Altered Scrapie Infectivity Estimates by Titration and Incubation Period in the Presence of Detergents

By ROBERT A. SOMERVILLE*† AND RICHARD I. CARP
New York State Institute for Basic Research in Developmental Disabilities, 1050 Forest Hill Road, Staten Island, New York 10314, U.S.A.

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SUMMARY

During experiments on the purification of scrapie infectivity, changes were found in the dynamics of scrapie titration. After exposure to detergent, infectivity estimates by both endpoint titration and incubation period were altered. The addition of detergent to the diluent used in titration resulted in at least a 100-fold increase in the infectivity estimate. This suggests that the amount of scrapie in a sample, as measured by serial dilution and titration, may be underestimated to different extents, depending on the biochemical milieu of the inoculum. Membrane fractions treated with detergents before dilution exhibited longer incubation periods than untreated fractions for the same number of infectious units of scrapie. This demonstrates that detergent treatments and possibly other biochemical manipulations can cause changes in the response of the host to the inoculum that are not detectable if incubation periods alone are used to estimate scrapie ‘titre’.

Scrapie is an infectious disease of the central nervous system of sheep and goats. Experimental transmission of scrapie to mice has allowed the large scale measurement of scrapie by the standard microbiological technique of titration to a limiting dilution to give an LD_{50}. It was apparent from early studies in mice that incubation periods were extended as the amount of infectivity was reduced (Chandler, 1963; Hunter et al., 1963; Mould et al., 1967). When all variables such as strain, sex and age of mouse, strain of scrapie agent, and the amount and chemical treatment of inoculum are held constant, then titre estimates and incubation periods are remarkably reproducible (Outram, 1976). Comparative measurements of incubation periods of strictly equivalent samples have been performed on many occasions (e.g. Dickinson et al., 1969; Kimberlin & Walker, 1980).

Attempts have also been made to use estimates of titre derived solely from measurements of incubation period to compare samples that are not strictly equivalent (Hunter & Millson, 1964; Prusiner et al., 1980a, b, 1982). This involves the assumption that a dose-response curve established for the starting homogenate could be used as the standard from which to determine the titre from the incubation period, even for samples subjected to different biochemical manipulations.

If certain of the parameters for measuring scrapie infectivity are changed then the estimated titre and the relationship between titre and incubation period are altered. For example, titration of infectivity using an intraperitoneal route of infection rather than an intracerebral route can lead to at least a 1 log_{10} unit decrease in the titre estimate and a prolongation of incubation period for the same dose (i.e. number of infectious units measured) (Kimberlin & Walker, 1978). Several treatments of brain homogenate have previously been found to affect the expected relationship between incubation period and dose of infectivity. Longer incubations for equivalent doses of scrapie infectivity were found after boiling the inoculum (Dickinson & Fraser, 1969). Although Prusiner et al. (1982) found no effect on the titre estimate when inoculum had been heated to temperatures of 100 °C, there was a 1 log_{10} unit difference between...
Fig. 1. Effect of detergents on dose-response curves. Brains from C57BL/6J mice infected intracerebrally with the 139A strain of scrapie were fractionated according to the method of Cohen et al. (1977) as modified by R. A. Somerville, P. A. Merz & R. I. Carp (manuscript in preparation). SPM fractions were treated with detergent and either subjected to differential centrifugation or overlaid on 25 to 55% metrizamide gradients and centrifuged at 80,000 g for 16 h. Aliquots of the following fractions were titrated: brain homogenate, synaptosomal fraction and SPM fraction (△—△); synaptosomal fraction plus S3-14, SPM fraction plus octyl glucoside and overlay from the top of metrizamide gradients after both detergent treatments (O----O); fractions from the gradients after centrifugation and the pellet obtained after octyl glucoside treatment and centrifugation (●⋯●). Samples were titrated by intracerebral injection of 0.03 ml of 10-fold serial dilutions in PBS. Mice were monitored twice weekly from day 130 to day 200 and once weekly until day 280 post-infection for clinical signs of scrapie, the incubation period being designated the time at which definite clinical signs of scrapie were first observed. Histological examination was performed on two occasions to confirm when a clinical diagnosis of scrapie was not clear. Titres were calculated by the Reed–Muench method (Dougherty, 1964) and the dose for each dilution determined. The regression lines indicated for each group of data were calculated for all values with less than 10^2 infectious doses.

the LD50 and the incubation period estimate of titre after treatment at 121 °C. SDS or LiCl treatments of brain homogenate have also produced skewing of the dose-response curve (Kimberlin, 1977). It has also been noted that measurement of infectivity after its partial purification with lysolecithin resulted in intracerebral estimates of titre similar to those obtained before treatment, but intraperitoneal measurements were reduced by 3 log units after treatment (G. C. Millson, personal communication). Here, we present data obtained in biochemical experiments (involving the use of detergents) in which the treatments changed the measured LD50 or altered the relationship between incubation period and dose.

In two experiments on the purification of scrapie infectivity, fractionations were performed to yield synaptosomal and synaptic plasma membrane (SPM) fractions (Fig. 1). Detergent [n-octyl-β-D-glucopyranoside (octyl glucoside) or sulphobetaine 3-14 (S3-14)] was added to these
Table 1. Effects of detergent in the diluent on infectivity titres and incubation periods

<table>
<thead>
<tr>
<th>Dilution</th>
<th>Addition of NLS (0·1%)</th>
<th>No detergent added</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mice/group with scrapie</td>
<td>Incubation period (± S.E.M.)</td>
</tr>
<tr>
<td>10⁻²</td>
<td>6/6 133 ± 0·6</td>
<td>5/5 133 ± 1·2</td>
</tr>
<tr>
<td>10⁻⁵</td>
<td>6/6 142 ± 0·8</td>
<td>6/6 154 ± 0·6</td>
</tr>
<tr>
<td>10⁻⁶</td>
<td>6/6 159 ± 2·7</td>
<td>6/6 181 ± 8·7</td>
</tr>
<tr>
<td>10⁻⁷</td>
<td>6/6 169 ± 10·0</td>
<td>1/5 210</td>
</tr>
<tr>
<td>10⁻⁸</td>
<td>4/6 175 ± 3·14</td>
<td>0/6 --</td>
</tr>
<tr>
<td>Titre§</td>
<td>≥8·3</td>
<td>6·4</td>
</tr>
</tbody>
</table>

(b) Titration of a detergent-treated membrane fraction*

| Dilution | Mice/group with scrapie | Incubation period (± S.E.M.) | P‡ |
| 10⁻²     | 6/6 139 ± 2·0           | 6/6 144 ± 2·0       | NS                   |
| 10⁻⁵     | 4/4 197 ± 9·0           | 0/6 --              | —                    |
| 10⁻⁶     | 5/5 238 ± 5·2           | 0/6 --              | —                    |
| 10⁻⁷     | 0/1 --                 | 0/6 --              | —                    |
| Titre§   | ≥6·5                    | ≥2·5, ≤4·5         | —                    |

* Scrapie-infected brain was fractionated as described for Fig. 1. The synaptosomal fraction was treated with S3-14 and overlaid on a metrizamide gradient. Brain homogenate and a 2 ml fraction from the centre of the gradient were titrated as in Fig. 1. NLS was added to the diluent for the titration of one aliquot of each sample and omitted from the other.

† S.E.M., Standard error of the mean.

‡ Probability that differences in incubation periods at the same dilution occurred by chance (Student's t-test).

NS, Not significant.

§ Titre, log₁₀LD₅₀ per 0·03 ml.

fractions which were then subjected to differential or density gradient centrifugation. Aliquots of these fractions were titrated for scrapie infectivity and the infectious dose at each dilution calculated (Fig. 1). The relationship between dose-response curves and incubation period was compared by analysing three groups of data pooled from these experiments: group 1, before detergent treatment (homogenate, synaptosomal and SPM fractions); group 2, samples containing detergent (membrane fractions containing detergent and samples from the overlay of density gradients where detergent remains after centrifugation); group 3, samples previously exposed to detergent (the pellet obtained after centrifugation and samples from the density gradient). For each group the relation between infectious dose and incubation period was compared. Data in each group were analysed by means of a multiple regression procedure (Cohen & Cohen, 1975), with groups represented by dummy coding. In common with most other scrapie dose-response curves a point in inflection occurred between 10² and 10³ units of infectivity. Since the majority of the values were below 10² units only these data were included in the regression analysis.

There was a significant linear relationship between dose of scrapie and incubation time (Fig. 1) which did not vary with group (r = 0·92, 0·94, 0·98 respectively). Regardless of dosage, significant differences were obtained in incubation period between groups 1 and 2 (P < 0·01) and between groups 1 and 3 (P < 0·001). The mean incubation period for samples containing detergent (group 2) was 11 days longer than for untreated material (group 1) for the same operational doses of infectivity. A 19 day difference was found when untreated fractions (group 1) were compared with fractions previously exposed to detergent (group 3).

In a separate experiment, the effect of the detergent N-lauroyl sarcosinate (NLS) added to the diluent was tested (Table 1). Two samples were titrated each with and without detergent included in the diluent. Titration of a scrapie-infected brain homogenate with 0·1% NLS included in the diluent (phosphate-buffered saline, PBS) resulted in a titre estimate at least 2 log₁₀ units higher than that obtained from the sample which was diluted in PBS alone (Table 1 a). Dilutions to the endpoint were not performed in the samples with unexpectedly high titres. It is probable that a complete LD₅₀ estimate of the dilution series with NLS would give a value
higher than $10^{10}$ infectious units/g. In the second part of this experiment (Table 1b), detergent (S3-14)-treated synaptosomal material obtained from the scrapie brain homogenate was titrated in the presence and absence of NLS. Again, the addition of detergent to the diluent resulted in an LD$_{50}$ estimate at least 100-fold higher than in the sample in which detergent was omitted.

Comparison of the incubation periods of each dilution shows that, whereas at low dilution (Table 1a and b; $10^{-2}$ dilution) the incubation periods are similar despite a 2 log$_{10}$ unit difference in effective dose, the incubation periods at higher dilutions in NLS (Table 1a; $10^{-5}$, $10^{-6}$) were significantly shorter than those obtained in the absence of NLS. Because an endpoint was not reached in two of these titrations it is not possible to compare fully the dose-response curves from this experiment. Assuming the value obtained for the homogenate (Table 1a) with detergent is near to or a little higher than the true LD$_{50}$, it is clear that for similar doses at high dilution there is little difference in incubation period whether detergent is present or absent. However, at low dilution ($10^{-2}$) the incubation period is similar despite a 2 log$_{10}$ unit difference in dose. The detergent-treated membrane fraction showed, similarly to Fig. 1, a prolongation of incubation period for equivalent doses when compared to the homogenate. A few deaths occurred in animals injected with NLS soon after injection (Table 1b) presumably because of the toxic effect of the detergent.

It is clear from these results that the measurement of scrapie infectivity by titration can be profoundly affected by the chemical environment of the infectivity in the inoculum. A priori, chemical treatments may act on the infectious agent itself, for example by destroying it; they may alter the environment of the infectious unit such that its ability to infect the host is changed, and chemicals in the inoculum may act on the host to alter its response to infectious material. In Table 1, the elevated titre observed in the detergent-containing samples could be due to a combination of these factors. Specifically in these experiments, tissue disaggregation could be more effective in the presence of detergents, such that aggregates containing more than one potential infectious unit are dispersed, thereby increasing the effective number of infective units; the ability to infect the injected animal might be enhanced by the presence of detergent, possibly by opening up the blood-brain barrier. In addition, the adherence of infectivity to dilution tubes, syringes, etc. may be reduced in the presence of detergent. It has been found that significant losses of infectivity can occur when inocula are left to stand for a period of 4 h in glass bottles or syringes before injection (A. G. Dickinson, personal communication). Titres of brain homogenates have been elevated by extensive homogenization (Malone et al., 1978) and, similarly, sonication of a brain homogenate at each step in the dilution series elevated the titre 17-fold (Rohwer & Gajdusek, 1980).

The data obtained in these experiments also show changes in the relationship between dose and incubation period. Changes in dose-response curves have been noted in unfractionated tissue after physical or chemical treatments, and have recently been reported for partially purified fractions exposed to sodium deoxycholate (Lax et al., 1983). In one case, treatment of a partially purified fraction with Triton X-100 did not affect the titre estimate or the dose-response curve (Somerville et al., 1980). Two effects of detergent on incubation period were observed in the present experiments. For samples diluted in the presence of NLS the data show that the presence of detergent increases the calculated number of infective units present at low dilutions ($2.5 \times 10^4$ infectious units in the $10^{-2}$ dilution in the absence of detergent and at least $2 \times 10^6$ infectious units in the presence of NLS) without changing the incubation period. In contrast, there are significant differences in incubation period between the samples with and without detergent at higher dilutions of the same starting material. Presumably, these are due primarily to the higher effective dose present in the sample with detergent added to the diluent. Overall, the addition of detergent to the diluent in this experiment increased the number of infective units present in each dilution, altering the relationship between dose, incubation period and the degree of dilution. It would be impossible to predict the differences in titre illustrated here solely from a measurement of incubation period. The second effect produced on dose-response curves is demonstrated in Fig. 1 where a shift in the dose-response curve took place following chemical treatment of the sample, in this case exposure to detergent before dilution. The effects found here are probably comparable to those described by Lax et al. (1983). Since the samples in this
experiment were diluted under identical conditions it is unlikely that the mechanics of the dilution process played a major role in producing the effect shown. Nevertheless, it is possible that significant amounts of detergent in group 2 would be carried through the dilution process so that at low dilutions effects similar to those shown in Table 1 may have applied to a certain extent. Clearly, there was a significant shift in the dose-response curves after treatment, which can only be ascribed to changes in the presentation of the inoculum to the host and a consequential alteration in the pathogenesis of the disease.

In conclusion, addition of detergent to the diluent for scrapie titration enhanced the efficiency of the titration process such that titre estimates at least 100-fold higher were obtained. Biochemical manipulation of the samples by treating with detergents resulted in altered dose-response curves. These two results have fundamental implications for the measurement of scrapie infectivity. Of particular importance will be the re-evaluation of experiments in which various biochemical treatments were intended to inactivate infectivity, since those treatments may have altered the interaction of infectivity with its environment or changed the scrapie agent's ability to infect the host rather than affected the structure or properties of the infectious molecule per se. In addition, estimates of the relative specific activity of partially purified fractions from scrapie-affected brains may not be correct, if the titre in those fractions and the starting homogenate were underestimated to different extents. These results also compromise attempts to estimate titre solely from incubation period. Except where strictly equivalent samples are being compared, assumptions that dose incubation period relationships are constant cannot be made. Accordingly, the effect of any changes in dose-response curve must be accounted for, particularly in biochemical work, before attempting to use incubation periods as a 'quick' assay for scrapie infectivity.

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REFERENCES

SHORT COMMUNICATION


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