Eosinophil-derived neurotoxin and human liver ribonuclease

IDENTITY OF STRUCTURE AND LINKAGE OF NEUROTOXICITY TO NUCLEASE ACTIVITY*

(Received for publication, December 16, 1991)

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Eosinophil-derived neurotoxin (EDN) and human liver RNase were found to be indistinguishable from each other but distinct from the pancreatic ribonucleases in their nucleolytic activity on polynucleotides or small defined substrates. Antibodies to EDN and liver RNase showed identical cross-reactivities in assays of nuclease inhibition and in a radioimmunoassay. In each instance, EDN and liver RNase were easily distinguished from bovine or human pancreatic RNase. When injected intrathecally into rabbits, 5–10 μg of EDN or liver RNase each was neurotoxic as judged by induction of the Gordon phenomenon. Human pancreatic RNase was less neurotoxic, and up to 20-fold higher levels of bovine pancreatic RNase showed no effect. Treatment of EDN, liver RNase, and eosinophil cationic protein with iodoacetic acid at pH 5.5 resulted in inactivation of their RNase activity and also destroyed their neurotoxicity. EDN conformation was not greatly affected by iodoacetate treatment since interaction of the modified protein with antibodies was only slightly altered. We conclude that RNase activity is necessary but not sufficient to induce neurotoxic action.

Almost 60 years ago M. H. Gordon, in an attempt to identify a pathogenic agent for Hodgkin’s disease, developed a biological assay in which experimental material was injected intrathecally or intracerebrally into rabbits (1). The resulting neuromuscular response, now generally identified as the “Gordon phenomenon,” is characterized by stiffness, ataxia, and paralysis, concomitant with loss of Purkinje cells and degeneration of white matter in the cerebellum (2). The granules of human eosinophils contain two basic proteins, namely eosinophil cationic protein (ECP)† and eosinophil-derived neurotoxin (EDN), which are neurotoxic when assayed by induction of the Gordon phenomenon (3, 4). These molecules may be partly responsible for the neurologic abnormalities in patients with the hypereosinophilic syndrome (5). Even more striking, individuals with the eosinophilia-myalgia syndrome associated with L-tryptophan consumption (6, 7) frequently have neuritis (8–10), and some have other neurological complications (11–13) that may be due to hypereosinophilia. At least 27 deaths have been associated with this syndrome (14).

Both EDN and ECP bear a strong structural resemblance to proteins of the ribonuclease superfamily (4); both possess RNase activity, and this is particularly high in the case of EDN (15). Two major types of related but distinct human ribonucleases have been defined by structural and functional studies. Human liver ribonuclease is classified as a “nonsecretory” RNase (16, 17); it is structurally related to but distinct from human pancreatic RNase, a “secretory” enzyme (18). Several observations indicate that EDN is, at the least, very similar to human liver RNase. Partial amino acid sequence analysis showed that amino-terminal segments of liver RNase and EDN to be identical both to each other and to the terminal section of the nonsecretory RNase found in human urine (4, 16, 17). Nucleotide sequences of EDN cDNA (19, 20) also correspond to the complete amino acid sequence determined for the urinary RNase protein (17). Liver RNase, EDN, and the urinary RNase are all also glycoproteins; however, RNase glycosylation has been reported to be organ-specific (21).

We considered it likely that the liver RNase and EDN are identical polypeptides, but still questioned whether they could be identical in both nucleolytic and neurotoxic actions. In this paper we directly compare EDN and liver RNase and report that they are indistinguishable immunologically, as RNases, and as neurotoxic agents. Moreover, we find that their RNase activity is necessary but not sufficient to induce the Gordon phenomenon.

**EXPERIMENTAL PROCEDURES**

Proteins—The purification of EDN (3), ECP (4), and human liver (16) and pancreatic (18) RNases and the production and characterization of antibodies to each protein have been described. Antibody preparations were partially purified by ammonium sulfate fractionation, followed by passage through a column of DEAE-cellulose and carboxymethyl cellulose in order to remove serum ribonucleases (22). A sample of human pancreatic RNase was further fractionated (21)

† The abbreviations used are: ECP, eosinophil cationic protein; EDN, eosinophil-derived neurotoxin; MES, 2-(N-morpholino)ethanesulfonic acid; MOPS, 3-(N-morpholino)propanesulfonic acid; CpA, cytidyllyl (3′–5′) adenosine; N, any nucleoside; CM-RNase, carboxymethylated ribonuclease; Hepes, 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid.

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on a column of concanavalin A-saprose (Sigma). Enzyme that was not retained was assumed to be nonglycosylated; bound (glycosylated) protein was eluted with 10% acetic acid, dialyzed against several changes of distilled water, and lyophilized. Bovine RNases A and B were purchased from Sigma and Calbiochem. Biochemical and enzymatic characteristics of bovine RNases A and B are essentially identical. Reagents—The site-specific modification of RNases with iodoacetic acid was adapted from the conditions used by Plapp (23): protein (0.18 mg/ml) in 200 mM MES (adjusted to pH 5.5 with NaOH), 167 mM NaCl, 15 mM sodium iodoacetate, was incubated at 37 °C in the dark for up to 24 h. In parallel incubations iodoacetate was omitted (buffer control) or 50 mg/ml reduced glutathione was added (sham carboxymethylation). Aliquots were removed periodically and assayed for residual RNase activity. Preparations of carboxymethylated protein, usually showing less than 5% residual nucleolytic activity, were then dialyzed against several changes of distilled water and lyophilized. Protein denaturation by reduction and carboxymethylation of all cysteine residues has been described (24). Placental RNase inhibitor (RNasin) was obtained from Calbiochem and Promega, and Inhibit-ACE was purchased from Promega and Inc. Assays—Except when otherwise specified, RNase activity was quantitated through the formation of perchloric acid-soluble nucleotides from a wheat germ ribosomal RNA substrate under conditions optimized for the pancreatic (18) or liver (16) enzymes. One unit of activity in this procedure corresponds to about 2.5 ng of human pancreatic RNase or 8 ng of liver RNase. Other procedures were used for comparative purposes. The Kunitz (25) assay employed yeast RNA (Sigma) that had been further purified (26) to reduce amounts of small oligonucleotides. Spectrophotometric assays of the hydrolysis of polynucleotides, dinucleoside phosphates, and nucleoside cyclic 2',3'-phosphates have been described (16, 27-29); a temperature-activity in this procedure corresponds to about 2.5 ng of human pancreatic RNase. Other procedures were used to quantify the formation of small oligonucleotides. Spectrophotometric assays of the hydrolysis process is followed, and samples were withdrawn at intervals of up to 50 h, and levels of residual nuclease activity, were then assayed for RNase activity. The immunochemical reactivity of iodoacetate-modified protein with polyclonal antibody was not important in the experiments but not iodoacetate (buffer control), EDN and the two proteins functioned at comparable doses. The results of experiments comparing EDN and liver RNase complexes was measured using the approach of Lee et al. (30). Enzyme plus 1 or 10 eq of RNasin were incubated at 0 or 37 °C in a buffer containing 2 mg/ml bovine serum albumin plus 50 mM MOPS, pH 7.5. Samples were withdrawn at intervals of up to 50 h, and levels of uninhibited RNase activity were measured. As a control, RNase samples were incubated in the absence of RNasin under the same conditions and assayed at similar intervals. Measurement of antibody inhibition of RNase activity involved incubation of varying amounts of antibodies with 1 unit of enzyme in the same buffer for 2 h on ice, followed by elevation of the temperature to 37 °C and RNase assay. Inhibition in this assay by reduced and carboxymethylated EDN or by EDN that had been inactivated at pH 5.5 with iodoacetic acid measured the restoration of antibody-inhibited RNase activity. One unit of RNasin plus variable amounts of chemically modified EDN were mixed in assay buffer with sufficient antibody to inhibit 1 unit of RNase activity by 50%; samples were incubated for 15 min at 37 °C followed by 2 h on ice, and then assayed for RNase activity. The immunochemical reactivity of the RNases was also analyzed by a competitive binding double-antibody radioimmunoassay described previously (31). The measurement of neurotoxic activity (the Gordon phenomenon) followed established methods (3, 4). Rabbits that had been injected with 200 µl of protein in saline/citrate buffer (or buffer alone as a control) were observed daily for up to 12 days for forelimb stiffness, incoordination, and ataxia. Animals were evaluated by an individual who was not aware of the specific sample injected in each case and were scored positively if all of the symptoms described above were apparent.

RESULTS
Activity as RNases—EDN and liver RNase were compared with each other and with bovine pancreatic RNase A in assays procedures that used both macromolecular and low molecular weight substrates. As shown in Table I, with RNA and polynucleotide substrates EDN and liver RNase were essentially identical and clearly unlike RNase A. Under the conditions used EDN and liver RNase both showed a preference for polynucleotides that contained uridine rather than cytidine, while the opposite was true for pancreatic RNase. Both liver RNase and EDN showed an identical and generally low activity with simple polymeric substrates, whether single-stranded (poly(U) or poly(C)) or double-stranded (poly(A)·poly(U) or poly(I)·poly(C)). With small defined substrates EDN and liver RNase were again essentially identical; none of these compounds was hydrolyzed efficiently. Again this is in contrast to RNase A, which was reasonably effective in the hydrolysis of several small substrates.

Immunological Cross-reactivity—Polyclonal antibodies to human pancreatic RNase (18), to EDN (3), and to liver RNase (16) were previously characterized in our laboratories. These antibody preparations were examined for their ability to inhibit the nucleolytic activities of EDN and liver RNase. Antibodies to either EDN or liver RNase inhibited the RNase activity of both proteins to an equivalent extent, as shown in Fig. 1. As expected from earlier results (16) inhibition of human pancreatic RNase by antibodies to EDN or liver RNase was not detectable at the antibody levels used in these experiments. Similarly, in radioimmunoassays using 125I-labeled liver RNase as the radioligand, binding by either anti-EDN or anti-liver RNase was inhibited to an equivalent extent by an equal quantity of either EDN or liver RNase (results not shown). Antibodies to human pancreatic RNase show less than 1% cross-reactivity with human liver ribonuclease in a similar assay (16).

Inactivation and Inhibition of RNase Activity—When samples of EDN and liver RNase were incubated at pH 5.5 with iodoacetic acid, each protein lost nuclease activity at an identical rate (Fig. 2) that was essentially the same as the rate of inactivation of bovine and human pancreatic RNases (16, 18). In order to test whether protein conformation is affected by carboxymethylation, we investigated the cross-reactivity of iodoacetate-modified protein with polyclonal antibodies to EDN and liver RNase. As shown in Fig. 3, carboxymethyl-EDN was still an effective competitor in a radioimmunological assay for EDN using antibodies raised against either protein. In contrast, fully reduced and carboxymethylated EDN possessed less than 0.01% of its initial reactivity. Carboxymethyl-EDN (but not EDN that was denatured by reduction and carboxymethylation of its cysteines) also effectively competed with native EDN in assays based on measurement of antibody inhibition of RNase activity. As shown in Fig. 4 nuclelease inhibition by anti-EDN or anti-liver RNase antibodies was relieved by iodoacetate-inactivated EDN, while reduced and carboxymethylated protein had no effect on RNase activity measured in these experiments.

RNasin, a placentalbumin protein that efficiently inhibits pancreatic RNase activity, also inhibits EDN and liver RNase (Fig. 5). A different inhibitory protein, Inhibit-ACE, showed no effect on any of the enzymes of human origin, but less than 0.1% as much inhibitor strongly inhibited bovine pancreatic RNase. EDN and liver RNase were thus indistinguishable from one another in these experiments.

Neurotoxic Activity: Induction of the Gordon Phenomenon—The results of experiments comparing EDN and liver RNase as neurotoxins and of attempts to dissociate their nucleolytic and neurotoxic activities are summarized in Table II. Experiment 1 directly compared EDN and liver RNase as neurotoxins; both were active in induction of the Gordon phenomenon, and the two proteins functioned at comparable doses. The latter conclusion is also based on other observations included elsewhere in Table II. Experiment 2 demonstrates the linkage of neurotoxic and nuclease activities; RNase activity was reduced to 2–5% of normal by treatment with iodoacetic acid at pH 5.5; carboxymethyl (CM)-EDN, liver CM-RNase, and CM-ECP were all devoid of neurotoxic activity. Controls, all of which were neurotoxic, included native proteins, EDN that had been exposed to the buffer and incubation conditions of the experiment but not iodoacetate (buffer control), EDN
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TABLE I

Activity of RNases on various substrates

| Substrate        | Assay pH | RNase activity (units/mg protein)* |
|------------------|----------|-----------------------------------|
|                  |          | EDN                  | Liver RNase | Pancreatic RNase |
| Wheat germ RNA   | 7.5      | 118,000              | 122,500     | 366,000          |
| Yeast RNA*       | 5.0      | 65.0                 | 66.5        | 95.2             |
|                  | 7.5      | 110.7                | 112.2       | 475              |
| Bacteriophage f2 RNA | 7.0 | 1,300                | 1,260       | 7,940            |
| Poly(U)          | 7.0      | 98.7                 | 102.4       | 890              |
| Poly(C)          | 7.0      | 7.4                  | 7.8         | 13,300           |
| Poly(A)          | 7.0      | ND*                  | ND          | 0.13             |
| Poly(A,U)        | 7.0      | 5,160                | 5,240       | 165,990          |
| Poly(A,C)        | 7.0      | 4,810                | 4,720       | 25,940           |
| Poly(A)-poly(U)  | 7.0      | ND                   | ND          | 2.9              |
| Poly(I)-poly(C)  | 7.0      | ND                   | ND          | 30.2             |
| Cytidine cyclic 2',3'-phosphate | 6.5 | ND                   | ND          | 17.4             |
| Uridine cyclic 2',3'-phosphate | 6.5 | ND                   | ND          | 3.7              |
| CpA              | 6.5      | 316                  | 308         | 6,600            |
| CpC, CpG, CpA    | 6.5      | ND                   | ND          | 37-282           |
| UpA              | 6.5      | 185                  | 180         | 4,185            |
| UpC, UpG, UpA    | 6.5      | ND                   | ND          | 3-37             |
| ApN, GpN (all)   | 6.5      | ND                   | ND          | ND               |

*Unless otherwise noted units are defined as: change in A_{600} per min/total change in A_{600}.

Assay used in all other experiments in this paper; units are defined in Ref. 16.

ND, not detected.

Measured at 286 nm.

Measured at 280 nm.

Fig. 1. Inhibition of RNase activity by antibodies. A, antibodies to EDN; B, antibodies to human liver RNase. O, EDN; Δ, liver RNase. Activities are shown as percentage of control (no antibody).

Fig. 2. Inactivation of RNase activity by iodoacetic acid. Reaction mixtures included 15 mM iodoacetic acid, 167 mM NaCl, 200 mM MES, pH 5.5, and 180 µg/ml protein. O, EDN; Δ, liver RNase; △, human pancreatic RNase.

treated with iodoacetic acid in the presence of excess 2-mercaptoethanol (sham CM-EDN), and EDN that was simply shipped between laboratories with the other samples. In contrast, EDN that had been denatured by reduction and carboxymethylation was active in this assay. Experiment 3 of Table II shows that RNase activity alone is not sufficient to confer neurotoxicity. Neither bovine RNase A nor its glycosylated equivalent RNase B induced the Gordon phenomenon, even at levels 10-20-fold higher than were effective with EDN or liver RNase. Human pancreatic RNase gave a positive response, but only at a dosage that was significantly higher than that found effective with EDN or liver RNase. It is known that the human pancreatic RNase preparation contains both glycosylated and nonglycosylated forms of the enzyme (32), and we speculated that only one form might be neurotoxic. This may be true; although both forms showed RNase activity, the nonglycosylated (unadsorbed to concanavalin A-Sepharose) rather than the glycosylated (i.e. acid-eluted) fraction was active in the assay. However, the acid-eluted fraction readily precipitated from solution and lost enzymatic activity; this property could have influenced the results of this experiment.

In Experiment 4 of Table II we attempted to neutralize EDN with a small excess of the nuclease inhibitor protein RNasin, and found no inhibition. However, we were concerned that the inhibitor or the EDN-RNasin complex might be unstable and release RNase during the assay. We tested this hypothesis by directly measuring the release of RNase activity from complexes that were formed using either sufficient RNasin to inhibit about 90% of the nuclease activity or a 10-fold excess of inhibitor. At 0 °C the apparent release of RNase activity during the 50 h of incubation was no more than 15%, regardless of the RNasin level. At 37 °C 10-15% of the RNase was detectable after 30 and 50 h of incubation with the 10-fold excess of RNasin. More important to Experiment 4 of Table II, incubation at 37 °C of EDN with a near-equivalent level of RNasin was accompanied by the release of about 20% of the RNase activity after 3 h and almost total
loss of inhibition by 50 h. Identical measurements with human liver RNase gave an indistinguishable result. Free RNases lost less than 10% of their activity during a 50-h incubation at 37 °C. We estimate the half-life of the EDN-RNasin complex to be about 10 h at 37 °C. Similar measurements with human pancreatic RNase-RNasin mixtures gave a calculated half-life of about 16 h.

**DISCUSSION**

Identity of EDN and Liver RNase—Our results strengthen the supposition that EDN and liver RNase are the same entity. It was already known that the NH₂-terminal amino acid sequences of liver RNase (16) and EDN (4) are identical to each other and to the equivalent section of the full sequence of the nonsecretory RNase isolated from urine (17), and that the EDN cDNA sequence (19, 20) corresponds to the amino acid sequence of the urinary RNase. Our inability to differentiate EDN and liver RNase in immunological measurements suggests that their entire sequences are probably identical, and, in addition, that they are not likely to differ significantly in post-translational processing, including glycosylation; differences in these characteristics would be expected to affect antibody recognition, yet the proteins are indistinguishable by both RIA and activity inhibition measurements with two different polyclonal antibody preparations. Their sequence clearly places EDN and liver RNase within the superfamily of RNase proteins that also includes the pancreatic RNases (32, 33), ECP (34, 35), and a protein that induces the formation of blood vessels in solid tumors, angiogenin (36).

Like bovine or human pancreatic RNase, EDN and liver RNase cleave polynucleotides that contain pyrimidine residues but purine homopolymers are resistant to hydrolysis. Both EDN and liver RNase prefer uridine-containing polymers, and both show low activity (compared with pancreatic RNase) with dinucleoside phosphate substrates. Their action on polynucleotides indicates that secondary structure may be particularly important in substrate selection since only RNA and single-stranded homopolymers are easily hydrolyzed. These catalytic properties place EDN and liver RNase in the “cellular” or nonsecretory group of nucleases, rather than in the secretory group of enzymes typified by bovine pancreatic RNase (33, 37). ECP has also been placed in the nonsecretory category on the basis of its enzymatic characteristics (29). Angiogenin has very limited RNase activity (38), but its amino acid sequence is more similar to the enzyme inactivation and inhibition. Reaction of bovine RNase A with iodoacetic acid at pH 5.5 results in carboxymethylation at either histidine residue 12 or 119 of the catalytic site of the enzyme (23, 40), and there is extensive evidence that the overall conformation of the protein is conserved (41). The similar kinetics of inactivation of EDN or liver RNase by iodoacetate (Fig. 2), the apparent retention of secondary...
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### Table II

**Neurotoxic activity of RNases: induction of the Gordon phenomenon**

| Sample assayed     | Dose (mg) | Days of observation | Positive responses/total rabbits |
|--------------------|-----------|----------------------|----------------------------------|
|                    |           | Total | To symptoms |                        |
| Expt. 1            |           |       |             |                        |
| EDN                | 1         | 8     | 3-5         | 0/3                    |
|                   | 5         | 8     | 3          | 3/3                    |
| Liver RNase        | 1         | 8     |             | 0/3                    |
|                   | 5         | 8     | 5-6        | 2/3                    |
|                   | 10        | 8     | 4-6        | 2/3                    |
| Phosphate/saline   |           | 8     |             | 0/3                    |
| Expt. 2            |           |       |             |                        |
| EDN                | 10        | 11    | 4-6        | 3/3                    |
| CM-EDN             | 20        | 9     | 2-3        | 3/3                    |
| Sham CM-EDN*       | 20        | 9     | 2-3        | 3/3                    |
| Shipping control†  | 20        | 9     | 2-3        | 3/3                    |
| EDN-buffer control| 20        | 9     | 2-3        | 3/3                    |
| Liver RNase        | 10        | 11    | 4-6        | 3/3                    |
| CM-liver RNase     | 10        | 11    |             | 0/3                    |
| ECP                | 10        | 11    | 3-5        | 3/3                    |
| CM-ECP             | 10        | 11    |             | 0/3                    |
| Phosphate/saline   |           | 9-11  |             | 0/6                    |
| Expt. 3            |           |       |             |                        |
| EDN                | 10        | 7-10  | 2-4        | 8/8                    |
| ECP                | 10        | 10    | 2-3        | 2/2                    |
| Bovine RNase A     | 10        | 10    | 4-5        | 2/2                    |
|                   | 33        | 10    | 4          | 1/2                    |
|                   | 100       | 10    |             | 0/3                    |
|                   | 200       | 7     |             | 0/3                    |
| Bovine RNase B     | 10        | 10    |             | 0/3                    |
|                   | 100       | 10    |             | 0/3                    |
| Human pancreatic RNase |   |       |             |                        |
| Unfractionated     | 10        | 7     | 3-4        | 6/3                    |
|                   | 35        | 7     | 3-4        | 2/3                    |
| Fraction A (unglycosylated) | 30 | 8     | 3-5       | 3/4                    |
| Fraction B (glycosylated) | 30 | 8     |             | 0/2                    |
| Phosphate/saline   |           | 8-10  |             | 0/9                    |
| Expt. 4            |           |       |             |                        |
| EDN                | 6.25      | 9     | 3-4        | 2/3                    |
| EDN + RNasin‡      | 6.25      | 9     | 3          | 2/3                    |
| RNasin             |           | 9     |             | 0/3                    |
| Phosphate/saline   |           | 9     |             | 0/3                    |

*Glutathione present during iodoacetate treatment.
†Protein shipped between laboratories but not included in experimental manipulations.
‡Iodoacetate omitted from reaction mixture but sample carried through the experimental procedure.
§2,500 units as defined in Ref. 26, sufficient to completely inhibit this amount of EDN.

Structure by the carboxymethylated proteins (Figs. 3 and 4), and the appropriate incorporation of radiolabeled iodoacetate (16) indicate that the inactivation involves a similar reaction that results in the modification of EDN/liver RNase histidine residues 15 and 129. Inhibition of the nuclease activity of EDN/liver RNase by RNasin is similar to that seen with human pancreatic RNase (Fig. 4) and previously reported for RNase A (26) and angiogenin (30, 42). It has been shown that the placental inhibitor protein forms an equimolar complex with RNase A (26) angiogenin (30, 42), and a placental RNase (43), apparently by blocking the catalytic site and competing with substrate (26, 44). Calculation of the quantities of enzyme and inhibitor used to generate the data of Fig. 4 indicates that EDN and liver RNase are also inhibited in the form of equimolar complexes with RNasin. Measurements of complex stability suggest that the affinity of liver RNase/EDN for RNasin is similar to that found with pancreatic RNase A (26) or human pancreatic RNase, but possibly much lower than that reported for angiogenin (30) or a ribonuclease from placenta that appears closely related to the nonsecretory enzymes (43).

**Neurotoxic Activity and Its Dependence on RNase Activity**—
The data shown in Table II show that liver RNase is capable of inducing the Gordon phenomenon and that the potency of the liver enzyme and EDN are comparable. This is additional evidence that these are identical proteins. The data also link neurotoxic action to RNase activity; human pancreatic RNase is effective in this assay, although smaller amounts of EDN/liver RNase or ECP are required relative to the pancreatic enzyme. However, RNase activity is not sufficient to induce the Gordon effect; even much larger quantities of bovine pancreatic RNase A or B had no effect.

Two characteristics in which these proteins differ (glycosylation and surface charge) could affect their ability to enter brain cells and exert nuclease activity. The nonglycosylated form of human pancreatic RNase was toxic, while the concanavalin A-reactive glycosylated fraction was not (Table II). However, this observation is complicated by the observation...
that the glycosylated fraction of the enzyme appeared to aggregate and lose RNase activity; since the assay of the Gordon phenomenon takes several days, activity could have been lost in the process. It is also significant that liver RNase (16), EDN (4), and ECP (4, 45) are all neurotoxic glycoproteins, while both the glycosylated and nonglycosylated forms of bovine pancreatic RNase did not induce the Gordon phenomenon. It is thus difficult to directly link glycosylation and neurotoxicity. The basicity of these proteins is compared in Table III. Bovine RNase A has the lowest net positive charge and is not neurotoxic. Human pancreatic RNase is slightly more basic and is somewhat toxic; glycosylation would decrease this charge if sialic acid residues were present, possibly explaining our results with human pancreatic RNase fractions. Liver RNase/EDN are slightly more basic and are neurotoxic at lower levels. ECP is much more basic; it is less active as an RNase (4, 29), but at least as neurotoxic as EDN in rabbits (4) and more toxic in guinea pigs (45). Angiogenin, intermediate in basicity between EDN and ECP, is a poor nuclease (38); it would be interesting to know if angiogenin is able to induce the Gordon phenomenon.

The most exciting observation in Table II is that controlled inactivation of the RNase activity of EDN with iodoacetic acid also inactivates its neurotoxic action. The conformation of bovine RNase A is not greatly altered in this reaction (41). The ability of carboxymethylated EDN to efficiently react with antibodies and compete in both radioimmunological (Fig. 3) and RNase inhibition assays (Fig. 4), while the unfolded reduced and carboxymethylated EDN does not compete, indicates that the conformation of carboxymethyl EDN is also retained. The assays of Figs. 3 and 4 also measure somewhat different aspects of EDN conformation. The radioimmuno- logical assay involves antigenic determinants at many sites on the protein surface, while antibody inhibition of RNase activity primarily involves regions that are involved in substrate binding. Quantitative evaluation of the radioimmuno- logical assay of Fig. 3 or the RNase inhibition assays of Fig. 4 indicates that more than 50% of the CM-EDN is fully cross-reactive with each preparation of antibodies, yet Table II shows that 5 mg of native EDN is neurotoxic while 4 times as much CM-EDN is not. We thus conclude that there is a direct linkage between RNase activity and neurotoxic function.

Angiogenin, a protein of the RNase superfamily that induces vascularization of solid tumors, is also a nuclease with very limited activity (36). Both activities are lost upon controlled carboxymethylation with bromoacetic acid (36, 46, 47). Moreover, both activities are enhanced by modification of angiogenin at aspartate residue 116 (48). These results link angiogenesis closely to RNase activity. Other data (49) indicate that a peptide segment (residues 61–67) that is not involved in nuclease action is also required for angiogenesis. This peptide segment is part of the base of the molecule if it is oriented as EDN is in Fig. 6, and it is in a region that includes several charged residues (see also Ref. 16). It may be

![Amino acid sequence of EDN/liver RNase superimposed on the bovine RNase structure.](image)

**Table III**

| Amino acid     | Bovine RNase A* | Human pancreatic RNaseb | Human liver RNase/EDNc | ECPd | Human angiogenin*e |
|----------------|----------------|--------------------------|------------------------|------|--------------------|
| Arginine       | 4              | 10                       | 8                      | 19   | 13                 |
| Lysine         | 10             | 8                        | 4                      | 1    | 7                  |
| Total basic    | 14             | 18                       | 12                     | 20   | 20                 |
| Histidine      | 4              | 5                        | 5                      | 5    | 6                  |
| Aspartic acid  | 5              | 6                        | 1                      | 6    | 6                  |
| Glutamic acid  | 5              | 6                        | 4                      | 0    | 4                  |
| Total acidic   | 10             | 12                       | 5                      | 6    | 10                 |
| Basic amino acid excess | 4 | 6 | 7 | 14 | 10 |

*From Ref. 41.
*From Ref. 32.
*From Refs. 17, 19, and 20.
*From Refs. 34 and 35.
*From Ref. 36.
significant that the equivalent region of EDN (residues numbered 62-70 in Fig. 6) is in a region with several ionic residues and two glycosylation sites.

Shapiro and Vallee (42) also found both the RNase and angiogenic activities of angiogenin to be inhibited by RNasin, and Molina et al. (50) report RNasin inhibition of EDN toxicity toward Trypanosoma cruzi trypomastigotes. We attribute the absence of effect of RNasin in Table II to several factors. The inhibitor is unstable (26) and the measured half-life of the RNasin-EDN complex is short (10 h) compared with the several days needed for assay of the Gordon phenomenon; most of the RNase would be released early in the process. In contrast, the positive result of Molina et al. (50) arose from a 2-6-h exposure of the EDN-RNasin complex to trypomastigotes, and the half-life of an angiogenin-RNasin complex is about 60 days (30), while assay of angiogenesis takes much less time. We hoped to overcome the problems caused by instability of the EDN-RNasin complex with excess RNasin in the assay of neurotoxicity; our stability measurements showed little RNase activity released if a 10-fold excess of RNasin was present. Unfortunately, this experiment was not practical. It would have required intrathecal injection of more than 2 ml (about 0.5 mg) of the most concentrated solution of active RNasin we could obtain.

Major questions now center on the role of EDN in the manifestation of the Gordon phenomenon and on the relationship of this model to the symptoms of cryptophagia-injected eosinophilia-myalgia syndrome. Since liver RNase is an identical protein, one may also question the possible relationship of this enzyme to some symptoms of hepatic encephalopathy (51). Specifically, how does EDN enter brain cells and in what amounts? Where is the primary action exerted and what is the substrate?

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