Purification of a Human Red Blood Cell Protein Supporting the Survival of Cultured CNS Neurons, and Its Identification as Catalase

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We have previously reported that red blood cells contain high levels of a protein that supports the survival of a variety of CNS neurons *in vitro* for 24 hr. Here we report the isolation of this trophic activity from human red blood cells. The active material, purified over 1000-fold by ion-exchange chromatography and isoelectric focusing, has been identified as catalase based on its isoelectric point, subunit molecular weight, and ability to degrade hydrogen peroxide. Commercially produced bovine liver catalase, lactoperoxidase, HRP, and vitamin E all mimic the ability of the purified human protein to support neuronal survival *in vitro*. Pharmacological inhibitors of peroxidase activity inhibit the trophic effects of both commercial catalase and the purified blood-derived protein. These results suggest that peroxidase activity mediates the neuronotrophic activity of these agents. The bioassay culture medium itself generates peroxides in the absence of cells. Removal of this toxic material may be the basis for the trophic effects of catalase.

Central nervous system neurons have been postulated to require specific neuronotrophic factors (Varon and Adler, 1981), in analogy to the well documented *in vivo* and *in vitro* requirement of sympathetic neurons for NGF (Greene and Shooter, 1980). The occurrence of developmental neuronal cell death in the CNS, as in the PNS, supports this hypothesis (Cowan et al., 1984). The similarity of responses of CNS and PNS neurons to axotomy, including morphological changes and cell death (Barron, 1983), provides further encouragement. Insufficient supplies of such neuronotrophic factors have been implicated in neuronal death after CNS trauma and in degenerative diseases (Appel, 1981; Varon et al., 1984a).

Because it allows manipulation of neurons in isolation from their normal cellular and environmental relationships, neuronal cell culture has played a key role in studies of NGF, and in the recognition and purification of new peripheral neuronotrophic factors, the chick eye and rat nerve ciliary neuronotrophic factors (CNTFs) (Barbin et al., 1984a; Manthorpe et al., 1985) and the pig brain-derived neurotrophic factor (BDNF) (Barde et al., 1982). Similar cultures of isolated CNS neurons have been developed and used to demonstrate the presence of trophic activity in tissue extracts, serum, and glial cell conditioned medium (Banker, 1980; Barbin et al., 1984b; Kaufman and Barrett, 1983; Manthorpe et al., 1982, 1983b; Rudge et al., 1985). Some of this trophic activity toward a variety of CNS neurons is due to the presence of low-molecular-weight molecules, such as py-

ruvate, rather than to proteins (Müller et al., 1984; Selak et al., 1985; Varon et al., 1984b). Cell culture methods have also allowed the detection of protein neuronotrophic factors affecting CNS neurons. Partially purified BDNF also supports retinal ganglion cell neurons (Turner, 1985), and a 55,000 molecular weight protein has been reported to promote spinal motor neuron survival (Gurney and Apatoff, 1984; Kaufman and Barrett, 1983). Fluids collected from aspiration wounds in the rat entorhinal cortex also contain neuronotrophic factors for spinal cord neurons that appear to be proteins based on ultrafiltration behavior, and heat and trypsin sensitivity (Manthorpe et al., 1983b; Nieto-Sampedro et al., 1982). A similar activity was also found in cerebrospinal fluid (CSF) samples from human head trauma patients, but not in CSF from uninjured patients (Longo et al., 1984). CNS-competent trophic activity was similarly present in blood, which inevitably contaminates samples from traumatized tissue and might have been responsible for the activity. Cellular fractionation of blood indicated that the activity was contained in red blood cells, rather than in plasma or platelets (Williams et al., 1985).

Here we describe the purification of the neuronotrophic protein from human hemolysate and its identification as catalase. The ability of the protein to support neuronal survival correlates closely with its peroxidase activity, and other molecules capable of degrading peroxide are also able to support neuronal survival. Cell culture sources of peroxide are discussed.

Materials and Methods

Materials

Bovine liver catalase (Cat. no. B-10), bovine milk lactoperoxidase (L8257), HRP (P8250), vitamin E (T3634), aminotriazole (A4383), and phenylhydrazine (P7126) were obtained from Sigma Chemical Co. Immobiline isoelectric focusing gel materials were obtained from LKB Bromma. Ion-exchange and analytical gel electrophoresis chemicals were obtained from various sources as described (Barbin et al., 1984a).

Neuronal cell culture methods

Assays were performed using cultures of embryonic day (E) 8 chick forebrain neurons, a heterogeneous cell population deriving from regions homologous with the mammalian striatum, thalamus, and cortex. Intact E8 chick forebrain was incubated for 30 min with 0.08% trypsin in calcium/magnesium-free balanced salt solution and then washed and triturated in Hank's modified Eagle's basal medium with 1% ovalbumin (Barbin et al., 1984b; Selak et al., 1985). Undissociated tissue was removed by filtration through a 40 μm Nitex screen, and the cell concentration adjusted to 2×10^4 cells/ml. Aliquots (50 μl) of the cell suspension were plated into 96-well microtiter plates (Costar no. 3596) that had previously received 50 μl/well of test medium for a final cell density of 1000 cells per 100 μl/well. The substratum was polyornithine coated with schwannoma conditioned medium or purified laminin as previously described (Manthorpe et al., 1983a). All cultures were maintained in Hank's modified Eagle's basal medium, with additions of 2 mM glutamine, 100 U/ml penicillin, and the serum-free N1 supplement

(containing 830 nM insulin, 62.5 nM transferrin, 100 μM putrescine, 20 nM progesterone, and 30 nM sodium selenite) (Barbin et al., 1984b; Bottenstein et al., 1980). After 24 hr, the cultures were fixed with glutaraldehyde, and surviving neurons counted in a strip covering 10% of the well area by phase microscopy. Less than 5% of the plated cells survived under control conditions, while 60–90% survived in supported cultures. Previous characterization of these cultures using immunologic cell markers demonstrated that non-neuronal cells (tetanus-toxin negative) constituted less than 10% of cells present (Barbin et al., 1984b); large flat cells were not counted in the determination of neuronal survival. Trophic activity was determined by finding the concentration supporting half-maximal neuronal survival in serial twofold dilutions, and is expressed in trophic units (TU) as previously described (Barbin et al., 1984b; also see Fig. 1).

Preparation of hemolysate

Outdated human packed red blood cells were obtained from a local blood bank. The cells were resuspended in an equal volume of PBS, pelleted by centrifugation at 2200 × g for five min, and the supernatant and upper buffy coat portions discarded. This procedure was repeated twice, an equal volume of 10 mM phosphate buffer (pH 7.2) added, and the samples frozen. The thawed material, after addition of a second volume of phosphate buffer, was homogenized in a Waring blender at the highest speed for 2 min at 4°C, centrifuged at 100,000 × g for 1 hr, and the dark red supernatant collected. This procedure yielded about 400–500 ml of a 33% red blood cell extract. The extract was dialyzed overnight against two changes of 14 liters of phosphate buffer. The resulting material was adjusted, if necessary, to pH 6.2.

DEAE chromatography

Whatman DE-52 was prepared according to the manufacturer's instructions and equilibrated with 10 mM phosphate buffer (pH 6.2). A 1.7 × 20 cm column, containing approximately 40 ml bed volume of gel and eluted by gravity at a rate of 180–200 ml/hr, was prepared. The hemolysate was applied to the column and the nonadsorbing material collected. Column flow rates decreased during this run to about 75 ml/hr, so that several hours were usually required. The nonadsorbing material from the first column was adjusted to pH 7.2 with 1 M NaOH, and applied to a second identical DE-52 column adjusted to pH 7.2. The loaded column was then washed with about 250 ml of buffer and a 200 ml linear gradient of 0.0–0.2 M NaCl in 10 mM buffer (pH 7.2) applied, followed by 100 ml of 1 M NaCl. Active fractions were determined by bioassay, pooled, and frozen at −76°C. Protein was assayed as described (Bradford, 1976) using BSA as a standard.

Isoelectric focusing

A preparative scale, pH 5.7–6.7 Immobiline isoelectric focusing gel, was prepared according to the manufacturer's instructions (LKB Bromma, Application Note 321). A 5% acrylamide solution (30 ml) containing appropriate concentrations of pH 3.6 and 6.2 Immobilines were used to produce the desired pH 5.7 and 6.7 solutions, and glycerol was added to the acidic solution to increase its density. A silicone-coated gel mold with a backing of GelBond (FMC Corporation) was placed against one of the inner surfaces of two glass plates separated by a 6 mm spacer, and the acrylamide solution poured between the plates as a linear gradient from acidic to basic pH. The gel was polymerized for 90 min at 50°C, and washed in multiple changes of distilled water to remove the glycerol and unpolymerized materials. The gel was then dried back to its original weight using a fan. The gel was prefocused to remove impurities for 1 hr at 4°C at a constant power of 5 W.

Immobiline gels do not tolerate salt concentrations greater than 25 mM without distortion of focusing. Therefore, the pooled DEAE fractions were desalted by repeated concentration using an Amicon PM10 ultrafilter and the final volume adjusted to 20–30 ml with 10 mM phosphate buffer.

Desalted DEAE fractions were placed in a well (approximately 10 × 2 × 0.3 cm) precast in the Immobiline gel, and focused at a constant power of 5 W. The well could accommodate around 6–8 ml of sample, and a second 6–8 ml application was made after several hours, when the initial sample had entered the gel. Each gel could accommodate about half the DE-52 fraction.

After 24 hr, strips were cut from the edges of the gel and stained for four hours in Coomassie blue by the method of Blakesley and Boezi (1977). The remainder of the gel was kept under constant voltage during this period. The strips were then aligned with the body of the gel and regions of acrylamide containing the various bands and interspaces excised with a razor blade; strip thickness varied from 2 to 8 mm. Each gel strip was eluted overnight at 4°C into 4 ml of PBS containing 0.1% Triton X-100. Active fractions were identified by neuronal bioassay and pooled.

SDS-PAGE

Samples were prepared by overnight incubation at 37°C in 60 mM Tris-HCl (pH 6.8) plus 2.3% SDS and 5% mercaptoethanol. All gels were 7.5–20% acrylamide gradients with a 4.5% stacking gel. Methods of gel preparation, electrophoresis, and gel staining were performed as previously described (Barbin et al., 1984a).

Catalase assay

Catalase activity was assayed by a modification of a previously reported method (Brannan et al., 1981). The sample was preincubated for 10 min at 4°C in 0.5 ml 10 mM phosphate buffer (pH 7.0) and 0.2 M ethanol. One milliliter of 1.5 mM hydrogen peroxide (H_2O_2) was added and the samples incubated for 10 min at 4°C. The reaction was terminated by addition of 2 ml of a mixture of 0.1 mg/ml HRP and 0.1 mM dimethoxybenzidine. Optical densities were determined at 420 nm. Catalase-specific activity (in U/mg) was calculated as $[\ln(A_0 - A_{bl}/A_t - A_{bl})]$ (1/mg protein) (1/10 min), where A_0 is absorbance in the presence of 1 mM sodium azide, A_{bl} is absorbance of sample and reagents without hydrogen peroxide, and A_t is absorbance at 10 min.

Determination of peroxide concentrations in cell culture medium

Medium was aspirated from the culture dishes and frozen until assay. The assay method was as described (Wang and Nixon, 1978). The thawed medium (0.1 ml) was combined with 0.2 ml of 0.05 M acetic acid, 5 μl of saturated ammonium molybdate, and 0.1 ml of 15% potassium iodide. The optical density of the reaction product was determined at 360 nm and peroxide content (in mM) derived by comparison with a standard curve of H_2O_2 added to fresh medium.

Results

Fractionation of neuronotrophic activity

An example of the titration curves used to calculate trophic titers by serial twofold dilution is shown in Figure 1. The 33% human red blood cell lysate used as a starting material contains high concentrations of trophic material, on the order of 1–2 × 10^6 TU/ml. There was no evidence of toxicity at dilutions of only 1:10 (i.e., 10^5 TU/ml); at lower dilutions hemoglobin deposits interfered with visualization of individual neurons. The morphology of embryonic forebrain neurons was not visibly different over the range of hemolysate concentrations supporting survival (data not shown). About 60–90% of seeded neurons were supported by the trophic activity in hemolysate. The level of the plateau and slopes of the regression are comparable to those previously reported for rat red blood cell extract (Williams et al., 1985).

The purification process is summarized in Table 1. The first step in the purification of the trophic activity consisted of applying the hemolysate to a DEAE–cellulose column at pH 6.2 and collection of the trophic activity in the nonadsorbed fraction. This resulted in only a minor degree of purification of the neuronotrophic activity, but was useful for removing some proteins that otherwise migrated near the active material during the subsequent isolectric focusing step (data not shown).

The nonadsorbed material from this first DEAE column was adjusted to pH 7.2 and passed over a second, similar column. The results are shown in Figure 2A. At this higher pH, most of the trophic activity bound to the column, while hemoglobin, accounting for over 90% of the hemolysate protein, did not bind. The bound trophic material eluted with a linear salt gradient at about 0.035 M NaCl and was contained in two fractions preceding the major protein peak. Further elution with 1 M NaCl
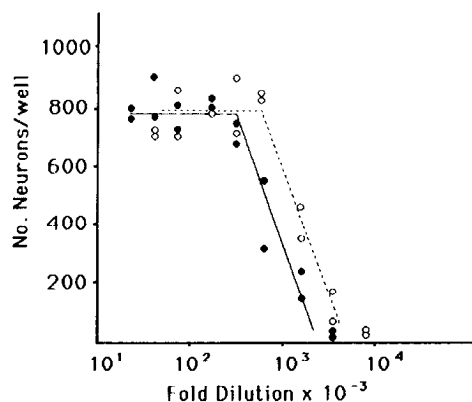
Figure 1. Dose–response curves for human CNS-directed neurono-trophic activities. Microwells were first provided with 50 μl serial two-fold dilutions of red blood cell lysate or a pooled fraction derived from isoelectric focusing gels. About 1000 embryonic chick forebrain neurons in 50 μl were then added to each well, cultured for 24 hr, fixed with glutaraldehyde, and the number of surviving neurons determined by phase-contrast microscopy. One trophic unit per milliliter of a given sample is read directly as the dilution eliciting a half-maximal response and is, for the hemolysate (●) and immobiline pool (O) samples, re-spectively, about 800 and 1600 TU/ml.

removed additional protein but no trophic activity. The active fractions were yellow-brown in color and exhibited 410 nm absorbance, characteristic of heme groups (data not shown).

The two most active fractions (arrow bar in Fig. 2*A*) from this second DEAE column were pooled, desalted, concentrated, and submitted to preparative scale gel isoelectric focusing, as described in Materials and Methods. The focused gel was cut into slices and eluted in Triton X-100 in PBS; the eluates were assayed for CNS neuronotrophic activity and protein. The re-sults are shown in Figure 2*B*. Nearly all the recoverable activity eluted from the pH 6.5–6.6 area (arrow bar in Fig. 2*B*) within a 5 mm region and separated from most of the recoverable protein. This purified material still exhibited a slightly yellowish color. The observed isoelectric point varied with the age of the DEAE preparation tested. Material that had been stored for several months displayed a more complex pattern with three to four peaks between pH 6.0 and 6.6 (data not shown).

A comparison of trophic activity and protein yields within the hemolysate and the pooled derivative fractions from each major step is shown in Table 1. The progression of the purifi-cation is also illustrated by SDS-PAGE of the samples from each step (Fig. 3). The final step (isoelectric focusing) of the purification procedure yielded a fraction containing 14 × 10^6 TU (2.5% of original hemolysate trophic activity) and 1.4 mg protein, with a specific activity of 10^7 TU/mg, about 1200-fold higher than the starting material. This final fraction (called "RBC
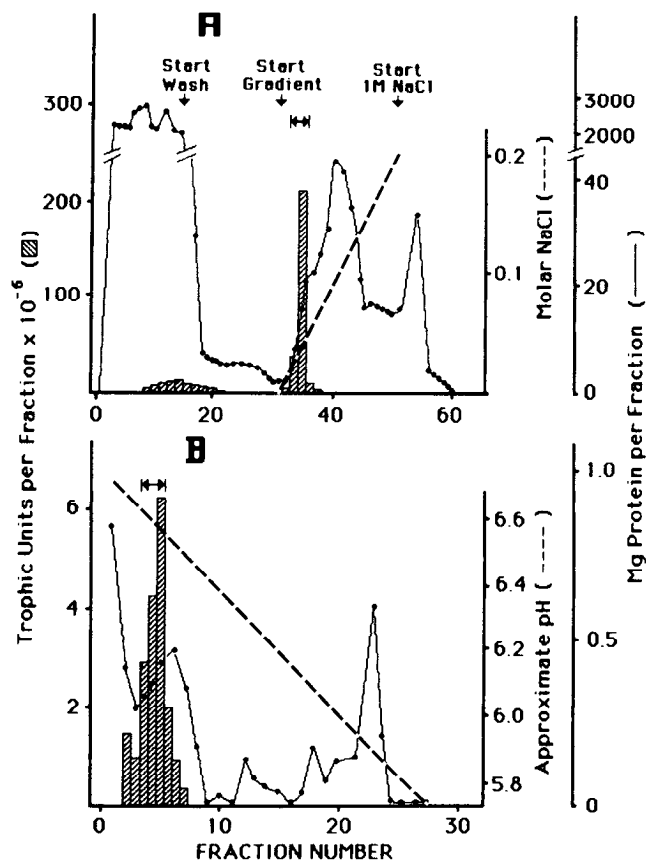

Figure 2. Fractionation of human CNS-directed neuronotrophic ac-tivity. *A*, Ion exchange (pH 7.2). Red blood cell extract (450 ml) was prepared at pH 6.2, passed through a DEAE column, the eluate pool adjusted to pH 7.2 and then submitted to a second DEAE column. Each fraction was assayed for protein and neuronotrophic activity; fraction sizes were 16.5 ml for eluate, 10 ml for gradient, and 1 M NaCl. A substantial portion of the recoverable trophic units (*histogram*) was eluted in two fractions with about 0.035 M NaCl (*dashed line*) and separated from most of the recovered protein (*solid lines*). *B*, Isoelectric focusing (pH 5.7–6.7). One-half of the salt-eluted pool (*arrow bar*) was submitted to polyacrylamide gel isoelectric focusing over a very narrow pH range (5.7–6.7). Slices were taken over the length of the focused gel, eluted, and assayed for protein and trophic activity (see Materials and Methods). Most of the recovered activity (*histogram*) focused in the pH 6.5–6.6 region and separated from most of the protein (*solid lines*).

trophic protein") exhibits one 60 kDa Coomassie blue-stained band after SDS-PAGE (Fig. 3, lane 4) but exhibits additional minor bands when a more sensitive silver stain is used (Fig. 4, lane 1).

Table 1. Purification of human red blood cell-derived neuronotrophic activity for CNS neurons

| Fraction	ml	TU × 10⁻⁶	Protein (mg)	TU/mg × 10⁻³	Increase (*n*-fold)	Recovery (%) TU	Recovery (%) mg
Hemolysate	450	560	67,000	8	1	100	100
DEAE, pH 6.2 eluate	450	460	45,000	10	1.2	82	67
DEAE, pH 7.2 pool	28	220	50	4400	550	39	0.075
Isoelectric focusing pool ("RBC trophic protein")	16	14	1.4	10,000	1200	2.5	0.002

Fractions were prepared and assayed for trophic units (TU) and protein (mg), as described in Materials and Methods. Values are averages of duplicate determinations for each of four separate bioassays of fractions from one representative purification run.
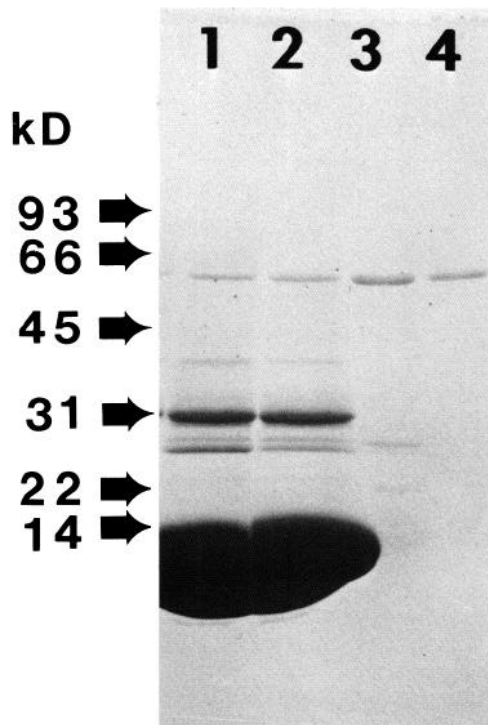
Figure 3. SDS-PAGE of selected fractions generated during the purification of the human RBC trophic protein. Lanes *1* and *2* were loaded with 2.5×10^4 TU, and lanes *3* and *4* with 5×10^4 TU. The lanes correspond to each of the four fractions shown in Table 1, as follows: Lane 1, hemolysate; lane 2, pH 6.2 DEAE eluate; lane 3, pH 7.2 DEAE pool; and lane 4, isoelectric focusing pool ("RBC trophic protein"). The gel was stained with Coomassie blue. Molecular weight markers were phosphorylase b (93 kDa), BSA (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (22 kDa), and lysozyme (14 kDa).

We failed in numerous attempts to elute detectable neuronotrophic activity from SDS–PAGE gels, even after application of 10^7 TU/lane. Simply incubating the RBC trophic protein with the SDS and mercaptoethanol electrophoresis buffer irreversibly destroyed its activity. Attempts to reconstitute the activity by SDS removal (dialysis, 8 M urea, hydrophobic interaction chromatography or incubation with protein and buffer) were unsuccessful. Therefore, indirect methods were employed to determine whether the 60 kDa band represented the trophic activity. The pH 7.2 DEAE pool (Table 1) was submitted to (1) sucrose density gradient centrifugation according to Barbin et al. (1984a), (2) hydrophobic affinity chromatography using 2–12 carbon side chains (Shaltiel, 1974), and (3) cibacron blue, procion red, green A, orange A, and blue B dye affinity chromatography (Amicon Corporation, 1980; Porumb et al., 1982) and the trophic activity monitored by bioassay. After each fractionation protocol, the active fractions were pooled and submitted to analytical SDS-PAGE. In all cases, the bioactive fractions, but not selected inactive fraction pools, displayed a prominent 60 kDa band, accompanied by minor bands that tended to vary in their M_r value (data not shown). These observations support the contention that the RBC trophic activity resides with the 60 kDa protein band exhibited after SDS-PAGE.

The purification and biochemical characterization described above indicated that the human RBC trophic protein (1) binds weakly to DEAE–cellulose at neutral pH; (2) may contain heme groups, as indicated by 410 nm absorbance; (3) has an isoelectric point of about 6.6, with appearance of more acidic forms after storage; (4) has a subunit molecular weight of 60 kDa by SDS-
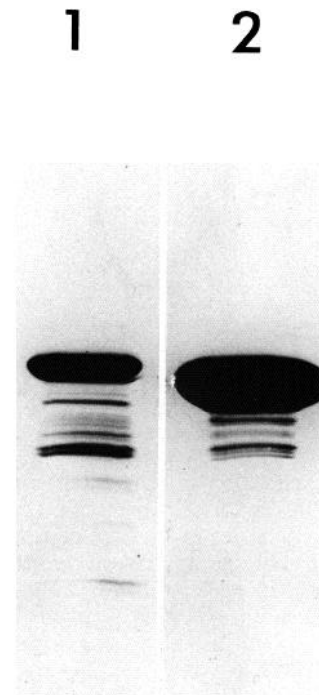
Figure 4. SDS-PAGE and silver staining of 50 μg (5×10^5 TU) of the purified human RBC trophic protein (lane *1*) and 100 μg (4×10^5 TU) of bovine liver catalase (lane *2*).

PAGE; and (5) is inactivated by dissociation into subunits with SDS and mercaptoethanol. An examination of the literature describing properties of previously purified RBC proteins revealed that the peroxide-degrading enzyme, catalase, shares with the RBC trophic protein all of the above properties (Aebi et al., 1974; Hennessey et al., 1962; Mörikofer-Zwez et al., 1969; Olofsson and Olsson, 1977; Porumb et al., 1982). Commercially available purified bovine liver catalase (purified human RBC catalase is not yet available commercially) exhibited a slightly faster migration on SDS gels than the RBC trophic protein (compare lanes 1 and 2, Fig. 4). Bovine catalase is composed of 506 amino acid residues/subunit compared to at least 520 residues/subunit for the human RBC catalase (Schroeder et al., 1982).

If the purified RBC trophic protein is indeed catalase, then it follows that (1) it should possess catalase enzymatic activity, (2) catalase enzymatic activity should copurify with the trophic activity using the present procedure, and, conversely, (3) catalase from another source should possess trophic activity for CNS neurons.

Catalase enzymatic activity was monitored during the purification of the human RBC trophic protein, and beef liver catalase was tested for neuronotrophic activity. The results are shown in Table 2. The purified RBC trophic protein indeed possesses the ability to degrade H_2O_2, and this enzymatic ac-

Table 2. Co-purification of neuronotrophic and catalase enzymatic activities

Fraction	Trophic activity (TU/mg × 10^{-3})	Increase (n-fold)	Catalase activity (U/mg × 10^{-3})	Increase (n-fold)	(TU/mg)/ (U/mg)
Hemolysate	8	1	12	1	0.7
DEAE, pH 6.2 eluate	10	1.2	13	1.1	0.8
DEAE, pH 7.2 pool	4400	550	4100	340	1.1
Isoelectric focusing pool ("RBC trophic protein")	10,000	1200	12,000	1000	0.8
Bovine liver catalase	4200	—	4000	—	1.1

Fractions were generated and assayed for trophic units (TU), catalase units (U), and protein (mg), as described in Materials and Methods. Catalase activity values are averages of six determinations on a single set of fractions. Trophic activity and protein are as in Table 1.

tivity was enriched 1000-fold during the purification protocol, compared to 1200-fold for the trophic activity. Purified bovine liver catalase is a potent supporter of neuronal survival. Microscopic inspection of the catalase-supported cultures showed identical maximal number and morphology of surviving neurons compared to cultures supported by the RBC trophic protein (data not shown). The liver catalase possesses about half the trophic activity (4.2×10^6 vs 10^7 TU/mg) and one-third the catalase activity (4.0×10^6 vs 12.2×10^6 TU/mg) of the human RBC trophic protein. A comparison of the ratio of trophic to catalase units reveals a relatively constant value (0.7–1.1), further suggesting an identity between the two properties of H_2O_2 degradation and neuronal survival promotion.

Identity of peroxidase activity and trophic activity of catalase

The only known function of catalase is degradation of H_2O_2. However, it is possible that its apparent trophic activity could reflect novel attributes of the enzyme. Aminotriazole and phenylhydrazine are both *irreversible* inhibitors of catalase enzymatic activity, and both bind to a conformation of the enzyme that occurs during interaction with H_2O_2 (Ortiz de Montellano and Kerr, 1983; Sinet et al., 1980). Aminotriazole is considered

to be a specific inhibitor of catalase. Although less specific, phenylhydrazine was also examined to provide verification of potential effects observed with aminotriazole. The two inhibitors of catalase enzymatic activity were tested for their effects on the trophic activity of the purified human RBC trophic protein and bovine liver catalase, as well as on crude hemolysates from human and rat. Samples were incubated in a solution of the appropriate inhibitor plus H_2O_2. Even at a dilution of 1:10^6, H_2O_2 was neurotoxic in the bioassay for neurons supported by pyruvate (Selak et al., 1985). However, dialysis of the samples overnight eliminated this toxicity, thus allowing detection of residual trophic activity. The results are shown in Table 3. Both inhibitors reduced by at least 80% the trophic activities of both the purified RBC trophic protein and purified bovine catalase. Also, 85–90% of the trophic activity present within crude hemolysates was eliminated, suggesting that nearly all of the trophic units lost during purification (i.e., from Table 1 the 2.5% TU yield indicates a loss of about 97.5% of the starting material) of the human RBC trophic protein may be attributable to catalase.

The effects of the catalase inhibitors were also examined directly in culture by adding either aminotriazole or phenylhydrazine to the bioassay medium. Both inhibitors had some toxicity (aminotriazole above 35 mM and phenylhydrazine above 1.2 mM) when presented to the neurons in the presence of pyruvate. Concentrations below these toxic levels were tested with both bovine liver catalase and the human RBC trophic activity (Table 3, lines 5 and 6). Again, trophic activity was considerably reduced by the enzymatic inhibitors. Since the inhibitors only bind in the presence of H_2O_2, this finding suggests the presence of this material in the culture system (Sinet et al., 1980).

If peroxides are killing the neurons during the neuronotrophic bioassay and catalase is supporting neuronal survival through its peroxide-degrading enzymatic activity, then other methods

Table 3. Effects of catalase inhibitors on the neuronotrophic activity directed to CNS neurons

Fraction	Saline[a]	TU remaining with Aminotriazole (%)	TU remaining with Phenylhydrazine
Pretreatment			
1. Human RBC trophic protein	100	4	4
2. Bovine liver catalase	100	18	4
3. Human hemolysate	100	10	15
4. Rat hemolysate	100	9	8
Inclusion in medium			
5. Human RBC trophic protein	100	4	4
6. Bovine liver catalase	100	4	18

A 100 μl aliquot of the indicated fraction was incubated for 1 hr at 37°C with 1 ml 10^{-4} M H_2O_2 in 0.1% Triton X-100 PBS with or without 40 mM aminotriazole or 0.5 mM phenylhydrazine, dialyzed overnight at 4°C against Triton X-100 PBS to remove toxic H_2O_2 and inhibitors, and assayed for trophic activity (Pretreatment). Recovery of units was 80–100% after incubation and dialysis in the absence of enzymatic inhibitors. Alternatively, the two inhibitors were included in the bioassay medium of the human RBC trophic protein or catalase (Inclusion in medium), at 5 mM for aminotriazole or 30 μM for phenylhydrazine. Concurrent cultures supported by pyruvate showed these concentrations to be nontoxic.

[a] One hundred percent values in TU/ml were (1 and 5) 4.7×10^5, (2 and 6) 8.0×10^5, (3) 5.3×10^5, and (4) 2.6×10^5.

Table 4. Antiperoxidants have neuronotrophic activity toward CNS neurons

Antiperoxidant	TU/mg (× 10^{-3})	TU/peroxidase (units × 10^{-3})
Bovine liver catalase	17,000	570
Lactoperoxidase	1000	570
HRP	1900	720
Vitamin E	0.4	200

The indicated commercially available antiperoxidant materials were tested for neuronotrophic activity and expressed in TU/mg protein or TU/mg α-tocopherol for vitamin E. Peroxidase units were derived from the manufacturers' specifications for the enzymes; international units (IU) are used for vitamin E.

of removing H_2O_2 should also support neuronal survival. Two commercially available antiperoxidant enzymes, lactoperoxidase and HRP, as well as the nonenzymatic antioxidant, vitamin E, were therefore tested for neuronotrophic activity. The results are shown in Table 4. Both enzymes and vitamin E were, by themselves, able to support neuronal survival. Estimates of the specific peroxidase activity (provided by the manufacturers) were used to calculate the ratios of specific trophic activity and specific peroxidase activity. It should be noted that these assays yielded different units than the catalase units used in Table 3. The relative constancy of this ratio (between 570–720 for the enzymes) again suggests that neuronotrophic and peroxidase activities are closely related, if not identical, properties.

These results also provide indirect support for the conclusion that catalase, rather than one of the impurities present in the RBC trophic protein preparation (see Fig. 4), represents the trophic activity. The various substances tested derive from very different sources (human blood, bovine liver or milk, radish root, and vegetable oil) and differ markedly in basic physicochemical properties such as size and isoelectric point (Bunt and Haschke, 1978; Malmgren et al., 1978; Nässel, 1983). Thus, it is highly unlikely that a common contaminant representing the trophic activity could be present in these different preparations.

Presence of peroxides in the culture medium used for neuronotrophic bioassays

If catalase promotes neuronal survival in vitro by removal of H_2O_2, then this cytotoxic material should be present within the cultures, and its levels should be reduced in the presence of catalase. To test these possibilities, neuronotrophic bioassay medium was incubated in microculture wells in the usual manner (see Materials and Methods) but without cells and with or without added catalase. At the indicated times the medium was removed and assayed for peroxide content in an assay that detects both H_2O_2 and organic peroxides. The results are shown in Figure 5. Two points are evident. First, peroxides accumulated in the culture medium to a concentration of about 40 mM between 4 and 12 hr of incubation and remained at that level for an additional 12 hr. Second, exogenously added catalase substantially reduced, but did not eliminate, peroxide accumulation at all three times. These results demonstrate that the culture medium itself is a source of measurable peroxides, and raise the possibility that catalase is primarily affecting the neuronal environment rather than directly interacting with the neurons themselves.

Discussion

By a combination of ion-exchange chromatography and isoelectric focusing, a neuronotrophic protein previously detected in red blood cells has been purified over 1000-fold. The final material possesses an activity of 10^7 TU/mg protein, which means that half-maximal activity occurs at 100 pg/ml, which compares favorably to the potency of other isolated trophic factors. This protein has been identified as catalase on the basis of its fractionation behavior on DEAE–cellulose columns; absorbance at 410 nm, indicating the presence of a heme group; isoelectric point; subunit molecular weight determined by SDS-PAGE; and its co-purification with peroxidase activity (Aebi et al., 1974; Hennessey et al., 1962; Mörikofer-Zwez et al., 1969; Olofsson and Olsson, 1977; Porumb et al., 1982; Schroeder et al., 1982). Furthermore, commercially prepared bovine liver catalase reproduces the effects of the purified human RBC trophic protein in supporting neuronal survival. Incubation of the RBC trophic protein in SDS and mercaptoethanol destroys its trophic activity, just as the dissociation of catalase into its subunits is recognized to disrupt enzymatic activity (Aebi et al., 1974). Two irreversible inhibitors of catalase enzymatic activity, aminotriazole and phenylhydrazine (Ortiz de Montellano and
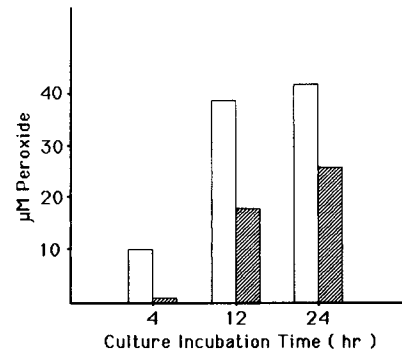
Figure 5. Catalase reduces spontaneous peroxide generation in neuronal culture medium. Aliquots of culture medium, 100 μl, were incubated in the absence of neurons but under standard neuronotrophic factor bioassay conditions (see Materials and Methods) in the absence (open histograms) or presence (hatched histograms) of 10^3 TU of human RBC catalase. At the indicated times, the medium was assayed for peroxide content (see Materials and Methods).

Kerr, 1983; Sinet et al., 1980), also block the ability of the RBC trophic protein and catalase to support the survival of cultured neurons. Besides catalase, two other peroxide-degrading enzymes, lactoperoxidase and HRP, are also potent supporters of neuronal survival. The ratio of trophic units to enzymatic units is quite similar for these three enzymes. In addition, vitamin E, a nonenzymatic antioxidant, can replace catalase as a trophic agent. Altogether, the above results not only demonstrate that the trophic material in hemolysates is catalase, but also justify the conclusion that catalase supports neuronal survival through its ability to degrade H_2O_2.

The effect of catalase in promoting the survival of cultured CNS neurons is not limited to those from chick forebrain. We previously reported (Longo et al., 1984; Williams et al., 1985) that hemolysates also support the survival of cultured 18 d fetal rat hippocampal, striatal, and septal neurons. The purified human catalase reported here, as well as the beef liver catalase, will also support all three rat neuronal types, as well as cortical neurons from the area V sensory motor region, at concentrations comparable to those required for chick forebrain neurons (data not shown). Although catalase has not been reported as a neuronotrophic agent, vitamin E has previously been observed to enhance cortical neuron survival in reaggregate cultures (Halks-Miller et al., 1982). Others have suggested that a variety of cultured non-neuronal cells show increased survival in the presence of catalase, including lymphoma cells, Erlich ascites tumor cells, transformed monkey kidney cells, and human xeroderma pigmentosa cells (Darfler and Insel, 1983; Koch and Bigalow, 1979; Parshad et al., 1978; Sagone et al., 1984; Wang and Nixon, 1984). The requirement for catalase can become more apparent when the cells are cultured at low cell densities (Darfler and Insel, 1983). Our neuronal cultures that require catalase at low seeding densities become capable of self-support at higher seeding densities (Barbin et al., 1984b).

Enhanced survival of non-neuronal cell types in the presence of catalase has generally been attributed to protection from H_2O_2 contained in the culture medium itself (Darfler and Insel, 1983; Koch and Bigalow, 1979; Parshad et al., 1978; Sagone et al., 1984; Wang and Nixon, 1978). Culture medium alone generates H_2O_2 and organic peroxides, after exposure to light, through interaction of photoactivated riboflavin with tryptophan and tyrosine (Hoffman and Meneghini, 1979; Wang and Nixon, 1978). The concentrations of oxygen used in culture incubators have been suggested (Hornsby and Gill, 1981; Parshad et al., 1978) to provide a source of superoxide ions, which could in turn mediate the formation of peroxides. Although the mech-

anisms of peroxide formation were not addressed here, we did demonstrate that our Hanks' modified Eagle's basal medium does contain peroxides even in the absence of the test neurons. Addition of catalase significantly reduced, though did not eliminate, the accumulation of peroxides, in agreement with previous findings using Dulbecco's modified Eagle's medium (Wang and Nixon, 1978).

Hydrogen peroxide and superoxide ion, the latter being converted to H_2O_2 by superoxide dismutase, are generated during a number of metabolic processes (Ramasarma, 1982). It seems likely that the cultured neurons might themselves generate H_2O_2 in addition to that produced by the medium. Such intracellular pools of peroxide could be particularly important in deciding cell viability. It is well known that lipid-soluble vitamin E (α-tocopherol) readily crosses cell membranes, and that HRP is taken up by neurons in a manner that allows retention of its enzymatic activity (LaVail and LaVail, 1974). These observations suggest the possibility of both intra- and extracellular sites of action in support of neuronal survival. Catalase and lactoperoxidase, however, may not be internalized by neurons (Bunt and Haschke, 1978; Malmgren et al., 1978; Nässel, 1983). The simplest hypothesis consistent with all the data is that catalase promotes neuronal survival by reducing culture medium peroxide levels. If so, its role would be one of improving the neuronal culture environment, and not of entertaining a direct neuronotrophic interaction with the nerve cells, as would be expected of a conventional "neuronotrophic factor."

Although this study failed to produce such a traditional neuronotrophic factor, it does have value in illuminating a common, though perhaps not widely appreciated, characteristic of the culture environment, namely, that of generating toxic peroxides. The serum-free conditions used are similar to those employed by a number of other laboratories, with the N1 additives of Bottenstein et al. (1980). Although serum-containing medium was not examined in these studies, reports in the literature indicate that it too generates peroxides (Hoffman and Meneghini, 1979; Parshad et al., 1978; Wang and Nixon, 1978). The presence of these toxic compounds is likely to complicate other studies of neuronal survival and growth *in vitro*, as well as studies of neuronal metabolism or membrane constituents *in vitro*. Routine inclusion of catalase in culture media could help to reduce peroxide-induced neuronal damage.

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