Reversible Chemical Modification of the Scrapie Agent

Abstract. *The scrapie agent causes a degenerative nervous system disease in sheep and goats. Studies with extensively purified preparations demonstrated that the agent contains a protein that is required for infectivity. Chemical modification of the scrapie agent by diethyl pyrocarbonate reduced the titer 1000-fold. Exposure of the inactivated agent to hydroxylamine, a strong nucleophile, resulted in complete restoration of infectivity. Presumably, nucleophilic residues within a scrapie agent protein undergo carbethoxylation on reaction with diethyl pyrocarbonate, and subsequent addition of hydroxylamine displaces these carbethoxy groups.*

Scrapie is a disease characterized by a progressive, fatal degeneration of the central nervous system. Its development follows a prolonged incubation period during which the animals are free of neurological dysfunction. Although a natural disease of sheep and goats, scrapie has been transmitted to laboratory rodents. It serves as a model for the transmissible disorders kuru and Creutzfeldt-Jakob disease, which afflict humans (*1*). The agents responsible for these neurological disorders have many unusual biological properties, which may be consequences of their small size and hydrophobicity (*2*, *3*).

Purification of the scrapie agent to homogeneity has not yet been accomplished. Low concentrations of the agent, its hydrophobic nature, and the necessity to purify it from brain tissue have hindered attempts to obtain a homogeneous preparation. In addition, a rapid assay for the agent is needed to aid progress in purification. Endpoint titrations in mice required holding 60 mice for up to 1 year in order to quantitatively ascertain the amount of agent in a single sample (*3*).

We have developed an incubation time interval bioassay for the scrapie agent in hamsters, which requires holding four hamsters for 60 to 150 days to quantify the amount of agent in a sample (*4*). This new assay has allowed us to monitor purification steps more quickly and thus develop an extensive purification protocol. With highly enriched preparations we have established that the agent contains a protein that is required for infectivity. Data demonstrating the loss of infectivity on treatment of the agent with phenol, sodium dodecyl sulfate, and chaotropic ions suggested the presence of an essential protein within the agent (*4*, *5*). However, the most compelling evidence for the presence of an essential protein is our observation that more than 99.9 percent of the infectivity of preparations that have been purified 100- to 1000-fold with respect to cellular protein can be destroyed by enzymatic hydrolysis (*6*).

To further probe the role of proteins in the structure and function of the scrapie agent, we have investigated the effects of reversible chemical modification by diethyl pyrocarbonate (DEP) on the agent. DEP is an electron-deficient electrophile that readily reacts with electron-rich nucleophiles having an unshared pair of electrons (*7*). The reaction yields a carbethoxylated (ethoxyformylated) adduct (Fig. 1A). DEP rapidly carbethoxylates proteins, especially histidyl residues, and reacts very slowly with nucleic acids (*8*, *9*). These carbethoxy groups are displaced from modified histidyl, tyrosyl, and seryl residues when exposed to hydroxylamine (*10*, *11*) (Fig. 1B).

Extensive purification of the agent involved the use of brain tissue from female, weanling, random-bred hamsters (LVG/LAK) which were inoculated intracerebrally with 10^7 ID_{50} (median infectious dose) units of the agent. After an incubation period of 60 days, the scrapie agent was purified by differential centrifugation, detergent extraction, enzymatic digestion, ammonium sulfate precipitation, and Sarkosyl agarose gel electrophoresis (*4*, *5*, *12*). Titers of the agent were determined by measuring the incubation time intervals from inoculation to onset of illness and to death in LVG/LAK female hamsters after intracerebral injection of samples (*13*). Protein was measured by a modification of the method of Lowry *et al.* with crystalline bovine serum albumin as a standard (*14*).

Exposure of the scrapie agent in an extensively purified preparation to increasing concentrations of DEP resulted in a dose-dependent inactivation (Fig. 2). No loss of infectivity was detected in samples exposed to DEP at concentrations below 5 m*M*; a marked reduction in agent titer was found with DEP concentrations of 10 m*M* or more. The titer of the agent was reduced about 50 percent on exposure to 5 m*M* DEP and more than 99 percent on exposure to 10 m*M* DEP. The $t_{1/2}$ for DEP under the reaction conditions used in our experiments was ~ 0.4 minute (*15*).

The scrapie agent in crude preparations was resistant to inactivation by DEP (*16*). In contrast, extensively purified agent was sensitive to modification by DEP as described above. These observations suggested that the higher protein concentrations in crude preparations protected the scrapie agent from chemical modification. To test this hypothesis, ribonuclease A, BSA, or L-histidine was added to extensively purified preparations of the agent prior to treatment with DEP. The infectivity of the agent was partially protected by the addition of these nucleophiles (Table 1).

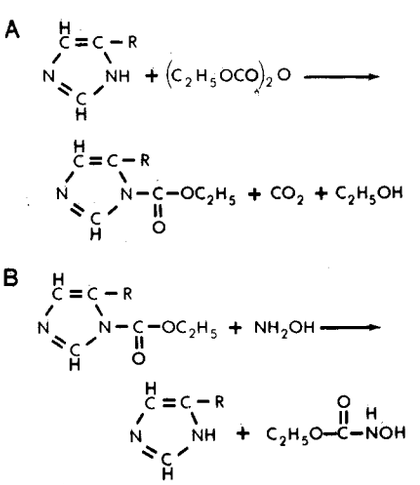

Fig. 1. Reversible chemical modification of a nucleophilic group. (A) Carbethoxylation of a nucleophile, such as an imidazole residue, by diethyl pyrocarbonate. (B) Proposed chemical reaction of decarbethoxylation by hydroxylamine, a strong nucleophile.

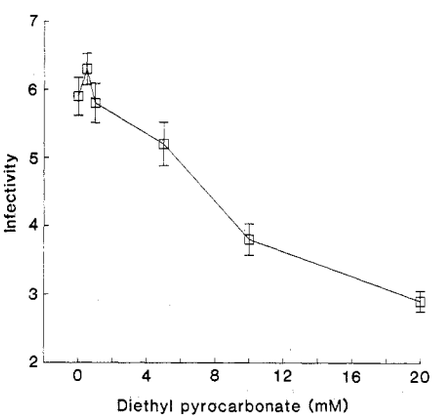

Fig. 2. Dose-dependent inactivation of the scrapie agent with increasing concentrations of DEP. Standard errors of titers are denoted by bars. An extensively purified fraction containing the scrapie agent and 75 μg of protein per milliliter was dialyzed overnight at 4°C against 20 m*M* tris-HCl, *p*H 7.4, containing 1 m*M* EDTA and 0.2 percent Sarkosyl. Freshly prepared aqueous DEP was added to give the desired final concentration of DEP in a 0.1-ml incubation mixture. All samples were mixed well and allowed to incubate at room temperature for 30 minutes. Reactions were terminated by plunging the vials into a dry ice–ethanol bath. Vials were then stored at −20°C for 3 to 4 weeks prior to bioassay. Infectivity is expressed as $\log_{10}$ ID_{50} units per milliliter.

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Table 1. Protection of the scrapie agent from chemical modification by diethyl pyrocarbonate (DEP). To 50 μl of an extensively purified sample, freshly prepared ribonuclease A, bovine serum albumin, or neutralized L-histidine was added to appropriate final concentrations from stock solutions. After the sample was mixed, freshly prepared aqueous DEP was added to a final concentration of 10 m*M*. Samples were incubated for 30 minutes at room temperature (see legend of Fig. 2).

Nucleophile addition	Concentration	Infectivity ($\log_{10}$ ID_{50} unit/ml ± S.E.) at DEP concentration	
		0 m*M*	10 m*M*
None		5.9 ± .28	3.8 ± .23
Ribonuclease A	50 μg/ml	6.2 ± .21	4.4 ± .17
	500 μg/ml	5.9 ± .33	5.1 ± .07
BSA	50 μg/ml	6.1 ± .21	4.0 ± .18
	500 μg/ml	6.2 ± .13	5.9 ± .27
L-Histidine	20 m*M*	6.4 ± .23	5.6 ± .33
	60 m*M*	6.5 ± .15	6.2 ± .24

The biological activity of some proteins inactivated by carbethoxylation can be restored by hydroxylamine (*10*, *11*). As shown in our experiments (Table 2), hydroxylamine restored the infectivity of the scrapie agent. Hydroxylamine alone had no significant effect on agent titer. An extensively purified preparation of the agent was treated with 20 m*M* DEP for 30 minutes at 25°C. Next, 100 or 500 m*M* hydroxylamine was added and the samples were held at 25°C for an additional 30-minute period prior to storage at −20°C. Such treatment reversed the DEP inactivation by restoring > 99 percent of the infectivity.

The most plausible explanation for the DEP-dependent inactivation of the scrapie agent is the carbethoxylation of a scrapie protein; however, further studies are required to define the mechanism of inactivation. DEP reacts with nucleophilic groups in proteins, such as histidyl residues, ~ 10^4 times more rapidly than with nucleotides (*17*). The return of infectivity after exposure of DEP-treated samples to hydroxylamine suggests that acylation of certain amino acid residues occurs during DEP inactivation of the agent. Chemical modification of histidyl, tyrosyl, or seryl residues must be considered since decarbethoxylation by hydroxylamine has been observed only for these amino acid residues (*10*, *11*). Hydroxylamine has not been reported to reverse DEP modifications of nucleic acids. However, 1*M* hydroxylamine at physiological *p*H has been used to produce base transitions in nucleic acids which lead to mutagenesis (*18*). The lack of a significant effect on scrapie infectivity by hydroxylamine alone is of interest.

Inactivation of some conventional viruses, such as T_4 and Qβ bacteriophages, by DEP has been described (*19*, *20*). Carbethoxylation of coat or capsid protein appears to be the mechanism of inactivation for each in that the infectivity of the isolated nucleic acid was unaltered (*21*). Other viruses, such as an intact tobacco-mosaic virus (TMV), are only slightly inactivated by DEP (*22*). This effect on intact TMV probably results from DEP reaction with viral protein moieties, since there was no loss in infectivity of RNA extracted from DEP-treated TMV (*22*). In addition, DEP has no adverse effect on either the isolated double-stranded DNA genomes of T_4 and 3NT bacteriophage or the double-stranded RNA of poliovirus replicative form, as demonstrated by transfection studies (*21*, *23*). However, similar studies on the single-stranded RNA genomes of TMV, poliovirus, and Qβ bacteriophage that had been treated with DEP showed marked reductions in titer as assayed by transfection (*20*, *22*, *23*). No information is available on the reversal of DEP inactivation of conventional viruses or their single-stranded genomes by treatment with hydroxylamine.

Studies with purified enzymes and membrane fractions have reported inactivation of biological activity on chemical modification with DEP, followed by reactivation after exposure to hydroxylamine. The catalytic activities of lactate dehydrogenase, ribonuclease, glutamate dehydrogenase, thermolysin, and chymotrypsin were all reduced after carbethoxylation with DEP (*10*, *11*, *24*, *25*). Decarbethoxylation by exposure to hydroxylamine restored the catalytic activities of these enzymes. For lactate dehydrogenase, ribonuclease, glutamate dehydrogenase, and thermolysin, reactivation of these enzymes was associated with displacement of a carbethoxy group from a critical histidyl residue. For chymotrypsin, reactivation was found after decarbethoxylation of a seryl residue at the active site (*9*). Acylation of membrane vesicles from *Escherichia coli* by DEP inhibited lactose translocation (*26*). Incubation of the acylated vesicles with hydroxylamine regenerated the lactose transport system. Similarly, carbethoxylation of rat α-fetoprotein inhibited estrogen binding while restoration of this binding function occurred on decarbethoxylation with hydroxylamine (*27*).

Table 2. Reversible chemical modification of the scrapie agent. Purified samples (50 μl) were treated with 10 or 20 m*M* DEP for 30 minutes at room temperature. Freshly prepared, neutral hydroxylamine hydrochloride was added to 0.1 or 0.5*M* final concentration. The samples were mixed and then incubated at room temperature for 30 minutes (see legend of Fig. 2).

DEP (m*M*)	Infectivity ($\log_{10}$ ID_{50} unit/ml ± S.E.) at hydroxylamine concentration		
	0.0*M*	0.1*M*	0.5*M*
0	5.9 ± .28	5.3 ± .38	4.9 ± .20
10	3.8 ± .23	5.4 ± .25	6.1 ± .23
20	2.9 ± .15	5.3 ± .35	5.6 ± .23

Our results demonstrate that chemical modification of the scrapie agent by diethyl pyrocarbonate can be prevented by the addition of protein or histidine and can be reversed by treatment with hydroxylamine. Although DEP reacts with various nucleophiles, the hydroxylamine result suggests that its target is a nucleophilic amino acid residue within a protein of the scrapie agent. Our findings should open new investigative approaches for probing the chemical structure of the scrapie agent and defining the molecular basis of its slow infectivity.

MICHAEL P. MCKINLEY
FRANK R. MASIARZ
STANLEY B. PRUSINER
Departments of Neurology and *Biochemistry and Biophysics, University of California, San Francisco 94143*

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Neuroleptic Drug–Induced Dopamine Receptor Supersensitivity: Antagonism by L-Prolyl-L-Leucyl-Glycinamide

Abstract. *An animal model of tardive dyskinesia was used to evaluate the potential antidyskinetic properties of the neuropeptide L-prolyl-L-leucyl-glycinamide (PLG). In rats, PLG administered concurrently with the neuroleptic drug haloperidol or chlorpromazine antagonized the enhancement of specific [^{3}H]spiroperidol binding in the striatum that is associated with long-term neuroleptic treatment. The results are discussed in relation to a possible functional coupling of the putative PLG receptor with neuroleptic–dopamine receptor complex and clinical implications for tardive dyskinesia.*

Antipsychotics belonging to the chemical classes of butyrophenone, phenothiazine, and thioxanthene are thought to exert their therapeutic effects by selectively blocking central dopamine receptors (*1*). Prolonged neuroleptic therapy in the management of psychiatric patients, however, has produced a variety of extrapyramidal motor disorders, tardive dyskinesia being the most prevalent. Animal models of tardive dyskinesia have been developed in rodents and nonhuman primates, and such studies suggest that supersensitivity of dopaminergic neuronal systems in the basal ganglia is the primary mechanism of tardive dyskinesia (*2*). Diverse pharmacological approaches have been attempted to reverse or prevent tardive dyskinesia, but none has yielded a consistent and favorable clinical outcome (*3*); differential modification of the sensitivity of dopamine receptors remains the preferred theoretical basis for designing therapeutic agents to alleviate tardive dyskinesia.

Clinical studies indicated that lithium and L-prolyl-L-leucyl-glycinamide (PLG) transiently but significantly reduced the intensity of dyskinetic symptoms associated with protracted antipsychotic therapy (*4*). In rats, prolonged lithium treatment in conjunction with neuroleptics abolished both the biochemical and behavioral manifestations of dopaminergic supersensitivity: such treatment enhanced neuroleptic–dopamine receptor binding and augmented stereotyped behavioral responses toward dopamine agonists (*5*). The potential antidyskinetic properties of PLG, a neuropeptide derived from the carboxyl terminal of oxytocin (*6*), have not been critically investigated. We found that PLG antagonized morphine- (*7*) and haloperidol-induced (*8*) catalepsy and selectively enhanced the affinity of the [^{3}H]apomorphine binding to neuroleptic-dopamine receptors in rat striatum (*8*). To investigate the possible desensitizing effect of PLG on dopaminergic supersensitivity, we studied the chronic effects of PLG, when administered concurrently with haloperidol or chlorpromazine, on dopamine receptor function, as measured by [^{3}H]spiroperidol binding in rat striatum.

Two series of drug studies were undertaken in male Sprague-Dawley rats to examine (i) PLG-haloperidol interaction and (ii) PLG-chlorpromazine interaction. The experimental protocols for the drug treatments of various groups of animals are described in Tables 1 and 2.

The procedure of Creese *et al.* (*9*) was adopted for the *l*-[phenyl-4-^{3}H]spiroperidol (25.64 Ci/mmole; New England Nuclear) binding assay. The specific binding of [^{3}H]spiroperidol was defined as the difference in binding in the presence and absence of 500 n*M* of unlabeled spiroperidol. The binding data were analyzed by the Scatchard plot from which the binding parameters, maximal number of binding sites (B_{max}), and dissociation constant (K_d) were derived by linear regression analysis. The biochemical data from different groups of animals were analyzed statistically by one-way analysis of variance followed by the Duncan multiple range test.

Our results indicate that Scatchard plots obtained from normal saline-control rats yielded a single class of noninteracting binding sites with a B_{max} of 317 ± 25 fmole per milligram of protein and K_d of 0.52 ± 0.20 n*M*. Protracted treatment with haloperidol and chlorpromazine resulted in significant ($P < .05$) elevation of the receptor density of [^{3}H]spiroperidol in rat striatum (Table 1), compared to that in the saline controls. Haloperidol (3 mg/kg, intraperitoneally) administered once daily for 21 days caused a mean increase of 58 percent in the B_{max} of [^{3}H]spiroperidol binding over the saline controls, whereas chlorpromazine (20 mg/kg, intraperitone-

Table 1. Blockade of haloperidol (HAL)-induced increase in specific [^{3}H]spiroperidol binding by L-prolyl-L-leucyl-glycinamide (PLG). Male Sprague-Dawley rats weighing 200 to 250 g were randomly assigned to six groups and received various drug dosages for 21 days according to the following protocol: group 1 was given isotonic saline (1 ml/kg, subcutaneously); groups 2 and 3 were administered PLG at the respective doses of 10 and 40 mg/kg, subcutaneously; groups 4 and 5 were dosed respectively with PLG at 10 and 40 mg/kg, subcutaneously, 10 seconds before administration of haloperidol (3 mg/kg, intraperitoneally); and group 6 received haloperidol (3 mg/kg, intraperitoneally) only. The animals were killed 5 days after the last drug session and [^{3}H]spiroperidol binding was carried out on striata. The striatum from each rat in the different treatment groups was used for one Scatchard plot of [^{3}H]spiroperidol binding from which the mean values and standard errors of B_{max} and K_d were determined.

Group	Treatment	*N*	B_{max} (fmole per milligram of protein)	K_d* (n*M*)
1	Saline	8	317 ± 25	0.52 ± 0.20
2	PLG	8	296 ± 44	0.50 ± 0.11
3	PLG	4	267 ± 29	0.38 ± 0.07
4	PLG and HAL	4	380 ± 14	0.66 ± 0.18
5	PLG and HAL	4	384 ± 20	0.74 ± 0.25
6	HAL	5	498 ± 22†	0.58 ± 0.20

*No statistically significant difference was found among the six treatment groups with respect to the K_d values at .05 level. †Significantly different ($P < .05$) from treatment groups 1, 2, 3, 4, and 5 by Duncan's multiple range test.

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