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Journal of Neurochemistry
Raven Press, New York

Axoplasmic Transport and Turnaround of Neurotoxic Esterase in Hen Sciatic Nerve

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Abstract: We have recently found that there is a proximo-distal delay in the recovery of neurotoxic esterase (NTE) following inhibition along the sciatic nerve of the hen. To determine whether this delay could be due to a requirement for the transport of newly synthesized NTE from the cell body, we investigated the transport of NTE by measuring the rate of accumulation of activity at either one or two ligations. Although rapid turnaround of accumulated protein confounds calculation of the transport rate, it appeared that NTE is transported down the hen sciatic nerve at a rate close to 300 mm/day. Acetylcholinesterase (AChE) was found to be transported at a rate of about 500 mm/day, which is close to the expected rate of fast axoplasmic transport in the chicken. The relatively rapid turnaround of NTE compared with the retrograde transport rate precluded the estimation of a retrograde transport rate. A model is presented that accounts for turnaround as a result of exchange between mobile and stationary transport pools. Exchange of NTE between pools may account for the rapid turnaround of NTE described in this paper and for the proximo-distal delay in recovery as a dilution of newly synthesized NTE in the anterograde fast transport pool by inhibited protein as it travels down the nerve. **Key Words:** Neurotoxic esterase—Hen sciatic nerve—Axoplasmic transport and turnaround. **Carrington C. D. and Abou-Donia M. B.** Axoplasmic transport and turnaround of neurotoxic esterase in hen sciatic nerve. *J. Neurochem.* **44**, 616–621 (1985).

A large number of organophosphorous compounds (OPs) are able to induce a neuropathy 2–3 weeks after a single dose in many species, including humans (Abou-Donia, 1981). This phenomenon is known as organophosphorous compound-induced delayed neurotoxicity (OPIDN). Although the mechanism of OPIDN is still unknown, Johnson (1969, 1982), has proposed that an enzyme assayed by phenylvalerate hydrolysis, called either neurotoxic esterase or neuropathy target enzyme (NTE), is the site at which the toxic effect is initiated.

One of the difficulties with the NTE hypothesis has been that considerable NTE activity returns in brain, spinal cord, and the sciatic nerve (Johnson, 1974; Caroldi and Lotti, 1982) before the onset of the neuropathy. However we recently found (Carrington and Abou-Donia, 1984) that there is a delay in the onset of the recovery of NTE activity in the peroneal branch of the hen sciatic nerve. A delay in the recovery of some function associated with NTE might account for the fact that the distal portions of long axons are the most sensitive to OPIDN (Cavanagh, 1964). Since most neuronal proteins are derived from the soma, it seems likely that a requirement for the transport of NTE down the axon may account for the delay in the recovery of the activity in the distal portions of the nerve.

In this paper we present an investigation of the transport of NTE in the hen sciatic nerve that was conducted by measuring the accumulation of activity at either one or two ligations at various times following surgery.

MATERIALS AND METHODS

Animals

White leghorn hens, 18 months old, were obtained from Featherdown Farms (Raleigh, NC). They weighed between 1.5 kg and 2.5 kg. The birds were kept in a humidity- (40–60%) and temperature- (21–23°C) controlled room with a 12-h light cycle before and during the experiment.

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Abbreviations used: AChE, Acetylcholinesterase; DFP, *O,O*-Diisopropyl phosphofluoridate; NTE, Neurotoxic esterase or neuropathy target enzyme; OPs, Organophosphorus compounds; OPIDN, Organophosphorus compound-induced delayed neurotoxicity; PMSF, Phenylmethylsulfonyl fluoride.

Materials

Paraoxon (*O,O*-diethyl *O*-4-nitrophenylphosphate), phenylmethylsulfonyl fluoride (PMSF), and chloral hydrate were obtained from Sigma Chemical Co. (St. Louis, MO). Mipafox (*N,N*-diispropyl diamidicphosphorofluoridate) was synthesized by Midwest Research Institute (Kansas City, MO). Sodium pentobarbital was obtained from Abbott Laboratories (Chicago, IL). Ketamine HCl was obtained from Parke-Davis (Morris Plains, NJ).

Nerve ligations

The surgeries for the single ligation experiments, the 24-h double ligations, and the acetylcholinesterase (AChE) accumulation experiments were performed on animals anaesthetized with 250 mg/kg of chloral hydrate. For all the other surgeries animals were anaesthetized with 20 mg/kg sodium pentobarbital and 50 mg/kg ketamine HCl. The use of chloral hydrate anaesthesia was discontinued because the onset and recovery were slow, and because it was frequently lethal. There did not appear to be any difference in accumulation rates following the two regimens. Following exposure, the sciatic nerve was ligated with 00 silk thread at distances between 14 and 53 mm from the point at which the nerve exited from the spinal cord. The distance between the double ligations ranged from 22 to 38 mm. Third ligations were made 12 h following the placement of the first two in the middle of the nerve segment isolated by the double ligations.

Tissue preparation

Animals were killed by asphyxiation with CO_2, and the sciatic nerves were removed. Six-millimeter segments were taken just proximal and distal to each ligation. Two segments taken between 18 and 30 mm proximal to the ligation were used as controls in the single-ligation experiments. In the double-ligation experiments, two to four additional segments were taken in the middle of the nerve section isolated by the ligations. Each segment was homogenized in 1 ml of 50 m*M* Tris-0.32 *M* sucrose with a polytron set at 7 for 5 s.

Enzyme assays

NTE activity was measured by the method of Johnson (1977) with three alterations. First the volume of the reaction was reduced by 50%. Second the incubation period was increased to 90 min. The reaction rate was found to be linear over this time period. Finally the reaction was performed with a 1000 *g* supernatant, rather than the whole homogenate. The first two alterations were introduced to increase the sensitivity of the assay, the third was necessary to remove the large particles of connective tissue that remained after homogenization. AChE assays were conducted by the method of Ellman et al. (1961), using nerve prepared in the same manner as the tissue used in the NTE assays with the exception that Tris was not included in the homogenization buffer.

Calculations

The activity in each segment of nerve was calculated. Transport rates were computed by the method of Partlow et al. (1972), with the exception that the apparent rate of transport was corrected for the transport of activity away from the ligation. The equations used to calculate the anterograde and retrograde transport rates were:

$$\text{Anterograde rate} = \frac{(PA + RMF) \times 6\text{ mm}}{AMF \times T\text{ days}}$$

$$\text{Retrograde rate} = \frac{(DA + AMF) \times 6\text{ mm}}{RMF \times T\text{ days}}$$

PA and *DA* are the percentage increases in activity in first segment (*PA*) or distal (*DA*) to the isolated nerve section. *AMF* and *RMF* are the apparent anterograde (*AMF*) and retrograde (*RMF*) mobile fractions. *T* is the time in days.

RESULTS

Accumulation of NTE activity at either a single ligation or outside a section of nerve isolated by double ligations was found to increase over a 24-h period (Fig. 1). However the rate of accumulation decreased with time. NTE levels proximal to an unrestricted ligation rose by 10.3%/h during the first 3 h, by 8.2%/h between 3 and 12 h, and at a rate of 4.7%/h from 12 to 24 h. During the same time periods the distal accumulation rates were 6.3%, 4.5%, and 1.7%/h.

A higher initial rate of accumulation of proteins at a ligation is a common finding (Lubinska and Niemarko, 1971; Couraud and Giamberardino, 1980). Lubinska and Niemarko (1971) suggested that the more rapid accumulation that they observed in the first 3 h following ligation was an artifact resulting from nerve injury. However we found no difference in activity in short isolated segments of nerve (12–24 mm) and control segments 12 mm proximal to the isolated section 3 h following the ligation of the nerve (inside/outside activity/mm = 0.95 ± 0.07, n = 6). A more likely explanation of the reduced rate of accumulation of NTE at later

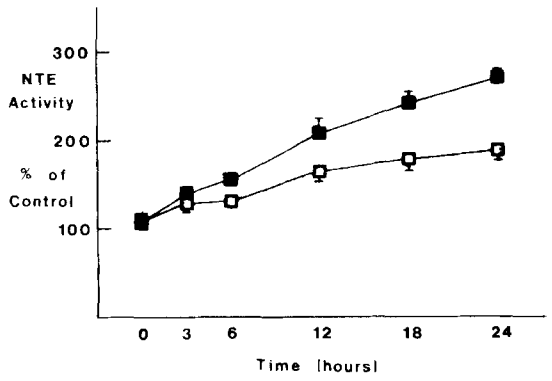

FIG. 1. Accumulation of NTE at a ligation of the hen sciatic nerve. The accumulation of NTE in hen sciatic nerve at a single ligation, or outside an isolated nerve segment, over a 24-h period. (■), Proximal or anterograde accumulation; (□), distal or retrograde accumulation. The values are the average of the percentage of the control NTE activity in each nerve in the segments either proximal or distal to the ligation. There were 11–16 measurements for each point. The error bars denote SEM.

time points is that some of the accumulated protein undergoes a reversal of the direction of transport (turnaround; Bisby and Bulger, 1977; Schmidt et al., 1980) so that it is transported away from the ligation.

The calculation of the rate of transport of a protein based on the rate of accumulation at a single ligation would be valid only if all of the protein were moving toward the ligation at an equal rate. The pattern of accumulation of both NTE and AChE at the distal and proximal ends of an isolated nerve section demonstrates that this is not the case (Fig. 2). First proximal and distal accumulation between the ligations was not as great as that observed outside of the ligatures. Were NTE transported at a rate of less than 15 mm/day, accumulation should not be restricted by ligations 30 mm apart in less than 24 h. The fact that proximal accumulation of NTE is significantly decreased by a second ligation after only 6 h (Table 1) indicates that the anterograde transport rate is greater than 120 mm/day (30 mm/0.25 days). Second the activity in the middle of the section of nerve isolated by the ligatures was not greatly diminished. These observations may be explained by supposing that only a fraction of the esterase activity is transported at a rapid rate (Lubinska and Niemarko, 1979).

Mobile fractions have been estimated by measuring proximal and distal accumulation in an isolated nerve section. To demonstrate that all of the fast-transported protein has cleared from the interior of the segment, the mobil fraction is estimated at a time point where maximal accumulation has occurred (Bisby, 1982). For instance Fonnum et al. (1973) found no significant difference in AChE accumulation at 22 and 35 h following the double ligation of a rabbit hypoglossal nerve. Using this criterium the mobile fractions for NTE may be estimated at 24 h since there is no change in distal (anterograde) accumulation after 12 h, or proximal (retrograde) accumulation after 18 h (Table 2). The estimation of the magnitude of the mobile fractions using this method neglects the possibility of exchange between stationary and mobile transport pools. Consequently the accuracy of the transport rate calculation depends on a slow exchange rate relative to the rate of transport.

It is apparent that the exchange between the stationary and mobile pools of NTE is fairly rapid for two reasons. First, there is a substantial turnaround of NTE activity at a ligation. As has already been noted, turnaround is likely to be responsible for the dimunition in the rate of accumulation of NTE at a ligation over time. More conclusive evidence is the fact that the placement of a third ligation in the middle of an isolated nerve segment reduces the amount of accumulation inside the first two ligations (Table 2). This would not occur if the distri-

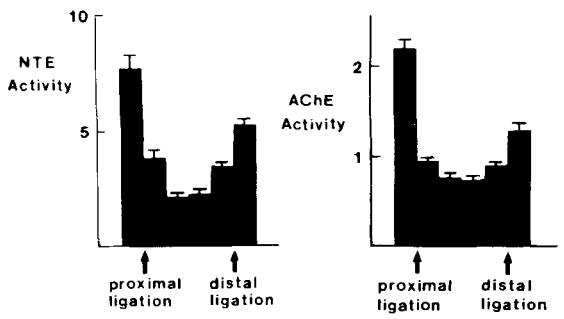

FIG. 2. The accumulation of NTE and AChE activity in doubly ligated hen sciatic nerve. NTE activity, in n*M*/segment-min (mean ± SEM; n = 11), and AChE activity, in μ*M*/segment-min, (mean ± SEM; n = 10) in 6-mm segments of hen sciatic nerve doubly ligated 24 h before measurement.

TABLE 1. *Accumulation ratios*

Time (h)	n	Proximal	Distal	Segment length (mm)
NTE				
0	6	0.99 ± 0.10	0.88 ± 0.02	31.0 ± 1.0
3	10	0.88 ± 0.07	0.89 ± 0.05	27.1 ± 1.1
6	9	0.74 ± 0.06*	0.97 ± 0.09	34.2 ± 1.0
12	7	0.64 ± 0.08*	0.84 ± 0.03	28.7 ± 1.2
18	7	0.47 ± 0.06*	0.90 ± 0.10	27.4 ± 0.5
24	11	0.45 ± 0.02*	0.73 ± 0.04*	24.2 ± 0.5
AChE				
3	6	0.78 ± 0.08	1.00 ± 0.03	25.3 ± 1.2
24	10	0.40 ± 0.02	0.77 ± 0.06	26.7 ± 1.1

The proximal and distal ratios of the activities of NTE and AChE at a ligation inside over the activities outside an isolated nerve segment. *Differs significantly ($p < 0.05$) from zero time control by unpaired *t* test.

TABLE 2. *Calculated transport rates for NTE*

Time (h)	n	Apparent transport rate	Apparent mobile fraction	Corrected rate (mm/day)
Anterograde				
0	6	—	−0.1 ± 0.9	—
3	10	18.2 ± 3.4	6.0 ± 1.0	328
6	9	12.7 ± 1.9	4.1 ± 0.6	345
12	7	13.3 ± 1.7	10.0 ± 2.6	145
18	7	11.4 ± 1.3	7.7 ± 1.4	164
12,6*	8	—	2.4 ± 0.9	503
24	11	10.1 ± 0.7	9.6 ± 1.0	113
12,12*	7	—	6.3 ± 1.3	170
Retrograde				
0	6	—	0.7 ± 1.2	—
3	10	13.0 ± 2.9	3.4 ± 1.1	466
6	9	7.7 ± 1.4	6.1 ± 1.2	142
12	7	8.0 ± 1.0	10.1 ± 2.0	91
18	7	6.3 ± 1.2	16.1 ± 2.0	43
12,6*	8	—	9.0 ± 1.7	72
24	11	5.2 ± 0.5	13.0 ± 1.8	45
12,12*	7	—	9.8 ± 1.6	57

The apparent transport rates and mobile fractions, and the calculated transport rates for NTE in hen sciatic nerve in both anterograde and retrograde directions. All values are means ± SEM. *A third ligation was tied in the middle of an isolated nerve segment 12 h after the placement of the first two.

bution between the mobile and stationary fractions were stable. Second, inhibition of activity 72 h prior to ligation with PMSF does not greatly alter the mobile fraction estimates. Schmidt et al. (1980) observed that pretreatment with *O,O*-diisopropyl phosphorofluoridate (DFP), an irreversible esterase inhibitor, reduced the apparent stationary phase of AChE by about 50%. This suggests that newly synthesized AChE enters the fast-transport phase and is not in rapid equilibrium with at least a portion of the stationary phase. Preinhibition of NTE by PMSF does not produce the same result (Table 3). Although there were slight increases in anterograde accumulation and in the magnitude of the apparent mobile fractions, the difference is small compared with the reduction in total activity (>50%). Consequently it appears that although NTE does enter the nerve in the fast transport pool, exchange with stationary pools is fairly swift. The lower retrograde accumulation following PMSF is expected considering the low levels of NTE in the branches below the sciatic 3 days following preinhibition (Carrington and Abou-Donia, 1984).

The calculated anterograde transport rate for AChE (Table 4) in hen sciatic nerve is close to the 500 mm/day value that would be expected in an animal with a body temperature of about 40°C (Ochs and Smith, 1975). As with NTE we found a higher initial rate of AChE accumulation. Couraud and Giamberardino (1980) also found a higher initial rate in their study of AChE transport in chick sciatic nerve. The lower estimate of AChE transport rate using the data resulting from 24 h of accumulation, compared with 3 h, may also be due to turnaround. The fact that the reduction in the rate estimate with time is greater for NTE than for AChE indicates that turnaround is a bigger problem in estimating the transport rate of NTE. However the fact that a second ligation appears to reduce proximal accumulation of AChE after 3 h, whereas NTE accumulation is affected very little (Table 1), suggests that the difference in the calculated transport rates for NTE and AChE at 3 h is due to actual differences in transport rate rather than turnaround.

TABLE 3. *Calculated transport rates for AChE*

Time (h)	n	Apparent transport rate	Apparent mobile fraction	Corrected rate (mm/day)
Anterograde				
3	6	20.2 ± 5.8	4.2 ± 1.6	537
24	10	10.2 ± 0.6	3.3 ± 0.7	320
Retrograde				
3	6	9.1 ± 0.5	7.4 ± 1.0	149
24	10	3.2 ± 0.4	5.4 ± 1.3	62

The apparent transport rates and mobile fractions, and the calculated transport rates for AChE in hen sciatic nerve in both anterograde and retrograde directions. All values are means ± SEM.

TABLE 4. *Calculated transport rates for NTE following preinhibition with PMSF*

Time (h)	n	Apparent transport rate	Apparent mobile fraction	Corrected rate (mm/day)
Anterograde				
3	6	19.7 ± 7.7	7.4 ± 1.6	299
6	8	17.8 ± 3.1	4.7 ± 1.6	426
Retrograde				
3	6	11.5 ± 2.9	4.8 ± 1.6	314
6	8	4.3 ± 2.6	9.2 ± 2.1	71

The apparent transport rates and mobile fractions, and the calculated transport rates for NTE in hen sciatic nerve in both anterograde and retrograde directions 72 h after the administration of 30 mg/kg PMSF. All values are means ± SEM.

DISCUSSION

The movement of NTE between transport pools appears to be sufficiently fast to warrant a model in which rates of exchange are included. Such a model for axonal transport in an intact nerve is presented in Fig. 3A, while Fig. 3B depicts transport in an isolated nerve segment. The model uses exchange between anterograde (A), retrograde (R), and stationary (S) pools to account for turnaround. The constants (K_1–K_4) represent drop-off and drop-on rates or fractions. K_1 is equivalent to the anterograde drop-off fraction (α) measured by Munoz-Martinez et al. (1981).

If the model is correct then the occurrence of turnaround is not unique to a ligation or nerve terminal but is instead a natural consequence of the drop-off of material as it is transported down the axon. The ratio between the drop-off and drop-on rates would determine how much of the protein is transported in a particular direction at equilibrium. In other words the magnitudes of the mobile fractions are dependent on the affinity of the protein for the transport system. This idea is similar to, and compatible with, the unitary hypothesis of axonal transport (Ochs, 1975), which maintains that transport rate is a function of the affinity of the protein for the transport system.

The equilibrium model should be better at describing accumulation of a protein for which the drop-off and drop-on rates are rapid compared with the transport rate. If the transport rate is rapid enough so that some of the protein reaches the terminal before an equilibrium is established, then the concentration of the protein at the distal end of the nerve would be greater than the equilibrium condition. The model could perhaps be refined further by supposing that only a part of the stationary fraction is in rapid (hours) equilibrium with the mobile fractions, while the rest is more stable.

According to the equilibrium model turnaround time, as measured by Bisby and Bulger (1977), should be dependent on the drop-off rate of the mo-

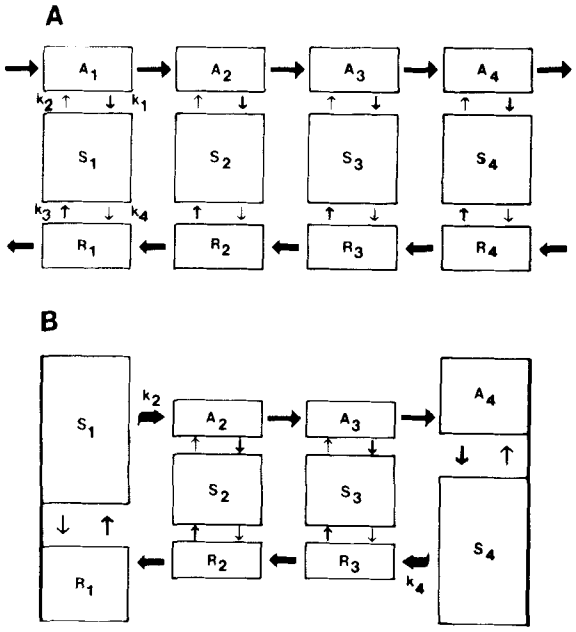

FIG. 3. An equilibrium model of axonal transport. An equilibrium model of axonal transport in intact nerve **(A)** and in an isolated nerve segment **(B)**. See text for further explanation.

bile fraction moving towards the ligation and the drop-on rate of the mobile fraction moving away from the ligation. Thus in a double ligation the rate of turnaround inside the distal ligation is determined by K_1 and K_4, whereas the rate of reversal at the proximal ligation is a function of K_3 and K_2. The size of the stationary fraction in equilibrium with the mobile fractions would determine which constant is more important.

Although the equilibrium model is useful for accounting for the pattern of NTE accumulation over time, it does not provide a means of calculating the transport rate since it introduces a number of unknown variables. Nonetheless there are two pieces of evidence that indicate that NTE is transported down the hen sciatic nerve at a rate close 300 mm/day. First, there is only a slight and statistically insignificant reduction in accumulation at a distal ligation caused by placing a second ligation about 30 mm proximal to the first after 3 h while there is a large difference after 6 h (Table 1), which suggests that the rate of transport is slightly greater than 240 mm/day. Second, the calculated rates of anterograde transport from the 3- and 6-h data are both close to 300 mm/day. Turnaround should have a minimal confounding effect at earlier time points. If it is assumed that the retrograde transport rate is at least half the anterograde rate, then 6 h should be sufficient for both the initial mobile fractions to be cleared from the isolated nerve segment. The earlier time points should also provide the most accurate estimates of the true mobile fractions.

On the other hand, there is no basis for estimating the retrograde transport rate from the data presented here. Unlike the anterograde transport data, none of the time points up to 18 h yield a calculated retrograde rate that predicts that sufficient time had elapsed for clearance of the retrograde mobile fraction in one of the earlier measurements. Furthermore, placement of the more distal ligation did not appear to reduce retrograde accumulation at the proximal ligation until 24 h following surgery.

There are two reasons why the retrograde calculations may be low. First, the NTE levels below the distal ligation in the sciatic bifurcations are less than those in the sciatic (Carrington and Abou-Donia, 1984). Second, there appears to be a difference in the anterograde-retrograde and retrograde-anterograde turnaround rates. If it is assumed that the accumulation observed at the ends of an isolated nerve section after 18 and 24 h represents equilibrium conditions, then there is a significantly greater amount of retrograde accumulation at equilibrium. Since at equilibrium an equal quantity of NTE activity must be leaving and arriving at both ends of the nerve section, the difference in accumulation must be due to a difference in turnaround rate, with anterograde-retrograde turnaround occurring faster than retrograde-anterograde turnaround. This might account for the observation that while NTE accumulation increases continuously for 18 h at the proximal end of an isolated nerve segment, there is a lag in accumulation between 3 and 6 h at the distal end. Anterograde-retrograde accumulation may be fast enough relative to the retrograde transport rate to maintain retrograde flow, whereas the anterograde NTE fraction is cleared before there is significant retrograde-anterograde turnaround. The fact that placement of a third ligation 12 h after the first two reduces anterograde accumulation to a greater extent than retrograde accumulation also supports the notion that turnaround inside the proximal ligation is slower.

Chemnitius et al. (1983) recently reported that there are two esterases (NTE_A and NTE_B) in hen brain that are more sensitive to mipafox than to paraoxon and that may be differentiated by their rates of inhibition by mipafox. We have found that the two species are present in similar proportions (80% NTE_B and 20% NTE_A) in the sciatic nerve (unpublished data). Since detection of the minor component requires a mipafox concentration curve, it is not possible to detect NTE_A in a small tissue sample. Consequently it is not possible presently to determine the distribution of the NTE isozymes in the fast-transport pools.

The ligation method does not appear to be a particularly accurate method for the estimation of the rates of NTE transport. However the data do reveal something of the nature of the proximo-distal delay in the recovery of NTE activity following inhibition.

Although the transport rate appears to be fast enough for newly synthesized NTE to be transported to the most distal portions of the branches of the sciatic nerve from the cell body in under 24 h, a substantial portion is dropped off and exchanged with the inhibited NTE in the retrograde and stationary pools. The greater the distance from the cell body, the greater will be the dilution of the newly synthesized NTE in the anterograde mobile fraction. This fact may be related to the greater susceptibility of longer axons to OPIDN.

Acknowledgment: This study was supported in part by NIEHS grant ESO-2717 and NIOSH grant OHO 2003.

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