Protection by Anoxia of the Scrapie Agent and some DNA and RNA Viruses Irradiated as Dry Preparations

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SUMMARY

Freeze-dried preparations of the scrapie agent and of 5 strains of virus were used to determine the extent of protection afforded against ionizing radiation by anoxia. The five viruses tested were bacteriophages T1 and T2 (DNA) and μ2 (RNA); and the animal viruses herpes simplex (DNA) and yellow fever (RNA). Oxygen enhancement ratios (measured in terms of doses required to give the same effect in the absence and presence of oxygen) varied from about 1.4 for the scrapie agent to 2.5 for yellow fever virus. With the viruses there was no obvious correlation of oxygen enhancement ratio with type or size of nucleic acid core.

'Target sizes' of the viruses were calculated from the doses required to give an average of one lethal event per particle. These agreed well with independent estimates of size of nucleic acid core; except that for bacteriophages T1 and T3 the calculated molecular weight was 0.3 of that determined by Bresler et al. (1967). Results with the scrapie agent confirmed the previous estimate (Alper, Haig & Clarke, 1966) of a molecular weight of about 1.5 × 10^6 daltons.

INTRODUCTION

We have previously reported results of radiobiological investigations on the agent of scrapie (Alper et al. 1966; Alper et al. 1967). These demonstrated that the 'target size' of the agent was considerably less than that of the smallest viruses, and that its viability was not affected by large doses of ultraviolet light of wavelengths from 230 to 330 μm. Our results suggested that the replication of the agent did not depend on a nucleic acid moiety. We have made further experiments with ionizing radiation in an attempt to obtain inferential evidence on the nature of the agent.

A phenomenon of interest in radiobiology is the protection afforded against ionizing radiation by the absence of oxygen. This may be ascribed to interaction of oxygen with radicals induced by radiation; in the absence of oxygen, some radicals may be restored to normal functioning, so that there is a reduction in the probability that each quantum of energy absorbed will result in biological inactivation (Alper, 1956). It is convenient to define the extent of protection by the ratio of doses needed to give the same effect in the absence and presence of oxygen, and to refer to this as the oxygen enhancement ratio (o.e.r.). It was shown by Alexander (1957) that an
enzyme irradiated in the dry state is protected by anoxia when loss of activity is the
test of damage. The o.e.r.'s for several enzymes, irradiated dry, have now been
measured (see review by Hunt, Till & Williams, 1962), the values for the majority
falling in the range 1·5 to 2. However, we have been able to find only one paper
giving information on the o.e.r. for nucleic acid irradiated dry, when damage is
assessed in terms of biological function. Tanooka & Hutchinson (1965) examined
the destruction by radiation of the transforming ability of DNA from spores of
Bacillus subtilis, and the o.e.r. deduced from their data appears to be about 2·3.

It seemed possible that the o.e.r. for macromolecules with biological activity
might reflect their chemical nature, and we set out to compare the o.e.r. for the
scrapie agent with those for DNA and RNA viruses irradiated likewise as freeze-dried
preparations. We selected one animal virus of each type, namely herpes simplex virus
and yellow fever virus. These could be grown in, and be harvested from, the brains
of mice, so preparations could be treated before irradiation, and tested for activity
afterwards, in the same way as preparations containing the scrapie agent. We also
used three bacterial viruses, coliphage T1 and T3 (DNA) and μ2 (RNA). These were
likewise irradiated as freeze-dried preparations, samples of phage suspensions having
been added to extracts of mouse brain.

We have found that the o.e.r.'s for the various viruses are not obviously correlated
with the type of nucleic acid core. While the o.e.r. for the scrapie agent is lower than
for the viruses, it is not significantly different from that for μ2 bacteriophage or herpes
simplex virus. Our results have confirmed the previous estimate (Alper et al. 1966) of the
"target size" of the scrapie agent. We have also confirmed the good agreement between
radiation target size of RNA viruses and independent estimates of the size of the
nucleic acid cores (Ginoza, 1967). Our results with double-stranded DNA viruses
show better agreement than many of those reviewed by Ginoza.

METHODS

Virus strains

Bacteriophages. Stocks of phages T1 and T3 have been carried in our laboratories
for some years. The RNA phage, μ2, which is virulent for F+ strains of Escherichia
coli K12, was isolated by Dettori, Maccacaro & Piccinin (1961) and kindly given
to us by Dr S. Glover, M.R.C. Microbial Genetics Research Unit.

Animal viruses. Herpes simplex virus strain 1074 was supplied by Dr M. S. Pereira,
Colindale, London. Yellow fever virus, FN strain, was supplied by Dr C. C. Draper,
Wellcome Research Laboratories, Beckenham, Kent.

Scrapie agent

The strain used was that of Chandler (1963), adapted to mice.

Preparation of material for irradiation

Suspensions of scrapie-affected mouse brain in distilled water were prepared as
previously described (Alper et al. 1966). Brains of mice infected with herpes simplex
virus were homogenized in a suspension of 0·75 g. bovine plasma albumin in 100 ml.
phosphate buffered saline. Yellow fever virus treated in the same way lost considerable
viability after freeze-drying; better results were obtained when brains were homogenized with ten times the concentration of bovine plasma albumin (Draper, personal communication). A preliminary test with bacteriophage T1 showed that the radiation sensitivity and o.e.r. were the same for preparations with the higher concentration of bovine plasma albumin as for those with no additive.

The bacteriophage preparations were made by adding small volumes of bacterial lysates to suspensions of normal mouse brain made in the same way as those from scrapie-affected brains.

One ml. volumes of the various suspensions were dispensed in ampoules and then freeze-dried. Before they were sealed, oxygen was added to about half the ampoules in each case. The remainder were flushed with oxygen-free nitrogen (British Oxygen Company) and re-evacuated twice, then filled with nitrogen and sealed.

Irradiation technique and dosimetry

Samples were irradiated by the electron beam from the M.R.C. linear accelerator (Batchelor et al. 1959). The energy of the electrons at the samples was estimated to be 5 to 6 Mev. Dose rates were about 6 to 10 x 10⁴ rad/min. with the viruses. Since the agent of scrapie requires much greater doses to give good inactivation curves, however, we used higher dose rates, about 2 to 3 x 10⁵ rad/min. Doses were measured for most irradiations by exposing strips of specially prepared Perspex (Boag, Dolphin & Rotblat, 1958) enclosed in glass containers which matched the ampoules containing the freeze-dried material. For the high doses (several megarads) given to the scrapie material, doses were checked also by measuring the bleaching of 'Cellophane' containing blue dye (Henley & Richman, 1956).

Assay procedures

For assays of the scrapie agent, the material from irradiated ampoules, and un-irradiated controls, were resuspended in distilled water. Serial 'half-log.' dilutions were used, and volumes of 0.05 ml. injected into the brains of mice, 8 to 12 being used to test each dilution. Yellow fever virus preparations were assayed in the same way, but 7 mice were used for each dilution. Herpes simplex virus suspensions were assayed by inoculating 0.1 ml. of each of the 'half-log.' dilutions on to each of 5 monolayer cultures of secondary pig kidney cells. These were scored as positive or negative for the virus after 7 days' incubation.

Bacteriophage activity was assayed by the soft agar layer technique. The dried material was reconstituted, the contents of 3 or 4 ampoules which had received the same dose were pooled, and appropriate dilutions were made. An F⁺ strain of Escherichia coli K 12 was used for μ2 bacteriophage. For phages T1 and T3, we used as hosts E. coli B, the sensitive mutant ('non host-cell reactivating') B8-1 (Hill, 1958) and a resistant mutant B–H. Plaque counts were the same in a given experiment, whichever host was used.

RESULTS

Table 1 gives the raw data from one experiment with the scrapie agent. When mice had been used for the assay of activity, the value of LD₅₀ for each radiation dose was calculated by probit analysis, for which a computer program had been written.
Table 1. *Raw data from an experiment on the scrapie agent*

<table>
<thead>
<tr>
<th>Electron dose (megarads)</th>
<th>Ampoules containing oxygen</th>
<th>Ampoules containing oxygen-free nitrogen</th>
<th>Cases of scrapie/total no. of mice in group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Log. conc. of injected suspension</td>
<td>0 12.6 14.7 32.1</td>
<td>0 14.7 22.9 33.3 44.3</td>
<td></td>
</tr>
<tr>
<td>-3</td>
<td>— — — — 6/7</td>
<td>— — — — —</td>
<td>7/7</td>
</tr>
<tr>
<td>-3.5</td>
<td>— — — — 8/8</td>
<td>— — — — —</td>
<td>7/8</td>
</tr>
<tr>
<td>-4</td>
<td>— — — 8/8 2/10</td>
<td>— 8/8 6/7 5/8 10/12</td>
<td></td>
</tr>
<tr>
<td>-4.5</td>
<td>— 6/6 7/8 1/10</td>
<td>— 7/7 6/6 3/6 3/10</td>
<td></td>
</tr>
<tr>
<td>-5</td>
<td>— 7/8 8/10 1/12</td>
<td>— 8/9 7/12 6/12 5/12</td>
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<td>7/7 4/11 5/10 0/8</td>
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<td>-6</td>
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<tr>
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<td>8/12 2/8 0/6 0/7</td>
<td>—</td>
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<td>0/6</td>
<td>— 0/8</td>
<td>— — —</td>
</tr>
<tr>
<td>-8.5</td>
<td>1/8</td>
<td>— —</td>
<td>0/7</td>
</tr>
<tr>
<td>LD 50 by probit analysis (log. conc.)</td>
<td>-7.00 -5.55 -3.83 -6.90 -5.79 -5.41 -4.77 -4.55</td>
<td>95% conf. interval</td>
<td>-5.20</td>
</tr>
</tbody>
</table>

Table 2. *Data for construction of dose-effect curves, scrapie agent, yellow fever and herpes simplex viruses*

<table>
<thead>
<tr>
<th>Electron dose (megarads)</th>
<th>Log. dose concentration for LD 50</th>
<th>Fraction of activity surviving</th>
<th>Electron dose (megarads)</th>
<th>Log. dose concentration for LD 50</th>
<th>Fraction of activity surviving</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scarepie agent</td>
<td></td>
<td></td>
<td>Yellow fever virus</td>
<td></td>
<td></td>
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<tr>
<td>0</td>
<td>-6.658</td>
<td>—</td>
<td>0</td>
<td>-5.102</td>
<td>—</td>
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<tr>
<td>4.7</td>
<td>-6.246</td>
<td>0.388</td>
<td>0.024</td>
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<td>9.7</td>
<td>-5.764</td>
<td>0.128</td>
<td>0.048</td>
<td>-4.383</td>
<td>0.191</td>
</tr>
<tr>
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<td>-5.036</td>
<td>0.0239</td>
<td>0.122</td>
<td>-3.832</td>
<td>0.0000685</td>
</tr>
<tr>
<td>Yellow fever virus</td>
<td></td>
<td></td>
<td>Herpes simplex virus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>-5.102</td>
<td>—</td>
<td>0</td>
<td>-3.952</td>
<td>—</td>
</tr>
<tr>
<td>0.024</td>
<td>-4.689</td>
<td>0.386</td>
<td>0.074</td>
<td>-3.247</td>
<td>0.074</td>
</tr>
<tr>
<td>0.048</td>
<td>-4.383</td>
<td>0.191</td>
<td>0.130</td>
<td>-2.664</td>
<td>0.130</td>
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<tr>
<td>0.074</td>
<td>-3.952</td>
<td>0.091</td>
<td>0.220</td>
<td>-1.327</td>
<td>0.220</td>
</tr>
<tr>
<td>0.129</td>
<td>-2.28</td>
<td>0.0000189</td>
<td>0.295</td>
<td>-0.076</td>
<td>0.295</td>
</tr>
</tbody>
</table>
Oxygen effect for irradiated viruses and scrapie

Fig. 1. Inactivation of the scrapie agent. ○, ●, Separate experiments under oxygen; □, ■, Separate experiments under nitrogen. Vertical lines show 95% confidence limits on assays.

Fig. 2. Inactivation of herpes simplex virus. ○, Under oxygen; □, under nitrogen.

Fig. 3. Inactivation of yellow fever virus. ○, Under oxygen; □, under nitrogen.

Fig. 4. Survival curves for bacteriophage T3. Open symbols, irradiated under oxygen. Closed symbols irradiated under nitrogen. Bacterial hosts: ○, *Escherichia coli* b; △, *E. coli* bs-1; □, *E. coli* b-h.
Table 3. Oxygen enhancement ratios, values of Do (‘inactivation dose’), values of target size on Lea theory and independent estimates of size of nucleic acid core

<table>
<thead>
<tr>
<th>Material tested</th>
<th>o.e.r. and 95% conf. limits</th>
<th>Do (megarads) and 95% conf. limits</th>
<th>95% conf. interval for target size</th>
<th>Estimate of size. Nucleic acid core of viruses</th>
<th>Reference, column 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scrapie</td>
<td>1.45 (1.18–1.71)</td>
<td>4.69 (3.92–5.83)</td>
<td>1.3–2.0* × 10⁶ daltons</td>
<td>1–2× 10⁶ daltons</td>
<td>Mitra, Ender &amp; Kaesberg (1963); Zinder (1965)</td>
</tr>
<tr>
<td>μ2 bacteriophage</td>
<td>1.62 (1.49–1.77)</td>
<td>0.344 (0.314–0.380)</td>
<td>1.8–2.2 × 10⁶ daltons</td>
<td>1–2× 10⁶ daltons</td>
<td>Bresler et al. (1967)</td>
</tr>
<tr>
<td>Phages T1 and T3</td>
<td>1.78 (1.64–1.92)</td>
<td>0.0830 (0.0658–0.1122)</td>
<td>6–11 × 10⁶ daltons</td>
<td>31–32× 10⁶ daltons</td>
<td>Bergold &amp; Weibel (1962)</td>
</tr>
<tr>
<td>Yellow fever virus</td>
<td>2.51 (2.00–3.40)</td>
<td>0.0221 (0.0184–0.0278)</td>
<td>41–48 μm</td>
<td>38±5 μm</td>
<td>Russell &amp; Crawford (1962)</td>
</tr>
<tr>
<td>Herpes simplex virus</td>
<td>1.84 (1.66–2.03)</td>
<td>0.0161 (0.0142–0.0186)</td>
<td>42–56 × 10⁶ daltons</td>
<td>45 × 10⁶ daltons</td>
<td>Russell &amp; Crawford (1964)</td>
</tr>
</tbody>
</table>

* This estimate is based on the assumption of a density of 1.35 g./cm³. For a nucleic acid preparation the corresponding molecular weights would be 1 to 1.5 × 10⁶ daltons.
† No direct determination of the molecular weight of the RNA of μ2 seems to have been made; but, according to Zinder (1965), the various strains of RNA phages specific for F² strains of E. coli K12 are of about the same size.
by Dr M. C. Pike and Mr P. Smith of the M.R.C. Statistical Research Unit. Probit analysis was also used to give the TCD\textsubscript{50} values for herpes simplex virus. The program also yielded ratios of the values of LD\textsubscript{50} for given radiation doses to the values for unirradiated material, together with the 95\% confidence limits on these parameters. Table 2 gives the values of LD\textsubscript{50}, and the fraction of surviving activity calculated therefrom, for the scrapie agent, herpes simplex and yellow fever viruses.

The corresponding curves for concentration to give the LD\textsubscript{50} or TCD\textsubscript{50} vs. dose are shown in Fig. 1 to 3. Inactivation curves for the bacteriophages were derived in the usual way from plaque counts. Fig. 4 represents an experiment with T\textsubscript{3} showing that the plaque counts were the same with resistant and sensitive mutants of \textit{Escherichia coli} \textit{B}. The results for T\textsubscript{1} were not significantly different from those for T\textsubscript{3}. In all cases, inactivation curves were exponential.

Oxygen enhancement ratios were calculated by the method of Pike & Alper (1964), for which a computer program was used. The results, together with 95\% confidence intervals, are given in Table 3. Also given are the calculated values of \textit{Do} (the dose to give survival of e\textsuperscript{-1} or 36.8\% in the presence of oxygen), the ‘target size’ from the calculations of Lea (1946), and independent estimates of the size of the nucleic acid cores of the viruses.

**DISCUSSION**

The protective action of anoxia during irradiation of the scrapie agent and three viruses is shown in Fig. 5, in which all inactivation curves for irradiation in oxygen have been superimposed by selecting appropriate dosage scales. Anoxia is seen to be less protective for the scrapie agent than for the viruses, though statistical analysis of the data shows that the 95\% confidence limits for the o.e.r. overlap with those for \(\mu_2\) phage and herpes simplex virus. The significance of the differences between the o.e.r.’s for the scrapie agent and the viruses could not be tested by conventional methods, since these would involve assumptions about the distribution of such ratios. We therefore computed the ratios of o.e.r.’s of the viruses to o.e.r. for the scrapie agent by comparing the slopes of the survival curves for anoxic irradiations, expressing the doses given in terms of \textit{Do} values for the aerobic curves, as in Fig. 5. The method yielded, as before, ratios of slopes together with 95\% confidence limits on these ratios. This analysis showed that the o.e.r.’s for \(\mu_2\) bacteriophage and herpes simplex virus were not different from that for the scrapie agent at the \(P = 0.05\) level, but the o.e.r.’s for the other viruses were different at a high level of significance. These data therefore add only slight support to others (Alper \textit{et al.} 1966, 1967) which have suggested that the scrapie agent is of a chemical nature other than nucleic acid.

Among the viruses tested, no pattern is discernible which might relate the magnitude of the o.e.r. to an obvious characteristic, such as the type of nucleic acid core, or its size. It should be noted that the radiobiological response of dried bacteriophage contrasts with that observed when they are irradiated in aqueous suspension. In dilute aqueous suspension, when the majority of inactivating events are due to the radiolysis products of water, the presence of oxygen is markedly \textit{protective} (Alper, 1954; Bachofer & Pottinger, 1954). Even when bacteriophage is irradiated (in suspension) in the presence of large amounts of radical-scavenging material, a slight protective action of oxygen is discernible (Hewitt & Read, 1950; Howard-Flanders, 1961). Shalek & Gillespie (1960) observed a comparable phenomenon with lysozyme, the
radiosensitivity of which was enhanced by oxygen, when it was irradiated dry; in aqueous suspension, however, there was no 'oxygen effect'.

Our calculations of target sizes from the radiation data are based on the theory of Lea (1946), in which the target volume is defined as that volume within which every energy-loss event will cause biological inactivation. As Lea pointed out, however, there may be a probability $p$, less than one, that an energy-loss event inactivates the macromolecule, in which case the estimate of target size will be only $p$ times the actual size. While it cannot be assumed that $p$ is always equal to one when oxygen is present, it is clearly less than one in anoxic conditions, when its maximum value would be the reciprocal of the o.e.r. Some calculations of target sizes of viruses have been based on data from irradiation in vacuo, and in such cases the target sizes have been underestimated. Ginoza (1967) reviewed the relationship between target molecular weight and molecular weight of the nucleic acid core of viruses and concluded that there is, roughly, a one-to-one correspondence for RNA viruses and single-stranded DNA bacteriophages, whereas with double-stranded DNA viruses the calculated target size is always less than that of the nucleic acid core, sometimes by a considerable factor. Of the results quoted by Ginoza, the largest discrepancy was found with phage T1, the results of Schambra & Hutchinson (1964) and of Fluke (1966) giving a value of $D_0$ of about 0.55 megarads. The target size calculated on the Lea theory from this dose is $1.2 \times 10^8$ daltons, which is only 0.04 of the observed

![Graph showing comparison of protection by anoxia of the scrapie agent and three viruses.](image)
value of about $30 \times 10^6$ daltons (Bresler et al. 1967). Schambra & Hutchinson (1964) and Fluke (1966) measured the inactivation dose for Ti dried and irradiated under high vacuum. If the oxygen ratio we observed applied also in the conditions of their experiments, $D_o$ under oxygen would have been 0.29 megarads, so there is still a large discrepancy between our own observations on Ti and those quoted by Ginoza.

It is relevant that Bachofer (1953) compared the inactivation doses for samples of Ti prepared by vacuum- and freeze-drying, and found them to be about 2.4 times greater with the vacuum-dried samples. We point out, also, that the standardization of the Perspex we used for dosimetry was based ultimately on calorimetric measurements (made by Drs D. K. Bewley and Elizabeth Blum), which is the fundamental method for measuring absorbed dose; and that all our scrapie and virus samples were prepared for irradiation in the same way, and treated similarly thereafter, so that any factor which would lead to an underestimate of the inactivation dose for Ti, in our experiments, would operate also for the other material used. If it is valid, therefore, that our results give roughly a one-to-one correspondence of target size and nucleic acid core for the RNA viruses, it is valid to conclude that for the double-stranded DNA virus, herpes simplex, the correspondence is also nearly one-to-one, and that with Ti the radiation data give a target molecular weight of about 0.3 times that of the observed molecular weight of the nucleic acid core.

We thank Drs C. C. Draper, S. Glover and M. S. Pereira for giving us some of the strains. We are most grateful to Dr Elizabeth Blum for the dosimetry and helping with the irradiations. We acknowledge with gratitude also the assistance given by Dr M. C. Pike in running the data on his program for fitting probits, and for fitting the regression lines shown in Figs 1 to 3; by Mr M. C. Clarke for the freeze-drying and titrations of the scrapie and animal viruses, and by Mrs Knowles and Miss S. Hopkins with the bacteriophage work.

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REFERENCES


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