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Addition of Reducing Agents to the Peroxidase-*o*-Phenylenediamine Buffer Reduces Background of Enzyme Immunoassays.

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The concentrations of *o*-phenylenediamine (OPD), H_2O_2, citrate and H^+ in a substrate buffer for peroxidase immunoassays were optimized for minimal background. The background was reduced 2-3 fold with 5.5 mM OPD, 3 mM H_2O_2, 150 mM citric acid/sodium citrate, pH 4.8, and the reproducibility interassay was increased. A further 3-5 fold reduction of the background was obtained by the addition of 1.5 mM acetanilide, 0.14 mM β-mercaptoethanol and 5 mM nitrilotriacetic acid to the substrate buffer. This low-background substrate buffer allows increased sensitivity and lowers the interassay variation coefficient. It has been used sucessfully in peroxidase immunoassays of human C-reactive protein, human antiestreptolysin and human rheumatoid factor.

Key words: ELISA, Peroxidase-*o*-phenylenediamine, Enzyme immunoassay, C-Reactive protein.

Horseradish peroxidase is one of the enzymes most used in enzyme immunoassays (ELISA)*. Horseradish peroxidase catalyses the reduction of H_2O_2 with the concurrent oxidation of another substrate producing an optically measurable colour. Among the many substrates tested, *o*-phenylenediamine (OPD) gives the highest absorbance readings for both positive and negative serum but with one of the highest backgrounds (1,9). When H_2O_2 and OPD are put together, the traces of metals and/or light initiate the reaction without peroxidase and in a few hours OPD is completely oxidized. Most probably this is one of the reasons why these ELISAS have the relatively high backgrounds which reduce their sensitivity and precision (3, 8).

To reduce the background of the peroxidase-*o*-phenylenediamine immunoassays, the influences of the concentrations of OPD, H_2O_2, citrate and H^+ were investigated. The addition of acetanilide and

* *Abbreviations used:* CRP, C-reactive protein; ELISA, enzyme linked immunoassay; PBS, phosphate buffered saline; OPD, *o*-phenylenediamine; NTA, nitrilotriacetic acid.

β-mercaptoethanol further delayed the oxidation rate of the H_2O_2 and OPD mixture. Nitrilotriacetate helped to neutralize the increase oxidation due to the Fe addition to the substrate buffer. The resulting low-background substrate buffer reduced about 10 fold the background in the C-reactive protein (CRP) quantification by ELISA (2, 5, 6).

Materials and Methods

Materials. — Sodium citrate and citric acid were purchased from Scharlau (Ferosa, Spain). Sodium phosphate was from Merck. Merthiolate tested were from E. Merck (Schuchardt, FRG), and Scharlau (Ferosa, Spain). Thirty percent H_2O_2 was obtained from Merck and percentage adjusted by measuring absorbance ε (1 M/240 nm) = 43. Acetanilides tested were from Probus (Spain) and May and Baker Ltd (Dagenham, England). Nitrilotriacetic acid was from Sigma. EDTA was from Merck. β-mercaptoethanol used was from Merck and Eastman Kodak (USA). Peroxidases were obtained from Sigma, and Seravac (Miles S.A.). *o*-phenylenediamine was obtained from Sigma and Dakopatts (Denmark).

Preparation of the substrate buffers. — All the components were mixed in a glass beaker without using any metalic parts and using plastic gloves to avoid contamination with skin peroxidases. Acetanilide was added by first dissolving it in 1 ml of ethanol. All water used was either twice destilled or milli Q grade water (Millipore). The mixtures, before adding H_2O_2 and β-mercaptoethanol were filtered through Millipore 0.22 μ and dispensed in sterile conditions under a laminar flow into sterile containers. All the mixtures were kept in tightly closed containers at 4 °C. Merthiolate was included in all formulations at 0.01 % (0.24 mM), since it did not have any effect on the enzyme immunoassay and allowed high stability of the formulations.

CRP enzyme-immunoassays. — Both a C-reactive protein (CRP) sandwich ELISA using polyclonal anti-CRP coated wells and CRP binding to phosphorylethanolamine (PE) assay (2, 5, 6), have been used.

Briefly, the conditions for the CRP-sandwich ELISA were as follows. Polystyrene wells were coated with 1 μg/well of polyclonal anti-CRP IgG obtained as described (2). Fifty μl of dilution buffer with 14 ng of purified CRP or without CRP were added to each well and incubated for 15 min. After washing with dilution buffer diluted 1/20 in water, they were incubated for 15 min with 50 μl of polyclonal anti-CRP conjugated with horseradish peroxidase (5).

After washing the wells 50 μl of substrate buffer was added and reaction stopped with 50 μl of 6 N H_2SO_4 after 15 minutes. Results were read in a Titertek Multiskan at 492 nm adjusting the zero to the air. Under these conditions 100 μl of water had an absorbance of 0.03. All the steps were carried out at room temperature.

The CRP binding to PE ELISA for the quantitative determination of CRP is a solid-phase enzymatic immuno test based on phosphorylethanolamine coated Nunc plates, CRP-Ca^{++} dependent binding and anti-CRP-peroxidase development (5). One hundred μl of dilution buffer with 14 ng of CRP or without it, were added to each well and incubated for 60 min. Then plates were washed and incubated with 100 μl of anti-CRP peroxidase for 30 min. After washing, 50 μl of substrate buffer was added and color development stopped with 50 μl of 4 N H_2SO_4 after 30 minutes. Results were measured in a SLT EAR 400 FW spectrophotometer at 492-620 nm (SLT, Austria). Under these conditions 100 μl of water had an

absorbance of 0.001. All the steps were carried out at room temperature.

Percentage of background was calculated by (average of duplicate absorbances without CRP/average of duplicate absorbances with 14 ng CRP) × 100.

Results

Optimization of the concentrations of OPD, H_2O_2, citrate and H^+. — Initial experiments were made in 25 mM sodium phosphate 25 mM sodium citrate, pH 5 buffer. After adding 1 mg/ml of OPD, the pH was lowered as much as 1 or 2 units, depending on the source of OPD. Further experiments showed that citric acid/citrate buffered the pH after OPD addition at 1 mg/ml (5.5 mM). The minimum molarity of sodium citrate maintaining 1 mg/ml of OPD at a pH of 4.8 was 70 mM (not shown).

Figure 1 A, shows the decrease of background when the OPD concentration is increased to 0.5 mg/ml. The optimal amount of OPD for minimal background was between 1 and 3 mg/ml (5.5 to 16.5 mM). The minimun background was obtained with H_2O_2 concentration of 3-6 mM (fig. 1 B). Sodium peroxide and sodium perborate were also tested between 1-16 mM, because of their possibility of being used in powder form. They had, however, 2.6 and 2.7 fold more background than H_2O_2, respectively. The minimum background was obtained with citrate concentrations between 50 to 300 mM (fig 1 C), and the minimum percentage of background was obtained with pH around 4.5 (fig. 1D).

By using each of the components to their optimal concentrations (5.5 mM OPD, 3 mM H_2O_2, 150 mM citric acid/sodium citrate, pH 4.8), the background was lowered 2-3 fold and the interassay variation coefficient decreased from 55-120 percent to 9.3-11.2 % (n = 8).

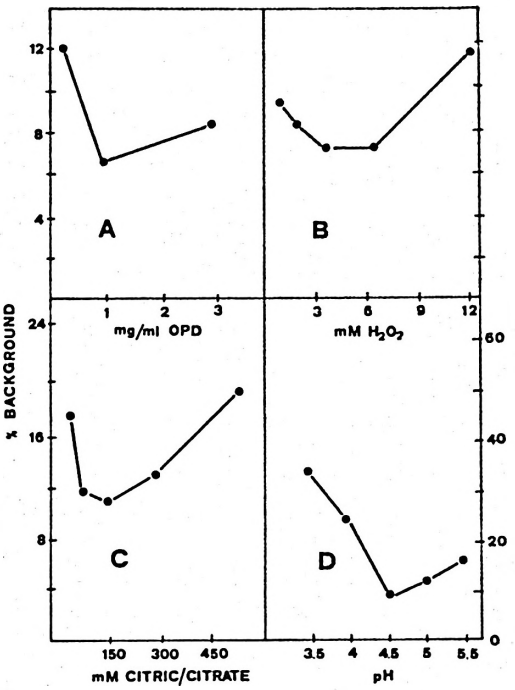

Fig. 1. *Influence of the concentrations of OPD (A), H_2O_2 (B), citrate (C) and H^+ (D), in the percentage of background.*
Experiments, except C, were made in 150 mM citric acid/citrate buffer, pH 4.8. The experiment A was made with 3 mM H_2O_2. The experiment B was made with 1 mg/ml OPD. Similar results were obtained by using phosphate/citrate buffers. Averages from duplicates were used to calculate percentage of background.

Addition of acetanilide, β-mercaptoethanol and nitrilotriacetic acid. — Figure 2 A shows that the effect of the addition of acetanilide on the background was concentration dependent. Optimal background was obtained in the range of 0.75-1.5 mM. Background was reduced about 2-3 fold with 0.5 mM β-mercaptoethanol (fig. 2 B). Dithioerythrol, ascorbic acid and sodium bisulphite were also tested between 0.4 to 1.8 mM but the background was 40 % higher in the three cases. $FeCl_3$, $CuSO_4$, $Pb(NO_3)_2$, $ZnCl_2$, $Al(NO_3)_3$ at final concentrations of 0.05 mM were added

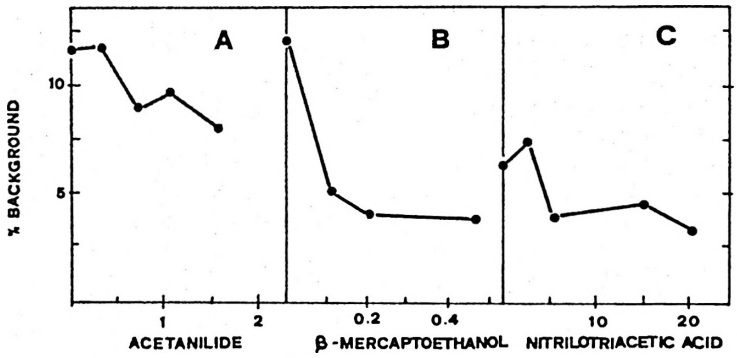

Fig. 2. *Influence of the concentrations of acetanilide (A), β-mercaptoethanol (B) and nitrilotriacetic acid (C), in the percentage of background.*
The experiments A and B were carried out in buffer adjusted to pH 5.5 after addition of 1 mg/ml OPD and 3 mM H_2O_2. Results from various experiments were combined in A. In experiment C the presence of trace metals was simulated by the addition of 0.01 mM $FeCl_3$ to the substrate buffer which was adjusted to pH 5.5 after addition of 1 mg/ml OPD, 3 mM H_2O_2, 0.75 mM acetanilide and 0.08 β-mercaptoethanol. Averages from duplicates were used to calculate percentage of background. Concentrations are in mM in all the experiments.

to the substrate buffer and their effects studied. Percentage of background was higher than controls after addition of Fe whereas with the other metals no increase in backgrounds could be detected. Therefore, the influence of addition of the iron chelant nitrilotriacetic acid (NTA) to substrate buffer in the presence of added ferric chloride was further studied. The addition of 5-10 mM NTA decreased to about half the background in the presence of 0.01 mM $FeCl_3$ (fig. 2 C).

Chequer-boards where some variables were compared against each other showed similar results to those in figure 2.

Performance of low-background substrate buffer in CRP binding to PE enzyme immunoassay. — The optimal mixture for low-background substrate buffer was: 150 mM citric acid/sodium citrate pH 4.8, with 0.01 percent merthiolate, 1.5 mM acetanilide, 5 mM nitrilotriacetic acid, 0.14 mM β-mercaptoethanol and 3 mM H_2O_2. OPD was added to a final concentration of 1 mg/ml.

Stability was studied at different temperatures. When this substrate buffer was kept at 0, 22 and 37 °C for two months, no alteration of performance on the CRP binding to PE assay was obtained. Backgrounds were $\leq$ 0.5 % which corresponds to absolute absorbances (A492-620 nm) from 0.001 to 0.01. In other experiment, substrates were prepared in the absence and in the presence of reducing agents, kept in closed containers protected from light at 4 °C for a year and then analysed. Straight lines were adjusted by minimum squares fit with six points each: $y = 0.81 \times + 0.3$ ($r = 0.994$) in the absence of reducing agents and $y = 0.60 \times + 0.15$ ($r = 0.984$) in the presence of reducing agents. Backgrounds were 1.1 % and 0.4 % for substrate buffer in the absence and in the presence of reducing agents, respectively.

The absorbance A492 nm-620 nm of the background values obtained in 11 assays were 0.013 ± 0.014 (mean ± S.D.). The sensitivity defined as the lowest concentration that can be detected above the zero

Table I. *Comparison of low-background substrate buffer with other commercial buffers.* Substrate buffer from Abbot (carcynoembryonic antigen kit) contained citrate/phosphate buffer pH 5.6 (pH 5 after addition of 1 mg/ml OPD). Substrate buffer from Pasteur (kit for hepatitis B) contained 50 mM citric/citrate buffer pH 5.5 (pH 5 after addition of 1 mg/ml OPD). Substrate buffer from Behring (kit for T 4) contained 100 mM citrate/phosphate buffer pH 5 (pH 4.5 after addition of 1 mg/ml OPD) it also contained sodium perborate, not H_2O_2. All substrates were assayed by the CRP sandwich assay. Averages from duplicates and ranges are given.

	Absorbances at 492 nm			
	Abbot	Pasteur	Behring	substrate buffer
ELISA + CRP	0.90 ± 0.01	0.67 ± 0.01	0.66 ± 0.04	0.69 ± 0.04
ELISA — CRP	0.20 ± 0.03	0.10 ± 0.01	0.12 ± 0.03	0.07 ± 0.01
% BACKGROUND	22	14.9	18	10

standard with 95 % confidence assuming a normal distribution, was 0.1 ng/well, which is about 3-fold higher than using the substrate buffer without reducing agents. The background of substrate buffers from Abbott, Pasteur and Behring were 1.5 to 2 fold higher than the substrate buffer with reducing agents described here (table I).

Within-run variation coefficient was between 4-7.5 percent for samples of low, medium and high CRP concentrations (12.5 to 160 mg/l), assayed 54 times. The between-run variation coefficient was between 9.3-11.2 percent for samples of low, medium and high CRP concentrations, assayed 8 times.

Discussion

A substrate buffer for the peroxidase-*o*-phenylenediamine reaction with low-background has been developed by optimization of OPD, H_2O_2, citrate and H^+ concentrations and the addition of acetanilide, mercaptoethanol and nitrilotriacetic acid.

Citric acid/citrate buffer 150 mM, pH 4.8 has been preferred over citrate/phosphate buffers because, one of its pK values is 4.8 which is optimal for peroxidase activity, it has enough buffering capacity at 150 mM for 1 mg OPD/ml and it acts as an iron chelating agent. The addition of OPD in the HCl form to the citrate/phosphate buffer but not to the citric/citrate buffer, decreased the pH and thus lowered the peroxidase activity, making it necessary to adjust the pH after dissolving the OPD. The horseradish peroxidase activity has a pH optimum which depends on the isoenzyme composition (4, 7). On the other hand the background is minimal at pH 4.5 (fig. 1 D). Therefore, horseradish peroxidase isoenzymes of optimal activity at pH 4.5 could probably be isolated and used for antibody conjugates to further lower the background.

Several reducing agents were tested to delay the spontaneous OPD oxidation rate. Of the compounds tested both acetanilide and β-mercaptoethanol were efficient enough even to stop oxidation. Concentrations had to be finely adjusted to allow reduction of background while preserving high enough signals in the peroxidase immunoassay. Optimal concentrations were 1.5 mM acetanilide (fig. 2 A) and 0.14 β-mercaptoethanol (fig. 2 B). The iron chelant NTA showed some effectiveness in preventing iron-accelerated absorbances at optimal concentrations of 5 mM (fig. 2 C). The addition of all these 3 additives was judged convenient, though the addition of NTA is probably not necessary.

By adjusting the concentrations of ace-

tanilide and/or β-mercaptoethanol, backgrounds might be lowered for any particular system to a convenient level while maintaining appropriated ELISA values as it has been demonstrated in enzyme immunoassays for human antiestreptolysin and for human rheumatoid factor (not shown).

By combining all the optimal conditions, the following buffer was tested for the CRP ELISA; 150 mM citric acid/sodium citrate pH 4.8, with 0.01 % methiolate, 1.5 mM acetanilide, 5 mM NTA, 0.14 mM β-mercaptoethanol and 3 mM H_2O_2. The inclusion of acetanilide NTA and β-mercaptoethanol reduced the relative background in the CRP ELISA. The new buffer was stable and excellent interassay variations (CV 5-10 %) were obtained when used in a CRP binding to PE ELISA Kit (5, 6).

Resumen

En enzimoinmunoensayos de proteína C-reactiva, antiestreptolisina y factor reumatoide humanos se optimizan las concentraciones de *o*-phenylenediamina (OPD), H_2O_2, citrato y H^+ en el tampón sustrato para los inmunoensayos que utilizan peroxidasa, para disminuir el fondo. Este se reduce de 2 a 3 veces utilizando 5,5 mM OPD, 3 mM H_2O_2, 150 mM ácido cítrico/citrato sódico, pH 4,8, y se incrementa la reproducibilidad interensayo. Se obtiene una reducción del fondo de 3 a 5 veces mayor, con la adición al tampón sustrato de acetanilida 1,5 mM, β-mercapto-etanol 0,14 mM y ácido nitrilotriacético 5 mM. Éste permite aumentar la sensibilidad y disminuir el coeficiente de variación interensayo.

Palabras clave: ELISA, Peroxidasa-*o*-fenilenediamina, Enzimoinmunoensayos, Proteína C-reactiva.

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