[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, UNIVERSITY OF WISCONSIN]

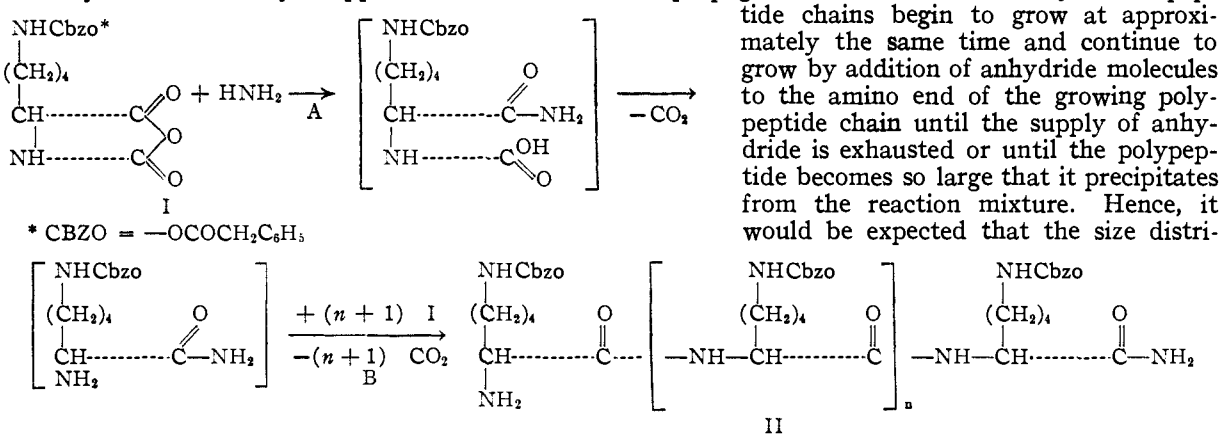
The Synthesis of High Molecular Weight Lysine Polypeptides[1,2]

BY R. R. BECKER AND MARK A. STAHMANN

The polymerization of ε-carbobenzoxy-α-carboxyl-lysine anhydride in dioxane was studied as a method for the synthesis of lysine polypeptides of varying chain length. By initiating the polymerization with ammonia and by varying the ratio of anhydride to initiator, polypeptide preparations containing an average from as few as 5 (molecular weight 1,500) to 240 (molecular weight 63,000) amino acid residues per polypeptide molecule were synthesized. The results of determinations of the average molecular weights of the resulting polypeptides as calculated from two end-group determinations, the relative rates of the polymerization reactions, and the optical rotation and viscosity of solutions of the poylpeptides all indicate that the average molecular weights of the synthetic polypeptides is dependent upon the ratio of initiator to anhydride.

A number of synthetic polypeptides have been prepared by the polymerization of the N-carboxyl anhydrides of amino acids.[3] The synthesis of a lysine polypeptide containing 32 amino acid residues was reported by Katchalski, Grossfeld and Frankel[4] who heated ε-carbobenzoxy-α-carboxyl-L-lysine anhydride in high vacuum. It has been pointed out that the polymerization of the N-carboxyl anhydrides is an initiated chain-polymerization reaction in which the molecular weight should be determined by the relative concentrations of monomer and initiator.[5,6] This has been emphasized by Coleman[3] who reported that one polymerization in dry benzene which was initiated by sodium phenylalaninate produced a polypeptide with the theoretical molecular weight as calculated from the mole ratio of initiator to anhydride. However, additional experimental data with molecular weight determinations to support the view that the molecular weight depends on the mole ratio of initiator to anhydride has not yet appeared. A ten-fold variation in the ratio of water, used as initiator, to anhydride did not significantly change the molecular weight of lysine polypeptides prepared by polymerization in benzene solution.[7] In this communication we wish to report the preparation of lysine polypeptides of widely different molecular weights which were obtained by polymerizing ε-carbobenzoxy-α-carboxyl-L-lysine anhydride with different ratios of ammonia (as initiator) to anhydride.

It is apparent from the reaction scheme that if the rate of the initiation reaction, reaction A, is rapid compared with the rate of propagation reactions that follow, *i.e.*, the decarboxylation and subsequent acylations, then the number of polypeptide chains that begin to grow at the start of the reaction will depend upon the amount of initiator. The molecular weight of the polypeptide that is formed will then be a function of the ratio of initiator to the monomeric anhydride. If the initiation reaction is sufficiently fast compared to the propagation reactions, then nearly all the peptide chains begin to grow at approximately the same time and continue to grow by addition of anhydride molecules to the amino end of the growing polypeptide chain until the supply of anhydride is exhausted or until the polypeptide becomes so large that it precipitates from the reaction mixture. Hence, it would be expected that the size distribution of the resulting polypeptide preparations should be also a function of the relative rates of the initiation and propagation reactions. When the initiation reaction is very rapid, a polypeptide preparation with a smaller spread in the molecular weight distribution should be obtained than when the initiation reaction is slow.



The failure to obtain polypeptides with different molecular weights when the amount of water used as the initiator was varied would indicate that the initiation reaction with water is much slower than the subsequent polymerization reactions. Since acid anhydrides generally react more rapidly with amino than hydroxy compounds, experiments were made in which the polymerization was initiated with

(1) Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by research grants from the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation and the National Institutes of Health, Public Health Service.

(2) Paper presented before the Division of Biological Chemistry, American Chemical Society, Chicago, September 5, 1950.

(3) H. Leuchs, *Ber.*, **39**, 857 (1906); T. Curtius and W. Sieber, *ibid.*, **55**, 1543 (1922); F. Wessely, *Z. physiol. Chem.*, **146**, 72 (1925); K. Meyer and Y. Go, *Helv. Chim. Acta*, **17**, 1488 (1934); M. Frankel and A. Berger, *Nature*, **163**, 213 (1949); W. E. Hanby, S. G. Waley and J. Watson, *ibid.*, **161**, 132 (1948); A. C. Farthing, *J. Chem. Soc.*, 3213 (1950); A. C. Farthing and D. Coleman, *ibid.*, 3218 (1950); D. Coleman, *ibid.*, 3222 (1950); W. E. Hanby, S. G. Waley and J. Watson, *ibid.*, 3009, 3239 (1950).

(4) E. Katchalski, I. Grossfeld and M. Frankel, THIS JOURNAL, **70**, 2094 (1948).

(5) R. B. Woodward and C. H. Schramm, *ibid.*, **69**, 1551 (1947).

(6) S. G. Waley and J. Watson, *ibid.*, **70**, 2299 (1948).

(7) M. A. Stahmann, L. Graf, E. Patterson, J. C. Walker and D. W. Watson, *J. Biol. Chem.*, **189**, 45 (1951).

various compounds including amines and ammonia.

To compare the rate of reaction in the presence of water and ammonia, the rate of carbon dioxide evolution was followed manometrically. Figure 1 shows that the reaction is much more rapid in the presence of small amounts of added ammonia than water, and also that there is a direct relationship between the reaction rate and the amount of ammonia added. When the molar ratio of monomer to ammonia was 5, the rate was approximately twice that when the ratio was 10. This would be expected since, at any one time, there should be twice as many growing chains in the reaction mixture containing the most ammonia. On the other hand, the rate of carbon dioxide evolution from those reaction mixtures with added water as initiator did not exceed that from the controls without added initiator.

Polymerizations for the synthesis of polypeptides with different molecular weights were run in dry dioxane solution, using anhydrous ammonia as the initiator at temperatures from 25 to 100°. The reaction mixtures were generally heated for a time considerably longer than that required for complete polymerization. The products which were obtained as white solids were dried and ground to pass a 60-mesh screen before analysis. Average molecular weights were determined by end-group analysis for the α-amino nitrogen content of the ε-carbobenzoxylysine polypeptides, using a modification of the van Slyke manometric nitrous acid method adapted for solids.[8]

The carbobenzoxy group was removed from the polypeptides by reduction with phosphonium iodide.[9] Katchalski, Grossfeld and Frankel[4] demonstrated that the chain length of their lysine polypeptides was not altered by this reduction. In the case of the shorter polypeptides, a second end group analysis for amide content was also made. Amide analysis of the lysine polypeptide hydrochlorides, obtained from the hydroiodides through the picrates, gave values for average chain length which agreed fairly well with those obtained by α-amino nitrogen analysis of the same carbobenzoxy polypeptide. This also indicates that removal of the carbobenzoxy group does not alter the chain length.

Experimental

ε-Carbobenzoxy-α-carboxyl-L-lysine Anhydride.—This was prepared according to the method of Bergmann[10] in about 80% yield, and recrystallized twice before polymerization. It was found that further recrystallizations did not improve the product or give better results in the polymerization studies; however, it was found necessary to use freshly prepared anhydride.

ε-Carbobenzoxy-L-lysine Polypeptides.—Polymerizations were usually run using from 5 to 25 g. of anhydride. A typical polymerization experiment is described below.

Twenty-five grams (82 mM) of ε-carbobenzoxy-α-carboxy-L-lysine anhydride was dissolved in 250 ml. of dry dioxane in a round-bottom flask equipped with a Hershberg stirrer and a reflux condenser. To this solution was immediately added 4.1 mM anhydrous ammonia dissolved in dry dioxane. The clear solution became slightly turbid on adding the initiator. Within 10 minutes a white product had started to collect on the sides of the flask. The flask was placed on a steam-bath after one hour. The solution became viscous, and formed a thick gel as the reaction proceeded. After 20 hours, 500 ml. of water containing 82 mM of hydrochloric acid was added to the flask and the mixture stirred for six hours to hydrolyze and dissolve any unreacted anhydride. The white amorphous product which separated upon the addition of water was filtered off, washed with water, and dried over P_2O_5 in a vacuum desiccator; yield 20 g., 93%.

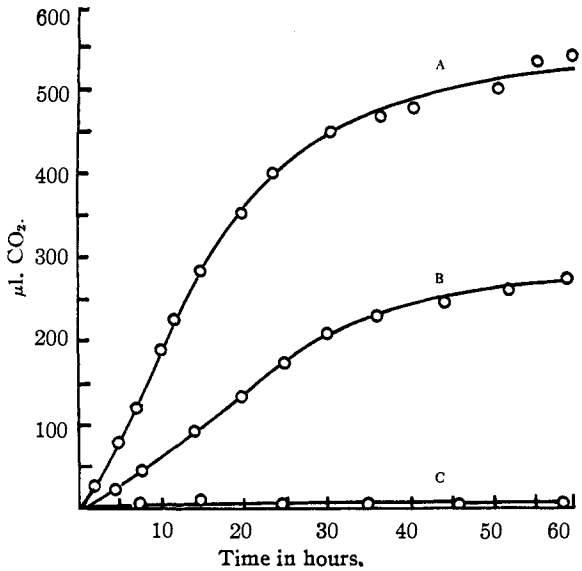


Fig. 1.—Rate of polymerization of ε-carbobenzoxy-α-carboxyl-L-lysine anhydride in dioxane with ammonia and water initiators: A, one-fifth mole ammonia; B, one-tenth mole ammonia; C, one-tenth mole water.

Reduction of ε-Carbobenzoxy-L-lysine Polypeptide.—Reductions were carried out by the method of Harrington and Mead[9] using phosphonium iodide. A typical experiment follows: 15 g. (57 mM) of ε-carbobenzoxy-L-lysine polypeptide (average chain length 19 units) was suspended in 220 ml. of glacial acetic acid under an atmosphere of hydrogen at 45–50°. Forty grams of phosphonium iodide was added in portions over a period of three hours, at which time evolution of CO_2 had ceased. The white suspension of lysine polypeptide hydroiodide was filtered off, washed with glacial acetic acid and ether, dissolved in 35 ml. of water, treated with activated carbon, and filtered. It was precipitated by addition of 150 ml. of absolute ethanol and 500 ml. of ether. This precipitation was repeated and the precipitate dissolved. The final aqueous solution was filtered, and concentrated to dryness in a desiccator. The dried film was ground to a powder for analysis. The yield was 13.9 g., 93%.

Anal. Calcd. for lysine polypeptide hydroiodide (av. chain length, 19 units): N, 10.6; I, 50.5. Found: N, 10.5; I, 49.7.

Lysine Polypeptide Hydrochloride.—To 6.7 g. of lysine polypeptide hydroiodide in 120 ml. of hot water was added 140 ml. of a hot saturated solution of picric acid. A yellow precipitate formed immediately. The mixture was allowed to stand at 4° overnight, and the yellow product collected by filtration, washed with cold water, and then suspended in 100 ml. of water containing 43 mM of HCl. The supension was extracted with ether until all the picric acid had been removed. The aqueous solution was filtered, concentrated *in vacuo*, and then dried to a clear film. The yield was quantitative based on the lysine polypeptide hydroiodide.

Anal. Calcd. for lysine polypeptide hydrochloride (av. chain length, 82 units); N, 17.0; Cl, 21.7. Found: N, 16.8; Cl, 22.0.

End-group Analyses.—For the analysis of α-amino nitrogen, a sample of an ε-carbobenzoxy-L-lysine polypeptide

(8) D. G. Doherty and C. L. Ogg, *J. Ind. Eng. Chem., Anal. Ed.*, **16**, 751 (1943).

(9) C. R. Harington and T. H. Mead, *Biochem. J.*, **29**, 1603 (1935).

(10) M. Bergmann, L. Zervas and W. F. Ross, *J. Biol. Chem.*, **111**, 245 (1935).

was ground to pass a 60-mesh screen, dried at 76° over P_2O_5, and analyzed according to the method of Doherty and Ogg[8] utilizing the reaction with nitrous acid in an auxiliary chamber of a manometric Van Slyke apparatus. About 90% of the nitrogen was liberated within five minutes; however, gas was collected and measured at intervals until nitrogen liberation had ceased. 51.2 and 50.8 mg. of the carbobenzoxy polypeptide from a reaction mixture initiated with one-fifth mole of ammonia gave 0.450 and 0.451 mg. of amino nitrogen, respectively, corresponding to a number average molecular weight of 1580 and average chain length of 6 amino acid residues.

In a typical analysis for amide nitrogen 142.4 mg. of a lysine polypeptide hydrochloride from a reaction mixture initiated with one-twentieth mole of ammonia were dissolved in 5 ml. of water and placed in the reaction chamber of a modified Markham micro Kjeldahl apparatus and 5 ml. of 8 *N* NaOH added. The mixture was refluxed for 30 minutes, and the ammonia then distilled into a 2% boric acid solution for ten minutes and titrated. Two additional fifteen-minute reflux periods and ten-minute distillation periods were used; the last distillation gave a titration value as low as the blank for the same period. 0.55 mg. amide nitrogen was found, corresponding to a molecular weight of about 3640, and an average chain length of 22. A duplicate analysis of 112.1 mg. yielded 0.43 mg. of amide nitrogen, a molecular weight of 3620 and an average chain length of 22. An α-amino nitrogen analysis of the carbobenzoxylysine polypeptide showed a chain length of 19 units.

Manometric Study of the Polymerization Reaction (Fig. 1).—Reactions initiated with sufficient water for a 10-unit polypeptide were followed by measuring the CO_2 release in a Warburg respirometer. Ten milligrams ϵ-carbobenzoxy-α carboxy-L-lysine anhyride dissolved in 1 ml. of dry dioxane was pipetted into each Warburg reaction vessel and the initiator dissolved in 0.5 ml. of dioxane added to the side arm. The flasks were then equilibrated in the Warburg bath (40.5°) for ten minutes, and the initiator was mixed with the anhydride solution and the CO_2 liberation measured for 59 hours. The reaction mixture with sufficient ammonia added for a 5 unit polypeptide had evolved 75% of the theoretical CO_2; the 10 unit 38%. The water initiated polymerizations had evolved only about 1% of the theoretical CO_2.

Discussion

The results of all the chemical studies of the products obtained from the polymerization of N-carboxyamino acid anhydrides indicate that the polymerization of these anhydrides results in the formation of high-molecular weight polypeptides.[3,4] It has also been shown that some of these synthetic polypeptides closely resemble certain proteins with respect to their X-ray diffraction pattern,[11-14] infrared absorption,[15] and attack by enzymes.[4] The chemical and physical properties of the products which were obtained in the present study of the ammonia initiated polymerization of ϵ-carbobenzoxy-α-carboxyllysine anhydride are consistent with their structure as lysine polypeptides which differ in their average molecular weights.

It will be noted from the data of Table I, that the average molecular weights of the polypeptides obtained from the polymerizations with different ratios of ammonia to anhydride agrees fairly well with those calculated from the ratio of anhydride to ammonia in those preparations with an average chain length of twenty lysine residues or less. The average molecular weight was lower than this ratio in the case of the higher molecular weight polypeptides. It was observed in this and other studies that polymerization of N-carboxyamino acid anhydrides does occur even in the absence of added initiator.[10,14,16] It would appear, therefore, that the polymerization may also be initiated by the thermal decomposition of the anhydride or by traces of impurities remaining in the anhydride preparation or in the solvents. This spontaneous initiation becomes much more important in the reactions in which only a small amount of initiator is added, and probably accounts for these molecular weights being lower than calculated from the ratio of monomer to initiator.

TABLE I

REPRESENTATIVE CARBOBENZOXYLYSINE POLYPEPTIDE PREPARATIONS

Ratio of anhydride to initiator	Temp., °C.	Polymerization time, hours	Average chain length	Average mol. wt.	Yield, %	$[\alpha]^{25}D$[a]
5	25	25	6, 7[b]	1,600	88	−37.6
5	100	1	5.5	1,450	84	
20	100	17	19, 22[b]	5,000	93	−70.2
20	65	24	22	5,800	99	
200	100	17	85	22,300	96	
200	100	44	82	21,600	93	
200	65	48	122	32,000	97	−78.8
500	100	44	158	41,400	99	−80.0
500 (DL)	65	56	240	63,000	95	

[a] The optical rotations were determined on the lysine polypeptides, concentration, 1.5 g./100 ml. 6 *N* HCl. [b] By amide analyses.

The difference between the α-amino nitrogen content of the carbobenzoxy polypeptide and the amide nitrogen content after the removal of carbobenzoxy groups indicate that in these cases, the products are about 85% straight chain polypeptides with a free α-amino group at one end and a carboxyamide group at the other end. It would seem probable that the remaining 15% consists of peptide chains with a free carboxyl group at one end which are formed by the spontaneous initiation mentioned above. These results and the variation in polypeptide size with change in ratio of initiator to anhydride support the view that the polymerization occurs through the initial reaction of one molecule of N-carboxyamino acid anhydride with a molecule of an initiator followed by decarboxylation and subsequent repeated additions (and decarboxylations) of anhydride molecules to the amino end of the growing polypeptide chain.

The optical rotations of some of the lysine polypeptide hydrochlorides are listed in Table I. These data show that the optical rotation increases as the molecular weight is increased. However, the data for these polypeptides do not support the suggestion by Brand[17] that "the optical rotation of peptides may be considered an additive function of the contributions of the asymmetric carbon atoms of the constituent amino acid residues." Instead, the rotation seems to approach a limit as the size of the polypeptide is increased. Viscosity measurements of aqueous solutions of the

(11) Y. Go and H. Tani, *Bull. Chem. Soc. Japan*, **14**, 510 (1939).

(12) W. T. Astbury, C. E. Dalgleish, S. E. Darman and G. B. B. M. Sutherland, *Nature*, **162**, 596 (1948).

(13) C. J. Brown, D. Coleman and A. C. Farthing, *ibid.*, **163**, 722 (1949).

(14) E. J. Ambrose and W. G. Hanby, *ibid.*, **163**, 483 (1949).

(15) I. Klotz, P. Griswold and D. M. Gruen, THIS JOURNAL, **71**, 1615 (1949).

(16) E. Miller, I. Fankuchen and H. Mark, *J. Applied Phys.*, **20**, 531 (1949).

(17) E. Brand and B. P. Erlanger, THIS JOURNAL, **72**, 3314 (1950).

lysine polypeptide hydroiodides showed an increase in viscosity with an increase in the molecular weight as calculated from the end-group analysis.

No attempt has yet been made to determine the molecular weight distribution of the synthetic lysine polypeptides. Waley and Watson[18] have calculated from kinetic data that the molecular weight distribution of polypeptides prepared by polymerization of N-carboxyamino acid anhydrides would be extremely sharp. Experiments in which the polypeptides were subjected to prolonged dialysis indicated that no significant quantity of the polypeptide containing an average of 158 lysine residues per molecule passed through a cellophane membrane. Microbiological assay of two polypeptide preparations containing an average of 6 and 158 lysine residues per molecule were carried out. These assays revealed that only 4% of the lysine of the shorter polypeptide was available for the growth of *Leuconostoc mesenteroides*. There was no growth even on high amounts of the larger polypeptide; furthermore, the polypeptide did not prevent utilization of added lysine. These results show that the peptide containing 158 lysine residues is essentially free of lysine, and small molecular weight polypeptides. The 6-unit polypeptide contains no more than 4% lysine and may actually be entirely free of lysine and be slowly utilized for growth.

Acknowledgment.—We express our appreciation to Mrs. Elaine G. Wood for technical assistance in the preparation of the anhydrides and the polypeptide analyses, and to Dr. R. J. Sirny for the microbiological assays.

(18) S. G. Waley and J. Watson, *Rec. trav. chim.*, **69**, 27 (1950).

MADISON 6 WISCONSIN RECEIVED MARCH 19, 1951

[CONTRIBUTION FROM THE CHEMICAL DEVELOPMENT DEPARTMENT OF SCHERING CORPORATION]

3β-Acetoxy-17α-hydroxynor-5-cholenic Acid Lactone, a By-product of the Oxidation of Sitosterol

BY AUGUST I. RYER AND WILLIAM H. GEBERT

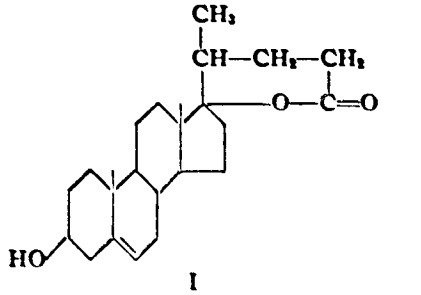
A lactone has been isolated from the products of the oxidation of sitosteryl acetate dibromide. The new compound, a lower homolog of the cholenic acid lactone described by Miescher and Fischer,[2] is shown to be 3β-acetoxy-17-hydroxynor-5-cholenic acid lactone. Evidence is presented which indicates that the configuration of the oxygen atom at C_{17} is probably α. A new 17-spiro compound has been prepared which throws some light on the stereochemistry of C_{20}.

Introduction

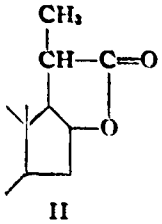
The oxidation of the dibromo acetates of cholesterol and sitosterol to produce dehydroepiandrosterone, a key intermediate for the synthesis of the sex hormones, is known to produce other ketones and acids as well.[1] Miescher and Fischer[2] reported the isolation of a lactone from the neutral products of the oxidation which was later shown by Billeter and Miescher[3] to be 3β,17-dihydroxy-5-cholenic acid lactone (I). Veer and Goldschmidt[4] isolated the same lactone but erroneously ascribed to it the structure of 3β,17-dihydroxy-5-norcholenic acid lactone (IV). This incorrect structure is also repeated in the patent literature.[5]

We isolated from the recrystallization mother liquors of dehydroepiandrosterone acetate, obtained by the oxidation of sitosteryl acetate dibromide, a new lactone. Carbon and hydrogen analyses of the product did not differentiate between a structure containing C_{22} or C_{23}. The infrared spectrum of the lactone showed a strong band at 5.58–5.62 μ which is typical of γ-lactones.[6]

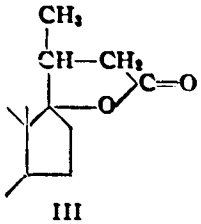
If the new product were a bisnorcholenic lactone, the point of attachment would be at C_{16} (II);



I



II



III

if a norcholenic lactone, the point of attachment would be at C_{17} (III). Since the hydroxyl group resulting from opening the lactone ring (XI) could not be readily acetylated, a tertiary hydroxyl located at C_{17} was indicated. The C_{22} structure was confirmed by converting the lactone to the known methyl, 3β-acetoxynor-5-cholenate (methyl 3β-acetoxy-Δ⁵-norcholenate).[7]

The new lactone was readily opened by treatment with Grignard reagents; with phenylmagnesium bromide the lactone acetate (V) gave the triol (VI). When the 3-acetate triol (VII) was treated with bromine in ethyl ether to form the 5,6-dibromo derivative, a dehydrated product was obtained

(1) L. F. Fieser and M. Fieser, "Natural Products Related to Phenanthrene," A. C. S. Monograph No. 70, Third Edition, Reinhold Publishing Corp., New York, N. Y., 1949, p. 364.

(2) K. Miescher and W. H. Fischer, *Helv. Chim. Acta*, **22**, 155 (1939).

(3) J. R. Billeter and K. Miescher, *ibid.*, **32**, 564 (1949); *ibid.*, **30**, 1409 (1947).

(4) W. L. C. Veer and St. Goldschmidt, *Rec. trav. chim.*, **66**, 75 (1947).

(5) Swiss Patent No. 236,582 (1945); L. Ruzicka, U. S. Patent No. 2,417,017 (1947).

(6) R. S. Rasmussen and R. R. Brattain, THIS JOURNAL, **71**, 1073 (1949).

(7) P. A. Plattner and J. Pataki, *Helv. Chim. Acta*, **28**, 1241 (1943).