[CONTRIBUTION FROM THE LABORATORY OF HIGH MOLECULAR CHEMISTRY, THE HEBREW UNIVERSITY]

Poly-condensation of α-Amino Acid Derivatives. III. Poly-lysine[1]

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In the previous papers of this series we have described the preparation and properties of some polymers of glycine[2a] and *d,l* alanine.[2b] As an extension of this work the study of the polymerization of poly-functional α-amino acids was undertaken. In the present paper the formation of poly-lysine by the polymerization of a 1-lysine derivative is described.

The following considerations had to be taken into account in the choice of a suitable monomer for polymerization: (a) the ε-amino group of the lysine had to be masked in order to prevent its participation in the polymerization and thus to insure that all the —CONH— bonds in the polymer are derived from the α-amino groups of the monomer. The ε-amino group was masked by Bergmann's method of introducing the carbobenzoxy group; (b) the monomer must consist of a lysine derivative having a pronounced tendency to polymerize. ε-Carbobenzoxylysine itself is comparatively stable owing to its zwitterionic structure, while its methyl ester yields mainly the corresponding diketopiperazine on heating;[3] both these lysine derivatives did not appear therefore to be the proper starting material. A more suitable monomer was eventually found in ε-carbobenzoxy-α-carboxyl-lysine anhydride.[4] It was found that this substance polymerizes on heating with the evolution of carbon dioxide in a manner similar to the polymerization of N-carboxyl anhydrides of other amino acids.[5]

The steps of preparation of poly-lysine from the chosen monomer are summarized in the first part of the scheme: ε-carbobenzoxy-α-carboxyl-1-lysine anhydride (I), yields on heating, under the catalytic action of water, poly-carbobenzoxy-lysine (II), carbon dioxide being evolved. The carbobenzoxy groups of (II) were removed by phosphonium iodide, and poly-lysine hydriodide (III) obtained.

For each polymerization experiment, the starting material, ε-carbobenzoxy-α-carboxyl-1-lysine anhydride, had to be freshly prepared from α,ε-carbobenzoxy-1-lysine kept in stock. This was necessary, as it was found that while the freshly prepared substance undergoes rapid polymerization at its melting point (100°), the same substance after having been allowed to stand for several weeks did not polymerize even at higher temperatures (160–170°), but decomposed slowly under such conditions. In this connection the observation of Bergmann, Zervas and Ross,[4] may be recalled that the melting point of freshly prepared ε-carbobenzoxy-α-carboxyl-1-lysine anhydride rises on standing several months to above 250°. Our attempts to recover the ε-carbobenzoxy-α-carboxyl-1-lysine anhydride with a melting point of 100° from the preparation having a high melting point by recrystallization were unsuccessful as no solvent for the latter was found.

During search for other precautions to be taken in the polymerization of ε-carbobenzoxy-α-carboxyl-1-lysine anhydride, the following findings were useful: (a) pure α,ε-di-carbobenzoxy-1-lysine, as well as pure carbobenzoxy-glycine, give no amino N values on using Van Slyke's[6] manometric method for amino N determination.

(b) ε-Carbobenzoxy-1-lysine yields practically the theoretical amount (96%) of carboxyl nitrogen in Van Slyke, MacFadyen and Hamilton ninhydrin–CO_2 method[7] when the reaction is carried out at *p*H 2.5.

(c) ε-Carbobenzoxy-α-carboxyl-1-lysine anhydride evolved an amount of carbon dioxide equivalent only to about 50% of its total α-nitrogen when analyzed by the usual ninhydrin–CO_2 method. The samples were boiled, after addition of citrate buffer, *p*H 2.5, for thirty seconds to remove performed carbon dioxide. The solution was chilled and the N-carboxyl determination carried out as usual after addition of ninhydrin.

Finding (a) shows that during the carrying out of the amino nitrogen determinations, no cleavage of the NH-carbobenzoxy bond, leading to the liberation of free NH_2 groups, occurs. It is thus possible to determine the free amino-N in materials containing NH-carbobenzoxy bonds, without fear that additional free amino groups, not present in the original material will be liberated on analysis. Finding (b) permits the determination of ε-carbobenzoxy-lysine in the presence of poly-carbobenzoxy-lysine. Furthermore, in view of (b) and (c), it is clear that high values of carboxyl-N (determined according to the ninhydrin–CO_2 method) indicate the presence of a considerable amount of either or both ε-carbobenzoxy lysine and ε-carbobenzoxy-α-carboxyl lysine anhydride. This conclusion enables us to estimate roughly the amount of "monomer" derivatives in the prepara-

(1) A manuscript under this title was received from these authors on January 21, 1946, for publication in the Journal. It was returned to them on February 21, 1946, for revision and further data.—The Editor.

(2) (a) Frankel and Katchalski, THIS JOURNAL, **64**, 2264 (1942); (b) Frankel and Katchalski, *ibid.*, **64**, 2268 (1942).

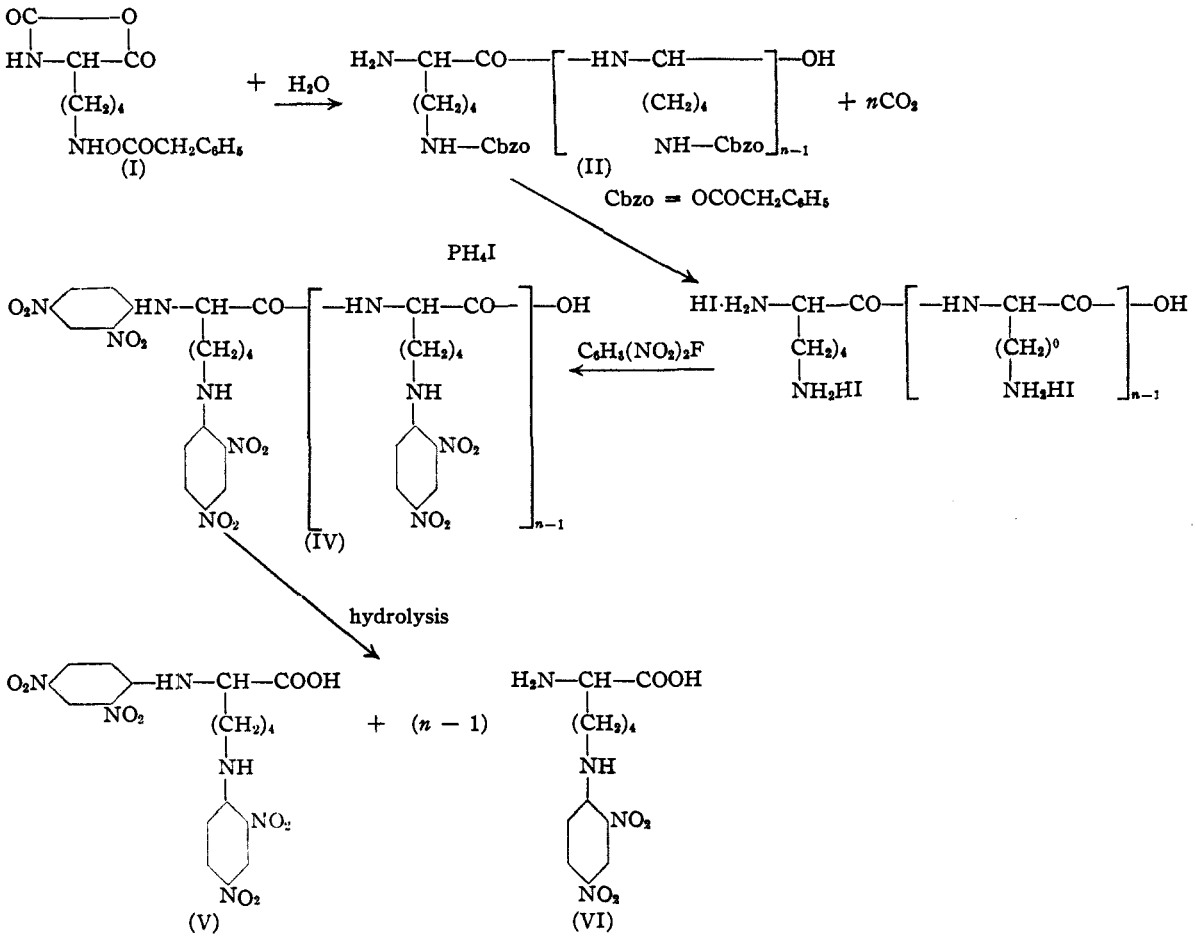
(3) Katchalski, Grossfeld and Frankel, *ibid.*, **68**, 879 (1946).

(4) Bergmann, Zervas and Ross, *J. Biol. Chem.*, **111**, 245 (1935).

(5) Leuchs, *Ber.*, **39**, 857 (1906); Leuchs and Manasse, *ibid.*, **40**, 3243 (1907); Leuchs and Geiger, *ibid.*, **41**, 1721 (1908); Curtius and Sieber, *ibid.*, **55**, 1543 (1922); Wessely, *Z. physiol. Chem.*, **146**, 72 (1925); Sigmund and Wessely, *ibid.*, **157**, 91 (1926); Wessely and Sigmund, *ibid.*, **159**, 102 (1926); Wessely and John, *ibid.*, **170**, 38 (1927); Meyer and Go, *Helv. Chim. Acta*, **17**, 1488 (1934); Go and Tani, *Bull. Chem. Soc. Japan*, **14**, 510 (1939); Woodward and Schramm, THIS JOURNAL, **69**, 1551 (1947).

(6) Van Slyke, *J. Biol. Chem.*, **83**, 425 (1929).

(7) Van Slyke, MacFadyen and Hamilton, *ibid.*, **141**, 671 (1941).



tions obtained on heating ε-carbobenzoxy-α-carboxyl lysine anhydride, even before removal of the carbenzoxy groups.

In Table I we summarize various analytical data concerning preparations obtained on heating ε-carbobenzoxy-α-carboxyl-lysine anhydride, purified in different ways and using different conditions of polymerization. Table II contains data concerning products derived on reduction of the corresponding preparations described in Table I.

Table I shows clearly that the purification of the ε-carbobenzoxy-α-carboxyl-lysine anhydride by recrystallization from ethyl acetate and petroleum ether and the thorough drying of freshly prepared monomer leads on heating to prepara-

TABLE I

ANALYTICAL DATA ON PREPARATIONS OBTAINED ON HEATING ε-CARBOBENZOXY-α-CARBOXYL-LYSINE ANHYDRIDE UNDER VARIOUS CONDITIONS

	Mg. of element (or group) per 100 mg. preparation					
Prepn.	Amino N	Carboxyl N	Total N	C	H	Remarks
(a)	2.00	1.09	10.7	63.5	6.9	Transparent brittle film, readily soluble in cold glacial acetic acid, insoluble in water
(b)	1.08	0.092	10.7	..	...	
(c)	0.22	0.025	10.8	64.2	6.8	Transparent film soluble in hot glacial acetic acid, insoluble in water
(d)	0.17	0.017	1.3	63.9	7.1	

(a) Obtained from ε-carbobenzoxy-α-carboxyl-1-lysine anhydride recrystallized once from ethyl acetate and petroleum ether and then dried for several days over sulfuric acid. The dried anhydride was left to polymerize in an open vessel at 105° for twenty-four hours.

(b) Obtained from ε-carbobenzoxy-α-carboxyl-1-lysine anhydride recrystallized twice from ethyl acetate and petroleum ether and dried in a 20-mm. vacuum over phosphorus pentoxide for forty-five minutes. Polymerization was carried out in the drying apparatus by raising the temperature to 104°, and maintaining this temperature for several hours.

(c) Obtained from ε-carbobenzoxy-α-carboxyl-1-lysine anhydride recrystallized three times from ethyl acetate and petroleum ether, and introduced immediately after the last recrystallization into a high vacuum apparatus containing phosphorus pentoxide and liquid air trap. The monomer was dried in this apparatus for three hours at 50° and then the temperature raised to 104° for two hours. A high vacuum (10^{-4} mm.) was maintained throughout. The polymeric preparation represents a hard, colorless transparent substance.

(d) The preparation of this product is described in detail under the heading preparation of poly-carbobenzoxy-lysine (*n* average = 32 carbobenzoxy-lysine units).

TABLE II

ANALYTICAL DATA ON PRODUCTS DERIVED FROM THE CORRESPONDING PREPARATIONS DESCRIBED IN TABLE I, ON REDUCTION WITH PHOSPHONIUM IODIDE

Product	Mg. of element (or group) per 10 mg. preparation: Amino, N	Carboxyl, N	Total, N	C	H	I	Remarks	Mg. group per 100 mg. preparate after hydrolysis: Amino, N	Carboxyl, N
(a')	5.40	1.120	10.8	28.4	5.3	50.8	Soluble in water	..	...
(b')	5.61	0.101	10.2	27.0	5.5	52.0		10.1	5.0
(c')	5.32	0.023	10.8	27.6	5.3	49.9	Transparent brittle film,	10.6	5.5
(d')	5.30	0.019	10.7	27.6	5.2	50.0	soluble in water	10.7	5.4

tions with a small percentage of free amino-N and carboxyl-N. In accordance, Table II shows that products obtained on reduction of the preparations prepared by heating purified and thoroughly dried ε-carbobenzoxy-α-carboxy-lysine anhydride, contain small amounts of free lysine. Some of the bulk products, (*cf.* b'c'd', Table II) obtained on reduction of the corresponding polycarbobenzoxylysine preparations, were totally hydrolyzed by boiling with 20% hydrochloric acid for twenty-seven hours. In the hydrolyzate, practically a quantitative yield of lysine was found.

The loss in weight of highly purified and thoroughly dried ε-carbobenzoxy-α-carboxyl-1-lysine anhydride during polymerization was measured. It was found that this loss is equivalent to 98–100% of the theoretical amount of carbon dioxide which could evolve on polymerization.

The presence of amino nitrogen in the preparations described in Table I and the fact that the amino N content of these preparations is considerably higher than that of the corresponding carboxyl-N's seems to indicate that water plays a certain role in the carbon dioxide cleavage of ε-carbobenzoxy-α-carboxyl-1-lysine anhydride, and its polymerization.

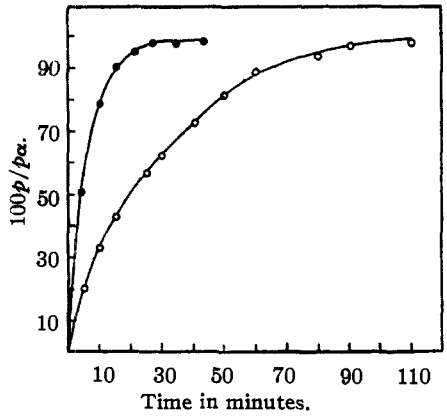


Fig. 1.—Per cent. of theoretical carbon dioxide, evolved on polymerization of ε-carbobenzoxy-α-carboxyl-lysine anhydride *versus* time at 102°: ●, monomer kept over water for forty minutes; ○, monomer dried over phosphorus pentoxide.

In order to evaluate the role of water in our case, the rate of carbon dioxide evolution of two samples of ε-carbobenzoxy-α-carboxyl-1-lysine anhydride containing different amounts of water was measured at 102° (*cf.* Fig. 1).

From these experiments, details of which are given in the experimental part, it became obvious that water acts as a catalyst during the polymerization of ε-carbobenzoxy-α-carboxyl-1-lysine anhydride. Catalytic action, but to a smaller extent, was found with methanol. This conclusion is in agreement with the findings of several previous authors,[5] who found that N-carboxyl anhydrides of various amino acids, while relatively stable in dry atmosphere, undergo rapid polymerization when exposed to moist air.

By taking into account the previously discussed precautions, we could obtain poly-carbobenzoxy-lysine polymers and poly-lysines virtually free of monomer and possessing a considerable chain length.

In the following, we describe in some detail the preparation and properties of one such polymer.

ε-Carbobenzoxy-α-carboxyl-lysine anhydride recrystallized six times, and dried in high vacuum (10^{-4} mm.) over phosphorus pentoxide at 60° for three hours, in an apparatus equipped with a liquid air trap, yielded on heating to 105° in high vacuum (10^{-4} mm.) for one hour, a transparent glassy hard polycarbobenzoxy-lysine. This polymer is insoluble in water and dissolves in hot glacial acetic acid. It contains free terminal amino groups determinable by Van Slyke's manometric method. Assuming that the above polymer is a mixture of poly-carbobenzoxy-lysine peptides of various chain lengths, an average chain length of 32-lysine units was calculated from the values of free terminal amino-N.

The carbobenzoxy groups of the poly-carbobenzoxylysine (n average = 32) were removed by reduction with phosphonium iodide according to the procedure used by Harington and Mead[8]; attempted reductions by the usual catalytic methods were unsatisfactory. Poly-lysine hydriodide (III) thus obtained, is very readily soluble in water, gives positive ninhydrin and biuret reactions, and negative picric acid test. The latter negative test indicated the absence of lysine anhydride in the poly-lysine hydriodide polymer obtained.

By making use of the ninhydrin–carbon dioxide method for determination of free amino acids it was found that (III n average = 32) contains a

(8) Harington and Mead, *Biol. Chem. J.*, **29**, 1603 (1935).

very small amount of free lysine (0.2 mg. free lysine per 100 mg. (III)).

Elementary analyses of poly-lysine hydriodide (*n* average = 32) and ratio of amino nitrogen to total nitrogen (found to be 1 to 2) are in agreement with the formula suggested. On hydrolysis, poly-lysine, yields lysine quantitatively.

Independent support for the proposed constitution for poly-lysine hydriodide (*n* average = 32) was obtained by using the procedure worked out by Sanger,[9] who found that 2,4-dinitrofluorobenzene reacts with the free amino groups of α-amino acids, peptides and proteins in the presence of sodium bicarbonate at room temperature with elimination of hydrogen fluoride. The dinitrophenyl-N-bond formed is relatively stable to acid hydrolysis. Thus, in the case of insulin, he was able, after coupling with 2,4-dinitrofluorobenzene and following acid hydrolysis, to isolate and determine quantitatively the 2,4-dinitrophenyl derivatives of those amino acids which in insulin bear the free amino groups.

On coupling poly-lysine hydriodide (*n* average = 32) with 2,4-dinitrofluorobenzene at room temperature, a yellow substance was obtained in almost quantitative yields. The low value of amino N (Van Slyke 0.03%) in the polymer (IV) indicates the blocking of all amino groups by 2,4-dinitrophenyl groups. Dinitrophenyl-poly-lysine (IV) was hydrolyzed in 50% (w/w) sulfuric acid during ten hours. The acid hydrolysate contains the α,ε-di-(2,4-dinitrophenyl)-lysine and ε-dinitrophenyl-lysine. The former is derived from the terminal lysine units of the poly-lysine containing two (α and ε) free amino groups, the latter from the other lysine units containing only one (ε) free amino group.

The α,ε-di-(2,4-dinitrophenyl)-lysine contained in this hydrolyzate was quantitatively extracted with ether, while the ε-2,4-dinitrophenyl-lysine remained in the aqueous acid solution. Both lysine derivatives were purified chromatographically; α,ε-di-(2,4-dinitrophenyl)-lysine by using butanol–chloroform 1% as developing solvent and ε-2,4-dinitrophenyl-lysine by using methyl ethyl ketone–ether (66%) as developing solvent. Water was the stationary phase in both cases. The R values found for α,ε-di-(2,4-dinitrophenyl)-lysine and ε-2,4-dinitrophenyl-lysine obtained from the acid hydrolyzate, were 0.6 and 0.2, respectively, under our conditions. The same values were obtained for the corresponding substances prepared from l-lysine. When each of the 2,4-dinitrophenyl-lysine derivatives, obtained from the polymer hydrolysate, was mixed with the corresponding synthetic substance, and each of the mixtures developed chromatographically by the suitable developing solvent only one yellow band was obtained which in each case showed the characteristic R value.

The quantitative estimations of α,ε-di-(2,4-dinitrophenyl)-lysine and ε-2,4-dinitrophenyl-lysine were carried out colorimetrically according to Sanger. Sanger found that during acid hydrolysis of 2,4-dinitrophenyl derivatives of α-amino acids, partial decomposition occurs. This is taken into account in the evaluation of the amount of the terminal amino acids with free amino groups in the insulin molecule. In order to determine the percentage decomposition of the two dinitrophenyl lysine derivatives, under the hydrolytic conditions applied in the present case, parallel experiments were carried out with the corresponding 2,4-dinitrophenyl-lysine derivatives obtained synthetically. It was found that on boiling with 50% (w/w) sulfuric acid for ten hours, 38% of α,ε-di-(2,4-dinitrophenyl)-lysine and 25% of ε-2,4-dinitrophenyl-lysine were decomposed. After correcting for the decomposition of each of the 2,4-dinitrophenyl-lysine derivatives during hydrolysis of 2,4-dinitrophenyl-poly-lysine, the amounts and ratio between α,ε-di-(2,4-dinitrophenyl)-lysine and ε-2,4-dinitrophenyl-lysine were determined; 4.80 mg. of α,ε-di-(2,4-dinitrophenyl)-lysine, and 98 mg. of ε-2,4-dinitrophenyl-lysine per 100 mg. of 2,4-dinitrophenyl-poly-lysine, were found. The ratio of the two compounds as determined in the hydrolysate was therefore: 31 moles of ε-2,4-dinitrophenyl-lysine per 1 mole of α,ε-di-(2,4-dinitrophenyl)-lysine.

The above data indicate that poly-lysine hydriodide from which the 2,4-dinitrophenyl-poly-lysine was obtained, is built up on the average of 31 lysine units. This conclusion is in satisfactory agreement with the average chain length ascribed to the above poly-lysine hydriodide from the value of the amino-N of the poly-carbobenzoxy-lysine from which it had been derived by reduction.

It must be emphasized, however, that the considerable destruction of both dinitrophenyl-lysine derivatives during hydrolysis on the one hand, and the low amino nitrogen content of poly-carbobenzoxy-lysine on the other, may lead to some uncertainty in the estimation of the average chain length. Nevertheless, the agreement between the two analytical methods justifies, in our opinion, the drawing of a conclusion concerning the order of magnitude of the average chain length of the poly-lysine derivatives synthesized.

From poly-lysine hydriodide (*n* average = 32), the picrate, picrolonate, hydrochloride and benzoyl derivatives were prepared.

An indication for the presence of polymers of various chain lengths was found by carrying out a rough fractionation experiment. Poly-carbobenzoxy-lysine, containing a very small amount of monomeric lysine derivatives, yielded three fractions which differed in terminal amino nitrogen content.

Preliminary enzymatic experiments were carried out with poly-lysine and lysine anhydride. It was found that poly-lysine is split by glycerol

(9) Sanger, *Biochem. J.*, **39**, 507 (1945).

extract of pancreatin as well as by crystalline trypsin. About 50% of the peptide bonds were split. Lysine anhydride is not split by pepsin.

Experimental

Preparation of ε-Carbobenzoxy-α-carboxyl-l-lysine Anhydride.—l-Lysine was prepared from the red corpuscles of ox blood according to Rice[10]; from it ε-carbobenzoxy-α-carboxyl-lysine anhydride was prepared according to Bergmann, Zervas and Ross.[4] The anhydride was recrystallized several times from ethyl acetate and petroleum ether, and the purified starting material used at once for polymerization experiments. The freshly purified product dissolves readily in ethyl acetate, and its m. p. is 100°.

Estimation of Carbon Dioxide from ε-Carbobenzoxy-carboxyl-lysine Anhydride on Heating to 102°.—1.140 g. of twice recrystallized ε-carbobenzoxy-α-carboxyl-lysine anhydride dried over phosphorus pentoxide *in vacuo* was kept at 102° for two hours. Carbon dioxide evolution started on melting and a transparent, glassy polymer residue was obtained. The weight of polymer residue was found to be 0.979 g. The weight of carbon dioxide evolved (161 mg.) represents 98.5% of the theoretical.

Rate of Carbon Dioxide Evolution at 102° from ε-Carbobenzoxy-α-carboxyl-lysine Anhydride (a) Kept over Phosphorus Pentoxide; (b) Kept over Water.—The rate of carbon dioxide evolution at 102° was measured in an apparatus similar to that described by Hinshelwood.[11] ε-Carbobenzoxy-α-carboxyl-lysine anhydride twice recrystallized was dried over phosphorus pentoxide *in vacuo* during two days at room temperature. One sample from the dried preparation was put at once into Hinshelwood's apparatus, the apparatus sealed, and placed in a constant temperature bath at 102°. The rate of carbon dioxide evolution was calculated from the manometric readings of the apparatus.

The second sample of the dried ε-carbobenzoxy-α-carboxy-lysine anhydride, weighing 67.85 mg., was put into a desiccator over water for forty minutes. After this period it was found to have gained in weight 0.870 mg. The wet product was put into Hinshelwood's apparatus and the rate of carbon dioxide evolution at 102° measured as above.

A comparison of the rate of carbon dioxide evolution in both cases is given in Fig. 1.

The increase in rate of carbon dioxide evolution in the presence of water may be at least partly explained by the catalytic action of water on the polymerization of ε-carbobenzoxy-α-carboxyl-lysine anhydride according to the first step of the general scheme. Nevertheless, it should be borne in mind that water may open the anhydride bond of the N-carboxyl anhydride, and thus lead to carbobenzoxy-lysine with carbon dioxide evolution. Regarding the latter possibility, it should be remarked, that for quantitative transformation of ε-carbobenzoxy-α-carboxyl-lysine anhydride into ε-carbobenzoxy-lysine, water in an amount of 5.9% of the weight of the anhydride is needed, whereas only 1.3% of water was taken up by ε-carbobenzoxy-α-carboxyl-lysine anhydride in our case. The fact that this amount of water is sufficient to shorten the time of cleavage, involving the loss of 95% of the theoretical amount of carbon dioxide in ε-carbobenzoxy-α-carboxyl-lysine anhydride, from ninety-five minutes to twenty minutes, justifies the conclusion that water acts under our experimental conditions mainly as polymerization catalyst. This is borne out also by the polymeric properties of the reaction products.

Preparation of Poly-carbobenzoxy-lysine (*n* average = 32 Carbobenzoxy-lysine Units).—The starting ε-carbobenzoxy-α-carboxyl-lysine anhydride for the polymerization, was recrystallized six times from ethyl acetate and petroleum ether. The purified product was introduced immediately into a glass vessel connected to an apparatus with phosphorus pentoxide and a liquid air trap and dried in high vacuum (10^{-4} mm.) at 60° for three hours. The temperature was then elevated to 105°; carbon dioxide evolution started at once with the melting of the anhydride. After one hour no further gas evolution was observed; a transparent, glassy hard residue remained in the reaction vessel.

The poly-carbobenzoxy-lysine preparation thus obtained is insoluble in water, ether or toluene. It is soluble in hot glacial acetic acid, and slightly soluble in hot alcohol. Part of the polymer precipitates from its solution in acetic acid on cooling, and from the supernatant solution a further precipitate of polymer may be obtained on adding water.

On heating the mixture of poly-carbobenzoxy-lysine in water with ninhydrin for half an hour, the polymers turn deep blue, while the water remains almost colorless.

The analytical data obtained correspond to a polymer having formula (II) with an average chain length $n = 32$.

Anal. Calcd. for (II) (*n*-average = 32 units): C, 64.0; H, 6.9; N, 10.6; amino N, 0.17. Found: C, 63.9; H, 7.1; N, 10.3; amino N, 0.17. The total nitrogen determinations, in this case as in other analytical data given in this paper, were carried out by the micro Dumas method.

Poly-lysine Hydriodide (*n* average = 32).—One gram of the above poly-carbobenzoxy-lysine (*n* average = 32) was dissolved in 25 ml. of hot glacial acetic acid and the solution kept at 50°, while a stream of dry hydrogen was passed through it; 4 g. of phosphonium iodide was added in portions of about 1 g. during one to one and one-half hours. The reduction of the carbobenzoxy groups was accompanied by a strong evolution of carbon dioxide. During the reduction, the phosphonium iodide dissolved, and a voluminous precipitate formed on the walls of the vessel. At the end of the reaction, the clear liquid was decanted off and the residue washed several times with dry ether and dissolved in 2 ml. of water; to this solution about 5 ml. of absolute alcohol and 70 ml. of ether were added. After standing overnight in the ice box, the supernatant fluid was decanted from the viscous material which had separated out. The latter was then dissolved in several ml. of water, and the solution filtered and evaporated to dryness in a vacuum desiccator over sulfuric acid and sodium hydroxide. The transparent, solid film-like polymer thus obtained was washed several times with ether and dried. It was obtained in almost quantitative yield. Poly-lysine hydriodide was dried in a micro vacuum desiccator over phosphorus pentoxide at 80° before analysis.

Anal. Calcd. for (III) (*n* average = 32): C, 27.7; H, 5.0; N, 10.7; amino N, 5.5; I, 50.2. Found: C, 27.6; H, 5.2; N, 10.7; amino N, 5.3; I, 50.0. The amino N value was obtained after shaking the polymer solution with HNO_2 for five minutes; this value was not altered on further shaking for half an hour.

Poly-lysine hydriodide dissolves very readily in water and its aqueous solution gives a positive ninhydrin reaction and a strong biuret reaction. A negative Abderhalden test was obtained on heating with an alkaline solution of picric acid.

Poly-lysine hydriodide does not dissolve in the usual organic solvents.

Search for Free Lysine in Poly-lysine Hydriodide (*n* average = 32).—The amount of free lysine in the poly-lysine hydriodide described above was determined by the ninhydrin-carbon dioxide method at *p*H 2.5. In 100 mg. of polymer product described in the previous section 0.2 mg. of free lysine was found.

Total Hydrolysis of Poly-lysine Hydriodide (*n* average = 32).—15.9 mg. of dried poly-lysine hydriodide was dissolved in 4 ml. of 20% hydrochloric acid and the solution boiled under reflux for twenty-four hours. The acid hydrolyzate was neutralized with sodium hydroxide and brought to 15 ml.

In 2 ml. of the final solution the amount of carboxyl N was determined by using the ninhydrin–carbon dioxide method[7]; in the other 2 ml., the total free amino N (Van

(10) Rice, *J. Biol. Chem.*, **131**, 1 (1941).

(11) Hinshelwood, *J. Chem. Soc.*, **117**, 156 (1920).

Slyke's manometric method—on shaking half an hour with nitrous acid—was determined. From the data obtained, the total amounts of carboxyl N and amino N in hydrolyzate were calculated. The amount of these groups per 100 mg. starting material are given below.

Anal. Calcd. for hydrolysis of 100 mg. poly-lysine hydriodide (*n* average = 32). Carboxyl-N 5.50 mg.; amino N, 10.60 mg. Found: carboxyl N, 5.4 mg.; amino N, 10.7 mg.; on hydrolysis of 100 mg. of starting material.

The analytical data show that the total amount of amino-N is equal to twice that of carboxyl N and thus indicates the presence of free lysine in the hydrolyzate. Furthermore, it is proved that the starting poly-lysine hydriodide is built up quantitatively of lysine units.

Preparation of 2,4-Dinitrophenyl-poly-lysine.—150 mg. of poly-lysine hydriodide (*n* average = 32) and 3.1 g. of sodium bicarbonate were dissolved in 30 ml. water; 30 ml. of ethanol and 5 ml. of 2,4-dinitrofluorobenzene were then added and the mixture mechanically shaken for two hours. A large excess of 2,4-dinitrofluorobenzene was necessary in order to assure complete coupling of the large number of free amino groups in poly-lysine hydriodide with the 2,4-dinitrophenyl reagent. The yellow precipitate of 2,4-dinitrophenyl-poly-lysine was centrifuged and washed several times with water and ethanol and dried in a vacuum desiccator. 2,4-Dinitrophenyl-poly-lysine is a yellow powder, insoluble in water, alcohol, ether, and glacial acetic acid. It is soluble in concentrated sulfuric acid, and can be precipitated from it by addition of water.

Anal. Calcd. for 2,4-dinitrophenyl-poly-lysine (*n* average = 32): amino N, 0.0; N, 18.9. Found: amino N, 0.03; N, 19.0.

The amino N value found indicates that practically all of the amino groups of poly-lysine reacted with 2,4-dinitrofluorobenzene.

Hydrolysis of 2,4-Dinitrophenyl-poly-lysine; Identification and Estimation of α,ε-Di-(2,4-dinitrophenyl)- and ε-2,4-Dinitrophenyl-lysine Formed.—2,4-Dinitrophenyl-poly-lysine is not hydrolyzed quantitatively in 20% hydrochloric acid even on boiling for extended periods, as clumps of polymer settle to the bottom of the vessel and do not enter into solution. We thus looked for another acid in which the polymer would dissolve more readily before hydrolysis. It was found that total hydrolysis of 2,4-dinitrophenyl-poly-lysine can be carried out in 50% (w/w) sulfuric acid; the following hydrolytic experiment was therefore carried out in this medium.

Twenty mg. of 2,4-dinitrophenyl-poly-lysine was boiled under reflux for ten hours in 20 ml. 50% (w/w) sulfuric acid. After cooling, the solution was extracted five times with ether. The first two ether extracts showed a clear yellow color which could be attributed to the presence of α,ε-di-(2,4-dinitrophenyl)-lysine in the hydrolyzate.

The ether extracts were washed with a small amount of water and the washings returned to the original aqueous solution. The collected ether extracts were reduced to dryness, and passed through an ether column prepared from 2 g. of silica, and again evaporated to dryness, and developed on a 1% butanol–chloroform column. There was a red band that moved rapidly (R = 1.5) and a yellow band which moved much more slowly (R = 0.6). After repeating the separation between the two bands on a 3 g. of 1% butanol–chloroform column, the red band was decanted and not explored further, as it seemed to represent a breakdown product of hydrolysis. The R of the yellow band was that found for synthetic α,ε-di-(2,4-dinitrophenyl)-lysine prepared from lysine and 2,4-dinitrofluorobenzene. Furthermore, in a parallel hydrolysis experiment, it was found that on mixing the fraction of yellow band with synthetic α,ε-di-(2,4-dinitrophenyl)-lysine, evaporating to dryness and developing with 1% butanol–chloroform one band only with the anticipated R was formed.

The solution of the yellow band is thus identified as the solution of α,ε-di-(2,4-dinitrophenyl)-lysine.

In order to estimate the α,ε-di-(2,4-dinitrophenyl)-lysine formed by the hydrolysis of 2,4-dinitrophenyl-poly-lysine quantitatively, the fraction of yellow band mentioned above was run out, taken to dryness, the residue dissolved in 50 ml. of chloroform and estimated colorimetrically, with a photoelectric absorptiometer after plotting the standard curve of synthetic α,ε-di-(2,4-dinitrophenyl)-lysine in chloroform; 0.596 mg. was found.

The acid solution, after extraction with ether, was diluted to 500 ml. with water, and the sulfuric acid removed quantitatively by means of barium chloride. The precipitate of barium sulfate was filtered off and washed several times with water. The combined filtrate and washings were made up to 1000 ml. A 100-ml. sample from this solution was withdrawn and evaporated to dryness. The residue was passed through a 66% methyl ethyl ketone ether column on silica gel. One yellow band was formed having R = 0.2. The same R was obtained when synthetic ε-2,4-dinitrophenyl-lysine prepared according to Sanger was passed through this column, and when the residue from aqueous solution of hydrolyzate, described above, in another hydrolytic experiment, was mixed with synthetic ε-2,4-dinitrophenyl-lysine and the mixture passed through a 66% methyl ethyl ketone ether column. The presence of ε-2,4-dinitrophenyl-lysine in the aqueous solution of hydrolyzate is thus proved.

In order to determine quantitatively the amount of ε-2,4-dinitrophenyl-lysine obtained, the yellow band in the original column was run out, taken to dryness and made up to 50 ml. with N hydrochloric acid. The amount of ε-2,4-dinitrophenyl-lysine in this solution was estimated colorimetrically. A total amount of 14.7 mg. of ε-2,4-dinitrophenyl-lysine was found in the 1000 ml. of aqueous solution.

In order to estimate the amount of α,ε-di-(2,4-dinitrophenyl)-lysine and ε-2,4-dinitrophenyl-lysine, decomposing under the conditions prevailing in the hydrolysis of 2,4-dinitrophenyl-poly-lysine, weighed amounts of synthetic α,ε-di-(2,4-dinitrophenyl)-lysine and ε-2,4-dinitrophenyl-lysine were kept in boiling 50% (w/w) sulfuric acid for ten hours. In the first case, the amount of α,ε-di-(2,4-dinitrophenyl)-lysine and in the second case, the amount of ε-2,4-dinitrophenyl-lysine which remained unaffected by the acid were determined as above.

A breakdown of 25% of ε-2,4-dinitrophenyl-lysine and a breakdown of 38% of α,ε-di-(2,4-dinitrophenyl)-lysine were observed under the hydrolytic conditions used.

If the values obtained on the hydrolysis of 2,4-dinitrophenyl-poly-lysine are corrected by the last data for the breakdown of ε-2,4-dinitrophenyl-lysine and α,ε-di-(2,4-dinitrophenyl)-lysine, the following yield from 100 mg. of 2,4-dinitrophenyl-poly-lysine of α,ε-di-(2,4-dinitrophenyl)-lysine and ε-2,4-dinitrophenyl-lysine, are obtained: 4.80 mg. of α,ε-di-(2,4-dinitrophenyl)-lysine and 98.0 mg. of ε-2,4-dinitrophenyl-lysine.

Although as already remarked by Sanger, "the correction may not be strictly valid, as the stability of the derivatives while still condensed in the protein or in peptide split products may not be the same as that found for the derivatives themselves," it seems that at least a good approximation for the real estimation of the two 2,4-dinitrophenyl-lysine derivatives is thus obtained.

It can be seen that 31 moles of ε-2,4-dinitrophenyl-lysine per 1 mole of α,ε-di-(2,4-dinitrophenyl)-lysine was obtained in the hydrolyzate. As pointed out previously, these data are in fair agreement with the suggested structure for poly-lysine hydriodide (*n* average = 32).

Proof for the Practical Absence of α,ε-Di-(2,4-dinitrophenyl)-lysine, in 2,4-Dinitrophenyl-poly-lysine.—In order to prove that the α,ε-di-(2,4-dinitrophenyl)-lysine is derived entirely from the original terminal lysine units of the 2,4-dinitrophenyl polymer, and does not partly originate from the coupling of 2,4-dinitrofluorobenzene with any free lysine monomer in the poly-lysine hydriodide (*n* average = 32), the following experiment was carried out.

A sample of 30 mg. of 2,4-dinitrophenyl-poly-lysine

was dissolved without heating in 10 ml. of concentrated sulfuric acid, 20 ml. of water was slowly added under strong cooling, and the mixture shaken vigorously with ether. No yellow color indicating the presence of α,ϵ-di-(2,4-dinitrophenyl)-lysine appeared in the ether extract.

On the other hand, when a sample of 2,4-dinitrophenyl-poly-lysine dissolved in 50% (w/w) sulfuric acid was boiled for about thirty minutes and the mixture then extracted with ether, the formation of small amounts of α,ϵ-di-(2,4-dinitrophenyl)-lysine due to hydrolysis was apparent from the faint yellow color of the ether extract.

Poly-lysine Picrate.—100 mg. of poly-lysine hydriodide (n average = 32) was dissolved in 2 ml. of hot water, and 2 ml. of hot saturated solution of picric acid added. After standing overnight in the ice box, the yellow picrate was filtered off and washed several times with cold water and ether. Yield was 85% of the theoretical.

The product was thoroughly dried in vacuum desiccator at 100° before analysis.

Anal. Calcd. for poly-lysine picrate (n average = 32): C, 40.5; H, 4.2; N, 19.5. Found: C, 40.1; H, 4.4; N, 19.4.

The picrate is soluble in hot water, slightly soluble in alcohol but insoluble in ether and benzene.

Poly-lysine Picrolonate.—100 mg. of poly-lysine hydriodide (n average = 32) was dissolved in 2 ml. of water, and 5 ml. of hot saturated solution of picrolonic acid in water added. A voluminous precipitate separated at once. After standing overnight in the icebox, the precipitate was filtered and washed several times with water and ether. The material was thoroughly dried in a vacuum desiccator at 100° before analysis.

Anal. Calcd. for polylysine picrolonate (n average = 32): C, 49.0; H, 4.9; N, 21.4. Found: C, 48.6; H, 5.1; N, 21.1.

It is sparingly soluble in water and insoluble in the usual organic solvents.

Benzoyl-poly-lysine.—100 mg. of poly-lysine hydriodide (n average = 32) was dissolved in 2 ml. of water, 400 mg. of sodium bicarbonate added and the mixture cooled to 0°. On further cooling, 0.5 ml. of benzoyl chloride in five portions was added with vigorous shaking. After forty-five minutes, the mixture was acidified to congo red with concentrated hydrochloric acid, and the white precipitate decanted and thoroughly washed with water, alcohol and ether. Benzoyl-poly-lysine was obtained in 80% yield as a white powder. It is soluble in hot glacial acetic acid and concentrated sulfuric acid, but insoluble in ether, alcohol and water. For analysis the polymer was dried in vacuum desiccator at 100°.

Anal. Calcd. for benzoyl poly-lysine (n average = 32): N, 11.86. Found: N, 11.60.

Poly-lysine Hydrochloride.—0.65 ml. of N hydrochloric acid was added to 150 mg. of poly-lysine picrate suspended in 5 ml. of water. The mixture was shaken several times with ether to remove the picric acid liberated. An equal volume of alcohol was then added to the aqueous solution and the mixture dried at room temperature in a vacuum desiccator over sulfuric acid and sodium hydroxide. The remaining hydrochloride was washed several times with dry ether and dried *in vacuo*. The yield was quantitative. The hydrochloride was further dried at 80° *in vacuo* over phosphorus pentoxide before analysis.

Anal. Calcd. for poly-lysine hydrochloride (n average = 32): Cl, 22.3. Found: Cl, 22.0.

Poly-lysine hydrochloride is very soluble in water and gives a strong violet biuret reaction. The ninhydrin reaction is positive, and when heated with an alkaline solution of picric acid, no red coloration is obtained. Polylysine hydrochloride is insoluble in usual organic solvents.

Preliminary Experiment in the Fractionation of a Poly-carbobenzoxy-lysine Preparation.—This experiment was carried out with the material described under Table IC; this preparation contains a very small percentage of carboxyl-N indicating a very small percentage of "monomers." 100 mg. of polymer was dissolved in 5 ml. of hot glacial acetic acid. The solution was allowed to cool and the precipitate (fraction 1) filtered. To the filtrate an equal amount of water was added and the turbid mixture formed allowed to stand overnight in the icebox. The white precipitate (fraction 2) was filtered off and the filtrate concentrated *in vacuo* to dryness (fraction 3). In the three fractions the free amino N (Van Slyke) was determined.

Fraction	Weight of fraction, mg.	Amino N, %
1	18	0.18
2	63	.23
3	19	.30

Discussion

The structure suggested for the polymeric derivatives obtained from ϵ-carbobenzoxy-α-carboxyl-lysine anhydride (*cf.* scheme) calls for proof of the presence of peptide bonds and free α-amino and α-carboxyl terminal groups.

The presence of the assumed –CONH– bonds is indicated by the positive biuret reaction, and proved by the formation of the expected equivalent amounts of α-amino and α-carboxyl groups on hydrolysis of poly-lysine hydriodide. The peptide structure of poly-lysine is also supported by the fact that crystalline trypsin causes cleavage of this polymer. This enzymatic result is in agreement with the findings of Bergmann and Fruton,[12] that a typical trypsin substrate has to contain lysine or arginine residues.

Furthermore, the method of synthesis of poly-lysine and the fact that it yields on hydrolysis lysine quantitatively, leave little doubt that the polymer is built up of lysine residues bound by normal peptide bonds.

The positive ninhydrin reaction in poly-lysine hydriodide and poly-carbobenzoxy-lysine indicates the presence of α-amino groups. The presence of the α-amino end-group in poly-carbobenzoxy-lysine is proved by the Van Slyke manometric amino nitrogen determination, while the presence of the same terminal group in poly-lysine hydriodide is proved by the isolation of α,ϵ-di-(2,4-dinitrophenyl)-lysine from 2,4-dinitrophenyl-poly-lysine. The formation of the terminal groups can be explained by the observed catalytic action of water on the polymerization of ϵ-carbobenzoxy-α-carboxyl-lysine anhydride.

The above facts support our view that the poly-lysine derivatives described in this paper represent mixtures of straight chains of poly-lysine peptides probably of various chain length; nevertheless the presence of high rings in the polymeric mixtures cannot be excluded.

In connection with our previous work on the polycondensation of esters of α-amino acids,[1,2] the absence of diketopiperazine in the product obtained on polymerization of ϵ-carbobenzoxy-α-carboxyl-lysine anhydride is of interest. While

(12) Bergmann and Fruton, "Advances in Enzymology," I, 75 (1941).

the methyl ester of ε-carbobenzoxy-lysine yields on heating mainly ε,ε'-dicarbobenzoxy-lysine anhydride,[3] the corresponding N-carboxyl anhydride yields a polymer without any diketopiperazine. This difference may be connected with the fact that polycondensation of α-amino acid esters proceeds slowly, while the polymerization of the corresponding N-carboxyl anhydrides is rapid. Thus the methyl ester of ε-carbobenzoxy-lysine condenses to ε,ε'-di-carbobenzoxy-lysine anhydride at 105° over a period of several days, while the polymerization of ε-carbobenzoxy-α-carboxyl-lysine anhydride is accomplished under the same conditions within an hour.

Summary

ε-Carbobenzoxy-α-carboxyl-lysine anhydride (I) when heated to 102–105° evolves carbon dioxide and yields a polymeric preparation to which the structure of poly-carbobenzoxy-lysine (II) is ascribed.

The products of polymerization of (I) obtained under various conditions were studied.

On polymerization of (I) under special precautions, a poly-carbobenzoxy-lysine was obtained with an average chain length of 32-carbobenzoxy-lysine units.

Poly-carbobenzoxy-lysine (II) (*n* average = 32) yields on reduction with phosphonium iodide poly-lysine hydriodide (III) (*n* average = 32).

Poly-lysine hydriodide (*n* average = 32) contains practically no free lysine or lysine anhydride. On hydrolysis it yields lysine quantitatively.

The following derivatives were prepared from (III) (*n* average = 32): picrate, picrolonate, hydrochloride, benzoyl and 2,4-dinitrophenyl-poly-lysine.

The suggested formula for poly-lysine (*n* average = 32) is supported by the analytical data, and by the fact that 2,4-dinitrophenyl-poly-lysine (*n* average = 32) yields the expected amounts of α,ε-di-(2,4-dinitrophenyl)-lysine and ε-2,4-dinitrophenyl-lysine on hydrolysis.

Independent support for the presence of peptide bonds in (III) is given by its cleavage with crystalline trypsin.

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[CONTRIBUTION FROM THE WESTERN REGIONAL RESEARCH LABORATORY[1]]

Phosphorylation of Proteins with Phosphoric Acid Containing Excess Phosphorus Pentoxide

BY ROBERT E. FERREL, HAROLD S. OLCOTT AND HEINZ FRAENKEL-CONRAT

When proteins are treated with cold concentrated sulfuric acid, the principal reaction is a transformation of the aliphatic hydroxyl groups to half-esters of sulfuric acid.[2,3] The present study was undertaken in order to determine whether analogous reactions occur when proteins are treated with phosphoric acid containing excess phosphorus pentoxide. Levene and Schormüller[4] had used such a reaction mixture for the preparation of *o*-phosphoric acid esters of serine, hydroxyproline and serine anhydride in small yield, and Plimmer,[5] using the same medium at elevated temperatures, had duplicated their findings and also reported the preparation of *o*-phosphoric acid esters of tyrosine, threonine and isoserine.

In general, the reaction was carried out by permitting a mixture of the material to be treated and the phosphoric acid–phosphorus pentoxide reagent to stand for three days at room temperature in a dry atmosphere (desiccator). The product was isolated by pouring the reaction mixture over cracked ice, neutralizing, and dialyzing, first against ion-free water, then against 10% sodium chloride, and finally against distilled water until free of inorganic phosphates. Recoveries ranged from 70 to 100% based upon nitrogen analyses. The extent of reaction was estimated from the phosphorus-to-nitrogen ratio of the product.

Of the many polar groups in proteins available for reaction, only the aliphatic hydroxyl groups of serine, threonine and hydroxyproline, and to some extent the aromatic hydroxyl group of tyrosine were found to bind phosphorus in a stable manner.[6]

The basic groups and the peptide bonds, however, appear to be responsible for an additional amount of phosphate, retained during dialysis against water but liberated by high salt concentration. Part of the peptide bonds were labile to

(1) Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture. Article not copyrighted.

(2) Reitz, Ferrel, Fraenkel-Conrat and Olcott, THIS JOURNAL, **68**, 1024 (1946).

(3) The product obtained from wheat gluten was gel-forming and appeared to have possible industrial significance (Reitz, Ferrel and Olcott, *Ind. Eng. Chem.*, **38**, 1149 (1944)).

(4) Levene and Schormüller, *J. Biol. Chem.*, **105**, 547 (1934); **106**, 595 (1934).

(5) Plimmer, *Biochem. J.*, **35**, 461 (1941).

(6) This specificity contrasts with the non-specific action of other phosphorylating agents which are known to react with amines, guanidyl compounds, etc., as well as with alcohols and phenols. Mayer and Heidelberger[7] phosphorylated horse-serum albumin in alkaline solution with phosphorus oxychloride. The derivatives contained 2–3% phosphorus, approximately half of which was accounted for by reaction with the amino groups. The reaction of egg albumin under similar conditions was described by Heidelberger, *et al.*[8]

(7) Mayer and Heidelberger, THIS JOURNAL, **68**, 18 (1946).

(8) Heidelberger, Davis and Treffers, *ibid.*, **63**, 498 (1941).