THE INACTIVATION OF SODIUM ETHYLMERCURITHIOSALICYLATE
IN STERILITY TEST MEDIA WITH SPECIAL REFERENCE
TO MYCOTIC STERILITY TEST

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(Received: June 27th, 1963)

In order not to invalidate the test for bacterial sterility of a biological product, the
growth inhibitory effect of added bactericides or bacteriostatics should be either neutralized
by some way or other or diluted out in a large volume of medium.

Organo-mercuric antiseptics are used in various kinds of biological products as the
preservative, and several procedures to make the antiseptic ineffective in sterility test
media have been proposed (Brewer, 1944; Pittman, 1946; Bonnel and Raby, 1957;
Didenko, 1957; Romanov et al., 1959). The medium established by Brewer (1940
and 1944) and Pittman (1946) is excellent and considered sufficient to inactivate the
mercuric antiseptic (National Institutes of Health, 1954). This medium has been
adopted in many countries (Public Health Service Regulations, 1962; Scheibel and
Bentzon, 1957; Requirements, 1960), and our minimum Requirements for sterility test
(Minimum Requirements of Biologic Products, 1962) was originally described on the
basis of their studies.

Our experiences, however, suggested that the method was not always effective to
some species of microorganisms sensitive to the mercuric antiseptics. On the other
hand, though fungi are known to be, in general, sensitive to mercuric preservatives,
no device has been proposed for use in the test for mycotic sterility of products
preserved with mercuric antiseptics, except that of Scheibel and Bentzon (1957).

In this situation, a study has been performed to directly follow up the inactivation
of the antiseptic in a sterility test medium.

For this study a method of measuring a minute amount of the antiseptic with
regard to its antiseptic activity is indispensable. The colorimetric method using dith-
izone solution in CC14 is inadequate for the purpose, not only because its sensitivity is
insufficient but also because it cannot detect the antiseptic activity.

Engley (1950) employed a paper disc method to compare mercuric antiseptics, but
it was only qualitative. More recently, Hara, et al. (1959) established a microbiological
assay method for organo-mercuric antiseptics, and proposed an analytical method for
determination of organo-mercuric antiseptics in biological products using the chemical
and microbiological assays collaterally. Their method overcame one of the weak points
of the chemical method, and has opened a way to directly pursue the antiseptic activity.
in media. But the sensitivity was much lower than that required for our purpose, the minimum concentration of the antiseptic detectable by their microbiological assay method being about 10 μg/cc.

The authors first looked for a method for determination of minute activity of the organo-mercuric preservatives using strains more sensitive to the mercuric antiseptics, and then, using the method established, followed up the antiseptic activity in some sterility test media under given experimental conditions.

The study is in progress and in the present paper the results obtained so far are presented.

MATERIALS AND METHODS

Organomercuric antiseptic: Sodium ethylmercurithiosalicylate (SEMT): thimerosal (Takeda, Japan) was used.

Ingredients for media: The followings were used, unless otherwise described.
- Peptones: Polypeptone (pancreatic digest of casein, Daigo, Japan) and Mikuni peptone for Toxin Production (peptic digest of meat, Mikuni, Japan).
- Water soluble extract of yeast (Oriental, Japan).
- Agar (granular, Hachiko, Japan).
- Thioglycollic acid (TGA), sodium thioglycollate (STG), L-cystine, resazurin (special grade, Wako-Junyaku, Japan).

All the above ingredients, except the meat digested peptone, meet our Minimum Requirement (1962).

Media: Fluid thioglycollate medium was prepared according to the Minimum Requirements of Biologic Products (1962).

Fluid Sabouraud medium was prepared by adding Polypeptone (5 g), Mikunipeptone (5 g), glucose (20 g) to 1,000 cc of distilled water. pH was not adjusted, unless otherwise described.

Sabouraud agar medium was prepared by adding agar to fluid Sabouraud medium, to make the gel strength 350 g/cm², for instance 1.5 % of Difco Agar or 1.2 % of Hachiko Agar.

Tube and amount of medium: The medium was distributed in 15 cc portions to tubes with an inner diameter of 20 mm and length of 150 mm (Minimum Requirements of Biologic Products, 1962).

Methods for measurement of the inhibition zone: In the majority of experiments, either or both of two systems, Ca. albicans (strain, 3147)-SAM system and Staphylococcus aureus (strain, 4496)-NAM system, was used, though the latter system was higher in sensitivity than the former. The former strain was obtained from the Department of Antibiotics of our Institute and the latter strain was isolated by the present authors from a contaminated biological product.

The procedures common to both the systems are as follows. The plate, on which the inhibition zone was to be formed, was prepared according to the Minimum Requirements of Antibiotic Products (1962). Twenty cc of the agar medium was poured into a Petri dish with an inside diameter of 900 mm. After the base layer had solidified, 4 cc of the mixture of the agar medium and a suspension of indicator cells was poured on each of the base layer to make the seed layer. The same agar medium was used for the base and seed layer in a pair. The sample to be tested was filled in a stainless-steel cylinder with an outside diameter of 8 mm (±0.1 mm), an inside diameter of 6 mm (±0.1 mm) and a length of 10 mm (±0.1 mm), which was placed on the seed layer 30 min. before the filling. Two cylinders were placed on each plate and 3 to 4 plates, in routine assay, were assigned for each dilution of one sample.

After the incubation specified below, the diameter of each zone of inhibition was measured twice, at right angles to each other, by a vernier calipers to the nearest 0.5 mm accurately.

1) Ca. albicans-Sabouraud agar medium (CA-SAM) system: The indicator strain grown on Sabouraud agar medium for 2 days at 25°C was suspended in saline at 1 mg/cc and 1.25 cc of the suspension was added to 100 cc of Sabouraud agar medium to make the seed layer. After filling a cup with a sample, the plate was incubated first for 1 day at 5°C and then for 2 days at 25°C.

In the present experiments Sabouraud agar medium was adjusted to pH 7.0, though there was little difference in the inhibition zone within the range of pH tested, from 6.0 to 7.0. The
above incubation method was proved to be best among the methods tested with regard to both
the sensitivity of the measurement and steepness of the log-dose response regression coefficient
(Ikuta, 1960a).

Though the cell concentration of the seed layer was not necessary to be kept strictly
constant, the lower the concentration, the larger the variance of the diameter.

2) *Staph. aureus*-nutrient agar medium (SA-NAM) system: Two-day culture at 31°C of
the indicator strain was suspended in saline at 1 mg/cc, and 0.31 cc of the suspension was added
to 100 cc of nutrient agar medium to make the seed layer. The plate was incubated for 2 days
at 31°C.

The minimum concentration of the antiseptic detectable was about 0.4 µg/cc by the CA-SAM
system and about 0.1 µg/cc by the SA-NAM system (Fig. 1). When *Ca. albicans* was combined
with nutrient agar medium, the minimum concentration detectable became 0.1–0.2 µg/cc, the
same order as with SA-NAM system.

Microbiological assay method and statistical analysis: Concentration of SEMT in a test
sample was estimated by the parallel line assay method (Finney, 1952). The reference solution
of SEMT was a 100 µg/cc of distilled water.

The parallelism of log-dose response regression line, its linearity, variance of the response,
homogeneity of the variance and so on were tested according to Finney (1952).

As shown in Fig. 1, in both the systems the log-dose inhibition-zone-diameter regression line
was linear over a rather wide range, while the inhibition zone less than 10 mm was apt to
deviate from the linearity. The regression coefficient was dependent on the medium, while
being independent of the strain, and varied from experiment to experiment within a range of
from about 15 to 20 (Table 4). Therefore, every trial must be designed so as to make it
possible to estimate the regression coefficient for each datum. The precision of the assay may
be figured out from the value of λ (Gaddum, 1933) in Table 1.

**Experiments and Results**

1. **Sensitivity of Various Species of Microorganisms to SEMT in Media**

Experiment 1. Sensitivity of various species of microorganisms to SEMT in fluid
thioglycollate medium without thioglycollate and in Sabouraud medium was determined
by measuring the maximum concentration of SEMT where the majority of micro-
organisms could grow. The experiment was carried out as follows.

Bacteria were cultivated on nutrient agar medium slant for 2 days at 37°C, except
psychrophilic strains L5, No. 22, No. 25 which were incubated at 25°C, and suspended in saline at the concentration of 1 mg/cc. *Ca. albicans* was cultivated on Sabouraud agar medium slant for 2 days at 25°C and the concentration of the suspension was the same as the bacteria. *Asp. oryzae* and *Pen. commune* were inoculated on Sabouraud agar medium slant and incubated first for 3 days at 25°C and then left at room temperature to form spores. The spores on the slant were washed off with saline to obtain a spore suspension. One tenth cc of $10^3$, $10^4$ and $10^6$ dilutions of the suspensions was inoculated in 6 cc of each of fluid thioglycollate medium containing SEMT at the concentration shown in the table. The number of cells inoculated was measured on plates simultaneously. As the concentration of the cell suspensions used for the inoculation varied very much among the strains, one tube was chosen from the three dilutions of each strain so that the growth might be compared among the strains with the tubes inoculated with similar number of cells, and the results of growth on the 7-day culture are tabulated in Table 1 and 2.

Table 1. Sensitivity of various species of microorganisms to sodium ethylthiosalicylate (SEMT) in fluid thioglycollate medium

<table>
<thead>
<tr>
<th>Strain</th>
<th>Number of cells inoculated</th>
<th>Temperature (°C)</th>
<th>Concentration of SEMT (µg/cc)</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pr. mirabilis</em> (OX-K)</td>
<td>45-50</td>
<td>37</td>
<td>6.25 3.13 1.56 0.78 0.39 0.195 0.098 0.049</td>
<td>++</td>
</tr>
<tr>
<td><em>Staph. aureus</em> (209P)</td>
<td>76-80</td>
<td>37</td>
<td></td>
<td>++</td>
</tr>
<tr>
<td><em>B. subtilis</em> (MY)</td>
<td>60-72</td>
<td>37</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td><em>Str. hemolyticus</em> (Animal C)</td>
<td>40-88</td>
<td>37</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>96-104</td>
<td>37</td>
<td></td>
<td>#</td>
</tr>
<tr>
<td><em>Staph. albus</em></td>
<td>116-144</td>
<td>37</td>
<td></td>
<td>#</td>
</tr>
<tr>
<td><em>Ps. pyocyanea</em> (A-3)</td>
<td>272-300</td>
<td>37</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>L 5 (G-B)*</td>
<td>96-98</td>
<td>25</td>
<td></td>
<td>#</td>
</tr>
<tr>
<td>No. 22 (G-B)*</td>
<td>350-360</td>
<td>25</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>No. 25 (G-B)*</td>
<td>130-134</td>
<td>25</td>
<td></td>
<td>#</td>
</tr>
<tr>
<td>No. 4996 (G+C)*</td>
<td>88-90</td>
<td>31</td>
<td></td>
<td>#</td>
</tr>
<tr>
<td>No. 5870 (G-B)*</td>
<td>?</td>
<td>37</td>
<td></td>
<td>#</td>
</tr>
<tr>
<td>No. 5868 (G-B)*</td>
<td>?</td>
<td>37</td>
<td></td>
<td>#</td>
</tr>
<tr>
<td><em>Ca.albicans</em> (3147)</td>
<td>44-46</td>
<td>25</td>
<td></td>
<td>#</td>
</tr>
<tr>
<td><em>Asp. oryzae</em></td>
<td>400-600</td>
<td>25</td>
<td></td>
<td>#</td>
</tr>
<tr>
<td><em>Pen. commune</em></td>
<td>400-500</td>
<td>25</td>
<td></td>
<td>#</td>
</tr>
</tbody>
</table>

* Isolated by the authors from contaminated biological products but the species have been identified.
  G+ : Gram positive
  G− : Gram negative
  B : Bacillary form
  C : Coccal form

** Obtained from Institute of Applied Microbiology, Tokyo University, Tokyo.

*** No growth was observed on the 7th day of incubation.

**** Growth was observed. Number of the symbol (+) shows grades of the turbidity.
As shown in Table 1, the majority of bacterial species tested grew in the media containing SEMT at the concentration of from 3 to 6 μg/cc, while part of bacterial species and all the species of fungi tested were found to grow first at the concentration of 0.1–0.2 μg/cc. Table 2 shows that the growth of one strain of Ca. albicans and two species of fungi in Sabouraud medium containing SEMT was the same as in fluid thioglycollate medium.

Table 2. Sensitivity of some fungi to SEMT in Sabouraud medium

<table>
<thead>
<tr>
<th>Strain</th>
<th>Number of cells inoculated</th>
<th>Temperature (°C)</th>
<th>Concentration of SEMT (μg/cc)</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca. albicans 3147</td>
<td>230–300</td>
<td>25</td>
<td>3.13 1.56 0.78 0.39 0.195 0.098 0.049 0.025</td>
<td>++</td>
</tr>
<tr>
<td>Asp. oryzae*</td>
<td>230–300</td>
<td>25</td>
<td>0.39 0.195 0.0975 0.0487 0.02437</td>
<td>+</td>
</tr>
<tr>
<td>Pen. commune*</td>
<td>200–300</td>
<td>25</td>
<td>0.39 0.195 0.0975 0.0487 0.02437</td>
<td>++</td>
</tr>
</tbody>
</table>

*, **, *** See Table 1.

The results in Table 3, where sensitivity to SEMT of several strains of Candida were compared in a similar way, show that growth of Candida was not always inhibited by SEMT at 0.2 μg/cc, but the majority of strains tested did not grow at this concentration.

Table 3. Sensitivity of various species of Candida to SEMT

<table>
<thead>
<tr>
<th>Strain</th>
<th>Concentration of SEMT (μg/cc)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.39</td>
</tr>
<tr>
<td>Ca. albicans</td>
<td>3147*</td>
</tr>
<tr>
<td>&quot;</td>
<td>YU*</td>
</tr>
<tr>
<td>&quot;</td>
<td>Toda*</td>
</tr>
<tr>
<td>&quot;</td>
<td>701**</td>
</tr>
<tr>
<td>Ca. tropicalis</td>
<td>7455*</td>
</tr>
<tr>
<td>Ca. pseudotropicalis</td>
<td>7494*</td>
</tr>
<tr>
<td>Ca. krusei</td>
<td>7492*</td>
</tr>
<tr>
<td>Ca. stellatoidea</td>
<td>&quot;</td>
</tr>
</tbody>
</table>

* Obtained from Department of Antibiotics, National Institute of Health, Tokyo.  
** Obtained from Institute for Infectious Diseases, Tokyo University, Tokyo.

2. Effects on the Microbiological Assay of the Ingredients in the Media

Experiment 2. To each of fluid thioglycollate medium containing neither thioglycollate nor L-cystine, Sabouraud medium, nutrient broth and distilled water, various concentrations of SEMT were added and their effects on the inhibition of growth of the indicator strain by SEMT were tested. As shown in Fig. 2, no ingredient contained in fluid thioglycollate medium, excepting thioglycollate and L-cystine, Sabouraud medium and nutrient broth showed effect upon the inhibition of growth by SEMT.
Fluid thioglycollate medium without thioglycollate and L-cystine.

* See Fig. 1.

Fig. 2: Effect of media containing no thioglycollate on the inhibition circle.

Experiment 3. Each of fluid thioglycollate medium, Sabouraud medium, nutrient broth and distilled water containing either TGA (400 µg/cc)* or STG (500 µg/cc)* and SEMT (6.25 µg/cc)** was diluted immediately after the addition of SEMT in distilled water and the diameter of the inhibition zone was measured, by both the systems. The results are shown in Fig. 3.

In fluid thioglycollate medium and distilled water, thioglycollate affected the inhibition and the diameter more or less decreased, while the log-dose response regression coefficient appeared not to be affected (Table 4). In Sabouraud medium and the nutrient broth there were little effect of thioglycollates on the inhibition by SEMT. There was little difference in the inhibition zone between TGA and STG.

In the following experiments, the concentration of SEMT in samples was estimated by either or both of the two systems of the microbiological assay method described under the METHOD.

3. Follow-up Studies on Inactivation of SEMT in Fluid Thioglycollate Medium

Experiment 4. SEMT was added to fluid thioglycollate medium either immediately after the preparation of medium or after a 7-day incubation at various temperatures, such as 5°C, 25°C and 37°C, and the activity of SEMT was pursued.

The inactivation of SEMT added in fluid thioglycollate medium containing STG (500 µg/cc) immediately after the preparation of the medium was followed up at 25°C, 31°C or 37°C, and the results were graphically summarized in Fig. 4. As clearly

* On the basis of our Minimum Requirements of Biologic Products (1962).
** According to the Requirement, 1.0 cc or 0.5 cc of a sample shall usually be added to 15 cc of fluid thioglycollate medium. In case where the sample contains SEMT at 100 µg/cc, when 1 cc of the sample is diluted in 15 cc of a medium, the concentration of SEMT in the medium becomes 6.25 µg/cc.
Fig. 3. Effect of media containing thiglycollate on the inhibition circle.

* See Fig. 1.  ** Sabourand medium.
*** Nutrient broth.

* See Fig. 1.  ** Fluid thiglycollate medium.
shown in the figure, the activity gradually decreased; the higher the incubation temperature and the lower the concentration of SEMT, the greater the tendency of inactivation of SEMT.

When SEMT was added in fluid thioglycollate medium incubated for 7 days at 5°C, 25°C or 37°C, the inactivation of SEMT was, in general, less than when SEMT was added immediately after the preparation of the medium, while the inactivation in fluid thioglycollate medium incubated at 5°C tended to be greater than that at the other two temperatures.

Anyhow, under the conditions specified in the Minimum Requirement, that is, when the initial concentration of SEMT was 6.25 μg/cc, the concentration of SEMT decreased down around the third day to such extent that the majority of bacterial species could tolerate. However, even on the 7th day, the concentration appeared too high to allow the growth of some bacterial species and the majority of fungi.

In a part of the experiments (for example, Fig. 4), when the initial concentration of SEMT was 1.56 μg/cc, the activity of SEMT decreased below the level where the species sensitive to mercuric preservatives could grow.

Experiment 5. Some of the ingredients in fluid thioglycollate medium considered to act upon the inactivation of SEMT in the sterility test media were studied.

1) STG and TGA: There was little difference in the inactivating capacity between STG (500 μg/cc) and TGA (400 μg/cc). With higher concentration, such as STG (2,000 μg/cc) and TGA (1,600 μg/cc), the inactivation of SEMT was demonstrable clearly; SEMT added at the initial concentration of 6.25 μg/cc did not show its activity on the 1st day at any incubation temperature.

2) Sodium hydrosulfite: Bonnel and Raby (1957) recommended sodium hydrosulfite for the sterility test media instead of thioglycollates. Sodium hydrosulfite inactivated certain preservatives (Requirements, 1960), especially “organomercuric-antiseptics” (Didenko, 1957; Romanov et al., 1959). One preparation of sodium hydrosulfite available in Japan (chemical use, Wako-Junyaku, Japan) was tested for the capacity in inactivating SEMT. No such capacity was found.

3) Agar: Agar seems to have an inhibitory effect on the inactivation of SEMT in fluid thioglycollate medium, when SEMT was added immediately after preparation of the medium. This effect was always observed both with STG and with TGA, and
Fig. 4. Effect of incubation temperature of the medium on inactivation of SEMT in fluid thioglycollate medium (STG 500 µg/cc).

Fig. 5. Effect of concentration of agar in the fluid thioglycollate medium (STG 500 µg/cc) on the inactivation of SEMT.

Fig. 6. Effect of incubation temperature of the medium on the inactivation of SEMT.
though not so much. The activity was larger with fluid thioglycollate medium containing L-cystine at 750 µg/cc than with fluid thioglycollate medium containing L-cystine at 500 µg/cc.

From the results in the above, the increase in concentration of STG or TGA seemed to be the most effective procedure. However, it must be taken into consideration that there are some bacterial species sensitive to STG at 2,000 µg/cc (Bonnel & Raby, 1957). At a concentration of STG, such as 2,000 µg/cc or above, the colour of resazurin became inadequate to show the oxidation reduction condition of the medium.

Experiment 6.

The Minimum Requirements of Biologic Products (1962) recommends the use of distilled water previously boiled to remove O₂. The meaning of this treatment was not described. Fluid thioglycollate medium prepared with distilled water previously boiled and kept at 60°C and fluid thioglycollate medium with distilled water without previous treatment were tested for the property of inactivating SEMT in each of the media and there was no difference in the activity between two kinds of media.

4. Follow-up Studies on Inactivation of SEMT in Sabouraud Medium

Experiment 7.

Sabouraud medium has been used for the test for fungi and yeast (Benkovic and Higy-Mandic, 1957; Scheibel and Bentzon, 1957; Requirements, 1960; and Public Health Service, 1962), and Scheibel and Bentzon (1957) recommended Sabouraud medium added with 0.05 per cent STG for products containing mercuric antiseptic.

SEMT was added to Sabouraud medium containing STG (500 µg/cc) immediately after the preparation of the medium, then the media were incubated at 25°C, 31°C or 37°C and the activity of SEMT was measured on the days indicated in Fig. 7. As shown in the figure, the result was similar to that with fluid thioglycollate medium, though the inactivation in Sabouraud medium was rather inferior to that in fluid thioglycollate medium. When strain 3147 was inoculated into Sabouraud medium containing SEMT at the initial concentration of 6.25 µg/cc, no growth was observed, coinciding with the results from the direct determination of the activity of SEMT in the medium by the microbiological assay method.

Experiment 8.

SEMT was added to Sabouraud medium containing STG (500 µg/cc) after incubation for 7 days at 25°C, 31°C or 37°C, and the activity of SEMT was measured periodically. In this case little inactivation of SEMT was demonstrated, showing that Sabouraud medium is very poor in preservation of the inactivating capacity of thioglycollate.

Experiment 9.

As the experiments with fluid thioglycollate medium, some ingredients used in fluid thioglycollate medium were tested for its property of inactivating SEMT in Sabouraud medium.

A slight capacity of L-cystine to inactivate SEMT and the protective effect of agar were observed also in Sabouraud medium. Sabouraud medium containing STG (500 µg/cc), L-cystine (750 µg/cc) and 0.075 % agar was divided into 4 parts. To one part, SEMT was added immediately after the preparation, and to the other three parts after the incubation for 7 days at 25°C, 31°C or 37°C. Then each medium was incubated at 37°C for measuring the activity of SEMT. Sabouraud medium containing both
Fig. 7. Effect of incubation temperature of the medium on inactivation of SEMT in Sabouraud agar medium (STG, 500 µg/cc).

STG and L-cystine but without agar was tested in the same way. In both cases of the interval between the preparation of medium and the addition of SEMT, Sabouraud medium without agar and incubated at a lower temperature showed higher activity in SEMT inactivation. However, the level where the SEMT was inactivated was much higher than the level where these strains sensitive to mercuric preservatives could grow.

Then, referring to the results with fluid thioglycollate medium, effect of higher concentrations of TGA or STG was tested. It was found that in Sabouraud medium containing thioglycollates at the concentration 4 times as high as specified by the Minimum Requirements of Biologic Products (1962) the inactivation of SEMT was very quick and sufficient, the growth of the strain 3147 inoculated into this medium was observed. However, when SEMT was added to the medium incubated for 7 days at 31°C after the preparation, little inactivation of SEMT was observed. Nevertheless, the growth of strain 3147 in the medium was observed, there being a contradiction between the results from the proposed microbiological assay method for the measurement of SEMT activity and the growth of the strain inoculated in the same medium.

Experiment 10.

Effect of pH on the inactivation of SEMT was observed. Each of Sabouraud medium containing STG (2,000 µg/cc) or TGA (1,600 µg/cc) was adjusted to pH=4.5, 5.5, 6.5 and 7.5 (pH after the sterilization was 4.4, 5.5, 6.1 and 7.2 in STG and 4.4, 5.5, 6.1 and 7.2 in TGA, respectively). Each of these was devided into 2 parts, and SEMT was added to one immediately after the preparation of the medium and to the other after the incubation for 7 days. The incubation temperature after the addition
Thioglycollates were added immediately after the preparation of medium. Thioglycollates were added 7-days at 25°C after the preparation of medium.

Fig. 8. Effect of pH of the medium on inactivation of SEMT in Sabourand medium (STG, 2000 μg/cc or TGA, 1600 μg/cc).

In this case, SEMT was inactivated quickly, when it was added to the medium immediately after the preparation. The inactivation was dependent on thioglycollates and pH. On the other hand, when SEMT was added to the medium after the incubation the inactivation was, in general, very little, and there was little difference in the inactivation between thioglycollates and among various values of pH, except pH 4.5 with TGA. Nevertheless, the growth of strain 3147 was demonstrated at pH 4.4 with STG and 4.5 and 5.3 with TGA.

The contradiction between the activity on the plate and the growth in the medium was observed again when SEMT was added after the incubation of the medium. This contradiction seems to appear at pH about 5 or above.

Experiment 11.

Next, by the use of Sabouraud medium containing 0.075 % agar the effect of pH on the inactivation was observed, and Fig. 9 was obtained. The inactivation of SEMT was similar between the two stages of SEMT addition, and dependent on pH as was the case in fluid thioglycollate medium, but the effect of pH was quite opposite to that in fluid thioglycollate medium.

All the concentrations of SEMT on the 3rd and 5th days were higher than 0.2 μg/cc, except that in the medium (pH 4.3). Nevertheless, the sufficient growth of strain 3147 was observed at any pH level (Table 5), without regard to the concentration of SEMT titrated on the plate.
Table 5. Effect of pH and some ingredients on growth of *Ca. albicans*, 3147 in Sabouraud medium

<table>
<thead>
<tr>
<th>Interval between the preparation of medium and the addition of SEMT</th>
<th>Dilution of cell suspension*</th>
<th>Agar (0%)</th>
<th>Agar (0.075%)</th>
<th>Agar (0%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>SEMT (6.25μg/cc)</td>
<td>TGA (1600μg/cc)</td>
<td>STG (2000μg/cc)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>pH</td>
<td>pH</td>
<td>pH</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4.5</td>
<td>5.3</td>
<td>6.1</td>
</tr>
<tr>
<td>Immediately after the preparation of the medium</td>
<td>12^</td>
<td>4**</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>10^</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>10^</td>
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<td>10^</td>
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</tbody>
</table>

* Twenty-four-hour culture in Sabouraud medium at 31°C was diluted ten-fold serially in saline. The inoculum was 0.1 cc per tube.

** Two tubes were used for each sample. Grades of the turbidity on the 7th day were represented by 3, 2, 1, 0.5 and 0 (no growth) and the sum of values of the two tubes was presented in the table.
Experiment 12.

Based on the above results, with media prepared under the conditions shown in Table 6, the growth of several representative strains of fungi was examined. As expected, in the media (pH was not adjusted) containing 0.075% agar and STG at the concentration of from 1,500 to 2,000 μg/cc, the growth of various species of fungi, without respect to the incubation of the media, was found to be fairly comparable to that in the control medium, to which SEMT was not added. The growth was better in the media without L-cystine.

Discussion

By selecting the indicator strain, a microbiological assay method was established, which made a minute amount of sodium ethylmercurithiosalicylate, such as 0.1 μg/cc, measurable with a fair accuracy. Under certain experimental conditions, the activity of SEMT titrated by this method did not always run parallel with the growth of some strains inoculated directly into the media, probably due to a loose combination of sodium ethylmercurithiosalicylate and thioglycollates.

The fact was demonstrated that the procedure, in which 1.0 cc or 0.5 cc of a sample containing sodium ethylmercurithiosalicylate at 100 μg/cc was inoculated into 15 cc of the fluid thioglycollate medium, was insufficient in that some bacterial strains sensitive to SEMT and the majority of fungi could not grow in this medium. It was suggested that by increasing the concentration of sodium thioglycollate in fluid thioglycollate medium to a range of 1,500 to 2,000 μg/cc, certain sodium ethylmercurithiosalicylate-sensitive strains grew, though this method was not considered adequate since such concentration of thioglycollate may be too high for certain thioglycollate-sensitive bacterial strains to grow (Bonnel & Raby, 1957).

The other ingredient or some conditions of preparation of the medium were investigated to get useful informations to modify the medium and the method of the test. The results suggested that only the increase of the amount of medium comparing to
Table 6. Concentration of thioglycollate in Sabouraud medium containing 0.075% agar and the growth of various species of fungi

<table>
<thead>
<tr>
<th>Interval between the preparation of medium and the addition of SEMT</th>
<th>Strain*</th>
<th>Number of cells inoculated</th>
<th>Sabouraud medium</th>
<th>Fluid thioglycollate medium</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>L-cystine (750 µg/cc)</td>
<td>L-cystine (0)</td>
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<tr>
<td></td>
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<td></td>
<td>SEMT (6.25 µg/cc)</td>
<td>STG (µg/cc)</td>
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<td></td>
<td></td>
<td></td>
<td>500 1000 1500 2000</td>
<td>500 1000 1500 2000 0</td>
</tr>
<tr>
<td>Immediately after the preparation of the medium</td>
<td>Ca. albicans 3147</td>
<td>50–60</td>
<td>0**</td>
<td>9/3</td>
</tr>
<tr>
<td></td>
<td>Ca. stellatoidea</td>
<td>65–75</td>
<td>3/1</td>
<td>9/3</td>
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<tr>
<td></td>
<td>Asp. oryzae</td>
<td>48–70</td>
<td>0</td>
<td>5/2</td>
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<td></td>
<td>Asp. niger ATCC 6275</td>
<td>54–64</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Pen. commune</td>
<td>70–84</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Rhiz. nigricans S. N. 32</td>
<td>54–65</td>
<td>3/1</td>
<td>8/3</td>
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<tr>
<td></td>
<td>Clad. herbarum IAMF 517</td>
<td>32–50</td>
<td>0</td>
<td>0</td>
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<tr>
<td></td>
<td>Chaet. globosum ATCC 9849</td>
<td>40–55</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Fus. moniliforme USDA 1004. 1</td>
<td>41–60</td>
<td>0</td>
<td>8/4</td>
</tr>
<tr>
<td>After the incubation for 7 days at 25°C</td>
<td>Ca. albicans 3147</td>
<td>50–60</td>
<td>3/2</td>
<td>11/4</td>
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<tr>
<td></td>
<td>Ca. stellatoidea</td>
<td>65–75</td>
<td>2/2</td>
<td>12/4</td>
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<td>Asp. oryzae</td>
<td>48–70</td>
<td>0</td>
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<td>Asp. niger ATCC 6275</td>
<td>54–64</td>
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<td>70–84</td>
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<td>Rhiz. nigricans S. N. 32</td>
<td>54–65</td>
<td>0</td>
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<tr>
<td></td>
<td>Fus. moniliforme USDA 1004. 1</td>
<td>41–60</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Obtained from Institute of Applied Microbiology, Tokyo University, Tokyo, except Ca. albicans.

** Four tubes were used for each of the combination of strain and medium. Grades of the turbidity and the value given to each symbol was the same as in Table 4. The numerator in the table is the sum of the four values, and the denominator is the number of tubes showing growth on the 7th day.
the inoculating amount of a sample was expected to improve the above-mentioned drawback of the present method, if it is considered necessary.

As the medium for mycotic sterility Sabouraud medium was investigated too. A method by which thioglycollate is added into Sabouraud medium has been employed in certain laboratories. The present experiments showed that a sufficient concentration of thioglycollates made the growth of the majority of fungi possible even in the ordinary ratio of the amount of medium to that of the sample to be inoculated. In this case, agar and pH were found to have a certain important role. Its meaning has not yet been clarified.

Thus, as far as the test for mycotic sterility is concerned, we could have a useful method by addition of a sufficient amount of thioglycollates and agar to Sobouraud medium.

**SUMMARY**

It was suggested that the fluid thioglycollate medium was insufficient for the test for mycotic sterility of biological products containing mercuric preservatives.

Employing a microbiological assay method for the antiseptic activity of sodium ethylmercurithiosalicylate, the inactivation of the antiseptic in the sterility test media was followed up. And some ingredients used in the sterility test media were tested for the effect of antiseptic on the inactivation. Though the proposed assay method in which the statistical method was applied and some strains highly sensitive to the mercuric antiseptics were used, was very sensitive and sufficiently accurate, it was found that there was a certain limit of availability.

From the results obtained in a number of experiments, the medium of choice for mycotic sterility test was a Sabouraud medium added with thioglycollate at a relatively high concentration and agar. The agar might not be an essential factor, but seemed to minimize the effect of pH on the inactivation of the antiseptic in the medium and to act on the medium to keep its inactivating activity.

The authors wish to thank Dr. H. Iizuka, Institute of Applied Microbiology, Tokyo University, Tokyo, and Dr. Y. Okami, Department of Antibiotics of this Institute for their kind supply of the strains. Thanks are due to Mr. S. Ishida for the statistical analysis.

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