Synthesis of Oligomeric L-Lysine Peptides by the Solid-Phase Method*

O. Grahl-Nielsen and G. L. Tritsch

ABSTRACT: The oligomeric peptides di- through deca-L-lysine were synthesized by the solid-phase method by the use of a newly developed apparatus. The peptide chain was elongated stepwise by starting with L-lysine covalently bonded to an insoluble copolymer of 98% styrene and 2% divinylbenzene. The α-amino group of lysine was protected with the *t*-butyloxycarbonyl group, and the ε-amino group was protected with the carbobenzoxy group. The *t*-butyloxycarbonyl group was selectively cleaved by 1 N HCl in acetic acid at room temperature for 30 min. After each coupling step, some peptide-resin was removed from the reaction vessel, dried, weighed, and deblocked with HBr gas in trifluoroacetic acid. It was found that 5 min of this treatment was sufficient to remove more than 90% of the peptide from the resin. The desired peptides were contaminated with lower homologs but chromatography on a carboxymethylcellulose column eluted with an exponential gradient of sodium chloride resulted in excellent separations. After lyophilizing and desalting on Sephadex G-15, the peptides were obtained in pure form.

The synthesis of peptides by the solid-phase method as developed by Merrifield (1964) has proved useful in preparing large biologically active peptides (Marglin and Merrifield, 1966). This method requires less time and gives higher yields than the various liquid-phase methods. In the work to be described, this method was applied to a homologous series of lysine peptides. Lysine was chosen because of the well-developed method of separating lysine peptides of different chain lengths on columns of CM-cellulose with an exponential gradient of NaCl (Stewart and Stahmann, 1962). Lysine peptides were also of interest as models in other investigations, which will be the subject of forthcoming papers.

Experimental Section

α-*t*-Butyloxycarbonyl-ε-carbobenzoxy-L-lysine was obtained from Schwarz BioResearch, Inc., as a syrup in methylene chloride (50% w/w). The chloromethylated copolystyrene–divinylbenzene was obtained from Cyclo Chemical Corp. CM-cellulose with a capacity of 0.53 mequiv/g dry weight was obtained from Bio-Rad Laboratories. All reagents and solvents were of highest purity available comercially. The optical density measurements were performed with a Beckman DU spectrophotometer in quartz cells of 1-cm light path.

*α-t-Butyloxycarbonyl-ε-carbobenzoxy-*L*-lysine-polymer.* The chloromethylated polymer (44.09 g), which contained 1.20 mequiv of chlorine/g, was added to a solution of 30.6 g (40 mmoles) of α-*t*-butyloxycarbonyl-ε-carbobenzoxy-L-lysine syrup and 5.6 ml (40 mmoles) of triethylamine in 80 ml of absolute ethanol, and the mixture was stirred at 78° for 48 hr in the apparatus shown in Figure 1. The esterified resin was removed by filtration, washed thoroughly in the filter with ethanol, water, and methanol, and then dried under vacuum over P_2O_5.

The amount of lysine bound to the resin was determined in the following manner. The dried lysine-resin (199 mg) was transferred to the deblocking funnel (Figure 2) and suspended in 5 ml of trifluoroacetic acid. Dry HBr gas was permitted to bubble through the suspension for 1 hr. The trifluoroacetic acid was then sucked into the lower tube, and the resin was washed three times with 5 ml of trifluoroacetic acid. The combined filtrates were evaporated under reduced pressure, and the oily residue was dissolved in water and transferred quantitatively to a 50-ml volumetric flask. Aliquots (1 ml) of this sample and five standard solutions of lysine were treated with ninhydrin by the procedure of Moore and Stein (1954). By interpolation from a calibration curve the esterified polymer was found to contain 0.234 mmole of lysine/g.

This is a far more accurate method than the ones used by Marshall and Merrifield (1965) and Khosla *et al.* (1967a) and is as fast as the latter. Furthermore, it can be employed for all amino acids.

*α-t-Butyloxycarbonyloligo(ε-carbobenzoxy-*L*-lysine)-polymer.* The α-*t*-butyloxycarbonyl-ε-carbobenzoxy-L-lysine-polymer (32.59 g) containing 7.63 mmoles of blocked L-lysine was transferred to the reaction vessel (Figure 3) and the steps shown in Table I were used to introduce each new L-lysine residue. The α-*t*-butyloxycarbonyl-ε-carbobenzoxy-L-lysine was added in

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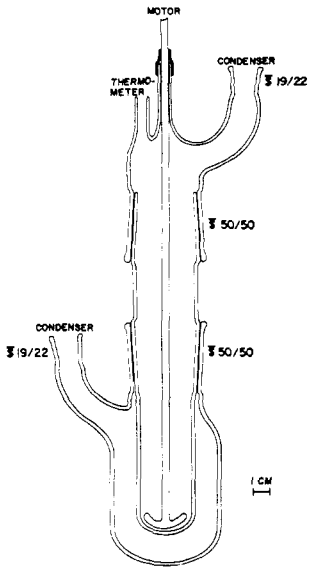

FIGURE 1: Apparatus for esterifying the first amino acid residue to the resin.

2.5-fold excess and the dicyclohexylcarbodiimide in equimolar amounts of this. After each cycle approximately 3 g of the resin was transferred to the deblocking funnel (Figure 2) in which it was dried under vacuum, and then suspended in 20 ml of trifluoroacetic acid. HBr gas was bubbled through the suspension for 1 hr. the trifluoroacetic acid was then sucked down in the lower tube, and the resin was washed threet imes with 10 ml of trifluoroacetic acid. The combined filtrates

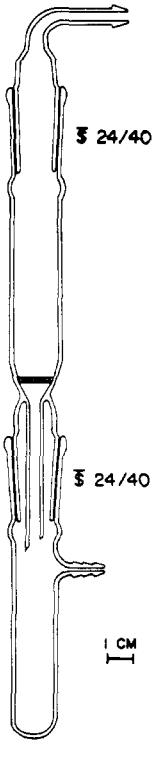

FIGURE 2: Resin-deblocking apparatus.

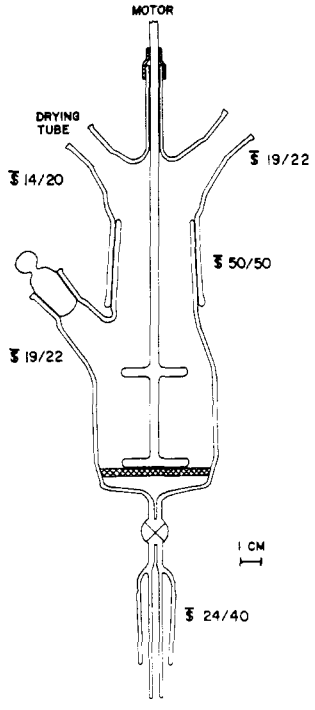

FIGURE 3: Reaction vessel for the solid-phase method.

were evaporated, and the residue was dissolved in 5 ml of methanol and precipitated by adding an excess volume of ether.

Purification of the Peptides. The dried crude product (200 mg) was dissolved in 0.5 ml of H_2O and transferred to the top of a 2 × 40 cm column of CM-cellulose. The column was eluted with an exponential gradient

TABLE I: One Cycle of the Solid-Phase Peptide Synthesis.

Step	Reagent	Purpose	Time (min)
1	Acetic acid, three times	Washing	10
2	1 N HCl in acetic acid	Deblocking	300
3	Acetic acid, three times	Washing	10
4	Ethyl alcohol, three times	Washing	10
5	DMF,[a] three times	Swelling	10
6	10% triethylamine in DMF	Deprotonating	10
7	DMF, three times	Washing	10
8	CH_2Cl_2, three times	Washing	10
9	New Lys-residue in CH_2Cl_2	Mixing	10
10	DCC[a] in CH_2Cl_2	Reaction	150
11	CH_2Cl_2, three times	Washing	10
12	Ethyl alcohol, three times	Washing	10

[a] DMF, dimethylformamide; DCC, dicyclohexylcarbodiimide.

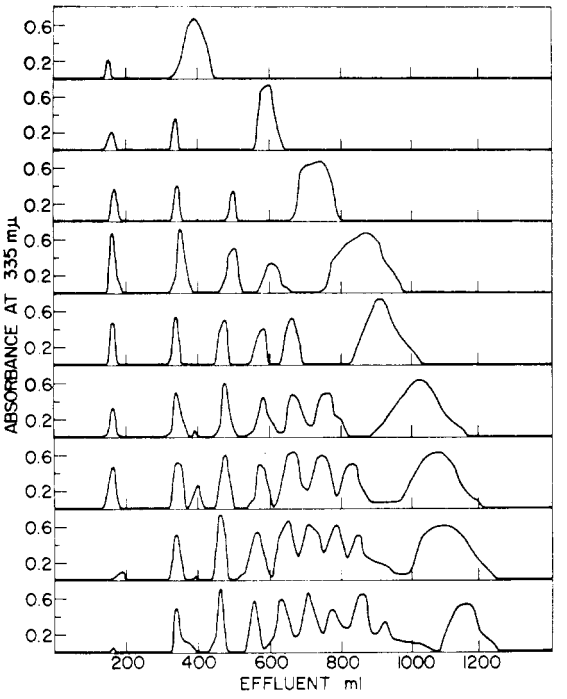

FIGURE 4: CM-cellulose chromatography of the crude products of oligo-L-lysines. The eluent patterns are from the top: Di-, tri-, tetra-, penta-, hexa-, hepta-, octa-, nona-, and deca-L-lysine. The main fraction is in each case represented by the broadest peak.

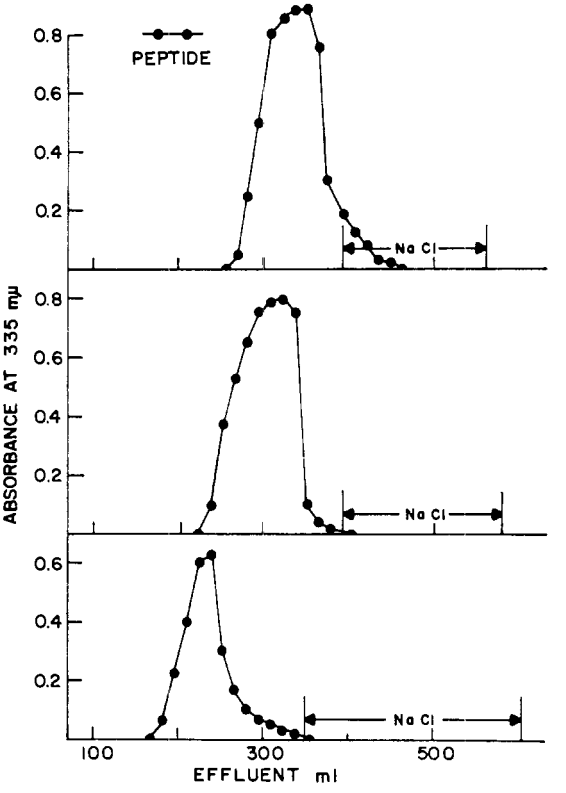

FIGURE 5: Desalting on Sephadex G-15. The peptides are: di-L-lysine (on top), hexa-L-lysine (in the middle), and deca-L-lysine (bottom).

of NaCl by replenishing a 1000-ml constant-volume mixing flask, initially filled with H_2O, with 0.82 M NaCl. Fractions of 50 drops (3.4 ml) were collected. The eluent pressure was kept constant at 100 cm with a Mariotte bottle, but the flow rate increased from approximately 30 sec/drop to approximately 10 sec/drop after 400 tubes (1360 ml).

Between each run the column was washed with H_2O, and essentially the same conditions were used for the purification of each product.

The peptides were detected the following way. Aliquots of 0.2 ml from every fourth tube were treated with 0.5 ml of 0.3 M Na_2HPO_4, 1.0 ml of 4% $NaHCO_3$, and 1.0 ml of 0.1% trinitrobenzenesulfonic acid in H_2O. The mixture was heated at 40° for 1.5 hr, then diluted with 2.0 ml of 0.25% HCl. The intensity of the color developed was determined at 335 mμ (DeLuca *et al.*, 1966).

The elution patterns are shown in Figure 4. The volatile solvent was removed from the desired fractions by lyophilization. The dried samples contained large amounts of NaCl; the larger the peptide, the larger the amount of NaCl.

Desalting. The peptide–salt mixture was dissolved in up to 15 ml of 0.2 N acetic acid and placed onto a 2.5 × 100 cm column of Sephadex G-15 equilibrated with 0.2 M acetic acid. The column was then developed with the same solvent, and 50 drops (3.5 ml) were collected in each tube at the rate of approximately 5 drops/sec. The eluent pressure was kept constant at 70 cm with a Mariotte bottle.

The peptide was detected with trinitrobenzenesulfonic acid as described above, and NaCl by evaluation of the intensity of the flame test (Figure 5). The volatile solvent was removed from the desired fractions by lyophilization. The acetates of the peptides thus obtained were very hygroscopic. Calculated from the 0.234 mmole of monolysine/g of the α-*t*-butyloxycarbonyl-ε-carbobenzoxy-L-lysine-polymer, the yields of the pure peptides were 45% of dilysine decreasing to 8% of decalysine.

Characterization of the Products. Samples of 5 mg of the desalted peptides were dissolved in 0.5 ml of H_2O and chromatographed on the same column of CM-cellulose as used earlier. This time the peptides were detected by ninhydrin. The ninhydrin reagent (1.0 ml) was transferred directly into each tube. The tubes were heated for 15 min in a boiling water bath and cooled, and the color intensity was immediately determined at 570 mμ. The results are shown in Figure 6.

Apparatus

Merrifield *et al.* (1966) have devised an automatic apparatus for solid-phase synthesis, and two manual setups have been described in a paper by Khosla *et al.* (1967b). In this work a modification of Merrifield's reaction vessel was used, which permitted better mixing of the two-phase reaction mixture (Figure 3).

It consists of a glass cylinder with a side arm of total

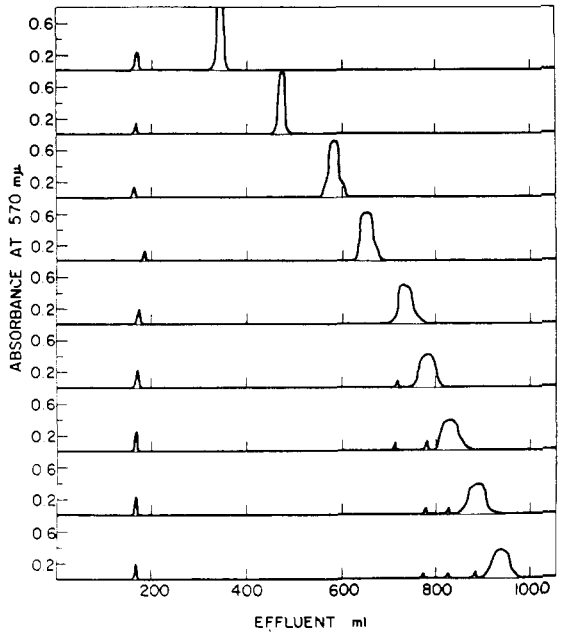

FIGURE 6: Analytical CM-cellulose chromatography of the purified oligo-L-lysines. The eluent patterns are shown in the same order as in Figure 4.

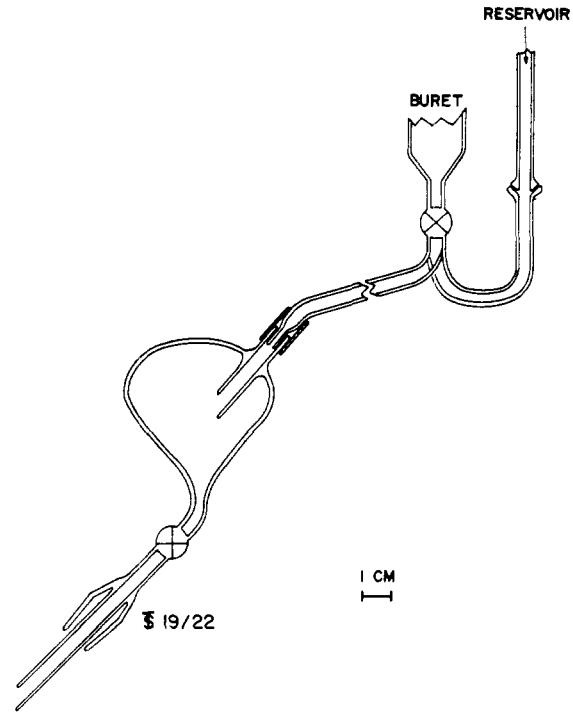

FIGURE 7: Inlet funnel for solvents and reagents. For simplicity only one of the connections to the funnel is shown.

volume of 250 ml, with a coarse fritted disk at the lower end. Sealed under the fritted disk is an inner glass joint with a connection to the vacuum reservoir. The upper end of the vessel is fitted with an inner glass joint which holds the lid. The lid has three openings: one for a mechanical stirrer, one for a $CaCl_2$ tube, and one for reagent and solvent inlet. The opening around the stirrer is made air tight with a greased rubber collar. The reagents and solvents are dispensed from self-filling burets connected with glass tubing both to the reservoirs and to a sealed funnel (Figure 7) which is held by a glass joint to the third opening in the lid. The whole apparatus is permanently mounted under a shelf where the reservoir bottles are placed. The reaction vessel and its lid are held by two different clamps so that the vessel can be removed without moving the lid and the inlet funnel with all its connecting glass tubing.

The operation of the apparatus is then very easy. For each step the operator only has to do the following. Turn the stopcock on the appropriate buret to have it filled to the desired volume, turn the stopcock 180° to let the solvent down in the reaction vessel, start the stirring motor, stop the stirring motor after the appropriate time, open the stopcock to the vacuum reservoir, and the sequence is ready for repetition. Each new amino acid and the dicyclohexylcarbodiimide can either be introduced through the side arm in the reaction vessel, or be taken from reservoir bottles through the same funnel as the solvents.

The first L-lysine residue was linked to the resin in the apparatus shown in Figure 1. The inner tube contained the reactants which were thoroughly mixed with a mechanical stirrer. The temperature was kept at 78° by refluxing ethanol in the outer tube.

A separate apparatus (Figure 2) was used for resin deblocking. The peptide-resin was suspended in trifluoroacetic acid above the fritted disk in the upper tube. The inlet in the lower tube was connected to a cylinder containing dry HBr gas, and the gas was bubbled into the suspension through the fritted disk. After the reaction was completed the lower tube was connected to a suction pump, and the trifluoroacetic acid solution of the peptide was collected in this tube.

Results and Discussion

From Figure 4 which shows the elution patterns of the crude products after removal from the resin, it can be seen that the desired peptide in each case was contaminated with the lower homologs.

The graphs cannot be used for quantitative evaluations because the trinitrobenzenesulfonates of the peptides higher than trilysine were slightly soluble and partially precipitated.

In an attempt to detect which of the steps in the procedure had given rise to the by-products, the *t*-butyloxycarbonyl removing step was first investigated. α-*t*-Butyloxycarbonyl-ε-carbobenzoxy-L-lysine (20 mg) was dissolved in 1 ml of the deblocking reagent, 1 N HCl in anhydrous acetic acid. At various time intervals at room temperature 3-μl aliquots were removed and spotted directly on a thin-layer plate. The starting line on the plate was impregnated with NaOH to stop the reaction.

The sensitivity of the system was tested by diluting two aliquots of the reaction mixture after 30 min 10- and 100-fold, respectively, and chromatographing

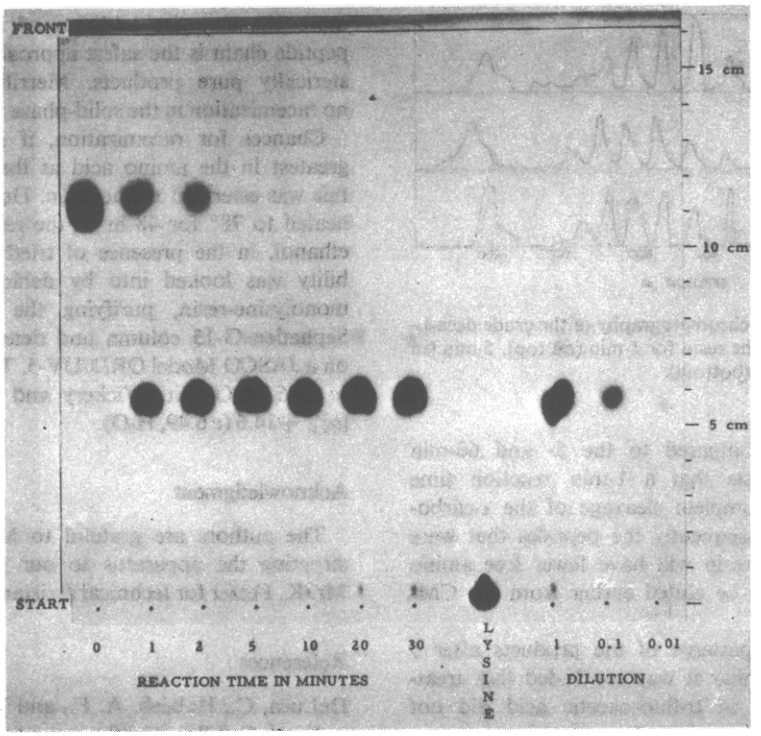

FIGURE 8: Thin-layer chromatogram showing the removal of the *t*-butyloxycarbonyl group from α-*t*-butyloxycarbonyl-ε-carbobenzoxy-L-lysine. Layer: 0.25-mm silical gel G (Merck). Solvent 1-butanol–acetic acid–chloroform–water (6:2:1:1, v/v). Running time, 100 min.

3 μl of these solutions parallel to 3 μl of the original solution.

After chromatography the plate was developed by heating it to approximately 130° for 15 min and spraying while still hot with a solution of 0.15 g of ninhydrin in 1.5 ml of acetic acid and 50 ml of 1-butanol (Ehrhardt and Cramer, 1962). This treatment is sufficiently rigorous to remove the *t*-butyloxycarbonyl group so the α-*t*-butyloxycarbonyl-ε-carbobenzoxy-L-lysine can react with the ninhydrin. The developed plate is shown in Figure 8. From the figure is seen that 10-min reaction time was enough to cleave all of the *t*-butyloxycarbonyl groups. However, a small amount of free L-lysine corresponding to the marker of L-lysine with R_F 0.04 appeared also in the reaction mixture. This means that some carbobenzoxy groups were cleaved by the HCl in acetic acid treatment. The amount of free L-lysine after 30-min reaction time was estimated to be less than 1% when the spot was compared with the diluted sample, also taking area of the spots and difference in color into consideration.

On the original plate could also be seen two other by-products with higher R_F values than the main product. These were not identified.

In the test described above the α-*t*-butyloxycarbonyl ε-carbobenzoxy-L-lysine was dissolved in the deblocking reagent. In the solid-phase synthesis, however, the *t*-butyloxycarbonyl group was removed from a lysine residue attached to the completely insoluble polymer. A deblocking time of 30 min should still be enough to cleave all the *t*-butyloxycarbonyl groups and leave a minimal amount of the ε-amino groups uncovered.

A free ε-amino group on a lysine residue will give rise to branched peptides. The branched peptides will, however, always have a higher number of lysine residues than the desired linear ones. Consequently they will be eluted later than the linear peptides on the CM-cellulose column. On the elution patterns in Figure 4 there are no peaks after the main peak. It is therefore concluded that the products do not contain branched peptides.

Another step in the procedure that might be responsible for the by-products is the resin deblocking with HBr gas in trifluoroacetic acid. Khosla *et al.* (1967a) assumed this step to be a weak point in the procedure. To investigate if this treatment could be damaging to the established peptide bonds, two different samples of decalysine-resin were deblocked as described, but only for 1 and 5 min, respectively. Decalysine-resin (1 g) gave after 1-min treatment 191 mg of crude product, and after 5-min treatment 207 mg of crude product.

Under the same conditions 25 mg of each of the crude products after 1 min and 5 min, and also 25 mg of the crude product obtained earlier after 60-min treatment, were chromatographed separately on the same column of CM-cellulose as described above. The fractions were treated with ninhydrin, and the resulting color intensities were plotted against fraction number (Figure 9). There is no striking difference in the three elution patterns, but it can be seen that the 1-min product contained less decalysine and more of the

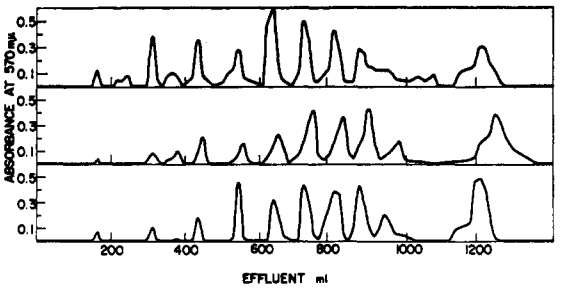

FIGURE 9: CM-cellulose chromatography of the crude deca-L-lysine deblocked from the resin for 1 min (on top), 5 min (in the middle), and 60 min (bottom).

lower peptides as compared to the 5- and 60-min samples. This suggests that a 1-min reaction time is not enough for complete cleavage of the ϵ-carbobenzoxy groups. Consequently the peptides that were deblocked from the resin will have fewer free amino groups and therefore be eluted earlier from the CM-cellulose column.

Since the elution patterns of the products after 5 and 60 min were similar it was concluded that treatment with HBr gas in trifluoroacetic acid did not break peptide bonds between the lysine residues.

The amount of crude product released from the resin after 5-min deblocking was 207 mg/g of decalysine-resin treated, whereas the 60-min treatment gave 220 mg of product from each gram of decalysine-resin. To check how much peptide was left on the resin after the short treatment it was treated for another 55 min. This gave only 20 mg of product/g of the original decalysine-resin.

From these deblocking experiments it is concluded: a deblocking time of 1 min is too short; 5-min treatment releases more than 90% of the peptide from the resin; extending the time to 60 min does not harm the product.

It can be seen in Figure 5 that in desalting even the smallest peptides, good separation from NaCl was achieved. On the other hand, the system Sephadex G-15 eluted with 0.2 M acetic acid would obviously not be effective in separating the peptides from each other.

The danger of racemization is very small when a urethan group is used for α-amino protection. Furthermore the stepwise addition of amino acids to the peptide chain is the safest approach to the synthesis of sterically pure products. Merrifield (1963) reported no racemization in the solid-phase method.

Chances for racemization, if any, were no doubt greatest in the amino acid at the carboxyl end when this was esterified to the resin. Here the reactants were heated to 78° for 48 hr in the relatively polar solvent ethanol, in the presence of triethylamine. This possibility was looked into by deblocking some of the monolysine-resin, purifying the resulting lysine on Sephadex G-15 column and determining the rotation on a JASCO Model ORD/UV-5. This gave $[\alpha]_D^{21}$ +13.8° (*c* 1.86, H_2O), lit. (Vickery and Leavenworth, 1928) $[\alpha]_D^{20}$ +14.6 (*c* 6.49, H_2O).

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References

DeLuca, C., Habeeb, A. F., and Tritsch, G. L. (1966), *Exptl. Cell Res. 43*, 98.

Ehrhardt, E., and Cramer, F. (1962), *J. Chromatog. 7*, 405.

Khosla, M. C., Smeby, R. R., and Bumpus, F. M. (1967a), *Biochemistry 6*, 754.

Khosla, M. C., Smeby, R. R., and Bumpus, F. M. (1967b), *Science 156*, 253.

Marglin, A., and Merrifield, R. B. (1966), *J. Am. Chem. Soc. 88*, 5051.

Marshall, G. R., and Merrifield, R. B. (1965), *Biochemistry 4*, 2394.

Merrifield, R. B. (1963), *J. Am. Chem. Soc. 85*, 2149.

Merrifield, R. B. (1964), *Biochemistry 3*, 1385.

Merrifield, R. B., Stewart, J. M., and Jernberg, N. (1966), *Anal. Chem. 38*, 1905.

Moore, S., and Stein, W. H. (1954), *J. Biol. Chem. 211*, 907.

Stewart, J. W., and Stahmann, M. A. (1962), *J. Chromatog. 9*, 235.

Vickery, H. B., and Leavenworth, C. S. (1928), *J. Biol. Chem. 76*, 440.