Exotoxin A of *Pseudomonas aeruginosa* (PA toxin) shares with diphtheria toxin the ability to inactivate mammalian elongation factor 2 by catalyzing the transfer of the ADP-ribosyl portion of NAD to elongation factor 2 (5). By means of specific binding to NAD, diphtheria toxin has been purified by affinity chromatography on NAD-linked agarose (3, 4). We now report that NAD specifically attached to agarose through an $N^6$-methyl group is efficient for binding PA toxin, whereas other NAD-agarose preparations are not. The binding of PA toxin to NAD-agarose provides a rapid, convenient, and efficient method for the preparation of purified toxin which we have demonstrated to be enzymatically, biologically, and immunologically active.

Toxin-producing strain PA-103 was provided by Liu (8) and a non-toxin-producing mutant, PAO-T1 was obtained from Ohman et al. (11). PA was grown in dialyzed tryptic soy broth (5) in a 1.5-liter fermentor. At the end of the logarithmic growth phase, bacteria were removed by centrifugation at 10,000 × g. Toxin was recovered from 3 liters of culture supernatant by batch-wise treatment with DEAE-cellulose (5) and was eluted in 0.25 M NaCl in 10 mM Tris-hydrochloride buffer (pH 8.0) (Tris buffer). This was followed by precipitation with 70% (NH$_4$)$_2$SO$_4$ and extensive dialysis against 5 mM Tris buffer (pH 8.0) containing 2 mM dithiothreitol. The dialyzed material was concentrated to 5 ml, applied to a column of agarose-$N^6$-(aminohexyl)carbamoylmethyl-NAD (Sigma Chemical Co., St. Louis Mo.; no. 9505), and eluted with Tris-dithiothreitol. When all UV-absorbing material had been washed off the column, elution was continued with Tris-dithiothreitol containing 0.15 M NaCl. Material which eluted in the salt fraction was immediately pooled and dia-
lyzed against Tris-dithiothreitol. Enzymatic activity of the toxin was measured by in vitro ADP ribosylation (5) by using adenine [2,8-$^3$H]NAD (New England Nuclear Corp., Boston, Mass.) and elongation factor 2 derived from rabbit reticulocytes. Biological activity was determined by a sensitive assay for the inhibition of protein synthesis in L-929 mouse fibroblast cell cultures. Immunological activity of the toxin was tested by reaction with patient and control sera in a radioimmunoassay. The molecular weight of the purified material was estimated by discontinuous sodium dodecyl sulfate-gel electrophoresis (6).

The results of the affinity chromatography of PA supernatant on NAD-agarose are depicted in Fig. 1. The ADP-ribosylating material from the supernatant of the toxin producing strain PA-103 was specifically bound to the column (Fig. 1B). This material was readily recovered from the column with an elution buffer of increased ionic strength. A small amount of ADP-ribosylating activity which eluted with the starting buffer might represent partially denatured toxin of low affinity for NAD. ADP-ribosylating material was not recovered from the supernatant of PAO-T1, a non-toxin-producing mutant (Fig 1A). In addition, no further UV-absorbing material was eluted from the column with 0.15 M NaCl. The affinity chromatography step resulted in an approximate 10-fold increase in the specific activity of the toxin (Table 1), measured as units of ADP-ribosylating activity per milligram of protein. Sodium dodecyl sulfate-gel electrophoretic analysis of the purified material revealed the presence of both whole toxin and fragment A (data not shown). A 65,900-molecular-weight band representing whole toxin was noted as was a broader band at 27,000 to 30,000 molecular weight depicting the more heterogeneous fragment A (2). Contaminating minor bands were not visible, even after the gel column was over-loaded by the addition of 50 μg of sample protein.

The purified material was found to be toxic to cultured cells and was able to produce a 50%
inhibiton of protein synthesis at a concentration of 0.8 ng/ml. This calculation is derived from a logarithmic curve produced from the data in Table 2. The toxicity for L-929 cells was completely neutralized by serum from a patient with PA infection but was not affected by serum from an uninfected control (Table 2). The serum of the infected patient showed an antitoxin reciprocal titer of \( \geq 12,800 \) when tested against purified toxin in a radioimmunoassay (Table 3). The control serum had an antitoxin titer of <10.

In addition to agarose-\( N^6 \)-(aminohexyl)carbamoylmethyl-NAD, several other resins were tested for their ability to bind PA toxin. These proved to be inefficient substrates for PA toxin purification in that only minute amounts of toxin were bound: (i) NAD attached through C8 with a 6-carbon spacer (Sigma no. 1008); (ii) two preparations of non-specifically linked NAD, probably attached through a ribose hydroxyl (10), which were Sigma no. 6130 and the NAD-Sepharose described in reference 3; and (iii) unsubstituted Sepharose.

The recovery of 25% of toxin ADP-ribosylating activity in this procedure (Table 1) and the protein synthesis inhibitory dose of purified toxin in (Table 2) are both within the same range as for other reported procedures which rely upon physical means of toxin purification (1, 5, 9). A 10-fold increase in specific activity of the purified product over the culture supernatant was achieved (Table 1). In another report of a PA toxin purification procedure which used ADP-ribosylation as a measure of specific activity, a 16-fold purification was demonstrated (7). Higher ratios have been reported when mouse lethality was used as a basis for specific activity (1). However, the comparison of toxin purification procedures by the increase in specific activity over the crude supernatant must be approached with caution, since the calculation is dependent on the amount of non-toxin protein present in the supernatant. The amount of non-toxin protein varies with extraneous factors such as culture conditions, the degree of bacterial autolysis, and the time of harvesting. The purified product from the affinity chromatography step,

**FIG. 1.** Affinity chromatography of PA supernatants from non-toxin-producing (A) and toxin-producing (B) strains on agarose-\( N^6 \)-(aminohexyl)carbamoylmethyl-NAD. A 20- by 2.5-cm column of resin was prepared in Tris-dithiothreitol at 4°C. It was eluted with the same buffer at the rate of 2 ml/min.

<table>
<thead>
<tr>
<th>TABLE 1. Specific activity of toxin at various stages of purification</th>
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<tr>
<td><strong>Stage</strong></td>
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<tr>
<td>Spent medium</td>
</tr>
<tr>
<td>Material applied to</td>
</tr>
<tr>
<td>NAD-Sepharose Purified toxin</td>
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\( \text{a} \) The ADP ribosylation (ADPR) unit is defined as the counts per minute obtained with 20 flocculation units of diphtheria toxin. One ADPR unit is equivalent to 14.5 \( \mu \)g of purified PA toxin.
which contained both intact toxin and fragment A, was appropriate for our purposes. For other procedures, whole toxin may be conveniently separated from its fragment by Sephadex chromatography in Tris buffer containing 1 mM EDTA (2).

Since this affinity chromatography method of purification relies on a biological property of the toxin (NAD binding) rather than on physical methods of separation, the purified product is especially suitable for use as an antigen in the study of the specific immune response to PA toxin without interference from other contaminating pseudomonas antigens. The product may also serve as an immunogen for the production of toxin-specific antibody which does not cross-react with other PA antigens.

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LITERATURE CITED


