The mixed melting point remained unaltered with an authentic sample of piplartine.

Isolation of Piperine.—The greenish-yellow deposit was dissolved in methanol and filtered hot. On cooling, more piplartine was obtained as fine needles, which were separated. The mother liquor was chromatographed over Brockmann alumina using benzene and benzene–chloroform mixture (1:3). Elution with benzene gave a steroid, while elution with benzene and chloroform mixture yielded piperine. After several crystallizations from benzene, light yellow crystals of piperine, m.p. 128–129°, were obtained. The test with concentrated sulfuric acid and gallic acid for methylene dioxy group was positive. A mixed melting point with an authentic specimen of piperine was not depressed.

Isolation of β-Sitosterol from Fatty Residue.—The fat was hydrolyzed with 0.5 *N* alcoholic potassium hydroxide, and the mixture of phytosterols was extracted with ether (10). The sterol mixture obtained after removal of solvent was chromatographed over alumina. The column was eluted with benzene and benzene–chloroform mixture (1:3). The benzene eluent was too small for further study. The residue obtained from benzene–chloroform mixture gave a positive Liebermann-Burchard test. On several crystallizations from methanol, the residue gave colorless needles, m.p. 136°, $(\alpha)_D^{25}$ −36.5° ($CHCl_3$). The sterol was freely soluble in benzene, chloroform, and petroleum ether and sparingly soluble in cold methanol and ethanol.

Anal.—Calcd. for $C_{29}H_{50}O$: C, 84.05: H, 12.09. Found: C, 83.84; H, 12.13.

The sterol acetate, benzoate, and digitonide were prepared in the usual manner; m.p. 127, 144, and 221° dec., respectively. β-Sitosterol and its acetyl and benzoyl derivatives did not show a change in melting point when admixed with the respective authentic specimen.

Thus, from a comparison of the data with those for known sitosterol, this sterol was identified as β-sitosterol. The characteristics of the sterol are in conformity with the earlier observation of the authors (11) on the sterol from *Ipomoea degitata* Linn. (*Convolvulaceae*).

REFERENCES

(1) Kirtikar, K. R., and Basu, B. D., "Indian Medicinal Plants," Vol. III, 2nd ed., Lalit Mohan Basu, Allahabad, India, 1933, pp. 2130–2131.

(2) Chopra, R. N., Nayar, S. L., and Chopra, I. C., "Glossary of Indian Medicinal Plants," Council of Scientific and Industrial Research, New Delhi, India, 1956, p. 194.

(3) Nadkarni, K. M., and Nadkarni, A. K., "Indian Materia Medica," Vol. I, 3rd ed., Popular and Dhootapapeswar Prakashan, Ltd., Panvel-Bombay, India, 1954, pp. 63, 964.

(4) Tewari, J. P., *et al.*, *L.J. Sci. Tech.*, **2**, 118(1964).

(5) Gokhale, V. G., Phalinikar, N. K., and Bhide, B. V., *J. Univ. Bombay*, **16A**, 47(1948); Spring, F. S., and Stark, J., *J. Chem. Soc.*, **1950**, 1177.

(6) Atal, C. K., and Banga, S. S., *Indian J. Pharm.*, **24** 105(1962).

(7) Atal, C. K., and Banga, S. S., *Current Sci. India*, **32** 354(1963).

(8) Chatterjee, A., and Dutta, C. P., *Sci. Cult. Calcutta* **29**, 568(1963).

(9) Rosenthaler, L., "The Chemical Investigation of Plants," 3rd ed., G. Bell and Sons Ltd., London, 1930, pp. 36–112.

(10) Chakravarti, D., *et al.*, *J. Indian Chem. Soc.*, **29**, 374 (1950); *J. Sci. Ind. Res.*, **15C**, 86, 89(1956).

(11) Tewari, J. P., Matin, M. A., and Mishra, S. S., *Indian. J. Appl. Chem.*, to be published.

Thimerosal as a Preservative in Biological Preparations. III. Factors Affecting the Concentration of Thimerosal in Aqueous Solutions and in Vaccines Stored in Rubber-Capped Bottles

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Inactivation of the antiseptic properties of thimerosal contained in vaccines stored in bottles which have been sealed with rubber closures is governed by the type of rubber used for sealing the containers, the temperature and duration of storage, and the ratio of volume of liquid to surface area of the rubber to which the thimerosal is exposed.

THE POLAROGRAPHIC method of estimating thimerosal[1] in dilute solutions has been applied to its determination in samples of pertussis vaccine and triple antigen[2] during storage of the preparations in contact with rubber closures of differing compositions for varied periods of time and temperature. The purpose of this investigation was to assess the suitability of thimerosal as a preservative in these vaccines and to provide a basis for their storage under optimum conditions.

There is no question that the preservative effect of thimerosal in the low concentration employed in biological preparations is appreciably diminished when those preparations are stored in containers sealed with rubber closures (1).

Considerations of safety in the use of vaccines dispensed in multidose containers which have been sealed with rubber caps make it important to know at what stage in the shelf life of the preparation the concentration of the preservative may be expected to fall below what can be regarded as an effective level.

As shown in a previous paper (2) the determination of thimerosal with the polarograph can be carried out rapidly and with no less accuracy than is possible with the customarily used biological method.

Received February 4, 1964, from the Commonwealth Serum Laboratories, Melbourne, Victoria, Australia.

Accepted for publication March 20, 1964.

The valued and helpful criticism of Dr. S. J. Leach, Principal Research Officer, Division of Protein Chemistry, C.S.I.R.O., Melbourne, is gratefully acknowledged. The authors also thank the Director, Commonwealth Serum Laboratories, for permission to publish this paper.

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

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

TABLE I.—EFFECT OF STORAGE TEMPERATURE, CONTAINER, AND SEAL ON THE THIMEROSAL AND ZINC CONTENT OF 1 ml. OF TRIPLE ANTIGEN STORED FOR 22 WEEKS[a]

Seal	Type of Container											
	Ampul						Bottle					
	6° C.		25° C.		37° C.		6° C.		25° C.		37° C.	
	Thimerosal, mcg./ml.	Zinc, mcg./ml.	Thimerosal, mcg./ml.	Zinc, mcg./ml.	Thimerosal, mcg./ml.	Zinc, mcg./ml.	Thimerosal, mcg./ml.	Zinc, mcg./ml.	Thimerosal, mcg./ml.	Zinc, mcg./ml.	Thimerosal, mcg./mg.	Zinc, mcg./ml.
Hermetic	58	Nil	40	Nil	32	Nil	Nil	8	Nil	10	Nil	11
White rubber cap	...	...	...	...	...	...	Trace	Nil	Below limit	Nil	Below limit	Nil
Gray rubber cap	...	...	...	...	...	...	Trace	Nil	Below limit	Nil	Below limit	Nil
Lacquered red rubber cap	...	...	...	...	...	...						

[a] Initial concentration of thimerosal, 100 mcg./ml.

With this instrument, the authors have been able to confirm the results of biological tests which show that zinc ions contributed by the rubber are not an important factor in the loss of antiseptic activity.

The present investigation was not designed to identify the substance responsible for the degradation of thimerosal, but to determine the conditions under which it occurs. There is reason to believe that sulfur, commonly incorporated into rubber mixes, reacts with thimerosal.

An experiment was carried out in which an aqueous solution containing 100 mcg. of thimerosal per milliliter was incubated at 37° for 72 hours with 1000 mcg./ml. of precipitated sulfur.

At the end of storage, polarographic assay indicated that only 42% of the thimerosal was present; this finding was confirmed by biological assay.

Rubber, of all compositions examined so far, is invariably blackened with mercury sulfide when left in contact with thimerosal solution.

The mercury-sulfur reaction, being irreversible, would account for the loss of activity of the antiseptic and explain why the loss is governed by the several factors noted in this paper.

EXPERIMENTAL

A series of three experiments was planned. In *Experiment A*, at the beginning and end of storage for several weeks in contact with rubber closures of differing compositions, the polarographic method was applied to the determination of the thimerosal content of samples of pertussis vaccine. The amount of antiseptic initially added was deliberately varied to give a series of concentrations of 80, 40, and 20 p.p.m.

The preparations containing the two higher concentrations of thimerosal gave characteristic polarographic waves; but those of the sample with an initial concentration of 20 p.p.m. showed only a wave characteristic of zinc ions.

Experiment B was carried out with samples of thimerosal and triple antigen, a biological preparation containing the antiseptic in an initial concentration of 0.01%.

The samples in 1-ml. quantities were stored for approximately 22 weeks in hermetically sealed ampuls and in bottles sealed with a selection of rubber closures. They were inverted at weekly intervals during storage in order to mix the contents and bring them into contact with the rubber. Samples of the triple antigen were held in three groups—at 6, 25, and 37° C. The results of the polarographic analysis of the contents are recorded in Table I.

No values for zero time assays are given, since the purpose of the experiments was to demonstrate differences which occurred between samples stored in all-glass and rubber-capped containers.

Experiment C was set up with triple antigen to determine to what extent the inactivation of thimerosal was affected by the ratio of volume of vaccine to surface area of rubber exposed to it.

The antiseptic was used in the same initial concentration of 0.01%. The vaccine was used in 5- and 10-ml. quantities distributed in hermetically sealed glass ampuls and in glass bottles closed with lacquered red rubber and gray rubber caps, both natural rubbers.

Thimerosal estimations were carried out periodically during storage at 6° for 18 months.

TABLE II.—EFFECT OF SAMPLE VOLUME, CONTAINER, AND SEAL ON THE STABILITY OF 100 mcg./ml. THIMEROSAL IN TRIPLE ANTIGEN STORED FOR 18 MONTHS AT 6° C.

		Type of Container					
		Ampul			Bottle		
Vol. of Stored Sample, ml.	Seal	Commencement Thimerosal, mcg./ml.	6 Mo. Thimerosal, mcg./ml.	18 Mo. Thimerosal, mcg./ml.	Commencement Thimerosal, mcg./ml.	6 Mo. Thimerosal, mcg./ml.	18 Mo. Thimerosal, mcg./ml.
5	Hermetic	...	...	83	...	...	...
	Gray rubber cap	...	...	...	89	85	71
	Lacquered red rubber cap	...	...	...	89	80	62
10	Hermetic	89	85	83	...	...	...
	Gray rubber cap	...	...	...	89	85	72
	Lacquered red rubber cap	...	...	...	89	85	72

Throughout the time of storage the containers were inverted at weekly intervals in order to bring the vaccine into direct contact with the rubber of the cap. The period of actual contact was 9 months in the aggregate—one-half of the full period of storage. The results of the test are recorded in Table II.

RESULTS AND DISCUSSION

Experiment A with pertussis vaccine indicated clearly that thimerosal will be an unsuitable preservative if the vaccine is dispensed in small volume quantities, such as 1-ml. containers sealed with rubber closures.

At the end of 22 weeks of storage at room temperature (region of 25°), the polarographic wave characteristic of the thimerosal ion was altogether absent from samples which had contained the preservative in the proportion of 20 p.p.m.

Although the pH of pertussis vaccine is within the range 7.2 to 7.4 the characteristic zinc wave was observed when white rubber stoppers were used as closures.

Table I summarizes the results of *Experiment B*. *Below limit* in Table I means that the concentration of thimerosal registered by the polarograph was less than 20 p.p.m.; no quantitative measure is possible below about 12.5 p.p.m., a value which is indicated by *trace*.

It will be noted that, irrespective of the temperature of storage, triple antigen in 1-ml. quantities retains a negligible proportion of the amount of thimerosal originally added after contact for a period totaling about 3 months with rubber of any of the three types used.

Apart from the fact that the white, zinc oxide-filled rubber yielded a measurable amount of zinc into the vaccine during storage, the interesting observation appears that even after prolonged storage under relatively adverse conditions more than 30% of the added thimerosal remained in the vaccine contained in hermetically sealed glass ampuls; of course, no zinc was detected.

No values for zero time assays are given because the purpose of the experiments was to demonstrate differences which occur between samples stored in rubber-capped and all-glass containers.

Experiment C is summarized in Table II. It was set up to determine to what extent the inactivation of thimerosal was affected by the ratio of volume of vaccine to surface area of rubber exposed to it.

The results, as tabulated, again demonstrate that in the absence of contact between liquid and rubber (as in the hermetically sealed glass containers) the diminution in the quantity of thimerosal is relatively small; but when rubber closures are used, a progressive loss occurs—a loss greater in the 5 ml. than in the 10-ml. volumes. However, even with the lower ratio of volume of vaccine to surface area of vaccine exposed to it, the maximum loss did not exceed 30%.

SUMMARY AND CONCLUSIONS

The inactivation of thimerosal contained in vaccines stored in bottles sealed with rubber closures is governed by the type of rubber used for sealing the containers, the temperature and duration of storage, and the ratio of volume of liquid to area of rubber surface to which the thimerosal is exposed.

Under the conditions of the experiment it is considered that the residual thimerosal in 5- and 10-ml. rubber-sealed multidose containers is adequate for the purpose it is intended to serve—that of a bacteriostat.

REFERENCES

(1) Weiner, S., *J. Pharm. Pharmacol.*, **7**, 118(1955).
(2) Birner, J., and Garnet, J. R., THIS JOURNAL, **53**, 1264 (1964).