The aesthetic advantage of the semisynthetic medium is that production of aromatic amines is minimal.

The semisynthetic medium devised promises to overcome some of the difficulties inherent in the present methods of phage typing *P. aeruginosa* and to provide a reproducible medium by which the typing procedure can be better standardized.

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**EFFECT OF CARBON DIOXIDE CONCENTRATION ON ABILITY OF COXSACKIE A-21 TO FORM PLAQUES**

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Coxsackie A-21 or Coe virus produces characteristic cytopathic effects in several human and animal cell lines. In most cells, the effect is insufficient for a plaque assay. Underwood (Am. J. Hyg. 76:124, 1962) showed that, in HeLa cells, the virus grew rapidly and produced high titers. He developed a plaque assay using a monolayer of HeLa cells grown in small bottles and overlaid with a gel of chicken plasma. HeLa cells have also been used in this laboratory to produce plaques with Coe virus by use of the Cooper (Virology, 1:397, 1955) plate technique.

For the purpose of routine laboratory investigation, the first system was considered too laborious and the second system too demanding on cells. Experience indicated that an assay based on the use of HeLa cells was not advisable due to frequent changes in the cell population. With these factors in mind, an assay was devised based on the cytopathic effects of Coe virus in a stable mouse liver cell line.

Coxsackie A-21 virus was obtained from two sources. One sample was received from D. A. J. Tyrrell of the Medical Research Council Common Cold Research Unit, Salisbury, Wilts, England, and another from E. H. Lennette, California State Department of Public Health, Berkeley. Each virus was passed twice in primary human amnion to provide a virus pool.

A continuous mouse liver cell line (ML) isolated from cultures of embryonic mouse liver and carried in this laboratory for over 200 transfers was used in these studies. This cell line has a marked susceptibility to Coe and other viruses and is sustained well under agar.

For use in the plaque assay and for other culture conditions, the cells were released from the glass by 0.25% trypsin, washed twice in Hanks’ balanced salt solution (BSS) by repeated sedimentation at low-speed centrifugation (150 × g for 20 min), and resuspended at 4 × 10^5 cells per ml in a medium of 0.5% lactalbumin, 10% inactivated calf serum, 79.5% Hanks’ BSS, 100 units per ml of penicillin, and 100 μg/ml of streptomycin. Plates or flasks were plated with 5 ml per 60-mm petri plate or 15 ml per T60 flask. Cell sheets were adequate for use after 48 hr of incubation at 37°C.

For plaque assay, confluent sheets of ML cells in 60-mm petri plates were washed with 5 ml of Hanks’ BSS, 0.5 ml of virus dilution was added, and attachment of virus to cells was allowed to proceed for 1 hr at 37°C in a humidified 8% CO₂-gassed incubator. (CO₂ concentration varied from 8 to 10%.) After attachment, 5 ml of overlay medium were added. The overlay medium consisted of Medium 199 containing Eagle’s vitamin, amino acid, and glutamine mixtures, 1.1% Noble’s agar, 100 units per ml of penicillin, and 100 μg/ml of streptomycin. After gelling of the agar, the plates were incubated at 37°C at various concentrations of CO₂ in air, and plaque counts were made at 48 hr.

During preliminary attempts to grow Coe virus in various cell lines, it was observed that in both HeLa and ML cells typical cytopathic changes due to virus took place in cultures grown in rubber-stoppered T60 flasks, but no changes occurred when infected T60 flask cultures were fitted with cotton plugs and incubated in our humidified, 8% CO₂-gassed incubators. Infected ML cultures in T60 flasks, when overlaid with agar, produced plaques only when rubber stoppered. Incubation of infected T60 cultures in humidified, 8% CO₂-
gassed incubators with loose-fitting cotton stoppers did not show plaque formation.

Since the only obvious difference between cultures was the concentration of CO₂, this variable was studied in more detail. Cultures of ML cells in 60-mm petri plates were infected, overlaid with agar, and incubated at 0, 2, 4, 5, 8, and 8 to 10% CO₂ concentrations in air for 48 hr.

With CO₂ absent, the production of minute plaques involving usually less than 10 to 15 cells was observed. The cell sheets readily took up neutral red and appeared viable. At 2% CO₂, 1- to 2-mm clear plaques were easily discernible against the deeply stained cell sheet (Fig. 1A). With increased CO₂, from 4 to 5%, the plaques reached a maximal size of 4 to 8 mm in diameter (Fig. 1B). Cultures gassed at 8% CO₂ showed plaques approximately 4 mm in diameter, (Fig. 1C). A slight increase in CO₂ concentration above 8% resulted in plaques of extremely small size, usually
involving fewer cells than observed in nongassed cultures. On several occasions, large plaques were obtained at 8% CO₂; however, on further investigation the tanks were found to contain substantially less than 8% CO₂.

The property of plaque formation by Coe virus in cultures of ML cells under 5% CO₂ has been obtained in this laboratory for the past year to assess viral concentrations in cell culture fluid, extracts of tissues infected with Coe virus, and nasal washings of infected human volunteers. The results have been equivalent to and more reliable than the usual TCID₅₀ determinations in roller-drum cultures. In contrast to the stable ML cell line, our HeLa cell culture produced 5- to 8-mm plaques (Fig. 1D) at the start of this investigation; after 22 serial passages, the culture would not produce plaques of any size. Recovery of the original HeLa line, frozen at the start of these experiments, showed that the property of plaque support had been retained. On two other occasions, it was observed that HeLa cells failed to support plaque formation by Coe virus.

The ML cell line under 5% CO₂ has been found to be superior to HeLa and other cell lines in the support of Coe virus and several group B Coxsackie viruses, affording a uniform system for recovery of virus from animal tissues and assessment of viral titers.

## LIVER NONPROTEIN SULFHYDRYL OF ENDOTOXIN-TREATED MICE

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The importance of tissue nonprotein sulfhydryl (NPSH), of which glutathione constitutes at least 90% (Jocelyn, Clin. Chim. Acta 3:401, 1958), is demonstrated by the role of glutathione as a cofactor of phosphoglyceraldehyde dehydrogenase (Barron, Advan. Enzymol. 11:201, 1951) and as a substrate in the detoxification of halogenated aromatic compounds (Booth et al., Biochem. J. 79: 516, 1961). The level of the liver NPSH has been shown to decrease after stress (Beck and Linkenheimer, Proc. Soc. Exptl. Biol. Med. 81:291, 1952) and injection of adrenaline (Bradley et al., J. Neuropsychiat. 2:175, 1961). Beck and Linkenheimer (Proc. Soc. Exptl. Biol. Med. 81:291, 1952) reported the NPSH in the livers of mice was markedly decreased 18 hr after an injection of endotoxin prepared from Serratia marcescens, but reported no observations at earlier times. The present work indicates that liver NPSH decreases as early as 2 hr after administration of endotoxin to mice.

Male ICR mice (20 to 25 g) from Rawley Farms, Plymouth, Mich., were maintained at the laboratory for at least 1 week before use. The mice were injected intraperitoneally with 100 µg (1.33 LD₅₀ doses) of endotoxin prepared from *Salmonella enteritidis* CDC 6001-59 by the method of Ribi et al. (J. Immunol. 82:75, 1959). After 1, 2, 4, and 18 hr, the mice were killed by cervical dislocation and the livers were excised and placed on crushed ice. Portions of liver from each mouse of a group were weighed rapidly on a Roller-Smith balance, pooled, and homogenized in 10 volumes of 0.25 M mannitol with a Potter-Elvehjem tissue homogenizer (Tri-R Instruments). The protein was precipitated by the addition of 0.25 volume of 20% sulfosalicylic acid, and the mixture was centrifuged at 10,000 × g for 15 min at 4 C. The NPSH in the supernatant fluid was determined

### Table 1. Liver nonprotein sulfhydryl (NPSH) of the mouse administered *Salmonella enteritidis* endotoxin

<table>
<thead>
<tr>
<th>Time after injection</th>
<th>Test animal</th>
<th>No. of determinations</th>
<th>NPSH (mole/kg ± SD × 10⁻⁶)</th>
<th>p*</th>
<th>Percentage of normal</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 hr</td>
<td>Intoxicated</td>
<td>6</td>
<td>7.61 ± 0.94</td>
<td>ns†</td>
<td>93.8</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>6</td>
<td>8.21 ± 0.74</td>
<td>.01</td>
<td>76.1</td>
</tr>
<tr>
<td>2 hr</td>
<td>Intoxicated</td>
<td>8</td>
<td>5.27 ± 0.88</td>
<td>&lt;.001</td>
<td>62.5</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>8</td>
<td>6.91 ± 0.62</td>
<td>.94</td>
<td>42.1</td>
</tr>
<tr>
<td>4 hr</td>
<td>Intoxicated</td>
<td>6</td>
<td>4.85 ± 0.51</td>
<td>&lt;.001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>6</td>
<td>7.76 ± 0.54</td>
<td>.01</td>
<td></td>
</tr>
<tr>
<td>18 hr</td>
<td>Intoxicated</td>
<td>6</td>
<td>3.29 ± 0.36</td>
<td>&lt;.001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>6</td>
<td>7.84 ± 0.63</td>
<td>.25</td>
<td></td>
</tr>
</tbody>
</table>

* Determined by Student’s t test.
† Mean of values calculated for paired determinations.
‡ Not significant.