for the differing periods of incubation, or for the color value of the two azo dyes produced. Calibration curves for the dye from 6-bromo-2-naphthol[12] and from β-naphthol[9] are given elsewhere.

Infrared Absorption Spectra.—The infrared spectra of the acetylated glycopyranosides were determined with a Baird Infrared Recording Spectrophotometer, Model B. The solvent chloroform was reagent grade.

BOSTON 15, MASS.

[CONTRIBUTION FROM THE NEW YORK STATE AGRICULTURAL EXPERIMENT STATION]

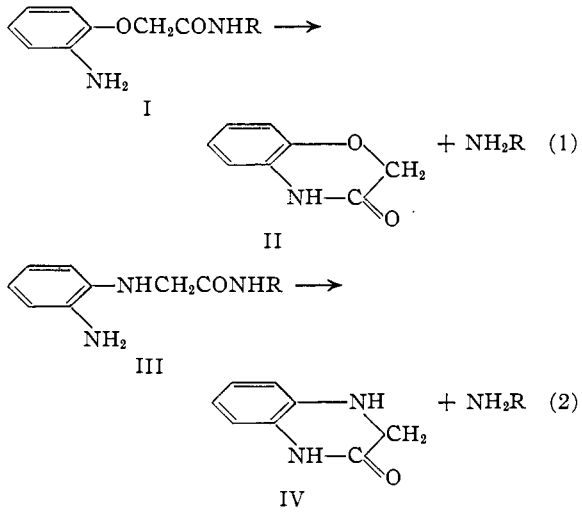
The Removal of N-o-Nitrophenoxyacetyl and N-Chloroacetyl Groups from Peptides[1]

BY ROBERT W. HOLLEY AND ANN D. HOLLEY

RECEIVED JANUARY 25, 1952

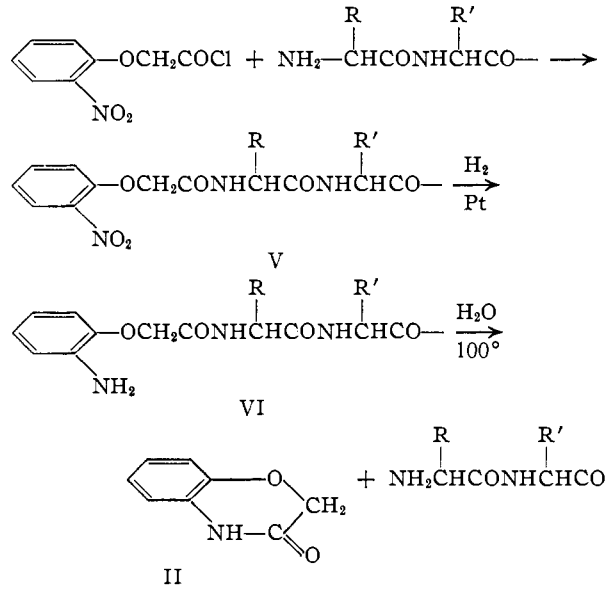
Methods have been developed for the removal of N-o-nitrophenoxyacetyl and N-chloroacetyl groups from peptides. These methods make possible the use of the o-nitrophenoxyacetyl and chloroacetyl groups as protecting groups during peptide synthesis.

Most γ- and δ-amino acids must be fused, or subjected to other dehydration conditions, in order to obtain the lactams. In contrast, a few, for example *o*-aminophenoxyacetic acid[2] and *o*-aminophenylglycine,[3] lactamize so readily that the free amino acids have never been obtained. It seemed of interest to know whether this ease of lactam formation extends to the amino acid amides, as in equations (1) and (2). That this might be the case was indicated by the report of Jacobs and



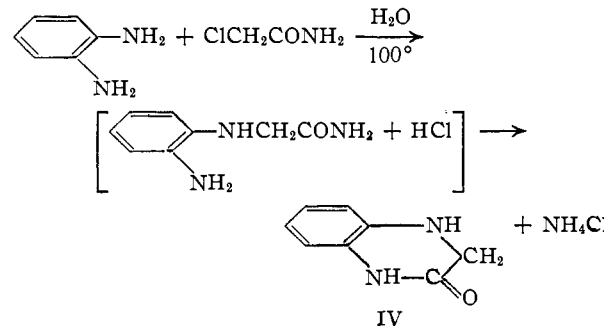
Heidelberger[4] that the lactam, II, of *o*-aminophenoxyacetic acid is obtained from ferrous sulfate–ammonia reduction of *o*-nitrophenoxyacetamide. If the conditions for this type of reaction were sufficiently mild, the reaction would have applications in peptide chemistry.

In order to investigate the reaction illustrated in equation (1), N-*o*-aminophenoxyacetylpeptides, VI, were prepared by catalytic reduction of the nitro compounds, V. The N-*o*-aminophenoxyacetylpeptides, VI, are insoluble in cold water, but they dissolve slowly in water at 100°. If the solution is heated a short time and then cooled, the compound which crystallizes is the lactam II. The peptide



remains in the aqueous solution and can be recovered in good yield. Using this series of reactions, the *o*-nitrophenoxyacetyl group has been removed from four N-*o*-nitrophenoxyacetylpeptides. The yields of once-recrystallized peptides, identical with authentic samples, were: glycylglycine, 73%; glycylglycylglycine, 76%; glycyl-L-alanyl-L-leucine, 65%; and L-phenylalanyl-L-leucine, 70%.

As a possible route to the synthesis of *o*-aminophenylglycine amide, which was desired for the study of the reaction shown in equation (2), the reaction of *o*-phenylenediamine with chloroacetamide, was studied. When these compounds were



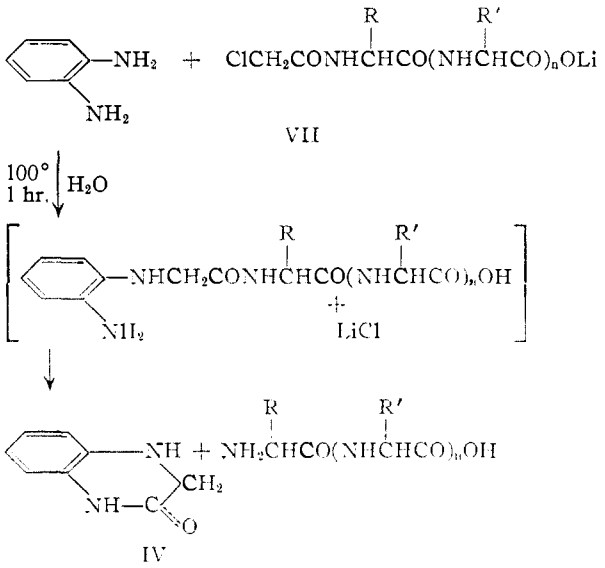
(1) Journal Paper No. 889, New York State Agricultural Experiment Station.

(2) A. Thate, *J. prakt. Chem.*, [2] **29**, 145 (1884).

(3) J. Plöchl, *Ber.*, **19**, 6 (1886).

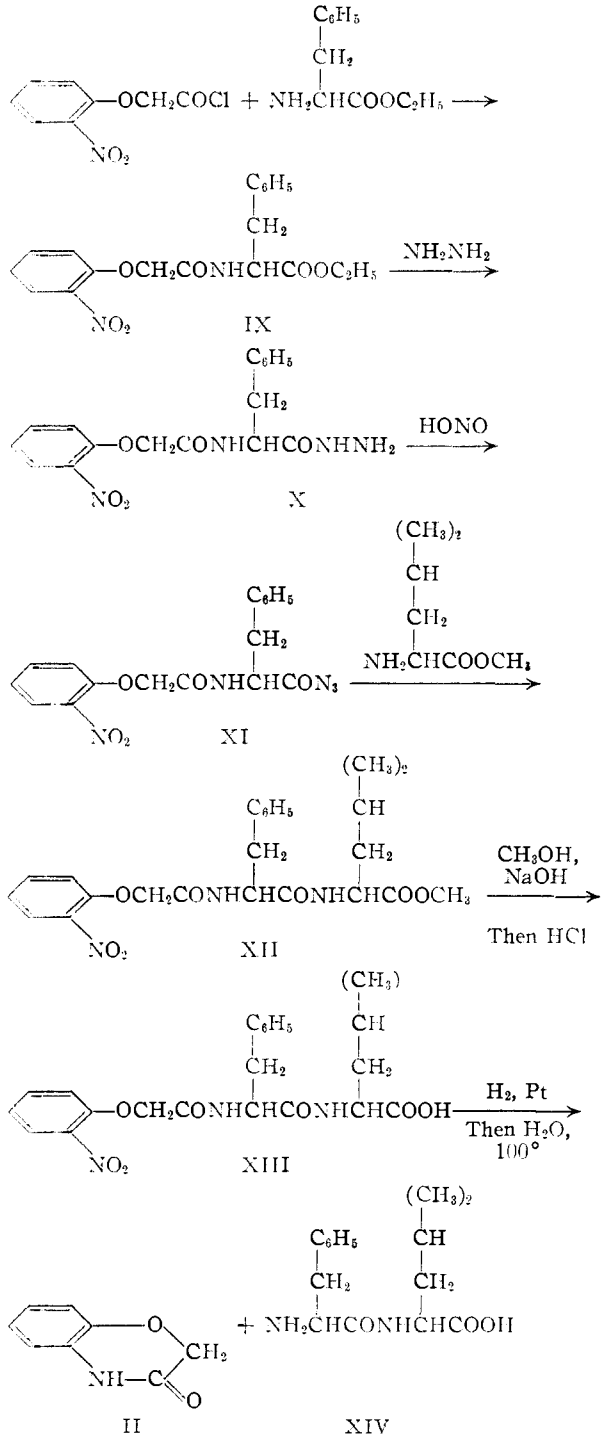
(4) W. A. Jacobs and M. Heidelberger, THIS JOURNAL, **39**, 2418 (1917).

heated in aqueous solution for one hour at 100°, the product which crystallized on cooling the solution was the lactam, IV, of *o*-aminophenylglycine. Presumably the initial product was *o*-aminophenylglycine amide, which lactamized under the conditions of the reaction.[5] This reaction was then studied with N-chloroacetylpeptides, VII. As indicated in the following equations, the products were the lactam, IV, and the peptide. The yields of once-recrystallized peptides prepared in this



manner from N-chloroacetylpeptides, and identical with authentic samples, were: glycylglycine, 76%; glycylglycylglycine, 68%; glycyl-L-alanyl-L-leucine, 59%; and L-phenylalanyl-L-leucine, 31%.

The ease of removal of the *o*-nitrophenoxyacetyl and chloroacetyl groups from peptides suggested that these groups might be used as protecting groups during peptide synthesis if there were suitable methods for lengthening the peptide chain. The choice was limited to those methods which would not cause racemization of optically active amino acids. Thus, the use of the "acid chlorides" was not investigated because these would presumably be racemized by way of the oxazolones.[6] It was found that the Curtius azide procedure[7] could be used in the synthesis of *o*-nitrophenoxyacetylpeptides, and an optically active peptide has been prepared. As indicated in the following equations, the N-*o*-nitrophenoxyacetyl-L-phenylalanine ethyl ester (IX) was converted by way of the hydrazide (X) into the azide (XI), and the azide was allowed to react with L-leucine methyl ester. The product, N-*o*-nitrophenoxyacetyl-L-phenylalanyl-L-leucine methyl ester (XII), was saponified, and the *o*-nitrophenoxyacetyl group was removed by the reactions described above. The



over-all yield of L-phenylalanyl-L-leucine (XIV) from L-phenylalanine ethyl ester hydrochloride was 31%.[8] N-*o*-Nitrophenoxyacetylglycylglycine

(5) The reaction of *o*-phenylenediamine with sodium chloroacetate in aqueous solution at 100° also gives the lactam IV. In contrast, the reaction of *o*-phenylenediamine with chloroacetic acid in aqueous *acid* gives 2-chloromethylbenzimidazole (ref. 14). Presumably the final product of the reaction depends on whether the conditions favor an initial alkylation or acylation of the *o*-phenylenediamine.

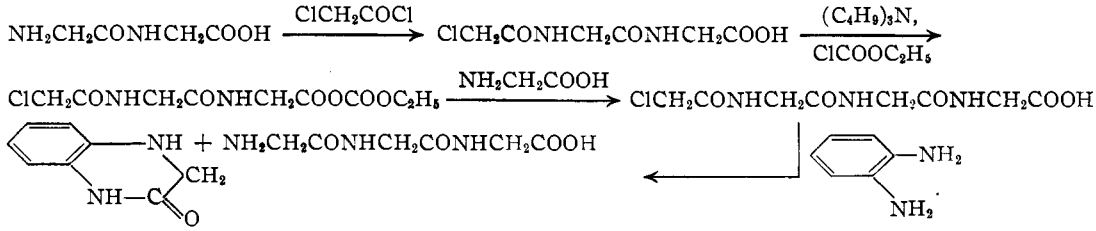
(6) H. T. Clarke, J. R. Johnson and R. Robinson, Editors, "The Chemistry of Penicillin," Princeton University Press, Princeton, N. J., 1949, p. 781.

(7) T. Curtius, *Ber.*, **35** 3226 (1902).

(8) C. S. Smith and A. E. Brown, (THIS JOURNAL, **63**, 2605 (1941)) synthesized D-phenylalanyl-D-leucine from D-phenylalanine in 12% yield by the carbobenzoxy method. During repetition of their work using L-amino acids, in order to obtain an authentic sample of L-phenylalanyl-L-leucine, it was observed that the N-carbobenzoxy-L-phenylalanine was impure, though it had the properties reported by Smith and Brown, and by Bergmann, *et al.* (ref. 21). Modification of the procedure to avoid the impurity (see Experimental) resulted in a 40% over-all yield of L-phenylalanyl L-leucine. This yield is more representative of the carbobenzoxy method, as is indicated by Erlanger and Brand (ref. 15), who reported over-all yields of 30 to 35% for six dipeptides.

ethyl ester was prepared in similar fashion from N-*o*-nitrophenoxyacetylglycine ethyl ester.

With the chloroacetyl as the protecting group, the Curtius azide procedure cannot be used because of the reaction of the chloroacetyl with hydrazine. The method of Boissonnas[9] does seem to be applicable, although the question of racemization has not been investigated. As shown in the following equations, glycylglycine has been converted to glycylglycylglycine. The over-all yield was 18%.



This work on peptide synthesis, though preliminary, demonstrates that the *o*-nitrophenoxyacetyl and chloroacetyl groups can be used as protecting groups during peptide synthesis. The yields using the *o*-nitrophenoxyacetyl group are comparable, in the instances studied, to the yields obtained using the carbobenzoxy group. Since *o*-nitrophenoxyacetyl chloride is a stable reagent and the *o*-nitrophenoxyacetyl derivatives crystallize well, the *o*-nitrophenoxyacetyl group may have some advantages. Nevertheless, considering that the carbobenzoxy group has been found satisfactory in many cases and that there are extensive data in the literature on its use, it seems best to consider the *o*-nitrophenoxyacetyl and chloroacetyl groups as alternatives to the carbobenzoxy group which may be of value in situations where the carbobenzoxy group has not been used or has been found to be unsatisfactory.

Experimental[10]

Removal of the *o*-Nitrophenoxyacetyl Group from N-*o*-Nitrophenoxyacetylpeptides. General Procedure.—A solution of 0.50 millimole of the *o*-nitrophenoxyacetylpeptide and 45 mg. (0.53 millimole) of sodium bicarbonate in 3 ml. of water, to which 20 mg. of platinum oxide[11] had been added, was hydrogenated in a micro hydrogenation apparatus. After the theoretical amount of hydrogen had been absorbed (30 to 40 minutes), the hydrogenation was stopped. The mixture was filtered, the catalyst was washed with 1 ml. of water, and the sodium salts were neutralized by the addition of 0.53 ml. of 1.00 *N* hydrochloric acid. The mixture was cooled and filtered, and the *o*-aminophenoxyacetylpeptide was washed with a little cold water and dried *in vacuo*. The *o*-aminophenoxyacetyl group was removed by heating the solid with 3 ml. of water in an oil-bath at 100° for approximately 1 hour. The solution was cooled, and the lactam of *o*-aminophenoxyacetic acid was collected by filtration. The lactam was identical with the lactam of *o*-aminophenoxyacetic acid described below. The aqueous filtrate was evaporated to dryness and the crude peptide was recrystallized. Special conditions and yields for the individual peptides are given below.

A. Glycylglycine.—The yield of crude peptide was 61 mg. (92%). It was recrystallized from 0.3 ml. of water and 0.6 ml. of ethanol, to give 48 mg. (73%). To convert it to its acetyl derivative, it was dissolved in 0.2 ml. of water, 0.1 ml. of acetic anhydride was added, and the mixture was heated at 80° for 5 minutes and evaporated to dryness. The residue was recrystallized from absolute ethanol; m.p. 178–181.5°[12]; mixed m.p. with authentic N-acetylglycylglycine undepressed.

B. Glycylglycylglycine.—The heating period was 30 minutes at 100° instead of 1 hour. The yield of crude peptide was 83 mg. (88%). It was recrystallized from 0.25 ml. of water and 0.25 ml. of methanol to give 71 mg. (76%). A sample was converted to N-*o*-nitrophenoxyacetylglycylglycylglycine; m.p. 213–218° (dec.); mixed m.p. with authentic material undepressed; mixed m.p. with the derivative of glycylglycine much depressed.

C. Glycyl-L-alanyl-L-leucine.—The *o*-aminophenoxyacetyl compound precipitated as a gelatinous material which could not be crystallized, so the crude product was heated in water at 100° for 30 minutes without first separating the sodium chloride. The crude peptide therefore contained sodium chloride, and was recrystallized from 2 ml. of 50% ethanol; 84 mg. (65%); $[\alpha]^{22}D$ −85° (*c* 0.6, 0.10 *N* hydrochloric acid). A sample was converted to its N-chloroacetyl derivative; m.p. 194–197°; mixed m.p. with the authentic material described below undepressed.

D. L-Phenylalanyl-L-leucine.—Because of the low solubility of the *o*-aminophenoxyacetyl derivative, it was heated in a mixture of 3 ml. of water and 1.2 ml. of ethanol for 2 hours. The peptide and the lactam of *o*-aminophenoxyacetic acid crystallized from the cold solution, so the mixture was extracted with ether to remove the lactam and then the aqueous mixture was evaporated to dryness. The crude peptide, 112 mg. (81%), was recrystallized from 10 ml. of absolute ethanol; weight 97 mg. (70%); $[\alpha]^{22}D$ −21° (*c* 1, 1% sodium bicarbonate solution); m.p. 257–260°; mixed m.p. with the L-phenylalanyl-L-leucine described below was undepressed.

Lactam of *o*-Aminophenoxyacetic Acid.—A solution of 197 mg. (1.0 millimole) of *o*-nitrophenoxyacetic acid[13] in 5 ml. of 95% ethanol was added to 20 mg. of platinum oxide, which had been reduced in 1 ml. of 95% ethanol, in a micro hydrogenation apparatus. The theoretical amount (3.0 millimoles) of hydrogen was absorbed in 20 minutes. The solution was filtered, to remove the catalyst, and was evaporated to dryness *in vacuo* at room temperature. The residue, 151 mg., m.p. 156–171°, was insoluble in sodium bicarbonate solution. It was recrystallized by dissolving it in 1 ml. of warm acetone and adding 5 ml. of water slowly; weight 105 mg.; m.p. 171–173°. It was recrystallized again for analysis.

Anal. Calcd. for $C_8H_7NO_2$: N, 9.39. Found: N, 9.28, 9.51.

***o*-Nitrophenoxyacetyl Chloride.**—A mixture of 5 g. of *o*-nitrophenoxyacetic acid and 25 ml. of thionyl chloride was heated under reflux for 30 minutes. The solution was cooled and evaporated to dryness *in vacuo*. The solid residue was washed with ligroin, filtered and dried *in vacuo*; weight 5.2 g. of tan-colored solid; m.p. 39–42°.

N-*o*-Nitrophenoxyacetylglycylglycine.—To a cold solution of 132 mg. (1.0 millimole) of glycylglycine in 1 ml. of 1 *N* sodium hydroxide solution was added 215 mg. (1.0 millimole) of powdered *o*-nitrophenoxyacetyl chloride and 1.1 ml. of 1 *N* sodium hydroxide solution. The mixture was stirred in the cold for 20 minutes. The solution was separated from the trace of solid which remained, and was acidified with 0.10 ml. of concentrated hydrochloric acid. The mixture was cooled in an ice-bath and filtered, and the solid was washed with a little cold water, and was recrystallized from 8 ml. of 50% ethanol. The yield of faintly yellow solid was 237 mg. (76%); m.p. 209.5–213.5° (dec.).

(9) R. A. Boissonnas, *Helv. Chim. Acta*, **34**, 874 (1951).

(10) All melting points were determined on a microscope hot stage and are corrected.

(11) Obtained from The American Platinum Works, Newark, N. J.

(12) A. H. Gordon, A. J. P. Martin and R. L. M. Synge, *Biochem. J.*, **37**, 79 (1943), report m.p. 178–179°.

(13) T. H. Minton and H. Stephen, *J. Chem. Soc.*, **121**, 1591 (1922).

Anal. Calcd. for $C_{12}H_{13}N_3O_7$: neut. equiv., 311. Found: neut. equiv., 313.

N-*o*-Nitrophenoxyacetylglycylglycylglycine.—The procedure was the same as for the glycylglycine derivative; yield 70%; m.p. 215–217° (dec.).

Anal. Calcd. for $C_{14}H_{16}N_4O_8$: N, 15.2; neut. equiv. 368. Found: N, 15.2, 15.4; neut. equiv., 372.

N-*o*-Nitrophenoxyacetylglycyl-L-alanyl-L-leucine.—In this preparation, 420 mg. (5 millimoles) of sodium bicarbonate in 5 ml. of water was used in place of the sodium hydroxide solution used above, and the aqueous mixture after acidification was washed with ether before the first filtration. The yield of recrystallized material was 76%; m.p. 184–186° (transition 179–181°).

Anal. Calcd. for $C_{19}H_{26}N_4O_8$: N, 12.8; neut. equiv., 438. Found: N, 12.7, 12.8; neut. equiv., 442.

Removal of the Chloroacetyl Group from N-Chloroacetylpeptides. General Procedure.—A mixture of 0.50 millimole of N-chloroacetylpeptide, 181 mg. (1.0 millimole) of *o*-phenylenediamine dihydrochloride and 60 mg. (2.5 millimoles) of lithium hydroxide was dissolved in 2.5 ml. of water and the solution was heated in a bath at 100° for 1 hour. The lactam of *o*-aminophenylglycine crystallized when the solution was cooled, and was collected by filtration. It was shown by m.p. and mixed m.p. to be identical with the lactam of *o*-aminophenylglycine described below. The aqueous mother liquors were evaporated to dryness and absolute ethanol was added and evaporated. The residue, a mixture of peptide, *o*-phenylenediamine, and lithium chloride, was mixed with 5 ml. of warm absolute ethanol. The mixture was cooled and filtered, and the solid was washed with absolute ethanol. Recrystallization of the peptides, the yields obtained, and any special conditions are described under the headings for the individual peptides below.

A. Glycylglycine.—The crude peptide, 74 mg., was recrystallized from 0.2 ml. of water and 0.5 ml. of absolute ethanol to give 50 mg. (76%). A small sample was converted to the N-acetyl derivative; m.p. 177–180°; mixed m.p. with authentic N-acetylglycylglycine undepressed.

B. Glycylglycylglycine.—The crude peptide, 103 mg., was recrystallized from 0.4 ml. of water and 0.4 ml. of methanol to give 64 mg. (68%). A small sample was converted to N-*o*-nitrophenoxyacetylglycylglycylglycylglycine; m.p. 212–217° (dec.); mixed m.p. with authentic material undepressed.

C. Glycyl-L-alanyl-L-leucine.—The crude peptide, 88 mg., was recrystallized from 1 ml. of water and 1 ml. of absolute ethanol to give 77 mg. (59%); $[\alpha]^{22}D$ −87° (*c* 0.6, 0.10 *N* hydrochloric acid). A small sample was converted to N-*o*-nitrophenoxyacetylglycyl-L-alanyl-L-leucine: m.p. 184–186° (transition 178–181°); mixed m.p. with authentic material undepressed.

D. L-Phenylalanyl-L-leucine.—Because of the low solubility of the chloroacetyl derivative, about 1 ml. of ethanol was added to the reaction mixture, and the mixture was heated 2 hours. When the mixture was cooled, the peptide and the lactam crystallized. Most of the ethanol was evaporated *in vacuo* and the aqueous mixture was extracted repeatedly with ether. The aqueous mixture was evaporated to dryness and the residue was washed with ether and absolute ethanol. Finally, the crude peptide, 60 mg., was recrystallized from 6 ml. of absolute ethanol to give 43 mg. (31%); m.p. 240–250°. Another recrystallization gave 31 mg.; $[\alpha]^{22}D$ −20° (*c* 0.6, 1% sodium bicarbonate solution); m.p. 252–257°; mixed m.p. with authentic peptide undepressed.

Lactam of *o*-Aminophenylglycine.—A mixture of 181 mg. (1.0 millimole) of *o*-phenylenediamine dihydrochloride and 94.5 mg. (1.0 millimole) of chloroacetic acid was dissolved in 3.0 ml. of 1.00 *N* sodium hydroxide solution, and the solution was heated at 100° for 1 hour. A trace of insoluble material was filtered from the hot solution, and the solution was cooled and filtered. The product was a tan-colored solid; weight 80 mg. (54%); m.p. 133–138°. Recrystallization from water, with treatment with charcoal, gave 40 mg. of almost colorless material; m.p. 136–138°. (A different crystalline form, melting at 190–193°, is sometimes obtained.)

Anal. Calcd. for $C_8H_8N_2O$: N, 18.9. Found: N, 18.6, 18.9.

The same product was obtained from a mixture of *o*-phenylenediamine dihydrochloride (1.0 millimole), chloroacetamide (1.0 millimole) and sodium hydroxide solution (2.0 milliequivalents) heated for 1 hour at 100°; yield 58%.

A mixed m.p of this lactam with 2-chloromethylbenzimidazole[14] was greatly depressed.

N-Chloroacetylglycyl-L-alanyl-L-leucine.—To a cold solution of 259 mg. (1.0 millimole) of glycyl-L-alanyl-L-leucine and 420 mg. (5.0 millimoles) of sodium bicarbonate in 3 ml. of water was added with stirring 0.12 ml. (180 mg., 1.6 millimoles) of chloroacetyl chloride, in four portions. When the odor of chloroacetyl chloride had disappeared, the solution was acidified to congo red and cooled. The solid was collected by filtration, washed with water and dried; weight 250 mg. (75%); m.p. 193–196°. It was recrystallized from 2 ml. of 50% ethanol to give 200 mg.; m.p. 198–201°; $[\alpha]^{22}D$ −47.5° (*c* 1, absolute ethanol).

Anal. Calcd. for $C_{13}H_{22}ClN_3O_5$: N, 12.5; neut. equiv., 336. Found: N, 12.5, 12.5; neut. equiv., 340.

N-Chloroacetyl-L-phenylalanyl-L-leucine.—To a cold solution of 148 mg. (0.50 millimole) of L-phenylalanyl-L-leucine in 0.30 ml. of 2 *N* sodium hydroxide solution was added, alternately and in five portions with stirring, 0.05 ml. (75 mg., 0.66 millimole) of chloroacetyl chloride and 0.15 ml. of 5 *N* sodium hydroxide solution during 15 minutes. When the odor of chloroacetyl chloride had disappeared, the solution was acidified with 0.30 ml. of 5 *N* hydrochloric acid. The chloroacetyl derivative was extracted with 3 ml. and 2 ml. of ethyl acetate, and the ethyl acetate solution was washed with 1 *N* hydrochloric acid and with water, and dried over anhydrous sodium sulfate. Evaporation of the ethyl acetate solution gave 102 mg. (58%), which was recrystallized from 0.5 ml. of methanol and 1 ml. of water; weight 74 mg.; m.p. 161.5–164°.

Anal. Calcd. for $C_{17}H_{23}ClN_2O_4$: neut. equiv., 355. Found: neut. equiv., 356.

Peptide Synthesis

N-*o*-Nitrophenoxyacetylglycine Ethyl Ester.—This was prepared on a 10-millimole scale from glycine ethyl ester hydrochloride and *o*-nitrophenoxyacetyl chloride using the procedure described by Erlanger and Brand for the preparation of carbobenzoxyglycine ethyl ester.[15] The crystalline product, obtained in 87% yield, melted at 91–93°. It was recrystallized (85% recovery) using 1 ml. of absolute ethanol per 100 mg.; m.p. 92–93°.

Anal. Calcd. for $C_{12}H_{14}N_2O_6$: N, 9.93. Found: N, 9.88, 10.1.

N-*o*-Nitrophenoxyacetylglycine Hydrazide.—A solution of 1.41 g. (5.0 millimoles) of N-*o*-nitrophenoxyacetylglycine ethyl ester and 0.70 ml. of 85% hydrazine hydrate in 25 ml. of absolute ethanol was heated at 45° for 5 hours. The solution was cooled, and the hydrazide was collected by filtration and washed with absolute ethanol; weight 1.19 g. (89%); m.p. 182–184.5°. For analysis a sample was recrystallized three times (recovery 70%) from 50% ethanol.

Anal. Calcd. for $C_{10}H_{12}N_4O_5$: N, 20.9. Found: N, 20.7, 21.0.

N-*o*-Nitrophenoxyacetylglycylglycine Ethyl Ester.—To a cold (0°) solution of 268 mg. (1 millimole) of N-*o*-nitrophenoxyacetylglycine hydrazide in 1.2 ml. of glacial acetic acid, 0.48 ml. of 5 *N* hydrochloric acid and 5 ml. of water, was added a solution of 90 mg. of sodium nitrite in 0.5 ml. of water. The solid azide precipitated at once. The mixture was extracted with 10 ml., 10 ml. and 5 ml. of ethyl acetate in the cold room. The ethyl acetate solution was washed with water, 5% sodium bicarbonate, and again with water, and dried briefly over sodium sulfate. The solution was filtered into an ethyl acetate solution containing 1.24 millimoles of glycine ethyl ester in 10 ml. (This solution was prepared by repeated extraction with ethyl acetate of a mixture of 200 mg. of glycine ethyl ester hydrochloride and 1.5 ml. of 50% potassium carbonate solution. The ethyl acetate solution was dried over sodium sulfate, and an aliquot was titrated, using sodium alizarinsulfonate as indicator, to determine the concentration of glycine ethyl ester.) The reaction mixture was allowed to stand at 25° for 20 hours. The solution was then washed with 1 *N* hydro-

(14) A. Bloom and A. R. Day, *J. Org. Chem.*, **4**, 14 (1939).

(15) B. F. Erlanger and E. Brand, THIS JOURNAL, **73**, 3508 (1951).

chloric acid, water, 5% sodium bicarbonate solution, and again with water, dried over sodium sulfate, and evaporated *in vacuo*. When the volume of the solution was approximately 15 ml., 50 ml. of ligroin was added, and the solution was cooled. The product was collected by filtration, washed with ligroin, and dried; weight 256 mg. (75.5%); m.p. 135.5–137.5°. It was recrystallized from 4 ml. of 95% ethanol with 87% recovery; m.p. 135.5–138.5°. A mixed m.p. with N-*o*-nitrophenoxyacetylglycylglycine ethyl ester prepared by acylation of glycylglycine ethyl ester was undepressed.

N-*o*-Nitrophenoxyacetylglycylglycine ethyl ester is obtained in a higher melting form, m.p. 142–143.5°, on fusing the low melting form and allowing it to resolidify, or on evaporation of a chloroform solution. Recrystallization of the high melting form from ethanol affords the low melting form.

N-*o*-Nitrophenoxyacetyl-L-phenylalanine Ethyl Ester.—This was prepared on a 6 millimole scale from L-phenylalanine ethyl ester hydrochloride and *o*-nitrophenoxyacetyl chloride using the procedure described by Erlanger and Brand for the preparation of carbobenzoxyglycine ethyl ester.[15] The crystalline product, obtained in 93.5% yield, melted at 85–90°. It was used without further purification in the preparation of the hydrazide. For analysis, a small sample was recrystallized from ethyl acetate–hexane; m.p. 86.5–90°; $[\alpha]^{25}D$ −12° (*c* 1.2, 95% ethanol).

Anal. Calcd. for $C_{19}H_{20}N_2O_6$: N, 7.52. Found: N, 7.53, 7.50.

N-*o*-Nitrophenoxyacetyl-L-phenylalanine Hydrazide.—To a solution of 712 mg. (1.91 millimoles) of N-*o*-nitrophenoxyacetyl-L-phenylalanine ethyl ester in 10.5 ml. of absolute ethanol was added 0.24 ml. of 100% hydrazine hydrate. The solution was heated for 90 hours at 45°. The mixture was cooled; and the product was collected and washed with absolute ethanol; weight 549 mg. (80.4%); m.p. 176.5–179.5°. This material was used without purification in the next step. Recrystallization of a small sample from ethanol for analysis did not raise the melting point.

Anal. Calcd. for $C_{17}H_{18}N_4O_5$: N, 15.6. Found: N, 15.7, 15.6.

N-*o*-Nitrophenoxyacetyl-L-phenylalanyl-L-leucine Methyl Ester.—To a solution of 1.49 g. (4.17 millimoles) of N-*o*-nitrophenoxyacetyl-L-phenylalanine hydrazide in 167 ml. of glacial acetic acid were added 4.16 ml. of 5 *N* hydrochloric acid and 83 g. of ice. When the temperature of the solution reached 0°, a solution of 305 mg. (4.4 millimoles) of sodium nitrite in 3 ml. of water was added with stirring. After 5 minutes, 650 ml. of ice-water was added and the mixture was transferred to a cold separatory funnel. In a cold room (0°), the aqueous mixture was extracted with three 150-ml. portions of ether. The ethereal solution was washed with water, 5% sodium bicarbonate solution (until the washes were basic), and again with water. The ethereal solution was dried briefly over sodium sulfate, and was filtered into an ethereal solution containing 6.0 millimoles of L-leucine methyl ester. (The solution of the amino ester was prepared by extracting a mixture of 1.1 g. of L-leucine methyl ester hydrochloride and 6.3 ml. of 50% potassium carbonate solution with ether.) The reaction mixture was allowed to warm up to room temperature. It was then evaporated *in vacuo* to a volume of about 100 ml. and allowed to stand for 20 hours at 25°. The product, which had crystallized, was collected by filtration, and was washed with ether and dried. The yield was 1.610 g. (82%); m.p. 150–165°. This was used in the next step without further purification. A sample of 150 mg. was recrystallized from 1.5 ml. of methanol to give 107 mg.; m.p. 164–167°. Further recrystallization did not raise the melting point.

By washing the ethereal mother liquors from the reaction mixture with 1 *N* hydrochloric acid, water, 5% sodium bicarbonate solution and again with water, drying over sodium sulfate, and evaporating the solvent a second crop of 136 mg. was obtained.

N-*o*-Nitrophenoxyacetyl-L-phenylalanyl-L-leucine.—A solution of 687 mg. (1.46 millimoles) of the ester in 3.1 ml. of methanol and 0.31 ml. of 5.1 *N* sodium hydroxide solution was heated at 45° for one hour. An equal volume of water was added, and the ethanol was evaporated *in vacuo*. The solution was diluted with 10 ml. of water, was extracted repeatedly with ether, and was acidified to congo red. The oil which separated was extracted into ether. The ethereal solution was filtered through a dry filter paper and was evaporated to about 10 ml. The acid soon crystallized and was collected by filtration and washed with ether; weight 391 mg.; m.p. 160.5–164.5°. A second crop was obtained by evaporation of the ethereal mother liquors to dryness and crystallization of the residue from ethanol–water; weight 127 mg.; m.p. 154–166°. Recrystallization of this material furnished 93 mg.; m.p. 161–164.5°. The total yield of material suitable for the next step was 484 mg. (72.5%). Recrystallization of a small sample from 50% ethanol did not raise the melting point; $[\alpha]^{23}D$ −23° (*c* 0.94, absolute ethanol).

Anal. Calcd. for $C_{23}H_{27}N_3O_7$: N, 9.18; neut. equiv., 457. Found: N, 9.10, 9.20; neut. equiv., 458.

L-Phenylalanyl-L-leucine was prepared from this material as described above under removal of the *o*-nitrophenoxyacetyl group. The over-all yield of L-phenylalanyl-L-leucine from L-phenylalanine ethyl ester hydrochloride was 31%.

Preparation of N-Chloroacetylglycylglycylglycine from N-Chloroacetylglycylglycine.—A solution of 417 mg. (2.0 millimoles) of N-chloroacetylglycylglycine[16] and 0.48 ml. of dry tributylamine in 2.0 ml. of dioxane (freshly distilled from sodium) was cooled to 10°, and 0.20 ml. of redistilled ethyl chlorocarbonate was added.[9] A colorless solid filled the mixture within a few minutes. After 15 minutes at 10°, a cold solution of 150 mg. (2.0 millimoles) of glycine in 1.0 ml. of 2.0 *N* sodium hydroxide solution was added. There was an immediate evolution of carbon dioxide. The solution was allowed to stand at 25° for 1 hour. The solution was evaporated *in vacuo* to about 1 ml. and 3 ml. of water was added. The solution was extracted twice with 4 ml. of ether and then was acidified with 0.20 ml. of concentrated hydrochloric acid. The solution was evaporated to about 2 ml. to remove dioxane, and was cooled. The solid was collected by filtration and was recrystallized from 8 ml. of 95% ethanol; weight 214 mg. (40%); m.p. 201–203°; mixed m.p. with authentic N-chloroacetylglycylglycylglycine[17] 202–210°.

Anal. Calcd. for $C_8H_{12}ClN_3O_5$: neut. equiv., 266. Found: neut. equiv., 272.

The chloroacetyl group was removed with *o*-phenylenediamine, as described above, in 63% yield (over-all yield of glycylglycylglycine from glycylglycine 18%). A sample of the glycylglycylglycine was converted to N-*o*-nitrophenoxyacetylglycylglycylglycine; m.p. 211.5–217° (dec.); mixed m.p. with authentic material undepressed; mixed m.p. with N-*o*-nitrophenoxyacetylglycylglycine much depressed.

Glycyl-L-alanyl-L-leucine.—This tripeptide was prepared by the carbobenzoxy method starting with carbobenzoxyglycyl-L-alanine ethyl ester prepared according to Erlanger and Brand.[15] **Carbobenzoxyglycyl-L-alanine hydrazide** was prepared according to the procedure of Bergmann and Zervas[18] but could be crystallized only when crystallization was started in warm ethanol solution; yield 81%; m.p. 158–160° (Bergmann and Zervas[18] reported m.p. 133°). The hydrazide was converted to **carbobenzoxyglycyl-L-alanyl-L-leucine methyl ester** using the general procedure given by Brand, *et al.*,[19] for carbobenzoxytripeptide esters, and the product was recrystallized from ethyl acetate–petroleum ether[18]; yield 65%; m.p. 111.5–113.5° (Bergmann and Zervas[18] reported m.p. 112°). Carbobenzoxyglycyl-L-alanyl-L-leucine methyl ester was converted to the tripeptide in 77% yield using the general procedures given by Brand, *et al.*[19] The glycyl-L-alanyl-L-leucine was recrystallized from water; $[\alpha]^{23}D$ −87 (*c* 1, water)[20]; $[\alpha]^{22}D$ −85° (*c* 0.6, 0.10 *N* hydrochloric acid).

Anal. Calcd. for $C_{11}H_{21}N_3O_4$: N, 16.2. Found: N, 16.0.

L-Phenylalanyl-L-leucine.—This was prepared using the procedure described by Smith and Brown[8] for the preparation of D-phenylalanyl-D-leucine except for modification of the preparation of carbobenzoxy-L-phenylalanine. When

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

(17) E. Fischer, *ibid.*, **37**, 2486 (1904), reported m.p. 224°. We found m.p. 210–212°.

(18) M. Bergmann and L. Zervas, *J. Biol. Chem.*, **113**, 341 (1936).

(19) E. Brand, B. F. Erlanger, H. Sachs and J. Polatnick, THIS JOURNAL, **73**, 3510 (1951).

(20) E. Abderhalden and A. Fodor, *Z. physiol. Chem.*, **81**, 1 (1912), reported $[\alpha]^{20}D$ − 90° (*c* 3, water).

this was prepared according to Smith and Brown[8] (also Bergmann, *et al.*[21]) a solid was obtained which recrystallized slowly from methanol–water as matted needles, m.p. 124–128°. This solid gave a neutralization equivalent of approximately 370 (calcd. for $C_{17}H_{17}NO_4$: 299) which was not lowered by prolonged drying *in vacuo*. If this solid was dissolved in ether, and the ethereal solution was washed with 1 *N* hydrochloric acid, and water, and dried over magnesium sulfate, evaporation of the ether gave an oil with a neutralization equivalent of approximately 310. **Carbobenzoxy-L-phenylalanine** was therefore prepared by acylation of L-phenylalanine in 2 *N* sodium hydroxide solution as described,[8,21] but after acidification the crude product was extracted into ether. This ethereal solution was washed with 1 *N* hydrochloric acid and water, and dried over anhydrous magnesium sulfate. Evaporation of the ether gave an oil. On the basis of the neutralization equivalent of the oil, the yield of carbobenzoxy-L-phenylalanine was 95%. This oil was then converted to the acid chloride as described,[8,21] and the remainder of the preparation of L-phenylalanyl-L-leucine was the same as the procedure described for D-phenylalanyl-D-leucine. The peptide was recrystallized from 50% ethanol (30 mg. per ml.) or absolute ethanol (10 mg. per ml.). Because of the above modification, the over-all yield from L-phenylalanine was 40% instead of 12%; m.p. 255–258°; $[\alpha]^{22}D$ +7.5° (*c* 1, 0.10 *N* hydrochloric acid); $[\alpha]^{22}D$ −21° (*c* 1, 1% sodium bicarbonate solution).

(21) M. Bergmann, L. Zervas, H. Rinke and H. Schleich, *ibid.*, **224**, 33 (1934).

GENEVA, NEW YORK

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, PURDUE UNIVERSITY]

The Chemistry of Diazo Compounds. III. The Reduction of Diazonium Salts by Phosphorous Acid[1]

BY NATHAN KORNBLUM, ALEC E. KELLEY[2] AND GLENN D. COOPER[2]

Treatment of diazonium salts with phosphorous acid results in replacement of the diazonium group by hydrogen; this reaction is subject to catalysis by certain oxidizing agents. It is a much less efficient way of replacing the diazonium group by hydrogen than the procedure employing hypophosphorous acid. The synthesis of pentabromobenzenediazonium hydrogen sulfate is described.

The reduction of diazonium salts by hypophosphorus acid is an excellent way of replacing the

$$\text{Aryl-}N_2^+ X^- \xrightarrow{H_3PO_2} \text{Aryl-H} + N_2$$

diazonium group by hydrogen.[1] Since phosphorous acid is also a strong reducing agent[3] its action upon four rather diverse diazonium salts was studied. Although phosphorous acid is able to replace the diazonium group by hydrogen, it turns out to be distinctly inferior to hypophosphorus acid for this purpose.

In every case studied the reduction with phosphorous acid is very much slower than with hypophosphorous acid. This provides an opportunity for competing processes to intervene, and notably in the case of the relatively unstable *p*-tolyldiazonium chloride[4] a large disparity in the yields of toluene results. With the more stable diazonium salts reduction by phosphorous acid is capable of giving good yields of reduced product, although it may be necessary to resort to catalysis (see below). In no instance, however, is there any advantage to the use of phosphorous acid in preference to hypophosphorous acid. Table I summarizes the best results obtained with phosphorous acid and compares them with those resulting from the use of hypophosphorous acid.

Since the hypophosphorous acid reduction of diazonium salts is strongly catalyzed by small amounts of potassium permanganate or copper sulfate,[1] the effect of these reagents on phosphorous acid reductions was briefly investigated. At 0° the reaction between pentabromobenzenediazonium hydrogen sulfate and phosphorous acid is unmistakably speeded up by the addition of as little as ten mole per cent.[5] of potassium permanganate; copper sulfate, however, has no effect at the ten mole per cent. level. Furthermore, when five mole per cent. of copper sulfate is added to a solution of *p*-tolyldiazonium hydrogen sulfate in phosphorous acid at 0° there is no catalysis.

TABLE I

REDUCTION OF DIAZONIUM SALTS BY H_3PO_2 AND BY H_3PO_3

Salt	Product	Yield with H_3PO_2, %	Yield with H_3PO_3, %
Pentabromobenzenediazonium hydrogen sulfate	Pentabromobenzene	81[a]	63[b]
p-Methoxybenzenediazonium chloride	Anisole	80[c]	71[d]
p-Nitrobenzenediazonium chloride	Nitrobenzene	65[c]	56[d]
p-Tolyldiazonium chloride	Toluene	72[c]	11[d]

[a] Reaction time 10 minutes at 13.8°. [b] Reaction time 4 hours at 25°. [c] Reaction time 24 hours at 0°. [d] Catalysis by ether peroxide required; see Table II.

Catalysis by a novel oxidizing agent was observed in the phosphorous acid reductions. Except for pentabromobenzene the various reduction products are liquids, and their isolation from the reaction mixtures entails extraction with diethyl ether. It was found that subsequent to this ether extraction further quantities of the various reduction products were formed.[6] Evidence in support of the hypothesis that ether peroxide is responsible for this catalysis is provided by the following experi-

(1) Paper II in this series: N. Kornblum, G. D. Cooper and J. E. Taylor, THIS JOURNAL, **72**, 3013 (1950).

(2) X-R Fellows of the Purdue Research Foundation.

(3) Don M. Yost and H. Russell, "Systematic Inorganic Chemistry," Prentice-Hall, Inc., New York, N. Y., 1946, p. 200.

(4) M. L. Crossley, R. H. Kienle and C. H. Benbrook, THIS JOURNAL, **62**, 1400 (1940).

(5) "Mole per cent." refers to the number of moles of catalyst per one hundred moles of diazonium salt.

(6) The H_3PO_2 reductions are so rapid that by the time the ether extractions were carried out the diazonium salts had been completely reduced. Hence there was no opportunity for catalysis.