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The recovery of bacteria from white soft paraffin

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Methods have been investigated for isolating and enumerating, by a membrane filtration technique, *E. coli* inoculated into white soft paraffin. Of three solvents used to prepare the ointment base for filtration, the best recovery (54%) was obtained with light liquid paraffin. Superior recovery levels (*ca* 75%) were obtained by homogenizing the ointment base with a Tween 80/peptone water mixture. Recovery levels were greatly affected by the weight of ointment base taken but the Tween 80/peptone water system permitted the estimation of the contamination in up to 0·25 g of white soft paraffin.

Tsuji, Stapert & others (1970) described the use of isopropyl myristate (IPM) as a solvent for recovering viable bacteria from eye ointments but there was evidence that the IPM became toxic if it was heat sterilized. This has been confirmed by Bowman, Knoll & others (1972), who also showed that the toxicity of IPM from different sources varied.

The unheated solvent may still be substantially toxic (Tsuji & others, 1970; Bowman, personal communication; Diding, personal communication). Tsuji & others (1970), reported that IPM exerted a lethal effect even against *Bacillus subtilis* spores though we were able to obtain complete recovery of *Bacillus megaterium* spores from white soft paraffin using IPM as the solvent (Hambleton & Allwood, 1972).

Other solvents used to prepare ointments for filtration are n-hexane (White, Bowman & Kirschbaum (1968) and light liquid paraffin British Pharmacopoeia (1968).

Aqueous non-miscible systems such as liquid growth medium (Woodward & McNamara, 1971) or mixtures of peptone water with non-ionic surfactant (Pederson & Szabo, 1968; Millipore Corporation, 1969) have also been used.

We have examined the efficiency of various isolation methods for the recovery of *Escherichia coli* from white soft paraffin.

MATERIALS AND METHODS

Preparation of inoculated ointment base. *E. coli* NCTC 9001 was grown for 18 h at 37° in nutrient broth (Oxoid Ltd., London, U.K.) supplemented with 0·5% w/v yeast extract. The culture was diluted in sterile water and 0·1 ml of a suitable dilution inoculated into 5 g of heat sterilized soft paraffin. The inoculated ointment base was mixed thoroughly with a sterile spatula to ensure an even distribution of cells of about 2×10^4 g^{-1}.

Solvent and dispersal systems. IPM was obtained from Schückardt, Munich, W. Germany and from Givaudan Ltd., Whyteleafe, Surrey. Light liquid paraffin was B.P.

These solvents were sterilized by filtration. Light liquid paraffin B.P., heat sterilized at 160° for 1 h was also tested.

The aqueous dispersal system used consisted of a 1·0% w/v solution of Tween 80 (Honeywill Atlas and Co., Carshalton, Surrey) in peptone water. This mixture was sterilized by autoclaving (Hambleton & Allwood, 1972).

Typical protocol

A known weight of the inoculated white soft paraffin was transferred to 10 ml of solvent or dispersing agent previously equilibrated at 42° and blended at high speed for 1 min with the aid of a homogenizer. Triplicate 1 ml samples of the resultant mixture were then added to 100 ml of a sterile filtration vehicle warmed to 42° (Hambleton & Allwood, 1972) and the mixture filtered through a 0·45 μm pore size membrane filter (Millipore type HAWP).

The membrane filter was rinsed with 100 ml of peptone water (also at 42°) and the membrane transferred for incubation to a Petri dish containing an absorbent pad moistened with nutrient broth supplemented with 0·5% w/v yeast extract. Viable counts were determined after 24 h incubation at 37°. Samples of ointment base dissolved in n-hexane and IPM or which had been homogenized in Tween 80/peptone mixture, filtered easily under negative pressure but samples in light liquid paraffin could be filtered and the membranes subsequently rinsed only under a positive pressure of 30–40 p.s.i.; a Millipore type XX40 047 filter holder was employed. The time between initial inoculation of the soft paraffin and completion of filtration was 3–4 min.

Both negative and positive filtration were slow if the temperature was below 40° and so solutions and homogenates were warmed to 42° before filtration, there was no loss of viability of *E. coli* cells suspended in water for 2 h at this temperature.

All viable counts were compared with a control count made on an aqueous dilution of organisms prepared at the same time as the inoculated ointment.

Toxicity of solvent and dispersal systems. 15 ml of each system equilibrated at 42°, was inoculated with 0·15 ml of a suitable aqueous dilution of *E. coli* culture and homogenized for 1 min. Samples (1 ml) were taken at intervals during storage of the homogenate and the viable count obtained as described above.

RESULTS

The recovery of viable *E. coli* from inoculated white soft paraffin varied greatly with the system used to prepare the ointment for filtration. Only the light liquid paraffin and the Tween 80/peptone system gave reasonably good recoveries; both n-hexane and IPM proved toxic (Table 1). The low recovery with n-hexane was not unexpected since previous findings had shown it to be toxic to *B. megaterium* spores

Table 1. *Recovery of* E. coli *from soft paraffin using different solvent/dispersal systems.*

Solvent/dispersal system	Mean % recovery from 0·025 g soft paraffin	Standard deviation
Isopropyl myristate[a]	5	—
n-Hexane	5	—
Light liquid paraffin B.P. ..	54	7·5
1·0% w/v Tween 80 in peptone water	74	6·4

[a] Recovery level with two samples of isopropyl myristate.

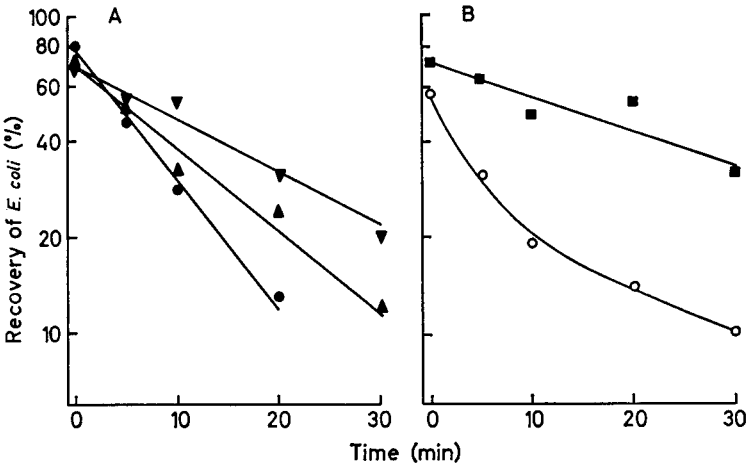

FIG. 1. (A) Inactivation of *E. coli* in isopropyl myristate (Schückardt) ▼—▼; isopropyl myristate (Givaudan) ▲—▲; and light liquid paraffin B.P., ●—●. (B) Inactivation of *E. coli* in soft paraffin ■—■, and soft paraffin dissolved in light liquid paraffin B.P. ○—○.

(Hambleton & Allwood, 1972). However, the low recovery with IPM contrasted with the results of Tsuji & others (1970) who reported a recovery of 49% of *E. coli* with this solvent.

The Tween 80/peptone system was non-toxic but both IPM and light liquid paraffin B.P. progressively inactivated *E. coli* cells at 42°. Fig. 1A shows that the rates of inactivation of *E. coli* in the two samples of IPM differed, although the variation was small during the initial 3–4 min sampling period.

The toxicity of unheated light liquid paraffin was slightly higher than that of IPM even though there was a higher recovery of *E. coli* with light liquid paraffin than with IPM. The toxicity of light liquid paraffin was greatly increased by heat sterilization. Less than 5% of cells inoculated into the soft paraffin were recovered when heated oil was used and its toxicity was such that cells inoculated into it were inactivated in about 10 min.

That white soft paraffin inactivated added *E. coli* was investigated by sampling at intervals during storage at 42°. Each sample (100 mg) was homogenized in the Tween 80/peptone water system before viable counting. The results (Fig. 1B) show that the percent recovery fell from 70 to 32% over a 30 min period.

That the low recovery (about 50%) of *E. coli* obtained with unheated light liquid paraffin might be due to a toxic action of the mixture was investigated by taking repeated samples of a homogenate of inoculated ointment base in light liquid paraffin stored at 42°. The results (Fig. 1B) show that over an initial 10 min period, the viable count fell at about the same rate in the homogenate as in the solvent oil alone (Fig. 1A). This indicates that the combination did not enhance the toxicity of the solvent.

The possibility that retention on the filters might be the cause of the low recovery was investigated by filtering increasing weights of inoculated soft paraffin, dissolved in liquid paraffin (the same number of organisms was used for each amount). Table 2 shows that recovery fell when weights of soft paraffin in excess of 50 mg (in 1 ml of oil) were filtered. Furthermore, it became increasingly difficult to filter

Table 2. *Effect of sample weight upon recovery of* E. coli *from soft paraffin.*

Solvent/dispersal system	% recovery of *E. coli* per weight of soft paraffin applied to filter					
	0·01 g	0·025 g	0·05 g	0·1 g	0·25 g	0·5 g
Light liquid paraffin ..	64	65	58	29	27	nil
1·0% w/v Tween 80 in Peptone water	75	75	85	65	84	nil

amounts in excess of 250 mg which only filter easily if dissolved in about 50 ml of light liquid paraffin.

The effect of increasing soft paraffin weight was less marked when the Tween 80/peptone water system was used. In this system the *E. coli* cells were released into the aqueous phase from the fatty base during homogenisation. Only a small proportion of the base remained suspended in the aqueous phase. When the proportion of base to Tween 80/peptone water exceeded 2·5% w/v, filtration of the aqueous phase was hindered because of the amount of finely divided base remaining suspended, thus blocking the filter.

DISCUSSION

The results indicate that a greater proportion of cells of *E. coli* inoculated into white soft paraffin may be recovered by homogenizing the base in an aqueous dispersal system and then filtering the aqueous phase than by direct filtration of the dissolved ointment. The use of a Tween 80/peptone water system also provided greater reproducibility of recovery than was obtained when the same ointment was dissolved in light liquid paraffin B.P. In contrast to the findings of Tsuji & others (1970) we found that IPM, even when unheated, was unsuitable as a solvent for recovering *E. coli* from soft paraffin. The combination of unheated light liquid paraffin with ointment base did not show the toxic effect found with IPM but heat sterilized light liquid paraffin is toxic to bacteria (Allwood & Hambleton, 1972).

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