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The measured level of prion infectivity varies in a predictable way according to the aggregation state of the infectious agent

Joanna Masel [a,*], Vincent A.A. Jansen [b]

[a] *Wellcome Trust Centre for the Epidemiology of Infectious Disease, Department of Zoology, University of Oxford, South Parks Road, Oxford OX1 3PS, UK*

[b] *School of Biological Sciences, Royal Holloway, University of London, Egham, Surrey TW20 0EX, UK*

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Abstract

Transmissible spongiform encephalopathies are believed to be caused by an infectious form of the prion protein, designated PrP^{Sc}. The concentration of PrP^{Sc} is often poorly correlated to the level of infectivity. Infectivity can be measured in two ways, namely endpoint titration and the incubation time assay, but patterns of infectivity vary depending on which method is used. These discrepancies can be explained by variation in the aggregation state of PrP^{Sc}. Both methods of measuring infectivity are modelled mathematically, and the theoretical results are in agreement with published data. It was found to be theoretically impossible to characterise prion infectivity by a multiple of a single quantity representing 'one prion', no matter how it is measured. Infectivity is instead characterised by both the number and sizes of the PrP^{Sc} aggregates. Apparent discrepancies arise when these complexities are reduced to a single number.

Keywords: Prion disease; Mathematical model; One-hit model; Incubation time assay; Protein aggregation; Amyloid

1. Introduction

It has been hypothesised that the infectious agent in transmissible spongiform encephalopathies may be an abnormal form of the prion protein (PrP), designated PrP^{Sc} [1]. This protein-only or 'prion' hypothesis has been extremely controversial, although it has now become widely accepted. There is often a poor correlation between the concentration of PrP^{Sc} protein and the corresponding number of infectious units [2–5]. This puzzling finding has sometimes been used as evidence against the prion hypothesis [4,6–9].

Theoretical kinetic work has shown that the prion infectious agent must consist, at minimum, of a PrP^{Sc} dimer or higher order oligomer [10]. In agreement with this, target analysis has shown experimentally that the minimum size of the infectious agent is a PrP^{Sc} trimer or tetramer [11–14] or dimer [15]. All prions are therefore aggregates of at least several subunits, but PrP^{Sc} is seen in many heterogeneous forms and is often much more substantially aggregated. Discrepancies between the concentration of PrP^{Sc} and the level of infectivity can be explained by invoking variation in the extent of PrP^{Sc} aggregation. A large number of small aggregates is usually assumed to have a different level of infectivity than a

* Corresponding author. Present address: Department of Biological Sciences, Stanford University, Stanford, CA 94305, USA. Fax: +1-650-725-7745; E-mail: masel@charles.stanford.edu

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smaller number of larger aggregates [16]. Thus, for a constant concentration of PrP^{Sc}, the measured level of infectivity will depend on the extent of aggregation. The number of infectious units is not an absolute physical quantity, related to the number of discrete, uniform infectious particles, but is instead a variable quantity that can be reversibly altered.

To understand the effect of aggregation on infectivity, and what infectivity really means in the prion context, we need to understand how infectivity is measured. The number of infectious units can be measured in two different ways, known as endpoint titration and the incubation time assay. For an endpoint titration, serial dilutions of a preparation are inoculated into animals, and the dilution that will kill half the animals is called an LD_{50} infectious unit [17–19]. The number of LD_{50} infectious units in the original preparation can be calculated from the dilution factor.

Endpoint titration is time-consuming and requires a large number of mice, so an alternative method, known as the incubation time assay, was developed [20]. The incubation time assay exploits the fact that the incubation time in an animal is highly reproducible under certain conditions, and is inversely related to the size of the inoculating dose [21,22]. A standard curve is generated for a given strain of mice and a given route of infection, and is calibrated by endpoint titration. The calibrated standard curve is then used to calculate the number of infectious units from the incubation time, and the results are usually expressed in terms of LD_{50} units.

Unfortunately, these two methods are not always equivalent [6,23–29]. It seems that the calibration curve can shift under certain conditions. For example, prion rods can be disaggregated into much smaller detergent-lipid-protein complexes and liposomes by sonication in the presence of detergents [30]. This leads to a 100-fold increase in infectivity as measured by endpoint titration [25], while it sometimes but not always leads to a mere 10-fold increase in infectivity as measured by the incubation time assay [30]. Similarly, sonication without the use of detergents leads to a 17-fold increase in infectivity as measured by endpoint titration [16], but no change in infectivity as measured by the incubation time assay [31].

Since the aggregation process may be complicated, and is not well understood, there is currently no straightforward definition of what constitutes a single prion particle. There is more than one physical quantity that could reasonably be taken for 'infectivity'. To explain discrepancies between the concentration of PrP^{Sc}, the level of infectivity measured by endpoint titration, and the level of infectivity measured by the incubation time assay, we need to know how measured infectivity depends on the size of the aggregates. In other words, we need to know the measured infectivity of a large number of small aggregates relative to a smaller number of larger aggregates. This is best done using a combination of the experimental data mentioned above and mathematical modelling. On the mathematical side, a suitable theoretical framework to describe prion replication has already been developed [10,32–36]. In this framework, aggregates take the form of macroscopically linear polymers or oligomers, corresponding to experimentally observed prion rods and scrapie-associated fibrils. Polymers or oligomers which contain more than n PrP molecules grow by incorporating new PrP monomers at the polymer ends, at a rate which is independent of the polymer size. Larger polymers eventually break into two smaller polymers, completing the replication cycle. A range of polymer sizes exists in any preparation.

In this paper, we explore what infectivity means within the context of a mathematical model of prion replication. We develop and use this mathematical model to predict how the observed level of infectivity should depend on the sizes of the prion polymers, and relate these predictions to experimental data.

2. Materials, methods and results

2.1. Modelling the incubation time assay

Consider the kinetics of prion replication immediately after inoculation. We follow a kinetic model presented elsewhere [36] and illustrated in Fig. 1. Let y be the total number of infectious PrP^{Sc} polymers of any size. The total number of PrP^{Sc} subunits incorporated into infectious polymers is z. PrP^{Sc} polymers are degraded at rate a, i.e., small polymers are degraded at the same rate as large polymers. A polymer of size i, i.e., containing i PrP^{Sc} subunits,

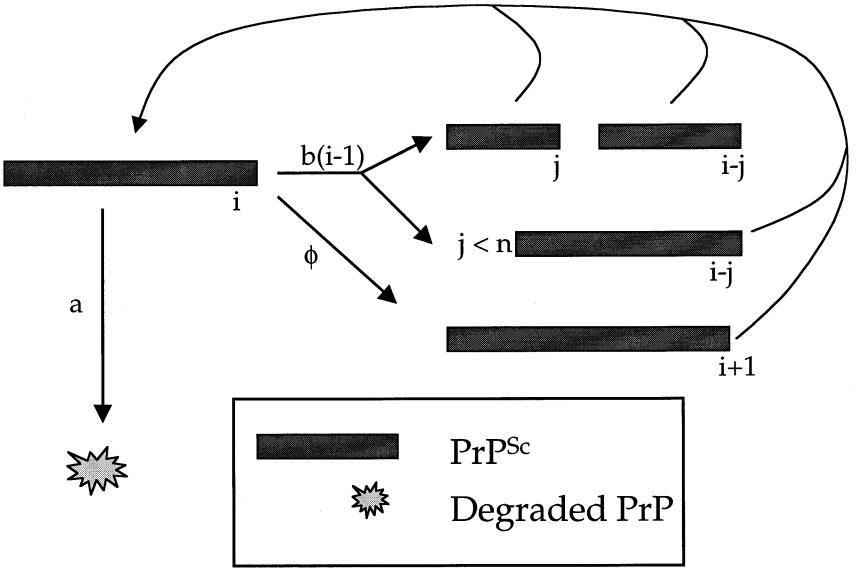

Fig. 1. Kinetic model of the early stages of prion replication, when monomer is not depleted. A polymer of length i is either degraded at rate a, incorporates an additional monomer at rate ϕ, or breaks at rate b at each of the $i-1$ joins along its length. Breakage results in two new polymers, one or both of which may fall apart if their size is smaller than n.

breaks at rate b at each of the $i-1$ joins along its length. Oligomers below the critical size n can be formed by breakage near a polymer end. These oligomers are unstable and disintegrate rapidly into monomers.

There is some controversy over which step is rate-limiting for the incorporation of PrP^C monomers into polymers [34,37]. The alternative nucleated polymerisation and template-assistance hypotheses postulate different levels of dependence on the monomer concentration. In the early stages of prion replication, however, the monomer concentration remains constant, so these two hypotheses are indistinguishable. We can assume that monomers are incorporated into polymers at rate ϕy, where ϕ depends in some way on the monomer concentration. We then obtain the following equations for the growth, degradation and breakage of the polymers, as has been shown elsewhere [36]

$$\begin{aligned}\frac{dy}{dt} &= -ay + bz - (2n-1)by \\ \frac{dz}{dt} &= \phi y - az - n(n-1)by\end{aligned} \tag{1}$$

These equations are linear, and can be solved explicitly. The amount of PrP^{Sc} as a function of time t is given by

$$y(t) = \frac{c_1(r+a)e^{rt} + c_2(r_2+a)e^{r_2 t}}{\phi_0 - bn(n-1)} \tag{2a}$$

$$z(t) = c_1 e^{rt} + c_2 e^{r_2 t} \tag{2b}$$

where

$$r = -a - bn + \frac{b}{2} + \frac{1}{2}\sqrt{b^2 + 4b\phi}$$

$$r_2 = -a - bn + \frac{b}{2} - \frac{1}{2}\sqrt{b^2 + 4b\phi}$$

and c_1 and c_2 are constants specified by the initial conditions. As t becomes large, the second terms in Eqs. 2a and 2b become negligible. A variety of simulations were performed using realistic parameter values [36] and a range of sizes in the initial inoculum. In each case, a value of t much lower than the incubation time was sufficient for the positive eigenvalue r to become dominant. After this point, the number of polymers grows exponentially at the rate given by the dominant eigenvalue r, in agreement with experimental observations of exponential prion growth [2,38,39]. The mean size of the polymers reaches a steady state given by [36]

$$\bar{s} = n - \frac{1}{2} + \sqrt{\frac{\phi}{b} + \frac{1}{4}} \quad (3)$$

and the progress of z is given by

$$z(t) = c_1 e^{rt}$$

The dependence of z on the initial conditions is given by c_1 rather than by $z(0)$. This is a very important point, and constitutes the definition of infectivity measured by the incubation time assay. The incubation time assay measures a quantity proportional to c_1, rather than to either the concentration of PrP^{Sc} $z(0)$ or the number of polymers $y(0)$. The logarithm of measured infectivity will be equal to log c_1 plus a constant. c_1 can be calculated from Eqs. 2a and 2b. Combining this result with Eq. 3 gives us

$$\text{infectivity} \propto c_1 = (z(0) + y(0)(\bar{s} - 2n + 1)) \frac{\bar{s}}{2\bar{s} - 2n + 1} \quad (4)$$

We can see from Eq. 4 that c_1, and hence the result of the incubation time assay, depends both on $z(0)$ and on $y(0)$.

2.2. Modelling endpoint titration

Consider a sample containing y prion polymers which is diluted by a factor of $1/y$, so that diluted preparations contain on average one polymer. Not all the diluted preparations will contain exactly one prion polymer. Instead, some of the diluted preparations will not contain any prions, and thus will never lead to infection, while other preparations will contain more than one prion polymer. Assume for now that the probability that a particular polymer is present in a specific dilution is independent of the presence of other polymers in that dilution. Then for a large number of polymers y in the initial preparation, the number of polymers present in a specific dilution by factor d will follow a Poisson distribution, i.e., the probability that i polymers are present is given by $(yd)^i/(i!e^{yd})$. A single polymer may initiate infection with probability p, or it may be cleared with probability $1-p$. Assume that each polymer in an inoculum is independently capable of initiating infection. Then for an inoculum diluted by factor d from a preparation originally containing y polymers, we find that

$$\text{probability of infection} = \sum_{i=0}^{\infty} \frac{(yd)^i}{i!e^{yd}} (1 - (1-p)^i)$$

Note that the dilution factor d is not given here on a log scale, as is normally the case. Since the sum of a Poisson distribution is always equal to one, we derive

$$\text{probability of infection} = 1 - \sum_{i=0}^{\infty} \frac{(yd)^i (1-p)^i e^{-ydp}}{i!e^{yd(1-p)}} = 1 - e^{-ydp} \quad (5)$$

This is the basic one-hit model [40], which has been used to calculate prion infectivity [41]. The basic one-hit model predicts that the proportion of animals infected as a function of the dilution factor should have the form given by Eq. 5. This is a falsifiable prediction that could be directly tested, given a large enough set of experimental data. An alternative hypothesis is that the one-hit model is not correct because prions work co-operatively rather than independently, and so prions can be 'diluted out' [41]. In this case, a plot of ln(1-proportion of animals infected) vs. the dilution factor would not give the straight line through the origin predicted by Eq. 5, and would instead curve to fall more steeply at high doses.

Setting Eq. 5 equal to 0.5, we find that endpoint titration gives

$$\text{infectivity}(LD_{50}\text{units}) = \frac{py}{\ln 2} \quad (6)$$

The number of LD_{50} units is therefore proportional to the number of aggregates in a preparation. It is not directly related to the size of the aggregates, but may be indirectly related via the probability that a single polymer will initiate infection p. If p is independent of the polymer size, then Eqs. 5 and 6 are valid as they stand. A more likely scenario is that the probability of infection $p(i)$ following inoculation by a single polymer depends on the polymer length i. In this case, the probability of infection resulting from a polymer of length i is given by $1 - e^{ydf(i)p(i)}$ where $f(i)$

is the frequency of polymers of length i. Overall, we find that

probability of infection =

$$1-\prod_i e^{-ydf(i)p(i)} = 1-e^{-yd\sum_i f(i)p(i)}$$

In other words, Eqs. 5 and 6 still hold, but now p is given by the weighted mean probability

$$p = \sum_i f(i)p(i)$$

From Eq. 6, we know that the number of polymers y' corresponding to one LD_{50} unit is equal to $(\ln 2)/p$. The number of PrP^{Sc} molecules z' corresponding to one LD_{50} unit, as measured by intracerebral inoculation, has been estimated as 10^4–10^5 [42], 10^4 [43], 10^5 [1,44,45] and $\geq 10^5$ [46]. If we know the mean size of polymers in the inoculum $s(0) = z'/y'$, we can then calculate

$$p = \frac{s(0)\ln 2}{z'}$$

For an inoculum consisting of prion rods, whose mean size has been estimated as 1000 PrP molecules [47], we take the consensus estimate of $z' = 10^5$, and calculate that $p = 0.007$. Obviously, since $s(0)$ and z' cannot currently be measured very accurately, this estimate of p is subject to substantial error.

We have assumed until now that the probability of a polymer being present in a particular dilution is independent of the presence of other polymers in that dilution. This may not be true. Polymers might clump together in the inoculum to form unstructured aggregates of multiple macroscopically linear polymers or prion rods. Our analysis is still valid if p is taken to represent the weighted mean probability of infection after inoculation with a single aggregate of polymers, and $s(0)$ is taken to represent the mean sum of the polymer sizes within such an aggregate. If polymers clump together, then the correct value of $s(0)$, and hence of p, will be substantially larger. Our estimate of $p = 0.007$ should be taken as a lower limit on p. The upper limit of $p = 1$ sets the upper limit for the mean size $s(0)$ of the aggregates of polymers to $z'/\ln 2 \approx 10^5$, equivalent to 100 prion rods each containing an average of 1000 PrP^{Sc} subunits. If this upper limit is approached, then it is reasonable to approximate $p(i)$ as constant.

Alterations in the aggregation state may occur during the dilution process itself, as polymers clump together, are broken up and/or dissociation occurs from the polymer ends. The speed with which the dilution protocol is followed, and how long dilutions stand on the bench before inoculation would then affect the results of endpoint titration. In agreement with this, it has been found that significant losses of infectivity measured by endpoint titration can occur when inocula are left to stand for a period of 4 h in glass bottles or syringes before injection [28].

Progressive dilution is likely to favour unclumping of aggregates and to tip the balance of polymer breakage and end-to-end polymer annealing in favour of breakage. Prion aggregates in more dilute preparations may therefore have a smaller mean size. In one study, the mean size of aggregates in a dilute preparation was found to be around 1000 [48]. This could mean either that no clumping of rods occurs so that the mean aggregate size is equal to the mean rod size, or that both rod size and the degree of clumping are low in a dilute preparation. If the mean aggregate size progressively decreases during successive dilutions, then the term yp in Eq. 5 will not stay constant for each dilution in an endpoint titration. A plot of ln(1−proportion of animals infected) vs. the dilution factor would then not be a straight line, and would instead curve to fall less steeply at high doses. This curve deviates from the straight line predicted by Eq. 5 in the opposite way to the case when prions act co-operatively and can be 'diluted out'.

2.3. Comparison with data

In this section we use the mathematical models presented above to explain data showing that sonication leads to a greater increase in titre as measured by endpoint titration than as measured by the incubation time assay. Before sonication, prion rods contain PrP^{Sc} in a fairly aggregated form of around 1000 PrP^{Sc} molecules per rod [47]. Clumps of these rods are even more aggregated. After sonication, liposomes contain only 2–4 PrP^{Sc} molecules on average [30]. This figure underestimates the mean size of the prion polymers in the liposomes, since some lipo-

somes may contain no functional prions. The small size of the liposomes, however, ensures that the mean size of prion polymers in liposomes is small.

Some PrP^{Sc} may irreversibly lose its infectious conformation during sonication. We assume for now that this loss is negligible, and that $z(0)$ therefore stays fixed while the mean polymer size is reduced. We also assume that the presence of liposomes has no effect on the speed or probability of infection other than through the indirect effect on polymer size. It should be noted that this a very stringent assumption, especially since the whole infection process might well occur at the membrane. Nevertheless, this assumption makes a suitable starting point for the mathematical analysis, which can then be considered a limiting case. We can then derive from Eq. 4 the expression for infectivity measured by the incubation time assay

$$\log \text{infectivity} = \log\left(1 + \frac{\bar{s} - 2n + 1}{s(0)}\right) + \text{constant} \quad (7)$$

where $s(0)$ is the mean size of polymers in the inoculum, i.e., $s(0) = z(0)/y(0)$ and $\bar{s}$ is the mean polymer size at the site of replication in the host. This equation can be used to calculate the increase in infectivity when liposomes are formed, as measured by the incubation time assay. This is shown in Fig. 2. Infectivity as measured by endpoint titration can be calculated from Eq. 6 as

$$\log \text{infectivity} = \log p - \log s(0) + \text{constant} \quad (8)$$

where in this case $s(0)$ is equal to the mean size of aggregates of polymers. The behaviour of this equation depends on how p varies with aggregate size. There are two main factors that are likely to influence this. The first factor is the intrinsic kinetics of prion replication immediately after inoculation. The model represented by Eq. 1 can be reformulated in a stochastic form to describe this process. In this stochastic model, the longer a polymer is, the more likely it is to break into two viable polymers before it is degraded. In addition, an aggregate of multiple polymers may provide multiple opportunities for infection. The probability of infection by a single aggregate therefore increases in some way with size. The second factor is that prions do not replicate uniformly everywhere in the host. To successfully initiate infection, a prion or its descendants must be transported to a highly effective site of replication. This transport may occur by diffusion, or it may be more actively mediated. In either case, a small polymer is more likely to be transported quickly, and is therefore more likely to cause infection than a large polymer.

It is not clear a priori what the balance between these two factors will be, and so p may either increase or decrease with polymer size. We consider the simplest case first, namely that p does not vary greatly, such that variation in $\log p$ is much less than variation in $\log s(0)$. This case is illustrated in Fig. 2. The rise in titre measured by endpoint titration depends only on the size of the prion aggregates before and after sonication into liposomes, and is therefore independent of the host. In contrast, the extent of the increase measured by the incubation time assay also depends on the minimum polymer size n and the mean polymer size $\bar{s}$ during prion replication at the site of inoculation, and is therefore dependent on the host animal, as shown in Fig. 2a. In practice, the incubation time assay is fairly insensitive to the size of the prion aggregates before sonication, unlike endpoint titration, as shown in Fig. 2b.

The calculated rise in titre is always larger as measured by endpoint titration than as measured by the incubation time assay, in agreement with the data. This makes intuitive sense. If p is constant, then two small polymers of size m are twice as infectious as a large polymer of size $2m$ in an endpoint titration. During the incubation time assay, it takes some time for the large polymer to break into two small polymers, but less time than it would for a small polymer to first grow and then break. The two smaller polymers are therefore less than twice as infectious as a single large polymer. Changes in size have less effect on the incubation time assay.

Now consider the case where the mean probability p of infection by a single aggregate varies according to the mean aggregate size. If p decreases with increasing aggregate size, then the line would shift further away from that of the incubation time assay. This case is consistent with the data, but seems intuitively unlikely. A large polymer may be less likely to be transported, but it should readily break to form multiple small polymers which will instead be trans-

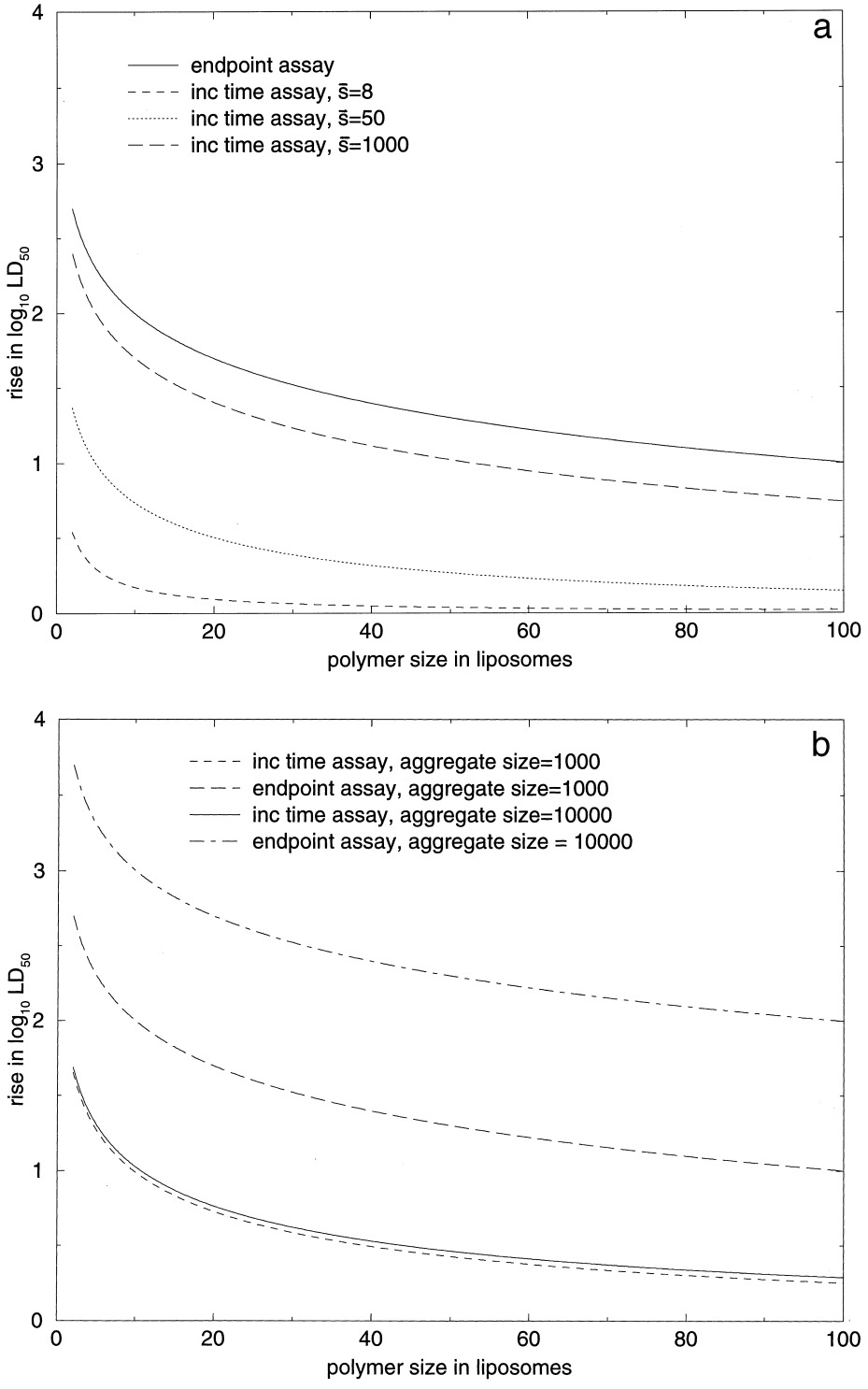

Fig. 2. The extent of the increase in titre is plotted against the size of the prion aggregates after sonication, according to Eqs. 7 and 8. The term p in Eq. 8 is constant. Prion rods are not clumped into aggregates of polymers. The endpoint titration assay shows a larger rise in titre than the incubation time assay. (a) The initial size of the prion rods is taken as 1000 PrP^{Sc} subunits. The incubation time assay shows a smaller rise in titre when the mean polymer size during in vivo replication $\bar{s}$ is small. (b) The initial size of the prion rods makes little difference to the incubation time assay, but substantially affects the endpoint titration. In this graph, $\bar{s}=100$. For both graphs, $n=2$.

ported. It is hard to imagine that the likelihood of immediate transportation will dominate the likelihood of breakage of a large polymer or dissociation of an aggregate into its constituent polymers followed by multiple opportunities for transportation.

If p increases with increasing polymer size, then the line for endpoint titration in Fig. 2 would shift closer to or past the line for the incubation time assay. In the extreme case with p approximately proportional to polymer size there would be no increase in titre at all. We know from the experimental data that titre does increase, and that the increase in titre is always greater as measured by endpoint titration than as measured by the incubation time assay, so we know that the endpoint titration curve in Fig. 2 cannot shift very far downwards. From the data, we can therefore conclude that any increase in p must be relatively small compared to an increase in the size of the prion aggregates. Eq. 7 must vary less with $s(0)$ than Eq. 8.

Following disaggregation into liposomes, an increase in titre is sometimes, but not always, seen using the incubation time assay [30]. This can be explained by the shape of the graphs in Fig. 2. The incubation time assay shows very little increase in titre when the size of the aggregates in the liposomes is significantly greater than the mean polymer length $\bar{s}$ during host replication. This small increase in titre could easily be offset by the irreversible inactivation of prions during sonication. When the prion aggregates in the liposomes become very small, the titre rises much more dramatically, and the increase may therefore be noticed. If the degree of aggregation changed slightly from one liposome preparation to another within a critical region, substantial variation in the incubation time could be seen. The location of this critical region is specified by $\bar{s}$, a parameter which is at least partly determined by the host. We therefore predict that different routes of inoculation, different strains of mice and different levels of PrP might all influence the location of this critical region.

In summary, our predictions are in qualitative agreement with the experimental data. Quantitative comparisons are limited by the small quantity of data available relative to the number of parameters in the models, and by the numerous simplifying assumptions that have been made.

3. Discussion

Prions, as unconventional infectious agents, cannot be characterised merely by a single number, no matter how it is measured. The infectivity of a given preparation should be specified by both the number of prion polymers and their mean size, if not by the entire polymer size distribution. It is possible to define a minimally infectious particle, which may be a PrP^{Sc} dimer, trimer or tetramer, but prion preparations are not simple integer multiples of this minimally infectious particle. It may not be correct to think of a long prion rod as composed of discrete minimally infectious particles. When a sample is subjected to simple manipulation which changes the aggregation state, the number of infectious units may not stay constant when only a single measurement is used. This poses a problem for both endpoint titration and the incubation time assay, and highlights the need for improved techniques that can accurately characterise amyloid sizes.

Endpoint titration is not always superior to the incubation time assay. For example, a substantial increase in titre measured by endpoint titration has been noted following especially vigorous homogenisation [49] or sonication [16]. Variation in the extent of homogenisation could be a source of error in some experiments, and could be minimised using the incubation time assay. Likewise, variation in the dilution protocol and the time before injection may also lead to significant error in endpoint titration experiments [28]. Understanding the basis of the differences between the two assays can help decide when it is worth the additional resources to perform an endpoint titration.

The comparison between the two assays can also yield information. For example, inocula taken from the spleen lead to a longer incubation period than the same number of LD_{50} units taken from the brain [22,27]. This may be because other components of the tissues alter the probability or speed of infection. Alternatively, it may be because the mean polymer size in the spleen is smaller.

Similarly, the level of infectivity measured by the incubation time assay rises faster than either the level of PrP^{Sc} in enriched fractions [2] or the level of proteinase K-resistant PrP [38] during the natural course

of infection. This may be because samples taken early in infection contain a higher ratio of other brain material to infectivity. The high level of other brain material may promote a greater non-specific response, causing infection to be cleared more effectively and therefore proceed more slowly in the test animals. Infectivity present early in infection is therefore underestimated, and the rate of increase of the infectivity is overestimated [36]. Alternatively, the mean polymer size may be progressively reduced during the natural course of infection, perhaps because small polymers with high breakage rates tend to grow faster than larger polymers, and therefore gradually come to dominate [36]. Repeating this experiment using endpoint titration would yield additional information. According to the second hypothesis, infectivity should rise still more rapidly when measured by endpoint titration. This is not necessarily the case under the first hypothesis. Hopefully, new biophysical techniques such as fluorescence correlation spectroscopy will be able to directly test the role of aggregate sizes in such phenomena.

We have described one reason why endpoint titration and the incubation time assay might give divergent results. It is also possible that chemical and/or heat treatments might modify the infectious agent in some way other than the extent of aggregation. Modified prions or inorganic prion templates [50] might be slower to initiate infection and may encounter something analogous to a species barrier. This can explain why many inactivation experiments show that titre is reduced more as measured by incubation time assay than as measured by endpoint titration [6,23,26,29]. It is also possible that smaller prions are more likely to survive the inactivation procedures than larger polymers, providing an alternative explanation for this data. The two explanations for the differences between the assays are not mutually exclusive, and each may be more or less relevant to specific experiments.

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