mg. of the spiro-lactone VIII, m.p. 213–217°, which was recrystallized to give 470 mg. of pure material, m.p. 219–220°, undepressed upon admixture with that described above and having an identical infrared spectrum.

Basification of the aqueous filtrate described above and treatment with benzoyl chloride afforded benzamide, m.p. 127.5–128°.

URBANA, ILL.

[CONTRIBUTION FROM THE NATIONAL INSTITUTE OF ARTHRITIS AND METABOLIC DISEASES, NATIONAL INSTITUTES OF HEALTH]

The Oxidative Cleavage of Tyrosyl–Peptide Bonds. I. Cleavage of Dipeptides and Some Properties of the Resulting Spirodienone-lactones

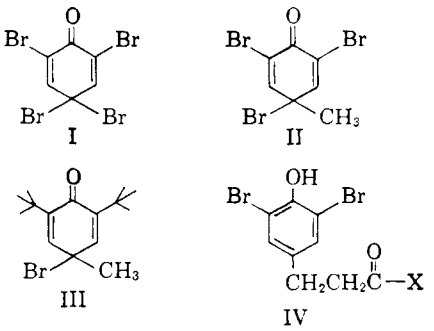
BY GASTON L. SCHMIR, LOUIS A. COHEN AND BERNHARD WITKOP

RECEIVED NOVEMBER 29, 1958

The reaction of phloretic acid with bromine or N-bromosuccinimide at *p*H 4.6 involves an oxidative participation between the phenolic ring and the carboxylate anion and leads to a spirodienone-lactone VII. A similar participation occurs with phloretylglycine (a tyrosyl–peptide model) leading to cleavage and release of glycine in 80% yield. The oxidative bromination of N-carbobenzyloxy-S-benzyl-L-cysteinyl-L-tyrosyl-L-isoleucine results in the formation of a spirolactone and the release of isoleucine in 40% yield. Aqueous hydrobromic or hydrochloric acid effects a reduction of the spirolactone to dibromophloretic acid while dilute sulfuric acid causes a rearrangement involving oxygen migration.

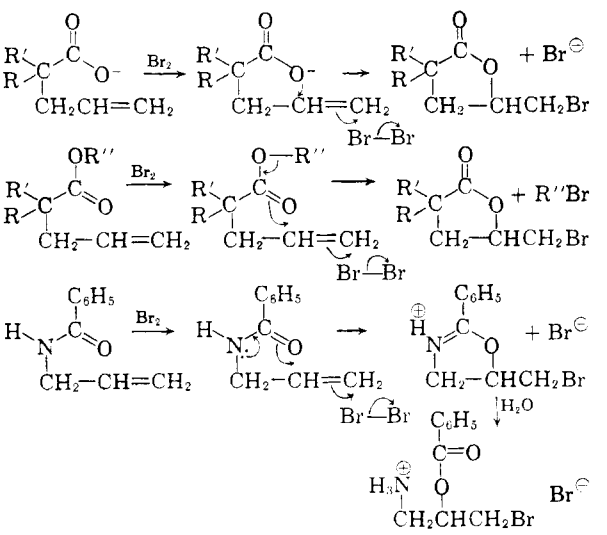
Recent studies in this Laboratory[1] have demonstrated the facile cleavage of tryptophyl–peptide bonds by the action of N-bromosuccinimide (NBS) in aqueous systems. We now wish to report some of our results from a study of the action of bromine and of NBS on derivatives of tyrosine and simpler analogs.

Under certain conditions the bromination of 2,6-disubstituted phenols leads to the formation of 4-bromo-2,6-disubstituted cyclohexadienones. For example, 2,6-dibromophenol forms I,[2] 2,6-dibromo-*p*-cresol forms II[3] and 2,6-di-*t*-butyl-*p*-cresol yields III.[4] Similar bromination of a phenol such as IV might lead to an interaction of the carbonyl function (CX) with the bromodienone *via* an internal displacement reaction. The facility of such displacements on double bonds has been amply demonstrated with carboxylic acids,[5] esters[5,6] and amides[7] as participating groups (Chart I).



The bromination of *p*-hydroxyphenylpropionic acid (phloretic acid) (V) in aqueous acetic acid led to the formation of the expected 3,5-dibromophloretic acid (VI) in high yield. However, quite different results were obtained in the reaction of V with bromine or NBS in acetate buffer at *p*H 4.6. The course of the reaction could be followed readily by measuring the rapid and extensive changes in the ultraviolet spectrum. The addition of three equivalents of brominating agent led to the appearance of a very intense peak at 260 mμ, the optical density attaining its maximum in less than three minutes. When the reaction was conducted on a preparative scale in acetonitrile–acetate buffer, a crystalline product began to separate shortly after the addition of reagent was begun. The same substance was obtained by the action of one equivalent of NBS on VI. The product, m.p. 174–176°, was a neutral compound whose infrared spectrum no longer indicated a phenolic hydroxyl group. The dienone-lactone structure VII (Chart II) was assigned on the basis of elemental analysis, infrared and ultraviolet spectra and subsequent transformations.

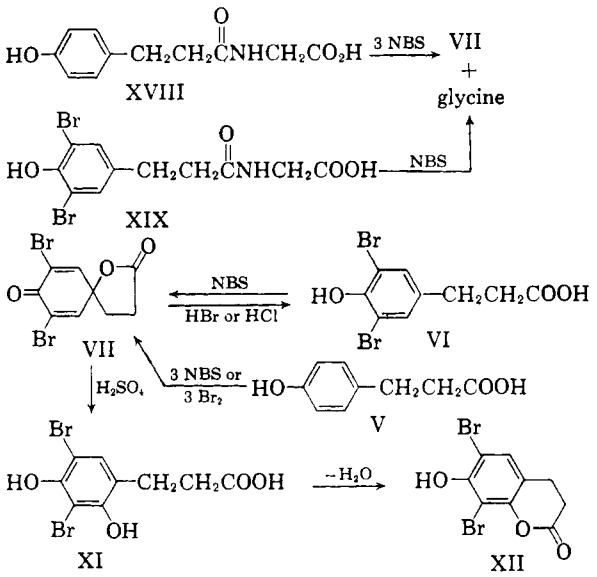
CHART I



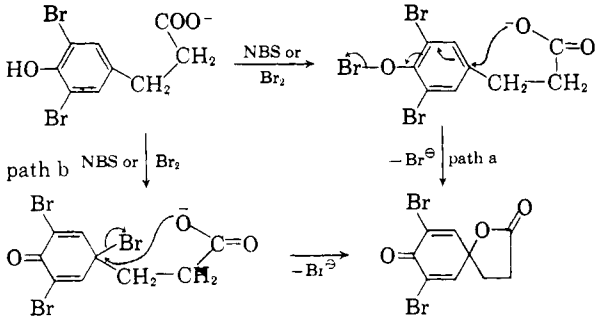
The course of the reaction may be considered to follow either path a or b. The occurrence of a concerted displacement reaction (path a) is postu-

(1) A. Patchornik, W. B. Lawson and B. Witkop, THIS JOURNAL, **80**, 4747, 4748 (1958).
(2) J. H. Kastle and A. S. Loevenhart, *Am. Chem. J.*, **27**, 32 (1902).
(3) K. Fries and G. Oehmke, *Ann.*, **462**, 1 (1928).
(4) C. D. Cook, N. G. Nash and H. R. Flanagan, THIS JOURNAL, **77**, 1783 (1955).
(5) R. T. Arnold, M. deMoura Campos and K. L. Lindsay, *ibid.*, **75**, 1044 (1953).
(6) W. P. Miller, Ph.D. Thesis, University of Minnesota, 1957.
(7) L. Goodman and S. Winstein, THIS JOURNAL, **79**, 4788 (1957).

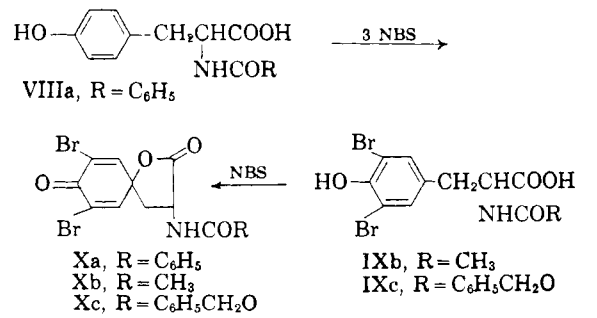
CHART II



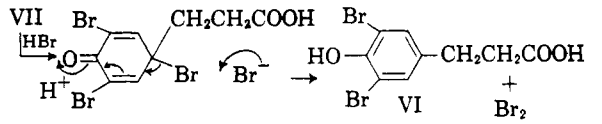
lated (*cf.* Chart I), although the formation of compounds such as I–III supports path b.



Analogous transformations were effected with N-acyl derivatives of tyrosine and 3,5-dibromotyrosine. Thus, N-benzoyl-L-tyrosine (VIIIa) and N-acetyl-(IXb) and N-carbobenzyloxy-3,5-dibromo-L-tyrosine (IXc) were converted in high yield into their respective N-acyldienone-lactones (X) by the action of NBS.



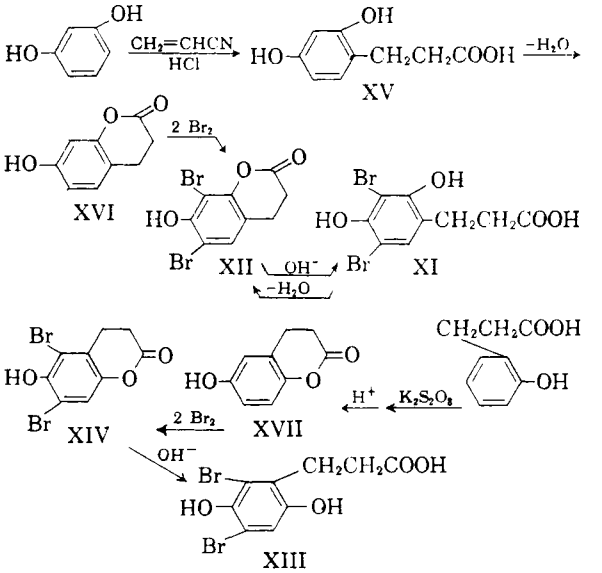
When the dienone VII was heated at reflux with 2 *N* hydrobromic acid for 1.5 hours, dibromophloretic acid (VI) was isolated in 40% yield from the reaction mixture. The identity of the reduction product was established by analysis and by comparison of melting point, infrared spectrum and paper chromatographic behavior with an authentic specimen. The reduction process is considered to proceed according to the scheme



This unusual reduction by halide ion of the vinylog of an α-acyloxyketone, presumably *via* an α-haloketone intermediate, finds analogy in the reduction of simple α-haloketones by halogen acid.[8]

To avoid reductive degradation, VII was refluxed with 4 *N* sulfuric acid for 3 hours. The crude product no longer showed any significant absorption at 260 mμ and a substance, isolated in 40% yield, was determined by analysis and infrared spectrum to be a phenolic acid of composition $C_9H_8O_4Br_2$. By fusion the acid was readily converted to a phenolic lactone. For comparison the isomeric dibromolactones XII, resulting from oxygen migration, and XIV, resulting from carbon migration, were synthesized as outlined in Chart III. The rearrangement product of VII

CHART III



and its lactone were found to be identical to the corresponding resorcinol derivatives XI and XII by mixed melting points and by infrared and ultraviolet spectra. The acid obtained by rear-

TABLE I

EFFECT OF ALKALI ON ULTRAVIOLET SPECTRA OF DIHYDRIC PHENOLS IN ALCOHOL[a]

Compound	λ_{max} (EtOH)	λ_{max} (EtOH + NaOH)	Δλ
Resorcinol	276	290	+14
Hydroquinone	295	283	−12
XVI	280	295	+15
XVII	286	265	−21
XII (synth.)	293	310	+17
XII (from H_2SO_4 rearr.)	292	310	+18
XIV	295	255	−40

[a] Alkaline shifts were measured after the addition of 0.1 ml. of 4 *N* sodium hydroxide to 2.5 ml. of 2 × 10^{-4} *M* ethanolic solutions.

(8) M. S. Newman, THIS JOURNAL, **73**, 4993 (1951); K. H. Meyer, *Ann.*, **380**, 212 (1911); J. V. Backes, R. W. West and N. A. Whiteley, *J. Chem. Soc.*, **119**, 359 (1921).

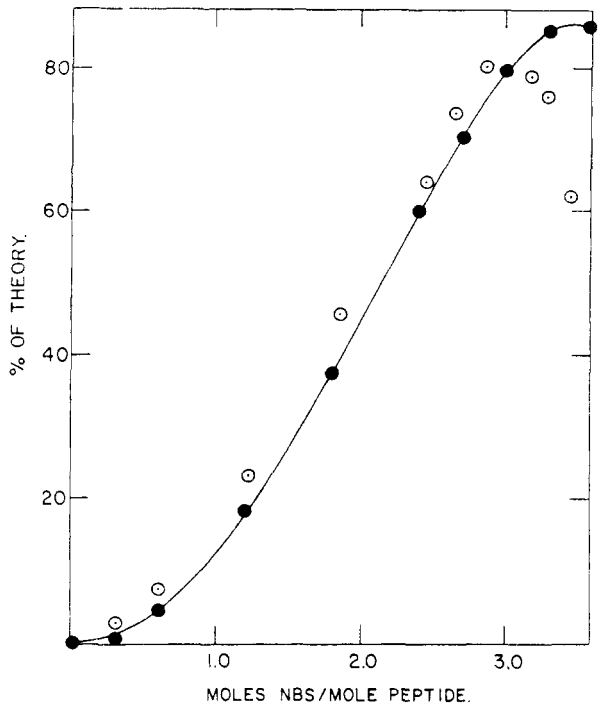


Fig. 1.—Reaction of phloretylglycine (XVIII) with N-bromosuccinimide: ●, ultraviolet absorption at 260 mμ; ⊙, ninhydrin color based on glycine.

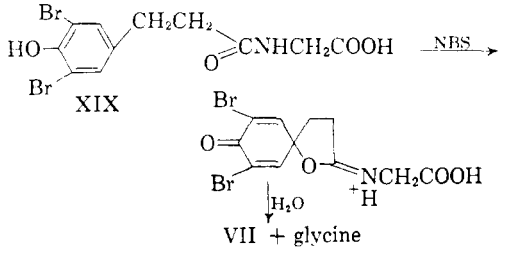
rangement and its lactone depressed the melting points of the corresponding hydroquinone derivatives XIII and XIV and showed different spectral properties. A study of the effect of alkali on the ultraviolet spectra of resorcinol and hydroquinone derivatives demonstrated the utility of this method for obtaining partial structural information. From Table I it may be seen that the addition of alkali to resorcinol or resorcinol lactones results in a bathochromic shift; on the other hand, the hydroquinone series consistently shows a hypsochromic shift.

The few analogies available from the literature[9] might lead one to expect carbon migration to occur. An inspection of molecular models suggests that the anomalous migration in this case may be due to the smaller steric interaction of oxygen–bromine than of methylene–bromine.

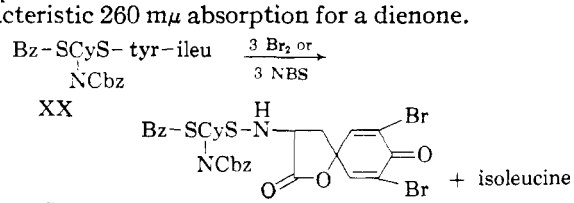
In 6 *N* hydrochloric acid at 25°, VII showed only a slight loss of 260 mμ absorption after 4 hours. When the reaction mixture was heated at reflux for one hour, the 260 mμ maximum had greatly decreased and a weak absorption maximum appeared at 290 mμ. A crude product, isolated in 50% yield, was shown to consist primarily of the reduction product VI. Examination of the crude material by paper chromatography revealed the presence of a small amount of the rearranged acid XI.

In contrast to the stability of the dienone VII to acid at room temperature, the addition of weak alkali caused a rapid decrease in 260 mμ absorption. In 0.01 *N* sodium carbonate or sodium hydroxide the reaction appeared to be complete in less than 2 hours and in 0.01 *N* sodium bicarbonate the same decrease required 24 hours. The alkaline degradation appears to be complex and the nature of the products is under investigation.

The ability of peptide bonds to participate in the lactonization reaction was demonstrated with phloretylglycine (XVIII) and 3,5-dibromophloretylglycine (XIX). The addition of NBS to a solution of the peptide in acetonitrile–buffer results in the formation of the lactone VII and the liberation of glycine. The correspondence between dienone formation and release of glycine was demonstrated by measuring ultraviolet absorption at 260 mμ and by quantitative ninhydrin assay following the addition of varying amounts of NBS (Fig. 1). The cleavage reaction shows a maximal yield, based on ninhydrin assay for glycine, of at least 80%. It was found in control experiments that glycine reacts rapidly with NBS. The drop in ninhydrin color yield when NBS is present in excess of theory (Fig. 1) may be attributed to this secondary reaction. In preparative experiments, VII was isolated in 48% yield from XVIII and in 49% yield from XIX. The cleavage reaction is presumed to follow the course outlined



The unusual cleavage of oxytocin and vasopressin by bromine water, reported by du Vigneaud and his associates,[10] is probably a reaction of the type described above. In a preliminary experiment using a tripeptide derivative related to oxytocin, it was found that bromine or NBS effected the expected cleavage. The liberation of a ninhydrin-reactive material in 40% yield from N-carbobenzyloxy-S-benzyl-L-cysteinyl-L-tyrosyl-L-isoleucine (XX)[11] was observed. Paper chromatography proved isoleucine to be the sole ninhydrin-reactive substance present. The ultraviolet spectrum of the reaction mixture showed the characteristic 260 mμ absorption for a dienone.



(9) E. Bamberger, *Ann.*, **390**, 164 (1912); S. Goodwin and B. Witkop, THIS JOURNAL, **79**, 179 (1957); F. Wessely and W. Metlesics, *Monatsh.*, **85**, 637 (1954). In the limited cases where oxygen migration has been observed, it has migrated as an acetoxy group and a neighboring-group participation is implicated. NOTE ADDED IN PROOF.—An alternative mechanism for forming a resorcinol derivative involves ring opening to the hydroxyacid, protonation of the ketonic carbonyl and β-addition of the carboxylate ion followed by elimination of water and aromatization. We are indebted to Prof. R. Huisgen for pointing out this alternative explanation.

(10) J. M. Mueller, J. G. Pierce and V. du Vigneaud, *J. Biol. Chem.*, **204**, 857 (1953); C. Ressler, S. Tripett and V. du Vigneaud, *ibid.*, **204**, 861 (1953); E. A. Popenoe and V. du Vigneaud, *ibid.*, **205**, 133 (1953); C. Ressler and V. du Vigneaud, *ibid.*, **211**, 809 (1954).

(11) C. Ressler and V. du Vigneaud, THIS JOURNAL, **79**, 4511 (1957).

In view of the oxidative cleavage of tryptophyl peptides[1] which is observed under experimental conditions similar to those for tyrosyl peptides, efforts are being directed toward a specific cleavage of the one bond in the presence of the other. In addition, the use of phloretic acid as a protective group for amines in peptide synthesis is being explored. More extensive studies on the selective cleavage of polypeptides and the chemistry of N-acylated dienone-lactones derived from tyrosine will be described in subsequent communications.

Experimental[12]

Phloretic Acid (V).—To a solution of 4.92 g. (0.03 mole) of *p*-coumaric acid in 70 ml. of 95% dioxane–water[13] was added 600 mg. of palladium chloride dihydrate. Hydrogenation was carried out at atmospheric pressure, and hydrogen uptake was complete in 1.5 hours at 100% of theory. The catalyst was removed by filtration, and the solvent evaporated under reduced pressure. The crystalline residue was recrystallized from water, yielding 3.92 g. (79%) of phloretic acid, m.p. 126–128° (lit.[14] 128–129°).

3,5-Dibromophloretic Acid (VI).—A solution of 565 mg. (0.0034 mole) of V and of 1.39 g. (0.010 mole) of sodium acetate trihydrate in 20 ml. of 50% acetic acid–water was cooled in ice. A solution of 1.09 g. (0.0068 mole) of bromine in 20 ml. of glacial acetic acid was added dropwise over a period of 30 minutes to the stirred solution. The reaction mixture was stirred 10 minutes longer, the solvent was removed under reduced pressure and a few ml. of water added to the residue. The flask was chilled briefly, the product collected and recrystallized from boiling water, yielding 850 mg. (77%) of colorless silky needles, m.p. 111.5–112.5° (lit.[15] 106–108°); VI was also occasionally recrystallized from benzene–ligroin (b.p. 90°).

7,9-Dibromo-1-oxaspiro[4,5]deca-6,9-dien-2,8-dione (VII).—To a solution of 240 mg. (0.00074 mole) of VI in 30 ml. of 20% acetonitrile–acetate buffer (0.20 *M*, *p*H 4.6) was added a solution of 135 mg. (0.00074 mole) of NBS in 20 ml. of the same solvent, in one portion. Within a minute, a crystalline substance began to separate. After 5 minutes, the reaction mixture was chilled briefly, and the product filtered, washed with cold water and dried *in vacuo* over P_2O_5 (193 mg., 81%, m.p. 167–177°). Two recrystallizations from acetonitrile–water yielded colorless needles, melting at 174–176° with decomposition.

The reaction filtrate was extracted with ether and the ethereal phase washed successively with 5% sodium bicarbonate and water, and then dried over magnesium sulfate. Removal of the solvent under reduced pressure yielded an additional 30 mg. (9%) of product. For analysis, the compound was dried over P_2O_5 at 65° for 16 hours; ultraviolet spectrum (20% acetonitrile–water): λ_{max} 260 mμ, ϵ_{max} 10,000; infrared spectrum (CH_2Cl_2): 5.55 μ (lactone C=O), 5.88 μ (conjugated C=O), 6.22 μ (conjugated C=C).

Anal. Calcd. for $C_9H_6Br_2O_3$: C, 33.57; H, 1.88; Br, 49.63. Found: C, 33.40; H, 2.01; Br, 49.80.

Compound VII also was obtained in 86% yield by reaction of phloretic acid (V) with three moles of NBS under the same experimental conditions, and in 62% yield by reaction with three moles of bromine.

3-(N-Benzoylamino)-7,9-dibromo-1-oxaspiro[4,5]deca-6,9-dien-2,8-dione (Xa).—To a chilled solution of 285 mg. (0.001 mole) of N-benzoyl-L-tyrosine[16] in 100 ml. of 20% acetonitrile–acetate buffer was added a solution of 534 mg. (0.003 mole) of NBS in 50 ml. of the same solvent. The reaction mixture was cooled for 2 hours, and the crystalline product was filtered and dried over P_2O_5 (420 mg., 95%, m.p. 198–203° with decomposition). Two recrystallizations from acetonitrile–water afforded colorless needles melting at 221–222° with decomposition. For analysis, the substance was dried over P_2O_5 at 65° for 17 hours; ultraviolet spectrum (ethanol): λ_{max} 228 mμ (ϵ 14,750), 259 mμ (ϵ 13,750); infrared spectrum (CH_2Cl_2): 2.92 μ (-NH-), 5.55 μ (lactone C=O), 5.89 μ (conjugated C=O), 5.97 μ (amide C=O), 6.23 μ (conjugated C=C).

Anal. Calcd. for $C_{16}H_{11}Br_2NO_4$: C, 43.57; H, 2.51; Br, 36.23. Found: C, 43.44; H, 2.63; Br, 36.24.

3-(N-Acetylamino)-7,9-dibromo-1-oxaspiro[4,5]deca-6,9-dien-2,8-dione (Xb).—To a solution of 381 mg. (0.001 mole) of N-acetyl-3,5-dibromo-L-tyrosine[17] (IXb) in 100 ml. of 20% acetonitrile–acetate buffer was added a solution of 178 mg. (0.001 mole) of NBS in 20 ml. of the same solvent. After a few minutes, the reaction mixture was placed in an ice-bath for 2 hours. The crystalline product was filtered, washed with cold water and dried over P_2O_5 (200 mg., 53%, m.p. 213–215° with decomposition). Two recrystallizations from acetonitrile–water yielded colorless needles, m.p. 219–221° with decomposition. For analysis, the compound was dried over P_2O_5 at 65° for 16 hours; ultraviolet spectrum (ethanol): λ_{max} 259 mμ, ϵ_{max} 10,600; infrared spectrum (CH_2Cl_2): 2.92 μ (-NH-), 5.55 μ (lactone C=O), 5.90 μ (conjugated C=O and amide C=O), 6.22 μ (conjugated C=C); $\alpha^{20}D$ −40.7° (*c* 0.6, acetonitrile).

Anal. Calcd. for $C_{11}H_9Br_2NO_4$: C, 34.84; H, 2.39; Br, 42.17. Found: C, 35.12; H, 2.41; Br, 42.11.

N-Carbobenzyloxy-3,5-dibromo-L-tyrosine (IXc).—To a solution of 1.69 g. (0.005 mole) of 3,5-dibromo-L-tyrosine in 40 ml. of water containing 1.5 ml. of 4 *N* NaOH was added, in alternating portions, 1.33 ml. of carbobenzyloxy chloride and 1.5 ml. of 4 *N* NaOH. The reaction mixture was kept cold and was shaken vigorously after each addition, while care was taken to maintain the *p*H at 9–10. The solution was extracted twice with ether and the aqueous phase was acidified in the cold to *p*H 1 with 6 *N* HCl. The oily product crystallized on cooling and scratching (1.70 g., 73%, m.p. 144°). Two recrystallizations from methanol–water yielded a product melting at 144–146°. For analysis, the substance was dried over P_2O_5 at 65° for 6 hours; $\alpha^{20}D$ +5.3° (*c* 1, methanol).

Anal. Calcd. for $C_{17}H_{15}Br_2NO_5$: C, 43.15; H, 3.20; Br, 33.78. Found: C, 43.38; H, 3.23; Br, 33.92.

The 3-(N-Carbobenzyloxyamino)-7,9-dibromo-1-oxaspiro-[4,5]-deca-6,9-dien-2,8-dione (Xc).—To a solution of 324 mg. (0.0007 mole) of IXc in 140 ml. of 20% acetonitrile–acetate buffer was added a solution of 128 mg. (0.007 mole) of NBS in 40 ml. of the same solvent. A crystalline substance separated within a few minutes. The reaction mixture was chilled for 30 minutes, and the product was collected and dried over P_2O_5 (310 mg., 97%, m.p. 201–214° with decomposition). Two recrystallizations from acetonitrile–water yielded colorless needles, m.p. 217–219° with decomposition. The product was dried over P_2O_5 at 65° prior to analysis; ultraviolet spectrum (ethanol): λ_{max} 259 mμ, ϵ_{max} 10,900; infrared spectrum (CH_2Cl_2): 2.92 μ (-NH-), 5.53 μ (lactone C=O), 5.78 μ (urethano C=O), 5.88 μ (conjugated C=O), 6.22 μ (conjugated C=C).

Anal. Calcd. for $C_{17}H_{13}Br_2NO_5$: C, 43.34; H, 2.78; Br, 33.92. Found: C, 43.32; H, 2.77; Br, 34.16.

6,8-Dibromo-7-hydroxychroman-2-one (XII).—To a solution of 1 g. (0.0062 mole) of 7-hydroxychroman-2-one[18] (XVI) and of 1.68 g. (0.0123 mole) of sodium acetate trihydrate in 30 ml. of 50% acetic acid–water was added dropwise a solution of 2 g. (0.0125 mole) of bromine in 30 ml. of glacial acetic acid. After stirring for 1 hour, the reaction mixture was concentrated to a small volume under reduced pressure, and the solid product collected by filtration (1.65 g., 83%, m.p. 175–184°). Two recrystallizations from benzene yielded large colorless prisms, m.p. 182–184°. Prior to analysis, the compound was dried over P_2O_5 at 80° for 12 hours; ultraviolet spectrum (90% ethanol–10% 0.3 *N* HCl): λ_{max} 293 mμ, ϵ_{max} 2800; infrared spectrum (CH_2Cl_2): 2.86 μ (phenolic -OH), 5.59 μ (lactone C=O).

Anal. Calcd. for $C_9H_6Br_2O_3$: C, 33.57; H, 1.88; Br, 49.63. Found: C, 33.58; H, 1.94; Br, 49.2.

(12) Melting points are uncorrected. Infrared spectra were run on a Perkin–Elmer recording spectrophotometer, model 21. Ultraviolet spectra were run on a Cary recording spectrophotometer, model 14.

(13) When the hydrogenation was performed in 95% ethanol–water, the product was contaminated with a small amount of phloretic acid ethyl ester.

(14) T. C. Bruice, *J. Org. Chem.*, **19**, 333 (1954).

(15) G. Habild, *Z. physiol. Chem.*, **285**, 127 (1950).

(16) S. W. Fox and C. W. Pettinga, *Arch. Biochem. & Biophys.*, **25**, 13 (1950); K. F. Itschner, E. R. Drechsler, C. Warner and S. W. Fox, *ibid.*, **33**, 294 (1954).

(17) D. G. Doherty and F. Vaslow, THIS JOURNAL, **74**, 931 (1952).

(18) W. D. Langley and R. Adams, *ibid.*, **44**, 2320 (1922).

3,5-Dibromo-2,4-dihydroxyphenylpropionic Acid (XI).—A solution of 250 mg. (0.00078 mole) of XII in 8 ml. of 1 *N* NaOH was allowed to stand at room temperature for 30 minutes. The reaction mixture was acidified in the cold to *p*H 1 with 6 *N* HCl, and the oil which appeared crystallized after scratching, yielding 260 mg. (98%) of needles, m.p. 148–150°. After two recrystallizations from water, the product melted at 149–150°. Prior to analysis, the substance was dried over P_2O_5 at 80° for 16 hours; ultraviolet spectrum (ethanol): λ_{max} 292 mμ, ϵ_{max} 2900; infrared spectrum ($CHCl_3$): 2.85 μ (phenolic –OH), 5.82 μ (carboxyl C=O).

Anal. Calcd. for $C_9H_8Br_2O_4$: C, 31.79; H, 2.37; Br, 47.0. Found: C, 31.77; H, 2.45; Br, 47.5.

The acid XI was reconverted to the lactone XII by heating it in an oil-bath at 150–155° for 1 hour, and then crystallizing the solid residue from benzene, m.p. 178–180°.

5,7-Dibromo-6-hydroxychroman-2-one (XIV).—To a solution of 1.64 g. (0.01 mole) of 6-hydroxychroman-2-one[19] (XVII) and of 2.72 g. (0.02 mole) of sodium acetate trihydrate in 30 ml. of 50% acetic acid–water was added dropwise with stirring a solution of 3.2 g. (0.02 mole) of bromine in 30 ml. of glacial acetic acid. The reaction mixture was stirred at room temperature for 1 hour. The precipitated product was filtered and washed with water (500 mg., 15%, m.p. 180–182°). Two recrystallizations from benzene–ligroin (b.p. 90°) yielded faintly yellow needles, m.p. 182–184°; ultraviolet spectrum (ethanol): λ_{max} 295 mμ, ϵ_{max} 3900; infrared spectrum (CH_2Cl_2): 2.86 μ (phenolic –OH), 5.63 μ (lactone C=O). On admixture with the isomeric lactone XII, the melting point fell to 150–160°. Concentration of the reaction filtrate under reduced pressure yielded an impure residue which was not examined further.

Anal. Calcd. for $C_9H_6Br_2O_3$: C, 33.57; H, 1.88; Br, 49.63. Found: C, 33.67; H, 1.98; Br, 49.44.

2,4-Dibromo-3,6-dihydroxyphenylpropionic Acid (XIII).—One hundred mg. (0.00031 mole) of XIV was dissolved in 100 ml. of 1 *N* NaOH and kept at room temperature for 5 minutes under a stream of hydrogen. After acidification with 6 *N* HCl, the reaction mixture was chilled briefly, and the crystalline product filtered and recrystallized from water (45 mg., 43%, m.p. 148–150°). Prior to analysis, the substance was recrystallized from chloroform–petroleum ether, and dried overnight with P_2O_5 at 80°, m.p. 149–150°; ultraviolet spectrum (ethanol): λ_{max} 302 mμ, ϵ_{max} 4800. On admixture with the isomeric acid XI, the melting point fell to 135–137°.

Anal. Calcd. for $C_9H_8Br_2O_4$: C, 31.79; H, 2.37. Found: C, 31.81; H, 2.68.

3,5-Dibromophloretylglycine (XIX).—To a cooled suspension of 303 mg. (0.0021 mole) of glycine ethyl ester hydrochloride in 15 ml. of methylene chloride was added 0.29 ml. (0.0021 mole) of triethylamine. The mixture was shaken vigorously and then poured into a suspension of 640 mg. (0.002 mole) of VI in 15 ml. of methylene chloride. After brief stirring, a solution of 420 mg. (0.0021 mole) of N,N'-dicyclohexylcarbodiimide in a small volume of methylene chloride was added. The reaction mixture was stirred at room temperature for 19 hours and then chilled for 5 hours. Following the removal of N,N'-dicyclohexylurea by filtration (370 mg. 81%), the filtrate was washed successively with 1 *N* HCl, 5% $NaHCO_3$ and water, and the solvent was removed under reduced pressure. The residue was dissolved in 10 ml. of methanol and 10 ml. of 1.25 *N* NaOH was added. After 3 hours at room temperature, the methanol was evaporated under reduced pressure and the reaction mixture filtered to remove a small amount of the urea. The filtrate was acidified to *p*H 7–8 with 6 *N* HCl and extracted twice with ether. Finally, the aqueous layer was acidified to *p*H 1 in the cold and the oil which separated crystallized on scratching. The product was collected by filtration, washed with cold water and dried *in vacuo* over KOH and P_2O_5 (500 mg., 66%, m.p. 152–155°). After recrystallization from methanol–water and then from ethyl acetate–ligroin (b.p. 90°), the product melted at 164–166°. Prior to analysis, the substance was dried for 6 hours at 65° over P_2O_5.

Anal. Calcd. for $C_{11}H_{11}Br_2NO_4$: C, 34.67; H, 2.91; Br, 41.96. Found: C, 34.66; H, 2.85; Br, 41.94.

Phloretylglycine (XVIII).—Condensation of glycine ethyl ester with phloretic acid was effected in a manner identical to the preparation of XIX. After acidification to *p*H 1, the clear solution was concentrated to a small volume under reduced pressure, and the product separated as colorless crystals. The yield, from 830 mg. of V, was 560 mg. (50%), m.p. 176–178° (lit.[20] 179°).

Reduction of Dienone-lactone VII with Hydrobromic Acid.—To a solution of 210 mg. (0.00065 mole) of VII in 5 ml. of acetonitrile was added 50 ml. of 2 *N* hydrobromic acid, and the reaction mixture was refluxed for 1.5 hours. The clear yellow solution was cooled to room temperature and the acetonitrile removed under reduced pressure. After addition of 10 g. of sodium chloride, the reaction mixture was warmed until a clear solution was obtained. Upon cooling overnight, colorless needles deposited (83 mg., 40%, m.p. 107–108°). After recrystallization from benzene–ligroin (b.p. 90°), the product melted at 108–109°.

Anal. Calcd. for $C_9H_8Br_2O_3$: C, 33.37; H, 2.49; Br, 49.34. Found: C, 33.28; H, 2.53; Br, 49.07.

No depression of melting point was observed on admixture with an authentic sample of 3,5-dibromophloretic acid (VI). The ultraviolet spectra (ethanol) and infrared spectra (chloroform) of the two substances were found to be identical. The reduction product was also compared to VI by chromatography on Whatman #1 paper in 1-butanol–dioxane–2 *N* NH_3 (4:1:5). Both substances gave single spots of R_f 0.18 upon spraying the air-dried chromatogram first with a 2% aqueous solution of phosphomolybdic acid, and then concentrated NH_4OH–ethanol (1:2).

Rearrangement of Dienone-lactone VII in 4 *N* Sulfuric Acid.—To a solution of 320 mg. (0.001 mole) of VII in 5 ml. of acetonitrile was added 54 ml. of 4 *N* sulfuric acid, and the reaction mixture refluxed for 3 hours. After concentration of the clear solution to about 20 ml., 4 g. of sodium chloride was added and the solution was cooled in ice for 3 hours. The solid product was filtered, washed with cold water and dried over P_2O_5 (130 mg., 40%, m.p. 135–140°). Recrystallization from water yielded 100 mg. of needles, m.p. 148–150°.

The product showed no depression of melting point on admixture with an authentic sample of XI. The ultraviolet (ethanol) and infrared (chloroform) spectra of the two substances were identical. The melting point of a mixture of the product with XIII was 135–136°.

This product, 65 mg., was heated in an oil-bath at 150–155° for 45 minutes. The solid residue was sublimed *in vacuo* at 140–150° (1 mm.), yielding 50 mg. of a colorless powder, which, after crystallization from benzene, melted at 180–181°. No depression of melting point was noted on mixing this substance with an authentic sample of the lactone XII, and the infrared spectra of the two compounds (CH_2Cl_2) were identical. The infrared spectra differed significantly in the fingerprint region from that of XIV, and mixed melting point of the product with XIV was 155–165°.

Anal. Calcd. for $C_9H_6Br_2O_3$: C, 33.57; H, 1.88; Br, 49.63. Found: C, 33.54; H, 2.16; Br, 49.26.

Reaction of Dienone-lactone VII with 6 *N* Hydrochloric Acid.—To a solution of 400 mg. (0.00125 mole) of VII in 5 ml. of acetonitrile was added 50 ml. of 6 *N* HCl. The mixture was refluxed for 1 hour, yielding a clear solution which was concentrated to a small volume under reduced pressure. The voluminous precipitate was filtered, washed several times with cold water to dissolve contaminating ammonium chloride, and dried (140 mg., m.p. 107–114°). The combined filtrate and washings were extracted with 3 35-ml. portions of ether. The ethereal phase was dried, concentrated under reduced pressure, and the residual oil was crystallized from water; yield 45 mg. of needles contaminated with a little oily material.

The infrared spectrum (chloroform) of the first product was almost identical to that of 3,5-dibromophloretic acid (VI). Paper chromatography of both fractions showed them to consist primarily of 3,5-dibromophloretic acid, together with a small amount of XI (1-butanol–dioxane–2 *N* NH_3 [4:1:5], upper phase, descending; development with

(19) O. Neubauer and L. Flatow, *Z. physiol. Chem.*, **52**, 376 (1907); G. Bargellini and L. Monti, *Gazz. chim. ital.*, **45** I, 90 (1915) [*C. A.*, **9**, 2239 (1915)]; S. M. Sethna, *Chem. Revs.*, **49**, 91 (1951). The lactone XVII was prepared by persulfate oxidation of dihydrocoumarin. A 12% yield was obtained after purification of the crude product by chromatography on neutral alumina in ethyl acetate solution.

(20) A. Sonn, *Ber.*, **46**, 4050 (1913).

2% aqueous phosphomolybdic acid spray, followed by 1:2 ammonia–ethanol).

Reaction of 3,5-Dibromophloretylglycine (XIX) with NBS.—To a solution of 200 mg. (0.0053 mole) of XIX in 30 ml. of 20% acetonitrile–acetate buffer (0.16 *M*, *p*H 4.6) was added a solution of 93 mg. (0.00053 mole) of NBS in 10 ml. of the same solvent. Colorless needles began to separate within 5 minutes. After 2.5 hours, the reaction mixture was chilled briefly and the dienone-lactone VII was collected by filtration (84 mg., 49%, m.p. 173–175°).

No melting point depression was observed on admixture with a specimen of VII prepared from 3,5-dibromophloretic acid (VI). The ultraviolet spectra of the two substances were identical. Paper chromatography of a sample of the reaction mixture on Whatman #1 paper in 1-butanol–acetic acid–water (4:1:5) showed the presence of a single ninhydrin-positive spot, corresponding in R_f to a glycine standard. Quantitative ninhydrin assay[21] of an aliquot of the reaction mixture gave a value of 76% cleavage, based on the color yield of a glycine standard.

Reaction of Phloretylglycine (XVIII) with NBS. A. Preparative Experiment.—To a solution of 334 mg. (0.0015 mole) of phloretylglycine in 25 ml. of 20% acetonitrile–acetate buffer was added, in 5 equal portions at one-minute intervals, a solution of 820 mg. (0.0046 mole) of NBS in the same solvent. Within 10 minutes, a crystalline solid had begun to separate. After 2 hours, the product was collected by filtration (230 mg., 48%, m.p. 166–173°). Recrystallization from acetonitrile–water yielded colorless needles melting at 172–174°. The product was shown to be identical to the dienone-lactone VII by ultraviolet spectrum and mixed melting point.

B. **Analytical Experiment.**—A series of reaction mixtures containing phloretylglycine (10^{-3} *M*) and NBS (0–3.6×10^{-3} *M*) in the same solvent as above were allowed to stand at room temperature for 30 minutes. At the end of this time, no NBS remained, as determined by iodometric titration. From each solution, aliquots were removed for spectrophotometric determination of the dienone-lactone VII (optical density at 260 mμ), and ninhydrin assay for glycine.[21] The results of this experiment are shown in Fig. 1.

In a control run, it was noted that glycine reacted rapidly with NBS under the conditions of the cleavage reaction. Using a solution containing glycine and NBS each at 10^{-3} *M*, it was found that less than half of the NBS remained after two minutes (iodometric titration). The decrease in ninhydrin color occurred more slowly.

Reaction of N-Carbobenzyloxy-S-benzyl-L-cysteinyl-L-tyrosyl-L-isoleucine (XX) with NBS.—A solution of N-carbobenzyloxy-S-benzyl-L-cysteinyl-L-tyrosyl-L-isoleucine[11] at 4×10^{-4} *M* was allowed to react with 3 equivalents of NBS or of bromine under the conditions described above for phloretylglycine (XVIII). After 30 minutes, aliquots were removed for ninhydrin assay, which indicated a cleavage yield of 40%. Paper chromatography on Whatman #1 paper in 1-butanol–acetic acid–water (4:1:5) gave a single ninhydrin-positive spot of R_f corresponding to that of a standard sample of isoleucine. Spectral examination of a sample of the reaction mixture showed the presence of an intense maximum at 260 mμ.

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(21) S. Moore and W. H. Stein, *J. Biol. Chem.*, **176**, 367 (1948).

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Study of Inhibition of Azaserine and Diazo-oxo-norleucine (DON) on the Algae *Scenedesmus* and *Chlorella*[1]

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The effects of azaserine and DON (diazo-oxo-norleucine) on the metabolism of the algae *Scenedesmus* and *Chlorella* during photosynthesis with $C^{14}O_2$ are presented and found to be largely the same for both inhibitors. DON appears to inhibit the formation of α-N-formylglycinamidine ribotide from α-N-formylglycinamide ribotide, as is known for azaserine. α-N-Formylglycinamide ribotide and glutamine accumulate with both inhibitors, being absent from the control experiment. A proposal is presented that a possible site of inhibition is the synthesis of glutamic acid from α-ketoglutaric acid. The relationship between glutamine and glutamic acid is discussed and the suggestion made that there may be a biosynthetic route to glutamine not involving glutamic acid as an intermediate. Indications for a still wider interference of the inhibitors are seen.

Introduction

In studies of the effect of azaserine on *Scenedesmus* during photosynthesis with $C^{14}O_2$ the inhibitor was found to cause many changes in the labeling of metabolic intermediates with C^{14}.[3] The transamination reactions were given as possible sites of inhibition. This work has been extended, and in this report a study on the inhibition effects of both azaserine and diazo-oxo-norleucine (DON) on the metabolism of the algae *Scenedesmus* and *Chlorella* is reported.

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(3) S. A. Barker, J. A. Bassham, M. Calvin and U. C. Quarck, THIS JOURNAL, **78**, 4362 (1956).

Experimental Procedures

The experiments were of two types: type A, in which first inhibitor and then, after 5 minutes, $C^{14}O_2$ is added to the algae suspension; and type B in which the algae are continuously in contact with $C^{14}O_2$, before, during and after the addition of inhibitor.

Experiments of type A show the effect of the presence of inhibitor for a fixed time on the distribution of radioactivity among the metabolic intermediates after 5 minutes photosynthesis. In experiments of type B the distribution of radioactivity among metabolites following introduction of the inhibitor can be followed over a longer period of time.

A brief description of experiments of type A and B with experimental details is given below, followed by tables and charts of the results of those experiments. Any deviation from the standard procedure is noted under the experiment concerned. Further experimental details have been published elsewhere.[4]

(4) P. Y. F. van der Meulen, Rijks Universiteit, Leiden, The Netherlands, Thesis, 1958.