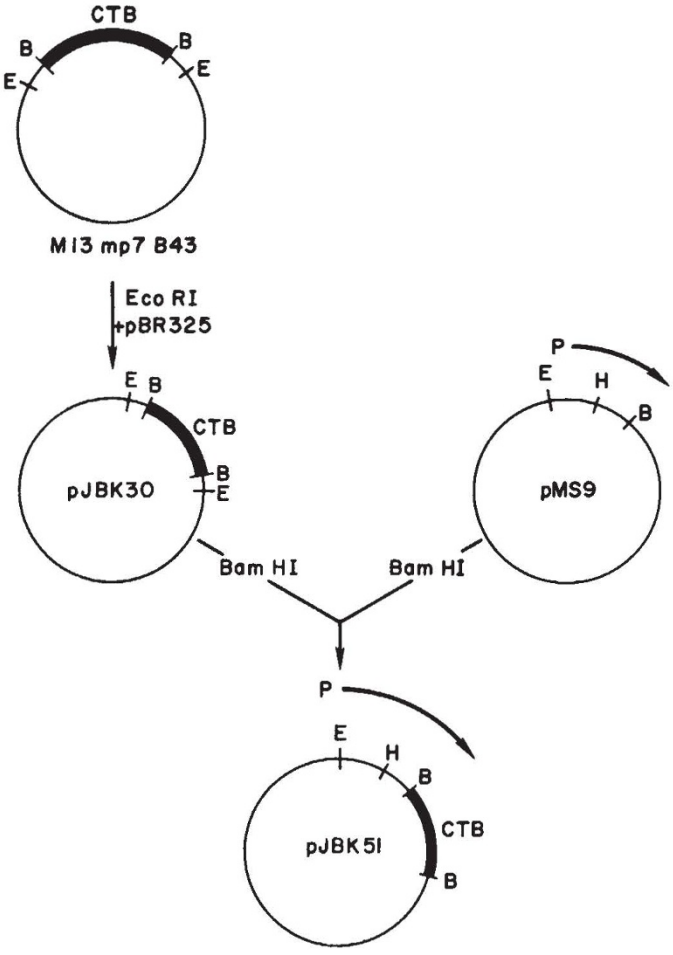

Fig. 4 Construction of *V. cholerae* JBK70 (pJBK51), an A^-B^+ derivative. During DNA sequencing studies of cholera toxin[13] a 1.3 kb *Hpa*II fragment containing the A_2 and B, but not the A_1 subunit genes, was cloned into the M13 mp7 vector, resulting in M13 mp7 B43. The placement in the M13 vector positioned *Bam*HI and *Eco*RI sites adjacent to this fragment which was subsequently cloned into pBR325, yielding pJBK30. The *Bam*HI fragment from pJBK30 was then cloned into pMS9, which contains the trp promoter from *Serratia marcescens*. This vector consists of a pBR32R vector plus 94 bp of *S. marcescens* DNA containing the promoter, but no attenuator region or ribosome binding site (ref. 26 and C. Yanofsky, personal communication). A putative ribosome binding site for the cholera toxin B subunit is found in the structural gene for the A_2 subunit[13] and when the *Bam*HI fragment from pJBK30 was cloned into the *Bam*HI site of pMS9, ~50% of the resulting clones produced B subunit (as measured by GM_1 ELISA), reflecting the orientation of the insert. The resulting plasmid, pJBK51, was mobilized into *V. cholerae* JBK70 by pRK2013 to produce an attenuated *V. cholerae* strain producing B subunit only.

Volunteer studies indicate that an antibacterial response is essential for immunity[4], so immunization with the non-toxinogenic strain may be sufficient for protection against pathogenic toxinogenic *V. cholerae*. Inhibition of the initial colonization of the vibrios along the mucosa of the small bowel is apparently the key immune mechanism in the protection that follows natural infection. Nevertheless, as antibodies to cholera toxin work synergistically in animal models to enhance immunity, a plasmid has been constructed which produces only the nontoxic B subunit (Fig. 4). This plasmid has been introduced into *V. cholerae* JBK70, after which it was found to produce B antigen but not holotoxin. A previously studied A^-B^+ *V. cholerae* strain, Texas Star-SR, attenuated by multiple rounds of nitrosoguanidine mutagenesis[16], stimulated infrequent and meagre antitoxin responses that appeared to be unrelated to successful protection against challenge with pathogenic *V. cholerae*[9]. Attenuated vaccine strains derived by such methods, however, suffer from a number of disadvantages: (1) the precise genetic mutation is unknown so the theoretical risk of reversion to toxinogenicity remains; (2) nitrosoguanidine may induce other unrecognized mutations affecting other antigens that contribute to immunity. The method we have described is free of these disadvantages as it eliminates the possibility of reversion to toxinogenicity, due to the complete deletion of the toxin genes, while it leaves unaffected all other antigens important for immunity. Clinical studies are underway at the Center for Vaccine Development to assess the safety and efficacy in man of the attenuated *V. cholerae* strain JBK70.

We thank Anne O. Summers, Charles Yanofsky, Walt Dallas and Ron Gill for providing plasmids, John P. Craig for rabbit skin permeability factor assays, Sue Trinker, Jane Michalski and Yu Lim for technical help and Jorge Flores for helpful discussion and criticisms. This work was supported by NIH grant R22AI19716 and contract N01AI12666.

Received 22 August 1983; accepted 19 January 1984.

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Scrapie infectious agent is virus-like in size and susceptibility to inactivation

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The virions of all known viruses are composed of small amounts of genomic nucleic acid enveloped by proteins and other macromolecules. The aetiological agents of scrapie disease and the other subacute spongiform virus encephalopathies (SSVE), a group of slow, fatal degenerative diseases of the central nervous system, are, based on their resistance to sterilization and on indirect measurements suggesting subviral size, thought to have non-viral structures (see refs 1–3 for reviews). The kinetic studies reported here demonstrate that scrapie's resistance to many inactivants is limited to small subpopulations of the total infectivity, the majority population being highly sensitive to inactivation. Moreover, control inactivations of conven-

tional viruses provide examples of both scrapie-like resistant subpopulations and complete insensitivity to virucidal agents, especially when those viruses, like scrapie, are suspended in hamster brain homogenate. Virus controls further establish that the ability of the scrapie agent to penetrate dilute agarose–acrylamide electrophoretic gels[4,5] is shared by conventional viruses. Direct comparison of scrapie's resistance to ionizing radiation with the resistances of other viruses places scrapie with the smaller viruses, as opposed to requiring a subviral size as claimed[6–10].

Scrapie infectivity is refractory to sterilization by many potent virucidal agents. However, since sterilization requires total destruction of infectivity, it measures only the most resistant subpopulation of a virus. In contrast, structural inferences must be deduced from the behaviour of the majority population of a virus as revealed in the initial rate of inactivation. All methods of quantitation of scrapie titres rely ultimately on end point dilution titration in animals, which, as typically used, can distinguish, at best, a $10^{0.5}$ difference in titre between two samples[11]. It is thus difficult to establish sensitivity of the scrapie agent to any inactivant that kills less than 90% even though it may be very active against the majority population. To be detected, a scrapie inactivation must be sufficiently first order in character and extensive enough to permit extrapolation to time zero.

Past scrapie inactivations have usually been conducted on crude brain suspensions. To control for the effects of brain homogenate on the inactivation kinetics, highly purified bacteriophages (ΦX174, PM2, λ and fd) were added to normal hamster brain homogenate and to phosphate-buffered physiological saline *p*H 7.2 (PBS) without brain homogenate and subjected to inactivation procedures identical to those for scrapie.

The majority of the scrapie infectivity was rapidly inactivated by 0.525% hypochlorite (Fig. 1*a*) and 0.01 M sodium metaperiodate *p*H 3.6 (Fig. 1*b*), leaving resistant subpopulations of 0.1–0.01%. Resistant subpopulations were also observed in the inactivations of bacteriophages ΦX174, fd and, in the case of periodate, phage λ, though only in the presence of 10% brain homogenate. The extent of the initial inactivation of scrapie infectivity by 2% aqueous iodine (Fig. 2*a*) is greater in 1% than in 10% brain homogenate, again indicating the role of brain homogenate in the apparent stability of the scrapie agent. (A better interpretation of the apparent increase in scrapie titre in the 1% brain sample is that both the 1% and 10% inactivations have plateaued within 15 min at a value approximated by the mean of the 15, 30 and 60 min points.)

The total extent of inactivation of the scrapie agent by 0.5% aqueous phenol (Fig. 2*b*), 3% hydrogen peroxide (Fig. 2*c*), or 0.1% potassium permanganate (Fig. 2*d*) is ≤90% even after 60 minutes of exposure. Therefore, the initial activation rate cannot be determined by these methods. Scrapie may undergo a rapid but limited initial inactivation like that observed for phage λ in the presence of 3% hydrogen perioxide and brain homogenate (Fig. 2*c*), or scrapie may be only slowly inactivated. However, even slow inactivation would not necessarily constitute a unique scrapie-specific property as evidenced by the unexpected insensitivity of bacteriophage ΦX174 to aqueous phenol (Fig. 2*b*).

Infectivity has been recovered from scrapie- and other SSVE-infected tissue specimens stored for months in formaldehyde solution[12–17]. In contrast, kinetic measurement reveals that 98% of the scrapie infectivity in a 10% brain homogenate is destroyed during the first 4 h of exposure to 10% formaldehyde solution (Fig. 3). Other viruses have shown remarkable resistance to complete inactivation by formaldehyde[18–20] and experience with vaccine production methods has revealed the sensitivity of the formaldehyde inactivation to the presence of contaminating substances[21]. The scrapie inactivation may be affected in a similar manner.

It has been reported that scrapie cannot be inactivated by boiling or even by autoclaving (see ref. 22 for review). However, in virus-controlled kinetic studies in which the sample temperatures were constantly monitored by an embedded thermistor, the vast majority of the scrapie infectivity was destroyed immediately on exposure to temperatures of 100 °C or above[22]. Thermal resistance reposed in small subpopulations, less than

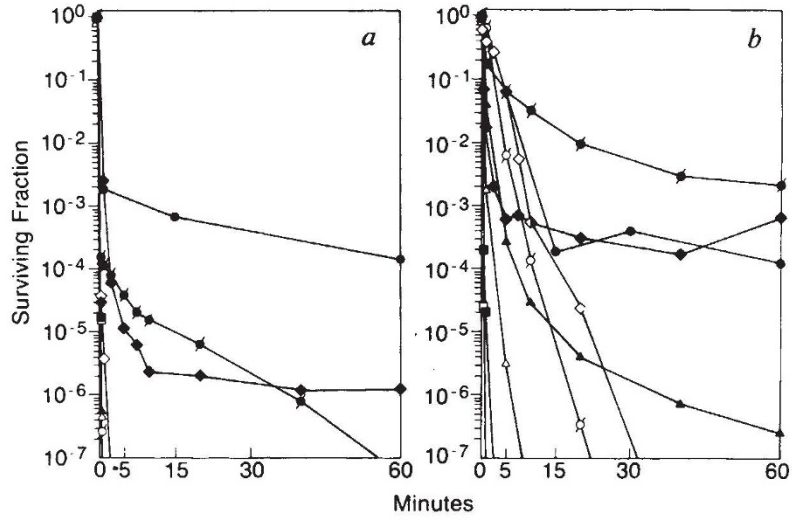

Fig. 1 Inactivation of scrapie and four control viruses by *a*, hypochlorite ion; *b*, periodate ions. Symbols: ●, scrapie; ●, ∅, ΦX174am3; ■, □, PM2; ▲, △, λ; ◆, ◇, fd. Closed symbols represent inactivation in 10% brain homogenate. Open symbols represent PBS.

Methods: Twenty per cent suspension of scrapie-infected (early clinical disease; hamster strain 263K obtained from R. Kimberlin[51,52] and passaged twice in this laboratory) and normal hamster brains were prepared in PBS *p*H 7.4 by exhaustive sonication and low-speed centrifugation (International PR-2, infected, and Sorvall HS4, uninfected, 2,000 r.p.m., 4 °C, 15 min), discarding the pellet. The scrapie suspension used in *a* and in Fig. 2*b–d* was stored at 4 °C, used the same day and titred 6.3×10^8 ID_{50} units ml^{-1}. That used in *b*, and in Figs 2*a* and 3 was frozen at −70 °C for 24 h, thawed and resonicated just before use and titred 1.59×10^9 ID_{50} units ml^{-1}. The several pools of normal brain homogenate used were stored at −30 °C until just before use. Filamentous bacteriophage fd and the lipid-containing phage PM2 were obtained from the American Type Culture Collection, along with their respective hosts, *Escherichia coli* strain Hfr and *Alteromonas espejiani*. Phage λ strain Y1 and host *E. coli* N720 were obtained from Howard Nash. Phage ΦX174am3 and host *E. coli* CQ2 were obtained from Clyde Hutchison. A λ resistant mutant of *E. coli* CQ2, designated CQ2 lamba^r, was selected from a λ-infected culture of CQ2. All phages could be titrated in the presence of one another. Purified, high-titre stocks, 10^{12}–10^{14} plaque forming units (PFU) per ml were prepared by CsCl bandings of polyethylene glycol precipitates of low speed supernatants of naturally lysed cultures of PM2, polyethelyene glycol precipitates of whole cell supernatants of productive stationary phase cultures of fd, virus pellets of low speed supernatants of chloroform lysed, lytic infections of λ and lysozyme lysates of cell concentrates of lysis-deficient ΦX174am3 grown on a non-permissive host. Small aliquots (≤1% of the total volume) of the phages were added to 20% normal hamster brain homogenate and PBS to give final titres approximating that of the scrapie agent, 10^9 PFU ml^{-1}. Brain homogenate alone did not inactivate the phages. Inactivations were conducted by sampling the 20% homogenate and, in the case of the phage experiments, the PBS suspension, for a time zero point. An equal volume of freshly prepared test reagent (reagent grade chemicals except *a* and Fig. 2*b*) was then added to give the final concentrations given in the figure legends. The scrapie samples were stirred continuously with magnetic stir bars is small beakers. The phage suspensions, except where noted, were continuously mixed in a Buchler vortex evaporator without evaporation. Samples were taken at the times indicated and immediately diluted serially in PBS (scrapie) or tryptone broth (phage) and inoculated into hamsters (scrapie) or onto agar petri plates with indicator bacteria (phage). Each of four weanling, female, golden Syrian hamsters (Charles River-Lakeview Hamstery) was inoculated intracerebrally in the left hemisphere with 0.05 ml at each dilution. 10–15 min were required to inoculate the complete dilution series in the case of scrapie, and, in the case of the phages, 3–8 min depending on the number of different phages being titrated. However, in every case the reaction mixture was diluted 100–1,000-fold and greater within moments of sampling, thereby effectively stopping the reaction. The inoculated hamsters were observed for 308 days (*a* and Figs 2*b–d*) or 291 days (*b* and Figs 2*a*, 3) and scored positive for scrapie only after histological confirmation of spongiform change in coded brain sections. Titres were calculated by the method of Reed and Muench[53]. *a*, 0.525% (wt/vol) sodium hypochlorite, a 1/10 dilution of Rainbow Bleach (J. L. Prescott Co., Passaic); *b*, 0.01 M sodium metaperiodate, 0.1 M sodium acetate (*p*H 3.6). The periodate solution and reaction mixtures were protected from light. Due to the low *p*H of the periodate solution a precipitate developed which was resuspended by trituration before inoculation.

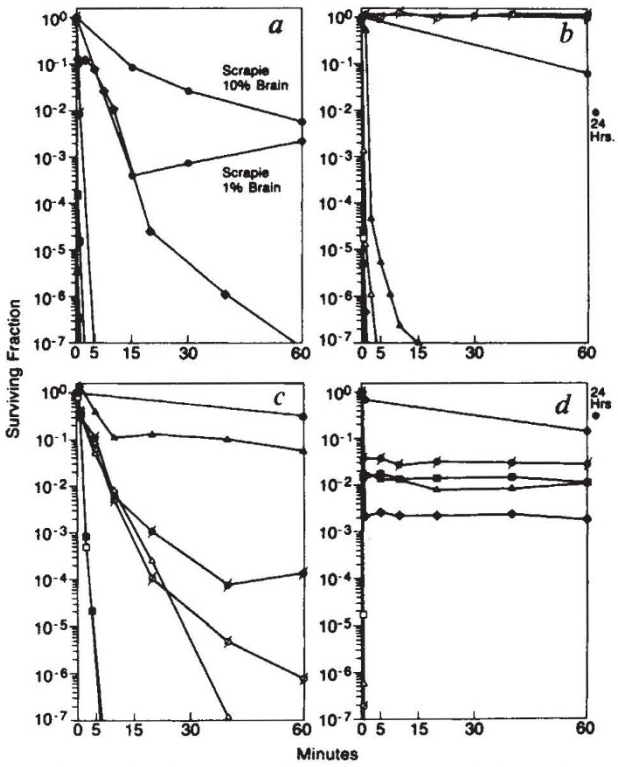

Fig. 2 Inactivation of scrapie and four control viruses by: *a*, molecular iodine; *b*, aqueous phenol; *c*, hydrogen peroxide; *d*, permanganate ions. *a*, 2% (wt/vol) iodine, 2.4% (wt/vol) sodium iodide. Scrapie was inactivated in both 10% and 1% suspensions of brain. Phage inactivations were in 10% brain homogenate or PBS. *b*, 0.5% (wt/vol) phenol (10% Lysol, National Laboratories, Montvale). *c*, 3% (vol/vol) hydrogen peroxide. The hydrogen peroxide reaction with brain homogenate foams making accurate volumetric sampling difficult. For this reason the volumes of the phage samples were checked gravimetrically. The increase in λ titre observed after 1 min of exposure to 3% hydrogen peroxide and brain homogenate was observed in three replicate experiments. *d*, 0.1% (wt/vol) potassium permanganate. An obvious chemical reaction as indicated by a colour change and the evolution of a distinctive odour occurs immediately on mixing permanganate with brain homogenate. Symbols: ●, scrapie; ●, ∅, ΦX174am3; ■, □, PM2; ▲, △, λ; ◆, ◇ fd. Closed symbols denote inactivations in 10% brain homogenate except in *a* where they also represent 1% brain homogenate. Open symbols represent PBS.

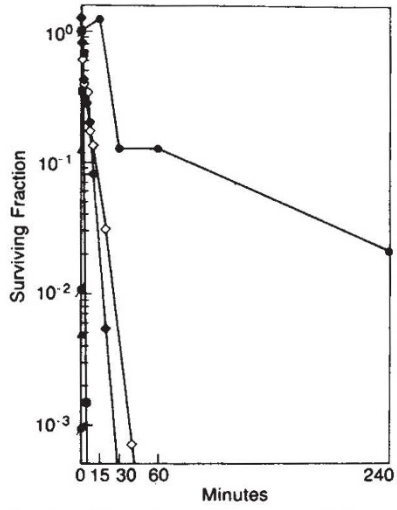

Fig. 3 Inactivation of scrapie and four control viruses by formaldehyde solution. Samples were mixed 1 : 1 with 20% formaldehyde solution F-79 (Fisher Scientific) for final concentrations of 3.7% wt/wt formaldehyde, 1 to 1.5% methanol. Symbols: ●, scrapie; ●, ∅, ΦX174am3; ■, □, PM2; ▲, △, λ; ◆, ◇, fd. Closed symbols denote inactivations in 10% brain. Open symbols denote PBS.

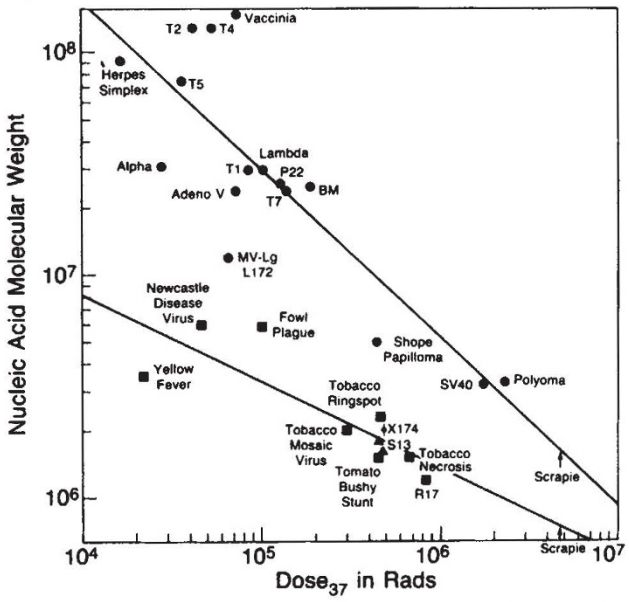

Fig. 4 Radiosensitivity of viruses as a function of the molecular weight of their nucleic acids. A literature search was conducted for all known inactivation rate constants of conventional viruses inactivated by ionizing radiation under conditions compatible with the scrapie data (first reference given below). These data, expressed as $dose_{37}$, the reciprocal of the inactivation rate constant, are plotted against the molecular weight of the nucleic acid component of the virus, obtained where possible from sequence or restriction enzyme data (second reference given below). ●, Double-stranded DNA viruses; ■, single-stranded RNA viruses; ▲, single-stranded DNA viruses. Separate regression lines have been drawn through the double-stranded and single-stranded virus data. References: polyoma[9,54], SV40[55,56], shope papilloma[27,57], phage MV-Lg-L172[58,58], phage T7[27,57], phage BM[27,27], phage P22[27,57], adenovirus 5[27,59], phage λ[27,57], phage T1[8,57], phage α[60,57], phage T5[28,57], herpes simplex[8,61], phage T2[9,27], phage T4[27,27], vaccinia[27,62], phage R17[27,57], tobacco necrosis virus[28,28], tomato bushy stunt[27,27], tomato mosaic virus[63,27], tomato ringspot virus[27,64], yellow fever virus[8,57], fowl plague virus[28,65], Newcastle disease virus[27,57], phage ΦX174[27,27], phage S13[9,27], scrapie (see text).

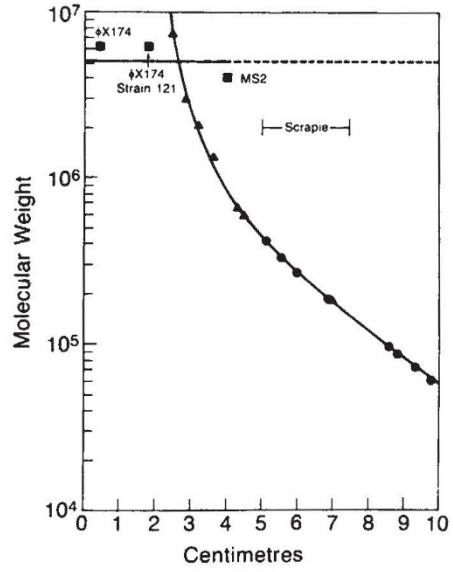

Fig. 5 Electrophoretic migration of viruses and nucleic acids as a function of their molecular weights. Three viruses of almost identical molecular weight and size (■) were co-electrophoresed with *Hin*dIII phage λ DNA restriction fragments (▲) and *Hae*III ΦX174 DNA restriction fragments (●) (restriction markers were obtained from Bethesda Research Laboratories). Gels were 0.5% agarose, 2.2% acrylamide, Tris acetate, *p*H 7.2 (4, 5). The region from which scrapie infectivity is recovered in this same gel system is indicated (ref. 4 and M. T. Borras and R.G.R., unpublished).

0.0001% of the total at 121 °C, and so cannot be used to draw structural conclusions about the agent.

The small size, 60,000–150,000 daltons of nucleic acid, inferred from scrapie's resistance to inactivation by ionizing radiation established[7–10,23,24] and continues to foster expectations of an unconventional structure. The 'target' theory used in this calculation was intended as a mechanistic description of the physical process of inactivation by ionizing radiation[25]; the size calculation itself depends heavily on parameters that are difficult to determine[9,18,25]. Other than nucleic acid strandedness, the only independent variables in a size comparison between viruses are the inactivation rate constants themselves[25]. A better size estimate can be obtained by simply comparing the scrapie inactivation rate constant directly with the inactivation rate constants of viruses of known nucleic acid size. This has been done in Fig. 4 where the log of the reciprocal of the inactivation rate constant (log $dose_{37}$) has been plotted against the log of the molecular weight of the nucleic acid component of all viruses for which comparable ionizing inactivation data could be found.

It is apparent from Fig. 4 that radiosensitivity of viruses is a strong function of both nucleic acid mass and strandedness[26–29]. However, since there is considerable scatter in this relationship, it cannot be an exclusive function of these parameters as is assumed in the 'target' theory[25]. Regression lines calculated separately for the single-stranded viruses and double-stranded DNA viruses have correlation coefficients of −0.832 and −0.841, respectively. The scrapie inactivation rate constant, computed from the regression of six separate inactivation experiments[7–9] over the entire dosage range of the experiments (correlation coefficient = −0.95), intercepts the single-stranded line at 0.75×10^6 daltons, well above the size of the smallest known single-stranded virus[30]. The double-stranded line is intercepted at 1.6×10^6 daltons, well within the range of what is conceivable for a virus and 10–20 times greater than estimates made with the same data by the target calculation.

The discovery that some scrapie infectivity co-migrates with low molecular weight DNA restriction fragments and viroid RNAs in dilute agarose–acrylamide electrophoretic gels[4] corroborated the results of the target calculation and seemingly secured the concept of subviral size. The electrophoretic gel system used in those experiments fractionated nucleic acids on the basis of their molecular weights. If scrapie were a naked nucleic acid, it must have the small molecular weight indicated by its co-migration with the viroid markers. However, if scrapie were a virus, nucleic acid markers would be inappropriate. In basic solutions, nucleic acids possess a large, uniform negative charge promoting migration, coupled with large hydrodynamic volumes and extended conformations impeding their movement through a gel matrix. In contrast, viruses are small and compact compared with the nucleic acids that they encapsulate, whereas their net charge is weak and can be negative, positive or neutral[29].

This difference is demonstrated in Fig. 5 where three small, icosohedral bacteriophages (MS2, ΦX174am3 and ΦX174strain 121, an electrophoretic mutant of ΦX174) of almost identical molecular weight have been electrophoresed with restriction endonuclease fragments of ΦX174 and phage λ DNA. The relative migrations of both the viruses and the nucleic acid fragments are plotted against the logs of their molecular weights. Whereas the nucleic acid fragments separate as a function of their molecular weights as predicted, virus migration is insensitive to this parameter. While none of the three test viruses migrated as far as the scrapie infectivity, there is no reason to believe that slightly more negative viruses, slightly smaller, or perhaps differently shaped viruses would not have done so.

If, as various authors have postulated, scrapie is a small infectious protein[3,31,32] instead of a virus, it should be capable of penetrating the more concentrated acrylamide gels that are commonly used to fractionate proteins as well as the dilute agarose–acrylamide gels used to date. An adequate demonstration of this property would require the co-migration of scrapie infectivity with protein markers of known size in an electrophoretic system that demonstrably excludes small highly charged viruses. To date, these criteria have not been met in any fractionation system[33] and, if scrapie has viral dimensions as predicted by the analysis of its inactivation by ionizing radiation given above, it will not be possible to do so.

There is ample evidence that scrapie aggregates readily[2,29,34,35] and an aggregated subpopulation of the scrapie agent could appear more resistant to a given reagent or treatment than would the unit virus[29]. This is because only a single inactivation event is required to kill a free virus particle (assuming that the inactivation of free virus proceeds by a first order process), whereas many inactivations are required (one for each virus) to kill an aggregate. The conditions in which the radiation experiments were conducted (samples were dried onto planchets) would be expected to promote aggregation which would lead to an underestimate of the inactivation rate constant and thereby the size of the virus[29].

Aggregation can also affect survival through the mechanism of multiplicity reactivation which has been demonstrated after virus inactivations by ionizing radiation[36], formaldehyde[19] and nitrogen mustard[37], when the virus mixture was aggregated either naturally or artificially before assay. When vaccinia virus is inactivated by any of these procedures, apparent survival increases 100-fold after aggregation[19,36,37].

Aggregation of dispersed viruses itself mimics inactivation. Subsequent disruption of such aggregates restores infectivity and may account for reports of the reversible inactivation of scrapie[38]. Since aggregation is a strong function of concentration, the role of aggregation can in part be surmised from the concentration dependence of an inactivation. Thus, the inactivation of scrapie infectivity by iodine (Fig. 2*a*) is unlikely to have been the consequence of aggregation as the more dilute suspension shows greater inactivation.

When the inactivation behaviour and size of the scrapie agent are compared directly with viral controls, there seems no justification for classifying the agent as non-viral. Studies of the scrapie agent's sedimentation behaviour[35,39,40], exclusion size in permeation chromatography[33,41] and buoyant density[42] also suggest a virus, and several ultrafiltration experiments have placed its size between 30 and 50 nm[41,43–45]. Furthermore, electron microscopic studies of SSVE-infected brains consistently reveal a filamentous structure[46–50] unusual for an animal virus but consistent in size and morphology with the structures of known viruses affecting plants and bacteria.

The unconventional biological properties of the subacute spongiform encephalopathies leave little doubt that this is a unique disease class, but the evidence strongly suggests that the scrapie agent, while perhaps representing a new taxon, is nevertheless, a small virus with conventional sensitivities to heat and numerous chemical inactivants.

I thank P. Brown[12] for collaboration in the chemical inactivations of scrapie presented in Figs. 1–3, E. Green for supervision of the animal colony, G. Sokol and J. Etherton for assistance in phage inactivations, M. H. Edgell and C. A. Hutchison for providing ΦX174 strain 121, E. Elliott and J. Goudsmit for critical reading of the manuscript and D. C. Gajdusek for advice and discussion.

Received 3 July 1983; accepted 1 February 1984.

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Secretion of a wheat α-amylase expressed in yeast

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The translocation of secretory proteins across the endoplasmic reticulum involves the recognition and cleavage of an amino-terminal extension called the signal sequence[1]. The structure of signal peptides appears to be ubiquitous in having a very hydrophobic central core[2], so that the signal sequence in secretory proteins from one organism could possibly be recognized by the processing and transport apparatus of another. We therefore wished to investigate whether a protein, α-amylase, one of several hydrolytic enzymes secreted from the aleurone of wheat into the endosperm during germination, could be processed and secreted in an active form from the yeast *Saccharomyces cerevisiae*, secretion being dependent upon the plant signal sequence. Here, synthesis of α-amylase was by inserting a cDNA clone coding for the entire α-amylase structural gene[3] into a yeast expression vector[4]. The α-amylase protein coded for by this gene fusion has the signal sequence located internally, not at the N-terminal end of the polypeptide. Nevertheless, it is processed and the processed form is secreted into the medium in an active form. There are potential industrial applications for yeast that secrete a functional α-amylase.

A previously isolated cDNA clone for a wheat α-amylase gene was inserted into a yeast expression vector as shown in Fig. 1*a*. The yeast expression vector pMA230 codes for the phosphoglycerate kinase (PGK) promoter as well as the first 12 amino acids of the PGK polypeptide[4]. The α-amylase cDNA was constructed using the dG/dC homopolymer tailing method and cloned into the *Pst*I site of pBR322[3]. The plasmid 2128 contains the entire protein-coding sequence as well as 5′ and 3′ untranslated regions. The insertion of the cDNA coding for the α-amylase gene into the single *Bam*HI site of pMA230, making the plasmid 520 (Fig. 1*a*), creates a hybrid gene coding for a translational fusion product as shown in Fig. 1*b*. From the sequence of the α-amylase gene (C.M.L., unpublished results), the polypeptide synthesized will have a total of 38 extra amino acids at its N-terminal end, coded for by the PGK gene, the normally untranslated α-amylase leader (in which there are no nonsense codons), the dG/dC homopolymer tail and DNA linker fragments (see Fig. 1*b*). The α-amylase signal peptide is therefore not at the N-terminal end of the polypeptide.

Another plasmid (528) was constructed in which the dG/dC tails, the 5′ untranslated region and almost all of the signal peptide coding region were deleted (Fig. 1*a*). As shown in Fig. 1*b*, the polypeptide coded for by 528 should be 23 amino acids longer than the wild-type mature wheat α-amylase. Plasmid 521 (Fig. 1*a*) is the same as plasmid 520 except that the α-amylase gene was inserted in the wrong orientation to be transcribed by the PGK promoter. Yeast cells carrying this plasmid were used as non-α-amylase producing strains.

Yeast cells transformed with the plasmid 520 were labelled with ^{35}S-methionine, the intracellular protein was isolated, immunoprecipitated with anti-α-amylase serum and electrophoresed on a SDS-polyacrylamide gel. Two distinct polypeptide bands can be seen on the resulting autoradiograph shown in Fig. 2*a*. The larger polypeptide has an apparent molecular weight (MW) of 49,000 which is the expected value for the predicted fusion protein coded for by 520. The smaller polypeptide is the processed form of the 49,000-MW protein (S.J.R., unpublished results) and is almost identical in molecular weight to mature *in vivo* labelled wheat α-amylase (42,000 MW), although the mobilities of the two differ slightly (Fig. 2*b*, tracks A, B). This small difference may be caused by differences in other post-translational events such as methylation or glycosylation. When cells transformed with the plasmid 528 were labelled with ^{35}S-methionine a single polypeptide of the expected molecular weight (44,400) was immunoprecipitated by anti-α-amylase serum. As expected since the signal peptide is absent, no cleavage to a smaller protein is seen (Fig. 2*c*). The incorporation of label into α-amylase in cells carrying plasmid 528 is considerably greater than that seen for cells containing plasmid 520. To demonstrate that this is not due to a general increase in incorporation of the label into protein, the β subunit of the yeast mitochondrial ATPase was co-immunoprecipitated with

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