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

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(Received 25 July 1983)

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 caused 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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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>Grist (1980)</td>
</tr>
<tr>
<td>S(70)</td>
<td>streA</td>
<td>Derived from the cross mex95 × A6SQ</td>
</tr>
<tr>
<td>r2SQ(70)</td>
<td>ery−r2, streA, opt−r2</td>
<td>Derived from the cross Am11-r2 × A6SQ</td>
</tr>
<tr>
<td>A6SQ</td>
<td>amp−A, amp−B, streA, opt−r2</td>
<td>Butler &amp; Nicholas (1973)</td>
</tr>
<tr>
<td>mex95</td>
<td>mex−95, AMP−A, AMP−B, streA, opt−r2</td>
<td>Hex− derived from the cross r2SQ(70) × 401</td>
</tr>
<tr>
<td>Am11-A6SQ</td>
<td>amA−r1, AMP−A, AMP−B, streA, opt−r2</td>
<td></td>
</tr>
<tr>
<td>Tc12Q(69)</td>
<td>tet−A, opt−r2</td>
<td></td>
</tr>
<tr>
<td>Am1−r2</td>
<td>amA−r1, ery−r2</td>
<td></td>
</tr>
<tr>
<td>Am1−r2SQ</td>
<td>ery−r2, streA, opt−r2</td>
<td></td>
</tr>
<tr>
<td>401</td>
<td>Hex−, sensitive to the antibiotic markers used</td>
<td>Tiraby &amp; Sicard (1973)</td>
</tr>
<tr>
<td>401-r2SQ</td>
<td>ery−r2, streA, opt−r2</td>
<td></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 D2O-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.

† streA confers resistance to streptomycin (2 mg ml⁻¹) (Hitchcock, 1951); ery−r2 confers resistance to erythromycin (1 µg ml⁻¹) (Green, 1959); opt−r2 confers resistance to optochin (5 µg ml⁻¹) (Ephrussi-Taylor, 1958); tet−A confers resistance to tetracycline (12 µg ml⁻¹) (Butler & Smiley, 1973); AMP−A, AMP−B confers resistance to ampicillin (0.06 µg ml⁻¹) (Butler & Smiley, 1970); amA−r1 and amA−r1 confers resistance to 1 × 10⁻⁵ m-aminopterin (Sicard, 1964); mex−95 confers resistance to 1 × 10⁻⁵ m-aminopterin or methotrexate (0.06 µg ml⁻¹) (Grist, 1980): Hex−, no discrimination between markers which are all transformed as very high efficiency markers (Lacks, 1970).

**Media.** The peptone medium 'P' was prepared as described by Sicard (1964) and Butler (1965). Deuterated 'P' medium (D2O-medium) was prepared by replacing the glass-distilled water by D2O (Koch-Light) containing 99.7 atom% D. All solutions added to the basic 'P' medium were also prepared in D2O but the pH was adjusted to 7.6 by a solution of NaOH dissolved in D2O. 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 D2O-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% (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 ¹⁴C-count due to the incorporation of [¹⁴C]thymidine. A culture of the strain which carried the streA marker was first subcultured in H2O-medium labelled with [²⁻¹⁴C]thymidine (60 mCi mmol⁻¹; 2.2 GBq mmol⁻¹) at 0.5 µCi ml⁻¹ before transferring it into H2O- or D2O-medium also containing [¹⁴C]thymidine. During the subsequent incubation at 37 °C, samples were periodically removed and assayed for the c.f.u. count, for the ¹⁴C-count, and for the transforming ability of lysates. The relative transforming activity of a sample was then obtained by dividing the number of streA transformants by the ¹⁴C-count of the sample.

The integration efficiency of a marker was measured relative to the streA marker present on the same chromosome using lysates as donor DNA. When necessary the streA marker was first introduced into the strain by transformation. The number of transformants which carried the mutant marker relative to the number of streA 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 H2O-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 J m⁻² 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.
Growth of pneumococcus in $D_2O$-medium

Determination of the amount of $H_2O$ present in samples of $D_2O$. $D_2O$ is hygroscopic so it was necessary to routinely check the $H_2O$ content of all samples of $D_2O$. 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 $D_2O$ samples contaminated with varying known amounts of $H_2O$. A linear relationship exists between the absorption at this wavelength and the amount of $H_2O$ present and hence the percentage of $H_2O$ present in an unknown sample could be determined. Various samples examined were contaminated with up to 5% (v/v) $H_2O$.

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 $D_2O$, it was found that the amount of $D_2O$ in the medium could be increased to 90% without any effect on the growth of cultures of strains C13(70) and A6SQ but that the subsequent transfer of the culture into medium containing 100% $D_2O$ always caused a death phase. The viable count declined by a factor varying from 10 to $10^4$ 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% $D_2O$-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 $H_2O$-medium and also back into $D_2O$-medium, suggesting that ‘adaptation’ was a permanent trait involving the selection of mutants capable of tolerating and growing in $D_2O$-medium. Prolonging the period of growth in media at the lower levels of $D_2O$ 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 $D_2O$-medium.

The effect of repeated subculturing in $D_2O$-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 $1–2 \times 10^7$ c.f.u. ml$^{-1}$ but this titre gradually increased till it eventually reached $1 \times 10^8$ 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. 1. Viability of strain A6SQ diluted 1 in 1000 into $D_2O$-medium.

Fig. 2. Effect of continual subculturing in $D_2O$-medium on the growth of strain A6SQ. □, 1 subculturing; ●, 7 subculturings; ■, 47 subculturings; △, 57 subculturings.
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>
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<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 10⁻¹</td>
<td>1718</td>
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<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 10⁻⁴</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>
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<tr>
<td>Novobiocin</td>
<td>5</td>
<td>A6SQ</td>
<td>200</td>
<td>2.3 x 10³</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 10³</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>
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<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>0.5</td>
<td>C13</td>
<td>24</td>
<td>5.9 x 10⁶</td>
<td>4.1 x 10⁻⁶</td>
<td>28*</td>
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<td>Ampicillin</td>
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<td>40</td>
<td>1.1 x 10⁷</td>
<td>3.6 x 10⁻⁶</td>
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<td>3.5 x 10⁶</td>
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<td>7-6</td>
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<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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<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>
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<td></td>
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<td>C13</td>
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<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>
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<tr>
<td></td>
<td>5</td>
<td>A6SQ</td>
<td>24</td>
<td>8.8 x 10⁷</td>
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<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$_2$O on the 'adaptation' process

It had been observed that some preparations of D$_2$O-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$_2$O in the D$_2$O. An 'adapted' culture of strain A6SQ was grown in medium prepared with 'pure' D$_2$O and with D$_2$O contaminated with 3-4% (v/v) H$_2$O; whilst the culture in the 'pure' D$_2$O gave a typical 'adaptation' curve, the presence of the H$_2$O was sufficient to prevent the need for the 'adaptation' process to occur. The sample of 'pure' D$_2$O was found by IR spectroscopy to contain 0.1% H$_2$O 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$_2$O-medium prepared from particular samples of D$_2$O.

The process of 'adaptation' could be mimicked by transferring cultures which were not 'fully-adapted' into H$_2$O-medium. When a sample of an 'adapting' culture of strain A6SQ incubating in D$_2$O-medium was removed at a point nearing the end of the death phase and diluted 1 in 40 into either H$_2$O-medium or D$_2$O-medium, the viability of the culture in H$_2$O-medium declined after it had undergone one generation of growth whilst that of the culture in the D$_2$O-medium exhibited the expected increase. Furthermore, the H$_2$O-medium culture showed a rise in the frequency of colonies resistant to methotrexate, which did not occur in the culture of D$_2$O-medium. It appeared, therefore, that the cultures incubating in H$_2$O-medium were undergoing a process analogous to that of 'adaptation' in D$_2$O-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$_2$O-medium or H$_2$O-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 µg 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$_2$O-medium was subcultured again in H$_2$O-medium (data not shown).

Behaviour of other C13-derived strains incubating in D$_2$O-medium

Not all strains needed a period of 'adaptation' before they could be successfully subcultured in D$_2$O-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$_2$O-medium and compared with those growing in D$_2$O-medium, using strain C13 as recipient (Fig. 5). The activities in the H$_2$O-medium
Growth of pneumococcus in D$_2$O-medium

Fig. 4. Effect on an 'adapted' culture of strain C13 kept in D$_2$O-medium with regard to viability (triangles) and frequency of mutation (circles) when transferred from (a) D$_2$O-medium (filled symbols) and then to H$_2$O-medium (open symbols); (b) H$_2$O-medium (open symbols) and then to D$_2$O-medium (filled symbols).

Fig. 5. Comparison of the number of str-r41 transformants ($\Delta$), c.p.m. due to the uptake of [$^{14}$C]thymidine (○), and the specific transforming activities (●) of lysates of 'unadapted' cultures of strain A6SQ growing in H$_2$O-medium (a) and D$_2$O-medium (b). Strain C13 was used as recipient.

remained constant during the whole of the growth curve whilst those in the D$_2$O-medium remained constant for 4 h and then decreased although the $^{14}$C-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 $^{14}$C-count began to drop. The early drop in the specific activity indicated damage to the DNA which interfered with its transforming activity, whilst
the later drop indicated the beginnings of a more fundamental degradation of the DNA resulting in the loss of the precipitable ¹⁴C-count.

**Integration efficiency of the opt-r2 marker of cultures undergoing 'adaptation'**

A low-efficiency marker may be more sensitive to possible inactivation than an high-efficiency marker and hence it was of interest to follow the integration efficiency of the low-efficiency marker opt-r2 compared to the high-efficiency marker str-r4l using lysates prepared at various times of incubation of an 'unadapted' culture in D₂O-medium. A control series was carried out using lysates of three 'unadapted' strains grown in H₂O-medium as donor to both a Hex⁺ recipient strain, C13, and a Hex⁻ recipient strain, 401; the values of the integration efficiency of the opt-r2 marker relative to the str-r4l marker are given in Fig. 6(a). It can be seen that little variation occurred in the integration efficiencies using either strain as recipient. However, when the 'unadapted' strain A6SQ was incubated in D₂O-medium the integration efficiencies had different profiles according to whether a Hex⁺ or a Hex⁻ recipient was used (Fig. 6b). With the Hex⁺ recipient, the values of the integration efficiency, although somewhat higher than those obtained in the H₂O-medium, showed very little variation, but with the Hex⁻ recipient there was first a lowering of the values corresponding with the short period of limited growth, followed by a return towards the initial values associated with the onset of the death phase before 'adaptation' was complete. This marked variation in the integration efficiencies using the lysates of cultures grown in D₂O-medium compared to those obtained from cultures grown in H₂O-medium strongly indicated that the DNA had been damaged.

**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 D₂O-medium and incubated until 'adaptation' had occurred. This took some 39 h. It was then subcultured in H₂O-medium.
Growth of pneumococcus in $D_2O$-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 CI3(70) (○), a newly ‘adapted’ strain CI3(70) (□) and a fully ‘adapted’ strain CI3(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 CI3(70).

from which frozen precultures were prepared before the preparation of cultures in which competence developed. Competent cultures were prepared after one subculturing in $H_2O$-medium and also after several subculturings over several months, referred to as the ‘newly-adapted’ and ‘fully-adapted’ cultures respectively. The subculturings in $H_2O$-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 $D_2O$-medium over a period of several months were first subcultured in $H_2O$-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 CI3(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 D₂O-medium with fully deuterated acetic acid as their only carbon source had a raised protein : DNA ratio and on transfer to H₂O-medium they initially grew faster than normal. This behaviour was not observed by Firshein & Schwenfeier (1969) with cultures of *S. pneumoniae* grown in D₂O-medium, nor from the results shown in Fig. 2, since they show that cultures growing in D₂O-medium were able to resume growth in H₂O-medium at their normal growth rate. It is therefore unlikely that growth of pneumococcal strains in D₂O-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 D₂O-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 D₂O-medium even after a passage through H₂O-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 D₂O 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 D₂O-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 D₂O-medium (Fig. 1). Transfer of 'adapting' cultures back into H₂O-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 D₂O 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 D₂O-medium after transfer to H₂O-medium; Laser (1959) observed that cells grown in non-deuterated medium were three times more sensitive to X-rays when irradiated in D₂O buffer, whilst a similar effect was observed by De Giovanni (1961) who found that cells incubated in D₂O-medium were more sensitive to UV-irradiation when irradiated and washed in physiological saline prepared in H₂O instead of D₂O, and, conversely, cells grown in H₂O-medium but irradiated in D₂O-saline were more sensitive than those irradiated in H₂O-saline.

The decrease in the relative transforming activities of an 'unadapted' culture of strain A6SQ during incubation in D₂O-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 D₂O-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 D₂O-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 CI3, 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


