UNIVERSITY OF ILLINOIS

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THIS IS TO CERTIFY THAT THE THESIS PREPARED UNDER MY SUPERVISION BY

THERESE CATHERINE NUELLE

ENTITLED THE NINTH AMINO ACID OF GLUTAMINE PHOSPHORIBOSYL-

PYROPHOSPHATE AMIDOTRANSFERASE IN BACILLUS SUBTILIS

IS APPROVED BY ME AS FULFILLING THIS PART OF THE REQUIREMENTS FOR THE

DEGREE OF Bachelor of Science

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APPROVED: Lawrence P. Hager

HEAD OF DEPARTMENT OF Biochemistry

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THE NINTH AMINO ACID OF GLUTAMINE PHOSPHORIBOSYLPYROPHOSPHATE AMIDOTRANSFERASE IN *BACILLUS SUBTILIS*

BY

THERESE CATHERINE NUELLE

THESIS

for the

DEGREE OF BACHELOR OF SCIENCE

IN

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FOR

ROBERT L. SWITZER

and

MY DAD

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I. Introduction

The first step in purine biosynthesis *de novo* is catalyzed by glutamine phosphoribosylpyrophosphate amidotransferase (amidotransferase), which catalyzes the reaction of glutamine and phosphoribosylpyrophosphate (PRPP) in the presence of Mg^{2+} to form glutamate, phosphoribosylamine (PRA), and pyrophosphate. The glutamine analogue, 6-diazo-5-oxo-L-norleucine (DON), inactivates amidotransferases by reacting covalently with the glutamine binding site (Hartman, 1963). Studies of amidotransferase from *Bacillus subtilis* confirmed that DON reacts with its glutamine site and inhibits its ability to catalyze the breakdown of glutamine to glutamate and ammonia. The DON-modified enzyme still catalyzes the reaction of ammonia and PRPP to form PRA (Vollmer *et al.*, submitted). Partial sequencing of amidotransferase showed DON-modified cysteine was released on the first cycle of Edman degredation. When amidotransferase that had been reacted with [^{14}C]DON was degraded, all the radioactivity was removed, showing that no other site reacts with DON.

The partial sequencing was performed in M. Hermodson's laboratory at Purdue University (Vollmer *et al.*, submitted). The first twenty-four amino acids from the amino terminus were identified. However, the phenylthiolhydantoin of the ninth amino acid was not extracted by the organic solvents. This suggests that it had a hydrophilic side chain, but otherwise this residue could not be identified. One tryptophan residue was found in this sequence at the ninth position from the amino

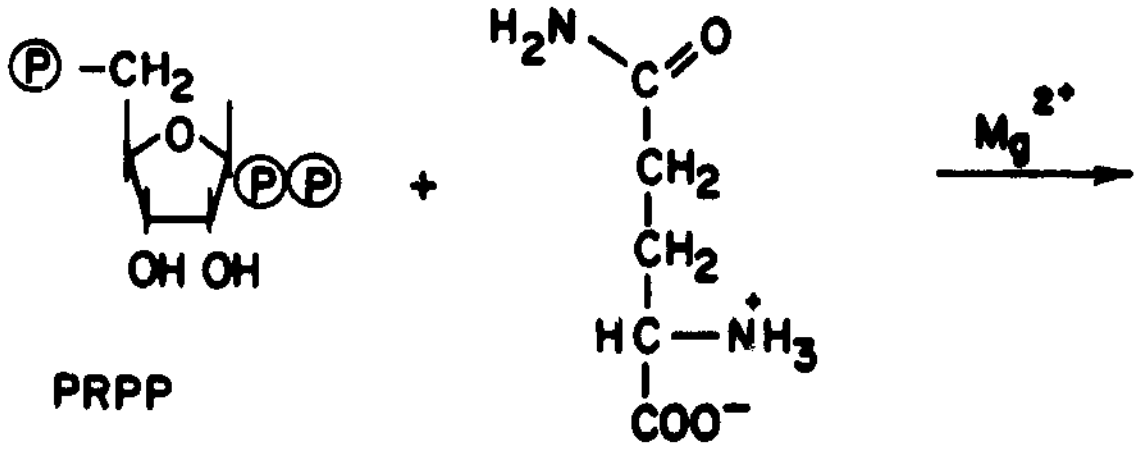
terminus. The amino acid composition of amidotransferase indicated the presence of only one tryptophan per subunit (Wong et al., 1981).

The focus of this project is to determine the identity of the ninth residue. Because no residue was identifiable from sequencing, there is reason to suspect it may be post-translationally modified. Bernlohr and Switzer (1983) also hypothesized that a covalent modification might be involved in the selective inactivation of amidotransferase. Bernlohr's work clearly shows two populations of amidotransferase in cells that were starved and refed glucose. These were newly synthesized enzyme, which is stable, and previously synthesized enzyme, which is inactivated even as new enzyme is being synthesized. A covalent modification would be an obvious way for B. subtilis to earmark the amidotransferase molecules for inactivation. Such covalent modification presumably occurs during glucose starvation.

The tenth amino acid from the amino terminus is glutamate, which can be selectively cleaved with Staphylococcus aureus V8 protease (Drapeau, 1972). A radioactive label can be incorporated into DON, which then provides a sensitive method to label the amino-terminal cysteine residue. Isolation of the amino terminal decapeptide can be monitored by following the [^{14}C] label and the absorbance of tryptophan at 280 nm. Analysis of the decapeptide should enable identification of the ninth residue. This should in turn allow identification of the nature of the post-translational modification, if there is one,

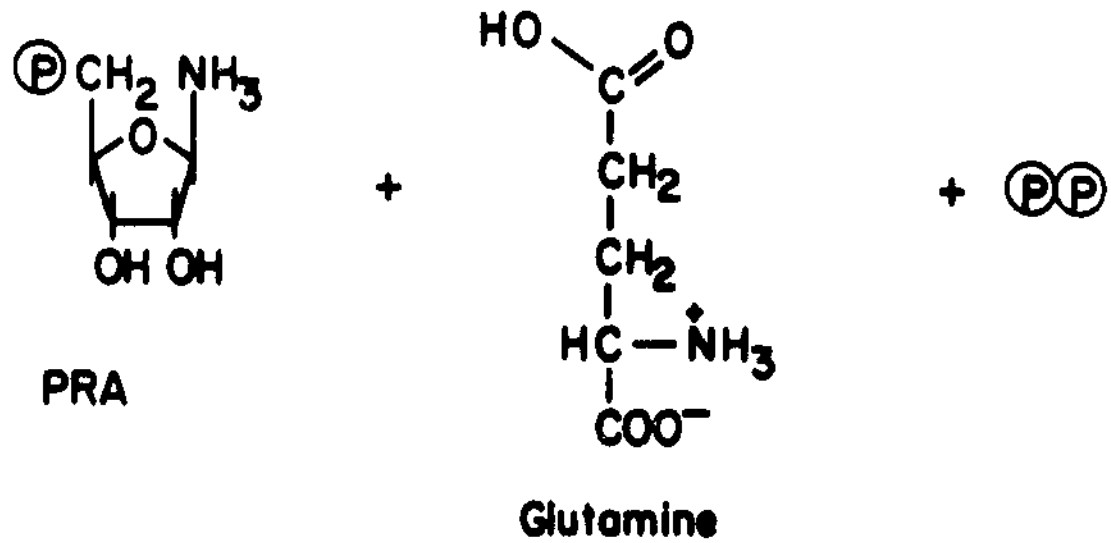
and provide the basis for further studies of the role of the putative modification in regulation of amidotransferase inactivation *in vivo*.

Figure 1. The structures of DON (6-diazo-5-oxo-L-norleucine) and of the substrates and products of the reaction catalyzed by amidotransferase. Ⓟ indicates an orthophosphate group.



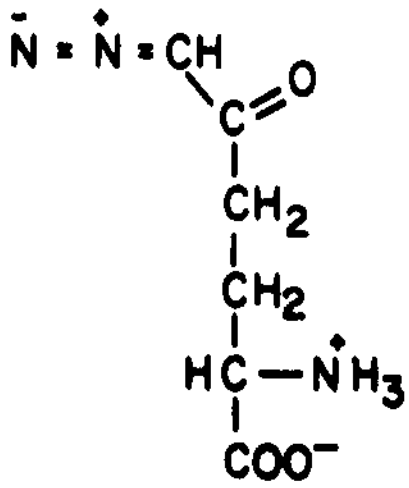
PRPP

Glutamine



PRA

Glutamine



DON

Figure 2. The sequence of the 24 amino-terminal amino acids of amidotransferase determined by automated Edman degradation.

$$H_2N\text{-}\ cys\text{-}gly\text{-}val\text{-}phe\text{-}gly_{5}\text{-}ile\text{-}trp\text{-}gly\text{-}\ X\ \text{-}glu_{10}$$

$$glu\text{-}ala\text{-}pro\text{-}gln\text{-}\ ile_{15}\text{-}thr\text{-}tyr\text{-}\ tyr\text{-}gly\text{-}leu_{20}$$

$$his\text{-}ser\text{-}\ leu\text{-}gln_{24}$$

II. Materials and Methods

A. Methods

1. Purification. Amidotransferase was isolated from B. subtilis strain 168 by the method of Wong et al. (1981).

2. [^{14}C]DON Labeling of Amidotransferase. Amidotransferase was removed from storage in liquid nitrogen and dialyzed at 0°C against 500 ml of 50 mM Tris-HCl pH 7.9 at 4°C under argon flushing. The reaction mixture contained 5 mg/ml ATase (25 mg total), 100 mM [^{14}C]DON (21,570 dpm/nmole), 7.5 mM $MgCl_2$, and 5 mM PRPP in a total of 5 ml. The reaction proceeded at room temperature for one hour under argon flushing. The solution was the dialyzed extensively against 50 mM Tris-HCl.

A reduced and alkylated form of the [^{14}C]DON-labeled amidotransferase was also prepared. After the reaction mixture containing amidotransferase, [^{14}C]DON, $MgCl_2$, and PRPP (in a total of 5 ml at the concentration as above) had proceeded for one hour, as described above, 2.85 g of guanidine HCl was added to make a 6 M solution. The pH was then adjusted to 8.5 with Tris base. An excess of $NaBH_4$ (50 mg) was slowly added, followed by the addition of 150 µl of previously distilled 4-vinylpyridine. The mixture was incubated for one hour in cool water at 10°C with stirring. Then it was dialyzed against 250 ml of 25% formic acid for 24 hours with 6 changes of formic acid. The reduced and alkylated [^{14}C]DON-labeled amidotransferase was lyophilized to dryness.

3. Digestion with S. aureus Protease. Digestion of the reduced and alkylated [^{14}C]DON-labeled amidotransferase was carried out in 50 mM ammonium acetate (pH 4.0) with 2 mM EDTA and 4 M urea. The final protein concentrations were 1 mg/ml DON-labeled amidotransferase and 33 µg/ml S. aureus protease. The reaction mixture was incubated at 37°C for 24 hours.

Digestion of the unreduced [^{14}C]DON-labeled amidotransferase was performed using 50 mM Tris-HCl (pH 7.9 at 37°C) with 2 mM EDTA. The [^{14}C]DON-labeled amidotransferase concentration was 1 mg/ml. S. aureus protease was used at 1:30 (w/w) with amidotransferase. The mixture was incubated at 37°C. Optimal digestion required 3.5 hours.

4. Polyacrylamide Gel Electrophoresis. Slab and tube polyacrylamide gels containing 10 or 15% acrylamide were prepared following the method of Laemmli (1970), except that they were not acid washed. The gels were stained with 0.05% Coomassie Brilliant Blue R-250 in 25% isopropanol and 10% acetic acid. Gels were destained in 5% methanol and 7% acetic acid.

Tube gels that were not stained were cut into 1.5 mm slices and dissolved overnight at 75°C in 15% hydrogen peroxide. After cooling to room temperature, 5 ml scintillation fluid (Anderson et al., 1973) was added and counted.

5. Electrophoresis in Polyacrylamide Gels Containing Urea to Separate Low Molecular Weight Peptides. A procedure developed by Bethesda Research Labs (1981) was used. The separating gel contained 15% acrylamide (N,N'-methylene-bis-

acrylamide:acrylamide, 0.8:30.0 w/w), 0.1 M sodium phosphate (pH 7.2), 0.1% sodium dodecyl sulfate (SDS), and 6 M urea. An upper gel of 3.5% acrylamide was used with the same buffer. The running buffer was 0.1 M sodium phosphate (pH 7.2) and 0.1% SDS. Samples were diluted into sample buffer to a final value of 0.01 M sodium phosphate (pH 7.2), 7 M urea, 1% SDS, 5% 2-mercaptoethanol, and 0.01% bromophenol blue. Samples were boiled 2.5 minutes prior to application on the gel. The separating gel was 24 cm long and was run at 100 V for 17 hours at room temperature. The gels were stained with Coomassie stain as previously stated, except that 0.1% cupric acetate was added to improve staining of small peptides. Destaining was as described above.

6. Fluorography. Detection of radioactivity in slab gels was accomplished using fluorography (Chamberlain, 1979). The gel was soaked in 1 M sodium salicylate for 30 minutes. It was then placed on water-wetted Whatman 3 MM paper and dried for 1 hour on a Bio-rad gel dryer. The gel was exposed to X-ray film at -70°C for at least 24 hours.

7. Ninhydrin Assay. The protein eluting from gel filtration columns was assayed using ninhydrin (Hirs, 1967). From each fraction to be tested, 0.1 ml was added to 0.5 ml 4N NaOH. This was autoclaved for 20 minutes and allowed to cool. Glacial acetic acid (0.24 ml) was added to neutralize the NaOH. Ninhydrin solution (0.5 ml) was added, and the solutions were vortexed. They were covered and heated for exactly 15 minutes

in a boiling water bath. Each sample was cooled for 10 minutes in an ice water bath, and 2.5 ml of 50% ethanol was added to each. The absorbance was read at 570 nm. The ninhydrin reagent was prepared as follows: 250 ml of 4N sodium acetate (pH 5.5) and 750 ml methyl cellosolve was mixed with 20 g of ninhydrin, followed by 0.4 g $SnCl_2 \cdot H_2O$.

8. Scintillation Counting. All counting was done with a Beckman LS 7000 scintillation counter. The scintillation fluid consisted of 0.4% diphenyloxazole (PPO) in 75% xylene and 25% Triton X-114 (Anderson et al., 1973).

9. Precipitation with Trichloroacetic acid (TCA). To precipitate proteins with TCA, an equal volume of 10% TCA was mixed with the samples, which were then incubated on ice for one hour. The precipitate was collected by centrifuging 30 seconds in a Beckman Microfuge. All of the supernatant liquid was removed and counted. When the samples contained less than 150 µg of [^{14}C]DON-labeled amidotransferase, 100 µl of bovine serum albumin (BSA) (1 mg/ml) was added before TCA addtiion.

B. Materials

The [^{14}C]DON was a geneours gift from Dr. Robert R. Engle and was prepared by SRI International under a contract from the National Cancer Institute. All chemicals were reagent grade. Special materials and reagents were as follows: acrylamide, N,N'-methylene-bis-acrylamide, and ammonium persulfate (Bio-Rad); urea (Mallinckrodt); Blue dextran, Sephadex G-10, and G-25, fine (Pharmacia); XAR-5 X-ray film (Kodak); S. aureus

V8 protease (Miles Laboratories); ovalbumin, β-lactoglobulin, cytochrome c, bovine trypsin inhibitor, and trypsin (Sigma); bovine insulin (Schwarz/Mann); and [^{3}H] leucine (Amersham).

III. Results

A. Digestion of Reduced and Alkylated [^{14}C]DON-labeled Amidotransferase.

1. Digestion Conditions. Drapeau (1977) states that S. aureus V8 protease is fully active in the presence of 0.2% SDS and 50% active in 4 M urea. Because the reduced and alkylated amidotransferase was difficult to dissolve in the ammonium acetate buffer, incubation of amidotransferase with S. aureus protease in buffer containing 0.1% SDS, 0.2% SDS, 2 M urea, or 4 M urea was attempted. The incubation including urea showed fairly complete digestion, whereas the samples with SDS showed incomplete digestion by the protease after 24 hours, when the samples were examined on 15% polyacrylamide gel electrophoresis.

The amidotransferase proved difficult to dissolve in ammonium acetate buffer containing 2 M urea, so the following adjustment was made. One mg of amidotransferase was placed in one ml of 25% formic acid. This was heated in a water bath until amidotransferase dissolved. Once dissolved, it was dialyzed against 50 mM ammonium acetate (pH 4.0) containing 4 M urea, then subjected to proteolysis by S. aureus V8 protease. Samples that were analyzed by gel electrophoresis showed a large amount of amidotransferase fragmentation, even when no S. aureus protease was present or without incubation at 37°C. The fragments indicated that this method was too harsh to dissolve the reduced and alkylated amidotransferase and resulted in chemical cleavage of peptide bonds.

2. TCA Precipitation of [^{14}C]DON-labeled Amidotransferase. Reduced and alkylated amidotransferase was dissolved in ammonium acetate (pH 4.0) containing 4 M urea to make 1 mg/ml. A 24 µl sample was removed to determine the total dissolved radioactivity. A second 24 µl sample was removed, 100 µg of 1 mg/ml BSA was added, and the protein was precipitated with 124 µl of 20% TCA to measure the total amount of radioactivity that could be precipitated. This was done by counting the supernatant fluid. After protease was added, samples were taken at timed intervals up to 16 hours, BSA was added, and the supernatant fluid after TCA addition was counted. Only 13% of the radioactivity stayed in the supernatant fluid after 16 hours of digestion. This indicated that digestion either didn't occur, or the fragments released were still insoluble in TCA.

This same experiment was run with 'native' DON-labeled amidotransferase (i.e. material that was not reduced and alkylated), except that 150 µg of amidotransferase was used instead of 25 µg amidotransferase plus 100 µg BSA and no urea was present. In this case, 53% of the radioactivity was not precipitable. There was also considerable precipitation during the incubation, which probably resulted from oxidation and denaturation of amidotransferase.

3. Separation on Sephadex Columns. Since the protease digestions were carried out in 4 M urea, the urea was removed by gel filtration on a Sephadex G-10 column (54.5 x 2 cm). Protein was detected by its radioactivity. Urea was detected using a colorimetric ureide assay (Prescott et al., 1969,

Shindler et al., 1979). Pooled column fractions were lyophilyzed, redissolved in 3 ml ammonium acetate buffer, and loaded onto a Sephadex G-25 column (56.5 x 2.5 cm). Fractions of 2 ml each were collected. The amidotransferase was eluted at 15 ml/h and collected with an LKB fraction collector. All the amidotransferase eluted at the void volume of the column, which suggested aggregation of the peptides.

Because of the insolubility and fragmentation of the reduced and alkylated amidotransferase and because the amount of radioactivity released to TCA precipitation seemed more promising with amidotransferase that had not been alkylated, native DON-labeled amidotransferase was digested with S. aureus protease. I continued all subsequent experimentation with native amidotransferase as the substrate for degradation.

B. Digestion of Native [^{14}C]DON-labeled Amidotransferase

1. Digestion with Trypsin. Trypsin digestion was accomplished using 5 mM $CaCl_2$ and 1:100 trypsin (w/w to amidotransferase) in 50 mM Tris (pH 7.9) at 37°C. Duplicate samples were taken at various times up to 60 minutes of digestion. One of the samples was precipitated with TCA. Up to 71% of the radioactivity became TCA soluble after 40 minutes of trypsin digestion. The other sample was analyzed by polyacrylamide gel electrophoresis and stained. The stained bands were cut out, dissolved, and counted. There was a disappearance of radioactivity at the position of the undigested subunit, but counts did not appear elsewhere in the gel. The disappearance of counts at the position of

the undigested subunit suggests that amidotransferase was digested, but since the radioactivity did not appear elsewhere, the fragments were small and diffused from the gel during destaining.

2. Digestion with S. aureus Protease. The method adopted for following digestion of [^{14}C]DON-labeled amidotransferase was electrophoresis in SDS-containing polyacrylamide slab gels, followed by fluorography (Chamberlain, 1979). The [^{14}C]DON label in undigested amidotransferase was stable for at least 12 hours at 37°C in the absence of S. aureus protease. Because the desired decapeptide is at the amino terminus, it was desirable to determine whether that radioactive decapeptide could be cleaved from amidotransferase, leaving most of the protein in larger fragments. Analysis by polacrylamide gel electrophoresis and fluorography revealed radioactivity in every Coomassie Blue staining band on the polyacrylamide gel (Figure 3). This indicated the decapeptide could not be quantitatively separated from the rest of amidotransferase by digestion of amidotransferase for a short time and separation of a radioactive fragment from a large amount of non-radioactive protein.

3. Analysis of [^{14}C]DON-labeled Amidotransferase after Trypsin Treatment. M. Ruppen showed that digestion of amidotransferase with trypsin, when analyzed by polyacrylamide gel electrophoresis, formed a large Coomassie Blue staining protein band that could be isolated (unpublished results). Samples were taken at timed intervals during trypsin digestion of [^{14}C]DON-labeled amidotransferase at 37°C and analyzed by 10% polyacrylamide gel

Figure 3. Fluorogram (A) and Coomassie Blue stained SDS polyacrylamide gel (B) of digestion products of [^{14}C]DON-labeled amidotransferase with S. aureus protease.

Duplicate samples containing 10 µg of [^{14}C]DON-labeled amidotransferase were removed at timed intervals from a digestion with S. aureus protease and electrophoresed on 10% polyacrylamide gels as described in Methods.

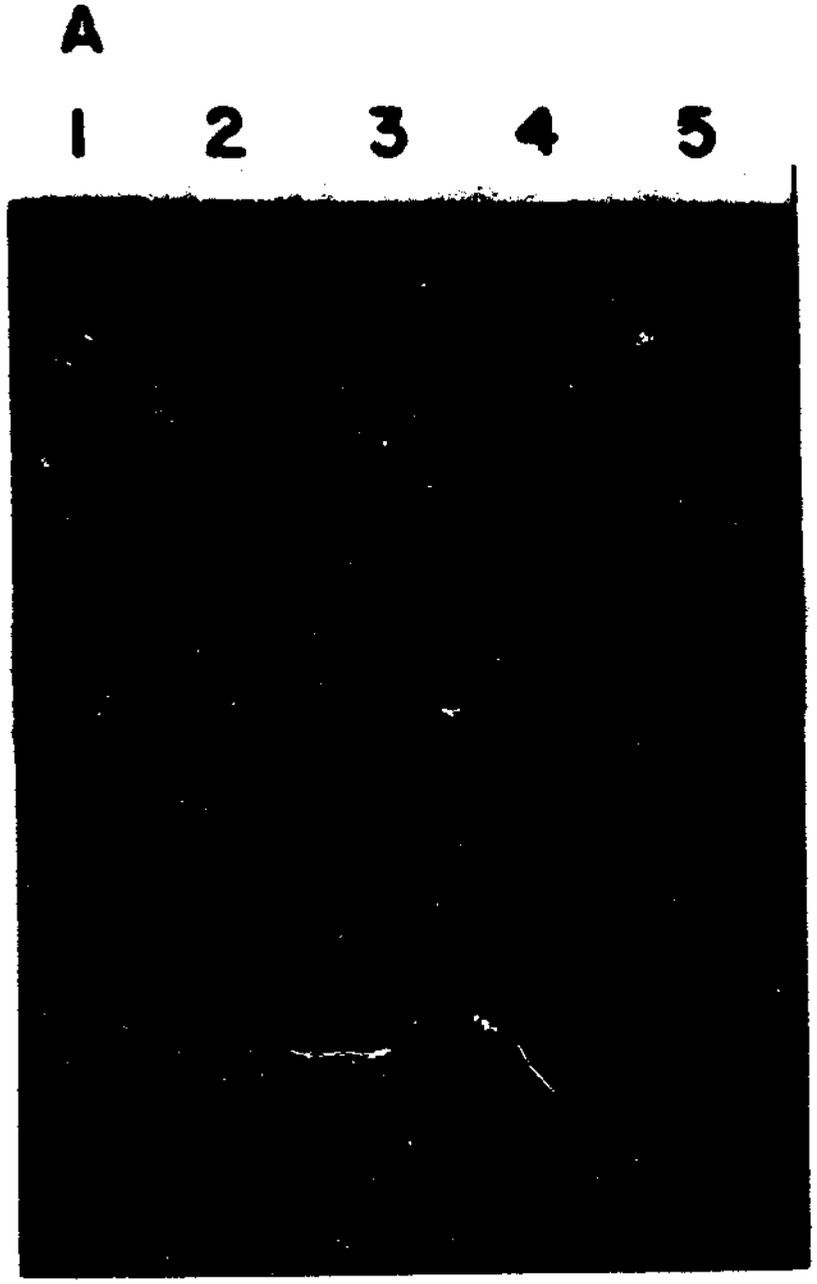
Lane 1: Amidotransferase with no S. aureus protease added, equilibrated at 37°C for 5 minutes.

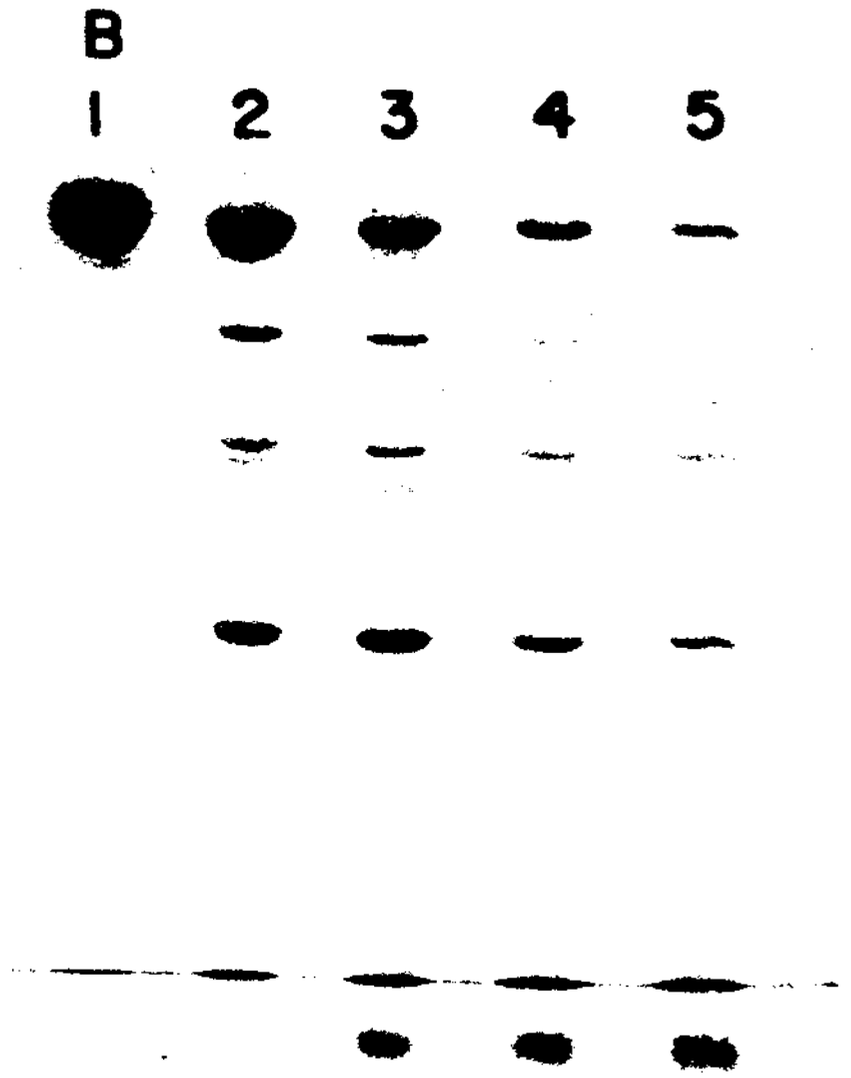
Lane 2: Amidotransferase with S. aureus protease, 0.5 hour digestion.

Lane 3: Amidotransferase with S. aureus protease, 1.0 hour digestion.

Lane 4: Amidotransferase with S. aureus protease, 1.5 hours digestion.

Lane 5: Amidotransferase with S. aureus protease, 2.0 hours digestion.


A
1 2 3 4 5


B
1 2 3 4 5

electrophoresis and fluorography revealed 2 Coomassie Blue staining bands that were also radioactive (Figure 4). These had molecular weights of 31,500 and 23,800.

4. Separation of Small Peptides for S. aureus V8 Protease Digestion by Electrophoresis on Urea-containing Polyacrylamide Gels. Because better resolution of the small peptides was desired, electrophoresis of urea-containing polyacrylamide gels was used. Digestion of [^{14}C]DON-labeled amidotransferase with either S. aureus protease or trypsin was analyzed on urea-containing polyacrylamide gels by Coomassie Blue staining and by fluorography. Digestion was at 37°C for 3.5 hours. The fluorogram showed that a majority of radioactive protein from the S. aureus protease digest migrated with a molecular weight of approximately 2600. This value was estimated from comparison to the positions of standard proteins. Several larger protein bands that were not radioactive were detected by Coomassie Blue staining (Figure 5). This appears to give the best conditions for digestion since several non-radioactive protein bands were detected. The smallest radioactive protein from the trypsin digest migrated with a molecular weight of approximately 5200, but appeared to be formed in poor yield. The resolution of peptides by urea-containing polyacrylamide gels was better than resolution obtained with 15% polyacrylamide gels prepared according to Laemmli.

Because it was desirable to separate peptides by gel filtration in the presence of 8.8% formic acid to prevent aggregation

Figure 4. Fluorogram (A) and Coomassie Blue stained SDS Polyacrylamide gel (B) of digestion products of [^{14}C]DON-labeled amidotransferase with trypsin.

Duplicate samples containing 10 µg of [^{14}C]DON-labeled amidotransferase taken at timed intervals from a trypsin digestion and analyzed by polyacrylamide gel electrophoresis.

Lane 1: Amidotransferase, trypsin present but reaction quenched at once.

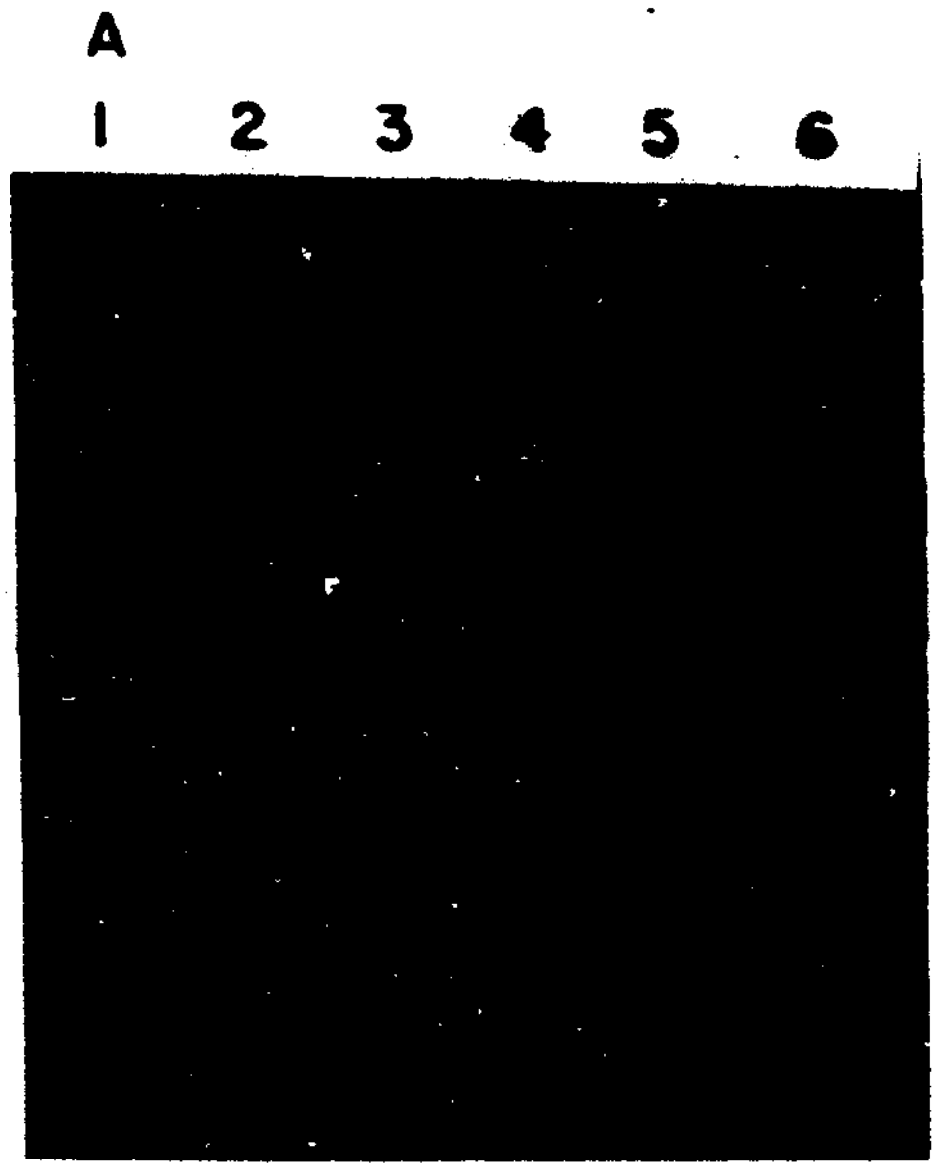
Lane 2: Amidotransferase with trypsin, 10 minutes digestion.

Lane 3: Amidotransferase with trypsin, 30 minutes digestion.

Lane 4: Amidotransferase with trypsin, 60 minutes digestion.

Lane 5: Amidotransferase with trypsin, 90 minutes digestion.

Lane 6: Amidotransferase with trypsin, 3 hours digestion.


A
1
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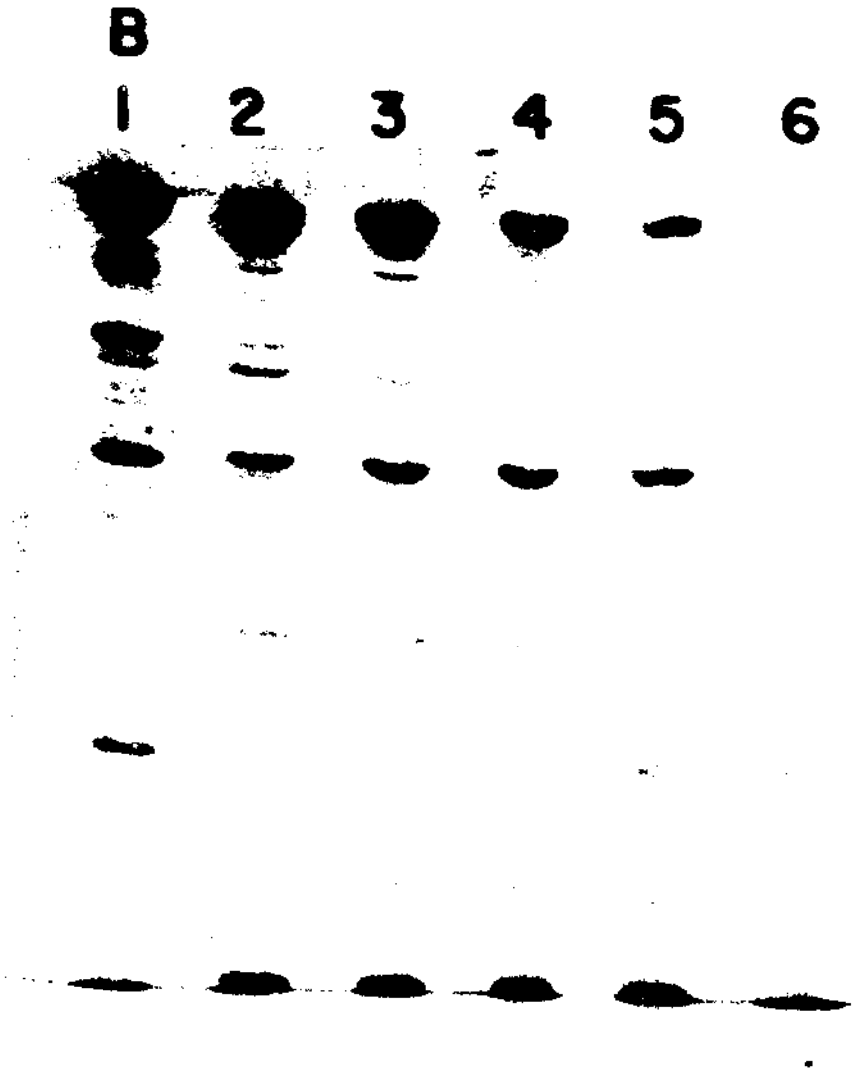
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Figure 5. Fluorogram (A) and Coomassie Blue stained urea-containing polyacrylamide gel (B) analysis of [^{14}C]DON-labeled amidotransferase after digestion with S. aureus protease or trypsin.

[^{14}C]DON-labeled amidotransferase was incubated with S. aureus protease or trypsin for 3.5 hours. Duplicate samples were removed and analyzed on urea-containing polyacrylamide gels.

Lane 1: Digestion of amidotransferase with S. aureus protease.

Lane 2: Digestion of amidotransferase with trypsin.

Lane 3: Molecular weight standards (BSA, 68,000; alcohol dehydrogenase, 37,800; chymotrypsinogen, 25,000; lysozyme, 14,400).

Lane 4: Molecular weight standards (ovalbumin, 43,000; β-lactoglobulin, 18,400; cytochrome c, 12,400, bovine trypsin inhibitor, 6,200; insulin, 2,300 and 3,400).


A
1
2
B
1
2
3
4

of peptides, the stability of the DON label to amidotransferase was determined under these conditions. Digested and undigested [^{14}C]DON-labeled amidotransferase were boiled in sample buffer for urea-containing polyacrylamide gel electrophoresis, except no urea was added to the sample buffer. Formic acid was added until each sample had 8.8% formic acid in it. The samples were allowed to sit at room temperature for 72 hours, then neutralized with concentrated sodium hydroxide. Urea was added to each sample, and they were analyzed on urea-containing polyacrylamide gel electrophoresis by fluorography and Coomassie Blue staining. The DON label on amidotransferase was stable for 72 hours in the presence of 8.8% formic acid (Figure 6).

5. Separation of Fragments from S. aureus Protease Digestion on Sephadex Columns. A small Sephadex G-25 column (50 x 1.0 cm) was equilibrated with 8.8% formic acid. A 0.5 mg sample of [^{14}C]DON-labeled amidotransferase which had been digested with protease for 3.5 hours was resolved on the column (17.7 ml/h, 0.78 ml fractions) into two radioactive peaks. A ninhydrin assay of the fractions also showed two peaks, but the ninhydrin peaks were shifted by a couple fractions from the radioactive peaks (Figure 7). The shift is probably due to an error in counting the fractions.

The Sephadex G-25 column previously described (56.5 x 2.5 cm) was re-equilibrated with 8.8% formic acid. Two one mg samples of S. aureus protease digestion of amidotransferase were analyzed on the column (83.7 ml/h, 2 ml per fraction), eluting

Figure 6. Fluorogram of [^{14}C]DON-labeled amidotransferase to show stability of the DON-amidotransferase bond in the presence of 8.8% formic acid.

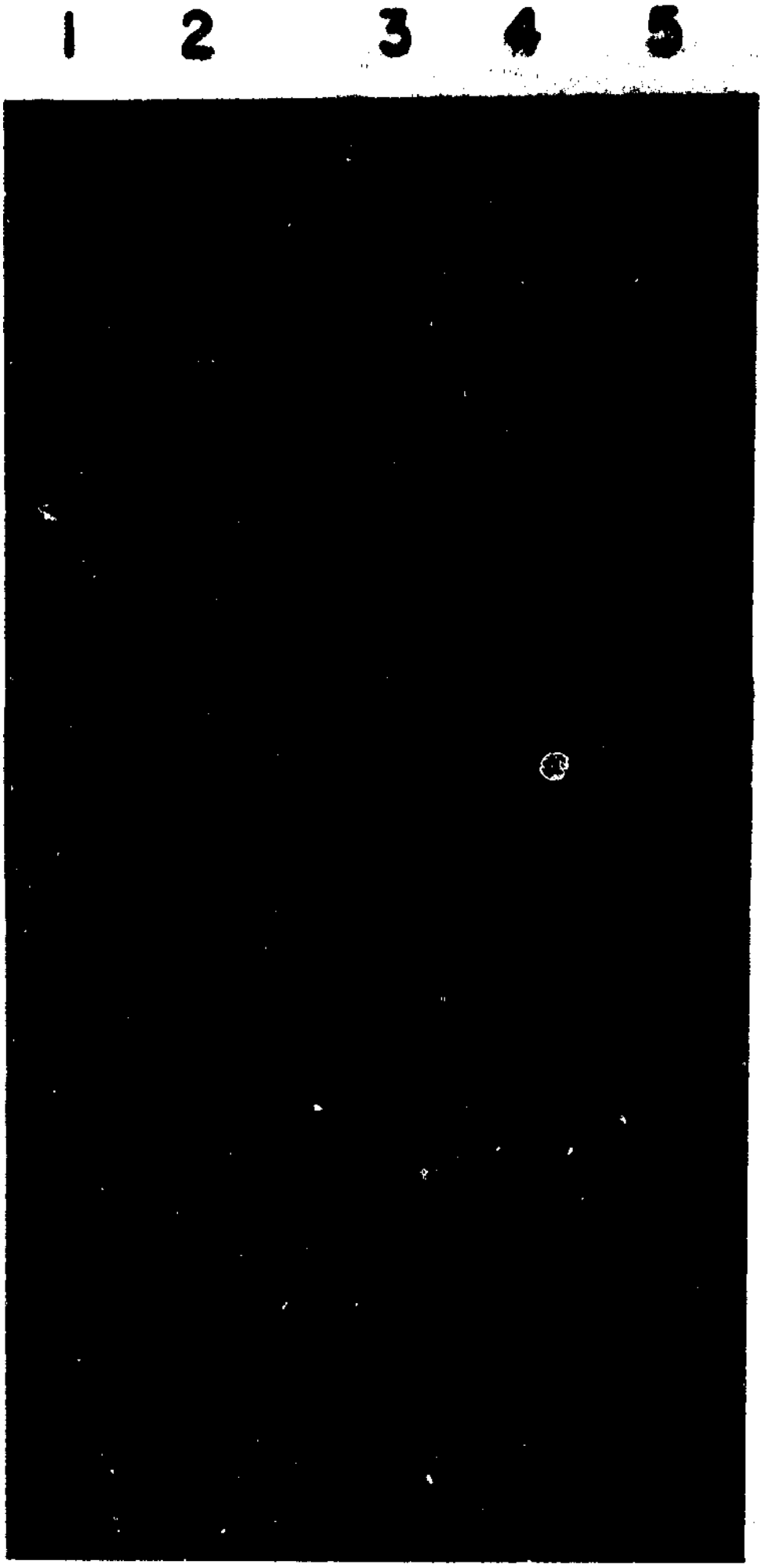
Duplicate samples of [^{14}C]DON-labeled amidotransferase containing 20 µg were treated as described in the text and analyzed on a urea-containing polyacrylamide gel.

Lane 1: Undigested amidotransferase with no formic acid.

Lane 2: Undigested amidotransferase with formic acid.

Lane 3: Amidotransferase from S. aureus protease digestion with no formic acid.

Lane 4 and Lane 5: Duplicate samples of amidotranferase from S. aureus digestion with formic acid.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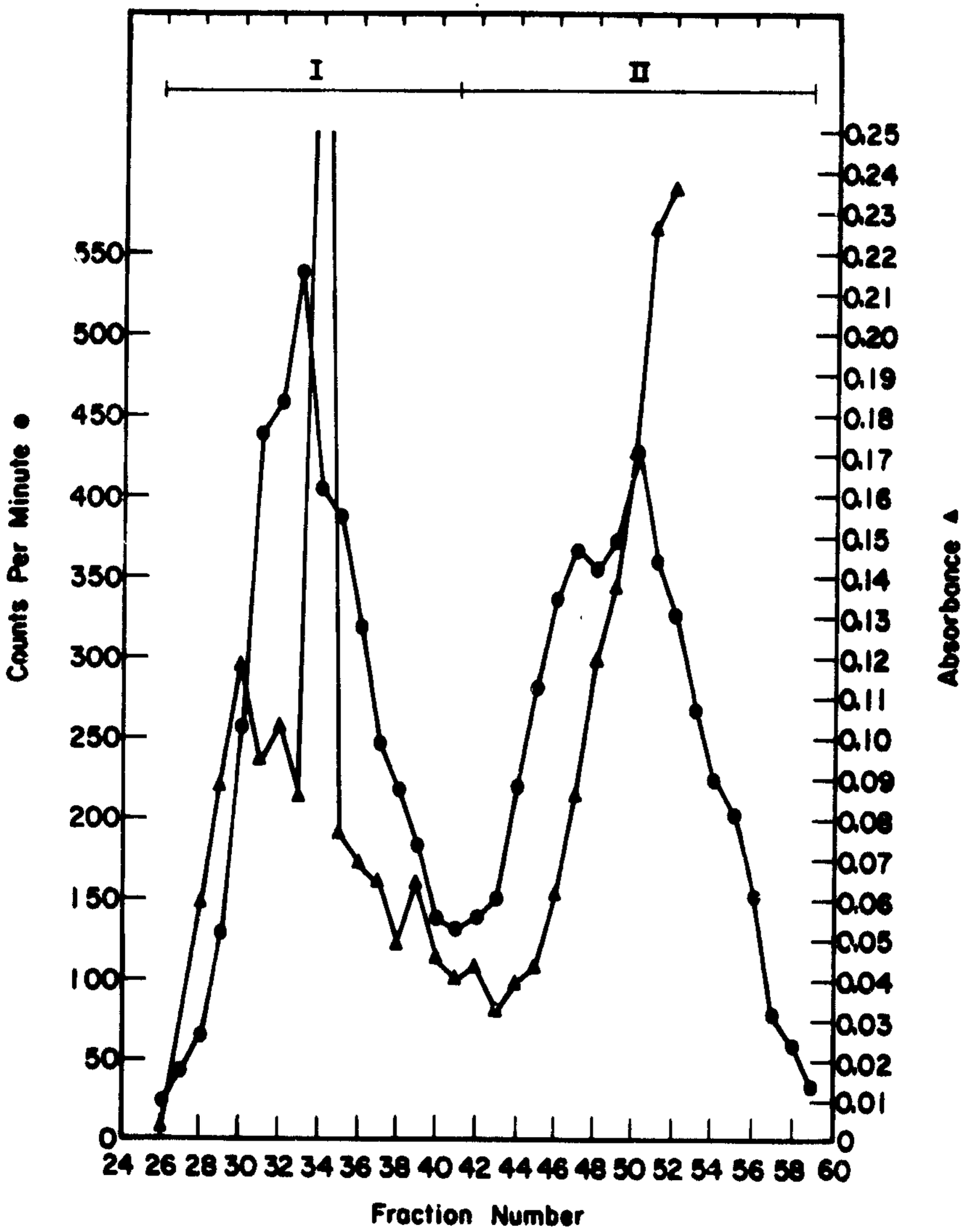
5

Figure 7. Separation of the products of S. aureus protease digestion of [^{14}C]DON-labeled amidotransferase by chromatography on Sephadex G-25. Radioactivity (●) and amino acid content by ninhydrin analysis after alkaline hydrolysis (▲) was determined in the fractions shown.

The bars indicate the pooled fractions.

The void volume was determined using Blue Dextran and corresponds to fraction 22. The salt volume was determined using [^{3}H] leucine and corresponds to fraction 51.


I
II
Counts Per Minute ●
Absorbance ▲
550
500
450
400
350
300
250
200
150
100
50
0
0.25
0.24
0.23
0.22
0.21
0.20
0.19
0.18
0.17
0.16
0.15
0.14
0.13
0.12
0.11
0.10
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
24 26 28 30 32 34 36 38 40 42 44 46 48 50 52 54 56 58 60
Fraction Number

with 8.8% formic acid. Both samples separated into two large radioactive peaks with a shoulder and some trailing on the second peak (Figure 8). Three fractions were pooled from each run on the column. Fraction I contained the first peak. Fraction II contained the second peak, and fraction III contained the shoulder and trailing edge of the second peak. Each pooled fraction was lyophilized to dryness and redissolved in a minimal amount of 50 mM Tris (pH 7.9 at 22°C), usually 50 to 100 µl. Duplicate samples of 20 µl each were removed from each fraction, heated at 100°C with sample buffer, and electrophoresed on duplicate urea-containing polyacrylamide gels. One gel was fluorographed, and the other was stained (Figure 9). The fluorogram showed substantial radioactivity in Fractions I and II. The gel shows a strong staining band of protein with a molecular weight of 3500. The stained band matches the radioactive band in the fluorogram for Fraction I. Fractions II and III were not detected in the destained gel. During the process of destaining, however, a number of protein bands appeared then disappeared. Small peptides may have diffused out of the gel during the destaining process. This is especially likely in the case of the bands that migrated past the dye front. Because the fluorogram shows the radioactivity of Fraction II migrates to the same position as Fraction I, it is unlikely that the protein in the Coomassie Blue stained gel diffused out in Fraction II and not Fraction I.

Figure 8. Separation of the products of S. aureus protease digestion of [^{14}C]DON-labeled amidotransferase by chromatography on Sephadex G-25. Radioactivity (●) was determined in the fractions as shown.

Bars indicate pooled fractions.

The void volume was determined using Blue Dextran. It corresponds to fraction 73. The salt volume was determined using [^{3}H] leucine and corresponds to fraction 123.

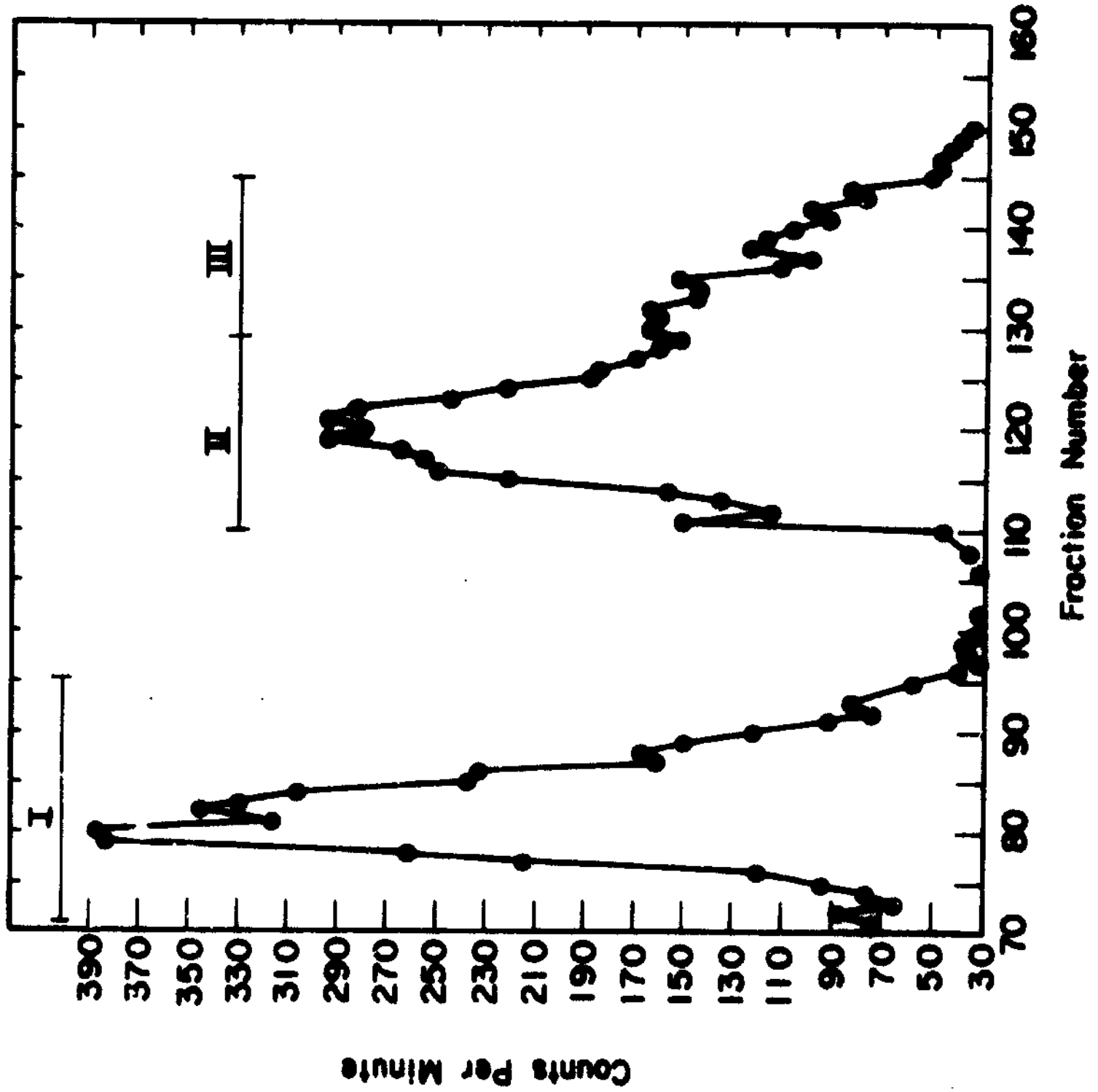

Counts Per Minute
390
370
350
330
310
290
270
250
230
210
190
170
150
130
110
90
70
50
30
I
II
III
70
80
90
100
110
120
130
140
150
160
Fraction Number

Figure 9. Analysis of pooled Sephadex fractions of digested [^{14}C]DON-labeled amidotransferase on urea-containing polyacrylamide gels.

Duplicate samples of 20 µl each were analyzed by fluorography (A) or Coomassie Blue staining (B) after gel electrophoresis as described in the text.

Lane 1: Undigested amidotransferase.

Lane 2: Fraction I from first run on large Sephadex G-25 column.

Lane 3: Fraction II from first run on Sephadex G-25 column.

Lane 4: Fraction III from first run on Sephadex G-25 column.

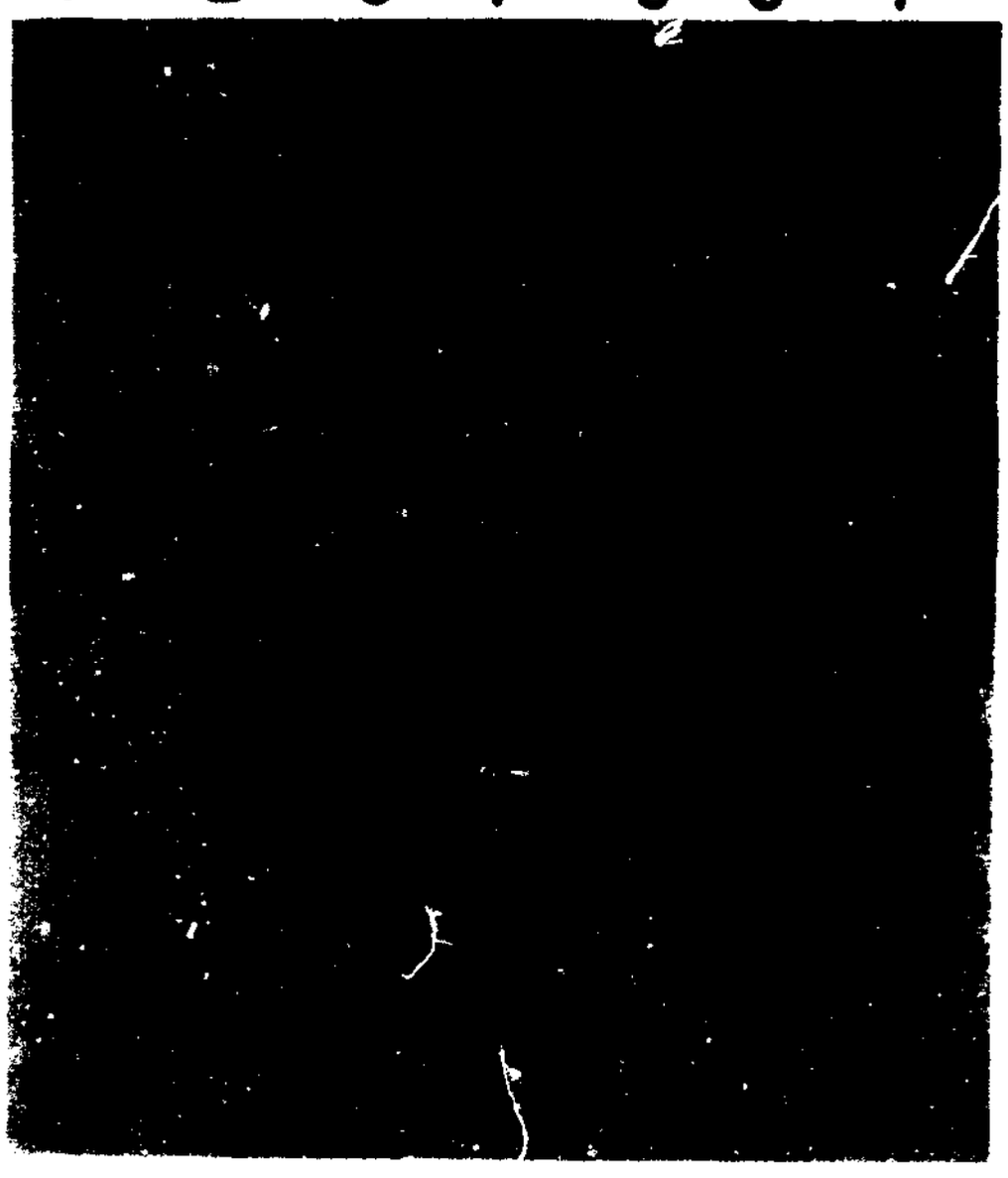
Lane 5: Fraction I from second run (Fig. 8) on Sephadex G-25 column.

Lane 6: Fraction II from second run on Sephadex G-25 column.

Lane 7: Fraction III from second run on Sephadex G-25 column.

Lane 8: Molecular weight standards (ovalbumin, 43,000; β-lactoglobulin, 18,400; cytochrome c, 12,400; bovine trypsin inhibitor 6,200; insulin, unresolved mixture of A and B chains, 2,800).

A



B

IV. Discussion:

The B. subtilis amidotransferase gene has been recently cloned in Escherichia coli by C. Makaroff and H. Zalkin (Makaroff et al., submitted). The plasmid-encoded amidotransferase was purified by a modification of the procedure of Wong et al. (1981). The purified amidotransferase obtained from E. coli cells containing the cloned B. subtilis gene was subjected to 24 cycles of automated Edman degradation. The amino-terminal sequence was identical to that of the enzyme purified from B. subtilis (Vollmer et al., submitted; see also Fig. 2). The phenylthiohydantoin of the ninth residue was isolated in an unusually low yield, but it was tentatively identified as histidine DNA sequencing of the amidotransferase gene also identified the codon of the ninth residue as histidine.

I have shown that optimal digestion of amidotransferase with S. aureus V8 protease occurs after 3.5 hours under the conditions used. Amidotransferase that had not been reduced and alkylated was a better substrate because of its solubility in buffer and its rapid degradation by S. aureus protease. The DON label was also shown to be stable to 8.8% formic acid, which allowed the digested peptide to be separated on Sephadex columns. However, it seems that 8.8% formic acid did not prevent aggregation of the peptides.

Analysis of the products of digestion of amidotransferase with S. aureus on urea-containing polyacrylamide gels revealed peptides containing radioactivity with molecular weights ranging

from 2600 to 3500. The molecular weight of the decapeptide with the DON label is approximately 1400. This suggests that the S. aureus protease cleaved the enzyme at a glutamate residue other than residue 10. An examination of the amino acid sequence obtained by DNA sequencing reveals glutamate residues as the twenty-ninth and thirty-ninth residues from the amino terminus. If the S. aureus protease cleaves at the twenty-ninth residue from the amino terminus, the peptide released would have a molecular weight of approximately 3600. This is comparable to the radioactive fragment detected on urea-containing polyacrylamide gels. Because the amino terminus is involved in the catalytic activity of amidotransferase, the glutamate at the tenth or eleventh positions may be inaccessible to S. aureus protease. The twenty-ninth amino acid may be closer to the surface of the amidotransferase, providing an easily accessible site for proteolysis. In light of this, the best procedure might be to isolate this larger peptide and subject it to a second digestion with S. aureus protease.

More work needs to be done to isolate an amino-terminal peptide. Probably use of a higher formic acid concentration would prevent aggregation. Once a large amount of peptides of fairly homogeneous sizes has been collected, a high performance liquid chromatography column can be used to separate the peptide mixture. When a homogeneous preparation of the decapeptide has been obtained, it can be subjected to amino acid analysis. Careful analysis could identify several possible modified histidine residues.

If the ninth residue is covalently modified, it would be very interesting to determine the phase of growth in which the histidine is modified in B. subtilis and whether the modification affects the stability of amidotransferase to inactivation or degradation.

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