Thimerosal as a Preservative in Biological Preparations II. Formation of a Thimerosal-Zinc Complex

By J. BIRNER and J. ROS. GARNET

Thimerosal reacts with zinc ions to form a zinc-thimerosal complex which possesses antiseptic properties comparable to those of thimerosal itself. The kinetics of the reaction can be demonstrated using polarography.

THIS PAPER discusses a previously undescribed reaction in which a thimerosal[1]–zinc complex is formed. The subject matter of the paper stemmed from an investigation of the effect of storage on the thimerosal content of certain biological preparations held in containers sealed with various types of rubber closure.

In the course of the investigation it was observed that generally, after an initial small and rapid fall, the concentration of thimerosal in dilute solution in rubber-capped glass containers continued to decrease at a rate which appeared to be related to the type of closure used.

The estimations were carried out by the polarographic technique mentioned in a previous paper (1).

After only a brief period of storage, in some instances the characteristic polarographic wave at −0.75 v. half-wave potential for thimerosal could not be detected. Instead, there appeared another at −1.0 v. half-wave potential which subsequently was found to be due to the zinc ion.

Reznek (2) has observed that zinc ions are leached from rubber closures when the rubber is allowed to remain in contact with acid substances. Davisson *et al.* (3) reported that traces of copper ion present in the water used in the preparation of poliomyelitis vaccine inactivate thimerosal. The addition of ethylenediamine tetracetic acid (EDTA) to chelate the copper was recommended.

EXPERIMENTAL

Extraction of Zinc from Rubber.—Three types of rubber combination cap and stopper of the pattern used as closures for vaccine multidose containers were used in the experiments: (*a*) a red natural rubber with the inner surface lacquered with a film of epoxy resin polymer, (*b*) a black carbon-filled natural rubber and, (*c*) a grey natural rubber.

Five of each type were boiled in 15 ml. of glass-distilled, deionized water. A portion of the extract was used for the Rush and Yoe (4) colorimetric test for zinc, and the rest was polarographed by the procedure described in a previous paper (1).

Formation of Thimerosal-Zinc Complex.—To investigate the possibility of a reaction between thimerosal and zinc ions, polarograms were recorded for a series of solutions consisting of approximately 10 ml. of test solution together with supporting electrolyte and suppressor in the proportions previously indicated (1).

A, nothing added..................	See Fig. 1
B, 100 mcg. of zinc ion in 10 ml. of aqueous solution (without thimerosal)	See Fig. 2
C, 100 mcg. of zinc ion in 0.1 ml. of aqueous solution................	See Fig. 3
D, 200 mcg. of zinc ion in 0.2 ml. of aqueous solution................	
E, 300 mcg. of zinc ion in 0.3 ml. of aqueous solution................	
F, 400 mcg. of zinc ion in 0.4 ml. of aqueous solution................	See Fig. 4
G, 10 mg. of solid EDTA disodium salt	See Fig. 5
H, 10 mg. of solid EDTA + 400 mcg. of zinc ion in 0.4 ml. of aqueous solution........................	See Fig. 6

All but solution *B* in the series above contained 1000 mcg. of thimerosal furnished by 10 ml. of a 0.01% aqueous solution of the antiseptic with additions as indicated. Solution *B* was 10 ml. of an aqueous solution of zinc sulphate.

Antiseptic Quality of the Thimerosal-Zinc Complex.—To test a supposition that the thimerosal and zinc might form a complex which is itself capable of functioning as an antiseptic in the manner of thimerosal, an additional series of experiments was undertaken.

Samples of 0.01% solutions of thimerosal in water and triple antigen[2] were stored for 10 months at 25°. They were separated into five groups: (*a*) unaltered solutions to serve as controls, (*b*) solutions to which 40 mcg./ml. of zinc ion had been added, (*c*) solutions to which solid zinc oxide been added, (*d*) to which fragments of white rubber caps had been added, and (*e*) solutions to which fragments of black rubber caps had been added.

At the conclusion of storage, the thimerosal content of each of the solutions was determined by both the polarographic and the biological methods. For the biological estimation, two different test organisms were used according to the recognized standard procedure mentioned in a previous paper (1). The results of both tests are recorded in Table I.

RESULTS AND DISCUSSION

Zinc was present in all three extracts obtained by boiling the rubber closures in distilled water, an indication that these ions are readily extracted from the rubber.

An inspection of the polarograms of solutions *C*, *D*, *E*, and *F* reveals that a reaction between thimerosal and zinc occurred.

The zinc wave at −0.1 v. is neither apparent in the polarogram of solution *C* (Fig. 3) nor in those of solutions *D* and *E*. Its appearance in solution *F* (Fig. 4) indicates the presence of an excess of zinc and a consequent alteration in the pattern of the thimerosal wave.

The polarogram of solution *H* (Fig. 6) shows the chelating action of EDTA on zinc ions. Comparison

Received February 4, 1964, from the Commonwealth Serum Laboratories, Melbourne, Victoria, Australia.
Accepted for publication March 20, 1964.

[1] Thimerosal, sodium ethylmercurithiosalicylate, is official in the "British Pharmacopoeia" as Thiomersal. It is trademarked as Merthiolate.

[2] Triple antigen is an alternative name for diphtheria, tetanus, and pertussis vaccine B.P.

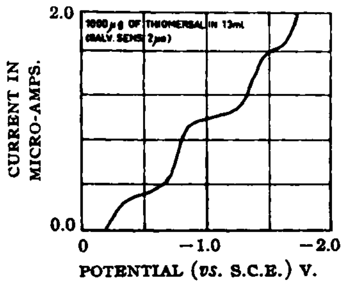

Fig. 1

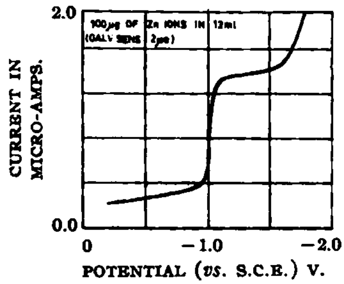

Fig. 2

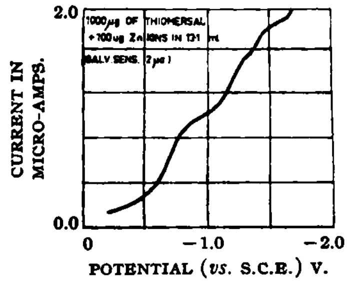

Fig. 3

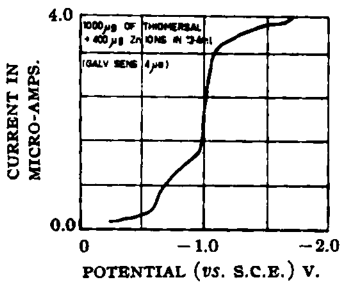

Fig. 4

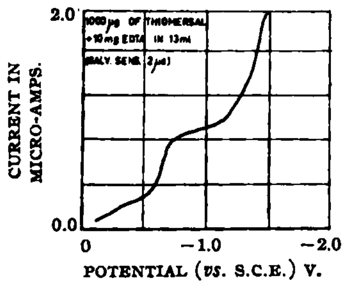

Fig. 5

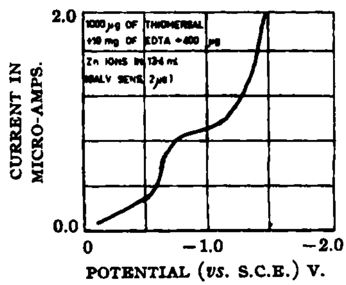

Fig. 6

TABLE I.—ACTIVITY OF 100 mcg./ml. OF THIMEROSAL

	After 10 Mo. Storage at 25°C.[a]			
	Water Soln.		Triple Antigen	
Added to Soln.	Biological Assay Against *B. subtilis* and *S. aureus*	Polarographic Assay	Biological Assay Against *B. subtilis* and *S. aureus*	Polarographic Assay
Nil	+	+	+	+
$ZnSO_4$	+	+	+	+
ZnO (solid)	+	+	+	+
White rubber cap	−	−	−	−
Black rubber cap	−	−	−	−

[a] +, Thimerosal present; −, thimerosal absent.

of Figs. 5 and 6 shows that the shape and height of the first thimerosal wave are identical, irrespective of the presence or absence of zinc ions.

The appearance of a second thimerosal wave is obscured by the presence of EDTA which, in solutions *G* and *H*, was used in a concentration of 0.075%. Both of the solutions produced polarograms in which the first thimerosal wave appeared at a lower half-wave potential than was apparent in the other polarograms. This is consistent with the observations of Benesch and Benesch (5) concerning the effect of pH on the solution.

The supposition that the thimerosal-zinc complex behaves as an antiseptic appeared to be confirmed by the results of the comparative assays given in Table I. Loss of antiseptic activity is apparent only in those solutions of thimerosal which were stored in contact with rubber; hence, the inference is warranted that the loss of activity which occurs is not due to the presence of zinc released from the rubber.

SUMMARY

The addition of zinc ions to dilute solutions of thimerosal results in the formation of a thimerosal-zinc complex which behaves as an antiseptic in much the same manner as uncomplexed thimerosal.

REFERENCES

(1) Birner, J., and Garnet, J. R., THIS JOURNAL, 53, 1264(1964).
(2) Reznek, S., *ibid.*, 42, 288(1953).
(3) Davisson, E. D., *et al.*, *J. Lab. Clin. Med.*, 47, 8(1956).
(4) Rush, R. M., and Yoe, J. J., *Anal. Chem.*, 26, 1345 (1954).
(5) Benesch, R., and Benesch, R. E., *J. Am. Chem. Soc.*, 73, 3391(1951).