burst. The probability of opening after the burst [$P_o(W_2)$] was computed within a time window (W_2) that could be set at several times the value of W_1. The expected negative correlation was not observed (Fig. 3B). The relationship between $P_o(W_1)$ and $P_o(W_2)$ (burstergram) seemed to show a positive correlation resulting from the carry-over of activity from the higher levels in W_1 to the lower levels in W_2.

The results of the burstergram may also be compared with predictions of the calcium-entry hypothesis for inactivation (*8*). The prediction is for a quadratic relation between the value of P_o during a burst and the subsequent change in P_o after the burst (Fig. 3C). The predictions were not proved by the results. Burstergrams for patches with two channels showed similar results.

We determined that inactivation occurred in averaged single calcium channel currents during a step change in potential and that it was due to a decrease in the rate of opening. Inactivation was not due to generalized calcium entry, and effects of localized calcium entry were excluded. The process appears to include a voltage-dependent component (*3*), although we have not extensively investigated this aspect at the single channel level. Nevertheless our results do not rule out an indirect, modulatory effect that calcium currents may have on transitions among closed states. In this case the channel, once activated, might be free to pass through the remaining states in the usual way. This implies that the effect of calcium entry is significant for a longer time than that encompassed by our experiments. This could explain the effect of changes in calcium current amplitude on recovery from steady-state inactivation (*9*).

Our results indicate that inactivation need not obligatorily be coupled to activation, as the calcium-entry hypothesis requires. These observations are not restricted to neuronal calcium channels. Similar results were obtained in rat and guinea pig ventricular myocytes and avian dorsal root ganglionic cells (*10*), and we suggest that they could apply generally to the behavior of single calcium channels.

H. D. LUX
Max-Planck-Institut für Psychiatrie, Am Klopferspitz 18A, 8033 Planegg-Martinsried b. München, Federal Republic of Germany
A. M. BROWN
Department of Physiology and Biophysics, University of Texas Medical Branch, Galveston 77550

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8. Calcium entering the cell reacts with a site S to form a complex CaS that inactivates the channel (*5*). The equilibrium, with dissociation constant K, is assumed to be instantaneous on the time scale of calcium channel activation. The probability P of a channel being open is expressed by

$$P = \frac{K}{[\mathrm{Ca}] + K}$$

where [Ca] is the intracellular calcium concentration. The change of [Ca] by entry through a channel relates to the probability that a channel, having been opened, will continue to be open; that is,

$$\frac{d[\mathrm{Ca}]}{dt} = D_P$$

where D is a constant relating to initial [Ca] and geometric factors. The solution to the differential equation is

$$[\mathrm{Ca}] = K \pm (2L)^{1/2}\{(t - t_0) + ([\mathrm{Ca}]_0 + K)^2(2L)^{-1}\}^{1/2}$$

where $L = DK$ and t_0 and $[\mathrm{Ca}]_0$ are integration constants. [Ca] is proportional to the current I and thus

$$\frac{I(t)}{2F^{-1}} = -0.5(2L)^{1/2}\{(t - t_0) + ([\mathrm{Ca}]_0 + K)^2(2L)^{-1}\}^{1/2} = \frac{i}{2F^{-1}} P_o(t)$$

for the probability of channel openings (F is Faraday's constant). After differentiating,

$$\frac{dP}{dt} = -RP^3$$

which relates opening probability with its change via calcium entry. The constant R ($A^{-2} \cdot sec^{-1}$) defines the initial conditions for each evaluation and applies, since sample periods with different occupation times were preceded by similar average opening activity.
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Sedimentation Field Flow Fractionation of DNA's

Abstract. *Sedimentation field flow fractionation (SFFF) is a method for purifying and providing mass or size distribution information on samples containing particulates or soluble macromolecules. Since SFFF separations are based on simple physical phenomena related to first principles, molecular weight (or particle sizes) can be determined without calibration standards. SFFF is a gentle technique suited for fractionating biomolecules. Studies with the fragile λ DNA (molecular weight, 33×10^6) and smaller supercoiled plasmids have shown that these materials are not altered during SFFF separation; molecular weights and conformation remain unchanged, and biological activity is not reduced. Recoveries of nucleic acids approach 100 percent. Typically, components with about 20 percent difference in mass can be separated essentially to baseline if required. Fractionation time is usually independent of molecular weight, and separations often can be carried out within an hour.*

Sedimentation field flow fractionation (SFFF) is capable of the high-resolution separation of a variety of soluble macromolecules and organic and inorganic colloids with molecular weights ranging from 10^6 to 10^{13} (*1, 2*). Separations are performed in a thin open channel with a single, continuously flowing carrier liquid under the influence of an external centrifugal force field. The laminar flow profile generated within this thin channel causes mobile phase velocity to reach a maximum in the channel center and to approach zero velocity at the walls. Interaction of suspended or dissolved molecules with the external centrifugal force field that is applied perpendicular to the channel flow forces larger or more dense particles into slower flow streams nearer the wall so that they elute later than smaller or less dense particles. Thus, species elute in the order of increasing mass or particle density in the form of high-resolution "fractograms" similar to chromatograms.

The theoretical basis, equipment, and techniques of SFFF have been described (*1, 2*). The general application of this method to the particle-size distribution analyses of colloids and other suspended particulates also has been demonstrated (*3, 4*). Initial experiments have shown

that whole bacteria, various intact viruses, viral DNA's, mammalian RNA, and liposomes can be separated by SFFF; observed molecular weights agree with reported values (*1, 2, 5, 6*).

These and other studies suggested that SFFF potentially had significant advantages as a gentle, high-resolution method for nucleic acids used in recombinant DNA studies. A particular advantage is that no standards are required if the densities of the components are known, since SFFF is a mass-separating method based on first-principles physics (*1*). We now attempt to define the applicability of SFFF in DNA fractionations.

Several basic issues regarding the behavior of nucleic acids during SFFF separation needed to be addressed. The first issue was the possibility of hydrodynamic shear acting during SFFF separations. The large, linear, and fragile λ DNA molecule (molecular weight, 33×10^6) was selected as a test case since this molecule should be more susceptible to hydrodynamic shear than would the smaller and often compactly coiled DNA's commonly used in recombinant DNA techniques. Samples of λ DNA (6 μg) (New England Biolabs, Beverly, Massachusetts) were subjected to SFFF conditions typical of those experienced in practical fractionations (Fig. 1A). The total unretained fraction and the entire retained λ DNA peak, which were detected by absorption at 260 nm, were collected separately. No other peaks were noted in the fractogram. The isolated fractions were lyophilized and redissolved in mobile-phase buffer. Portions of each peak were separated electrophoretically overnight on a 0.4 percent agarose gel and visualized with the fluorescent dye ethidium bromide (*7*). No evidence of λ DNA was seen in the unretained peak (Fig. 1B), indicating the expected separation of the channel dead volume peak from the retained λ DNA peak. Absorption of the unretained fraction (Fig. 1A) was due to EDTA, which was present in the injected sample but not in the mobile phase. The band in the separated and control samples exhibited the same mobility. No other bands of lower molecular weight appeared in the separated sample, indicating that no shear degradation occurred.

Circular supercoiled DNA molecules varying in molecular weight from 2.8 to 6.8×10^6 also gave no indication of undergoing shear degradation to the corresponding linear or relaxed conformers as judged by their electrophoretic mobilities on 1 percent agarose gel. Furthermore, even weakened, relaxed open-circular forms were not converted by shear to the linear form.

A second issue was the possibility of alterations in the biological properties of DNA not related to shear. Effects of exposure to SFFF were assessed by monitoring the ability of λ DNA to lyse bacteria after isolation. Samples of the λ DNA were fractionated by SFFF, and the isolated λ DNA peaks were transfected into bacteria (*Escherichia coli* strain LE392) (*8*). Within experimental reproducibility, the activity of λ DNA isolated by SFFF was equivalent to that of a control sample and to that of λ DNA passed through the SFFF apparatus with no force field (Table 1). Thus, the viability of λ DNA was unaffected either by the SFFF channel or by the effects of the applied gravitational force.

A similar viability study was performed with plasmid pBR322 as a model (Table 1). This DNA is a common cloning vehicle that has the easily monitored ability to confer resistance to ampicillin and tetracycline on bacteria induced to accept the DNA. Identical samples of pBR322 plasmid obtained commercially were subjected to SFFF and collected as described (see legend to Table 1). The collected fractions were incubated with equal numbers of *E. coli* HB101 cells (*9*) and plated on medium containing ampicillin. The control for spontaneous mutation consisted of cells that were incubated without plasmid pBR322 and plated identically. Within the limits of experimental error, equivalent numbers of colonies were found whether the pBR322 was subjected to a force field or not (Table 1). Essentially no colonies were found in the control test. These results indicate that the SFFF separation pro-

Table 1. Biological activities for λ DNA and pBR322 after fractionation by SFFF. Activity of λ DNA was assessed by plaque formation on "lawns" of transfected *Escherichia coli* on nutrient agar. Activity of pBR322 was assessed by formation of colonies of mutated *E. coli* cells in medium containing ampicillin. Measurements were made by ultraviolet absorbance at 260 nm [absorption coefficient (0.1 percent), 20]. The conditions for the λ DNA test were: sample size, 10 μg of λ DNA in 100 μl of injected fluid; initial rotor speed, 15,000 rev/min; time delay–decay constant (*11*), 10 minutes; relaxation time, 10 minutes; flow rate, 0.5 ml/min; and mobile phase, 10 m*M* tris (*p*H 7.6). The conditions for the pBR322 test were the same as above except for initial rotor speed (30,000 rev/min), relaxation time (5 minutes), and time delay–decay constant (12 minutes).

Treatment	Biological activity	
	λ DNA (plaques per nanogram)	pBR322 (CFU/μg)*
No SFFF	14 ± 2.3 (two tests)	
SFFF without force field	15 ± 5.2 (four tests)	$(7.4 \pm 2.5) \times 10^4$
SFFF with force field†	18.7 ± 3.8 (four tests)	$(5.6 \pm 3.6) \times 10^4$
Control (no nucleic acid)	0	4

*CFU, colony-forming units. †For λ DNA test, the ratio of channel dead volume (V_0) to peak retention volume (V_r) was 0.16; for pBR322 test, the ratio was 0.1.

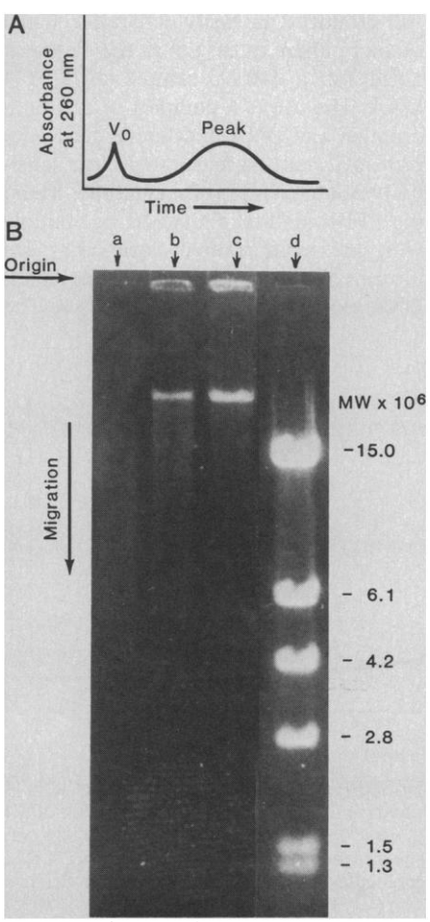

Fig. 1. (A) Fractogram of λ DNA. Conditions for SFFF were: sample size, 6 μg of λ DNA in 100 μl of injected fluid; rotor speed, 15,000 rev/min; relaxation time, 10 minutes; time delay–decay constant (*11*), 10 minutes; flow rate, 0.50 ml/min; and separation time, 30 minutes. (B) Agarose gel (0.4 percent) electrophoresis of fractionated λ DNA. The buffer contained 4 m*M* tris (*p*H 7.4), 36 m*M* phosphate, and 1 m*M* EDTA. (Lanes a and b) Fractions isolated for the unretained (V_0) and retained SFFF peaks, respectively; (lane c) untreated control λ DNA; and (lane d) 1 μg of a λ DNA Hind III digest used as a standard.

cess did not inhibit either transformation or expression of the β-lactamase gene on pBR322 that confers antibiotic resistance.

Another important issue was the yield obtained by the SFFF method. To test the degree of recovery, we measured concentrations of isolated material by ultraviolet absorption at 260 nm. We found that DNA samples could be passed through a channel at rest and recovered quantitatively. Samples of DNA of known concentrations were then separated by SFFF, collected, and measured by ultraviolet absorption. In some studies, the unretained and retained peaks were isolated separately. The results of studies for pBR322 and λ DNA (Table 2) showed essentially quantitative isolation yield for both nucleic acids, indicating that no significant amount of DNA was irreversibly retained during SFFF. This result is in keeping with the sample's being exposed only to an empty channel with a low surface area. This feature of SFFF is in contrast to separations carried out in chromatographic columns or on electrophoretic gels, which have relatively large surface areas for possible sample adsorption.

Of considerable interest was whether measurements of molecular weight made by SFFF are affected by sample concentration as a result of unwanted enhanced molecular interaction of highly charged molecules as they are forced close to the analytical wall by the external centrifugal force field. To test the effects of sample concentration on retention, we subjected samples of ϕX174 relaxed form 1 (RF1) (0.5 to 50 μg in 100 μl of injected solution) to normal SFFF analytical conditions (*10*). The observed retention times for samples of these concentrations indicated a calculated molecular weight of about $(3.46 \pm 0.06) \times 10^6$, in agreement with the reported value of 3.5×10^6. In another experiment, samples of λ DNA (0.6 to 10 μg) were similarly subjected to SFFF separation. Again, the observed retention times were constant within experimental error. These results suggest that, under proper operating conditions, concentration does not affect DNA molecules over a sample range adequate for analysis.

Table 2. Recovery of pBR322 and λ DNA after separation by SFFF. Sample size was 7.3 μg of pBR322 and 6.3 μg of λ DNA for each experiment. Yields were determined by ultraviolet absorbance at 260 nm.

Experiment	Yield (percent)	
	pBR322	λ DNA
1	112 (103 in peak)	92
2	97 (82 in peak)	101
3		104
Average	104 (93 in peak)	99

Another consideration was whether SFFF was sufficiently sensitive to measure DNA's in concentrations that are typically used in recombinant DNA techniques. Although SFFF bands are broader than those in high-performance liquid chromatography, the high-sensitivity ultraviolet detector used in these studies still produced an easily detectable signal of more than three times the detector noise for a 500-ng sample of ϕX174 DNA. The use of a complex of ethidium bromide and DNA and fluorescence detection should increase analytical sensitivity another order of magnitude. Thus, the SFFF method should be compatible with the small sample sizes that are typical of those used for recombinant DNA experiments.

We were also interested in the degree of resolution that could be obtained in SFFF separations. Earlier SFFF studies have indicated that, under optimum conditions, peak resolution almost to baseline can be expected for a mixture of two components of equal density having a 20 percent difference in molecular weight (*11*). To test for this feature, we separated a mixture of ϕX174 RFI DNA (molecular weight, 3.5×10^6) and ϕX174 virion DNA (molecular weight, 1.75×10^6) (Fig. 2). Although resolution for this pair of compounds was almost to baseline, separation could be significantly increased, if needed, with an increase in separation time. If the components were collected separately, with the arrow between the two peaks used as the point of distinction (see Fig. 2), each fraction would exhibit a purity of 98 percent.

The observed molecular weights of both components in Fig. 2 agree with reported values, and the separation time of less than 1 hour is typical of SFFF fractionations. If the approximate range of molecular weights for the sample is known, instrumental conditions can be established so that components differing either little or greatly in molecular weight often can be separated within about 1 hour. This situation applies to both analytical and preparative applications and is in contrast to gel electrophoresis and size-exclusion chromatography, where it is often more difficult or inconvenient to manipulate the relative separation times between components. Our studies suggest that SFFF has potential as a mild, rapid, and quantitative method for resolving and isolating DNA's and as such may prove useful in recombinant DNA studies.

L. E. SCHALLINGER
W. W. YAU
J. J. KIRKLAND
E. I. du Pont de Nemours & Company, Central Research and Development Department, Experimental Station, Wilmington, Delaware 19898

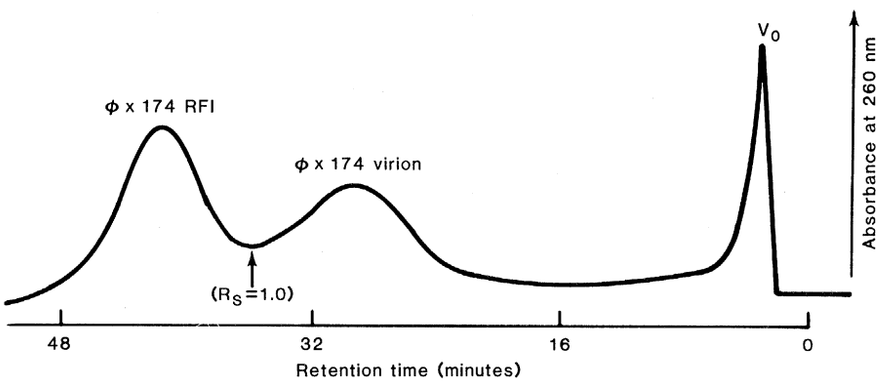

Fig. 2. Separation of DNA mixture. Conditions for SFFF were: sample sizes, 6 μg of ϕX174 RFI and 6 μg of ϕX174 virion in 100 μl of injected fluid; mobile phase, 0.1*M* tris and 0.1*M* NaCl (*p*H 7.6); flow rate, 0.5 ml/min; initial rotor speed, 30,000 rev/min; rotor thickness, 0.024 cm; and time delay–decay constant (*11*), 12 minutes. The molecular weight reported for ϕX174 RFI is 3.5×10^6 and is identical to that observed. The molecular weight reported for ϕX174 virion is 1.75×10^6 and that observed was 1.68×10^6.

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10. SFFF conditions: equipment and technique as in (*2*); rotor speed, 15,000 rev/min; relaxation time, 1 minute; time delay–decay constant, 10.0 minutes; flow rate, 0.5 ml/min; mobile phase was 0.1*M* tris and 0.2*M* NaCl at *p*H 7.6.
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Infection-Specific Particle from the Unconventional Slow Virus Diseases

Abstract. *Scrapie-associated fibrils, first observed in brains of scrapie-infected mice, were also observed in scrapie-infected hamsters and monkeys, in humans with Creutzfeldt-Jakob disease, and in kuru-infected monkeys. These fibrils were not found in a comprehensive series of control brains from humans and animals affected with central nervous system disorders resulting in histopathologies, ultrastructural features, or disease symptoms similar to those of scrapie, kuru, and Creutzfeldt-Jakob disease. These fibrils are also found in preclinical scrapie and in the spleens of scrapie-infected mice; they are a specific marker for the "unconventional" slow virus diseases, and may be the etiological agent.*

Scrapie in sheep and goats and Creutzfeldt-Jakob disease (CJD) and kuru in humans cause fatal infectious encephalopathies after long periods of inapparent disease. The causal "slow viral" agents have yet to be identified by electron microscopy. Scrapie-associated fibrils (SAF) are distinct particulate structures first observed in scrapie-infected mouse brain preparations by negative stain electron microscopy (*1*). They are composed of two to four twisted filaments, each filament 4 to 6 nm in diameter and of variable length (*1*). Merz *et al.* have reported these structures in brain extracts from three strains of mice infected with any of six different strains of scrapie (139A, ME7, 22A, 87V, 22L, and 79A); from hamsters infected with the 263K strain of scrapie; from mice, guinea pigs, and hamsters infected with a single isolate of CJD; and from a human case of CJD and a human case of Gerstmann-Straussler syndrome (*2*), thought to be a variant of CJD (*3*).

There has so far been a good correlation between the presence of SAF and the "unconventional" slow virus diseases. We have now extended this correlation by the identification of SAF in experimental kuru and in additional cases of human CJD and scrapie. We have established the specificity of SAF by their absence in a comprehensive assortment of other human and animal diseases exhibiting either similar histopathologies, ultrastructural features, or clinical courses similar to those of scrapie, kuru, or CJD. In so doing we have also demonstrated the capability of negative stain electron microscopy to distinguish between SAF and similar structures observed in other disorders of the central nervous system (CNS).

The principal neuropathological features of Alzheimer's disease and senile dementia of the Alzheimer type (AD-SDAT) are neuritic amyloid plaques and neurofibrillary tangles composed of paired helical filaments (PHF). Neurofibrillary tangles are also associated with Guam parkinsonism dementia complex and amyotrophic lateral sclerosis (ALS) on Guam and occasional cases of ALS elsewhere. The ability to distinguish SAF from amyloid fibrils and PHF observed together or separately in these different human CNS diseases, and to corroborate cases of mixed diagnosis on the basis of these ultrastructural features, may make it possible to use ultrastructural analysis to distinguish types of CNS involvement in this complex disease spectrum.

Specimens coded with respect to species, treatment, and diagnosis from 44 separate brains were examined in three separate experiments (two at The National Institutes of Health and one at the Institute for Basic Research) with the code broken after each experiment by a third individual after all the results were tabulated. Crude mitochondrial synaptosomal preparations were prepared from 0.5 to 3 g of cerebral cortex from human (*4*) or sheep (*5*) or squirrel monkey brains (*5*) or from whole brains of mice (*6*) or hamsters (*7*). Each preparation was then treated with octyl-β-D-glucopyranoside and sedimented through a discontinuous sucrose gradient; a band was collected and analyzed for the presence of SAF by negative stain electron microscopy (*1*, *2*).

Scrapie-associated fibrils were present in the three scrapie-infected hamsters, two scrapie-infected squirrel monkeys, one kuru-infected squirrel monkey, and in all six human CJD brain extracts (Table 1 and Fig. 1). These fibrils were not observed in six AD-SDAT cases, four cases of parkinsonism dementia, two cases of ALS, and four other human controls. The SAF were expected but not found in one case of naturally occurring scrapie in sheep, two cases of kuru,

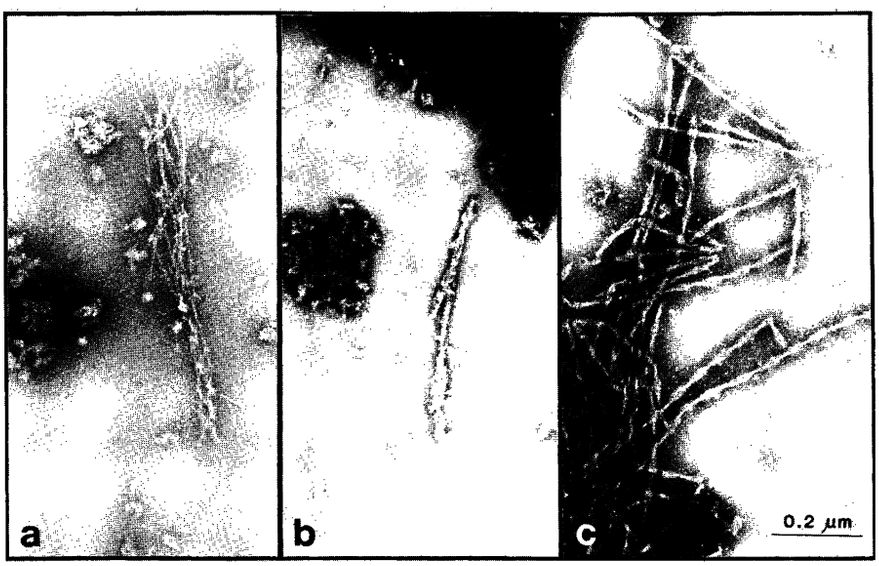

Fig. 1. An example of (a) amyloid fibrils observed in one of the natural CJD cases; (b) SAF from a natural CJD case. The SAF's are a mixture of type I and type II (*2*); (c) PHF observed in extracts from a sporadic Alzheimer case (×90,000). Samples are stained with 3 percent sodium phosphotungstate, *p*H 7.2.