Protease activity has been demonstrated in culture supernatants of Clostridium tetani at various stages of fermentation. Gel chromatography of the concentrated filtrates revealed the presence of three enzymatically active fractions eluting at separate positions off the column. The smallest protease was found to "nick" the single chain intracellular tetanus toxin, producing the extracellular, two-chain structure of the molecule. As little as 3 ng of active protease were sufficient to cleave 50 μg of intracellular tetanus toxin, suggesting that this enzyme is responsible for the observed structural change of the toxin molecule during its release into the culture medium. By comparison, the second protease, eluting at an intermediate position, exhibited only marginal activity towards intracellular toxin. The third, largest, enzyme was not active under the conditions of the assay. However, the latter protease effectively hydrolyzed low molecular weight histidyl peptides, and it is concluded that this enzyme is similar to the one described by Miller, P. A. Gray, C. T., and Eaton, M. D. (1960) J. Bacteriol. 79, 95–102.

The properties of the partially purified enzymes, including their differential behavior towards a number of protease inhibitors, are reported.

Recent work focused on the structure of tetanus toxin has helped to establish the existence of two molecular forms of this protein (1–6). Apparently, the toxin molecule is elaborated by Clostridium tetani as a single polypeptide chain with a molecular weight of about 140,000. This single chain molecule has been termed intracellular toxin and may be obtained by interrupting the fermentation prior to lysis, followed by extraction of the harvested bacteria with salt solutions. Alternatively, an extracellular form of tetanus toxin may be recovered from the filtrates of autolyzed cultures. Craven and Dawson (1) showed that extracellular toxin was separable into two polypeptide chains (heavy chain and light chain), which were linked to each other by a disulfide bond. Matsuda and Yano (6a) confirmed and extended these findings, reporting the conversion of the intracellular toxin to the extracellular form by digestion with trypsin (2). The two fragments, termed α- and β-fragment, were apparently similar to the light chain and heavy chain polypeptide, respectively.

New data on the N-H-terminal structure of tetanus toxin seem to confirm the present concepts regarding its polypeptide chain composition (6). Thus, proline was the sole amino acid detected in NH2-terminal position of the intracellular toxin, whereas the extracellular species contained NH2-terminal leucine in addition to proline. Furthermore, isolated light chain was found to exhibit proline as its NH2-terminal amino acid, whereas leucine occupied the equivalent position of the heavy chain polypeptide. These data suggest that the light chain fragment constitutes the NH2-terminal region of the original protein (6).

Since treatment of the intracellular toxin with trypsin resulted in the conversion to a structure very similar to that seen in toxin preparations derived from the culture filtrate, it is conceivable that a proteolytic enzyme of C. tetani is involved in this alteration of the molecular structure. Previously, protease activity in cultures of C. tetani has been demonstrated (7–10) and there is some indication that the formation of a histidyl peptidase occurs concomitantly with tetanus toxin (8). In the present communication, three separate proteases were demonstrated in the bacterial culture filtrate. Only one of these was highly active in generating the heavy chain-light chain framework of tetanus toxin. A second proteolytic enzyme exhibited substantial activity towards general protease substrates but produced only a marginal quantity of "nicked" tetanus toxin from the intracellular species. Finally, a protease lacking the capacity to convert the toxin to its extracellular form, but displaying properties consistent with the previously described histidyl peptidase (8), was separated from the other two enzymes.

MATERIALS AND METHODS

Chemicals—Sephadex G-100 gel was purchased from Pharmacia, Uppsala, Sweden. Ultrogel AcA 44 gel was ordered from LKB, Bromma, Sweden. Soybean trypsin inhibitor, benzamidine, and PMSF were delivered by Sigma, St. Louis, Mo. TPCK-trypsin was obtained from Worthington, Freehold, N. J. Azocoll was a product of Calbiochem-Behring Corp., San Diego, Calif. Protease inhibitors derived from pooled human sera (α-2-macroglobulin, α-1-antitrypsin, and inter-α-trypsin inhibitor) were kindly supplied by Mr. Haupt, Behringwerke AG. Equine tetanus antitoxin (different lots) were stock preparations of Behringwerke AG.

Tetanus Toxin—The extracellular form of the toxin was derived from culture filtrates and purified as described previously (5). Intracellular tetanus toxin was prepared after interrupting the fermentation process at 48 h. The live cells from 2-liter cultures were harvested by centrifugation, washed twice in 0.1 M NaCl, and suspended in 1/10 of the original volume in a buffer containing 1 M NaCl and 0.1 M sodium citrate (3). The buffer was supplemented with benzamidine (2 g/liter) to minimize any proteolytic activity present. After incubating at 4°C for 5 days, the cells and debris were separated from the extracted protein by centrifugation. The supernatant was dialyzed against several changes of 0.01 M phosphate buffer and applied to a column (2 x 10 cm) of DEAE-cellulose, eluted with a gradient mixture

1 The abbreviations used are: PMSF, phenylmethanesulfonyl fluoride; IgG, immunoglobulin G fraction, produced by gel chromatography of whole serum on Sephadex G-150; SDS, sodium dodecyl sulfate.
gel chromatography, on a column (2 X 100 cm) of Ultrogel AcA 44, to
xin prepared in this fashion yielded a single polypeptide band on
hard to $0.15^1$ to $0.15^1$ portions to a column (5 X 100 cm) of Sephadex G-100, eluted with 0.1
ered by gel electrophoresis under reducing conditions.

Demonstration of Proteolytic Activity in Cultures of C. tetani—
Samples (50 ml) of actively growing cultures were taken every 24 h
and separated by centrifugation into a cell fraction and a supernatant
fraction. The cells were washed with 0.15 M NaCl and resuspended in
2 ml of 0.1 M Tris-HCl buffer, pH 8.0. The supernatant fraction was
also concentrated to 2 ml by ultrafiltration prior to enzymatic analysis.
Proteolytic activity was determined as described below.

Separation and Partial Purification of Bacterial Proteases—
After preliminary experiments (11), the following procedure was
adopted to separate the three proteases. The culture filtrate (6000 ml)
was concentrated to 100 ml by ultrafiltration and applied in two
portions to a column (5 X 100 cm) of Sephadex G-100, eluted with 0.1
M Tris-HCl buffer, pH 8.0, 1 M NaCl. Aliquots (0.05 ml) of each
fraction were diluted to 1 ml with the same buffer and incubated for
6 h with Azocoll (5 mg/tube). Appropriate pools were formed and
purified by a second chromatographic run on a column (2.5 X 250 cm)
of Ultrogel AcA 44, eluted as above.

Determination of Proteolytic Activity—Column eluents were
monitored by incubating each fraction with Azocoll and measuring the
release of dye from the insoluble carrier colorimetrically (12). For
convenience, some kinetic experiments and most inhibition assays
were also performed using this substrate. General protease activity
was also determined by following the liquefaction of fibrin in agar
plates (13). Conversion of the intracellular form of tetanus toxin to
the nicked species was demonstrated by SDS-gel electrophoresis
under reducing conditions following incubation with various enzyme
pools and using the intracellular toxin as the substrate. The disappear-
ance of the toxin band at 140,000 daltons and the appearance of
the composite polypeptide chains at approximately 93,000 and 48,000
daltons, respectively (5), indicated the presence of converting enzyme
activity.

Histidyl peptidase activity was demonstrated by incubating the
enzyme preparation with glycyl-L-histidine (8) followed by high volt-
age paper electrophoresis of the digest.

Other Analytical Methods—Gel electrophoresis in SDS buffer was
performed by the procedure of Weber and Osborn (14). Estimation of
the molecular size was achieved by determining the elution position
of the enzyme on Ultrogel AcA 44. The column had been calibrated
by a number of globular proteins and the approximate molecular size
of each protease was computed as described by Andrews et al. (15).

Antisera—Rabbit antisera against fractions of the culture supernat-
ant from C. tetani were produced by three subcutaneous injections
of the partially purified enzyme. Prior to injection, the protease with
an activity equivalent to 0.5 µg of trypsin was mixed with an equal
volume of Freund's complete adjuvant. In order to eliminate traces of
tetanus toxin contaminating the preparations, two methods were
employed. Preliminary treatment of each fraction with formaldehyde
(0.1% HCHO, pH 6.5, 96 h at room temperature), or with rabbit antibodies against Fragment B of tetanus toxin (16) rendered all
materials nontoxic. In some cases, the immunoglobulin fraction was
separated from serum protease inhibitors by gel chromatography on
Sephadex G-150.

RESULTS

Demonstration of Protease Activity—Fig. 1 shows the re-
lease of proteolytic activity into the extracellular medium of

FIG. 2. SDS-gel electrophoretic analysis after digestion of
intracellular tetanus toxin with the crude culture filtrate. A, 
intracellular toxin after incubation with the culture filtrate (sample
taken at 120 h and concentrated 25-fold) overnight; B, the correspond-
ing culture filtrate without added intracellular tetanus toxin; and C,
intracellular toxin used as substrate. In each incubation the
concentration of the components was kept constant. Preliminary
experiments indicated the presence of extracel-
lar tetanus toxin in the enzyme preparation interferes with the
interpretation of the gel patterns. However, the disappearance of the
single chain polypeptide due to intracellular toxin after digestion
with the culture filtrate is indicative of the presence of converting enzyme
activity (cf. Gel A +Me and C +Me).

Histidyl peptidase activity was demonstrated by incubating the
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FIG. 1. Formation of proteases by C. tetani as a function of
fermentation. Every 24 h, samples (50 ml) were withdrawn from the
culture and filtered. The culture filtrate was concentrated to 2 ml and
 aliquots (0.05 ml) were incubated with Azocoll at pH 8.0. After 3 h at
37°C, the tubes were read at 520 nm. O——O, samples taken from
nonstirred 1-liter cultures; ——, samples from a 10-liter fermenter.
In the latter case, the growth rate is always slightly below that
observed in still cultures and maximal release of protease activity is
obtained after 120 h (instead of 96 h) into the fermentation.

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C. tetani cultures as fermentation proceeds. The overall protease activity peaked concomitant with the attainment of maximal cell mass, but measurable activity was demonstrable at all stages during fermentation. Incubation of intracellular toxin with the crude protease from the culture supernatant also resulted in the conversion to the extracellular form; however, the interpretation of the stained gels was complicated by several protein bands which may be attributed to contaminants present in the culture filtrate (Fig. 2). In contrast, no converting enzyme activity was detected after removing the culture supernatant and incubating the harvested bacterial cells with intracellular toxin. Therefore, the culture filtrate was selected as the starting material for the purification of the protease activity.

Purification of C. tetani Proteases—Fig. 3 shows the elution pattern obtained after ultrafiltration of the culture filtrate and gel chromatography on a column (5 x 100 cm) of Sephadex G-100. Three Azocoll-positive peaks, eluting behind tetanus toxin and denoting P-1, P-2, and P-3, respectively, were separated by the gel column. The fractions corresponding to each peak were pooled and subjected to rechromatography on long columns (2.5 x 250 cm) of Ultrogel AcA 44 (Fig. 3). These semipurified materials (P-1R, P-2R, and P-3R) were used for most studies reported here. The yields of each protease expressed as trypsin equivalents at pH 8.0 are summarized in Table I.

Substrate Specificity—Protease P-1R did not effect the conversion of intracellular tetanus toxin (Fig. 4) but caused the hydrolysis of glycyl-L-histidine as shown by paper electrophoresis after incubating the enzyme with the dipeptide overnight (Fig. 5). In contrast, proteases P-2R or P-3R exhibited no significant activity towards glycyl-L-histidine. However,
the latter enzymes were found to modify the chain structure of tetanus toxin. Under the conditions of the assay, protease P-2R only partially degraded intracellular toxin (Fig. 4). By comparison, protease P-3R was by far the most active converting enzyme. As little as about 3 ng of protease P-3R (expressed in terms of trypsin equivalents) adequately cleaved 50 μg of intracellular toxin, producing the composite polypeptide chains. At higher concentration, extensive degradation of the toxin occurred (Fig. 4a). The partially purified enzymes did not interfere with the interpretation of the gel patterns.

All enzymes exhibited activity towards Azocoll or fibrin (13). Their approximate molecular sizes, based on gel chromatography data, are listed in Table I.

**Inhibition Studies**—Fig. 6 summarizes data from representative experiments carried out to determine the sensitivity of the three enzymes to several inhibitors. Protease P-1R was only marginally affected by general inhibitors of serine proteases, such as benzamidine or PMSF. Both P-2R and P-3R, respectively, showed greater susceptibility; thus, at 2 mg/ml

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Inhibitor added</th>
<th>Protease activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-1R</td>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>α-2-Macroglobulin</td>
<td>104</td>
</tr>
<tr>
<td></td>
<td>α-1-Antitrypsin</td>
<td>104</td>
</tr>
<tr>
<td></td>
<td>Inter-α-trypsin inhibitor</td>
<td>104</td>
</tr>
<tr>
<td>P-2R</td>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>α-2-Macroglobulin</td>
<td>98</td>
</tr>
<tr>
<td></td>
<td>α-1-Antitrypsin</td>
<td>67</td>
</tr>
<tr>
<td></td>
<td>Inter-α-trypsin inhibitor</td>
<td>96</td>
</tr>
<tr>
<td>P-3R</td>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>α-2-Macroglobulin</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td>α-1-Antitrypsin</td>
<td>122</td>
</tr>
<tr>
<td></td>
<td>Inter-α-trypsin inhibitor</td>
<td>112</td>
</tr>
</tbody>
</table>

The effect of solvent alone was also investigated. As is seen, P-3R was not affected by ethanol itself. However, P-1R and in particular, P-2R were strongly impeded by ethanol. Thus, the contribution of PMSF to the inhibition of these two enzymes is limited, and in the case of P-2R, possibly negligible. O—O, enzyme P-1R; ■—■, enzyme P-2R; and ×—×, enzyme P-3R.

**Fig. 5.** High voltage paper electrophoresis of digests after incubating glycyl-β-histidine (2 μmol) with proteases P-1R, P-2R, and P-3R (cf. Fig. 3, b to d). Each enzyme was adjusted to a final concentration of 0.2 μg/ml (trypsin equivalents, Azocoll assay) and kept overnight at room temperature in a total volume of 200 μl. Aliquots (10 μl) were spotted on Whatman No. 3MM paper and subjected to paper electrophoresis on a Gilson model D high voltage electrophorator (3500 V, 30 min). The dried papers were stained with ninhydrin. A, glycyl-β-histidine standard; B, D, and F, glycyl-β-histidine incubated in the absence of proteolytic enzymes; C, glycyl-β-histidine + enzyme P-1R; E, glycyl-β-histidine + enzyme P-2R; G, glycyl-β-histidine + enzyme P-3R; H, L-glycine standard; and I, standard mixture of L-glycine and L-histidine. The analysis was performed in pyridine/acetate buffer at pH 6.4.

**Fig. 6.** Inhibition of C. tetani proteases by (a) benzamidine, (b) PMSF, or (c) ethanol. Each protease was adjusted to a final concentration of 0.2 μg/ml (trypsin equivalents) and the inhibitor was added in the final concentration indicated. After 10 min at 37°C, Azocoll (5 mg) was added and the incubation continued for 60 min. The activity is expressed as per cent color yield compared to controls without added inhibitor. Since PMSF was added dissolved in ethanol,
TABLE III

Inhibition patterns of C. tetani proteases

+ indicates significant inhibition at the maximal concentration of inhibitor tested. − indicates absence of inhibition within the concentration range tested. ± indicates a marginal inhibitory effect.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Equine antitoxin</th>
<th>PMSF (2 mg/ml)</th>
<th>Benzamidine (8 mg/ml)</th>
<th>Urea (2 M)</th>
<th>Ethanol (4%)</th>
<th>(tetracycline)</th>
<th>(diphteria)</th>
<th>(tetracycline)</th>
<th>(diphteria)</th>
<th>Soybean trypsin inhibitor (0.5 mg/ml)</th>
<th>α-2-Macroglobulin (0.5 mg/ml)</th>
<th>α-1-Antitrypsin (0.5 mg/ml)</th>
<th>Inter-alpha-trypsin inhibitor (0.5 mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-1R</td>
<td>+ + + + +</td>
<td>+ +</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>±</td>
<td>−</td>
<td>−</td>
<td></td>
</tr>
<tr>
<td>P-2R</td>
<td>+ + + + + +</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>±</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td></td>
</tr>
<tr>
<td>P-3R</td>
<td>− + + + +</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td></td>
</tr>
</tbody>
</table>

*Values in parentheses represent maximal concentration tested.

FIG. 7. Dependence of the enzymatic activity on pH. Each enzyme was diluted to 0.2 μg/ml (trypsin equivalents) with the appropriate buffer solutions and incubated with Azocoll (5 mg) for 60 min at 37°C. Triplicate tubes were read at 520 nm. The buffers used were: 0.1 M acetate (pH 5 to 6) or 0.1 M Tris/acetate (pH 7 to 11). O—O, enzyme P-1R; □—□, enzyme P-2R; and X—X, enzyme P-3R.

When tested against proteinase inhibitors derived from pooled human sera, only P-3R was significantly impeded by α-2 macroglobulin at 600 μg/ml inhibitor concentration (Table II). This enzyme was also partially inhibited by inter-α-trypsin inhibitor. Furthermore, slight inhibition of P-2R by α-1-antitrypsin was noted (Table II). Normal rabbit sera had no effect on the proteolytic activity of P-1R. However, the activity of protease P-2R was moderately affected by the serum. In keeping with the sensitivity of enzyme P-3R against α-2-macroglobulin, this protease was strongly inhibited by normal rabbit serum.

Equine tetanus antitoxin effectively inhibited all three proteases. Furthermore, the capacity of the antiserum to block enzymatic activity towards Azocoll was independent of prior absorption of the serum with highly purified tetanus toxin. Nonspecific horse serum did not interfere with the enzymatic activity of the proteases. A summary of the inhibition patterns is given in Table III.

Dependence on pH—Proteases P-1R and P-2R showed similar pH-profiles with a broad maximum in the range of pH 6.0 to 9.0. The third protease exhibited maximal activity below pH 7.0 and was distinguished from the other two enzymes in this assay (Fig. 7).

Immunochemical Distinction between the Three Proteases—Table IV shows the effect on the enzymatic activity of the three proteases after preliminary incubation with antibodies raised in rabbits against each enzyme preparation. Each enzyme was immunogenic and elicited antibodies affecting the catalytic center, in agreement with the data obtained with polyvalent equine antitoxin (Table III). In addition to inhibiting the homologous protease, anti P-1R serum also interfered with the activity of protease P-2R. Enzyme P-3R was strongly impeded by preimmune sera, presumably due to the presence of serum inhibitors (cf Table II). However, after preparing the corresponding immunoglobulin fraction, this enzyme was affected by the homologous serum only.

DISCUSSION

The thorough study of Miller et al. (8) indicated a close association between the formation of a histidyl peptidase by C. tetani, and the appearance of the toxin in culture filtrates. However, the relationship between this proteolytic enzyme and the structural features of tetanus toxin has remained unclear, although recent work on the polypeptide chain composition of the toxin has suggested an important role for a bacterial protease in transforming intracellular toxin to its extracellular counterpart (2, 6, 11).
From the data presented in this communication, it appears highly likely that the histidyl peptidase activity demonstrated by Miller et al. (8) is very similar to the P-1R protease eluting immediately after tetanus toxin on gel chromatography. This enzyme catalyzed the hydrolysis of glycyl-L-histidine but did not, under the reaction conditions employed, induce the characteristic modification of the intracellular toxin. However, with general protease substrates, two further enzymes were detected and separated from P-1R by gel chromatography. The two additional enzymes failed to hydrolyze low molecular weight histidyl peptides in the standard assay but converted intracellular tetanus toxin to the extracellular form. Whereas only slight converting activity was associated with P-2R, enzyme P-3R was highly efficient in this capacity. Thus, three enzymes with differential proteolytic activities may now be distinguished in culture filtrates of C. tetani. The yield of each enzyme (Table I) was considerably below that of the tetanus toxin released by the bacteria, but sufficient protease P-3R is clearly available for conversion of the toxin to the extracellular form during cultivation.

Since the intracellular form of tetanus toxin may be isolated as an intact polypeptide chain with a molecular weight of about 140,000 (1-4, 6, 11), one might tentatively conclude that the toxin is excreted into the culture medium in the single chain form and is subsequently hydrolyzed by the extracellular proteases. Furthermore, the finding that bacteria isolated from the growing cultures by centrifugation and subsequently incubated with intracellular tetanus toxin were unable to effect hydrolytic cleavage of the polypeptide chain suggests the absence of significant activity of the relevant proteases on the surface of the bacterial membrane. However, at least traces of the extracellular toxin was present in all preparations derived by extraction of early cultures with NaCl-citrate buffer. In a few such "intracellular" toxin preparations, the quantity of nicked protein amounted to 50% of the total amount of tetanus toxin isolated, although Azocoll analyses routinely carried out prior to purification confirmed the absence of detectable proteolytic activity. Therefore, under certain culture conditions, at least, the cleavage of the polypeptide chain of tetanus toxin may possibly occur prior to the release of the molecule into the medium. Thus, the site where proteolytic cleavage occurs remains unsettled and this problem will require further attention.

Apart from separate elution positions on gel chromatography, the three proteases were also distinguished by their behavior towards various inhibitors (Tables II to IV). Only enzyme P-3R was markedly inhibited by α-2-macroglobulin and PMSF, but not by benzamidine. The inhibitory action of α-2-macroglobulin is presumably mediated by a conformational change following interaction with the protease (17). Due to sterical interference, the proteolytic activity of the trapped enzyme is impeded. It is conceivable that the sizes of proteases P-1R or P-2R were too great to permit a close association and entrapment by this inhibitor. Each enzyme was inhibited by the homologous antiserum derived in rabbits (Table IV). Interestingly, anti P-2R serum showed no inhibitory effect against protease P-1R, whereas the antiserum obtained after immunization with the latter enzyme also impeded the activity of P-2R. Thus, it cannot, at this stage, be ruled out that the two enzymes may express common antigenic determinants. An alternate explanation for this finding would be given by assuming the presence of aggregated P-2R molecules contaminating enzyme P-1R. Such aggregates may be highly immunogenic and induce anti P-2R antibodies upon immunization. The latter hypothesis is supported by the observation that culture filtrates which were kept for extended periods of time tended to lose some activity at the elution position of P-2R, although this enzyme, once isolated, was stable for months at 4°C.

All three enzymes were inhibited most effectively by hyperimmune sera produced against crude toxoid preparations. However, some lot-to-lot variation was observed and it is conceivable that toxoid preparations of slightly variable purity may influence the yield of specific antibody against the three proteases.

A possible role of the bacterial protease in the establishment of infection has previously been discussed (8), and the potential value of anti-protease antibody to help control the spread of pathogens in the host tissue was implicated. Furthermore, the elaboration of proteases by the bacteria may be necessary for the conversion of tetanus toxin to the extracellular form. There is some indication that this conversion is required for the full expression of toxicity of the molecule (2, 18). Since only partial inhibition of protease P-3R was obtained with serum factors, it is clear that host tissue infected with C. tetani may contain active proteases of probable significance for the pathogenesis of tetanus during the initial stages of disease.

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