Separation of Plasma Thromboplastin Antecedent from Kallikrein by the Plasma α2-Macroglobulin, Kallikrein Inhibitor

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ABSTRACT Plasma thromboplastin antecedent (PTA, factor XI) is an important intermediate in the intrinsic coagulation system, and plasma kallikrein has been implicated as a mediator of the inflammatory process. Whereas their biologic activities are functionally distinct, their identity as separate entities in plasma has not been fully established, and the nature of their plasma inhibitors has not been completely characterized. A partially purified preparation containing the clotting, tosyl arginine methyl ester (TAME) esterase and kinin-producing activities of these substances has been prepared by DEAE-cellulose chromatography of a Celite eluate obtained from acid-treated human plasma. These activities were not separable by acrylamide gel electrophoresis nor by isoelectric focusing, their pI being approximately 8.7. Human plasma α2-macroglobulin has been shown to inhibit the proteolytic activity of kallikrein and to inhibit partially its TAME esterase activity. An α2-macroglobulin, PTA, kallikrein incubation mixture was separated by gel filtration chromatography. The α2-macroglobulin formed a high molecular weight complex with kallikrein and appeared in early chromatographic fractions. The PTA-clotting activity was not inhibited by the α2-macroglobulin; 64% of the initial PTA activity was isolated in later fractions free of kallikrein-induced kinin-like activity. In contrast, clotting, TAME esterase, and kinin-forming activities were inhibited after gel filtration chromatography of an incubation mixture of these activities and partially purified CI inactive (CI esterase inhibitor). Electrofocusing of an incubation mixture of an activated PTA, kallikrein preparation, and α2-macroglobulin resulted in the isolation of a PTA fraction free of kallikrein proteolytic activity, and with 4% of the original TAME esterase activity. In this manner, activated PTA and plasma kallikrein have been shown to be distinct substances, and methods have been introduced for the further purification of active coagulation factor XI.

INTRODUCTION

The exposure of human plasma to glass results in the initiation of the coagulation process and the generation of kinin-like activity (1, 2). The sequence of events responsible for the generation of these activities has been intensively investigated (3, 4). It is generally agreed that activation of Hageman factor (coagulation factor XII) is the first event which occurs when cell-poor plasma contacts glass. Activated Hageman factor functioning as an enzyme, changes plasma thromboplastin antecedent (PTA, factor XI)1 into its activated form (5). The activation of Hageman factor is also necessary for the conversion of plasma prekallikrein into the esterolytic and kinin-producing enzyme, kallikrein (6-9). The complete separation of activated PTA and plasma kallikrein has not been reported previously, and before the present study, no human activated PTA preparation has been shown to be free of kinin-producing activity.

Human plasma α2-macroglobulin has been shown to be a major inhibitor of plasma kallikrein (10), thrombin (11), and plasmin (12, 13). The α2-macroglobulin forms a complex with these enzymes, inhibiting their proteolytic activity and only partially inhibiting their esterolytic activity. The possible interaction between activated PTA and the α2-macroglobulin has not been previously investigated. Another plasma kallikrein inhibitor, CI inactive, has recently been found to

1Abbreviations used in this paper: ALMe, N-α-acetyl-l-lysine methyl ester; PF/dil, plasma permeability factor; PTA, plasma thromboplastin antecedent; TAME, tosyl arginine methyl ester.

This study was presented in part at the 13th Annual Meeting, American Hematology Society, San Juan, P.R., 7 December 1970.
Dr. Harpel is an American Cancer Society Scholar.
Received for publication 4 February 1971.

2084 The Journal of Clinical Investigation Volume 50 1971
inhibit activated PTA and Hageman factor (14). This inhibitor, however, accounted for only a part of the total plasma activated PTA inhibitory activity.

In view of the biological importance of PTA and plasma kallikrein in coagulation and inflammatory reactions and of the uncertainty regarding the characterization of these enzymes and their plasma inhibitors, the present studies were undertaken. It has been found that these enzymes share physical chemical properties which make separation difficult. The separate identity of these two enzymes has, however, been established, and preparations of activated PTA have been obtained free of kinin-producing activity. The plasma α2-macroglobulin, while inhibiting kallikrein, failed to inactivate PTA. The ability of the plasma α2-macroglobulin to bind with kallikrein and to form a stable, high molecular weight complex has made it possible to separate kalli-

kirein from activated PTA. In addition, the activity of C1 inactivator in inhibiting activated PTA and kallikrein has been confirmed.

METHODS

Substrates. N-α-acetyl-L-lysine methyl ester (ALMe) and tosyl arginine methyl ester (TAME) were obtained from Cyclo Chemical Corp., Los Angeles, Calif.

Activated human plasma PTA and kallikrein were prepared as described in a previous study (10). The procedure consisted of removal of coagulation factors II, VII, IX, and X from outdated plasma, followed by acidification to pH 2.0 and neutralization. The plasma was then incubated with Celite (Celite analytical filter aid; Johns-Manville Celite Division, Cleveland, Ohio), and an eluate was prepared by extraction at one of two pH’s. The eluate obtained at pH 6.0 had significantly lower total activated PTA and kallikrein activity than did a pH 10 eluate. The specific activity of the pH 6.0 eluate was approximately 4 times greater than the eluate obtained at pH 10.0. The eluates were further purified by DEAE-
cellulose chromatography, a procedure which increased the specific cloting activity twofold, and removed a number of trace contaminants. The material not adsorbed to the column contained activated PTA and kallikrein activities and was used as the PTA, kallikrein preparation described in this study. PTA activity was quantitated by a minor modification of a previously described method (15) using PTA-deficient plasma. 0.1 ml of the test preparation was added with a polypropylene pipette to 0.1 ml cephalin (Platelin; Warner-Chilcott Laboratories, Morris Plains, N. J.) in a silicone-coated glass test tube. After 80 sec of incubation at 37°C, 0.1 ml factor XI-deficient plasma and 0.1 ml of 0.025 M calcium chloride solution were rapidly added. The tubes were tilted continually at 37°C until clot formation was observed. The concentration of activated PTA in the partially purified preparations was compared with a standard pool of 10 normal human plasmas. 0.1 ml of a 1/4 dilution of the pooled standard was added with a polypropylene pipette to 0.1 ml of a Celite, cephalin mixture (activated Platelin, Warner-Chilcott) and incubated at 37°C, 10 min. 0.1 ml of PTA-deficient plasma and 0.1 ml of 0.025 M calcium chloride were then added, and the clotting time was observed. A standard calibration curve of varying dilutions of the pooled plasma was plotted on double logarithmic paper. Units of activated PTA were obtained by reference to this curve. 1 ml of the pooled normal plasma arbitrarily contained 100 PTA units, a unitage selected to facilitate graphing of the activity recovered after gel filtration chromatography. The specific PTA activity of the pH 6.0 eluate after DEAE-
cellulose chromatography was 1 PTA unit/2–3 μg protein representing an approximate 350-fold purification as compared with the starting plasma, and at pH 10, 1 PTA unit/8 μg protein. The PTA, kallikrein preparation contained no detectable thrombin as assayed by its ability to clot purified fibrinogen, plasminogen, plasmin (16), C1 (C1 esterase) (17), or clotting factors II, V, VII–X (18), or X activity (19). No factor VIII or IX activity was demonstrated using specifically deficient plasma (20). Although assays could not be performed for Hageman factor due to the presence of activated PTA, it is unlikely that significant concentrations were present in the PTA preparations as human Hageman factor is adsorbed to DEAE-cellulose (21, 22).

Plasma α2-macroglobulin and C1 inhibitor were prepared as described earlier (10). Concentrations of α2-macroglobulin were measured by radial immunodiffusion (23). The preparation of α2-macroglobulin was used homogenous by immuno-
electrophoresis (24) using polyvalent rabbit whole human serum antiserum obtained from Behring Diagnostics, Inc., Woodbury, N. Y. It possessed insignificant TAME esterase activity. C1 inhibitor activity was determined using TAME as the substrate for purified C1s as has been previously described (25). Inhibitor preparations contained 50 inhibitor units/ml, 1 unit of C1 inhibitor being defined as that amount which neutralized the esterolytic activity of 10 units of C1. The C1 inhibitor preparation, although containing several other components by acrylamide gel electrophoresis, possessed no immunologically identifiable α2-macroglobulin and no trypsin or thrombin inhibitory activity (26). The α2-macroglobulin and C1 inhibitor preparations had no procoagulant activity as assayed in the recalculated clotting system described below.

TAME esterolytic assays of fractions of the activated PTA, kallikrein preparation were carried out in a manner previously detailed (10). 1 unit of activity was defined as that amount which hydrolyzed 1 μmole TAME/hr.

The evolution of kinin-like activity was assayed by measuring the contractions of a rat uterine horn suspended in a 10 ml chamber after the addition of a preincubation mixture consist-
ing of the sample and a heated plasma substrate as de-
scribed earlier (10).

The coagulant activity of the activated PTA fractions ob-
tained from the electrofocusing and chromatographic columns was assayed by a minor modification of the method of Nossel (27). The material to be tested was diluted in polypropylene tubes with 0.05 M Tris-HCl buffer, pH 8.0, containing 0.02 M NaCl. 0.1 ml of this solution was added with a polypropylene pipette to 0.1 ml platelet-poor, noncontact plasma, preincu-
bated with 0.1 cephalin (Platelin) for 80 sec. The contents were immediately recalcified with 0.1 ml of 0.025 M calcium chloride solution and incubated at 37°C. A standard calibration curve was established relating arbitrary units of activated PTA to clotting time by determining the activity of the purified PTA preparations relative to pooled normal plasma activated with Celite using a PTA-deficient patient plasma substrate as previously described (15). A plot on double logarithmic paper of clotting time vs. PTA units produced a straight line (27). Serial dilutions of a standard partially purified PTA preparation were tested with each experiment to standardize the PTA activity on different days.

The inhibition of activated PTA by C1 inhibitor was as-
sayed by incubating 12 units of PTA with Tris-NaCl buffer or with 20 units of C1 inhibitor in polypropylene tubes at 37°C. Portions of the mixtures were removed for assay of PTA
activity at varying time intervals. Residual PTA activity was expressed as a per cent of the initial PTA activity by reference to a calibration curve on logarithmic paper of serial dilutions of the test sample at zero time.

Sephadex G-200, 40–120 μ (Pharmacia Fine Chemicals Inc., Piscataway, N. J.), was swollen and packed by gravity in 0.05 M Tris-HCl buffer, pH 8.0, containing 1.5 M NaCl. All chromatographic procedures were carried out at 4°C using a peristaltic pump. The eluate was passed through a continuous flow counter current dialysis apparatus (Biomed Instruments, Inc., Chicago, Ill.), and dialyzed against 0.05 M Tris-HCl buffer, pH 8.0, before fraction collection.

Blue Dextran 2000, aldolase, chymotrypsinogen A, ovalbumin, and ribonuclease A (Pharmacia Fine Chemicals Inc.) were used as markers to calibrate the Sephadex G-200 column. The apparent molecular weights of activated PTA and kallikrein were estimated from a plot of the elution volumes vs. the logarithm of the molecular weight of the markers.

Isoelectric focusing of an activated PTA, kallikrein preparation was accomplished by the separation procedure as described by Vesterberg and Svensson (28). A sucrose density gradient was established according to the manufacturer’s instructions using a 110 ml focusing column (LKB Instruments, Inc., Rockville, Md.). The anodal compartment at the top of the column contained sulfuric acid and the cathodal compartment, ethanalamine. 4.0 mg of protein, containing 1440 units of activated PTA and 2880 TAMe esterase units, was applied to the column. The run was performed at a maximum of 1 w for 3 days. 2-ml fractions were collected, and the pH of each fraction was measured immediately at 4°C with a Radiometer type PHM 4C meter and a type GK2322C electrode (Instrumentation Associates, Inc., New York). The fractions were dialyzed and assayed for coagulant, TAMe esterase, and rat uterine-contracting activities.

Isoelectric focusing of an activated PTA, kallikrein preparation incubated with purified plasma α2-macroglobulin. Preliminary experiments established that the α2-macroglobulin formed a precipitate at its isoelectric point. The column electrodes were reversed for experiments utilizing the α2-macroglobulin, thus the precipitate formed at the bottom of the column and did not disturb the PTA focused at the middle of the column. 48 mg of purified α2-macroglobulin in 6.0 ml Tris- NaCl buffer was incubated 15 min at 37°C with 2.0 ml of an activated PTA, kallikrein preparation. This mixture, as well as the PTA, kallikrein preparation alone, were electrofocused 72 hr in a pH 3–10 amphyolyte gradient. The fractions were assayed as has been described.

Disc acrylamide gel electrophoresis was performed at alkaline pH as detailed by Reisfeld, Lewis, and Williams (29). 0.05 ml of a PTA, kallikrein preparation eluted from Celite at pH 6.0 and further purified by DEAE-cellulose chromatography was applied per gel. After a 90 min separation at 5 ma/tube at 4°C, the gels were stained for protein or were cut into 1 mm slices. The slices were then eluted with 1.0 ml 0.05 M Tris-HCl buffer, pH 8.0, containing 0.15 M NaCl, and assayed for coagulant, TAMe esterase, and uterine-contracting activities.

Protein was measured by the Lowry, Rosebrough, Farr, and Randall procedure (30) using bovine serum albumin (Pentex Biochemical, Kankakee, Ill.) as the reference standard or by transmittance at 280 μg in a Beckman model D.B. spectrophotometer.

**RESULTS**

Isoelectric focusing of activated PTA and kallikrein. PTA coagulant activity, TAMe esterase, and kallikrein-like kinin-producing activities were recovered after electrofocusing of a PTA, kallikrein preparation in a carrier amphyolyte gradient, pH 3–10 (Fig. 1). No separation of these activities was achieved. Repeat focusing of the active fractions from Fig. 1 in a 7–10 pH gradient resulted in no apparent separation of the several activities. The approximate isoelectric point (pI) of activated PTA and kallikrein was found to be 8.7.

Disc acrylamide gel electrophoresis of activated PTA and kallikrein. A major protein band, which migrated in the gamma globulin region, and a minor component with faster mobility were identified after electrophoresis of a PTA, kallikrein preparation. Analysis of the eluates obtained from gel slices demonstrated no separation of the coagulant, TAMe esterase, or kinin-producing activities. All activities were in a location corresponding to the gamma globulin protein band.

Separation of activated PTA from kallikrein activity by Sephadex G-200 column chromatography. Partial separation of clotting activity, TAMe esterase, and kinin-producing activity was achieved after Sephadex G-200 column chromatography (top panel, Fig. 2). The eluting buffer contained 1.5 M NaCl, as preliminary experiments had shown that recovery of PTA and kallikrein activities was poor at lower molarities, and co-
agulant activity was widely spread over the Sephadex G-200 column, a finding previously noted by Lewis (31). Elution at 1.5 M NaCl produced a relatively sharp peak of PTA activity, with approximately 75% recovery. Protein markers established the apparent molecular weight of activated PTA as approximately 170,000 and kallikrein as 100,000 confirming the findings of previous workers (31-33). An incubation mixture of purified α2-macroglobulin and an activated PTA, kallikrein preparation were separated by chromatography on Sephadex G-200 (bottom panel, Fig. 2). No inhibition of PTA activity by the α2-macroglobulin was demonstrated; however, about 7% of the starting PTA coagulant activity was cochromatographed with the α2-macroglobulin fractions. Activated PTA was recovered free of kinin-producing activity and with less than 10% of the TAMe esterase activity of the starting material. Approximately 64% of the initial PTA activity was recovered in this kallikrein-free peak. This peak also contained immunoglobulin G as identified by immunoelectrophoresis. Although kallikrein was removed from the PTA peak, no significant increase in the specific clotting activity was achieved by gel filtration chromatography. No TAMe esterase or kallikrein-induced kinin-forming activity was identified in chromatographic fractions where these activities were found after chromatography of the activated PTA, kallikrein preparation without the α2-macroglobulin. As described in earlier studies (10), more than 90% of the TAMe esterase activity of the PTA, kallikrein preparation became associated with the α2-macroglobulin peak. Fractions obtained by Sephadex G-200 chromatography of the purified α2-macroglobulin preparation used in this study had no TAMe esterase activity.

Sephadex G-200 column chromatography of a CI inactivator, activated PTA, kallikrein incubation mixture. PTA clotting, TAMe esterase, and kinin-like activities were found to be absent in fractions obtained from Sephadex G-200 column chromatography of a CI inactivator, activated PTA, kallikrein incubation mixture (Fig. 3). Incubation of activated PTA with CI inactivator (Table I) demonstrated, in agreement with recently reported findings (14), that the inactivation of PTA was time dependent.

Electrofocusing of an α2-macroglobulin, activated PTA, kallikrein incubation mixture. Electrofocusing of a mixture of an activated PTA, kallikrein preparation incubated with purified plasma α2-macroglobulin resulted in separation of clotting and kallikrein-induced uterine-contraction activity (Table II). TAMe esterase activity was identified in association with the α2-macroglobulin which focused at pH 5.1. This complex possessed the esterolytic activity of kallikrein, but the kinin-producing activity of kallikrein was completely
**TABLE I**

**The Time-Dependent Effect of C1 Inactivator in Inhibiting the Clotting Activity of Activated PTA**

<table>
<thead>
<tr>
<th>Incubation time (min)</th>
<th>PTA + buffer*</th>
<th>PTA + C1 inactivator</th>
<th>Residual PTA %</th>
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<tr>
<td>0</td>
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<td>100</td>
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<tr>
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<td>8</td>
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<tr>
<td>120</td>
<td>65.0</td>
<td>124.8</td>
<td>5</td>
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</tbody>
</table>

Buffer control 171.2 sec

* 0.05 ml activated PTA preparation containing 12 PTA units was incubated at 37°C in polypropylene tubes with 0.5 ml Tris- NaCl buffer or with 0.5 ml C1 inactivator (20 inactivator units). 0.1 ml portions were removed, diluted 1:25 in Tris-NaCl buffer, and assayed for coagulant activity.

inhibited. The α2-macroglobulin, as well as the α2-macroglobulin, kallikrein complex, formed a precipitate at its pI. These precipitates became soluble after dialysis. The TAME esterase activity of the fraction containing the most PTA-coagulant activity from the electofocusing column containing the α2-macroglobulin was compared with the PTA peak fraction focused in the absence of the kallikrein inhibitor. The α2-macroglobulin removed 96% of the TAME esterase activity associated with the PTA coagulant activity, and no kinin-forming activity was demonstrable.

**DISCUSSION**

Plasma thromboplastin antecedent (factor XI) after activation by Hageman factor (factor XII), activates Christmas factor (factor IX), thus playing a key role in the intrinsic blood coagulation mechanism (3, 5, 34). Hageman factor activation is also required for the conversion of plasma prekallikrein into the bradykinin-releasing enzyme, kallikrein (4). Bradykinin has been implicated in the mediation of the inflammatory reaction in a variety of pathophysiologic states (35). The sequence of events leading to the activation of kallikrein by Hageman factor is not clear and may be mediated by a plasma permeability factor (PF/dil) (3, 6, 36–38). Recent reports have suggested an alternate hypothesis, that Hageman factor or a breakdown product derived from Hageman factor directly converts plasma prekallikrein into kallikrein (7–9).

Evidence has been previously presented that PTA and plasma kallikrein have functionally distinct activities (33, 39). It has not been unequivocally shown, however, that these substances exist in nature as separate entities. The studies reported here have shown that activated PTA and plasma kallikrein are distinct substances and have established methods for the preparation of PTA, free of kallikrein activity. Activated PTA and kallikrein have been found to share a number of biophysical and biochemical properties. Purification, without separation of both activities, was achieved by Celite adsorption of plasma followed by ion exchange chromatography. The present studies have demonstrated that both activities migrate similarly in acrylamide gel electrophoresis and correspond to the gamma globulin region of serum. Previous investigators have noted that kallikrein has the electrophoretic mobility of a gamma globulin (32, 33, 38–40). Furthermore, the present study has found that the isoelectric point of both activities is similar.

The biochemical similarities of these substances prompted an examination of the possible interactions between the purified plasma α2-macroglobulin and activated PTA as well as between serum C1 inactivator and this procoagulant activity. In addition to the ability of the α2-macroglobulin to form a high molecular weight complex with thrombin and plasmin (11–13) and to inhibit the proteolytic activity of these enzymes, it has recently been found that the α2-macroglobulin is a major plasma inhibitor of kallikrein (10). This study has demonstrated that incubation of purified human plasma α2-macroglobulin with a partially purified activated PTA, kallikrein preparation followed by gel filtration chromatography, results in the isolation of an activated PTA peak, entirely free of kallikrein-induced rat uterine-contracting activity. Both the kinin-producing and TAME esterase activities associated with the
kallikrein peak were completely inhibited. The α2-macroglobulin chromatographic fractions showed an increase in TAME esterase activity which was quantitatively similar to the TAME esterase activity of the kallikrein peak. These findings are consistent with the formation of an α2-macroglobulin, TAME esterase, high molecular weight complex. The identity of the α2-macroglobulin–bound TAME esterase as kallikrein is suggested by prior studies which have established that plasma kallikrein is an arginine esterase (32, 41, 42) with a characteristic ratio of esterolytic activity toward several different substituted amino acid ester substrates (42). The ratio of esterolytic activity of the biologically active kallikrein peak after chromatography was found to be similar to the activity of the α2-macroglobulin–bound enzyme (10). A relatively small amount of PTA procoagulant activity was found in association with the α2-macroglobulin after incubation with the PTA, kallikrein preparation. The nature and possible physiologic significance of this interaction between the α2-macroglobulin and activated PTA remains to be determined. Kallikrein was also separated from activated PTA by electrofocusing a mixture of a PTA, kallikrein preparation with α2-macroglobulin. Kallikrein remained complexed to its inhibitor under the low ionic strength conditions necessary for electrofocusing. The pI of the enzyme, inhibitor complex was 5.1, similar to that found for the purified α2-macroglobulin. This permitted separation of kallikrein from activated PTA with a pI of 8.7.

C1 inactivator is an enzyme inhibitor of broad specificity, inhibiting C1 esterase (43, 44), plasma kallikrein, PF/dil (38, 45, 46), plasmin, and C1r subcomponent of the first component of complement (47). In contrast with the ability of the α2-macroglobulin to inhibit plasma kallikrein but not activated PTA, C1 inactivator inhibited both enzymes. These observations confirm the recent findings of Forbes, Pensky, and Ratnoff (14), that the C1 inactivator inhibited activated PTA and that this inhibition was time dependent. A similar time-dependent inactivation of C1 esterase (48), Hageman factor (14), and kallikrein (10, 48) by this inhibitor has been reported.

Kingdon, Davie, and Ratnoff found that partially purified preparations of activated PTA hydrolyzed TAME (49). These authors demonstrated that the coagulant and esterase activity of this preparation was inhibited by diisopropylphosphofluoridate, indicating that activated PTA is a hydrolytic enzyme. The identity of the enzyme responsible for the TAME esterase activity was not established nor were these preparations assayed for kallikrein activity. As indicated by the authors, the PTA preparations examined were impure. The arginine esterase activity which is activated in plasma by contact with kaolin is due to kallikrein (10, 42). Although PTA is activated in kaolin-treated plasma, its contribution toward the total plasma TAME esterase activity was found to be minimal, as the arginine esterase activity generated in normal plasma and in congenitally deficient PTA plasma was found to be similar (10, 42). The present study has demonstrated that most of the TAME esterase activity of a partially purified PTA preparation was contributed by plasma kallikrein. Electrofocusing of a PTA, kallikrein preparation incubated with α2-macroglobulin yielded an activated PTA fraction, free of kallikrein-induced kinin-like activity and with only 4% of the TAME esterase activity of the starting material. These data suggest that the residual TAME esterase activity in the PTA preparation may have been produced by another, as yet unidentified, esterase. These experiments provide evidence for the separate identity of activated PTA and plasma kallikrein and establish methods for the further purification and characterization of activated coagulation factor XI.

ACKNOWLEDGMENTS

These studies were performed with the invaluable technical assistance of Mr. Tsun-san Chang and Mr. Ben Denson. We wish to thank Dr. Ralph Nachman for his review of the manuscript.

This study was supported in part by U. S. Public Health Service Research grant NB-03346 (National Institute of Neurological Diseases and Blindness).

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