Giardia cysts.

Standard assay

Assay conditions were essentially those described by Bos et al (4). TMB was dissolved in DMSO to give a final concentration of 10 g/l. 1 ml of this solution was added to 100 ml of 0.1 mol/l sodium acetate-citric acid buffer pH 6.0 followed by hydrogen peroxide to a final concentration of 1.3 mmol/l immediately prior to use. 25 ul of varying concentrations of HRP (1.25, 2.5, 5.0 uu/ml) were added to each well either as native enzyme or as an HRP-antibody conjugate (10, 50 uu/ml). This was followed by 150 ul of TMB solution. After incubation, the reaction was stopped by the addition of 25 ul of 4.7 N sulphuric acid. Absorbance at 450 nm was determined using a through-the-plate spectrophotometer (Minireader II, Dynatech Laboratories, Billinghurst, U.K.)

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TMB stability

Experiments were designed to determine optimal substrate storage conditions of TMB during a three month period. TMB was stored as concentrated stock solution (10 g/l) or diluted in buffer (0.1 g/l). In addition, the effects of temperature (4^0C and 22^0C) and light (combined fluorescent and natural light for

12 hours on a laboratory bench or total darkness in a light-excluding box) on TMB stability were also examined. Finally, we examined the effect on TMB stability of storage pH and of removal of dissolved gases by de-gassing under negative pressure. TMB solutions were stored in air-tight containers without a gas phase. All assays were performed in quadruplicate.

Optimal assay conditions

The HRP-TMB assay was optimised with regard to HRP concentration, time of incubation and reaction product stability using both the standard assay described above and an ELISA for *Giardia* antigen. The ELISA is a non-competitive indirect assay using trophozoites or faecal antigen. 50 ul of antigen preparation was adsorbed on to the microplates overnight at $37^{o}C$. After washing with phosphate buffered saline (PBS) containing 0.05% Tween 20 (PBS-T), 50 ul rabbit antiserum (diluted 1:1000 in PBS-T) was added and incubated at $37^{0}C$ for 60 minutes. The washing was repeated and 50 ul antirabbit-IgG-HRP conjugate (diluted 1:1000 in PBS-T) was added and incubated for a further 60 min at $37^{0}C$. After a final wash, TMB was added and the standard assay performed as described above.

Using both a standard TMB assay and *Giardia* antigen ELISA, experiments were performed to determine the rate of reaction product formation at intervals during a two hour incubation

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period and also to determine the stability of reaction product at room temperature by measuring absorbance at intervals for up to 18 hours after termination of the reaction by acidification. All assays were performed in quadruplicate.

RESULTS

Effect of light, temperature, air and pH on TMB stability

Stored as concentrated stock solution (10 g/l), TMB was found to lose 10% of its reactivity in the dark and 22.5% in daylight after one week. Following this, absorbance readings remained unchanged despite darkening of the stock reagent from pale yellow to dark orange. However, storage as a dilute solution in buffer (0.1 g/l) led to a rapid loss of reactivity, decreasing by more than 20% in 24 hours and by more than 70% after one week (Figure 1). This loss of reactivity of TMB in dilute solution occurred similiarly when stored at $4^{o}C$ and $22^{0}C$, and also when protected from light. Similarly, de-gassing and altering pH of TMB storage solutions failed to influence TMB stability (data not shown).

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Effect of HRP concentration and duration of incubation

As shown in Figure 2, the absorbance of all three concentrations of HRP reached a peak at 30 min. With the two

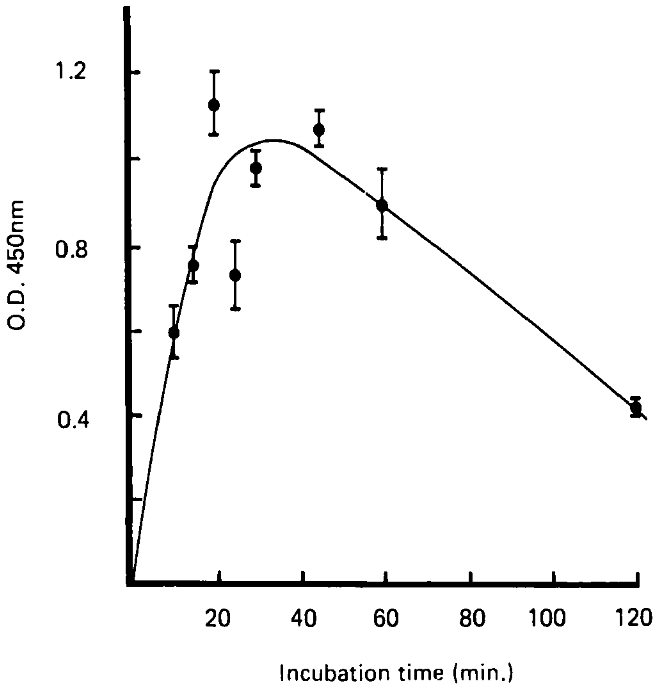

FIGURE 1

TMB stability on storage; effect of concentration and exposure to light. Assays perforemd with 2.5 uu/ml HRP.

higher concentrations, however, there was a rapid reduction in absorbance during the following 60 min. The lowest HRP concentration (1.25 uu/ml) remained stable for up to 60 min and then declined slowly thereafter. Similar absorbance profiles were obtained when HRP was provided as an HRP-antibody conjugate (data not shown) and when incorporated into the Giardia-antigen ELISA (Figure 3).

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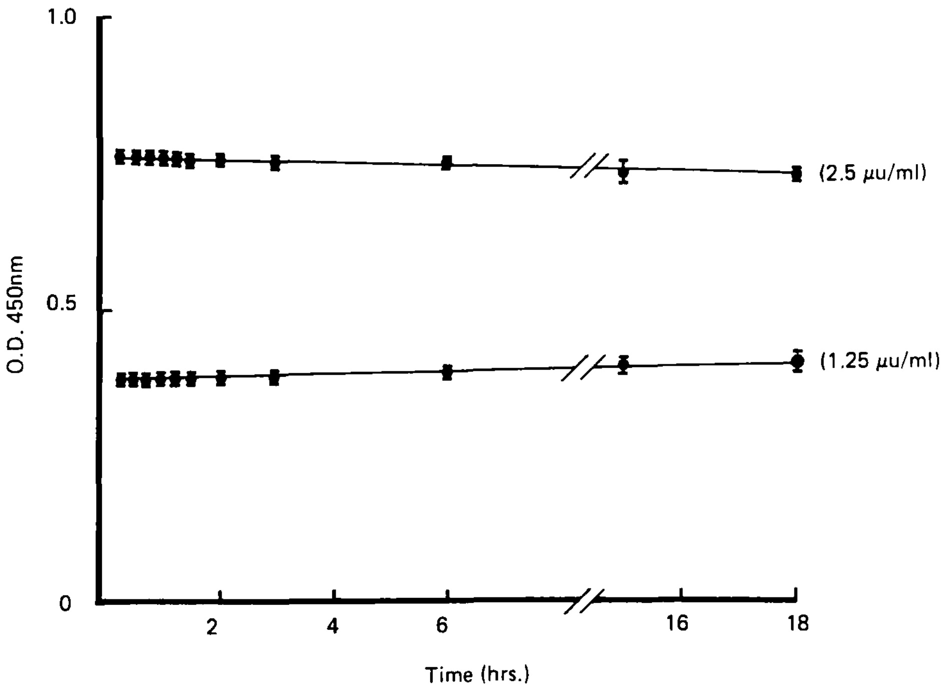

FIGURE 2

Effect of duration of incubation on TMB reaction product formation using native HRP.

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Stability of reaction product after acidification

The stability of the reaction product after termination by sulphuric acid was investigated by measuring absorbance of the same microplate at intervals of up to 18 hours. As shown in Figure 4, the values remained quite stable for low and middle range readings over this time, when the plates were kept uncovered at room temperature.

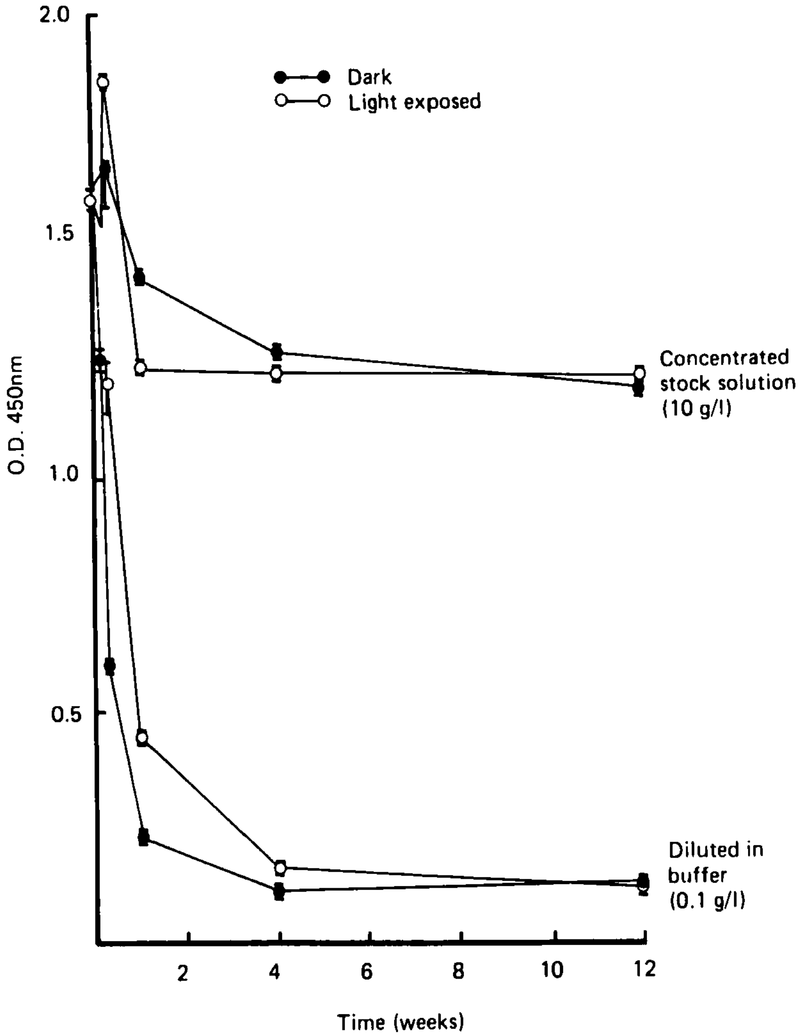

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FIGURE 3

Effect of duration of incubation on TMB product formation in Giardia antigen ELISA.

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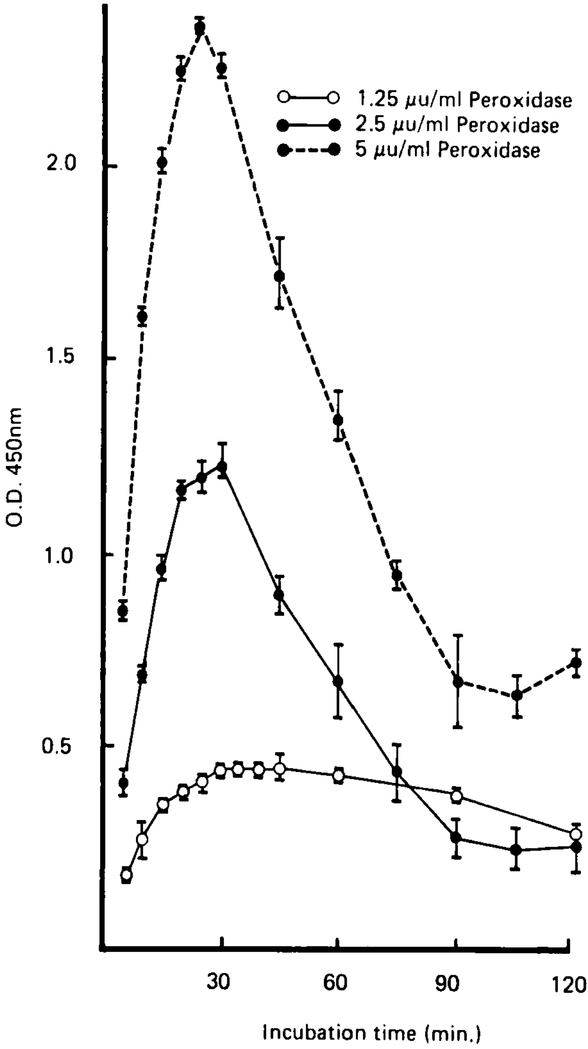

FIGURE 4

Stability of TMB reaction products after acidification with 4.7 N sulphuric acid using 1.25 and 2.5 uu/ml HRP (native).

DISCUSSION

We have successfully used TMB as HRP substrate in ELISA. During the developmental stages of the assay, we had some difficulties with TMB, notably that the deep blue colour that developed soon after the addition of the substrate to the wells faded as the recommended incubation time of 60 min progressed. We also noticed that the stock solution darkened appreciably on the bench. The earlier recommendation was for TMB to be prepared fresh for each assay (4,10). In micro-ELISA as we have described, the amount of TMB used for the plate is 1 ug. Should this be prepared fresh every time an assay is run there would be a marked waste of time and reagent. The original description by Bos et al was in a macro-ELISA. It was therefore important for us to determine whether the stock solution could be kept, and if so, to identify the appropriate storage conditions.

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We have shown that the 10 g/l stock solution remained relatively stable on the bench, losing less than 25% of this activity over the first week, after which activity remained unchanged for up to 3 months. Diluted preparations, on the other hand, lost their activity very rapidly and we would recommend that once diluted, TMB should be used immediately. We now routinely use TMB that has been prepared and kept for one week which we have found gives highly consistent ELISA readings. The

reagent is not highly photosensitive despite the visual colour change which occurs with storage. Storage at 4°C did not prevent the loss of reactivity in the diluted solution and made no appreciable difference to the stock solution. It is, however, very inconvenient to store TMB in DMSO at 4°C since thawing is prolonged, the freezing point being 18°C. Other physical factors relating to storage of TMB such as exposure to air and variable pH failed to alter TMB stability in dilute solution, although exposure to light probably accounted for some of the early loss of TMB reactivity when stored as a concentrated stock solution.

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Bos et al (4) recommended 60 minutes incubation of TMB with HRP. We have found 60 min to be too long and would recommend that the reaction be stopped after 30 minutes incubation.

TMB is a relatively safe chromogen that works reproducibly in micro-ELISA and should replace the currently-used chromogens. We have shown that it can be stored in stock solution at 10 g/l for up to 3 months on the bench, and that the reaction is not photosensitive. The stopped reaction can be read immediately or many hours later as the yellow colour of the 'stopped' reaction is quite stable. This would remove time lapse errors if many microplates have to be processed.

ACKNOWLEDGEMENTS

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