Synthesis of a Polypeptide with Lysozyme Activity

J. J. Sharp,*[1] A. B. Robinson, and M. D. Kamen

Contribution from the Department of Chemistry, University of California, San Diego, La Jolla, California 92037. *Received January 27, 1973*

Abstract: The results of two Merrifield solid-phase syntheses of hen egg-white lysozyme are reported. The first synthesis essentially duplicated those procedures used by Gütte and Merrifield for the synthesis of ribonuclease A. 2-Mercaptoethanol was incorporated into the Boc removal solution (TFA–CH_2Cl_2–2-mercaptoethanol–anisole) for tryptophan protection but this reagent proved unsatisfactory. Further studies led to the choice of 1,2-ethanedithiol for tryptophan protection. Much of the synthetic product exhibited a molecular weight less than that of native lysozyme (as evidenced by Sephadex G-100 chromatography). The *S*-benzyl protecting group of cysteine proved difficult to remove with anhydrous HF, but once conditions were found for its removal, the resultant crude synthetic product showed ~0.05% specific enzymatic activity of the native molecule. Experiments were carried out which indicated poor coupling of amino acids resulted in the low molecular weight peptide. In addition, 93% of the initially esterified material had been removed during the course of the synthesis. Consideration of which chains were being lost led to the decision to use a milder Boc removal solution which removed less peptide from the resin (4 *N* HCl–dioxane–anisole–ethanedithiol). Examination of ϵ-NH_2 deprotection of lysine also led to the choice of this HCl–dioxane system for Boc removal. The synthetic procedures for a second synthesis were altered in accordance with these and other results. The changes resulted in a product in the correct molecular weight range (elution position on Sephadex G-75) as well as a nonreducible (by dithiothreitol or 2-mercaptoethanol) high molecular weight fraction. Only the former fraction had enzymatic activity; for the crude product this was 0.5–1% that of native lysozyme. Following chromatography on chitin, the specific activity was 2–3% that of native lysozyme (or 9–25% that of native lysozyme subjected to the same HF conditions and purification as the synthetic product). Three HF cleavage conditions were compared and the effect of one of these conditions on native, reduced lysozyme was examined. The results of these two lysozyme syntheses are discussed.

The methodology of Merrifield solid phase peptide synthesis has gained wide acceptance and given rise to expectations that facile syntheses of large biologically active polypeptides might be in prospect.[2,3] Of the syntheses attempted, the most successful has been that of ribonuclease A by Gütte and Merrifield.[4]

However, at present there is no universal methodology which will guarantee such success.[5–7] Certain difficulties associated with a synthesis (poor coupling, removal of side chain blocking groups, etc.) assume increasing magnitude as the peptide length increases. The resulting problems of product purity, isolation, and yield also increase with increasing length of the desired product. Thus it may be expected that considerable developmental research will be required before the potential of solid phase peptide synthesis is realized.

We have attempted the total synthesis of hen egg-white lysozyme and have found it necessary to concentrate on definition of some of the problems involved in the synthesis of a large polypeptide. We report herewith details of the synthetic procedures and difficulties encountered in the hope that this information may prove useful for future syntheses of large molecules.

In this paper, we present the results of two attempts to synthesize lysozyme. The methodology employed in the first synthesis essentially duplicated that used by Gütte and Merrifield in their synthesis of ribonuclease A. Our experiences resulted in investigations of some of the solid-phase chemistry, results of which also are presented. These results led to the conclusion that the incorporation of several synthetic alterations would improve the possibility for a successful lysozyme synthesis. The improved strategy of this second attempt to synthesize hen egg-white lysozyme centered on improvements in the coupling procedures. Attempts were made to drive each coupling reaction to completion, and if these proved inadequate, to acetylate the remaining chains, thereby eliminating their participation in further reactions. There was no added consideration given to either the Boc removal or the neutralization steps.

A preliminary report of this work has appeared elsewhere.[8]

Experimental Section

Synthetic Conditions. First Synthesis. Crosslinked polystyrene beads (1%) (200–400 mesh) obtained from Bio-Rad (SX-1 Bio-Beads) were washed, chloromethylated, and esterified by the usual procedures.[9] The chloromethylated resin contained 0.7 mequiv of chloride/gram of resin. Resin-leucine (1.5 g) was used in the synf thesis. The esterification of Boc-leucine was 0.41 mmol/gram o-resin.

The *tert*-butyloxycarbonyl (Boc) amino acid derivatives used were the following: Ala, Arg (NO_2), Asp (β-OBzl), Asn-ONp, Cys (*S*-Bzl), Glu (γ-OBzl), Gln-ONp, Gly, His, Ile, Leu, Lys (ϵ-carbobenzoxy (Z)), Met, Phe, Pro, Ser (Bzl), Thr (Bzl), Trp, Tyr (Bzl), and Val. These derivatives were either synthesized in our laboratory or purchased from Schwarz BioResearch Co. The purity of each derivative was checked by thin-layer chromatography and melting point determination.

Amino acid derivative (1.5 mmol) was used for each coupling

(1) Address correspondence to this author at the Department of Biochemistry, Dartmouth Medical School, Hanover, N. H. 03755. National Institutes of Health Research Fellow (1 F01 GM-38214-01A1) 1969–1971.

(2) R. B. Merrifield, *Fed. Proc., Fed. Amer. Soc. Exp. Biol.*, **21**, 412 (1962).

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

(4) B. Gütte and R. B. Merrifield, *J. Biol. Chem.*, **246**, 1922 (1971).

(5) S. Sano and M. Kurihara, *Z. Physiol.*, **30**, 1183 (1969).

(6) C. H. Li and D. Yamashiro, *J. Amer. Chem. Soc.*, **92**, 7608 (1970).

(7) E. Bayer, G. Jung, and H. Hagenmaier, *Tetrahedron*, **24**, 4853 (1968).

(8) J. J. Sharp, A. B. Robinson, and M. D. Kamen, *Fed. Proc., Fed. Amer. Soc. Exp. Biol.*, **30**, Abstract 1287 (1971).

(9) J. M. Stewart and J. D. Young, "Solid Phase Peptide Synthesis," W. H. Freeman, San Francisco, Calif., 1968.

whether mediated by dicyclohexylcarbodiimide (DCC) or a *p*-nitrophenyl ester (ONp). Single 5-hr couplings were used in all cases except for later couplings of the ONp derivatives.

Boc removal was accomplished with a mixture of trifluoroacetic acid (TFA)–CH_2Cl_2–2-mercaptoethanol–anisole, 45:50:5:2 (this and future solution compositions are on a volume basis). This solution will be referred to as "TFA." Anisole was added to prevent possible alkylations of labile groups (*i.e.*, unprotected Met) by *tert*-butyl cations present during Boc removal. The TFA was injected into the reaction vessel twice: first it was rocked for 1 min and then for 30 min.

Neutralization was accomplished with a mixture of triethylamine (Et_3N)–$CHCl_3$, 12.5:87.5. This solution was injected into the reaction vessel twice: the first was rocked for 1 min and the second for 10 min.

Synthetic Conditions. Second Synthesis. Crosslinked polystyrene beads (1%) (200–400 mesh) were obtained from Bio-Rad (SX-1 Bio-Beads). Following chloromethylation there was 0.9 mequiv of chloride/gram of resin. The esterification of Boc-leucine was 0.4 mmol/gram of resin; 4.0 grams was used for the synthesis, an amount anticipated to be sufficient to meet the requirement that resin-peptide would be removed for monitoring of coupling.

The Boc amino acid derivatives were the same as the first synthesis with the following exceptions: Cys (*S*-(*p*-methoxy)Bzl) and His (*im*-Tos). The derivatives were either synthesized in our laboratory or purchased from Fox Chemical Co. The purity of the derivatives was checked as before. Amino acid derivative (4.0 mmol) was used for each coupling. The DCC was injected in two 2-mmol increments. Each DCC injection was followed by a 3-hr reaction time. There were three coupling solvents used throughout the synthesis: CH_2Cl_2, dimethylformamide (DMF), and DMF saturated with recrystallized urea (DMF–urea). In all cases, the amino acid was dissolved in one of the three above solvents, the DCC solvent always being CH_2Cl_2. Recoupling times (when necessary) were variable and were generally between 1 and 6 hr. The *p*-nitrophenyl ester couplings (ONp) of glutamine and asparagine were between 16 and 24 hr in length and 5 mmol of the derivative was used for each coupling. Boc[^{14}C]glycine was purchased from Schwarz Bio-Research.

Acetylations were performed using 6 mmol of glacial acetic acid with two equal injections of 3 mmol of DCC. The reaction times were from 30 min to 2 hr. The solvent system for acetylation varied, being either CH_2Cl_2, DMF, or DMF–urea. When acetylimidazole was used for acetylation, 5 mmol of this compound in CH_2Cl_2 was injected directly into the reaction vessel. The reaction time varied between 1 and 3 hr.

Boc removal was accomplished with 4 *N* HCl–dioxane–anisole–ethanedithiol, 98:1:1 (v/v). This solution will be referred to as "HCl–dioxane." The HCl–dioxane injection procedure and reaction times were the same as for TFA in the first synthesis. Anisole was redistilled before use. Dioxane used for the washes prior to, and succeeding, the Boc removal reaction, as well as for the preparation of the HCl–dioxane, was purified by the method of Fieser and Fieser[10] and was stored under 1.2 atm of argon.[11]

Neutralization was accomplished as in the first synthesis. The triethylamine (Et_3N) used for neutralization was purified by the method of Fieser and Fieser.[12]

The DMF used in this synthesis was "Sequenal Grade" DMF purchased from Pierce Chemical Co. It was used without further purification. Saturated urea (recrystallized) in DMF was prepared weekly.

Sequence. The amino acid sequence used was that of Canfield[13] and Jollès.[14]

Automation. The design of the automated machine, as well as that of the reaction vessel (A. B. Robinson and P. Yeager), is described elsewhere.[11,15]

Hydrolysis and Amino Acid Analysis. Resin-peptide samples were hydrolyzed at 130° for 2 hr in 50:50 (v/v) concentrated hydrochloric acid–propionic acid in anaerobic, vacuum-sealed tubes.[16] This procedure removes all blocking groups except the benzyl group of cysteine. Before hydrolysis the resin-peptide sample was thoroughly washed with TFA–CH_2Cl_2, 50:50 (v/v), CH_2Cl_2, and ethanol to remove any ion-exchange adsorbed amino acids from the resin. All other peptide and protein samples were hydrolyzed at 110° for 20–24 hr in constant-boiling HCl in anaerobic, vacuum-sealed tubes.

Amino acid analyses were performed on a modified Beckman Spinco amino acid analyzer. Benzylcysteine was determined on the short column (elution at 20 min in the system used). Tryptophan was determined by titration with *N*-bromosuccinimide.[17]

Karl–Fisher titrations for water were determined by the method of Fieser and Fieser.[18]

Elemental analysis was performed by Huffman Laboratories in Wheaton, Colo.

HF Conditions. The specific HF conditions are given in the following text. The construction of the HF line is described elsewhere.[9,15]

Following treatment with anhydrous HF the peptide was taken up in TFA–ethanedithiol, 98:2, and filtered. This solution was cooled to 0° and 4° ether was added to precipitate the product. The precipitate was washed twice with 4° ether and dried under vacuum. Tryptophan destruction of native lysozyme in the TFA–ethanedithiol had previously been followed spectrally (decrease in A_{280} and increase in A_{335}). There was no evidence of tryptophan destruction after 5 hr at room temperature in this solvent. TFA–ethanedithiol, 98:2, was routinely used to recover the cleaved, deblocked peptide from the dried HF reaction mixture.

Reduction and Reoxidation of Disulfide Bonds. Reduction and reoxidation of disulfide bonds was carried out by one of three methods. In the first, the method of Epstein and Goldberger[19] was followed. In the second procedure, the protein was reduced in 6 *M* guanidine hydrochloride (Matheson), 0.03 *M* dithiothreitol (Pierce),[20] pH 8.0, for 1–3 hr at 37°. After reduction, the solution was passed through a Sephadex G-10 column equilibrated with 0.1 *M* acetic acid. Reoxidation was effected by diluting the protein–acetic acid solution 30- to 50-fold with 0.025 *M* Tris–HCl, 10^{-6} *M* $CuCl_2$, pH 8.0 buffer, and stirring at room temperature for 24 hr. Isolation of the oxidized protein was accomplished by adsorption onto a 1 × 4 cm carboxymethylcellulose (CMC) column equilibrated with 0.025 *M* Tris–HCl, pH 8.0, and elution from the column with 0.5 *M* ammonium bicarbonate. Thirdly, the rapid reoxidation procedure of Saxena and Wetlaufer[21] was used on occasion. All three procedures gave similar results with native lysozyme.

Enzymatic Assay for Lysozyme Activity. The assay for lysozyme activity was performed according to the method of Epstein and Goldberger,[19] which involved adding 1–500 μl of protein solution to 3.0 ml of a *Micrococcus lysodeikticus* cell suspension and monitoring the absorption decrease at 450 nm. The assay was carried out at 30° in a temperature controlled cuvette holder. A Gilford spectrophotometer (Model 2000) was used to follow the 450-nm absorbance decrease.

The specific activity of a protein solution was defined either as the 450-nm absorbance decrease per second per mg of protein (A_{450}/sec per mg) or the 450-nm absorbance decrease per second per A_{280} per milliliter of the solution assayed (A_{450}/sec per A_{280}/ml). The per cent lysozyme activity of a particular synthetic sample was defined as the per cent specific activity the sample exhibited when compared with the specific activity of native lysozyme (Miles Laboratories, 6X crystallized lysozyme, Lot no. 902) measured under the same conditions, *i.e.*, when the same amount of native lysozyme in the same volume was simultaneously assayed.

Determination of Protein Concentration. Protein concentration was determined by one of two methods. In the first, the concentration was determined by assuming an $E_{1\text{ cm}}^{1\%}$ of 26.9 at 280 nm[22] for both native and reduced material. In the second, a portion of the sample was removed, dried, and hydrolyzed, and an amino acid analysis was performed. The concentration was determined from the amino acid recovery.

Mixed Disulfide of Lysozyme. The procedure of Bradshaw, *et al.*[23] (with minor modification), was used to form the cysteine-

(10) L. Fieser and M. Fieser, "Reagents for Organic Synthesis," Wiley, New York, N. Y., 1967, p 333.
(11) J. J. Sharp, Ph.D. Thesis, University of California, 1971.
(12) Reference 10, p 1198.
(13) R. E. Canfield and A. K. Liu, *J. Biol. Chem.*, **240**, 1997 (1965).
(14) J. Jauregui-Adell, J. Jollès, and P. Jollès, *Biochim. Biophys. Acta*, **107**, 97 (1965).
(15) A. B. Robinson, Ph.D. Thesis, University of California, 1967.
(16) J. Scotchler, R. Lozier, and A. B. Robinson, *J. Org. Chem.*, **35**, 3151 (1970).
(17) A. Patchoenik, W. B. Lawson, and B. Witkop, *J. Amer. Chem. Soc.*, **80**, 4747 (1958).
(18) Reference 10, p 528.
(19) C. J. Epstein and R. F. Goldberger, *J. Biol. Chem.*, **238**, 1380 (1963).
(20) W. W. Cleland, *Biochemistry*, **3**, 480 (1964).
(21) V. P. Saxena and D. B. Wetlaufer, *Biochemistry*, **9**, 5015 (1970).
(22) K. Imai, T. Takagi, and T. Isemura, *J. Biochem.* (*Toyko*), **53**, 1 (1963).
(23) R. A. Bradshaw, L. Kanarek, and R. L. Hill, *J. Biol. Chem.*, **242**, 3789 (1967).

Table I. Amino Acid Ratios[a] of Selected Resin-Peptide Samples

	No. 1	No. 2	No. 3
Asp	1.0 (1)	6.7 (8)	17.8 (21)
Thr	0.8 (1)	1.3 (2)	4.6 (7)
Ser	0 (0)	3.0 (5)	6.5 (10)
Glu	1.0 (1)	1.5 (1)	5.6 (5)
Pro	0 (0)	Trace (1)	1.1 (2)
Gly	2.3 (2)	5.1 (4)	16.1 (12)
Ala	1.0 (1)	5.9 (6)	14.7 (12)
Cys		Trace	
Val	0.8 (1)	4.0 (4)	8.0 (6)
Met	0 (0)	0.4 (1)	1.2 (2)
Ile	0.8 (1)	3.1 (4)	5.9 (6)
Leu	1.3 (1)	2.9 (3)	9.1 (8)
Tyr	0 (0)	0 (0)	2.6 (3)
Phe	0 (0)	0 (0)	2.8 (3)
His	0 (0)	0 (0)	0.9 (1)
Lys	0 (0)	3.3 (3)	9.8 (6)
Arg	*b* (2)	4.6 (4)	12.3 (11)
Bzl-Cys	*b* (1)	*b* (4)	9.2 (8)
Trp	*b* (1)	*b* (3)	*b* (6)
Total residues	13	53	129

[a] The calculations are based on the theoretical length of the peptide. The theoretical number of each residue appears in parentheses beside the calculated value. [b] No determination made.

mixed disulfide of lysozyme (lyso-*S*-Cys). Performic acid oxidation[24] and amino acid analysis of lyso-*S*-Cys and native lysozyme showed 14 and 7 residues, respectively, of cysteic acid. The difference spectrum (*vs.* native lysozyme) of the lyso-*S*-Cys agreed well with the published spectrum.[23]

Cellulose Acetate Electrophoresis. Barbitone buffer (pH 8.6) was used for the electrophoresis.[25] Cellulose acetate strips (1 × 12 in.) were employed. Electrophoresis time was 2 hr at 300 V. The strips were first stained in Ponceau S and then in Nigrosin.

HBr–TFA Cleavage. HBr–TFA cleavages were carried out in a 40-ml jacketed solid phase peptide synthesis reaction vessel.[8] The top of the vessel included a drying tube filled with Drierite. Gaseous HBr was dried by passage through P_2O_5 before introduction into the bottom of the reaction vessel. Constant temperature was maintained by pumping 0° or room temperature water through the jacket on the vessel.

Carboxymethylation was carried out by standard procedures.[26]

Chitin Column Chromatography. Chitin columns were run according to the procedures of Cherkasov and Kravchenko.[27,28] Chitin was purchased from Matheson Chemical Co. The material was screened and the 60–150 mesh particles were used.

Enzymatic digestion was performed by the procedure of Pisano, *et al.*[29] (with minor modification).

Synthetic Results. First Synthesis

The total synthesis time was 10 weeks. Amino acid analysis of the 24-residue resin-peptide showed that the first two asparagines were incompletely coupled (~60% complete). Hence, reaction times for the remaining ONp couplings were increased from 5 to 24 hr. A machine error was responsible for incomplete coupling of residue 51 (proline).[30]

After 2 weeks a precipitate formed in the Boc removal solution (TFA–CH_2Cl_2–anisole–2-mercaptoethanol). Elemental analysis of this precipitate was consistent with the composition $(-CH_2CH_2S-)_n$ which suggested that a reaction of the following type was occurring: $(HOCH_2CH_2SH)_n \xrightarrow{H^+} (-CH_2CH_2S-)_n + (H_2O)_{n-1}$. To test this possibility, water production in the Boc removal solution was followed by Karl–Fisher titrations. The results are shown in Figure 1. The apparent first-order rate constant for this reaction at room temperature is 0.063 hr^{-1}.[31]

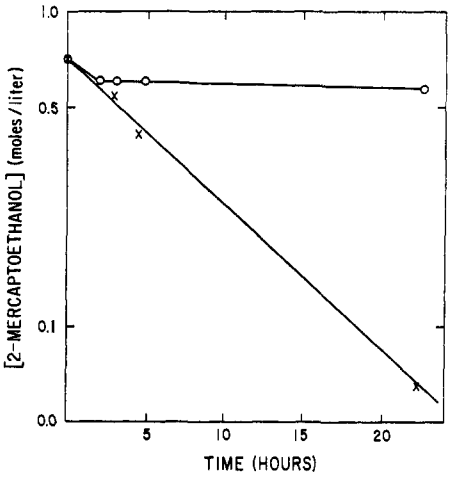
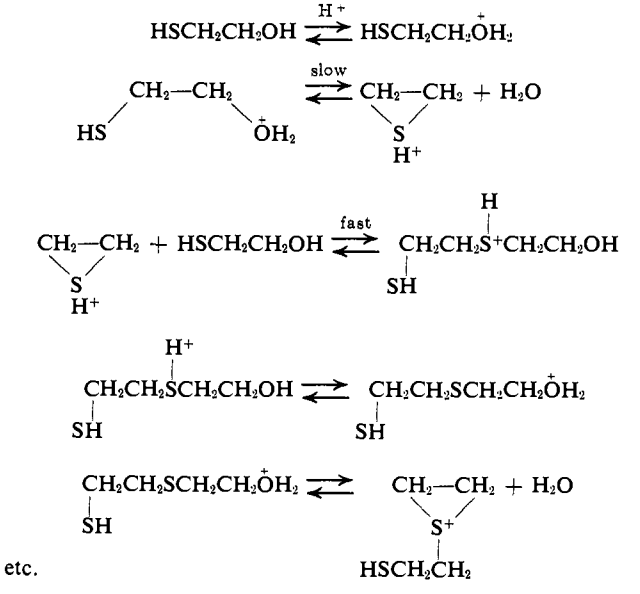


Figure 1. Kinetics of 2-mercaptoethanol decrease (water production) in TFA–CH_2Cl_2–2-mercaptoethanol–anisole, 45:50:5:2, at room temperature (×—×) and 0° (○—○). (The experimental points were determined by subtracting the concentration of water from the initial concentration of 2-mercaptoethanol; see text.)

It is obvious that water production in this solution is quite rapid and that water appears long before a precipitate becomes noticeable. To eliminate the potentially harmful conditions of water under acidic conditions, the reagents (TFA, CH_2Cl_2, anisole, 2-mercaptoethanol) were mixed at room temperature and then placed at 0°. Water production was again monitored and these results are shown in Figure 1. After an initial production of water, which took place while the solution was cooling to 0°, the rate was considerably retarded (first-order rate constant ≃0.003

(31) The first-order rate equation and considerations of carbonium ion stability are consistent with the following mechanism involving neighboring group participation.



(24) C. H. W. Hirs, *J. Biol. Chem.*, **219**, 611 (1956).
(25) C. B. Laurell, S. Laurell, and N. Skoog, *Clin. Chem.*, **2**, 59 (1956).
(26) F. Gurd in "Methods in Enzymology," Vol. 25, C. H. Hirs and S. N. Timasheff, Ed., Academic Press, New York, N. Y., 1972, p 424.
(27) I. A. Cherkasov and N. A. Kravchenko, *Biokhimiya*, **33**, 761 (1968).
(28) I. A. Cherkasov and N. A. Kravchenko, *Biokhimiya*, **34**, 1089 (1969).
(29) J. J. Pisano, J. S. Finlayson, and M. P. Peyton, *Biochemistry*, **8**, 871 (1969).
(30) For synthesis purposes, amino acids are numbered beginning with the C-terminal amino acid instead of the normal N-terminal amino acid.

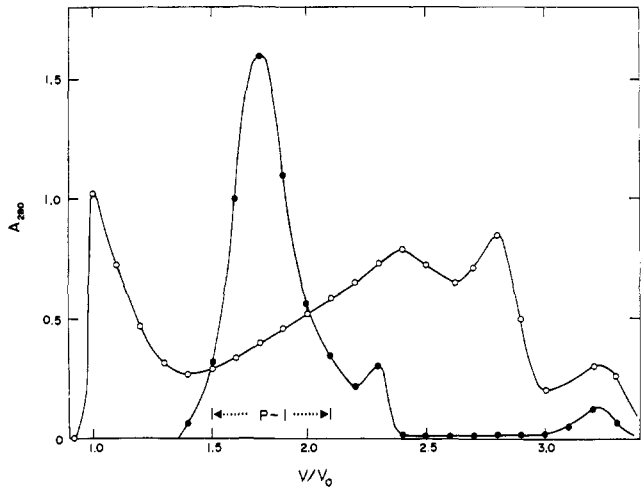


Figure 2. The A_{280} profile of native lysozyme (●—●) and the synthetic product (○—○) chromatographed on similar 2 × 60 cm Sephadex G-100 columns. The column was equilibrated with 8 *M* urea–0.1 *M* acetic acid. The lysozyme was reduced with 2-mercaptoethanol in 8 *M* urea before application. P-1 is the fraction of the product exhibiting the correct elution position. The high molecular weight material disappeared if the product peptide was reduced before application but the other characteristics of the profile remained constant. (V is the elution volume and V_0 the void volume of the column.)

hr^{-1}). On the basis of this result these reagents were stored separately at −10° for the remainder of the synthesis. They were mixed at this temperature and then stored at 0°. A solution was kept no longer than 4 or 5 days. Immediately prior to Boc removal the correct volume was brought to room temperature and injected into the reaction vessel.

Amino acid analyses of the resin-peptide were performed throughout the synthesis and results of three of these appear in Table I. It was difficult to draw any conclusions from these data. However, a few points could be made. The amino acid ratios became poorer as the synthesis progressed. Because of the heterogeneity of the product and the method used to calculate these data, molar ratios greater than theoretical undoubtedly reflected, in part, the ease with which an amino acid coupled rather than multiple additions during the coupling reaction. Conversely, ratios less than theoretical could be ascribed to amino acids which coupled poorly. Of those which survive the hydrolysis conditions, the low aspartic acid value probably indicated the poor coupling of ONp esters (13 of the 21 "aspartic acids" are asparagine) as noted elsewhere.[32,33]

Upon completion of the synthesis the dried resin-product weighed 2.75 g, and amino acid analysis showed that the esterification of the peptide was 0.03 mmol/gram of resin, assuming a molecular weight of 19,000 daltons for the blocked peptide and an average molecular weight of 147 daltons for the blocked amino acids. On this basis, 93% of the initially esterified material had been removed during the course of the synthesis. The blocked peptide constituted 50% of the total weight of the resin-peptide, or 1.38 g.

The peptide was deblocked and removed from the resin by reacting 200 mg of the resin-product in 5 ml of anhydrous HF and 0.5 ml of anisole for 90 min at 0°. A large portion of the product (~80%) exhibited a molecular weight less than that of native lysozyme, as evidenced by Sephadex G-100 chromatography in 8 *M* urea–0.1 *M* acetic acid (Figure 2). The recovery of peptide with the correct molecular weight was always about 20% of the material recovered from the column and there was never a peak corresponding to the reduced native molecule. Following isolation of product with the correct molecular weight (Figure 2) and reoxidation, the peptide showed no enzymatic activity, based on the whole cell assay method (~0.05% native specific activity could have been observed). Amino acid analysis showed that 2.7 residues of benzylcysteine were present in the product and only a trace of cystine.

The same HF reaction conditions were repeated at room temperature in an attempt to completely deblock the benzylcysteine. The peptide product of the correct molecular weight again exhibited no enzymatic activity. Amino acid analysis indicated the level of benzylcysteine had been reduced to 0.5 residue but there was still only a trace of cystine present.

In an attempt to clarify the cystine recovery problem 0.1 mmol of Boc-Cys (*S*-Bzl) was treated with HF using the room temperature reaction conditions. After recovery from HF the material was subjected to normal HCl hydrolysis and amino acid analysis. The major product (~85% of the material recovered from the column assuming a normal proline analyzer constant for this material) was present as a peak and shoulder eluting at the normal proline position. The A_{440} was higher than the A_{560} value, as with proline, indicating it was possibly an imine. On reexamination of previous analyses this peak and shoulder were present and could, therefore, be used as an indication of cystine destruction during the HF reaction. About 5% of the total material recovered from the analysis was cystine and 10% was benzylcysteine.

In an attempt to improve the cystine recovery, 0.1 g of resin-product was reacted in 10 ml of anhydrous HF and 2 ml of anisole for 90 min at room temperature. This represented a fivefold dilution of peptide in HF and a twofold increase in anisole concentration. After collection of the correct molecular weight fraction from Sephadex G-100 (8 *M* urea–0.1 *M* acetic acid) chromatography, reduction, and reoxidation, the peptide-product had ~0.05–0.1% native lysozyme activity. Amino acid analysis showed 4.6 residues of cystine and 0.5 residue of benzylcysteine present in the active fraction. The cysteine degradation product was also considerably reduced. Peptide was removed from the resin several times by this method. The yield of material with 0.05–0.1% native activity was typically about 0.1%.[34] The low molecular weight fractions never exhibited any activity.

No attempt was made to further purify the product. The known errors that occurred during the chain assembly did not account for the poor amino acid ratios of the product or for the large percentage of low molecular weight peptide. Several lines of investigation were undertaken in an attempt to better understand the results of this synthesis.

(32) M. Bodansky and R. J. Bath, *Chem. Commun.*, 1259 (1969).
(33) S. Karlsson, G. Lindeberg, and U. Ragnarsson, *Acta Chem. Scand.*, **24**, 337 (1970).
(34) The basis of the per cent yield calculation is the following: 100% yield would be a 129 amino acid peptide esterified to the resin (0.41 mmol/gram of resin). The yield prior to HF treatment was therefore 7%.

Results of Investigations into the Synthetic Procedure. The Coupling Reaction

Incomplete coupling of amino acids is the most obvious explanation for the large percentage of low molecular weight peptide and poor amino acid ratios found during the lysozyme synthesis. To investigate this possibility the coupling efficiency was examined by employing the ninhydrin test of Kaiser, *et al.*[35] This test indicated less than 90% coupling in several instances when coupling procedures of the lysozyme synthesis were used. Subsequent couplings could drive the reaction to completion.

The coupling reaction was also investigated under conditions in which a molar excess of Boc amino acid to DCC was used and under conditions which will be referred to as "incremental DCC additions." If an amino acid were to be coupled incrementally, the DCC was added to the reaction vessel in 2 or 3 equal increments, the final concentration being equimolar with the Boc amino acid. The reason for provision of a molar excess of Boc amino acid and for incremental DCC addition was to promote *in situ* symmetrical anhydride formation.[36]

The rates of *O*-acyl to *N*-acyl isomerization in DCC mediated couplings are not well known, and coupling rates most probably decrease as the resin-peptide increases in length. The coupling efficiency could therefore come under greater influence of *O*-acyl to *N*-acyl isomerization rates as the chain length increases. By the *in situ* generation of the symmetrical anhydride of the Boc amino acid the influence of isomerization is reduced. Since the soluble product of the solid phase aminolysis of a symmetrical Boc amino acid anhydride is the Boc amino acid, DCC could be added later to utilize this product. This was the basis for incremental DCC addition.

To determine the usefulness of a molar amino acid to DCC excess and of incremental DCC addition, several resin-peptide and Boc amino acid combinations that had previously been difficult to couple were examined. The results of one such experiment are given in Table II. There was a definite advantage when using a molar excess of Boc-Leu to DCC in these experiments. The result was quite reproducible for this and other resin-peptide–Boc amino acid combinations. In several instances (*i.e.*, Boc-Gly and Boc-Phe), a twofold excess of Boc amino acid–DCC, with and without incremental DCC injection, gave the same result as an equimolar coupling. However, an amino acid–DCC excess was never found to couple less completely than an equimolar coupling.

Table II. The Coupling of Boc-Leu to Resin-Asp-Phe-Ala under Various Conditions

Coupling conditions			Amino acid analysis[b]			
Expt	Mole ratio of Boc-Leu/DCC	Coupling time, hr	φ-Asp	-Phe	-Ala	-Leu
1	1:1	3	1.0	1.0	0.9	0.4
2	1.3:1	3	1.0	1.0	0.9	0.5
3	1.6:1	3	1.0	1.0	0.9	0.6
4	2:1	3	1.0	1.0	0.9	0.7
5	1:1	5	1.0	1.0	0.9	0.5
6	Incremental[a]	5	1.0	1.0	0.9	0.8

[a] The initial ratio was 1.6:1. After 3 hr DCC was added to the vessel to bring the ratio to 1:1, and coupling continued for the remaining 2 hr. [b] The amino acid analysis was performed after hydrolysis of the resin-peptide as described in the Experimental Section.

The effect of solvent on the *O*- to *N*-acyl migration is well known and the incremental addition of DCC might prove useful in increasing coupling efficiency when DCC couplings are carried out in DMF.[9]

Removal of Peptide from the Resin. Several investigations, including ours, have shown that peptide is removed from the resin throughout the course of a synthesis.[4,7,37] Resin-Leu-Ala-Val-[^{14}C]Gly was carried through several synthetic cycles and the washes were collected and examined for radioactivity. As expected, peptide was removed from the resin during the acidic Boc removal reaction. The amino acid ratios were unchanged even though there was an accompanying 40% reduction in esterification, indicating that intact peptide had been removed.

It is known that the resin sites are not homogeneous with respect to coupling rates.[38–40] This heterogeneity is presumably owing to the local environment created by the resin matrix.[37] Since there are heterogeneous coupling rates, one might also expect heterogeneity in rate of removal of peptide from the resin. It may be that the peptides at resin sites which participate faster in the synthetic reactions are also the peptides most easily removed from the resin sites. The resin would, therefore, gradually become enriched in poor coupling sites. This difficulty would be of greater importance as the chain length increased and would tend to favor the production of a heterogeneous low molecular weight product if accompanied by incomplete coupling. The possibility of heterogeneous rates of removal from the resin made us consider it advisable to reduce this source of loss by minimizing rates of removal.

The peptide removal rates of the acidic Boc removal solvents currently in use were compared. (Because of previous Boc removal difficulties, 1 *N* HCl–acetic acid was not included.[9,38]) The solvent systems compared were: TFA–CH_2Cl_2 (50:50); TFA–CH_2Cl_2–anisole–mercaptoethanol, 45:50:2:5; TFA–CH_2Cl_2–anisole–ethanedithiol, 45:50:2:5; and 4 *N* HCl–dioxane–anisole–ethanedithiol, 98:1:1. Resin-Leu-Ala-Val[^{14}C]Gly was reacted successively with the four solvents for 30-min periods. Following each reaction all radioactivity was washed from the resin with either CH_2Cl_2 or dioxane. The per cent of peptide removed from the resin was calculated from the cpm recovered and specific activity of the peptide. HCl–dioxane removed less peptide per 30-min reaction time (1.1% of the total peptide was removed in 30 min) (TFA–CH_2Cl_2 = 2.9%; TFA–CH_2Cl_2–anisole–mercaptoethanol = 1.6%; TFA–CH_2Cl_2–anisole–ethanedithiol = 2.1%) than any of the other solvents. These

(35) E. Kaiser, R. L. Colescott, C. D. Bossinger, and D. I. Cook, *Ann. Biochem. Exp. Med.*, **34**, 595 (1970).
(36) H. Smith, J. G. Moffatt, and H. G. Khorana, *J. Amer. Chem. Soc.*, **80**, 6204 (1958).
(37) R. B. Merrifield, *Advan. Enzymol. Relat. Areas Mol. Biol.*, **32**, 221 (1969).
(38) R. B. Merrifield, *Recent Progr. Horm. Res.*, **23**, 451 (1967).
(39) J. Rudinger and U. Gut, *Peptides, Proc. Eur. Peptide Symp., 8th, 1966*, 89 (1967).
(40) M. Monahan, A. B. Robinson, and M. D. Kamen, unpublished results.

data could be used to calculate the yield of peptide following a 129 amino acid synthesis. For TFA–CH_2Cl_2–anisole–mercaptoethanol the theoretical yield would be 9.3% (found 7%). For 4 *N* HCl–dioxane–anisole–mercaptoethanol the theoretical yield would be 18.1% (found 30%).

The peptide removal rate might be reduced further by shortening the reaction time in the Boc removal solvent. However, because the kinetics of Boc removal in solid-phase synthesis are not well known (especially for large peptides), the 30-min reaction time was considered minimal for any of the solvents used.

Tryptophan Protection during Boc Removal. 2-Mercaptoethanol had previously been used for tryptophan protection during Boc removal,[41] but not when TFA was the deblocking agent. The polymer and water formation in TFA necessitated either the use of another tryptophan protecting agent and/or the use of an acid other than TFA. Even though no visible polymer formation had been observed when 2-mercaptoethanol was used in other acidic solvents, it was considered desirable to find another reagent for tryptophan protection. 1-Mercaptobutane and 1,2-ethanedithiol were tested for their ability to protect tryptophan under acidic conditions.

The acid catalyzed resin-tryptophan degradation product was used as an indicator of tryptophan destruction (the resin becomes dark purple). Resin-Trp (0.4 mmol/gram of resin) (0.2 g) and resin-Val-Asp-Met-Asn-Asn-Ile-Ala-Trp-Glu (0.4 mmol/gram of resin) (0.2 g) were stored covered at room temperature in 2 ml of the following solutions: TFA–CH_2Cl_2–anisole–ethanedithiol, 43:50:2:5; TFA–CH_2Cl_2–anisole–mercaptobutane, 43:50:2:5; TFA–CH_2Cl_2–anisole–mercaptoethanol, 43:50:2:5 (this solution contained the polymer); TFA–CH_2Cl_2, 50:50. Both resin samples turned dark purple after 8 hr in TFA–CH_2Cl_2. After 5 days only the samples containing ethanedithiol were uncolored. The samples containing mercaptoethanol were a moderate purple and those with mercaptobutane were dark purple.

1,2-Ethanedithiol was also tested for tryptophan protection in 4 *N* HCl–dioxane–anisole, 99:1, using the same resin-peptides; 0, 1, 2, 3, 4, and 5% ethanedithiol was examined. After 2 weeks at room temperature none of the samples containing 1,2-ethanedithiol were colored while those without this reagent were dark purple.

Lysine ε-Amino Protection. There have been reports that Z protection of the ε-amino group of lysine is inadequate[37,38] and that trifluoroacetyl (TFA) ε-amino protection of lysine is better.[42] However, removal of the TFA group requires strong basic conditions (1 *M* piperidine). We found that the cysteine mixed disulfide of native lysozyme, when treated with 1 *M* piperidine (5 mg in 0.5 ml), had lost considerable enzymatic activity after regeneration of the native molecule. An untreated sample had 63% native lysozyme activity, a sample treated at 0° 28%, and a room temperature treated sample 5% (2-hr reaction time). Because the conditions for removal of the TFA group are variable[9] and might require room temperature treatment, TFA protection of lysine was considered unacceptable for the solid phase synthesis of lysozyme. Therefore, investigations of ε-Z stability were performed.

Initial experiments accomplished by incubation of α-Boc-ε-Z-lysine in TFA–CH_2Cl_2–anisole–ethanedithiol, 43:50:2:5, followed by silica gel thin-layer chromatography of an aliquot of the reaction mixture (developed with chloroform–methanol–acetic acid, 85:10:5), showed that free lysine appeared in the reaction mixture after 1.5 hr. After 75 hr, more than 75% of the lysine was present in the free form.

To obtain further data on the removal of the ε-Z group, α-Boc-ε-Z-lysine was incorporated into two peptides: φ-Asp-Leu-Val-Phe-Lys and φ-Asp-Leu-Val-Phe-Lys-Ala. These resin-peptides were reacted for 24 hr with TFA–CH_2Cl_2–anisole–ethanedithiol, 43:50:2:5, and 4 *N* HCl–dioxane–anisole–ethanedithiol, 98:1:1. Glycine was then coupled to completion (negative ninhydrin test). If a molar excess of glycine was found in the 24-hr treated samples, this reflected ε-amino deprotection (*i.e.*, glycine would be coupled at both the α- and ε-amino positions). The results (Table III) show that the Z group was removed during the 24-hr TFA treatment (about 40%). Within the sensitivity of this procedure there was no detectable removal of the ε-amino Z group with HCl–dioxane. In TFA there was perhaps greater removal of the Z group when lysine was N-terminal than when alanine was N-terminal.

Table III. Deprotection of ε-Z-Lys in Acidic Solution

Peptide[a]	Treatment	Amino acid analysis Lys	Ala	Gly
1	None[c]	1.0		1.0
1	HCl–dioxane;[b] 24 hr	1.0		1.0
1	TFA–CH_2Cl_2;[b] 24 hr	1.0		1.4
2	None[c]	1.0	1.0	1.0
2	HCl–dioxane;[b] 24 hr	1.0	1.1	0.9
2	TFA–CH_2Cl_2;[b] 24 hr	1.0	1.1	1.2

[a] Peptide 1 = φ-Asp-Leu-Val-Phe-Lys-Gly. Peptide 2 = φ-Asp-Leu-Val-Phe-Lys-Ala-Gly. [b] The composition of these solutions is given in the text. [c] These samples were deblocked in TFA–CH_2Cl_2 (50:50) for 30 min.

Cystine Sulfhydryl Protection. Because the benzyl sulfhydryl protecting groups of cysteine proved difficult to remove during this synthesis, the *S*-(*p*-methoxy)-benzyl group was considered more applicable for cystine sulfhydryl protection.[43]

Boc-*S*-(*p*-methoxy)Bzl-L-cysteine was used to test the *p*-methoxy-Bzl group stability in TFA–CH_2Cl_2–anisole–ethanedithiol, 44:50:1:5, and 4 *N* HCl–dioxane–anisole–ethanedithiol, 98:1:1. The same thin layer chromatographic procedure that was used for α-Boc-ε-L-lysine was employed. There was no evidence of S-protection removal in either solvent after 75 hr.

(41) G. R. Marshall, "Milan Symposium on Peptides and Proteins," N. Back, R. Padetti, and L. Martini, Ed., Plenum Press, New York, N. Y., 1968.

(42) M. Ohno and C. B. Anfinsen, *J. Amer. Chem. Soc.*, 89, 5994 (1967).

(43) S. Sakakibara, *Bull. Chem. Soc. Jap.*, 38, 1412 (1965).

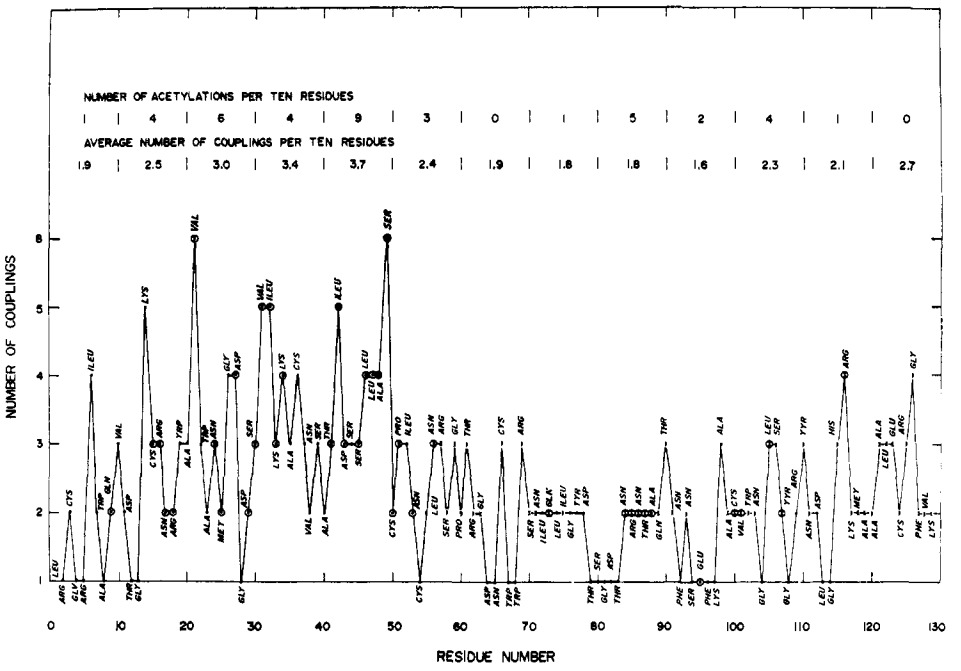


Figure 3. A graphical representation of the number of times each amino acid had to be coupled, the number of acetylations/every 10 amino acids, and the average number of couplings/every 10 amino acids. If an amino acid on the graph is represented by a · then CH_2Cl_2 and/or DMF was used as the coupling solvent, by an X then DMF–urea was used at least once, and if the · or X is circled then that particular amino acid was acetylated.

Synthetic Results. Second Synthesis

The strategy for a second synthesis was based on our investigations which indicated that the most probable cause of low molecular weight product was incomplete coupling of amino acids. It was suggested that this incompleteness of coupling was worsened because the fast coupling chains were also fastest removed from the resin. For this reason it was considered desirable to reduce the peptide removal rate through the choice of a milder Boc removal solution (HCl–dioxane). A consideration in reducing the loss of peptide from the resin is the effect of a resultant greater number of growing sites. It is possible that greater crowding and steric requirements would result from this situation and that coupling efficiency thus might be reduced, especially when synthesizing a large peptide. The fact that HCl–dioxane did not remove the Z ϵ-amino blocking group of lysine while the TFA solvent did favored the use of HCl–dioxane.

The synthetic alterations therefore included the use of 4 *N* HCl–dioxane for Boc removal, of 1,2-ethanedithiol for tryptophan protection in the 4 *N* HCl–dioxane, of incremental DCC injection during the coupling of amino acids, of the *p*-methoxybenzyl group for S-protection of cysteine, and of the ninhydrin test to monitor degree of completion of the coupling reaction. Also, in an attempt to drive each coupling reaction to completion, the coupling solvent (CH_2Cl_2) was varied when necessary. Residues which proved difficult to couple in CH_2Cl_2 (as evidenced by the ninhydrin test) were coupled in dimethylformamide (DMF) and if necessary in DMF saturated with urea (DMF–urea).[44] These solvent systems had been shown to be of value when coupling in CH_2Cl_2 was difficult.[11,44]

Residues which still proved difficult to react to a sufficient degree were acetylated (either by DCC-mediated coupling of acetic acid or reaction with acetylimidazole). Thus, the unreacted chains would be terminated and easily separated by gel filtration during purification. Obviously this modification of the synthetic procedure would be of decreasing value as the synthesis progressed to completion.

The total time spent on this synthesis was about 6 months. The numerous recouplings and often lengthy coupling times reduced the assembly rate to less than one residue per day.

A summary of the number of couplings per residue, coupling solvents, and acetylations is presented in Figure 3.

The most easily coupled portion, as expected, was from residues 1 to 13. These reactions were driven to completion in CH_2Cl_2 and acetylation was not necessary. An attempt was made to complete the coupling in CH_2Cl_2 before using DMF or DMF–urea. In general, coupling in CH_2Cl_2 became more difficult as the chain length increased. After 40 amino acid additions it was apparent that repeated couplings in CH_2Cl_2 failed to yield better than about 80% completion (as evidenced by the ninhydrin test). After residue 40 the initial coupling of an amino acid was the only one carried out in CH_2Cl_2. Subsequent couplings were done in either DMF or DMF–urea. (Exceptions to this were residues 64–68, 79–83, and 92–97 for which the initial coupling solvent was DMF and for which CH_2Cl_2 was never used.) The choice between DMF and DMF–urea as a second coupling solvent depended on the amino acid involved and on the results obtained in coupling the amino acids immediately prior to the one in question.

The summary in Table IV also indicates expected trends for the coupling of the individual amino acids. Although coupling conditions (solvent, time, etc.)

(44) F. C. Westall and A. B. Robinson, *J. Org. Chem.*, 35, 2842 (1970).

Table IV. Average Couplings/Residue and the Number of Acetylations Employed for Each Amino Acid during the Synthesis

Amino acid	No. of each amino acid	Average couplings/ residue	Total acetylations for each amino acid
Ala	12	2.4	2
Arg	11	2.4	4
Asp	8	2.1	3
Asn	13	2.2	6
Cys	8	2.4	3
Glu	2	2.0	1
Gln	3	2.0	1
Gly	12	1.6	0
His	1	3.0	0
Ile	6	3.5	2
Leu	8	2.7	3
Lys	6	2.8	2
Met	2	2.0	1
Phe	3	1.3	0
Pro	2	2.5	1
Ser	10	2.7	3
Thr	7	2.0	2
Trp	6	2.0	1
Tyr	3	2.3	1
Val	6	3.3	3
Total	129	2.4	39

ing for the fact that the hydrolysis conditions led to poor recoveries of serine, cysteine, methionine, and tyrosine, these analyses appeared very satisfactory through the 99 amino acid peptide. We did not consider these favorable amino acid ratios to indicate homogeneity in the product, however. Comparison of these data with similar data from the first synthesis (Table I) seemed to indicate a significant improvement in synthesis.

The amino acid ratios in subsequent analyses became gradually poorer. This was of minor concern because the same coupling and testing procedures were being used. It seemed more likely that the analyses through the 99 amino acid peptide gave a poor indication of product homogeneity than that the coupling and testing procedures suddenly deteriorated at residue 100. We suspect that a combination of product heterogeneity, some incomplete coupling and removal of peptide from the resin are responsible for the unsatisfactory amino acid ratios after residue 99.

The 126th amino acid to be added was incorporated as [^{14}C]Boc-glycine. This was used to examine the rate of removal of peptide from the resin. Both 4 *N* HCl–dioxane–anisole–ethanedithiol, 98:1:1, and TFA–CH_2Cl_2–anisole–ethanedithiol, 47:50:1:2, were reacted with the resin-peptide for 30 min and the washes collected and counted (three washes with each solution were carried out). The TFA under these conditions removed about five times as much peptide from the resin as did the HCl–dioxane. Using a specific activity of 0.01 mci/mmol and average molecular weight of 19,000 daltons for the blocked resin-peptide, about 0.4% of the peptide was removed by the HCl–dioxane and 1.9% by the TFA. The resin-peptide was also considerably more swollen in the TFA than the HCl–dioxane. This fact plus a comparison of the removal rate data obtained on the 4 and 126 residue peptide indicate TFA was better able to penetrate a large resin-peptide than was HCl–dioxane. It was unknown if the relative rates of Boc removal would be similarly affected and/or if the 30-min reaction time was suffi-

Table V. Amino Acid Ratios[a] of Selected Intermediate Resin-Peptide Samples

Amino acid	No. 1	No. 2	No. 3	No. 4	Lysozyme
Asp	0.9 (1)	6.9 (8)	18.6 (18)	18.1 (21)	20.1 (21)
Thr	0.7 (1)	1.9 (2)	6.4 (7)	5.1 (7)	6.5 (7)
Ser		3.9 (5)	5.5 (9)	5.0 (10)	6.5 (10)
Glu	0.8 (1)	0.9 (1)	4.4 (4)	6.2 (5)	5.3 (5)
Pro		1.2 (1)	2.4 (2)	1.9 (2)	2.1 (3)
Gly	2.0 (2)	3.7 (4)	8.4 (8)	14.2 (12)	12.0 (12)
Ala	1.1 (1)	6.5 (6)	11.7 (9)	15.6 (12)	12.5 (12)
Cys	1.5 (2)	3.2 (4)	3.4 (6)	4.1 (8)	4.0 (8)
Val	0.8 (1)	3.2 (4)	3.4 (4)	6.0 (6)	5.9 (6)
Met		0.2 (1)	0.5 (1)	1.5 (2)	1.5 (2)
Ile	1.1 (1)	3.9 (4)	6.4 (6)	5.1 (6)	5.1 (6)
Leu	1.3 (1)	4.0 (3)	6.4 (5)	10.6 (8)	7.7 (8)
Tyr			1.3 (1)	2.9 (3)	2.3 (3)
Phe			2.7 (2)	4.1 (3)	2.7 (3)
His				1.0 (1)	1.0 (1)
Lys	1.0 (1)	2.7 (3)	4.7 (4)	7.6 (6)	5.6 (6)
Arg	2.2 (2)	4.1 (4)	7.7 (8)	8.4 (11)	10.4 (11)
Trp	*b* (1)	*b* (3)	*b* (5)	*b* (6)	*b* (6)
Total residues	15	53	99	129	129

[a] The calculations were based on the theoretical length of the peptide. The theoretical number of each residue appears in parentheses beside the calculated value. [b] No determination made.

were not identical for each amino acid, glycine obviously coupled very easily (presumably owing to minimal steric requirements). Phenylalanine also coupled easily. Conversely, the sterically hindered amino acids (valine, isoleucine) were more resistive to complete reaction.

A total of 38 residues were acetylated during the synthesis. In this respect the ninhydrin test never gave a completely negative test after residue 15. Acetylation with either acetic acid–DCC or acetylimidazole in any of the three coupling solvents failed to completely eliminate the residual slightly positive ninhydrin value, as did a thorough washing of the resin before testing.

Amino acid analyses were performed on the resin-peptide every 10 residues throughout the synthesis. Representative samples appear in Table V. Allow-

cient for 4 *N* HCl–dioxane to deblock a 126 residue resin-peptide.

At the completion of the synthesis the resin-peptide was vacuum dried and weighed. Its weight was 14.8 g and amino acid analysis indicated that 7.4 g of this weight was peptide. This was a substantial yield but was complicated by the fact that the synthesis was begun with 4.0 g of resin-leucine. In addition it was estimated that an equivalent of about 1.5 g of the starting resin was removed throughout the synthesis for amino acid analysis, ninhydrin tests and removal at critical residues which later might be examined for activity and binding properties. This meant that approximately 35% of the final weight (~5 g) was due to material other than peptide or resin. In an attempt to identify this unknown material, HCl–propionic acid hydrolyses for extended periods of time were carried out but failed to release any more peptide from the resin. Extensive washing by various solvents (dimethyl sulfoxide, TFA, TFA–CH_2Cl_2 (50:50), chloroform, glacial acetic acid, methanol, ethanol) also failed to remove anything from the resin. Elemental analysis of the resin-peptide was inconclusive although it did indicate that no Cl, F, or S was contained in this unknown material. It did seem that the material was removed during the HF cleavage. Although no positive identification was made, it is possible the unknown material is tightly bound solvent (although this problem was not observed with the first resin-product) and/or urea (DMF–urea was used extensively as a coupling solvent). Perhaps related to the presence of this unknown material is the fact that the resin-product was resistant to all attempts at cleavage by HBr–TFA.

The final esterification of the peptide was 0.15 mmol/gram of resin (60% of the originally esterified material was removed during the synthesis). As shown below, a large portion of the product consisted of a nonreducible (by mercaptoethanol or dithiothreitol) high molecular weight peptide. Assuming one-half of the product had a molecular weight twice that of the native peptide (and the other half had the correct molecular weight) the corrected esterification was 0.12 mmol/gram of resin (70% of the original material was removed). In either case the amount of peptide removed was less than that predicted from the results on a 4 amino acid resin-peptide (82% removal would have been expected). This, in addition to the removal rate obtained on the 126 residue resin-peptide, suggests that the rate of removal of peptide from the resin decreased as the chain length increased when 4 *N* HCl–dioxane is used for Boc removal. Again it appeared that 4 *N* HCl–dioxane was less able to penetrate a large resin-peptide than was TFA (for TFA the theoretical removal rate based on a 4 amino acid resin-peptide agreed well with what was found during the first synthesis, 91% *vs.* 93%, respectively).

To obtain information on the optimal HF conditions, three different cleavage conditions were examined. In the first (A), 200 mg of resin-product was reacted in 20 ml of anhydrous HF and 4 ml of anisole for 1 hr at 0°. The reaction vessel was then allowed to warm to room temperature for 30 min, at which time the HF and other volatile products were removed under vacuum. The second conditions (B) were identical with the first except that 200 mg of methionine was added to the reaction mixture. In the third (C), 200 mg of methionine, 200 mg of glutamine, and 200 mg of tryptophan were added to the reaction mixture. Evidence has been presented that deamidation of asparagine and glutamine occurs during the HF cleavage and that this deamidation can be eliminated by the addition of glutamine to the reaction mixture.[45] Hence we also examined the addition of methionine and tryptophan to the reaction mixture in order to protect these possibly labile amino acids.

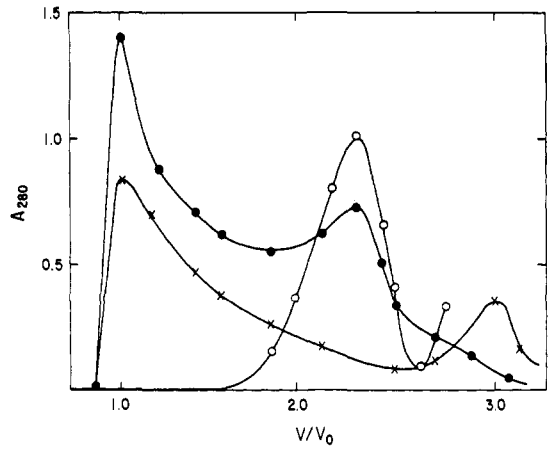


Figure 4. The A_{280} profile of native lysozyme (O—O), the synthetic product removed from the resin by the A cleavage conditions (●—●), and A-h following reduction with dithiothreitol (×—×) on Sephadex G-75 (see text). (V is the elution volume and V_0 the void volume of the column.)

The dried samples were taken up in 4 ml of 0.1 *M* acetic acid and any precipitate was removed by centrifugation. The peptide product was chromatographed on a 2 × 60 cm Sephadex G-75 column equilibrated with 0.1 *M* acetic acid. The chromatography profile of A appears in Figure 4 along with the profile of reduced native lysozyme chromatographed on a similar column.

The products from the three HF reaction conditions exhibited similar profiles on G-75. In contrast to the first synthesis, about 30–50% of the product has eluted at the same position as native reduced lysozyme. There was very little low molecular weight product. There was, however, a large amount (50–70%) of high molecular weight peptide.

Each of the samples was divided into a high molecular weight and a correct molecular weight (elution position) fraction and freeze dried. The weights of the A fractions were: high molecular weight (A-h) = 17 mg, correct molecular weight (A-c) = 14 mg (yield = 31%). The weights of the B fractions were: B-h = 19 mg, B-c = 13 mg (yield = 32%). The weights of the C fractions were C-h = 7 mg, C-c = 10 mg (yield = 17%). From amino acid analyses the amount of peptide expected to be recovered from each of these cleavages was 100 mg.

We attempted to resolve further the high molecular weight fractions by reduction with mercaptoethanol or dithiothreitol in 6 *M* guanidine–HCl. Following reduction the samples were rechromatographed on Sephadex G-75. The A_{280} profiles of the initial A re-

(45) J. McKerrow, Ph.D. Thesis, University of California, 1972.

action mixture and the A-h fraction after reduction are shown in Figure 4. Reduction failed to change the molecular weights (elution position) of any of the high molecular weight fractions.

Samples of all fractions from this experiment (A-h, A-c, B-h, B-c, C-h, and C-c) were taken for amino acid analysis following both HCl and enzymatic hydrolysis. These data failed to show any improvement in amide yield when glutamine was present in the HF reaction mixture. There was also no improvement in tryptophan recovery when free tryptophan was added to the HF. The same was true for methionine. The amino acid analyses of the enzymatic digests were examined for the presence of Glu (γ-OBzl), Asp (β-OBzl), Ser (Bzl), Thr (Bzl), and Tyr (Bzl). None of these derivatives were found indicating the peptide had been completely deblocked in all three HF reaction mixtures.

All fractions were reduced with dithiothreitol, reoxidized, concentrated on CMC, and assayed for enzymatic activity. None of the high molecular weight fractions were enzymatically active. The specific activity of A-c was 0.5% that of native lysozyme and 2.0% that of similarly reduced and reoxidized native lysozyme. Corresponding data for B-c were 0.06% of native and 0.2% of reduced and reoxidized native specific activity. For C-c the figures were 0.1% of native and 0.4% of reduced and reoxidized native specific activity. Free amino acid additions to the HF reaction mixture seemed to decrease the enzymatic activity of the product. Subsequent cleavage procedures were therefore performed using method A.

The results of the amino acid analyses of both the enzymatic and HCl hydrolysis for the A fractions and native lysozyme appear in Table VI. Few conclusions can be drawn from these data. As expected the amino acid ratios did not match native lysozyme, and were similar to those of the first synthesis (Table I). Most noticeable were the low recoveries of Asp, Thr, Ser, and Ile.

Table VI. Amino Acid Ratios[a] of the Synthetic Product

Amino acid	A-h HCl	A-h Enzymatic	A-c HCl	A-c Enzymatic	Native lysozyme HCl[b]	Native lysozyme Enzymatic
Asp	18.3	3.5	15.8	3.6	21.3 (21)	5.7 (8)
Thr	5.7	4.2	4.9	3.7	6.9 (7)	6.2 (6)
Ser	9.3		7.5		9.4 (10)	
Glu	5.5	3.1	6.5	4.6	5.3 (5)	2.3 (2)
Pro	2.0		1.9		2.1 (2)	
Gly	14.2	10.9	15.6	12.7	13.5 (12)	9.9 (12)
Ala	15.3	15.1	16.5	15.7	13.4 (12)	13.1 (12)
Cys	5.0	6.3	3.7	5.5	6.9 (8)	8.8 (8)
Val	5.9	6.4	6.3	6.5	5.6 (6)	6.6 (6)
Met	0.6	2.2	1.1		1.2 (2)	1.8 (2)
Ile	5.3	4.2	3.9	2.5	5.2 (6)	4.8 (5)
Leu	10.3	11.8	10.3	9.1	7.8 (8)	8.5 (8)
Tyr	1.9	3.4	2.7	3.7	2.4 (3)	4.0 (3)
Phe	3.6	4.6	4.4	5.3	2.9 (3)	3.3 (3)
His	1.3	2.1	1.8	2.8	1.0 (1)	1.2 (1)
Lys	6.4	4.4	8.6	7.1	5.8 (6)	6.1 (6)
Arg	12.5	10.8	14.3	10.7	12.3 (11)	10.6 (11)
Trp	c	c	4.8[d]	c	6.4[d] (6)	c

[a] The basis of the calculations was the same as those in Table II. [b] The numbers in parentheses are the theoretical number of each residue. [c] No determination made. [d] Determined by *p*-toluenesulfonic acid hydrolysis: T. Y. Liu and Y. H. Chang, *J. Biol. Chem.*, **246**, 2842 (1971).

Following the information obtained on HF cleavage conditions we decided to examine milder conditions to see if a more active product could be recovered. In this case 200 mg of resin-product was reacted in 20 ml of anhydrous HF and 4 ml of anisole for 90 min at 0°. The HF and other volatile products were again removed under vacuum. The peptide was recovered and chromatographed as previously described. Under these milder conditions only the high molecular weight product was cleaved from the resin. These data offered support to our proposal that heterogeneity in resin esterification sites was directly related to heterogeneity in the coupling sites (*i.e.*, chains more easily removed from the resin were also the fastest coupling sites). It seemed probable that the high molecular weight peptide was the result of overzealous concern for completing each coupling reaction. Deblocked amino acid side chains (incurred during the synthesis or from impure amino acid derivatives) branched as a result of the extreme coupling conditions. It followed that most of this branching occurred in the more accessible chains. The fact that these were the only chains removed by mild HF conditions supported our contention of a direct relationship between coupling and cleavage efficiency.

One further aspect of the HF cleavage conditions remains to be reported. Before beginning the first synthesis the stability of native lysozyme in HF was examined. The reaction conditions employed were essentially those of method A. After recovery from the HF this material had 70% of the native specific activity. On the basis of these data it was concluded that the molecule was sufficiently stable to withstand the cleavage conditions. Following this second synthesis the stability of *reduced* (*i.e.*, unfolded) native lysozyme was tested when the A cleavage conditions were used. After recovery from HF, Sephadex G-75 chromatography, and reoxidation the product had 10% the specific activity of the native molecule. This result prompted us to examine the HF stability of the reduced native molecule in the presence of the correct molar ratios of the blocked free amino acids. We attempted to more closely simulate the actual conditions a blocked synthetic product would experience during the HF reaction. Reduced native lysozyme (3.5 μmol) was treated by the A cleavage conditions in the presence of 38 μmol of Arg (NO_2),28 μmol of Cys (*S*-(*p*-methoxy)Bzl), 7 μmol of Glu (γ-OBzl), 28 μmol of Asp (β-OBzl), 21 μmol of Lys (ϵ-Z), 3.5 μmol of His (*im*-TOS), 35 μmol of Ser (Bzl), 25 μmol of Thr (Bzl), and 10 μmol of Tyr (Bzl). On the basis of results obtained with added amino acids in the HF reaction mixture (B and C cleavage conditions) it was requisite to be concerned with a similar effect of these amino acids. However, it should be noted that in the B and C cleavage conditions the amino acids were present in a two- and sixfold weight excess, respectively, over the product peptide while in this experiment the native lysozyme was present in a twofold weight excess over the amino acids.

Following recovery from the HF, Sephadex G-75 chromatography, and reoxidation, the product had 2–3% the specific activity of the native molecule. It

is interesting that the specific activity of the crude product was 9–25% that of native lysozyme treated with HF in the presence of the blocked amino acids. Based on amino acid analysis and product characterization it seems unlikely that the crude product could have been so homogeneous. However, these results indicated that problems existed with the HF cleavage conditions and that better synthetic results might result from more thorough examination of the HF reaction.

The synthetic product (A-c) was examined for purity by CMC chromatography and by cellulose acetate electrophoresis. In both cases most of the product was more acidic than native lysozyme. It is possible that this increased acidity arose from an unfavorable balance between the ratios of glutamic acid (Glu) + aspartic acid (Asp) and glutamine (Gln) + asparagine (Asn). From the amino acid analyses in Table VI it was calculated that for native lysozyme the recovery of Glu + Asp was 42% of the total of Glu + Asp + Gln + Ans (theoretical 39%) while the same figure for the synthetic product was 46%. In addition, if some chain branching or acetylation did occur at the ϵ-amino group of lysine the apparent acidity of the product would be further increased.

We have previously reported[46] the results of fingerprinting of the crude product and native lysozyme (in cooperation with L. E. Barstow, V. J. Hruby, T. Shimoda, and J. A. Rupley at the University of Arizona). The synthetic product fingerprint showed a similarity to native lysozyme except for one peptide and low yields of several large peptides. Although encouraging, this type of analysis was inconclusive without an extensive characterization of the peptides.

Chitin column chromatography and ammonium sulfate fractionation were employed in an attempt to further purify the synthetic product. Following chitin chromatography the synthetic product had 4–6% the specific activity of native lysozyme that had been similarly reduced, reoxidized, and chromatographed (2–3% the specific activity of native lysozyme). Ammonium sulfate precipitation failed to improve the specific activity. Both native lysozyme and the synthetic product precipitated in saturated ammonium sulfate and in neither case did peptide precipitate before the saturating concentration was reached.

The specificity of the enzymatic bond breakage was examined and has previously been reported.[46,47] The synthetic product exhibited the proper specificity in the hydrolysis of a hexamer of *N*-acetylglucosamine $(GlcNAc)_6$ and in the glucosyl transfer from $(GlcNAc)_6$ to [^{14}C]GlcNAc. The specific activity of the crude product was 1% that of native lysozyme.

Further characterization of the product was not attempted. Since the completion of this synthesis, two further syntheses have been carried out by the Arizona group.[48] The crude activities of these products were approximately those of this second synthesis. A product of high specific activity (~70%) has been obtained with the use of affinity chromatography, although with low yield. Neither of the products discussed in this paper were subjected to the affinity chromatographic procedures.

(46) L. E. Barstow, V. J. Hruby, A. B. Robinson, J. A. Rupley, J. J. Sharp, and T. Shimoda, *Fed. Proc., Fed. Amer. Soc. Exp. Biol.*, **30**, Abstr. 1292 (1971).

(47) This assay was carried out by L. E. Barstow, V. J. Hruby, T. Shimoda, and J. A. Rupley at the University of Arizona.

(48) L. Barstow, D. Cornelius, V. Hruby, T. Shimoda, J. Rupley, J. J. Sharp, A. B. Robinson, and M. D. Kamen in "Chemistry and Biology of Peptides," J. Meienhofer, Ed., Ann Arbor Science Publishers, Ann Arbor, Mich., 1972, p 231.

Discussion

A polypeptide(s) with lysozyme activity was synthesized using Merrifield solid-phase peptide procedures. The yield[34] after HF treatment and Sephadex G-75 chromatography was ~10%. The yield of the correct molecular weight fraction after reduction, reoxidation, and concentration on CMC was ~1%. The total possible recovery of peptide with the correct molecular weight was ~210 mg (if the total resin-product was reacted in HF by method A). The specific activity of this crude product was 0.5–1% that of native lysozyme (or 9–25% that of native lysozyme which had been similarly treated with HF in the presence of blocked amino acids). Following chitin chromatography the specific activity of the correct molecular weight fraction was 2–3% that of native lysozyme. This material exhibited the correct enzymatic specificity with regard to the hydrolysis of $(GlcNAc)_6$ and in the glucosyl transfer from $(GlcNAc)_6$ to [^{14}C]GlcNAc.

The problems encountered during these syntheses will be discussed below.

Coupling Reaction. From the results of the first synthesis and following experiments it was concluded that poor coupling and selective removal of esterified peptide were the reasons for the large excess of low molecular weight peptide. By using the ninhydrin test[4] for estimating the coupling efficiency, the amount of low molecular weight material was considerably reduced in the second synthesis. The possibility that the number of couplings performed were unnecessary and/or harmful has been discussed.

Boc Removal. The possibility that poor Boc removal during the first synthesis resulted in the low molecular weight product was never ruled out. Rather, the difficulty was attributed to poor and variable coupling. The results of the second synthesis indicate that Boc removal was not a problem. Even with a milder Boc removal reagent (HCl–dioxane) there was very little low molecular weight product in the second synthesis. (There is a report[49] that HCl–dioxane is a much poorer reagent for Boc removal than TFA–CH_2Cl_2, or even than HCl–acetic acid.)

It is possible that there was ϵ-amino deprotection of lysine during the second synthesis and that this served as a chain branching point resulting in the high molecular weight polymer. The lysine deprotection experiments were performed by rocking the appropriate resin-peptides in the acid solution for 24 hr and then examining the amount of ϵ-amino deprotection. To reproduce the synthetic conditions the acid should have been withdrawn and fresh acid injected in 30-min time intervals. These experiments did show, however, that TFA–CH_2Cl_2 was unacceptable for Boc removal if Z was used for the ϵ-amino protection of lysine. Certainly any Z removal was compounded by the fact that each coupling reaction was driven to completion.

(49) S. Karlsson, G. Lindeberg, J. Porath, and U. Ragnarsson, *Acta Chem. Scand.*, 24, 1010 (1970).

HF Reaction. The HF cleavage conditions are not ideal as demonstrated by the activity measurements of native lysozyme after this reaction. Certainly ribonuclease was not affected so severely by the HF conditions. The severity of this reaction undoubtedly varies from protein to protein.

Aspartic Acid. The α-β peptide bond migration of aspartic acid is well known. This reaction has been reported when glycine is C-terminal to aspartic acid.[50] There are three Asp-Gly sequences in lysozyme but unfortunately no simple method exists to determine the extent of cyclization and/or β migration in a peptide of this size.

Asparagine and Glutamine. The deamidation of asparagine and glutamine during HF cleavage has been reported.[51] It is not known how much, if any, deamidation occurs during the chain assembly process. Although asparagine and glutamine are usually used unblocked in the solid-phase procedures, blocking groups for these amino acids are available (for example, ref 52). Nor is it known what effect these groups will have in reducing any HF caused deamidation.

From the results of these two lysozyme syntheses it follows that there is need for milder conditions and a better solid support when attempting a long-term solid-phase synthesis. It has been demonstrated that cleavage conditions milder than HF would be desirable. Milder cleavage conditions, however, necessitate more easily removable side chain blocking groups, which in turn require less severe conditions for α-amino deprotection removal.

If the goal of protein synthesis had been to obtain a lysozyme-like native protein with high specific activity, the purification of the product obtained from this synthesis could have been further pursued. However, the synthesis of the native molecule was only the initial stage of researches leading to the production of synthetic analogs for use in structure–function investigations. We feel that the heterogeneity exhibited by the products obtained from these two lysozyme syntheses (and from other syntheses) underlines the necessity for further development of solid-phase procedures as applied to the synthesis of large molecules before attempts based on analog syntheses can be mounted. In spite of the remarkable success in the syntheses of ribonuclease A[4] and acyl-carrier protein,[53] it seems that the method is, as yet, not reliable enough for general applicability to the synthesis of any large molecule and its significant structural analogs. A problem obviously exists in the purification of analogs (affinity chromatography purification of structural analogs may or may not be of value, depending on their substrate binding properties). At the present time thorough characterizations of the analog are required to establish its nature. For a large molecule with one or two amino acid replacements (the remainder of the molecule being identical with the "native" product) this is a significant undertaking. For this reason it is felt that the development of solid-phase procedures specifically aimed at improving the syntheses of large molecules is crucial.

Acknowledgments. Financial support provided by grants from the National Institutes of Health (HD-01262 to M. D. K. and AM-14879 to A. B. R.) and the National Science Foundation (GB-7033X to M. D. K.) is gratefully acknowledged.

(50) E. E. Haley, B. J. Corcoran, F. E. Dorer, and D. L. Buchanan, *Biochemistry*, **5**, 3229 (1966).
(51) A. B. Robinson, J. H. McKerrow, and P. Cary, *Proc. Nat. Acad. Sci. U. S.*, **66**, 753 (1970).
(52) P. G. Pietta and G. R. Marshall, *Chem. Commun.*, 650 (1970).
(53) W. S. Hancock, D. J. Prescott, G. R. Marshall, and P. R. Vagelos, *J. Biol. Chem.*, 247, 6224 (1972).

Communications to the Editor

Transport of Amino Acids through Organic Liquid Membranes[1]

Sir:

Research in transport phenomena through membranes has reached a very broad development in recent years, above all in the biological field. The study of synthetic thin membranes[2] has also been actively pursued, especially in the case of cation transport using natural or synthetic macrocyclic[2,3] or macrobicyclic[4] carriers. On the other hand, much attention has been directed toward the passive selective and specific transport of inorganic ions through organic bulk liquid membranes[5–7] between two aqueous phases. Transport of sodium cations against their concentration gradient pumped by coupling to a movement of protons in the opposite direction has been described.[8] *Via* studies in cation transport[4] by cryptate[9] type carrier complexes, we have become interested in the general area of transport processes of organic molecules. Indeed, the development of transport systems for organic molecules may have wide consequences in the study of transport

(1) Transport in Organic Chemistry. I.
(2) P. Läuger, *Angew. Chem.*, 81, 56 (1969); *Angew. Chem., Int. Ed. Engl.*, 8, 42 (1969).
(3) B. C. Pressman and D. H. Haynes in "The Molecular Basis of Membrane Function," D. C. Tosteson, Ed., Prentice-Hall, Englewood Cliffs, N. J., 1969, p 221.
(4) J. M. Lehn and M. Kirch, unpublished results.
(5) H. L. Rosano, J. H. Schulman, and J. B. Weisbuch, *Ann. N. Y. Acad. Sci.*, **92**, 457 (1961).
(6) K. Sollner and G. M. Shean, *J. Amer. Chem. Soc.*, **86**, 1901 (1964).
(7) K. Sollner in "Diffusion Processes, Proceedings of the Thomas Graham Memorial Symposium, University of Strathclyde," Vol. 2, J. N. Sherwood, A. V. Chadwick, W. M. Muir, and F. L. Swinton, Ed., Gordon and Breach, London, 1971, p 655.
(8) J. H. Moore and A. S. Schechter, *Nature (London)*, **222**, 476 (1969).
(9) B. Dietrich, J. M. Lehn, and J. P. Sauvage, *Tetrahedron Lett.*, 2889 (1969); J. M. Lehn and J. P. Sauvage, *Chem. Commun.*, 440 (1971).