tions of ether. The ether extracts and the top layer were then combined and dried over potassium carbonate. Ether was removed on a steam-bath, and 126 g. of 3-diethylamino-1-propyne, distilling at 119–120°, was obtained (76% yield, $n^{25}D$ 1.4288). This procedure has obvious advantages over the literature method which requires diethylamine acetate.[7]

3-(4-Morpholino)-1-propyne.—3-(4-Morpholino)-1-propyne, b.p. 89–92° (38 mm.) $n^{25}D$ 1.4723, was obtained in 66% yield by utilizing the procedure described above. This propyne was identified by infrared and nuclear magnetic resonance spectra and analytical data. *Anal.* Calcd. for $C_7H_{11}NO$: C, 67.2; H, 8.9; N, 11.2; mol. wt., 125. Found: C, 67.7; H, 8.9; N, 10.9; mol. wt., 123, 128. The infrared spectrum indicated ≡CH (3.0 μ), saturated CH (3.4, 3.5 and 3.7 μ), and —C≡C— (4.75 μ). The nuclear magnetic resonance spectrum indicated acetylenic hydrogen and two types of methylene hydrogen.

3-Diethylamino-3-methyl-1-propyne, b.p. 126–128°, $n^{25}D$ 1.4273, was prepared in 33% yield from acetylene and diethylamine by a published procedure.[8]

(7) Ref. 1a, p. 110; also, Reppe, *Ann.*, **596**, 1 (1955).

(8) C. Gardner, V. Kerrigan, J. D. Rose and B. C. L. Weedon, *J. Chem. Soc.*, 780 (1949).

Reaction of 3-Diethylamino-1-propyne with Dicobalt Octacarbonyl.—3-Diethylamino-1-propyne (0.19 g.) in acetone (25 ml.) was added to dicobalt octacarbonyl (0.57 g.) in the standard Orsat apparatus for measuring gases. There was collected 63.3 ml. of carbon monoxide, making the necessary corrections for acetone vapor (87% of theory).

Commercial-grade acetylene was purified according to a previously described procedure.[9] The infrared spectra were determined on a Perkin–Elmer 21 double-beam spectrometer. The ultraviolet spectra were determined on a Cary model 11 spectrophotometer. The proton magnetic resonance spectra were obtained using a Varian high-resolution n.m.r. spectrometer and electromagnet at frequencies of 40 Mc. and fields of 10,000 gauss, respectively. The spectra were calibrated in terms of displacements in cycles per second (c.p.s.) from the proton resonance of water. Positive values are on the low field side of water, and negative values are on the high field side. Calibration was accomplished by superimposing an audiofrequency on the sweep field to produce side band peaks to the water resonance. Yields of the dihydrofuramides were calculated on the basis of the amount of dialkylamino-propyne consumed.

(9) J. C. Sauer, THIS JOURNAL, **79**, 5314 (1957).

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[CONTRIBUTION FROM THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH]

Characterization Studies with Subtilin

BY ALFRED STRACHER[1] AND LYMAN C. CRAIG

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Further purification studies of subtilin A by countercurrent distribution (c.c.d.) are reported. Molecular weight determination by the method of partial substitution has conclusively shown that subtilin A approximates 3300 in molecular weight. This and quantitative amino acid analyses are consistent with an amino acid formula of Asp,Pro,Gly_2,Ala,Val,Ileu,Leu_4,-Phe,Lys_3,Lan,β-MeLan$_4$,Glu_3,Try,Sar_2. Sarcosine, not heretofore reported, has been shown to be an N-terminal group. Subtilin A is a pentacyclic peptide with a side chain.

A number of different polypeptide antibiotics[2] in the molecular weight range up to 1500 have now been well characterized chemically and reasonably certain cyclic structures have been proposed for them. Although larger ones are known they have not thus far been studied as carefully. Subtilin[3] produced by a particular strain of *Bacillus subtilis* is the best characterized and perhaps the most readily available member of the larger size group.

A preliminary survey of the excellent chemical work already done with subtilin[4–9] indicated that it probably was sufficiently well characterized for further structural study. Nonetheless, because of the effort required in such an undertaking it seemed wise to repeat part of the work using different methods. This paper will report studies of this nature and some new observations bearing on the structure of the peptide.

Experimental

Materials.—Three samples of subtilin all received from the Western Regional Laboratory have been studied thus far. One was a 5-g. sample (Lot 317) received from Dr. Harold Olcott in 1950. Two more samples were received from Dr. Alderton in 1957. One of these, 152F, was similar to 317 but freshly prepared whereas 317 had been stored for about 10 years. The other was relatively pure subtilin A obtained by silica gel partition chromatography of 152F. Dr. Alderton had found it to give a single band by countercurrent distribution at 180 transfers (system = 20% acetic acid, 5, *n*-butanol, 4) with close agreement to a calculated curve. It behaved the same way in our hands.

Fractionation Studies.—Fractionation was accomplished by countercurrent distribution in a 1000 tube (2 ml. lower phase) automatic train of the type previously described[10] in the Alderton system. The charge was 1 g. of sample 152F initially scattered in the first twenty tubes of the train. Each transfer required 2.5 minutes including 5 strokes for equilibration and 1.5 minutes for separation of the phases. 5 strokes seemed to be sufficient for equilibration. The temperature of the train was 25°.

After 1000 transfers the upper patterns of Fig. 1 were obtained by optical density measurement at 288 mμ of the upper and lower phases in a Beckman quartz spectrophotometer. Several small bands as indicated and a larger one on the right, C_3, were removed from the train. After these tubes were filled with fresh phases the distribution was continued to 2540 transfers by the recycling procedure.

Analysis by optical density now gave the lower patterns of Fig. 1. A plot of the partition ratio across the main band is given above the distribution curve.

Cuts labeled A_3 and B_3 were taken as indicated on the chart. They were concentrated in a rotary evaporator until all the butanol phase was removed and lyophilized from the aqueous solution. The preparation from cut A_3 was the material used for the characterization studies reported in this paper. It corresponds to subtilin A.

Anal. The sample (from the run of Fig. 1) lost 18.2% on drying at 100° *in vacuo*. Found: C, 52.97; H, 6.99; N, 16.06; S, 4.76; N-CH_3, 1.92.

(1) Fellow of the National Foundation for Infantile Paralysis.

(2) L. C. Craig, Proc. 3rd Int. Cong. Biochem., Brussels, 1955.

(3) K. P. Dimick, G. Alderton, J. C. Lewis, H. D. Lightbody and H. L. Fevold, *Arch. Biochem.*, **15**, 1 (1947).

(4) J. C. Lewis and N. S. Snell, THIS JOURNAL, **73**, 4812 (1951).

(5) N. G. Brink, J. Mayfield and K. Folkers, *ibid.*, **73**, 330 (1951).

(6) G. Alderton and H. L. Fevold, *ibid.*, **73**, 463 (1951).

(7) J. F. Carson, *ibid.*, **74**, 1480 (1952).

(8) G. Alderton, *ibid.*, **75**, 2391 (1953).

(9) H. L. Fevold, K. P. Dimick and A. A. Klose, *Arch. Biochem.*, **18**, 27 (1948).

(10) L. C. Craig, W. Hausmann, E. H. Ahrens, Jr., and E. F. Harfenist, *Anal. Chem.*, **23**, 1236 (1951).

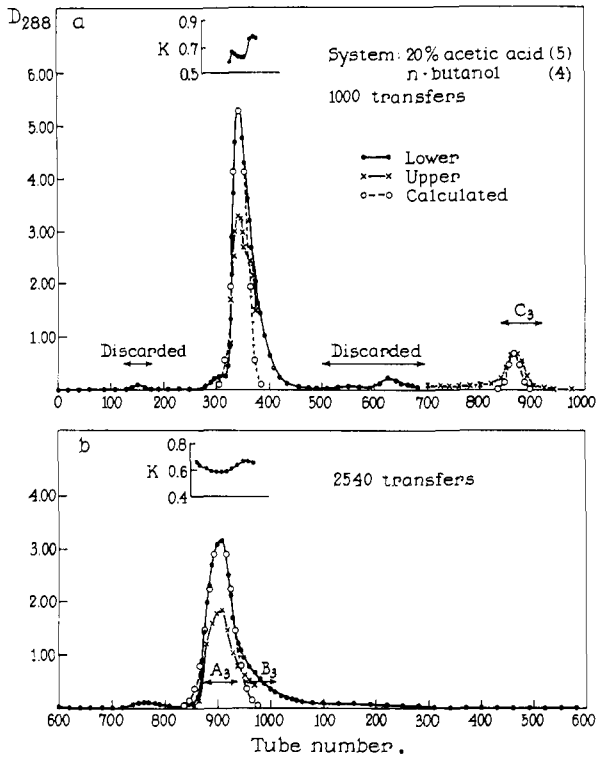

Fig. 1.—C.c.d. patterns of subtilin, Lot 152F.

The sample was suspected to contain acetic acid since it is a basic peptide lyophilized from a dilute acetic acid solution. The acetate content was determined in a modified acetyl apparatus by distillation from a solution acidified with *p*-toluenesulfonic acid. When titrated (iodometrically) the distillate from 16.47 mg. of dried sample required 1.185 ml. of 0.01 *N* sodium thiosulfate. Calcd. for 2 equivalents of acetate per molecular weight of 3361.9; 1.00.

Distribution studies of the same sample 152F in a second entirely different system gave a result very much like that given in Fig. 1. The system here was made from *n*-butanol, pyridine, 0.1% acetic acid (18,6,33). This gave a *p*H approximating 6.7 and a *K* of the main component of about 4. Because of the higher *K* the volume of the upper phase was held at a little more than half that of the lower. It required nearly six minutes for the phases to separate after equilibration at the beginning but less time later on.

The original sample, 317, first obtained gave a much more complex pattern when distributed in this system. Figure 2 shows the result obtained in a 440 tube (10 ml. lower phase) train at 1100 transfers (recycling) and at 2730 transfers. After 1100 transfers the three faster moving overlapping bands were permitted to emerge from the train at the transfer numbers shown in pattern c. The three overlapping bands on the left of pattern a were kept in the machine and recycled to 2730 transfers as shown in pattern b. A small amount of solute occurring between the two lower patterns b and c was discarded and is not shown. The *K* values in pattern a were calculated from the band position. They were found to be higher with a lower solute concentration as in Fig. 3b, probably because of a non-linear partition isotherm.

Distribution of the older sample (317) also gave a much more complex pattern than the newer one (152F) when distributed in the system used in Fig. 1. Figure 3, pattern a, shows the result at 383 transfers. This separation was made in a 200 tube, 10 ml. lower phase, train. When the cut S was recovered and distributed in the pyridine system to 500 transfers, pattern b was obtained.

Amino Acid Analysis.—Samples were hydrolyzed in 6 *N* HCl in sealed tubes at 108° for two different periods of time, 22 and 70 hr. The amino acid composition of the hydrolysate was determined by the most recent ion-exchange chromatographic method of Moore, Spackman and Stein[11] with an XE-69 resin. The procedure recommended, in which the entire fractionation is done at 50°, however, was not found suitable for resolving the glutamic acid, lanthionine, β-methyllanthionine and proline bands. A slight modification in which the column is operated at 30° until these amino acids have emerged and then at 50° for the remainder of the run gave good resolution.

Color yields for all the residues were known[11] except lanthionine and β-methyllanthionine. Samples of these were not available but the color yield of the closely related cystathionine[12] was assumed to be the same. Tryptophan was destroyed by the acid hydrolysis. Therefore, a separate barium hydroxide hydrolysis in the presence of starch was used for it.[13]

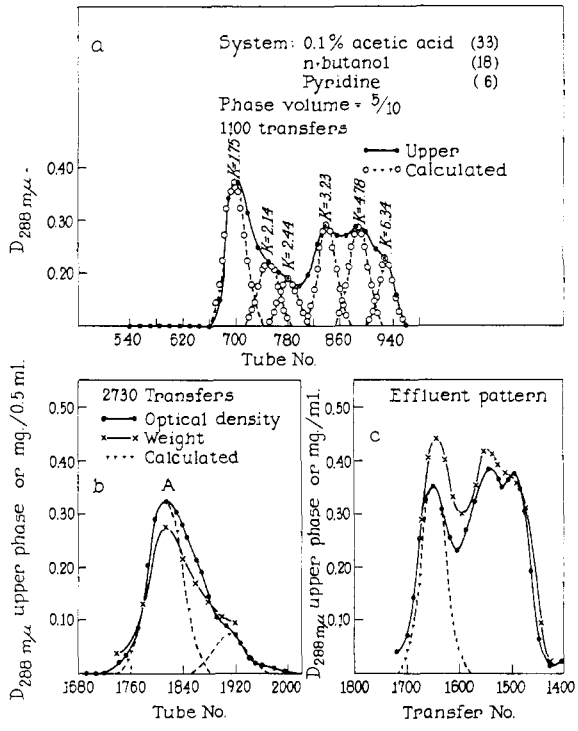

Fig. 2.—C.c.d. patterns of subtilin, Lot 317.

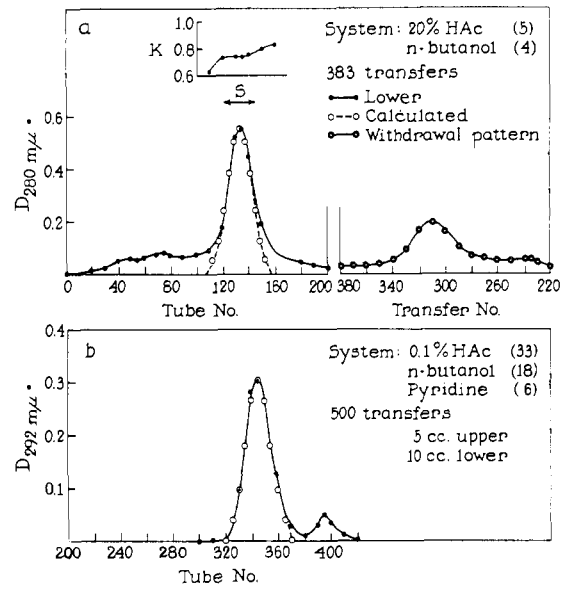

Fig. 3.—C.c.d. patterns of subtilin, Lot 317.

(11) S. Moore, D. H. Spackman and W. H. Stein, *Anal. Chem.*, **30**, 1185 (1958).

(12) H. H. Tallan, S. Moore and W. H. Stein, *J. Biol. Chem.*, **230**, 707 (1958).

(13) A. Drèze, *Biochem. J.*, **62**, 3p. (1956).

A sample weighing 5.27 mg. was treated with 150 mg. of $Ba(OH)_2$, 25 mg. of starch and 0.125 ml. of H_2O in a sealed, evacuated tube. After heating at 120° for 20 hours, water was added, the silica gel was centrifuged off and an aliquot placed on a starch column. The tryptophan was determined in the effluent by the ninhydrin procedure.

The results in terms of amino acid residues per molecular weight of 3361.9 for the peptide are given in Table I.

TABLE I

Amino acid	22 hr.	70 hr.	Compn. given by Lewis and Snell[14]
Aspartic acid	0.600	1.02	1
Proline	1.19	1.19	1
Glycine	1.66	1.96	2
Alanine	1.00	0.97	1
Valine	0.81	0.96	1
Isoleucine	1.20	1.00	1
Leucine	3.88	3.85	4
Phenylalanine	0.91	0.94	1
Lysine	2.80	2.80	3
Sarcosine	0.80	2.20	..
Glutamic acid	2.60	2.90	3
Lanthionine	0.75	0.80	1
β-Methyllanthionine	3.95	4.10	4
Tryptophan	...	0.76	1
Ammonia	...	8.20	..

Isolation of Sarcosine from Hydrolysate.—A 350-mg. sample of subtilin A was hydrolyzed for 60 hr. in 6 *N* HCl in a sealed tube. The solution was evaporated to dryness and treated with 2 g. of sodium nitrite and 5 ml. of concentrated hydrochloric acid. The brown solution was permitted to stand overnight. The N-nitroso derivatives were extracted with ethyl ether and the ether extract evaporated to dryness. The residue was hydrolyzed by refluxing in 6 *N* HCl for 1 hr. and evaporated to dryness.

The residue was distributed to 90 transfers in the system 2% HCl, phenol (16–15). Two bands were obtained (K = 0.73 and K = 2.7). The one with K = 0.73 corresponded to proline and behaved as proline in paper chromatography in the system 2-butanol–formic acid–water. Two weak spots were also observed.

The solute in the band of K = 2.7 would agree with the determined K of sarcosine in this system. It was recovered and spotted on paper in the 2-butanol–formic acid–water system. The single spot travelled with an R_f corresponding to sarcosine (0.55).

Part of the solute with the K of 2.7 was converted to the DNP derivative with excess 1-fluoro-2,4-dinitrobenzene (FDNB) in alkaline solution. The reaction products were distributed in the system chloroform—glacial acetic acid—0.1 *N* HCl (2–2–1). After 85 transfers two solutes with a low K were visible and a third satisfactory band with a K of 0.67. This corresponded to the K of DNP sarcosine. It was recovered and found to have the same R_f as DNP sarcosine in paper chromatography with *t*-amyl alcohol, 3% ammonia as the solvent.

Optical Activity.—The optical activity of subtilin A in a 1.01% aqueous solution at 20° at different wave lengths was determined in a Rudolph photoelectric polarimeter. The result is given in the curve of Fig. 4 $[\alpha]^{20}D$ −32° (*c* 1.01, in H_2O). A dispersion curve determined in 6 molar urea is also given.

Dialysis Experiments.—Membrane diffusion experiments were made by the method recently published by Craig, King and Stracher[14] using Visking cellophane. With $^{18}/_{32}$ cellophane and 0.01 *N* acetic acid as the solvent it gave a straight line[14] indicating homogeneity with respect to size.

Absorption Spectra.—Absorption spectra in an aqueous solution were determined by a Cary model 11 recording spectrophotometer with a 1 cm. cell. A curve is given in Fig. 5. Comparative curves of tryptophan and lanthionine are also given. Studies of the effect of *p*H on the absorption spectrum were made.

(14) L. C. Craig, T. P. King and A. Stracher, THIS JOURNAL, **79**, 3729 (1957).

Reaction with FDNB.—A mixture of the partial substitution products of 1-fluoro-2,4-dinitrobenzene with subtilin A was prepared by permitting 100 mg. of the peptide to react in a solution of 14 mg. of FDNB in 10 ml. of water, 10 ml. of ethanol and 0.2 ml. of triethylamine. After 5 minutes at 25° the mixture was acidified with acetic acid and quickly evaporated in a rotary evaporator to a small volume. This was extracted three times with isopropyl ether and lyophilized.

The residue was distributed to 50 transfers in the system chloroform–acetic acid–water (2–2–1) with a fifty tube hand operated train. Analysis was made by optical density at 350 mμ. The result is given in Fig. 6, pattern a. Residue weights per ml. were determined for the lower phases of tubes 4 (0.151 mg.) and 6 (0.189 mg.) and for the upper phases in tube 36 (0.692 mg.). The molecular weights given on the pattern were calculated from the optical density–weight relationship assuming a molecular extinction coefficient of 14,500 in this solvent.[15] The value of 14,500 was determined on ε-DNP lysine in this system.

Such a weight–optical density relationship for the material in the right-hand band was too high to be the result of a single solute and it was suspected that unchanged peptide was present. Accordingly, a cut, as indicated, of this band was recovered in a rotary evaporator. The residue was distributed to 40 transfers in the system used for the original purification of subtilin, 20% acetic acid–*n*-butanol (5–4) using 5 ml. upper, 10 of the lower. Analysis of the colorless lower phases from tube 0 to 20 was made by optical density at 280 mμ. Analysis of the upper phases was made by optical density at 350 mμ. The single yellow band had its maximum at tube 31. The analysis is shown in Fig. 6, pattern b. Residue weights per ml. of upper were taken for tubes 28 (0.326 mg.), 30 (0.430 mg.) and 31 (0.376 mg.).

Completely substituted DNP subtilin A was prepared by permitting excess FDNB to react in a sodium bicarbonate solution for 3 hr. in the dark at room temperature. The product had too low a partition coefficient to be distributed in the system used in Fig. 6. Instead a system made from chloroform–acetic acid–benzene–water (1.3–2.6–1.0–1.0) was used. A rather satisfactory distribution at 45 transfers was obtained with a K of 1.65 and a constant weight–optical density relationship at several points across the central part of the band (0.135 mg./ml. gave an optical density of 1.88 at 350 mμ). Mol. wt. calcd. for 1 DNP = 1040 or 4160 for a tetrasubstituted derivative.

Hydrolysis of the Tetra-DNP Derivative.—A sample of 50 mg. was hydrolyzed in 2 ml. of glacial acetic acid plus 2 ml. of concentrated hydrochloric acid in a sealed evacuated tube for 24 hr. at 108°. The excess acid was evaporated under reduced pressure and the residue was taken up in a little water. Extraction of this solution gave no yellow color in the ether extract. The aqueous layer was evaporated again and distributed to 50 transfers in a system made from 2-butanol–0.1 *N* HCl. A single yellow band with the K (1.38) of ε-DNP lysine was found. The experimental band showed perfect agreement with the calculated and corresponded to a recovery of 2.9 moles of lysine.

When the hydrolysis was repeated under identical conditions except the hydrolysis time was 1 hour, a different result was obtained. In this case the ether extract was highly colored. It was evaporated and spotted on a paper chromatogram with the system *t*-amyl alcohol saturated with 3% ammonia. The R_f value was 0.3 and agreed with that of a sample of DNP sarcosine. The yellow spot was cut out and eluted with a system made from chloroform, glacial acetic acid, 0.1 *N* hydrochloric acid. The K in this system was 0.78 and showed good agreement with that of DNP sarcosine.

Esterification Experiments.—A sample of 75 mg. of subtilin A was suspended in dry acetone and treated with excess diazomethane in acetone. After 1 hr. at room temperature the solvent was evaporated and a thirty transfer distribution made on the residue in the system used to purify subtilin A. Two bands of K 0.50 and 3.3, respectively, were obtained. The more polar one was the larger and was obviously not homogeneous. The less polar one was recovered and the residue analyzed for $-OCH_3$ by the Zeisel method. Calcd. for 2 OCH_3 assuming the mol. wt. to be 3361.9; 1.88. Found: OCH_3, 1.92.

(15) A. R. Battersby and L. C. Craig, *ibid.*, **74**, 4023 (1952).

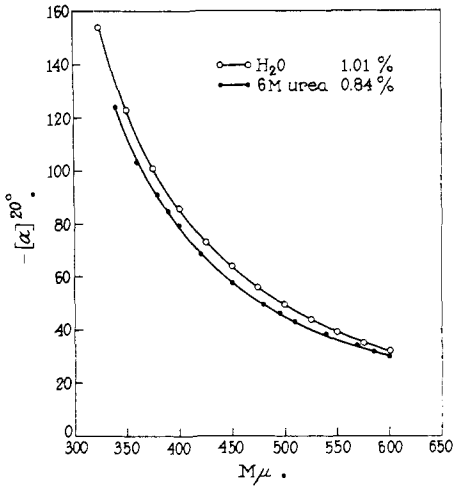

Fig. 4.—Rotary dispersion curves of subtilin A.

Discussion

Inspection of Figs. 1 and 2 would seem to indicate that the strain of *B. subtilis* which produces subtilin probably does not synthesize a single antibiotic but instead a family of peptides. This would be expected from the previous experience[2] with polypeptide antibiotic producing organisms. If not, the heterogeneity must arise from secondary transformation. Irrespective of the cause, it now seems entirely possible to separate and isolate a single chemical individual from the mixture. This can be accomplished by countercurrent distribution or by the silica gel chromatography method used by Alderton.[16] The peptide composition of the crude antibiotic may vary with the strain of organisms or method of culture since an older sample proved to be much more complex than a recent one.

A search was made for suitable systems other than the *n*-butanol–acetic acid system developed by Alderton.[16] One providing a different *p*H and a practical partition ratio range was developed by combining *n*-butanol, pyridine and dilute acetic acid. This system was found to have a different selectivity than the first since comparison of the patterns of Figs. 2 and 3 will show that the pyridine system indicated more components to be present in the older sample than the other. Also a cut from the main band of the acetic acid run in Fig. 3, pattern a, was clearly resolved into two components in the pyridine system, pattern b. In spite of the apparent greater selectivity of the pyridine–acetate system, it should be used with a certain reservation until complete retention of antibiotic potency has been demonstrated.

In view of the fact that a family of peptides must be considered, the individual members have been called subtilin A, B, C, etc. A fraction called "subtilin A" was isolated by zone electrophoresis by Sacks and Pence.[17] It apparently corresponds to the most abundant member thus far and that which accounts for the majority of Lot 152F. It also corresponds to the most polar band of Fig. 2, the one on the extreme left of patterns a and b.

The thesis that material from the distribution of Fig. 1 is a single component is not only supported by silica gel chromatography and countercurrent distribution in two different systems but by conversion to DNP derivatives as well and further distribution of these derivatives. In paper electrophoresis with a pyridine–acetate buffer at 5.6, a single spot travelling as a positively charged solute was obtained. A small amount of tailing was observed. The buffers suggested by Sacks and Pence[17] were less satisfactory.

The method of partial substitution[15] has given clear and interpretable results. From the data given in Fig. 6 and the experimental section, it is certain that a mono-, di-, tri- and tetra-DNP can be prepared. For each of these bands weight–optical density relationships have been obtained which are consistent with four amino groups capable of reacting with FDNB and indicate a molecular weight in the range of 3300. This is in accord with the earlier work of Lewis and Snell.[4]

(16) Unpublished results.

(17) L. E. Sacks and J. W. Pence, *Anal. Chem.*, **29**, 1802 (1957).

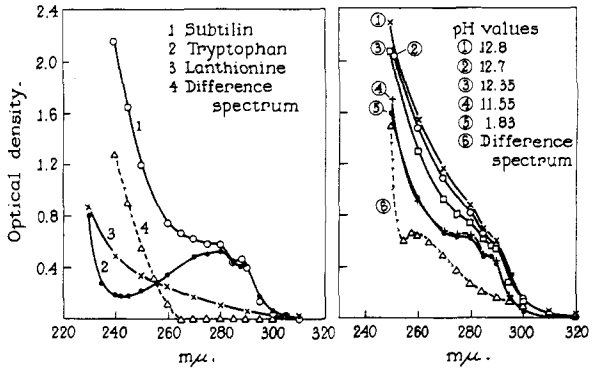

Fig. 5.—Absorption spectrum curves.

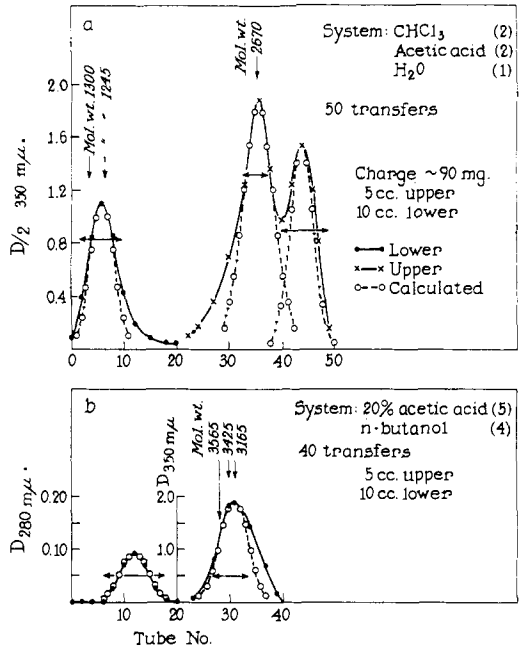

Fig. 6.—C.c.d. patterns of DNP derivatives of subtilin.

The amino acid composition of the peptide has been determined by complete acid hydrolysis and ion-exchange chromatography according to the method of Moore, Spackman and Stein.[11] Bands corresponding to all the amino acids reported by Lewis and Snell[4] and the β-methyl lanthionine reported by Alderton[8] were found except tryptophan which was destroyed by the acid. Tryptophan was isolated from an alkaline hydrolysate. However, an additional band from the column occurring in the position of sarcosine was found. This had not been reported by previous workers. The likelihood that it was sarcosine was supported by an N-methyl determination made on the intact peptide.

An attempt was made to isolate the supposed sarcosine from the hydrolysate of subtilin. In this experiment the amino acids with a secondary nitrogen were removed from the others as the nitroso derivatives. These were then hydrolyzed and fractionated by countercurrent distribution in a phenol system. A band, not entirely pure, with the K of sarcosine was found which behaved like sarcosine in paper chromatography. Because of the small amount of material available, it was converted to the DNP derivative and again fractionated by countercurrent distribution and paper chromatography. The partition ratio and R_f value were identical with the values for DNP sarcosine.

The composition of subtilin A hydrolysate was studied at two different hydrolysis times, 22 and 70 hr. This has shown that there probably are several linkages, presumably peptidic, which are very resistant to hydrolysis. After 70 hr. as compared to 22 hr., the results given in Table I show that the aspartic acid, glycine, sarcosine and glutamic acid

values increase. The sarcosine increase is particularly puzzling and may suggest a type of linkage other than a normal peptide bond.

The amino acid analyses given in Table I are consistent with the thesis of a practical degree of purity. In order to reach an over-all empirical formula from these figures, it is first necessary to determine the amide composition and the numbers of free carboxyl groups. These data will permit the number of rings to be deduced.

From the FDNB studies reported in the experimental part it seems clear there are four free NH_2 groups. Three of these emerge after complete substitution with the FDNB reagent as ϵ-DNP lysine. No free lysine emerges, in agreement with the previous report of Carson.[7] The fourth has been found to emerge from hydrolysis of the tetra-DNP derivative as DNP-sarcosine. A shorter hydrolysis time (1 hr.) was required to reveal clearly the DNP-sarcosine since on longer hydrolysis it was completely converted to dinitrophenol. An experiment with authentic DNP-sarcosine alone showed that in 1 hr. even these hydrolysis conditions converted 80% of it to dinitrophenol.

Esterification experiments with diazomethane gave an ester which was analyzed for methoxyl content. On the basis of a molecular weight of 3241.9 for subtilin A the amount of methoxyl found would agree closely with two free carboxyl groups. The esterification experiments of Carson, Jansen and Lewis[18] by methanolic hydrochloric acid at 0° would correspond to a little above two carboxyls, but the identity of their starting material as compared to ours may not be certain. Two carboxyls would leave an excess of 2 amino groups in subtilin A.

This balance is supported by the observation that in paper electrophoresis at a *p*H of 5.6 the free peptide migrates strongly as a positively charged solute. The mono-DNP derivative migrates only half as fast but the di-DNP derivative does not migrate because the positive and negative charges now are the same in number.

From Table I it can be seen that there are five residues each with two carboxyl groups and two amino groups, four each with two carboxyl groups and one amino group and three each with one carboxyl group and two amino groups. Assuming all the residues indicated in Table I to be linked in a linear peptide with no amide groups, there would be a total of ten free carboxyl groups and nine free amino groups including the C carboxyl and the N amino group.

All of the five amino groups of the sulfur amino acids must be bound, since in the FDNB experiments reported here they failed to be substituted. This was supported by amino acid analysis of the hydrolysate of the totally substituted DNP derivative. Here no free lysine was found. There appeared to be no appreciable decrease in the amounts of any of the other amino acids liberated with the exception of sarcosine. Therefore the five extra amino groups of the five sulfur amino acids must be covered by carboxyl groups and thus account for five of the ten to be accounted for.

On the basis of two free carboxyl groups there remains three to be covered. These could be present as amide groups. However, we have consistently found slightly more than five in a standard amide determination with the Conway method and a hydrolysis time of four hours at 108°. This agrees with the value reported by Lewis and Snell.[4] There is reason to suspect that ammonia is liberated from amino groups by decomposition under hydrolytic conditions since the ammonia values in the amino acid analysis were always far too high. Amide values considerably too high have also been found in our work with bacitracin, another sulfur containing peptide. We are therefore inclined to accept the likelihood of three amide groups in spite of the amide determination. An amide content higher than this is also difficult to rationalize with the over-all nitrogen analysis now to be discussed.

If the empirical formulas for all the 26 residues indicated by Table I are combined by the loss of 25 molecules of water for the hypothetical linear peptide, then three ammonia molecules added with the loss of three more molecules of water and finally five more molecules of water deducted as indicated above to supply the required coverage of carboxyl and amino groups, the empirical formula $C_{144}H_{226}N_{38}O_{37}S_5$ is derived.

However, the sample of subtilin A used for analysis was prepared by lyophilization of an acetic acid solution of the peptide. Since there are two excess strong basic groups, it would be expected that at least two acetic acid molecules would be bound. Direct determination of free acetate has confirmed this supposition. Therefore the sample analyzed is the diacetate $C_{148}H_{234}N_{38}O_{41}S_5$. The calculated composition for this formula is C, 52.9; H, 7.00; N, 15.8; S, 4.76; $N\text{-}CH_3$,[2] 1.74. The found values reported in the experimental part are C, 52.97; H, 6.99; N, 16.06; S, 4.76; $N\text{-}CH_3$, 1.92.

In summary subtilin A is a cyclic basic polypeptide of molecular weight 3241.9 which contains 5 ring structures. It contains a single N-terminal amino acid which is sarcosine. The conclusion that none of the sulfur containing amino acids have a free amino group is not in accord with the finding of Carson,[7] but the discrepancy here may arise from a difference in starting material.

In trying to carry the study of the structure further, desulfurization of subtilin A with Raney nickel catalyst seemed a promising approach. This would theoretically open all five rings if a thio ether linkage were involved in each ring as might be supposed. However, conditions which permitted the removal of sulfur from the β-methyllanthionine[3] did not seem to be very effective with the intact subtilin A. The recovered product was found to contain about 80% of its original sulfur.

However, countercurrent distribution of this material gave two main bands. The smaller of the bands on total hydrolysis and amino acid analysis was devoid of either of the sulfur containing amino acids present in subtilin. The other contained all the amino acids present in subtilin A.

This work has not progressed sufficiently at present to give a final report and will be made the subject of a future contribution. However, the experimental data do strongly suggest certain structural features which can be mentioned.

No aminobutyric acid was found in the hydrolysate of either band. This would indicate that β-methyllanthionine was not appreciably split. Alanine was found in the hydrolysate of both bands and in a higher amount in the larger band than in subtilin A. Lanthionine was found in an amount lower than in the starting material. The larger peptide was nearly devoid of sarcosine.

The hydrolysate of the smaller peptide contained leucine, alanine and glutamic acid in about equimolar amounts. Sarcosine in double the molar amount was found and aspartic acid in one half molar quantity.

While the amount of the aspartic acid is disturbing, the results seem to suggest that subtilin A is a cyclic peptide with a side chain-Sar·(Glu,Leu,Sar,Asp)-Lan with the lanthionine serving as the bridge-head for attachment of the side chain. Sarcosine is the N terminal amino acid but the order of the others is not known.

From over-all considerations, if the thioether of the single lanthionine is the bridgehead, then one of the five rings in subtilin A must be completely peptidic while the others could be peptide chains joined by the thioether linkages of the four β-methyllanthionines present.

A definite suggestion that linkages other than peptidic and thioether linkages hold the molecule together in what appears to be a cyclic rather rigid configuration can be derived from absorption spectrum measurements. From the data given in Fig. 5 it can be seen that the sum of the absorption offered by the single tryptophan, the β-methyllanthionines, the lanthionine[19] and phenylalanine is not sufficient to account for the total absorption of subtilin A. In Fig. 5 the difference curve, No. 2, was determined by using the calculated concentrations of these substances as the blank.

Moreover, the spectrum is markedly influenced by *p*H in a strongly alkaline range as shown in Fig. 5. The lowest curve of the right-hand pattern is the absorption in water subtracted from that in strong alkali. An enolic or weakly phenolic grouping with a pK in the range of 12 could be indicated, but poor stability in this alkaline range makes this interpretation uncertain. The probable unsaturation does not seem to be conjugated to the indole nucleus of the tryptophan. An attempt to remove this unsaturation by hydrogenation with Adams catalyst failed. However, the failure may have been a result of the sulfur of the sulfur-containing residues.

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(18) J. F. Carson, E. F. Jansen and J. C. Lewis, THIS JOURNAL, **71**, 2318 (1949).

(19) In a private communication, Dr. Lewis reported less absorption for lanthionine in the range of 250–270 mμ than we found.