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Identification of a Protein That Purifies with the Scrapie Prion

Abstract. *Purification of prions from scrapie-infected hamster brain yielded a protein that was not found in a similar fraction from uninfected brain. The protein migrated with an apparent molecular size of 27,000 to 30,000 daltons in sodium dodecyl sulfate polyacrylamide gels. The resistance of this protein to digestion by proteinase K distinguished it from proteins of similar molecular weight found in normal hamster brain. Initial results suggest that the amount of this protein correlates with the titer of the agent.*

Scrapie is a transmissible, degenerative nervous system disease of sheep and goats. Several features of scrapie closely resemble two human diseases, kuru and Creutzfeldt-Jakob disease. Research over the last two decades has demonstrated that the scrapie agent is extremely resistant to physical and chemical treatments that inactivate bacteria, viruses, and viroids (*1, 2*). Although the agent is known to be a small, hydrophobic particle (*2–4*), its precise structure and biochemical composition have eluded definition. A scrapie-specific structural macromolecule has not yet been identified.

Studies with substantially purified preparations of the scrapie agent have shown that a protein is required for infectivity. Based on these studies, a new term, "prion," was introduced to describe and identify these unusual infectious particles (*2*). The agent in the purified preparations was inactivated by protease digestion and by reversible chemical modification with diethyl pyrocarbonate (DEP) (*4, 5*). In addition, the agent was inactivated by chemical reagents that denature proteins; these reagents included sodium dodecyl sulfate (SDS), urea, guanidinium thiocyanate, and phenol (*2, 6–8*). The most likely explanation for these observations is that inactivation results from modification of a functional protein within the scrapie agent.

The agent was obtained from weanling random-bred (LVG/LAK) hamsters that were given an intracerebral inoculation with 10^7 ID_{50} (mean infectious dose) units of the scrapie agent (*6*). Sixty days later, the infected brains were collected and homogenized. The agent was purified by low-speed centrifugation, polyethylene glycol precipitation, enzyme digestion, ammonium sulfate precipitation, and sedimentation into a discontinuous sucrose gradient (25 to 60 percent) (*9*). Samples collected throughout the purification were analyzed for infectivity by the incubation time interval assay (*6, 10*) and for protein concentration by the Peterson-Lowry method (*11*).

Infectivity analysis of discontinuous sucrose gradients (Fig. 1A) showed that approximately 50 percent of the infectious agent was found near the bottom of the gradient in fraction 2. This fraction represented the interface of the 25 and 60 percent sucrose solutions. It was enriched for the scrapie agent since most of the applied protein remained in the top portion of the gradient. This procedure produced a 100- to 1000-fold purification of the agent; the specific infectivities of fraction 2 ranged from 3.8×10^9 to 1.2×10^{11} ID_{50} units per milligram of protein (*9*).

Analysis of ^{125}I-labeled sucrose gradient fractions by gel electrophoresis revealed a diffuse protein band with a molecular size of 27,000 to 30,000 daltons in those fractions enriched for the infectious agent (Fig. 1B). This band was readily detected in fraction 2 and to a lesser extent in fractions 1 and 3. It was also present in fraction 15, but was difficult to visualize because of the high concentration of contaminating proteins.

The presence of this protein in fractions containing the substantially purified agent suggested there might be a specific relation between this protein and the infectious particle. For this reason, we attempted to determine whether or not the protein was present in fractions analogously purified from brains of age-matched, uninoculated hamsters. Samples of fraction 2 from six different preparations of scrapie-infected hamster brain and three different preparations of normal hamster brain were ^{125}I-labeled and analyzed by SDS polyacrylamide gel electrophoresis. The protein of interest was found in all the samples from scrapie-infected brains but appeared to be absent from samples of normal brains (Fig. 2). These observations were complicated by the presence of discrete protein bands that migrated within the 27,000- to 30,000-dalton region in some scrapie and normal samples (Fig. 2, lanes 4 to 8).

Subsequent investigations showed that the protein was readily degraded by proteases if it was denatured prior to digestion. After denaturation at 100°C in the presence of 1.25 percent SDS and 1.25 percent β-mercaptoethanol, the protein was hydrolyzed by proteinase K, pronase, and *Staphylococcus aureus* V-8 protease. These results confirmed that this macromolecule is indeed a protein.

Attempts to digest the protein prior to denaturation were not successful. Instead, we found that this protein was resistant to digestion by proteinase K under nondenaturing conditions. Fractions prepared from scrapie and normal hamster brains were ^{125}I-labeled with Bolton-Hunter reagent (Fig. 3A) or ^{14}C-labeled DEP (Fig. 3B) and incubated with proteinase K. The electrophoretic profiles of those samples for which the

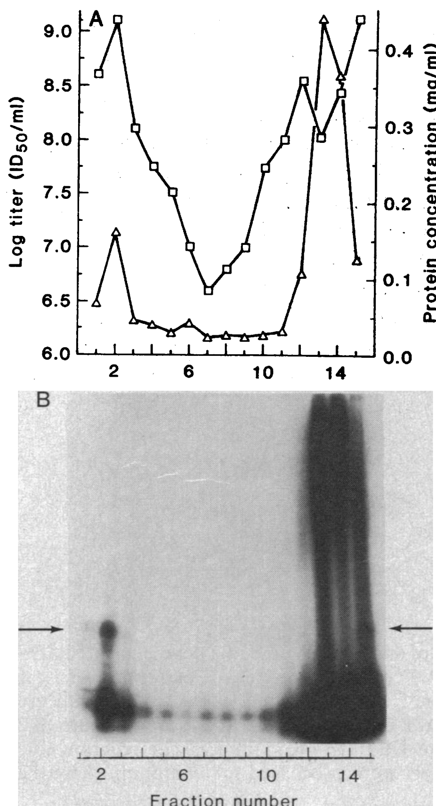

Fig. 1. Scrapie infectivity and ^{125}I-labeled proteins in sucrose gradient fractions. The partially purified scrapie agent (4 ml in 20 m*M* tris-acetate, 1 m*M* EDTA, 1 m*M* dithiothreitol, 0.2 percent Sarkosyl, 2 percent TX-100, and 0.8 percent SDS, *p*H 8.3) was layered on a discontinuous sucrose gradient consisting of 60 percent sucrose (5 ml) and 25 percent sucrose (30.5 ml) in 20 m*M* tris-acetate, 1 m*M* EDTA, *p*H 8.3, containing no detergents. The agent was sedimented into the gradient at 50,000 rev/min (Beckman VTi 50 rotor) for 2 hours at 4°C. Gradient tubes were fractionated from the bottom (fraction 1) into 15 samples of 2.5 ml each (*9*). (A) Infectious scrapie titers (□) and protein concentrations (△) were determined for each fraction (*6, 10, 11*). (B) Portions of each gradient fraction were concentrated tenfold and labeled with *N*-succinimidyl 3-(4-hydroxy, 5-[^{125}I]-iodophenyl)propionate as described (*9, 13, 14*). The samples were subjected to electrophoresis through a 5 to 20 percent linear gradient polyacrylamide gel (*15*). Radioautographic exposure of the dried gel was performed at room temperature for 1.5 hours.

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proteinase K was omitted are shown in lanes 1 to 4. Digestion with proteinase K prior to electrophoresis removed most of the labeled bands from both scrapie and control fractions; however, the resistant protein described above remained intact in scrapie samples (lanes 5 and 6). The protein was not found in control samples prepared from uninfected hamster brains (lanes 7 and 8). Although the molecular basis for the resistance of this protein to degradation by proteinase K is unknown, we presume it results from the primary structure of the molecule. In initial studies, one-dimensional peptide maps have shown that the protein of interest and the proteins of similar molecular size from uninfected hamster brain generate distinctly different peptide fragments (*12*).

In addition to the data presented in Figs. 2 and 3, we have confirmed the presence of the protease-resistant protein in purified fractions from six other preparations of scrapie-infected hamster brain. The protein was not found in analogous fractions from five other preparations of normal hamster brain, including four preparations made from the brains of animals which were inoculated with normal hamster brain and killed approximately 60 days later. Since this proteinase K-resistant protein has not been found in purified fractions from normal hamster brains, we conclude that the protein is specifically associated with scrapie infection.

Our data provide evidence for a protein which is associated with scrapie infection. There are at least two hypotheses that satisfactorily explain our observations: (i) the scrapie-associated protein may be a pathological product of scrapie infection, or (ii) it may be a structural component of the scrapie agent. If the first hypothesis were true, several different models could explain the appearance of a specific protein in the brain during the course of the disease. First, a pathological process, such as astrocyte proliferation, might increase the synthesis of a protein normally present in the brain at a concentration below our limit of detection. Second, scrapie infection could induce the synthesis of a protein which is not normally expressed postnatally in the brain. Third, generation of this protein from an existing brain protein might occur as a consequence of proteolytic degradation, glycosylation, fatty acid acylation, or other chemical modification induced by scrapie infection. Although these models could account for the appearance of such a protein in scrapie-infected brains, they provide no obvious explanation for the copurification of this protein and the scrapie agent.

The second hypothesis, that this protein is a structural component of the scrapie agent, is supported by several lines of evidence. The protein has been detected in all highly purified scrapie agent fractions examined and was shown to be distinct from purified normal brain proteins. The physical and chemical properties of the associated protein are similar to those of the scrapie agent, as demonstrated by their copurification through a series of procedures which result in a specific enrichment for the agent (*9*). In our purest fractions, this protein accounted for as much as 10 percent of the total protein. The protein can be isotopically labeled by DEP, which has been shown to react with a component of the agent, thereby causing inactivation (*7*).

Resistance of this protein to proteinase K–catalyzed degradation may appear to be inconsistent with the sensitivity of the agent to this enzyme (*4*). However, significant inactivation of the agent by proteinase K was observed only after incubation at 37°C for three or more hours (*4*). Thus, the resistance of this protein to proteinase K digestion is consistent with the behavior we would predict for a component of the scrapie agent. Further investigations will be required to conclusively demonstrate whether or not the protein described above is required for the infectivity of the agent.

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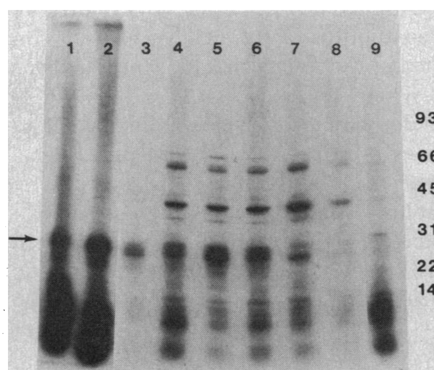

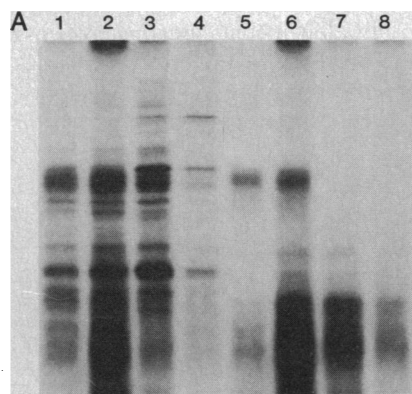

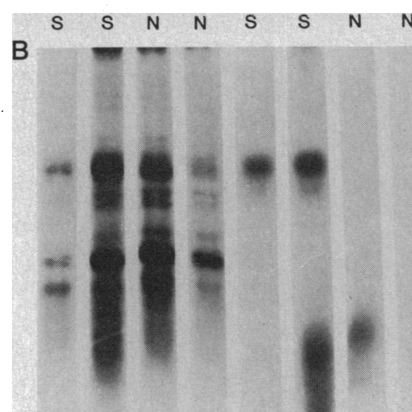

Fig. 2 (left). Analysis of scrapie and normal fraction 2 by gel electrophoresis. Portions of fraction 2 from six different scrapie-infected brain preparations (lanes 1 to 6) and three different normal brain preparations (lanes 7 to 9) were subjected to electrophoresis in a 5 to 20 percent linear gradient polyacrylamide gel after ^{125}I-labeling as described in Fig. 1. Radioautographic exposure of the dried gel was performed at room temperature for 30 minutes; lanes 1, 2, and 9 were enhanced by exposure for 20 hours. Fig. 3 (right). Proteinase K digestion of concentrated scrapie and normal fraction 2 preparations. Concentrated and labeled samples of scrapie (*S*) and normal (*N*) fraction 2 were incubated for 30 minutes at room temperature in 10 m*M* tris-Cl, *p*H 7.4 (lanes 1 to 4) or the same buffer containing proteinase K (100 μg/ml) (lanes 5 to 8). The digestion was terminated by the addition of an equal volume of 2× concentrated SDS electrophoresis sample buffer and heating to 100°C for 2 minutes. Electrophoresis was performed in 15 percent polyacrylamide gels by the Laemmli method (*15*). (A) Samples were ^{125}I-labeled as described above. (B) Samples were labeled in 60 m*M* sodium phosphate, 0.2 percent Sarkosyl (*p*H 7.2) with 10 m*M* ^{14}C-labeled DEP (53 mCi/mmole). The reaction was performed at room temperature for 1 hour. The gels were processed for fluorography by the method of Bonner and Laskey (*16*).

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Virus-Induced Corticosterone in Hypophysectomized Mice: A Possible Lymphoid Adrenal Axis

Abstract. *Infection of hypophysectomized mice with Newcastle disease virus caused a time-dependent increase in corticosterone and interferon production. Prior treatment with dexamethasone completely inhibited the virus-induced elevation in corticosterone concentration, but did not significantly alter the interferon response. Lymphocytes appear to be the most likely source of an adrenocorticotropin-like substance that is responsible for the increased corticosterone, since spleen cells from the virus-infected, but not from control or dexamethasone-treated, hypophysectomized mice showed positive immunofluorescence with antibody to adrenocorticotropin-(1–13 amide). Thus the adrenocorticotropin-like material and interferon appear to be coordinately induced and differentially controlled products of different genes. These findings strongly suggest the existence of a lymphoid-adrenal axis.*

Increases in corticosteroid concentrations are usually considered to be mediated by the pituitary-adrenal axis and to result from increased release of adrenocorticotropin (ACTH) from the pituitary gland (*1*). Recently, we demonstrated in vitro that substances that induce interferon (IFN)-α, such as viruses, cause production of an ACTH-like substance from human lymphocytes (*2*) that is strongly associated with IFN-α. The ACTH-like substance and IFN-α were dissociated by acid (*p*H 2) and were never associated if lymphocytes were induced in the presence of tunicamycin (*2*). These findings strongly suggest that the ACTH-like substance and IFN-α are coordinately induced products of different genes and become associated through a carbohydrate interaction. These findings imply that certain stimuli, which effect lymphocyte production of IFN, should not require the participation of the pituitary gland for an ACTH-mediated increase in corticosteroids. Furthermore, they suggest that lymphocyte production of ACTH in vivo might be dissociable from IFN production. These concepts were tested by determining (i) whether virus-infected hypophysectomized mice would have an increase in ACTH-like activity, thus showing an elevated corticosterone concentration and (ii) whether this putative response is dissociable from IFN production.

Hypophysectomized, female Swiss Webster mice were infected with Newcastle disease virus (NDV) (*3, 4*). These mice showed a time-dependent increase in corticosterone production (Fig. 1A). Peak steroid concentrations occurred at 8 hours after infection and thereafter declined. Serum IFN increased coordinately with corticosterone (data not shown). Thus the production of corticosterone was related to the induction of IFN and, by inference, induction of the ACTH-like substance. Corticosterone concentrations at 8 hours (10 ± 2.6, mean ± standard error, $N = 13$) differed from those at 0 hour (2.76 ± 0.11, $N = 19$) at a probability of < .01 (two-tailed Student's *t*-test). Whereas hypophysectomized animals did not respond to a classical "stressor" (cold water) they did respond to ACTH (Fig. 1B). The response to a pharmacologic dose of ACTH was not maximal since there was probably some adrenal atrophy in the 5 days between the hypophysectomy and the experiment (*4*). This suggests that under optimal conditions the adrenal response to virus might be even higher than the 3.5-fold increase that was observed.

The production of ACTH by the pituitary gland, and thus the production of steroid by the adrenal glands, is controlled by negative feedback via adrenal corticosteroids (*1*). The virus-induced increases in corticosterone concentration in hypophysectomized mice appear to be similarly controlled, because prior treatment with the synthetic steroid dexamethasone completely inhibited the increase in corticosterone at 8 hours after NDV infection (Fig. 1B). That lymphocytes are a likely source of an

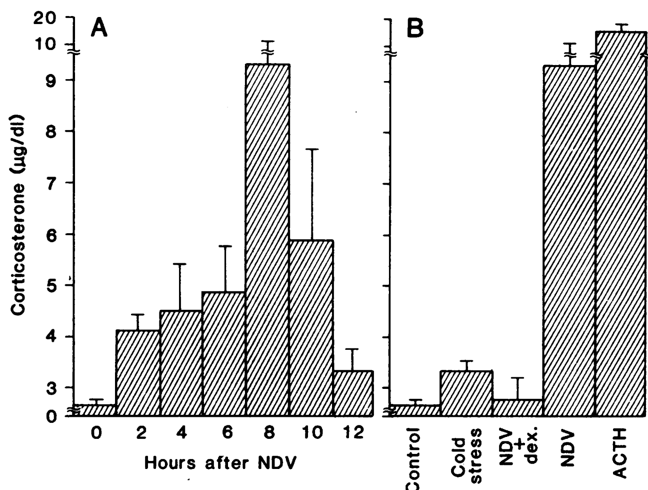

Fig. 1. (A) The kinetics of corticosterone production after NDV infection of hypophysectomized mice. Mice were injected intraperitoneally with 800 hemagglutination (HA) units of NDV in 0.2 ml. At the indicated times, the mice were decapitated and trunk blood was collected for corticosterone determination by radioimmunoassay (*7*). Mouse heads were dissected and examined under a dissecting microscope, and animals with any remnant of the pituitary gland were discarded. The numbers of mice for 0, 2, 4, 6, 8, 10, and 12 hours were 19, 2, 9, 16, 13, 12, and 5, respectively. (B) Dexamethasone (*dex.*) suppression of corticosterone production by NDV-infected hypophysectomized mice. The mice were given free access to water with or without dexamethasone (20 µg/ml) for approximately 24 hours. Control (NDV, $N = 13$) or dexamethasone-treated (NDV + dex, $N = 5$) mice were then injected, as before, with NDV. Eight hours after infection the mice were killed and treated as in (A). Other controls without dexamethasone included: controls ($N = 19$), in which saline (0.2 ml) was injected intraperitoneally and the mice were killed 8 hours later; cold stress ($N = 5$), in which the mice were placed in ice water for 45 seconds and killed 20 minutes later, and ACTH ($N = 9$), in which the mice received an intramuscular injection of 25 µg of Cortrosyn (Organon, Inc., West Orange, New Jersey) in 0.2 ml and were killed 2 hours later. Corticosterone concentrations are expressed as means ± standard error.

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