The Effect of D₂O on the Growth and Transforming Activities of
Streptococcus pneumoniae

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After an initial period of growth in medium made up in D₂O, most strains of pneumococcus
tested dramatically lost viability, the extent of the loss depending on the strain and on the
amount of contaminating H₂O in the D₂O. This was followed by a recovery period. Once a
strain was ‘adapted’, the ability to grow in D₂O-medium without cell death was inherited, even
after passage through H₂O-medium, indicating the selection of mutants. Cultures that had not
reached ‘full adaptation’ also exhibited cell death if transferred into either D₂O-medium or
H₂O-medium, supporting the conclusion that the presence of hydrogen and deuterium together
cause the toxicity.

‘Adapted’ cells exhibited an increased mutation frequency to a variety of antibiotic
resistances, the propensity for this appearing in the death phase of ‘adaptation’. The specific
transforming activity of DNA preparations from cultures undergoing ‘adaptation’ decreased
before DNA synthesis ceased indicating damage to the DNA. The integration efficiency of a
low-efficiency marker also dropped during ‘adaptation’ before returning to the initial value
when measured in a Hex⁻ recipient, but remained constant in a Hex⁺ recipient, suggesting that
the Hex system may be involved in repair of the DNA damage. ‘Adapted’ organisms showed
evidence of possessing higher Hex activity and were also able to repair lesions caused by UV-
irradiation better than the wild-type.

INTRODUCTION

It has been reported that numerous species of bacteria have been successfully cultured in
media made up in 100% D₂O with deuterated ingredients, and a number of studies have also
shown that growth in deuterated medium was mutagenic for Escherichia coli (De Giovanni,
1960, 1961; De Giovanni & Zamenhof, 1963), for Proteus mirabilis (Jung, 1968), for Salmonella
typhimurium (De Giovanni, 1961), and for the T4 bacteriophage (Konrad, 1960).

Cultures of Streptococcus pneumoniae which had been incubated in media which contained
either D₂O and/or nutrients which were partially substituted with deuterium have been used in a
number of previous studies (Lester et al., 1960; Martin & Ephrussi-Taylor, 1964; Firshein &
Schwenfeier, 1969; Butler & Smiley, 1973). Butler & Smiley (1973) used such cultures for
mapping the position of genetic markers by the density-shift method, and in the course of re-
assessing the suitability of using such pneumococcal cultures for this purpose the factors
involved in ‘adaptation’ were investigated. This paper reports on the nature of the ‘adaptation’
process and its effects on mechanisms related to the genetics of the resulting ‘fully-adapted’
cultures.

METHODS

Bacterial strains. Table 1 shows the strains of Streptococcus pneumoniae used. The relationship between various
wild-type laboratory strains has been summarized by Tiraby et al. (1975). Stock cultures were maintained as
previously described (Butler, 1965; Butler & Smiley, 1970). Subcultures from H₂O-medium were diluted 1 in 100 or
1 in 1000 into D₂O-medium.

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theses) with the date that they were frozen for storage in order to distinguish between them.

For the study of the \( D_1O \)-medium shown by the different derivatives of strain C13, they are labelled (in parentheses).

- \( \text{ampicillin} \) confers resistance to tetracycline (I)
- \( \text{macrolide} \) confers resistance to erythromycin (\( \text{I} \))
- \( \text{apomycin} \) confers resistance to optochin (\( \text{I} \))
- \( \text{tetracycline} \) confers resistance to tetracycline (\( \text{I} \))
- \( \text{streptomycin} \) confers resistance to streptomycin (\( \text{I} \))

Medium. The peptone medium 'P' was prepared as described by Sicard (1964) and Butler (1965). Deuterated 'P' medium (\( D_2O \)-medium) was prepared by replacing the glass-distilled water by \( D_2O \) (Koch-Light) containing 99.7 atom\(^{-1}\) \( D \). All solutions added to the basic 'P' medium were also prepared in \( D_2O \) but the pH was adjusted to 7.6 by \( 0.5 \text{M NaOH} \) dissolved in \( D_2O \). Defibrinated horse blood, normally present in stock culture medium, was omitted.

Assay of the mutation frequency. Mutants were scored directly on agar plates supplemented with an appropriate concentration of antibiotic. Preliminary experiments showed that the average chain length of cultures incubating in \( D_2O \)-medium remained in the range 1.5 to 2.0 for both 'adapting' and 'adapted' cultures. For this reason, the c.f.u. count was not corrected for the average chain length.

Measurements of transforming activity and of the integration efficiencies of markers. The general transforming procedure was essentially as described by Butler & Nicholas (1973) except that M1 medium (Moynet, 1976) supplemented with 0.125 \( \mu \text{g} \) (w/v) bovine serum albumin (Armour Pharmaceuticals) and 0.1% (w/v) charcoal-absorbed Difco yeast extract replaced the NS medium (Grist & Butler, 1981).

The specific transforming activity of a crude lysate was measured by relating the number of transformants obtained to the amount of DNA present quantified by the \( ^{14} \text{C} \)-count due to the incorporation of \( ^{14} \text{C} \)thymidine. A culture of the strain which carried the \( str-r41 \) marker was first subcultured in \( H_2O \)-medium labelled with \( [2^{14} \text{C}] \)thymidine (60 mCi mmol\(^{-1}\) 2.2 GBq mmol\(^{-1}\)) at 0.5 \( \mu \text{Ci ml}^{-1} \) before transferring it into \( H_2O \) or \( D_2O \)-medium also containing \( [2^{14} \text{C}] \)thymidine. During the subsequent incubation at 37 °C, samples were periodically removed and assayed for the c.f.u. count, for the \( ^{14} \text{C} \)-count, and for the transforming ability of lysates. The relative transforming activity of a sample was then obtained by dividing the number of \( str-r41 \) transformants by the \( ^{14} \text{C} \)-count of the sample.

The integration efficiency of a marker was measured relative to the \( str-r41 \) marker present on the same chromosome using lysates as donor DNA. When necessary the \( str-r41 \) marker was first introduced into the strain by transformation. The number of transformants which carried the mutant marker relative to the number of \( str-r41 \) transformants was determined. Lysates were prepared from cultures concentrated 5× after washing and resuspending in chilled sodium citrate saline and lysed with sodium deoxycholate.

UV-irradiation. Early exponential growth phase cultures in \( H_2O \)-medium were centrifuged, washed and diluted to 10 times their original volume in the mineral part of the synthetic medium of Sicard (1964). Samples of 10 ml were then irradiated in open 9 cm Petri dishes at a distance of 82 cm from a 30 W TUV Philips germicidal lamp giving an incident dose rate of 0.58 mJ \( \text{m}^{-2} \text{s}^{-1} \) as measured by a model J-225 ultraviolet meter (Ultra-violet Products Inc., kindly lent by Dr R. Pinney, London School of Pharmacy) and plated to determine their sensitivity.

### Table 1. Strains of S. pneumoniae

<table>
<thead>
<tr>
<th>Strain*</th>
<th>Properties†</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>C13(68)</td>
<td>Hex⁻, sensitive to the antibiotic markers used</td>
<td>Ephrussi-Taylor (1951)</td>
</tr>
<tr>
<td>C13(70)</td>
<td>Hex⁻, sensitive to the antibiotic markers used</td>
<td>Ephrussi-Taylor (1951)</td>
</tr>
<tr>
<td>S(70)</td>
<td>ery⁻, r str-r41 opt-r2</td>
<td>Grist (1980)</td>
</tr>
<tr>
<td>r2SQ(70)</td>
<td>amp-A amp-B str-r41 opt-r2</td>
<td>Derived from the cross mex95 × A6SQ</td>
</tr>
<tr>
<td>A6SQ</td>
<td>amp-A amp-B str-r41 opt-r2</td>
<td>Derived from the cross Am11-r2 × A6SQ</td>
</tr>
<tr>
<td>mex95</td>
<td>mex⁻, Amp-A and -B</td>
<td>Butler &amp; Nicholas (1973)</td>
</tr>
<tr>
<td>mex95-A6SQ</td>
<td>mex⁻, Amp-A and -B</td>
<td>Butler &amp; Nicholas (1973)</td>
</tr>
<tr>
<td>Am11-A6SQ</td>
<td>amn-r11 Amp-A and -B</td>
<td>Butler &amp; Nicholas (1973)</td>
</tr>
<tr>
<td>Tc6(9)</td>
<td>tet-A opt-r2</td>
<td>Butler &amp; Nicholas (1973)</td>
</tr>
<tr>
<td>Am1-r2</td>
<td>amn-r1 ery⁻, r</td>
<td>Butler &amp; Nicholas (1973)</td>
</tr>
<tr>
<td>Am1-r2SQ</td>
<td>amn-r1 ery⁻, r str-r41 opt-r2</td>
<td>Derived from the cross r2SQ(70) × 401</td>
</tr>
<tr>
<td>401</td>
<td>Hex⁻, sensitive to the antibiotic markers used</td>
<td>Derived from the cross r2SQ(70) × 401</td>
</tr>
<tr>
<td>401-r2SQ</td>
<td>ery⁻, r str-r41 opt-r2</td>
<td>Derived from the cross r2SQ(70) × 401</td>
</tr>
</tbody>
</table>

* Apart from strains 401 and 401-r2SQ, all the strains listed are derivatives of strain C13. In view of the variable pattern of growth in \( D_2O \)-medium shown by the different derivatives of strain C13, they are labelled (in parentheses) with the date that they were frozen for storage in order to distinguish between them.

† \( str-r41 \) confers resistance to streptomycin (2 mg ml\(^{-1}\)) (Hottchkiss, 1951); \( ery-r2 \) confers resistance to erythromycin (1 \( \mu \text{g ml}^{-1}\)) (Green, 1959); \( opt-r2 \) confers resistance to optochin (5 \( \mu \text{g ml}^{-1}\)) (Ephrussi-Taylor, 1958); \( tet-A \) confers resistance to tetracycline (12 \( \mu \text{g ml}^{-1}\)) (Butler & Smiley, 1973); \( amp-A, amp-B \) confers resistance to ampicillin (0.06 \( \mu \text{g ml}^{-1}\)) (Butler & Smiley, 1970); \( amn-r11 \) and \( amn-r1 \) confer resistance to \( 1 \times 10^{-5} \) M-aminopterin (Sicard, 1964); \( mex-r95 \) confers resistance to \( 1 \times 10^{-5} \) M-aminopterin or methotrexate (0.06 \( \mu \text{g ml}^{-1}\)) (Grist, 1980): \( Hex⁻ \), no discrimination between markers which are all transformed as very high efficiency markers (Lacks, 1970).
Determination of the amount of H₂O present in samples of D₂O. D₂O is hygroscopic so it was necessary to routinely check the H₂O content of all samples of D₂O. A sample was loaded into a silver chloride cuvette, path length 0.1 mm, and the spectrum was recorded at room temperature using a Perkin-Elmer 157 silver chloride IR spectrophotometer. The absorbance of the peak at 3400 cm⁻¹ was measured and compared to values obtained from spectra of pure D₂O samples contaminated with varying known amounts of H₂O. A linear relationship exists between the absorption at this wavelength and the amount of H₂O present and hence the percentage of H₂O present in an unknown sample could be determined. Various samples examined were contaminated with up to 5% (v/v) H₂O.

RESULTS

The process of ‘adaptation’

Using the procedure described by Butler & Smiley (1973) in which cultures were grown in media containing progressively higher concentrations of D₂O, it was found that the amount of D₂O in the medium could be increased to 90% without any effect on the growth of cultures of strains Cl3(70) and A6SQ but that the subsequent transfer of the culture into medium containing 100% D₂O always caused a death phase. The viable count declined by a factor varying from 10 to 10⁸ before the culture entered a lag phase from which growth was eventually resumed. A typical example is shown in Fig. 1. Cultures that had been through this ‘adaptation’ were now able to grow in 100% D₂O-medium without a death phase, and are referred to as ‘adapted’, and also were now able to grow at a normal rate when shifted firstly into H₂O-medium and also back into D₂O-medium, suggesting that ‘adaptation’ was a permanent trait involving the selection of mutants capable of tolerating and growing in D₂O-medium. Prolonging the period of growth in media at the lower levels of D₂O had no effect on the duration or severity of this death phase, and cultures entering the death phase could not be rescued by subculturing them into fresh D₂O-medium.

The effect of repeated subculturing in D₂O-medium of one strain over a period of a year can be seen from Fig. 2. After one subculture, the newly-adapted culture entered the stationary phase at a titre of 1–2 × 10⁷ c.f.u. ml⁻¹ but this titre gradually increased till it eventually reached 1 × 10⁸ c.f.u. ml⁻¹ after 57 sub-culturings. This had been accomplished with a reduction in the generation time from 50 to 40 min.
Fig. 2. Frequency of mutants resistant to aminopterin (3.3 µg ml⁻¹) (●) during eight subculturings of an 'unadapted' strain A6SQ in D₂O-medium in relation to the c.f.u. count (○). Except for the first three, samples were removed only at the beginning and end of the incubation of subcultures 4 to 8 and hence the plot of c.f.u. ml⁻¹ for these subcultures is not intended to represent the true pattern of growth.

**Induction of antibiotic-resistant mutants**

It was of interest to compare the frequency of mutation exhibited by 'adapted' strains growing in D₂O-medium with that of 'unadapted' strains growing in H₂O-medium. Consequently, the presence of mutants resistant to a variety of different antibiotics in strains that had been 'adapted' and subcultured for varying periods of time in D₂O-medium was measured and compared to their frequency in the parental 'unadapted' strains incubated in H₂O-medium. The results from several experiments are summarized in Table 2. In all cases, the frequency of mutants was greatest in 'adapted' cultures incubated in D₂O-medium (last column), the extent of this increase varying according to the antibiotic used, and sometimes depending on its concentration. In order to ascertain when the propensity to increased mutation frequency had occurred, the frequency of mutants resistant to 3.3 µg aminopterin ml⁻¹ was monitored during the 'adaptation' of strain A6SQ to growth in D₂O-medium. The results (Fig. 3) indicated that the bulk of the increase in mutation frequency occurred during the death phase as a result of which the frequency rose 300-fold, whilst further incubation stabilized the mutation frequency at an overall increase of about 500-fold. The strain had then become 'adapted' and hence capable of sustained growth in the D₂O-medium. No expression time for the mutant character was given but allowance for the usual expression time of 2.5 h for the aminopterin marker would still locate the region of increased mutation frequency in the death phase.

It was possible that the observable characteristic possessed by a mutant provided a selective advantage to an organism growing in D₂O-medium, and therefore one of these characters, namely aminopterin resistance, was chosen for study. A strain carrying the marker amia-r1 together with an unlinked marker ery-r2 was mixed with a related strain carrying the str-r41 marker and the growth of both strains was monitored in D₂O-medium. At various times during the incubation samples were removed and plated on medium containing (a) aminopterin plus erythromycin (for the intrinsic aminopterin count), (b) streptomycin, and (c) aminopterin plus streptomycin (for aminopterin mutants). The results showed that the intrinsically aminopterin-resistant strain maintained a constant frequency in the total population although the frequency of the aminopterin-resistant mutants increased by 100-fold. It can be concluded that the presence of the aminopterin-resistance gene in a strain did not confer any selective advantage to that strain.

It could also have been possible that growth in D₂O-medium had altered the sensitivity of the wild-type organism, allowing growth in the presence of higher concentrations of the
Table 2. Comparison of the frequencies of mutation to antibiotic resistance in D₂O-medium of 'unadapted' cultures with those of 'adapted' cultures

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Conc (µg ml⁻¹)</th>
<th>Strain</th>
<th>Total no. of mutants scored</th>
<th>Total no. of organisms plated</th>
<th>Mutation frequency A</th>
<th>Total no. of mutants scored</th>
<th>Total no. of organisms plated</th>
<th>Mutation frequency B</th>
<th>B/A</th>
</tr>
</thead>
<tbody>
<tr>
<td>d-Cycloserine</td>
<td>100</td>
<td>A6SQ</td>
<td>349</td>
<td>2.5 x 10⁷</td>
<td>1.4 x 10⁻⁵</td>
<td>113</td>
<td>4.8 x 10³</td>
<td>2.3 x 10⁻²</td>
<td>1718</td>
</tr>
<tr>
<td></td>
<td>150</td>
<td>A6SQ</td>
<td>1</td>
<td>9.5 x 10⁷</td>
<td>1.0 x 10⁻⁸</td>
<td>88</td>
<td>5.3 x 10⁶</td>
<td>1.7 x 10⁻⁵</td>
<td>1700</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>A6SQ</td>
<td>2</td>
<td>2.3 x 10⁷</td>
<td>8.7 x 10⁻⁸</td>
<td>866</td>
<td>5.3 x 10⁶</td>
<td>1.6 x 10⁻⁴</td>
<td>1840</td>
</tr>
<tr>
<td>Novobiocin</td>
<td>0.5</td>
<td>A6SQ</td>
<td>200</td>
<td>2.3 x 10⁴</td>
<td>8.7 x 10⁻⁴</td>
<td>&gt;5000</td>
<td>4.8 x 10⁴</td>
<td>&gt;1.0 x 10⁻¹</td>
<td>&gt;120</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>A6SQ</td>
<td>216</td>
<td>2.3 x 10⁸</td>
<td>9.4 x 10⁻⁴</td>
<td>&gt;5000</td>
<td>4.8 x 10⁴</td>
<td>&gt;1.0 x 10⁻¹</td>
<td>&gt;120</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>C13</td>
<td>ND</td>
<td>2.2 x 10⁷</td>
<td>&lt;4.5 x 10⁻⁸</td>
<td>5</td>
<td>7.0 x 10⁶</td>
<td>7.1 x 10⁻⁷</td>
<td>&gt;15-9</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>0.5</td>
<td>A6SQ</td>
<td>5</td>
<td>2.3 x 10⁷</td>
<td>2.2 x 10⁻⁷</td>
<td>346</td>
<td>5.3 x 10⁶</td>
<td>6.5 x 10⁻⁵</td>
<td>295</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>A6SQ</td>
<td>ND</td>
<td>2.3 x 10⁷</td>
<td>&lt;4.3 x 10⁻⁸</td>
<td>2</td>
<td>4.8 x 10⁶</td>
<td>4.2 x 10⁻⁷</td>
<td>&gt;9-7</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>C13</td>
<td>24</td>
<td>5.9 x 10⁶</td>
<td>4.1 x 10⁻⁶</td>
<td>28*</td>
<td>3.9 x 10⁵</td>
<td>7.2 x 10⁻⁵</td>
<td>17-5</td>
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<tr>
<td>Ampicillin</td>
<td>0.02</td>
<td>C13</td>
<td>40</td>
<td>1.1 x 10⁷</td>
<td>3.6 x 10⁻⁶</td>
<td>96</td>
<td>3.5 x 10⁶</td>
<td>2.7 x 10⁻⁵</td>
<td>7-6</td>
</tr>
<tr>
<td>Optochin</td>
<td>42</td>
<td>C13</td>
<td>82</td>
<td>6.4 x 10⁷</td>
<td>1.3 x 10⁻⁶</td>
<td>289</td>
<td>7.2 x 10⁶</td>
<td>4.0 x 10⁻⁵</td>
<td>31</td>
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<tr>
<td>Aminopterin</td>
<td>5</td>
<td>C13</td>
<td>132</td>
<td>6.4 x 10⁷</td>
<td>2.0 x 10⁻⁶</td>
<td>1576*</td>
<td>7.2 x 10⁶</td>
<td>2.2 x 10⁻⁴</td>
<td>110</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>C13</td>
<td>132</td>
<td>6.4 x 10⁷</td>
<td>2.0 x 10⁻⁶</td>
<td>3711*</td>
<td>8.0 x 10⁶</td>
<td>4.6 x 10⁻⁴</td>
<td>230</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>A6SQ</td>
<td>24</td>
<td>8.8 x 10⁷</td>
<td>2.7 x 10⁻⁷</td>
<td>62</td>
<td>1.8 x 10⁷</td>
<td>3.4 x 10⁻⁶</td>
<td>12.5</td>
</tr>
</tbody>
</table>

ND, None detected. * 'Newly adapted' strain. † Strain C13* after it had been subcultured twice in H₂O-medium.
antibacterial substances. However, when streaks of 'adapted' and 'unadapted' strains were made on agar medium containing various concentrations of optochin or aminopterin no difference in the level of resistance was found although the 'adapted' strain gave higher numbers of discrete resistant colonies than the 'unadapted'. This observation taken together with the non-selectiveness of the mutant characteristic strongly indicates that the observed higher frequency of organisms resistant to the variety of antibacterial agents was the result of mutation.

Effect of \( H_2O \) on the 'adaptation' process

It had been observed that some preparations of \( D_2O \)-medium allowed the growth of cultures of 'unadapted' strains C13(70) and A6SQ without exhibiting the process of 'adaptation', and this led to an investigation of the effect of trace amounts of \( H_2O \) in the \( D_2O \). An 'adapted' culture of strain A6SQ was grown in medium prepared with 'pure' \( D_2O \) and with \( D_2O \) contaminated with \( 3.4\% \) (v/v) \( H_2O \); whilst the culture in the 'pure' \( D_2O \) gave a typical 'adaptation' curve, the presence of the \( H_2O \) was sufficient to prevent the need for the 'adaptation' process to occur. The sample of 'pure' \( D_2O \) was found by IR spectroscopy to contain 0.1\% \( H_2O \) whilst other samples contained higher amounts, explaining the anomalous observation of the growth of the 'unadapted' strains of C13(70) and A6SQ in the \( D_2O \)-medium prepared from particular samples of \( D_2O \).

The process of 'adaptation' could be mimicked by transferring cultures which were not 'fully-adapted' into \( H_2O \)-medium. When a sample of an 'adapting' culture of strain A6SQ incubating in \( D_2O \)-medium was removed at a point nearing the end of the death phase and diluted 1 in 40 into either \( H_2O \)-medium or \( D_2O \)-medium, the viability of the culture in \( H_2O \)-medium declined after it had undergone one generation of growth whilst that of the culture in the \( D_2O \)-medium exhibited the expected increase. Furthermore, the \( H_2O \)-medium culture showed a rise in the frequency of colonies resistant to methotrexate, which did not occur in the culture of \( D_2O \)-medium. It appeared, therefore, that the cultures incubating in \( H_2O \)-medium were undergoing a process analogous to that of 'adaptation' in \( D_2O \)-medium. This result showed that 'adaptation' is not simply the result of the difficulty of the culture to grow in deuterated medium, but rather may stem from the presence of both deuterated and non-deuterated products in the cells. Once the 'adaptation' process was complete, the resultant 'fully-adapted' cells were able to grow with normal characteristics in either \( D_2O \)-medium or \( H_2O \)-medium and could be shifted back and forth between the two media with no loss in viability, although some fourfold increase in the frequency of mutants resistant to 4 \( \mu \)g optochin ml\(^{-1}\) occurred during the first generation of growth in the new medium (see Fig. 4). No increase was observed when an 'adapted' culture which had been grown once in \( H_2O \)-medium was subcultured again in \( H_2O \)-medium (data not shown).

Behaviour of other C13-derived strains incubating in \( D_2O \)-medium

Not all strains needed a period of 'adaptation' before they could be successfully subcultured in \( D_2O \)-medium, and these fell into three groups: (a) those exhibiting no death phase but an increased mutation frequency (to some 40-fold) during the initial growth period, exemplified by strain r2SQ(70); (b) strains showing a less severe loss of viability than strain A6SQ but with an increased mutation frequency similar to group (a), exemplified by strains Tc12Q(69) and C13(68); (c) the Hex\(^-\) strain 401-r2SQ which showed a death phase but with only a small increase in mutation frequency, but did show a very marked loss of expression of the \( opt-r2 \) marker after some 5 h incubation, this expression beginning to recover after some 8 h incubation.

Although none of the C13-derived strains tested were totally immune to the effects of this medium, the behaviour of each strain was consistent throughout the period of study, showing it to be genetically determined.

Specific transforming activity of DNA from cultures undergoing 'adaptation'

The specific transforming activities were determined at various incubation times for an 'unadapted' culture of strain A6SQ growing in \( H_2O \)-medium and compared with those growing in \( D_2O \)-medium, using strain C13 as recipient (Fig. 5). The activities in the \( H_2O \)-medium
Growth of pneumococcus in D₂O-medium

remained constant during the whole of the growth curve whilst those in the D₂O-medium remained constant for 4 h and then decreased although the ¹⁴C-count continued to increase. When the DNA synthesis halted, the values of the specific activities formed a plateau but then dropped steeply at least 1 h before the ¹⁴C-count began to drop. The early drop in the specific activity indicated damage to the DNA which interfered with its transforming activity, whilst...
Integration efficiency of the low-efficiency marker *opt-r2* relative to the high-efficiency marker *str-r41* of DNA in lysates of cultures grown in H$_2$O-medium (a) or D$_2$O-medium (b) with the Hex$^{-}$ strain 401 and the Hex$^{+}$ strain C13 as recipients. (a) Strain 401 (filled symbols) and strain C13 (open symbols) as recipients to donor DNA in lysates of strains grown in H$_2$O-medium: ○, ●, strain A6SQ (average values of integration efficiencies 0.69 ± 0.069 and 0.14 ± 0.014 respectively); □, strain 401-r2SQ (0.84 ± 0.24); △, strain Am1-r2SQ (0.14 ± 0.012). (b) Strain 401 (filled symbols) and strain C13 (open symbols) as recipients to donor DNA in lysates of strain A6SQ grown in D$_2$O-medium: ○, ●, ○.66 ± 0.13 and 0.20 ± 0.015 respectively.

comparison of DNA concentrations required for 'swamping'

Comparison of DNA concentrations required for 'swamping'

The changes in the integration efficiencies indicated that the Hex$^{+}$ recipient strains may be able to effect some repair of the damage to the DNA from cells undergoing 'adaptation', and also that the 'adapted' cells themselves had also effected repair. It was of interest to ascertain whether the 'adapted' cells possessed higher Hex activity. Therefore comparison was made of the concentrations of DNA required to effect 'swamping' of the Hex system, thereby rescuing low-efficiency markers from Hex attack, in 'unadapted' and 'adapted' cells (Guild & Shoemaker, 1974; Butler et al., 1977; Butler & George, 1981).

An 'unadapted' culture of strain C13(70) was diluted 1:250 in D$_2$O-medium and incubated until 'adaptation' had occurred. This took some 39 h. It was then subcultured in H$_2$O-medium...
Growth of pneumococcus in D₂O-medium

Fig. 7. Effect of DNA concentration on the integration efficiency of opt-r2 relative to str-r41 when DNA prepared from strain mex95-A6SQ was transformed into an 'unadapted' strain C13(70) (○), a newly 'adapted' strain C13(70) (□) and a fully 'adapted' strain C13(70) (●). The arrow denotes the lowest concentration of DNA that gave saturating numbers of opt-r2 transformants.

Fig. 8. Effect of UV-irradiation on the survival of an 'unadapted' culture (○) and an 'adapted' culture (●) of strain C13(70).

from which frozen precultures were prepared before the preparation of cultures in which competence developed. Competent cultures were prepared after one subculturing in H₂O-medium and also after several subculturings over several months, referred to as the 'newly-adapted' and 'fully-adapted' cultures respectively. The subculturings in H₂O-medium served to rid the cells of deuterated products. At the same time, competent cultures were prepared from the original 'unadapted' culture and all three sets of cultures were then treated with various concentrations of the same transforming DNA prepared from an 'unadapted' culture of strain mex95-A6SQ. All recipients gave, for a given marker, identical dilution curves with this donor DNA. The transformants for the str-r41 and opt-r2 markers were scored, and the integration efficiencies for the opt-r2 marker calculated (Fig. 7). A rise in the integration efficiency with increasing concentration of transforming DNA indicates 'swamping' of the Hex system; this occurred at a concentration of DNA which was lowest with the 'unadapted' culture as recipient, whilst the 'fully-adapted' culture required the highest concentration, indicating that the cells of this culture possessed the most Hex system since they needed more of the DNA to cause 'swamping'. Furthermore, the value of the integration efficiency at the lowest DNA concentrations was lowest with the 'fully-adapted' culture, indicating that the attack on the integrated opt-r2 marker was the greatest, again supporting the view that these cells possessed the highest amounts of the Hex system.

UV-sensitivity of 'adapted' strains

Because of the possession of the higher amounts of a repair system by the 'adapted' cells, it was of interest to discover whether this had any effect on their UV-sensitivity. 'Adapted' strains which had been subcultured in D₂O-medium over a period of several months were first subcultured in H₂O-medium to remove deuterated products from the cells, then washed and suspended in the mineral-salts solution (see Methods) and their sensitivities to UV-irradiation measured. Comparisons were made with suspensions of the appropriate parental 'unadapted' culture. The results for strain C13(70) are shown in Fig. 8, from which it can be seen that the 'adapted' strain exhibited a significantly higher resistance to UV-irradiation than its parental 'unadapted' strain. Similar results were obtained with strain A6SQ (data not shown).
Mann & Moses (1971) reported that cultures of *Escherichia coli* grown in D$_2$O-medium with fully deuterated acetic acid as their only carbon source had a raised protein : DNA ratio and on transfer to H$_2$O-medium they initially grew faster than normal. This behaviour was not observed by Firshein & Schwenfeier (1969) with cultures of *S. pneumoniae* grown in D$_2$O-medium, nor from the results shown in Fig. 2, since they show that cultures growing in D$_2$O-medium were able to resume growth in H$_2$O-medium at their normal growth rate. It is therefore unlikely that growth of pneumococcal strains in D$_2$O-medium resulted in extensive substitution of deuterium into their cell components, a conclusion expected from the observation made by Sicard (1964) that pneumococci are unable to synthesize many of their essential nutrients, although Orgel (1964) proposed that extensive deuterium substitution occurred in *E. coli*. However, transfer of some strains into D$_2$O-medium did result in the onset of a death phase from which only a selected population of cells could survive. Once a culture had been ‘adapted’ it was capable of sustained growth in D$_2$O-medium even after a passage through H$_2$O-medium showing that ‘adaptation’ was a permanent trait involving the selection of mutants. The substantial increase observed in the frequency of mutation to a variety of antibiotic resistances (Table 2) suggested that the D$_2$O had an highly mutagenic effect, i.e. the process of ‘adaptation’ was mutagenic. In the case of methotrexate-resistant mutants the bulk of this increase occurred during the death phase (Fig. 3) and was due neither to the mutation conferring a selective advantage to cells undergoing ‘adaptation’, nor to higher levels of resistance in the wild-type population. The high incidence of mutation could account, therefore, for the rapid increase in growth after the end of the death phase.

The amount of deuterium that would be expected to be incorporated into pneumococcal cells growing in D$_2$O-medium would increase during the incubation as the non-deuterated substrates present in the broth were used up. This may explain why the onset of ‘adaptation’ was delayed in cultures newly transferred to D$_2$O-medium (Fig. 1). Transfer of ‘adapting’ cultures back into H$_2$O-medium brought back all of the features associated with ‘adaptation’, whilst a similar transfer of a fully ‘adapted’ culture resulted only in an increase in its mutation frequency. It is apparent from these results that ‘adaptation’ should not be ascribed just to the toxicity of D$_2$O but rather that it results from the presence of both deuterium and hydrogen together. A number of other studies have also reported that the mixture of deuterated and non-deuterated products may be the detrimental agent; Borek & Rittenberg (1960) observed distortion of normal-sized cells of *E. coli* K12 grown in D$_2$O-medium after transfer to H$_2$O-medium; Laser (1959) observed that cells grown in non-deuterated medium were three times more sensitive to X-rays when irradiated in D$_2$O buffer, whilst a similar effect was observed by De Giovanni (1961) who found that cells incubated in D$_2$O-medium were more sensitive to UV-irradiation when irradiated and washed in physiological saline prepared in H$_2$O instead of D$_2$O, and, conversely, cells grown in H$_2$O-medium but irradiated in D$_2$O-saline were more sensitive than those irradiated in H$_2$O-saline.

The decrease in the relative transforming activities of an ‘unadapted’ culture of strain A6SQ during incubation in D$_2$O-medium showed that the *str-r41* marker was being inactivated in the process of ‘adaptation’. Evidence for damage to the DNA of pneumococcal cells incubating in D$_2$O-medium was first reported by Martin & Ephrussi-Taylor (1964). However, no evidence for DNA damage or repair was seen by Firshein & Schwenfeier (1969) or by Butler & Smiley (1973) during the course of density-shift analyses, but both these studies used strains that were capable of sustained growth in D$_2$O-medium and were therefore ‘adapted’.

Confirmation of the inactivation of chromosomal markers was obtained by the behaviour of the integration efficiencies of the *opr2* marker which decreased in ‘adapting’ cultures when the marker was transformed into the Hex$^+$ recipient strain 401. It was significant, however, that the values of the integration efficiencies returned towards their initial values as the strain became ‘adapted’, indicating that repair of the damage to the DNA was occurring. This repair was evident when the marker was transformed into the Hex$^-$ strain C13, suggesting that the Hex system itself was active in carrying out this repair. The higher level of the transforming DNA required to effect ‘swamping’ of the Hex system in the ‘adapted’ culture indicated that these cells
possessed higher levels of the Hex system, and this was further supported by their exhibiting the lowest value of the integration efficiency for the opt-r2 marker. Hence, it can be concluded that the incubation of an 'unadapted' strain in D₂O-medium caused damage to the DNA which is repairable by 'fully-adapted' cells, these cells possessing higher amounts of a repair system which has characteristics possessed by a Hex+ strain. If the Hex system is implicated in this repair process it might explain the marked loss of gene expression shown by the Hex− strain during the 'adaptation' process. Furthermore, the greater resistance to UV-irradiation exhibited by the 'adapted' strains also indicated that these strains have repair capabilities not possessed by the 'unadapted' cells. However, it is not yet possible to identify the Hex system itself as this repair enzyme, since the cells may possess other repair mechanisms: indeed evidence has already been presented which suggested that pneumococcal cells may exhibit an SOS repair mechanism (Grist & Butler, 1983).

REFERENCES


