found for carrying out absorption measurements on solutions of the carbanilates in the ultraviolet.

Amylose, amylopectin, and starch carbanilates have similar dispersions; approximately parallel curves are obtained if wave length is plotted *versus* specific rotation of the various compounds. This is also true for the polysaccharides and their acetates. Molecular structural factors do not enhance the differences among the optical rotations of the various polysaccharides at any particular wave length, to an extent that advantage accrues from the use of light other than that of the sodium D-line.

The use of trade names in this publication does not necessarily constitute endorsement of these products nor of the manufacturers thereof.

Acknowledgment.—We are grateful to Duncan Macmillan for his advice and assistance in the course of this work.

PEORIA, ILLINOIS

[CONTRIBUTION FROM THE CHEMICAL LABORATORY OF HARVARD UNIVERSITY, DEPARTMENT OF SURGERY OF BETH ISRAEL HOSPITAL AND HARVARD MEDICAL SCHOOL]

Preparation of Naphthyl β-D-Glycopyranosides as Chromogenic Substrates for β-D-Glucopyranosidase[1]

BY KWAN-CHUNG TSOU AND ARNOLD M. SELIGMAN

RECEIVED DECEMBER 28, 1951

For the study of β-D-glucopyranosidase activity colorimetrically, 2-naphthyl β-D-glucopyranoside, 1-bromo-2-naphthyl β-D-glucopyranoside, 4-chloro-1-naphthyl β-D-glucopyranoside, 2-naphthyl β-D-galactopyranoside and 6-bromo-2-naphthyl β-D-ribopyranoside were synthesized and subjected to enzymatic hydrolysis by mammalian tissues. Their rates of hydrolysis were found to be slower than those of 6-bromo-2-naphthyl β-D-glucopyranoside and 6-bromo-2-naphthyl β-D-galactopyranoside. The riboside was not susceptible to hydrolysis by any organ including those which were able to hydrolyze 6-bromo-2-naphthyl β-D-glucopyranoside. Not only was the absence of β-D-ribopyranosidase demonstrated in these organs but evidence was thereby provided that β-D-glucopyranosidase requires a hydroxyl group at C_3 *cis* to the glucosidic oxygen for attachment to the substrate.

Synthetic substrates which yield naphthols after enzymatic hydrolysis have been employed in both histochemical and colorimetric methods for the demonstration of a variety of hydrolytic enzymes. The liberated naphthol was converted to an insoluble azo dye by coupling with an appropriate diazonium salt. In this way enzymatic activity has been localized histochemically within sections of tissue[2-7] or measured quantitatively in homogenates by extraction of the azo dye with an organic solvent for measurement of the color density.[7-11]

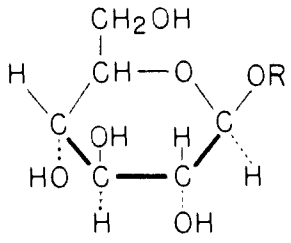


Fig. 1.

Based on these principles, the synthesis of 6-bromo-2-naphthyl β-D-galactopyranoside provided both a colorimetric and histochemical method for demonstrating β-D-galactopyranosidase activity in mammalian tissue.[12] The synthesis of 6-bromo-2-naphthyl β-D-glucopyranoside provided a colorimetric method for measuring β-D-glucopyranosidase activity in mammalian tissue.[13] In the course of this study, 1-bromo-2-naphthyl β-D-glucopyranoside (I), 4-chloro-1-naphthyl β-D-glucopyranoside (II), 2-naphthyl β-D-galactopyranoside (III) and 6-bromo-2-naphthyl β-D-ribopyranoside (IV) were also synthesized. Their preparation and susceptibility to enzymatic hydrolysis by mammalian tissues is reported below.

Helferich[14] and Pigman[15] have shown that the specificity of β-D-glucopyranosidase for various glucosidic substrates resides in the sugar moiety of the substrates. They have also shown that formation of an enzyme–substrate complex requires the hydroxyl groups at both C_2 and C_4 to be beneath the plane of the ring (Fig. 1). The configurational requirements of C_3 for enzyme–substrate complex formation are less well understood.[16] Since there is evidence that the pentosides are hydrolyzed by the same enzymes that hydrolyze corresponding hexosides,[17] and since the hydroxyl group at C_3 in D-ribopyranose is on the lower side of the plane

(1) This investigation was supported by a research grant from the National Cancer Institute of the National Institutes of Health, Public Health Service, Federal Security Agency.

(2) M. L. Menten, J. Junge and M. H. Green, *J. Biol. Chem.*, **153**, 471 (1944).

(3) L. H. Manheimer and A. M. Seligman, *J. Nat. Cancer Inst.*, **9**, 181 (1948).

(4) M. M. Nachlas and A. M. Seligman, *ibid.*, **9**, 415 (1949).

(5) A. M. Seligman and L. H. Manheimer, *ibid.*, **9**, 427 (1949).

(6) A. M. Seligman, M. M. Nachlas, L. H. Manheimer, O. M. Friedman and G. Wolf, *Ann. Surg.*, **130**, 333 (1949).

(7) H. A. Ravin, K.-C. Tsou and A. M. Seligman, *J. Biol. Chem.*, **191**, 843 (1951).

(8) M. M. Nachlas and A. M. Seligman, *ibid.*, **181**, 343 (1949).

(9) A. M. Seligman and M. M. Nachlas, *J. Clin. Invest.*, **29**, 31 (1950).

(10) A. M. Seligman, H. H. Chauncey, M. M. Nachlas, L. H. Manheimer and H. A. Ravin, *J. Biol. Chem.*, **191**, 7 (1951).

(11) H. A. Ravin and A. M. Seligman, *ibid.*, **191**, 391 (1951).

(12) R. B. Cohen, K.-C. Tsou, S. H. Rutenburg and A. M. Seligman, *ibid.*, **195**, 239 (1952).

(13) R. B. Cohen, S. H. Rutenburg, K.-C. Tsou, M. Woodbury and A. M. Seligman, *ibid.*, **195**, 607 (1952).

(14) B. Helferich, *Ergeb. Enzymforsch.*, **7**, 83 (1938).

(15) W. W. Pigman, *Advances in Enzymol.*, **4**, 41 (1944).

(16) Gottschalk in the chapter on "The Specificity of Glycosides" in *Adv. in Carbohydrate Chem.*, **5**, (1951), postulated without experimental proof that "the action of glycosidase is initiated by chemisorption, at the enzyme surface, of substrate molecule, with the glycosidic oxygen contacting the attacking group of the enzyme and with hydroxyl groups *cis*-disposed to the glycosidic oxygen, in juxtaposition to hydrogen bond forming groups of the enzyme." Such a hydroxyl group occurs at C_3 in β-D-glucopyranosides.

(17) Reference 14, p. 46.

of the molecule, 6-bromo-2-naphthyl β-D-ribopyranoside (IV) was tested for susceptibility to enzymatic hydrolysis by β-D-glucopyranosidase. Hydrolysis of IV was not observed in any organ of rats and guinea pigs (Table II). This meant that not only was there no β-D-ribopyranosidase capable of hydrolyzing aryl ribopyranosides in these tissues, but in addition that β-D-glucopyranosidase, which was able to hydrolyze aryl glucopyranosides in many of these organs and particularly fast in the pancreas of guinea pigs, was not able to hydrolyze the aryl ribopyranoside. Indirectly, these observations provide evidence that β-D-glucopyranosidase attaches to the substrate by the hydroxyl group at C_3 only when it is *cis* to the glucosidic oxygen.[16] There has been some controversy over whether galactosidase and glucosidase are distinct enzymes or not.[18] Evidence was provided that they are distinct enzymes in mammalian tissues with the chromogenic substrates prepared from 6-bromo-2-naphthol and with the aid of enzyme inhibitors.[12,13]

The importance of the 6-bromo group in facilitating enzymatic hydrolysis is shown in Table II. Glucopyranosidase hydrolyzed the 6-bromo derivative 7 times as fast as 2-naphthyl β-D-glucopyranoside,[19] whereas the 1-bromo derivative[20] (I) was hydrolyzed less than twice as fast. Galactopyranosidase hydrolyzed the 6-bromo derivative 10 times as fast as 2-naphthyl β-D-galactopyranoside (III).

The naphthyl tetraacetyl-β-D-glucopyranosides (V and VI) were prepared by fusing pentaacetyl-β-D-glucose and the corresponding naphthol in the presence of a trace of *p*-toluenesulfonic acid, according to the modified Helferich procedure of Montgomery, Richtmyer and Hudson.[21] 2-Naphthyl tetraacetyl-β-D-galactopyranoside (VII) and 6-bromo-2-naphthyl triacetyl-β-D-ribopyranoside (VIII) were prepared by a similar method, through the respective pentaacetyl-β-D-galactose and tetraacetyl-β-D-ribose. The former compound (VII) was not characterized due to its extreme sensitivity to moisture and failure to crystallize. Deacetylation of each polyacetyl glycoside was accomplished with dry ammonia in absolute methanol. The catalytic method[22] of Zemplén and Kunz frequently gave a non-crystallizable gel. The configuration of the ribopyranoside (IV) and its acetate (VIII) was assigned by the mode of synthesis and the fact that it was levorotatory.

Since the yield of 1-bromo-2-naphthyl tetraacetyl-β-D-glucopyranoside (V) from 1-bromo-2-naphthol was very poor, bromination of 2-naphthyl tetraacetyl-β-D-glucopyranoside[19] in glacial acetic acid was explored and found to give exclusively the 1-bromo derivative in good yield. The products of both reactions were identical by mixed melting points and infrared spectra, and 1-bromonaphthol was isolated after hydrolysis.

The infrared spectra of the 2-naphthyl tetraacetyl-β-D-glucopyranosides are given in Fig. 2 for comparison with the spectra of pentaacetyl β-D-glucose and 6-bromo-2-naphthyl triacetyl-β-D-ribopyranoside (VIII). A common strong band was seen in the 9.3 to 9.7 μ region. The 2-naphthyl groups differed in their relative intensity of the 6.1 and 6.2 μ bands. The spectra of VIII differed from the others in the 9.3 to 9.7 μ region, whereas

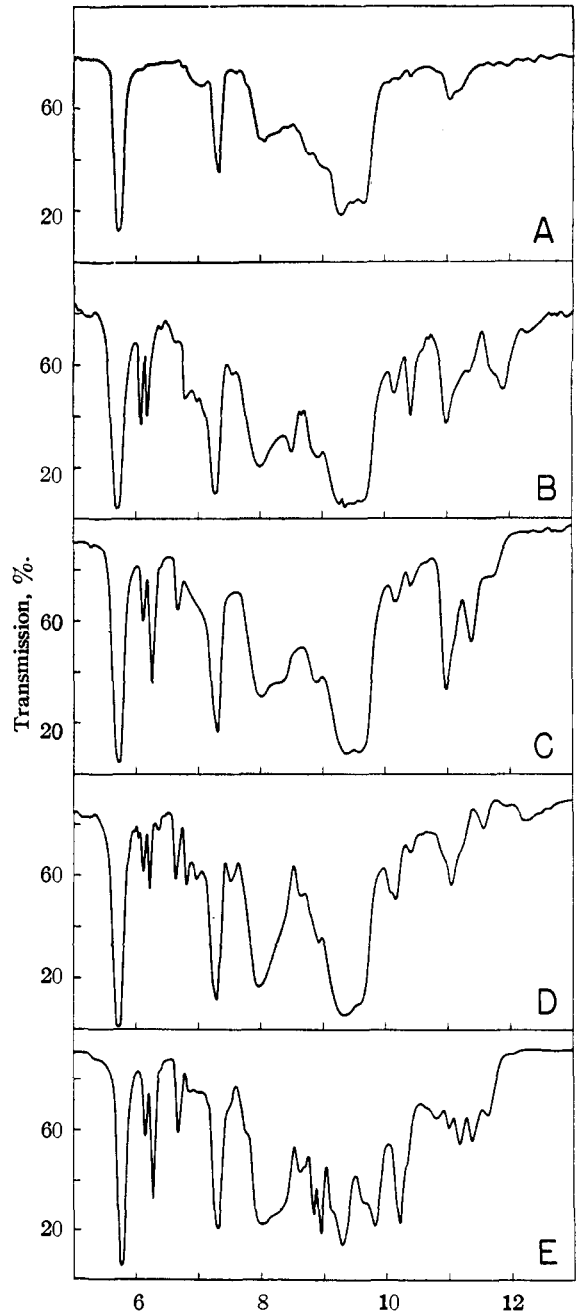


Fig. 2.—Infrared spectra: A, pentaacetyl β-D-glucopyranose; B, 2-naphthyl tetraacetyl-β-D-glucopyranoside; C, 6-bromo-2-naphthyl tetraacetyl-β-D-glucopyranoside; D, 1-bromo-2-naphthyl tetraacetyl-β-D-glucopyranoside; E, 6-bromo-2-naphthyl triacetyl-β-D-ribopyranoside.

(18) S. Veibel in "The Enzymes," Vol. I, edited by J. B. Sumner and K. Myrback, Academic Press, Inc., New York, N. Y., 1950, p. 583.

(19) B. Helferich and E. Schmitz-Hillebrecht, *Ber.*, **66**, 378 (1933).

(20) Joffe (*J. Gen. Chem. Russ.*, **6**, 1074 (1936); **7**, 2637 (1937)) showed that 1-bromo-2-naphthol coupled with diazotized nitroaniline by displacement of bromine at C_1. Since coupling occurred readily with tetrazotized diorthoanisidine, it was inferred that the azo dye obtained was identical to that derived from β-naphthol.

(21) E. M. Montgomery, N. K. Richtmyer and C. S. Hudson, THIS JOURNAL, **64**, 690 (1942).

(22) G. Zemplén and A. Kunz, *Ber.*, **56**, 1705 (1923).

TABLE I

NAPHTHYL β-D-GLYCOPYRANOSIDES

Compound, pyranoside	Yield, %	Rotational value $(\alpha)_D$[a]	M.p., °C.[b]	Formula	Analyses, %[c] Calcd. Carbon	Calcd. Hydrogen	Found Carbon	Found Hydrogen
1-Bromo-2-naphthyl tetraacetyl-β-D-gluco- (V)	60[d]	− 77.4, (c 2.0, chloroform)	172–173	$C_{24}H_{25}BrO_{10}$	52.09	4.55	51.90	4.49
1-Bromo-2-naphthyl β-D-gluco- (I)	80	− 32.6, (c 1.6, pyridine)	197.5–198.5	$C_{16}H_{17}BrO_6$	49.88	4.45	49.69	4.69
4-Chloro-1-naphthyl tetraacetyl-β-D-gluco- (VI)	75	− 63.9, (c 3.9, chloroform)	140–140.5	$C_{24}H_{25}ClO_{10}$	56.64	4.95	56.86	5.10
4-Chloro-1-naphthyl β-D-gluco- (II)	85	− 77.3, (c 1.1, dioxane)	214–215	$C_{16}H_{17}ClO_6$	56.39	5.03	56.22	5.04
2-Naphthyl β-D-galacto- (III)	53	+ 1.3 (c 1.3, dioxane)	190.5–192	$C_{16}H_{18}O_6$	62.74	5.92	62.50	6.14
6-Bromo-2-naphthyl triacetyl-β-D-ribo- (VIII)	50	− 53.8 (c 2.2, chloroform)	111.5–112.5	$C_{21}H_{21}BrO_8$[e]	52.40	4.40	52.54	4.61
6-Bromo-2-naphthyl β-D-ribo- (IV)	85	−108 (c 0.7, dioxane)	143–144	$C_{15}H_{15}BrO_5 + CH_3OH$	49.62	4.95	49.48	4.73

[a] Temperature varies from 23 to 27°. [b] All melting points are corrected. [c] Microanalyses by Mrs. Shirley Golden. [d] Prepared by the bromination method. [e] Anhydrous sample, m.p. 161–162°, loss of weight 8%.

the 6.1 and 6.2 μ bands were the same as those of 6-bromo-2-naphthyl tetraacetyl-β-D-glucopyranoside.[13]

Experimental

Naphthyl Tetraacetyl-β-D-glycopyranosides.—The naphthyl tetraacetyl-β-D-glycopyranosides were prepared according to the modified Helferich procedure.[21] Pentaacetyl-β-D-glucopyranose,[23] or pentaacetyl-β-D-galactopyranose[12,24] (0.01 mole), the naphthol (0.03 mole) and *p*-toluenesulfonic acid (0.2 g.) were fused *in vacuo* for 30 minutes at 100°. The melt was taken up in benzene or chloroform and the solution was washed with ice-water, 2% ice–sodium hydroxide solution, ice-water and dried over anhydrous calcium sulfate. The dried solution was evaporated under reduced pressure and the sirupy residue was crystallized from methanol and recrystallized again from methanol or ethanol until a constant melting point was obtained. The yields and physical data are given in Table I. 2-Naphthyl tetraacetyl-β-D-galactopyranoside (VII) could not be crystallized after numerous attempts.

1-Bromo-2-naphthyl Tetraacetyl-β-D-glucopyranoside (V).—2-Naphthyl tetraacetyl-β-D-glucopyranoside[19] was prepared according to the modified Helferich procedure[21] in 80% yield. It was dissolved in 10 cc. of glacial acetic acid (0.5 g.) and bromine (0.05 cc.) was added. The reaction mixture was allowed to stand for 2 hours at room temperature and then it was poured into 50 cc. of water. The milky solution was extracted with 2 portions of benzene (25 cc.) and the extract was washed with water, 2% ice-cold sodium hydroxide solution, again with water, and dried with anhydrous calcium sulfate. The dried benzene solution was evaporated to dryness under reduced pressure and the residue was crystallized twice from methanol (0.35 g.) and once from benzene–petroleum ether in white microcrystals (0.2 g.), m.p. 172–173°. A mixed melting point with a sample prepared from 1-bromo-2-naphthol as described above, showed no depression, 172–173.5°. The infrared spectra were identical (Fig. 2).

Isolation of 1-Bromo-2-naphthol.—A 0.1-g. sample of 1-bromo-2-naphthyl tetraacetyl-β-D-glucopyranoside (V), prepared by the bromination method, was suspended in 2 cc. of 2% sodium hydroxide solution and warmed slightly until solution was complete. The reaction mixture was cooled, acidified with dilute hydrochloric acid and extracted with chloroform. The extract was washed with saturated sodium chloride solution, dried and the solvent was evaporated to yield crude 1-bromo-2-naphthol which was purified by recrystallization from methanol, m.p. 83–84°. The melting point was not depressed by an authentic sample (m.p. 82–84°).[25]

6-Bromo-2-naphthyl Triacetyl-β-D-ribopyranoside (VIII).—A mixture of 1.0 g. of tetraacetyl ribopyranose,[26] 1.5 g. of 6-bromo-2-naphthol[27] and 0.05 g. of *p*-toluenesulfonic acid was heated *in vacuo* for 30 minutes at 100°. The melt was extracted with ether, washed, dried and evaporated to a sirup. After solution in methanol (5 cc.), it was allowed to stand for 3 weeks at 4°, to afford crystalline rosettes (0.9 g.), m.p. 109 to 110°. After recrystallization from methanol the melting point was 111.5 to 112.5°.

Naphthyl β-D-Glycopyranosides (I, II, III, IV).—The corresponding acetates (V, VI, VII, VIII) were suspended in absolute methanol and saturated with dry ammonia at 0°. The suspension was allowed to stand at 4° with occasional shaking for 1 to 2 days when solution occurred. The solutions were evaporated to dryness and the products were crystallized from methanol. Physical data are given in Table I.

Each product was shown to be free of traces of naphthol by treating a solution in dilute alcohol with sodium bicarbonate and tetrazotized diorthoanisidine.[28]

Enzymatic Hydrolysis.[29]—Fresh pancreas of guinea pig was homogenized in distilled water (5 mg. per cc.) with a motor-driven ground glass homogenizer for 2 minutes and centrifuged for 2 minutes at 2500 r.p.m. The supernatant (0.6 cc.) was incubated at 37° with a solution of each substrate (5 cc.) prepared as follows:

Each substrate (10 mg.) was dissolved in methanol (20 cc.) and water (20 cc.) at the boiling point, and phosphocitrate buffer[12] (20 cc., *p*H 4.95) and water (40 cc.) were added.

The period of incubation was 5 hours for the glucosides, 24 hours for the riboside, and 2 hours for the galactosides. Each solution was made alkaline with 0.2 *M* trisodium phosphate (0.5 cc.), coupled with 1 cc. of tetrazotized diorthoanisidine[28] (1 mg. per cc. dissolved immediately before use), acidified with 80% trichloroacetic acid (2 cc.), extracted with chloroform (10 cc.), and the color density measured in a photoelectric colorimeter (Klett) with a 540 mμ filter. The results are given in Table II. No correction was made

TABLE II

RELATIVE RATE OF ENZYMATIC HYDROLYSIS WITH GUINEA PIG PANCREAS[29]

Substrate, -pyranoside	Color density	Period of incubation (hours)
6-Bromo-2-naphthyl β-D-gluco-	640	5
1-Bromo-2-naphthyl β-D-gluco-	150	5
2-Naphthyl β-D-gluco-	90	5
4-Chloro-1-naphthyl β-D-gluco-	24	5
6-Bromo-2-naphthyl β-D-ribo-[a]	0	24
6-Bromo-2-naphthyl β-D-galacto-	242	2
2-Naphthyl β-D-galacto-	25	2

[a] The same result was observed in the following organs of guinea pig and rat: liver, kidney, spleen, heart, lung, stomach, large intestine, small intestine, brain, muscle, ovary, uterus, thyroid, adrenal and salivary gland. With dry yeast, no hydrolysis occurred after 6 hours of incubation and after 24 hours only a trace of color was noted.

(23) C. E. Redemann and C. Niemann, *Org. Syntheses*, **22**, 1 (1942). Supplied by Eastman Kodak Co., m.p. 132–134°.

(24) F. S. Bates and Associates, "Polarimetry, Saccharimetry and the Sugars," Circular C440 National Bureau of Standards 488 (1942), Washington Government Printing Office.

(25) A. J. Smith, *J. Chem. Soc.*, **35**, 789 (1879).

(26) H. Zinner, *Ber.*, **83**, 156 (1950).

(27) C. F. Koelsch, *Org. Syntheses*, **20**, 18 (1940).

(28) Available in powder form under the trade name du Pont naphthanil diazo blue B.

(29) The experiments on enzymatic hydrolysis were performed with the collaboration of Dr. Selma H. Rutenburg.

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]

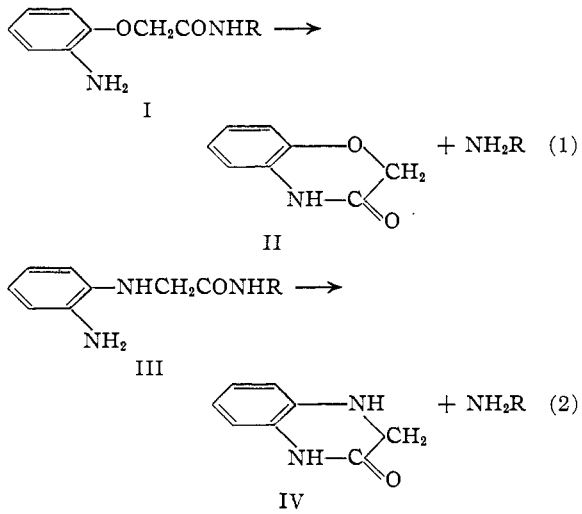
The Removal of N-o-Nitrophenoxyacetyl and N-Chloroacetyl Groups from Peptides[1]

BY ROBERT W. HOLLEY AND ANN D. HOLLEY

RECEIVED JANUARY 25, 1952

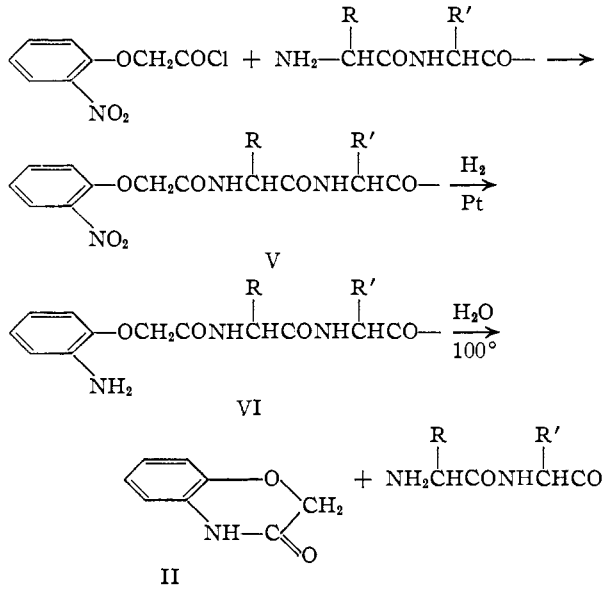
Methods have been developed for the removal of N-o-nitrophenoxyacetyl and N-chloroacetyl groups from peptides. These methods make possible the use of the o-nitrophenoxyacetyl and chloroacetyl groups as protecting groups during peptide synthesis.

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



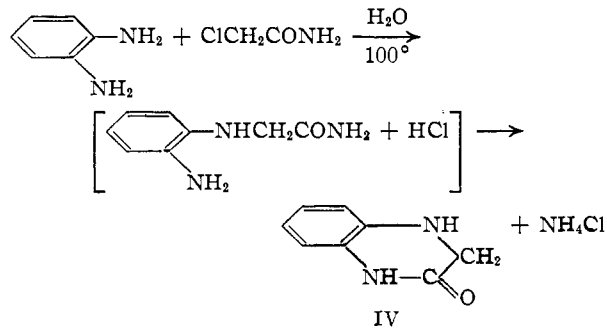
Heidelberger[4] that the lactam, II, of o-aminophenoxyacetic acid is obtained from ferrous sulfate–ammonia reduction of o-nitrophenoxyacetamide. If the conditions for this type of reaction were sufficiently mild, the reaction would have applications in peptide chemistry.

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).