THE INTERFERENCE OF RUBBER WITH THE BACTERIOSTATIC ACTION OF THIOMERSALATE

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Introduction

Apart from the presence of variable amounts of natural rubber, a botanical substance of no fixed composition (Davis[1]), rubber products contain variable quantities of different organic and inorganic substances which have been added during the process of compounding. It is not surprising that products having such variable and complex composition are not without influence on pharmaceutical preparations with which they may come into contact. In a recent publication Berry[2] quoted his own observations and those of other investigators which showed that rubber products may yield substances to a pharmaceutical preparation as well as extracting substances from it.

In order to prevent the multiplication of bacteria accidentally introduced into a pharmaceutical preparation suitable for injection, the B.P. requires that a bacteriostatic be added if such solutions are issued in containers which permit the withdrawal of successive doses on different occasions. At the present time the majority of such containers are sealed with rubber caps. It is a matter of great importance to be aware of and, if possible to prevent the removal of the protecting bacteriostatic from solution by the rubber cap. For this reason, the B.P. 1953 specifies that rubber caps should either be boiled under a reflux condenser for 30 minutes or stored for not less than 48 hours in a solution containing the same bacteriostatic in the same concentration or preferably in twice the concentration used in preparing the injection. The B.P. then adds the further caution that on prolonged storage rubber so treated is liable to absorb bacteriostatic from the injection.

No investigation appears to have been made to confirm the effectiveness of the treatment recommended by the B.P., nor is any indication given by the B.P. of the interval of time which is considered "prolonged storage," during which further amounts of bacteriostatic may be removed from solution. During an investigation of the action of thiomersalate on bacteria it was found that certain rubber caps interfered with its bacteriostatic action. An attempt was made to discover the cause of this interference. In the light of the findings to be presented here, there is little doubt that thiomersalate can be absorbed by rubber products. However, at the beginning of this investigation, the interference with the bacteriostatic action of thiomersalate by rubber caps was ascribed to inactivation by substances derived from the rubber.

EXPERIMENTAL

Methods and Materials.

As it was found that 10 per cent. of serum in broth interfered to some extent with the bacteriostatic action of thiomersalate in the higher dilutions, nutrient meat infusion broth containing only 1 per cent. of serum and serial dilutions of thiomersalate was prepared. The *p*H of the broth was 7·2 to 7·5. In such a medium, thiomersalate in a dilution up to and including 1:2 million will regularly inhibit the growth of *Staph. aureus* which was the organism used in this investigation.

Into 10 ml. of each dilution about 1·5 to 2 g. of different types of rubber material was introduced, 0·1 ml. of a 24-hour broth culture of *Staph. aureus* was added and the tubes incubated at 37° C. for 14 days. Earlier experience had shown that a latent period of several days usually elapsed before bacteria start to grow in the higher concentrations of thiomersalate. With organic mercurials Brewer[3] observed a similar effect. Results here recorded express the presence or absence of visible turbidity after 14 days' incubation at 37° C. Prior to use, all rubber materials were boiled in dilute hydrochloric acid, washed with distilled water and autoclaved. Sulphur estimations on rubber products were made according to the method for total sulphur determination of the American Society for Testing Materials[4]. Thiomersalate estimations involved the estimation of mercury according to Maren[5].

TABLE I

VISIBLE TURBIDITY 14 DAYS AT 37° C. AFTER INOCULATING WITH *Staph. aureus*, SERIAL DILUTIONS OF THIOMERSALATE IN 1 PER CENT. SERUM BROTH CONTAINING DIFFERENT RUBBER SAMPLES

Dilution of thiomersalate	No rubber	Black cap	Orange cap	Red rubber stopper	Red rubber tubing	Grey rubber tubing	Black rubber washer	Red rubber washer	Brown cap
1:10,000	–	–	–	–	–	–	+	–	–
1:20,000	–	+	–	–	+	–	+	–	–
1:40,000	–	–	–	+	+	–	+	–	–
1:80,000	–	+	–	+	+	–	+	–	–
1:100,000	–	–	–	+	+	–	+	–	–
1:320,000	–	+	–	+	+	–	+	+	–
1:1,000,000	–	+	+	+	+	+	+	+	–
1:2,000,000	–	+	+	+	+	+	+	+	–
Nil	+	+	+	+	+	+	+	+	+

RESULTS

I. The effect of different rubber products on the bacteriostatic action of thiomersalate.

Different rubber products were added to serial dilutions of thiomersalate in serum broth. After adding 0·1 ml. of a 24-hour broth culture of *Staph. aureus*, the tubes were incubated at 37° C. for 14 days. The results are summarised in Table I. It is evident that there was a great variation in the ability of different rubber products to interfere with the bacteriostatic action. The brown rubber caps showed no interference in the present test. Black rubber products appeared to be particularly effective. During the 14 days of contact, the orange rubber caps and grey rubber tubing did not interfere up to a dilution of 1 in 320,000.

Inquiry from the manufacturers revealed that the percentages of rubber in these products were as follows:—brown cap, 70; orange cap, 56; grey rubber tubing, 59; and black cap, 82.

II. The role of sulphur compounds.

At the beginning of this study the interference of rubber products with the bacteriostatic action of thiomersalate was attributed to the presence of sulphur compounds and, in particular, of thiol groups in the rubber compound. Attention was directed to these substances for several reasons. Sulphur and sulphur compounds are generally used

TABLE II

SULPHUR CONTENT AND THIOMERSALATE INHIBITION OF DIFFERENT RUBBER PRODUCTS

Sample	Total sulphur per cent.	Maximum concentration of thiomersalate inhibiting growth
Black rubber washer ..	2·4	1:10,000
Red rubber washer	2·9	1:100,000
Black rubber cap	1·5	1:10,000
Grey rubber tubing.. ..	1·6	1:320,000

for the compounding of rubber products. On several occasions when rubber caps were boiled in dilute hydrochloric acid, the odour of hydrogen sulphide was detected and its liberation confirmed by the blackening produced on lead acetate paper. Hydrogen sulphide is evolved after boiling of neoprene synthetic rubber caps for 1 hour in dilute hydrochloric acid. Many years ago it was shown that hydrogen sulphide will antagonise the action of mercurials on micro-organisms (Chick[6]). In addition, it has since been shown that substances containing thiol groups will antagonise the action of mercurials on bacteria (Fildes[7]). In fact, in the Technical Report Series No. 61 of the World Health Organisation, it is recommended that 1:5000 potassium iodate be added to a pharmaceutical product containing thiomersalate as the preservative, in order to inactivate any free thiol groups that may be present. Preliminary experiments have shown that the slight interference by 10 per cent. serum with the bacteriostatic action of thiomersalate to which reference was made earlier, is diminished by the addition of 1:5000 potassium iodate.

Tests for thiol groups in aqueous extracts of rubber products were consistently negative. In addition, treatment with strong oxidising agents for prolonged periods did not result in any diminution of the ability of rubber caps to interfere with the bacteriostatic action of thiomersalate. Total sulphur estimations of different rubber products showed no relationship between reduction of bacteriostatic titre and the sulphur content of the sample (Table II). It thus appeared unlikely that sulphur compounds could be incriminated.

III. Adsorption of thiomersalate by charcoal.

Carbon black is present in many rubber products and in appreciable quantities in those that are black in colour. It was decided to observe

if charcoal would interfere with the bacteriostatic action of thiomersalate. For this purpose, small quantities of purified charcoal were enclosed in small Cellophane bags and placed in serial dilutions in serum broth. It can be seen from Table III that charcoal is most effective in antagonising the action of thiomersalate.

TABLE III

VISIBLE TURBIDITY, IN THE PRESENCE AND ABSENCE OF CHARCOAL, IN SERIAL DILUTIONS OF THIOMERSALATE IN BROTH, INOCULATED WITH *Staph. aureus* AND INCUBATED AT 37° C. FOR 14 DAYS

Dilution of thiomersalate	No charcoal added	Charcoal added
1:10,000	−	−
1:20,000	−	+
1:40,000	−	+
1:80,000	−	+
1:100,000	−	+
1:320,000	−	+
1:1,000,000	−	+
1:2,000,000	−	+
Nil	+	+

In view of the known capacity of charcoal to adsorb substances, and in view of the purity of the charcoal used, it is reasonable to suppose that thiomersalate was adsorbed by charcoal and thus prevented from exerting its bacteriostatic effect on the medium. This was confirmed by showing that if Cellophane bags containing charcoal were soaked in a 1:100 solution for several days, washed, dried, and placed into nutrient broth, the broth became bacteriostatic. Furthermore, when such treated charcoal bags were placed in several changes of distilled water over a period of 4 days, they lost this capacity of rendering a medium bacteriostatic. In other words, the adsorption of thiomersalate by charcoal is a reversible process. Similar tests for adsorption with a suspension of aluminium phosphate, as used for the preparation of adsorbed toxoids, were negative.

IV. The adsorption of thiomersalate by crude rubber.

In view of the fact that interference with the bacteriostatic action occurred in the presence of rubber compounds which, according to the

TABLE IV

VISIBLE TURBIDITY IN THE PRESENCE OF CRUDE RUBBER IN SERIAL DILUTIONS OF THIOMERSALATE IN BROTH, INOCULATED WITH *Staph. aureus* AND INCUBATED AT 37° C. FOR 14 DAYS

Dilution of thiomersalate	Crepe rubber	Smoked sheet rubber
1:10,000	−	−
1:20,000	+	−
1:40,000	+	−
1:80,000	+	+
1:100,000	+	+
1:320,000	+	+
1:1,000,000	+	+
1:2,000,000	+	+
Nil	−	−

manufacturers, contained no carbon black, factors besides the presence of charcoal must play a part.

Strips of crude rubber in the form of smoked sheet and crepe were tested for interference. Table IV shows that crude rubber and particularly crepe rubber can interfere. It is of interest that, because of the absence of odour, crepe rubber is used by many manufacturers of rubber products in preference to smoked sheet. The presence of natural rubber in rubber products could thus alone account for most of the interference hitherto observed. However, one sample of synthetic rubber caps (Neoprene) which was also tested, interfered up to a dilution of 1:40,000. The cause of this interference has not been investigated.

V. Adsorption of thiomersalate by rubber caps.

In order to confirm the finding that rubber caps adsorb thiomersalate, estimations were performed. It was found that a single black rubber cap is capable of removing 750 μg. of thiomersalate from 10 ml. of a 1:10,000 aqueous solution in 3 days at 37° C. In 10 ml. of a 1:10,000 solution, there is 1000 μg. of thiomersalate. As 1:2 million regularly prevented the growth of *Staph. aureus*, in the absence of free thiol groups, it appears that as long as 5 μg. is free in 10 ml. of solution, the growth of these bacteria will be prevented. For this reason, growth was obtained in 10 ml. of a 1:20,000 solution in the presence of one black rubber cap. In such a solution only 500 μg. is present and the rubber cap is capable of removing it all. Although only 75 per cent. of the amount present was removed after 3 days' contact with one black rubber cap, all the thiomersalate contained in 10 ml. of a 1:10,000 dilution could be taken up by a similar cap on prolonged storage. In a vaccine to which 1:10,000 had been added 8 years previously, none was detectable. About 70 per cent. of the expected amount was detected in the rubber cap. This aspect is being further investigated. That rubber caps adsorb thiomersalate from solution was further shown by the fact that when such caps were soaked in thiomersalate solution they no longer interfered with the bacteriostatic action of thiomersalate.

Caps treated with thiomersalate were able to render bacteriostatic a nutrient broth into which they had been placed. After immersing such treated caps in several changes of distilled water for 2 to 3 days at 37° C., it was found that they lost the ability to render nutrient broth bacteriostatic and that they regained their former capacity to interfere with the bacteriostatic action of thiomersalate.

Table V shows that treatment of rubber caps with thiomersalate abolished further interference with the bacteriostatic action if such treated caps were introduced into the medium at the time of inoculation, the results being recorded as presence or absence of visible turbidity at the end of 14 days' incubation at 37° C.

VI. The efficacy of the treatment recommended by the B.P.

On the basis of the results shown in Table V, it appeared that the pre-treatment of rubber closures as recommended by the B.P. was

adequate. However, in order to imitate more closely conditions met in actual practice, the procedure hitherto used in testing for interference by rubber caps with the bacteriostatic action of thiomersalate was altered.

TABLE V

THE EFFECT OF TREATING GREY RUBBER CAPS ON THEIR ABILITY TO INTERFERE WITH THE BACTERIOSTATIC ACTION OF THIOMERSALATE. VISIBLE TUBIDITY AFTER INOCULATION WITH *Staph. aureus* AND INCUBATION AT 37° C. FOR 14 DAYS

Dilution of thiomersalate	Soaked for 2 days at 37° C. in:—			
	1:100 thiomersalate	1:1,000	1:10,000	Water
1:10,000	—	—	—	—
1:20,000	—	—	—	—
1:40,000	—	—	—	+
1:80,000	—	—	—	+
1:100,000	—	—	—	+
1:320,000	—	—	—	+
1:1,000,000	—	—	+	+
1:2,000,000	—	—	+	+
Nil	+	+	+	+

Black rubber caps were placed in 1:100, 1:1000, and 1:10,000 aqueous thiomersalate (10 ml. of each dilution per cap) and kept at 37° C. for 2 days. Another two series of black caps were boiled for 1 hour in 1:1000 and 1:5000 aqueous thiomersalate under a reflux condenser. The caps were quickly washed, dried and autoclaved. They were then used to close vaccine bottles containing 10 ml. of serial dilutions of thiomersalate in broth; the capped bottles, lying on their sides, were then incubated at 37° C. for 5 weeks. At the end of this storage period, 0·1 ml. of a 24-hour broth culture of *Staph. aureus* was introduced into each bottle through the rubber cap by means of a hypodermic needle.

TABLE VI

BLACK RUBBER CAPS TREATED WITH DIFFERENT CONCENTRATIONS OF THIOMERSALATE USED TO CLOSE VACCINE BOTTLES CONTAINING SERIAL DILUTIONS OF THIOMERSALATE. INOCULATED AFTER 5 WEEKS' STORAGE. VISIBLE TURBIDITY AFTER INCUBATION AT 37° C. FOR 14 DAYS AFTER INOCULATION

Dilution of thiomersalate	Not treated	Soaked in thiomersalate			Boiled in thiomersalate	
		1:100	1:1,000	1:10,000	1:1,000	1:5,000
1:10,000	—	—	—	—	—	—
1:20,000	—	—	—	—	—	—
1:40,000	+	—	—	—	+	—
1:80,000	+	+	+	+	+	+
1:100,000	+	+	+	+	+	+
1:320,000	+	+	+	+	+	+
1:1,000,000	+	+	+	+	+	+
1:2,000,000	+	+	+	+	+	+
Nil	+	+	+	+	+	+

All bottles were again incubated for 14 days. Table VI shows the results. It is obvious that treating rubber caps as suggested by the B.P. did not prevent further absorption of thiomersalate during 5 weeks' storage. Treating rubber caps even with 1:100 thiomersalate for 2 days was ineffective in preventing further adsorption.

In a similar experiment, vaccine bottles were filled with serial dilutions of thiomersalate in broth and closed with rubber caps which had been soaked at 37° C. in 1:100, 1:500 and 1:1000 aqueous thiomersalate for 2 months. The bottles were then placed upside down at 37° C. and at 4° C. for 5 weeks. In those bottles stored at 37° C. bacterial growth was obtained, after inoculation, when the concentration of thiomersalate was 1:40,000 or less. In the control series closed with untreated caps, growth occurred in 1:20,000 or less of thiomersalate. The bottles stored at 4° C. showed no growth after inoculation in up to 1:2 million of thiomersalate while in the control series growth occurred when the concentration was 1:100,000 or less. It appears that the extent of removal of thiomersalate from solution by rubber caps is reduced at low temperatures.

Some rubber caps which had been investigated previously by what may be called "the short range test" (see Table I) were again tested by the method of using vaccine bottles as described above. The results are given in Table VII. The brown caps which previously (Table I) showed no interference, now revealed definite ability to interfere with the bacteriostatic action of thiomersalate. The small orange cap which previously interfered only up to 1:1,000,000 now showed interference up to 1:80,000.

TABLE VII

UNTREATED RUBBER CAPS, USED TO CLOSE VACCINE BOTTLES CONTAINING SERIAL DILUTIONS OF THIOMERSALATE IN BROTH. INOCULATED AFTER 5 WEEKS' STORAGE

Dilution of thiomersalate	Small orange cap	Orange vial	Brown cap
1:10,000	--	--	--
1:20,000	--	--	--
1:40,000	--	--	±
1:80,000	+	--	+
1:100,000	±	±	+
1:320,000	±	±	±
1:1,000,000	+	+	+
1:2,000,000	+	+	+
Nil	±	+	+

DISCUSSION

It is important to remember that these experiments were conducted under the most favourable conditions for the growth of bacteria. A large inoculum and a highly nutrient medium were used. Even when a rubber cap was introduced into the medium, thus offering a maximal surface, no growth was ever obtained in a 1:10,000 dilution of thiomersalate. It is a tribute to the high bacteriostatic power of thiomersalate that even if its concentration is reduced from 1:10,000 to 1:1,000,000, i.e. a reduction of 99 per cent., it is still bacteriostatic. Berry[2] suggests that in the early stages, thiomersalate is adsorbed by the surface of the cap during which period the surface area of the cap plays the main part, whilst on prolonged storage the thiomersalate slowly diffuses into the rubber, when the whole mass of the cap participates. It is perhaps for this reason that, during the short range tests reported here, some types

of stoppers showed little interference, or their interference could be abolished by treatment with thiomersalate. During longer storage, i.e. storage for 5 weeks at 37° C., pre-treatment of rubber caps for a period much longer than that recommended by the B.P. will not prevent further removal of thiomersalate from solution.

From the present investigation one may conclude that treatment of rubber caps with thiomersalate for 2 days will prevent immediate further adsorption. Some of it may even leave the rubber cap and go into solution. During storage for 5 weeks, however, thiomersalate diffuses into the rubber and is progressively removed from solution. For practical purposes it would appear best to avoid the use of black rubber caps or caps containing carbon black. In order to limit adsorption, the smallest cap available and one which presents the smallest possible surface area to the solution should be selected for closing multiple dose containers. Apart from a transitory negative phase, treated caps are as capable of removing thiomersalate after several weeks' storage as are those which have not been treated. The extent of adsorption by rubber caps appears to be appreciably reduced at 4° C. In view of the variation in the ability of different types of rubber caps to remove thiomersalate from solution, samples of each batch of caps intended for use as closures for multiple dose containers should be tested for their capacity to adsorb that substance.

Conclusions

1. Certain rubber products are able to remove thiomersalate from solution. This capacity varies amongst the different types of rubber products which were tested.
2. Charcoal and crude rubber adsorb thiomersalate.
3. Rubber caps, pre-treated with thiomersalate and placed in contact with a solution for 5 weeks, are as capable as untreated caps of removing thiomersalate from solution.

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References

1. Davies, *The Chemistry and Technology of Rubber*, New York, 1937.
2. Berry, *J. Pharm. Pharmacol.*, 1953, **5**, 1008.
3. Brewer, *J. Amer. med. Ass.*, 1939, **2**, 2009.
4. *American Society for Testing Materials*, 1952, **6**, 63.
5. Maren, *J. Lab. clin. Med.*, 1943, **28**, 1511.
6. Chick, *J. Hyg.*, 1908, **8**, 92.
7. Fildes, *Brit. J. exp. Path.*, 1940, **21**, 67.