was increased only a limited extent because the increased phytic acid precipitated part of the protein and reduced the concentration of the total protein below the desired level of 1.2%. The precipitation of protein by phytin was confirmed by adding sodium phytate to a protein solution at a ratio of 1:3 on a dry weight basis and adjusting the system to the *p*H range of 2.0–3.0. About 96% of the protein was precipitated having a composition of nitrogen 14.52% and phosphorus 3.06%.

Two New Components.—Examination of the electrophoretic pattern of solution I of the above experiment taken after 60 minutes showed two fast-moving minor components which had not been identified in previous work. These two components are not in the photographs taken at 120 minutes, as they have moved outside the field of the camera. Also, comparison of the 60-minute patterns of solutions I and II, in this experiment indicated that another protein–phytin reaction was occurring. To examine this reaction, the experiment described in Fig. 2 was repeated, except that the four photographs were taken after 60 minutes of migration. The 60-minute photographs are in Fig. 3 where pattern no. I shows the two new fast-moving components A_1 and A_2, whereas in pattern no. II of dialyzed and resin-treated protein the A_2 component is not apparent. Pattern no. III, made from a dialyzed and resin-treated extract followed by the addition of sodium phytate to a level of about 50% above the original level of phytin as determined by phosphorus analysis, shows component A_2 is as prominent at least as in the original solution. Pattern no. IV is protein prepared as described for no. IV of Fig. 2, except that a slightly higher level of sodium phytate was used. This pattern also shows component A_2 in a much higher ratio than for sample no. I.

This series of patterns seems to indicate that component A_2, as it occurs in the acid-precipitated protein from the original untreated protein solution, may be a reaction product of protein and phytin, and that dialysis and resin treatment of the original water extract removes the phytin before a detectable reaction has occurred. There is some indication that component A_1 also reacts with phytate, but the differences between the patterns are not large enough for a satisfactory conclusion.

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[CONTRIBUTION FROM THE FULMER LABORATORY, DEPARTMENT OF CHEMISTRY, STATE COLLEGE OF WASHINGTON]

The Oxidative Cleavage of Phenylhydrazide Groups from Carboallyloxy-α-amino Acid Phenylhydrazides and Carboallyloxydipeptide Phenylhydrazides[1,2]

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A method is described for the quantitative estimation of phenylhydrazide groups in carboallyloxy-α-amino acid phenylhydrazides. A number of carboallylloxyamino acid phenylhydrazides have been oxidized with ferric chloride to yield the corresponding carboallyloxyamino acids. The enzymatic synthesis of L-leucyl-L-leucine is reported.

One of the limitations of the enzymatic synthesis of dipeptides by means of the Bergmann reaction is the difficulty of removing an amide or anilide group without splitting the peptide bond.[3] However, Waldschmidt-Leitz and Kuhn[4] prepared glycylglycine by the following reactions. They incubated carbobenzoxyglycine with glycine phenylhydrazide in the presence of papain and obtained carbobenzoxyglycylglycine phenylhydrazide, which reacted with hydriodic acid in the presence of acetic acid to give glycyclglycine phenylhydrazide. The phenylhydrazide was refluxed with copper acetate to yield glycylglycine.

It was of interest to see if the phenylhydrazide oxidation could be used with carboallyloxy-α-amino acid phenylhydrazides, which may be prepared by an enzymatic synthesis,[5] without oxidizing the carboallyloxy groups.

It was decided to try several oxidizing solutions to determine the best procedure for the oxidation of carboallyloxyamino acid phenylhydrazides. As the earlier work indicated that liberated nitrogen could be used as a measure of the extent of the reaction, a modified Van Slyke–Koch[6] micro-nitrogen apparatus was built.

In preliminary experiments using this apparatus, the nitrogen liberated from the oxidation of hippuric acid phenylhydrazide was measured. The hippuric acid phenylhydrazide was dissolved in a number of solvents and the solutions mixed with several different oxidizing agents. The highest yield of nitrogen was obtained with potassium permanganate using methyl Cellosolve as a solvent for the phenylhydrazide.

In the cases where the yield of nitrogen was low, an amorphous white solid precipitated from the reaction mixtures when they were diluted with water. This material would not react further with the oxidizing agent even upon refluxing for several hours. This product was not studied further, but is thought to be a diphenylhydrazide as reported by Tafel.[7]

As the potassium permanganate would probably react with the allyl group in the carboallyloxyamino acids, an attempt was made to improve the yields of nitrogen when copper acetate and ferric chloride were used as oxidizing agents. It was found that increasing the temperature of the reaction mixture greatly increases the yield of liberated nitrogen. When a representative group of carboallyloxyamino acid phenylhydrazides was oxidized (Table I) with copper acetate or ferric chloride at 96 to 97°, the

(1) This investigation was supported in part by a Grant-in-aid from The State College of Washington Research Fund.

(2) Presented in part at the St. Louis Meeting of the American Chemical Society, September 8, 1948.

(3) J. S. Fruton, "Advances in Protein Chemistry," Vol. V, Eds. M. L. Anson, J. T. Edsall and K. Bailey, Academic Press, Inc., New York, N. Y., 1949, p. 1.

(4) E. Waldschmidt-Leitz and K. Kuhn, *Ber.*, **84**, 381 (1951).

(5) H. B. Milne and C. M. Stevens, THIS JOURNAL, **72**, 1742 (1950).

(6) F. C. Koch, *J. Biol. Chem.*, **84**, 602 (1929).

(7) J. Tafel, *Ber.*, **25**, 413 (1892).

yield of nitrogen was nearly quantitative. Ferric chloride gave the better yield in each case. This method should be useful for the quantitative analysis of phenylhydrazides.

TABLE I

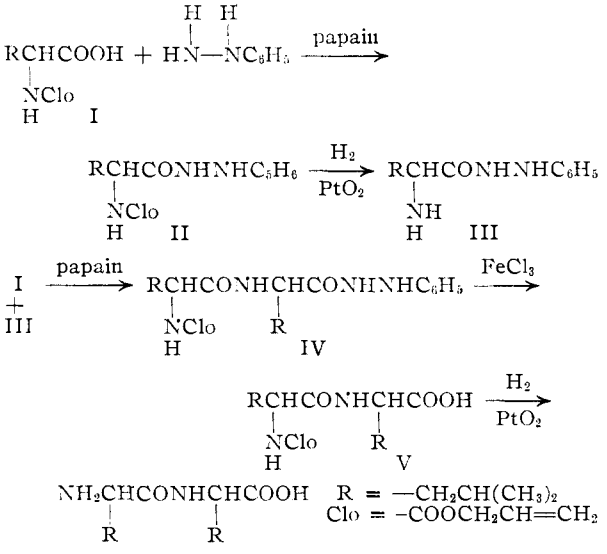
OXIDATION OF CARBOALLYLOXY-α-AMINO ACID PHENYLHYDRAZIDES

In each case 25 ml. of 0.01 *M* phenylhydrazide in methyl Cellosolve was oxidized with 15 ml. of 1 *N* oxidizing agent at 96–97°

Phenylhydrazide	Nitrogen liberated, % By $Cu(C_2H_3O_2)_2$	By $FeCl_3$
Carboallyloxy-glycine	94.8	99.2
Carboallyloxy-L-alanine	96.3	99.5
Carboallyloxy-L-phenylalanine	97.1	100.8
Carboallyloxy-L-leucine	96.0	99.4
N,N-Dicarboallyloxy-L-lysine	95.8	99.6
O,N-Dicarboallyloxy-L-tyrosine	96.1	100.2

Larger samples of carboallyloxy-L-leucine phenylhydrazide, carboallyloxy-L-lysine phenylhydrazide and carboallyloxy-L-tyrosine phenylhydrazide were oxidized with ferric chloride at 96 to 97°. The carboallyloxy-L-amino acids were recovered in 85 to 90% yields. The melting points and optical rotations of these carboallyloxy-L-amino acids were the same as authentic samples of carboallyloxy-L-amino acids prepared directly from the L-amino acids.

In order to test the applicability of the ferric chloride oxidation of phenylhydrazides in an enzymatic synthesis of a dipeptide, L-leucyl-L-leucine was prepared by the reactions



A variety of catalysts and experimental conditions were employed to find the best method for hydrogenolysis of the carboallyloxy group from amino acids and their phenylhydrazides. The procedure used by Stevens and Watanabe[8] gave the best results for the hydrogenolysis of carboallyloxyamino acids. However, in the case of carboallyloxyamino acid phenylhydrazides, hydrogenation with Adams platinum oxide[9] in acetic acid and hydrogen chloride gave a high yield of the corresponding carbopropyloxy derivative. When di-carboallyloxy-L-tyrosine phenylhydrazide was hydrogenated in glacial acetic acid with palladium-on-charcoal as a catalyst, an 85.4% yield of N-carbopropyloxy-L-tyrosine phenylhydrazide was recovered. On the other hand, when neutral methylal with Adams platinum oxide as a catalyst was used in the hydrogenation, a 70% yield of L-tyrosine phenylhydrazide and a 23% yield of N-carbopropyloxy-L-tyrosine phenylhydrazide was isolated. The highest yield of amino acid phenylhydrazide was obtained using Adams platinum oxide in neutral methylal. L-Leucine phenylhydrazide hydrochloride (III) was obtained in 76% yield from the carboallyloxy derivative II.

The hydrogenolysis of carboallyloxy-L-leucyl-L-leucine (V) proceeded satisfactorily in a solution of ethanol acidified with hydrogen chloride.

The same conditions were used for the enzymatic synthesis of carboallyloxy-L-leucyl-L-leucine phenylhydrazide as Bergmann and Fraenkel-Conrat[10] used for the preparation of benzoyl-L-leucyl-L-leucine anilide. The only difficulty in the reaction is the low solubility of carboallyloxy-L-leucine at a *p*H of 4.7.

The oxidation of the phenylhydrazide IV to give carboallyloxy-L-leucyl-L-leucine (V) proceeded smoothly without splitting the peptide bond or racemizing the product.

Experimental

Carboallyloxyamino Acids.—The carboallyloxy amino acids were prepared as described in a previous article.[8]

Papain.—Commercial papain (Merck and Co., Inc.) was purified by the general procedure of Grassmann[11] and Bergmann and Fraenkel-Conrat.[10] After three successive treatments with hydrogen sulfide followed by precipitation with methanol, the preparation was lyophilized, yielding a white powder.

Enzymatic Synthesis of Carboallyloxyamino Acid Phenylhydrazides.—The carboallyloxyamino acid phenylhydrazides were prepared by incubating carboallyloxyamino acids with phenylhydrazine in a buffered solution of papain and cysteine hydrochloride at 40° according to the method of Fraenkel-Conrat.[10] Certain of the results are listed in Table II.

Micro Oxidation of Phenylhydrazides. Measurement of Nitrogen.—The oxidation of the phenylhydrazides was followed by the evolution of nitrogen in an apparatus similar to a Van Slyke–Koch micro-nitrogen apparatus.

In each case, the phenylhydrazide was dissolved in a solvent to form a 0.01 *M* solution. The oxidizing agents were dissolved in water and were made of such a strength that 1 ml. of oxidizing solution had 10 times as much oxidizing agent as was theoretically necessary to oxidize 1 ml. of the phenylhydrazide solution. In using the apparatus, 15 ml. of oxidizing solution was added to 25 ml. of the solution containing the phenylhydrazide.

Oxidation of N-Carboallyloxy-L-leucine Phenylhydrazide.—A solution of 2.62 g. of carboallyloxy-L-leucine phenylhydrazide dissolved in 100 ml. of methyl Cellosolve was heated 96–97° in a boiling water-bath. To this solution was added 100 ml. of solution containing 16.2 g. of ferric chloride hexahydrate dissolved in 1 *N* hydrochloric acid. The resulting mixture was stirred vigorously until the nitrogen ceased bubbling from the solution. The heating was continued for 14 minutes; then the solution was cooled to room temperature. The solution was then diluted with water and extracted with 200 ml. of benzene in a continuous extraction apparatus. The solvent was evaporated under vacuum at 50–55° and the residue taken up in a minimum volume of benzene. The solution was washed with water

(8) C. M. Stevens and R. Watanabe, THIS JOURNAL, **72**, 725 (1950).

(9) Obtained from American Platinum Works, Jersey City, N. J.

(10) M. Bergmann and H. Fraenkel-Conrat, *J. Biol. Chem.*, **124**, 1 (1938).

(11) W. Grassmann, *Biochem. Z.*, **279**, 131 (1935).

TABLE II

ENZYMATIC SYNTHESIS OF PHENYLHYDRAZIDES

Substrate	Yield,[a] %	M.p., °C.	$[\alpha]^{22}D$	Formula	Nitrogen, % Calcd.	Found
N-Carboallyloxy-D,L-phenylalanine[b]	37	163–164	−26.5°[c]	$C_{19}H_{26}O_3N_3$	12.4	12.42
N-Carboallyloxy-D,L-alanine[b]	28.8	115–116	−60.3[d]	$C_{13}H_{17}O_3N_3$	16.0	15.60
O,N-Dicarboallyloxy-L-tyrosine	94	162–163	− 8.5[e]	$C_{23}H_{25}O_6N_3$	9.57	9.83
N-Carbopropyloxy-L-tyrosine	75	185–186	− 3.4[e]	$C_{12}H_{23}O_4N_3$	11.75	11.52

[a] Yield based on total substrate. [b] Because the enzyme is not always optically specific for carboallyloxy-L-amino acids,[5] the derivatives of phenylalanine and alanine may not be optically pure. [c] (*c* 1, chloroform). [d] (*c* 3, chloroform). [e] (*c* 1, 95% ethanol).

twice to remove any iron salts or hydrochloric acid present and then decolorized by boiling 15 minutes with charcoal. Upon filtering and evaporating the solvent a light yellow oil remained. The final product was dried at 2 mm. over phosphorus pentoxide for 24 hr.; yield 1.58 g. (85%), $[\alpha]^{22}D$ −14.6° (*c* 3.7, chloroform); for authentic sample carboallyloxy-L-leucine $[\alpha]^{22}D$ −14.5° (*c* 3.7, chloroform).[5]

Anal. Calcd.: neut. equiv., 215. Found: neut. equiv., 217.

Oxidation of N,N-Dicarboallyloxy-L-lysine Phenylhydrazide.—Dicarboallyloxy-L-lysine phenylhydrazide (6.22 g.) was oxidized in the same manner as carboallyloxy-L-leucine phenylhydrazide and the dicarboallyloxy-L-lysine recovered as a dark oil. The oil was taken up in benzene and boiled with charcoal for 15 minutes. The mixture was filtered and the solvent evaporated yielding a light yellow oil. The product was dried under 2 mm. over phosphorus pentoxide; yield 4.16 g. (86%), $[\alpha]^{22}D$ −8.2° (*c* 12, 11% concd. HCl and 89% methyl Cellosolve); authentic sample N,N-dicarboallyloxy-L-lysine, $[\alpha]^{22}D$ −8.4° (*c* 12, 11% concd. HCl and 89% methyl Cellosolve).[5,3]

Anal. Calcd.: neut. equiv., 314. Found: neut. equiv., 317.

Oxidation of O,N-Dicarboallyloxy-L-tyrosine Phenylhydrazide.—O,N-Dicarboallyloxy-L-tyrosine phenylhydrazide (0.44 g.) was oxidized in the same manner described above. The recovered light oil was dissolved in 95% ethyl alcohol and boiled with charcoal for 15 minutes. Upon filtration and evaporation of the solvent, crystals formed. The crystals were dissolved in hot 50% ethyl alcohol and the solution put in a refrigerator for 2 days; yield 0.32 g., m.p. 105° uncor., $[\alpha]^{22}D$ +28.9° (*c* 3, chloroform). A sample of O,N-dicarboallyloxy-L-tyrosine prepared directly from L-tyrosine had m.p. 104–105°, $[\alpha]^{22}D$ +29.2° (*c* 3, chloroform). A mixed melting point was 104°.

Anal. Calcd. for $C_{17}H_{19}O_7N$: C, 58.45; H, 5.48; N, 4.01; neut. equiv., 349. Found: C, 58.48; H, 5.46; N, 4.09; neut. equiv., 347.

Carboallyloxy-L-leucyl-L-leucine Phenylhydrazide.—Carboallyloxy-L-leucine phenylhydrazide (6.8 g.) was dissolved in 100 ml. of methylal and 650 mg. of platinum oxide was added. The mixture was shaken in a Parr hydrogenation apparatus under a pressure of 30 lb. of hydrogen for 2 hr. The catalyst was removed by filtration. The solvent was distilled under reduced pressure leaving a dark oil. The oil was dissolved in 100 ml. of dry ether and dry hydrogen chloride gas passed through the solution, yielding 4.35 g. of L-leucine phenylhydrazide hydrochloride which was used in the enzymatic reaction without further purification.

Carboallyloxy-L-leucine (5.00 g.), 4.35 g. of L-leucine phenylhydrazide hydrochloride and 2.0 g. of cysteine hydrochloride was dissolved in 300 ml. of 3 *M* acetate buffer. The *p*H of the solution was 4.7. Three grams of papain was added and the solution incubated at 40°. After 55 hr., the crystalline product was collected and recrystallized from alcohol to yield 4.49 g. (63.5% based on L-leucine phenylhydrazide hydrochloride) of carboallyloxy-L-leucyl-L-leucine phenylhydrazide, m.p. 185–186°, $[\alpha]^{22}D$ −91.0° (*c* 0.86, chloroform).

Anal. Calcd. for $C_{22}H_{34}O_4N_4$: C, 63.13; H, 8.13; N, 13.39. Found: C, 62.93; H, 8.08; N, 13.25.

Carboallyloxy-L-leucyl-L-leucine.—Carboallyloxy-L-leucyl-L-leucine phenylhydrazide (2.04 g.) was dissolved in 30 ml. of 95% ethanol and then warmed to 35°. To this solution 15 g. of ferric chloride hexahydrate dissolved in 25 ml. of water was slowly added. The mixture was stirred until nitrogen gas was no longer liberated. The solution was made basic to litmus with 1 *N* sodium hydroxide and the ferric hydroxide precipitate removed by centrifugation. The supernatant solution was acidified with concentrated hydrochloric acid and extracted with five 25-ml. portions of ether. Ligroin was added to the ether solution until it became turbid. The solution was then cooled and the resulting crystals collected; yield 0.69 g. (43%), m.p. 113–114°, $[\alpha]^{22}D$ −25.1° (*c* 2, chloroform).

Anal. Calcd. for $C_{16}H_{28}O_5N_2$: C, 58.5; H, 8.53; N, 8.53; neut. equiv., 328.4. Found: C, 58.74; H, 8.59; N, 8.40; neut. equiv., 329.5.

L-Leucyl-L-leucine.—Carboallyloxy-L-leucyl-L-leucine (0.44 g.) was hydrogenated with platinum oxide in ethanol, containing concentrated hydrogen chloride, in the usual manner. The peptide was recrystallized from ethanol and dried *in vacuo* over phosphorus pentoxide; 0.138 g. (42%), m.p. 259–261°, $[\alpha]^{22}D$ −13.5° (*c* 1.2, 1 *N* sodium hydroxide); Fischer[12] reported m.p. 270°, $[\alpha]^{22}D$ −13.4° (*c* 8.1, 1 *N* sodium hydroxide).

Anal. Calcd. for $C_{12}H_{24}O_3N_2$: N, 11.5. Found: N, 11.2.

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(12) E. Fischer, *Ber.*, **39**, 2893 (1906).

[CONTRIBUTION FROM THE FULMER CHEMICAL LABORATORY, THE STATE COLLEGE OF WASHINGTON]

The Use of Benzylsulfonyl Chloride in Peptide Syntheses[1,2]

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The use of benzylsulfonyl chloride as a reagent in peptide syntheses has been investigated. N-Benzylsulfonyl derivatives of 25 amino acids have been prepared and characterized. The N-benzylsulfonyl group is easily cleaved from amino acid derivatives by sodium in liquid ammonia or Raney nickel. It is slowly cleaved by hydriodic acid and by hydrobromic acid. Both the preparation and the cleavage of the N-benzylsulfonyl derivatives of optically active amino acids are accomplished without racemization. The application of this reagent to peptide syntheses through the preparation of L-leucyl-L-leucine and L-methionyl-D,L-methionine is reported.

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(2) Abstracted in part from a thesis presented to the Graduate Faculty of the State College of Washington by Chi-Hsieh Peng in partial fulfillment of the requirements for the degree of Doctor of Philosophy, June, 1956.