Technical Methods

Feeley (1964) except that the inoculum (500 colony-forming units of *V. cholerae* 12R) was injected into the duodenum exposed through a right subcostal incision. One animal was killed with ether when it developed diarrhoea 20 hours later. The watery fluid in its caecum was mixed with an equal volume of normal rabbit serum. The mixture was frozen after adding glycerol.

RESULTS AND CONCLUSIONS

The results of three consecutive experiments are presented in the table. It will be observed that there was usually a slight increase in viable count after freezing and thawing, presumably due to disaggregation of clumps; the contents of tubes processed simultaneously had comparable viable counts; and the addition of a freezing protectant was unnecessary.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Preservative</th>
<th>Count (Million Colony-Forming Units)</th>
<th>Before Freezing</th>
<th>After Freezing</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>V. cholerae</em> 12R</td>
<td>Nil</td>
<td>16-9</td>
<td>19-21</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Glycerol</td>
<td>21-5</td>
<td>21, 24</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dimethyl-sulphoxide</td>
<td>16, 16</td>
<td>18-1, 23-8</td>
<td></td>
</tr>
<tr>
<td><em>V. cholerae</em> 12R</td>
<td>Glycerol</td>
<td>18-4, 15-5, 18-6</td>
<td>18-1, 17, 16-5</td>
<td></td>
</tr>
<tr>
<td><em>V. eltor</em></td>
<td>Nil</td>
<td>5-5, 4-4, 4-5</td>
<td>4-2, 5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Glycerol</td>
<td>4-4, 4-5</td>
<td>5-2, 5-5, 6-1</td>
<td></td>
</tr>
</tbody>
</table>

1 Each count represents one tube, corrected for dilution by preservative. 
2 Counts after five months: 17-1, 14-1.

The count in the diarrhoea fluid was reduced from 1-3 million colony-forming units per milligram of the mixture with serum and glycerol to 0-4 million colony-forming units.

It thus seems practicable to store at low temperatures moderately dense suspensions of vibrio cultures without cell death. Perhaps even cholera stools can be preserved directly; it would be of interest to investigate various freezing protectants for this purpose.

We are indebted to Professor R. Cruickshank for his help and advice during the experiments and the preparation of the manuscript, and for providing the senior author with a research grant.

REFERENCES


Letter to the Editor

PREPARATION OF BILIRUBIN STANDARDS

Dear Sir,

The instability of bilirubin in strong alkali led to search for an organic solvent, miscible with water, which could be used to prepare a reliable bilirubin standard for routine use.

It was found that bilirubin is freely soluble in dimethyl-sulphoxide which is water-miscible and in which serum proteins are easily dispersed. A solution of bilirubin in this solvent shows an absorption peak at 453 nm with a curve shape similar to that of a chloroform solution.

The stability in this solvent, whilst not approaching that in non-polar solvents, is better than that in aqueous alkali. No change in optical density was noted within 15 minutes of dissolution, and the fall in optical density after 30 minutes in a 0-01 mM solution approximated a 0-25 µM/hour. This indicated that although a simple solution in dimethylsulphoxide would not be adequately stable for use as a standard, the solvent could be used to prepare a stable standard in aqueous albumin.

The diluent used is 5% bovine albumin in water buffered to pH 7-4 by phosphate buffer. A given weight of bilirubin of molar extinction 60,700 ± 800 is dissolved in 5 ml. of dimethylsulphoxide. When dissolution is complete this solution is made to 100 ml. with the diluent.

This standard is checked spectrophotometrically by adding 0-2 ml. to 3-8 ml. of dimethylsulphoxide, mixing and reading at 453 nm. A blank is prepared by adding 0-2 ml. of diluent to 3-8 ml. dimethylsulphoxide. The optical density is multiplied by 19-26 (based on E 60,700) to give the bilirubin concentration of the standard in mg./100 ml. The standard is then dispersed in small quantities for storage by deep freezing. The stability of the standard prepared in this way appears to be better than 30 days at −20°C.

The ability of dimethylsulphoxide to disperse serum proteins suggested the possibility of estimating bilirubin directly at 453 nm. As shown by Heilmeyer (1943), other coloured components in serum, principally haemoglobin and carotenoids, interfere considerably. It was noted that the addition of one drop of 100-volume hydrogen peroxide to the test as described above gave a 60% reduction in the non-bilirubin colour whilst reducing the optical density of a 3-5 mg % bilirubin solution by only 7%. Although obviously unsuitable for a true quantitative estimation, this technique may well prove of value in the rapid estimation of raised bilirubin levels in unhaemolysed sera.

K. G. GADDY

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