Low Molecular Weight Products in *p*-Polyphenyl.—Less than 0.2% of soluble material (brown tar) was obtained by successive extractions of the polymer with boiling ether, chloroform and *p*-xylene. There was no biphenyl or terphenyl in the extract on the basis of gas chromatographic analysis.

Benzene–Aluminum Chloride–Water.—A mixture of benzene (1 mole), aluminum chloride (0.5 mole) and water (1 ml.) was stirred at reflux under nitrogen for 1 hr. No *p*-polyphenyl was obtained.

Benzene–Cupric Chloride–Water.—A mixture of benzene (1 mole), cupric chloride (0.5 mole) and water (1 ml.) was stirred at reflux under nitrogen for 1 hr. No *p*-polyphenyl was obtained.

Acknowledgment.—Acknowledgment is made to donors of the Petroleum Research Fund, administered by the American Chemical Society, for support of this research.

[CONTRIBUTION FROM THE INSTITUTE FOR ENZYME RESEARCH, UNIVERSITY OF WISCONSIN, MADISON, WISC.]

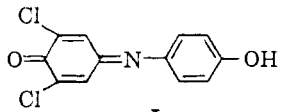
The Conjugation of Cysteine during its Oxidation by 2,6-Dichloroindophenol[1a]

BY HERBERT I. HADLER,[1b] MARY JANE ERWIN AND HENRY A. LARDY

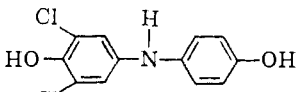
RECEIVED JULY 9, 1962

When cysteine is oxidized by 2,6-dichloroindophenol not only is the reduced dye produced, as hitherto accepted, but also a conjugate between cysteine and the dye is formed. The nature of the conjugate and the relative amount of reduced dye are determined by the initial molar ratio of dye to cysteine. Contrary to expectation, polyconjugation of the dye with cysteine was favored by an excess of dye. The term *oxidative addition* or *oxidative substitution* has been proposed to emphasize the role of oxidation in fostering addition or substitution.

The reduction of an oxidation–reduction indicator, such as 2,6-dichloroindophenol (I), has frequently been used to follow the course of many biochemical oxida-



I

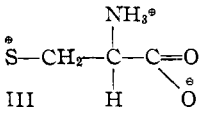


II

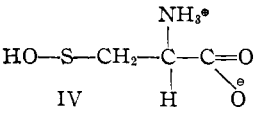
tion–reduction processes.[2] The stoichiometry has been represented by eq. 1 and the dye classed as a two-electron oxidant. The loss of color in the visible spectrum has been used as a convenient measure of the conversion of I to II.

$$\text{I} + 2 \text{ electrons} + 2\ \text{H}^+ \longrightarrow \text{II} \qquad (1)$$

Toderick and Walker[3] found that one mole of I was decolorized by one mole of cysteine. This observation became of interest when Basford and Huennekens[4] raised the question of the oxidation level of the altered thiol group. If cysteine had yielded the disulfide, cystine, two moles of cysteine would have reduced one mole of dye.[5] These authors observed that the rate of decolorization was first order with respect to the hydrogen ion concentration. Thus in order to comply with the stoichiometry of eq. 1 they concluded that the medium yielded one proton, while one proton and two electrons were derived from cysteine. Consequently they proposed that cysteine had been converted to the sulfenium ion III, which is the cation derived from cysteinesulfenic acid (IV).



III

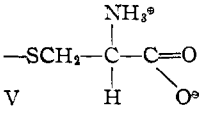


IV

Benesch and Benesch[6] studied the disulfide interchange between cystine and bis-(2,4-dinitrophenyl)-cystine in a strongly acidic medium (9.5 *N* hydrochloric acid). They believed the sulfenium ion III to be an intermediate. In accordance with the concept of Basford and Huennekens,[4] Benesch and Benesch[6] generated III from the dye and cysteine at *p*H 7.0; then lowered the *p*H to 4 and removed I and II from the aqueous medium by extraction into ether. The aqueous solution catalyzed disulfide interchange. Furthermore, Benesch and Benesch[6] found that the extracted aqueous solution retained its catalytic activity for several days when stored at 0°. As this was not in keeping with the expected instability of III, Benesch and Benesch[6] suggested that the extracted solution at *p*H 4.0 did not contain the sulfenium ion III, but a relatively stable compound which gave rise to III, in the presence of strong acid.

The oxidized form of the dye I resembles a *p*-quinone. The 1,4-addition of a thiol to a quinone has been well recognized.[7–11] Basford and Huennekens were cognizant of the possible 1,4-addition of cysteine to I, but believed that such a reaction did not complicate the oxidation–reduction process being measured because of the speed of the reaction, the dilution of the reactants and the observed stoichiometry.

An equation such as 1, while useful for the calculation of the thermodynamic parameters of two half-cells, need not necessarily describe the mechanism of a reaction occurring entirely in solution. This view as well as the data of Benesch and Benesch[6] prompted the search for a conjugate between cysteine and the dye I. In such a substance one of the hydrogens of II might be replaced by a cysteine residue V and thus the conjugate



V

would be colorless in the visible, absorb in the ultraviolet, and likely be non-extractable by ether.

Evidence which suggested adduct formation was obtained by reducing the dye with a series of graded amounts of cysteine (two cases are shown in Fig. 1) and with an excess of potassium borohydride (Fig. 1). Although partial re-oxidation occurred during the spectral scanning of the mixture reduced with potassium borohydride, this curve (tube 3) intersected the curve of the original dye at three points (256.5, 279.5 and 291.5 mμ). One of these points should be an isos-

(1) (a) Supported by a grant from the National Science Foundation and by a grant from the National Institutes of Health (H-2677). Presented at 142nd Natl. Meeting of the American Chemical Society, Atlantic City, N. J., September, 1962. (b) Supported by a Special Fellowship, Division of Medical Sciences, National Institutes of Health.

(2) W. M. Clark, "Topics in Physical Chemistry," 2nd ed., The Williams and Wilkens Co., Baltimore, Md., 1952, p. 469.

(3) A. Toderick and E. Walker, *Biochem. J.*, **31**, 292 (1937).

(4) R. E. Basford and F. M. Huennekens, *J. Am. Chem. Soc.*, **77**, 3873 (1955).

(5) Only the thiol group of cysteine participated in the reaction for cystine did not decolorize the dye.

(6) R. E. Benesch and R. Benesch, *J. Am. Chem. Soc.*, **80**, 1666 (1958).

(7) T. Posner, *Ann.*, **336**, 85 (1904).

(8) J. M. Snell and A. Weissberger, *J. Am. Chem. Soc.*, **61**, 450 (1939).

(9) R. Kuhn and H. Beinert, *Ber.*, **77**, 606 (1944).

(10) M. Shubert, *J. Am. Chem. Soc.*, **69**, 712 (1947).

(11) R. Cecil and J. R. McPhee, *Advan. in Protein Chem.*, **14**, 255 (1959).

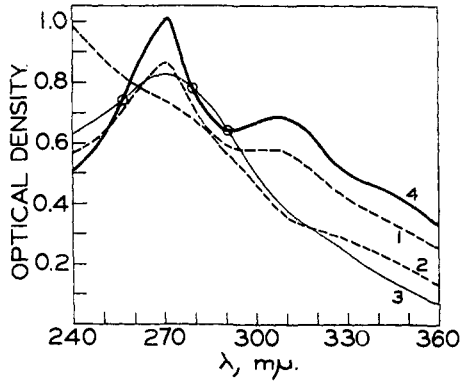


Fig. 1.—Comparative reduction of dye I by cysteine and potassium borohydride.

μ mole	1	2	3	4
Dye	0.4	0.4	0.4	0.4
Cysteine	0.10	0.8	...	...
Potassium borohydride	...	...	10	...

Each tube contained 2.0 ml. of 0.1 *M* potassium phosphate adjusted to *p*H 7.0 and as indicated: 0.4 ml. of 1×10^{-3} *M* dye, the appropriate volume of 5×10^{-4} *M* cysteine and 0.2 ml. of 5×10^{-2} *M* potassium borohydride in a total volume of 6.0 ml. The solutions with cysteine were kept at room temperature and during the second and third hour after mixing they were read against a blank of water and buffer. Controls with cysteine or potassium borohydride showed no absorbancy. Although tube 3 was decolorized immediately before being placed in the recording (continuous) spectrophotometer, some blue color had returned at the end of the 15 minutes required for scanning. The possible isosbestic points have been shown by the open circles.

bestic point for binary mixtures of I and II. It was found that none of these points of intersection was an isosbestic point for the dye solutions reduced by cysteine. Thus the dye solution to which cysteine had been added was not a simple mixture of I and II.

At *p*H 6.8 or lower, both I and II[12] were completely extractable by ether[13] (Table I, Fig. 2). On the other hand, after compound I had been decolorized with an excess of cysteine and the aqueous solution extracted with ether the aqueous solution absorbed in the ultraviolet (Table I, Fig. 3). Clearly cysteine and the dye I had formed a hydrophilic ultraviolet-absorbing conjugate. The ether extract contained ultraviolet-absorbing material whose chromophore was identical with that of the leuco dye (Fig. 2). The identity of the leuco dye in the ether extract from an experiment with cysteine was confirmed by air re-oxidation in buffer to the original dye. As shown (Fig. 2), cysteine generated less leuco dye than potassium borohydride because conjugation with cysteine had rendered some of the dye non-extractable into ether.

When the dye was decolorized with an excess of cysteine the amount of conjugate or leuco dye did not change with time (Table II). This led to the conclu-

TABLE I

EXTRACTION OF DECOLORIZED DYE

	1	2	3	4	5	6
μmoles of dye	0.3	..	0.3	..	0.3	0.3
μmoles of cysteine	0.6	0.6	..	..	..	..
μmoles of potassium borohydride	..	..	10	10	..	..
Ether extraction	Yes	Yes	Yes	Yes	Yes	No
*p*H of aqueous residue	6.7	6.7	6.8	6.9	6.8	6.7
U.v. absorbance of aq. residue	Yes	No	No	No	No	Yes
Visible absorbance of aq. residue	No	No	No	No	No	Yes
U.v. absorbance of ether extract	Yes	No	Yes	No	Yes	..
Visible absorbance of ether extract	No	No	No	No	Yes	..

Each 15 ml. calibrated centrifuge tube contained 2.0 ml. of 0.1 *M* potassium phosphate adjusted to *p*H 7.0 and as indicated: 0.3 ml. of 1×10^{-3} *M* dye, 0.3 ml. of 2×10^{-3} *M* cysteine and 0.2 ml. of 5×10^{-2} *M* potassium borohydride, in a total volume of 6.0 ml. After 20 minutes incubation in ice, 0.5 ml. of 0.1 *M* hydrochloric acid was added and the tubes extracted three times with ether (4 + 4 + 5 ml.). The ether extracts were adjusted to 12 ml. and read against an ether blank. The volume of the aqueous residue was recorded and the solution read against a blank of buffer, water and acid. Other data showed that there was no ultraviolet-absorbing material extractable into the water from the ether.

(12) The conversion of I to II in the aqueous medium was carried out with potassium borohydride.

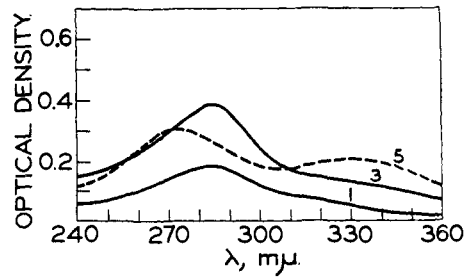


Fig. 2.—Ultraviolet spectra of an ether extract of dye I (Table I, tube 5), an ether extract of dye decolorized with potassium borohydride (Table I, tube 3), and an ether extract of dye decolorized with excess cysteine (Table I, tube 1).

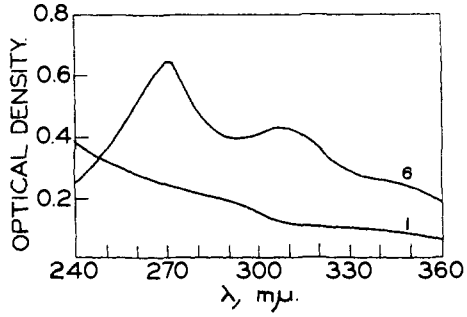


Fig. 3.—Ultraviolet spectra of a non-extracted aqueous solution of dye I (Table I, tube 6) and an extracted aqueous residue from a mixture of cysteine and dye I (Table I, tube 1).

TABLE II

STABILITY OF THE CONJUGATE

Period of incubation, minutes	Optical density of aqueous residue at 240 mμ	320 mμ	600 mμ
10	0.39	0.11	0.02
25	.39	.10	.02
55	.39	.12	.02
145	.40	.11	.02
170	.39	.11	.02

The incubation tube, which was kept in ice under an atmosphere of nitrogen, contained: 3.0 ml. of 1×10^{-3} *M* dye, 20 ml. of 0.1 *M* potassium phosphate *p*H 7.0 and 3.0 ml. of 2×10^{-3} *M* L-cysteine in a final volume of 60 ml. In control and standard tubes the dye and cysteine, respectively, were omitted. At the appropriate time beginning immediately after the dye was visibly decolorized, 0.5 ml. of 0.1 *N* HCl was added to a 6.0-ml. aliquot which then was extracted thrice with ether (4 + 4 + 5 ml.) and a continuous ultraviolet spectrum obtained against a blank of buffer and water. Representative points have been tabulated. The 145-min. sample was kept exposed to air at 23° during the final 65 minutes. There was no ultraviolet absorbancy in the control. The continuous ultraviolet spectra of the ether extracts of the 10-minute and 25-minute sample indicated identical amounts of leuco dye.

(13) Surprisingly, it was not necessary to lower the *p*H to 4, as Benesch and Benesch did in order to effect this extraction, even though the pK_a of the dye I is 5.7 (ref. 2, p. 467).

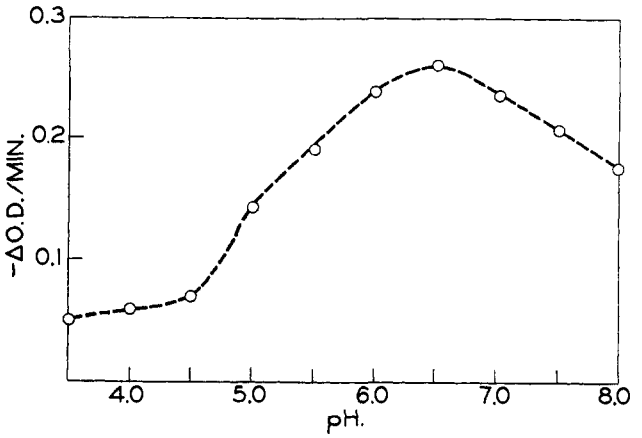
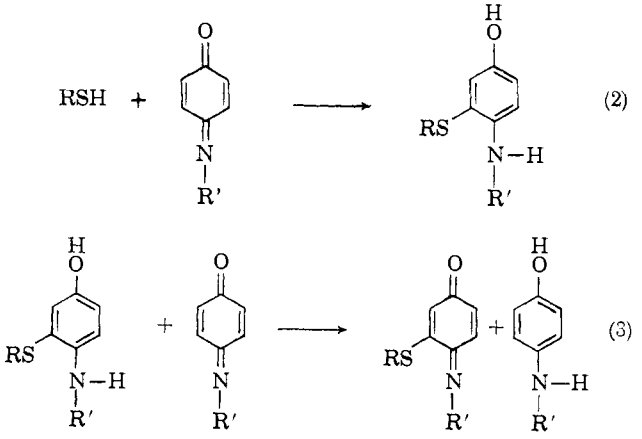


Fig. 4.—Variation of rate of decolorization with *p*H. Each tube contained 1.0 ml. of 0.1 *M* potassium phosphate adjusted to the appropriate *p*H with acetic acid, 0.3 μmole of dye and 0.05 μmole of cysteine in a final volume of 3.0 ml. The measurements were made at 524 mμ during the initial 30 seconds.

sion that there was no common intermediate which yielded conjugate and leuco dye; consequently another explanation for the formation of a conjugate and leuco dye was sought.

It is known that when an electron-donating group adds 1,4 to a quinone the new substituted hydroquinone is the reduction product of a substituted quinone whose oxidation potential is lower than the original unsubstituted quinone.[11,14] Consequently the new substituted hydroquinone may be oxidized to the substituted quinone by the original quinone with the simultaneous formation of an unsubstituted hydroquinone. More than one group may enter the original quinone by a repetition of these processes. When applied to a tautomer of the dye the addition and oxidation–reduction sequence would be represented by eq. 2 and 3.



On further reaction leading to polysubstitution the stoichiometric ratio of leuco dye to conjugate would vary directly with the degree of substitution as long as the dye present was capable of oxidizing all the reduced adduct.

Measurements[15] were made following the addition of a series of graded amounts of cysteine to a fixed amount of dye (Fig. 6 is typical of such an experiment). The

(14) L. F. Fieser and M. Fieser, "Organic Chemistry," Reinhold Publishing Corp., New York, N. Y., 3rd ed., 1956, p. 716.

(15) These experiments were conducted at *p*H 6.5 in a phosphate buffer adjusted with acetic acid as used by Benesch and Benesch.[6] In agreement with Basford and Huennekens,[4] the rate of decolorization of the dye by cysteine increased as the *p*H was lowered from 8.5 to 6.5 (Fig. 4). However, it was also observed that in the range from *p*H 6.5 to 3.5 (Fig. 4) the rate of decolorization dropped as the *p*H was lowered. The absorbancy for the rate studies was measured at the isosbestic point (524 mμ) for the spectral variation of I with *p*H (Fig. 5).

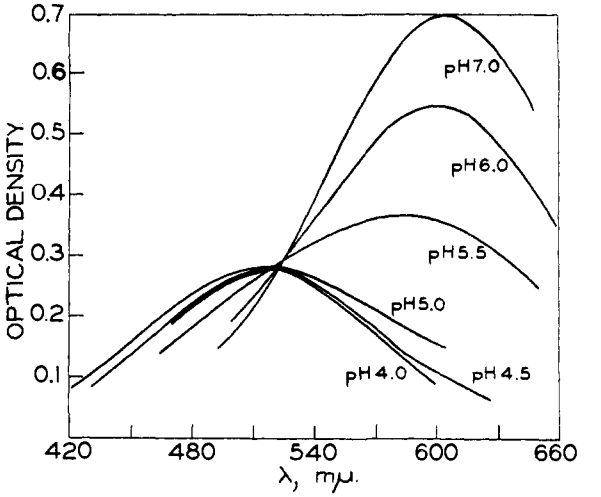


Fig. 5.—Variation in spectra of dye I with *p*H. Each tube contained 0.3 μmole of dye and 1.0 ml. of 0.1 *M* potassium phosphate adjusted to the appropriate *p*H with acetic acid in a final volume of 3.0 ml.

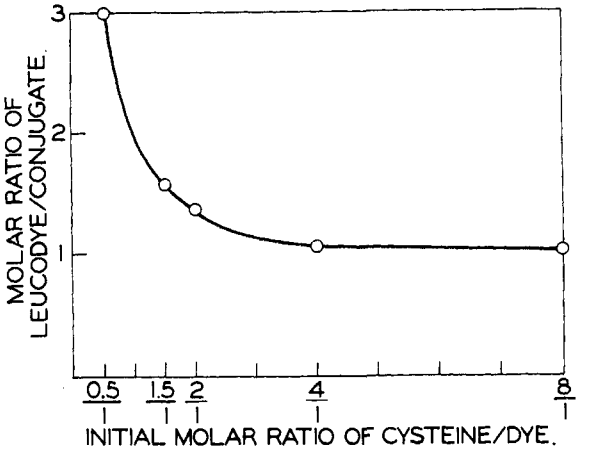


Fig. 6.—Variation of products from cysteine and dye I. Each tube contained 2.0 ml. of 0.1 *M* potassium phosphate adjusted to *p*H 6.5 with acetic acid, 0.3 μmole of dye and the appropriate amount of 1×10^{-3} *M* cysteine at *p*H 6.5; *viz.*, 0.15 ml., 0.45 ml., 0.6 ml., 1.2 ml., 2.4 ml. in a total volume of 6.0 ml. Standards for I and II (generated with 0.2 ml. of 5×10^{-2} *M* potassium borohydride) were included as well as corrections for the trace absorbancy introduced by the potassium borohydride. Incubation was 20 minutes in ice. Three ether extractions (4 + 4 + 5 ml.) were carried out. The aqueous residue was read at 240 and 600 mμ against a blank of water and buffer. The ether extract was adjusted to 12 ml. and read at 283 and 530 mμ against an ether blank.

absorbancy of standard amounts of I and II[12] in the ether extracts (measured at their respective maxima of 530 and 283 mμ) allowed the determination of I and II when mixed together in the ether extracts. The dye not accounted for as I and II in the ether extract was the amount of conjugated dye remaining in the aqueous solution. The data (Fig. 6) showed that the molar ratio of leuco dye II to conjugate varied with the ratio of reactants. The greater the original proportion of dye the greater the ultimate ratio of leuco dye to conjugate and consequently the greater the degree of substitution.

Without the preceding data first reflections might suggest that polysubstitution would be favored by an excess of cysteine.[16] The data demonstrate the op-

(16) A. Blackhall and R. H. Thomson (*J. Chem. Soc.*, 1138 (1953)) tacitly made this assumption when they attempted, unsuccessfully, to minimize polysubstitution of *p*-benzoquinone by thioglycolic acid by adding the thiol slowly to the quinone.

posite: *polysubstitution is favored by an excess of dye.* This reflects the preference of an oxidized adduct as the electrophilic acceptor for the nucleophilic cysteine.

A reaction leading to substitution through the generation of an oxidized adduct might be termed *oxidative addition* or *oxidative substitution* in order to emphasize that addition or substitution is driven by an oxidizing agent acting in appropriate sequence. The term could apply to several instances: (a) oxidation generates an electrophilic acceptor; (b) oxidation of the first adduct pulls the first addition reaction; (c) a combination of b and a.

This concept is useful in explaining the older results of Snell and Weissberger[8] and of Shubert.[10] It was evident from these two papers that experimental conditions controlled the degree of substitution of *p*-benzoquinone by thioglycolic acid. When Shubert[10] added, during a period of two and a half hours, an aqueous solution of thioglycolic acid to a well-stirred aqueous suspension of *p*-benzoquinone he in essence maintained a large excess of oxidizing agent during the initial part of the reaction and isolated tetrasubstituted product in the reduced form in 30% yield. Shubert[10] also reported, without experimental details, that when the addition was made in the reverse order the yield of tetrasubstituted product fell sharply. After the immediate addition of an aqueous solution of thioglycolic acid to a double molar portion of *p*-benzoquinone in alcoholic solution, Snell and Weissberger[8] isolated the monosubstitution product in the oxidized form in 32% yield. The yield fell if the reaction period was longer than thirty minutes. It is entirely reasonable that in this latter experiment the effective ratio of quinone to thiol was smaller than at the beginning of Shubert's experiment.

The formation of a conjugate between cysteine and 2,6-dichloroindophenol (I) does not eliminate the possible formation of cysteinsulfenium ion III or the possibility that the conjugate, especially in the oxidized form, is a source of electrophilic sulfur in the strongly acidic medium employed by Benesch and Benesch.[6,17]

Experimental[18]

Re-oxidation of Leuco Dye.—A colorless ether extract containing the leuco dye generated by the action of excess cysteine on the dye I remained colorless until all the ether slowly evaporated. After the addition of water and phosphate buffer (*p*H 7.0) and shaking, the blue color developed after several hours. The ultraviolet and visible spectra were quantitatively that of the nonconjugated amount of original dye.

(17) The scope and limitations of the reaction of compounds of biochemical interest with 2,6-dichloroindophenol will be published elsewhere.

(18) Absorption spectra were measured in a cuvette with a 1-cm. light path in a Cary (Model 11) recording spectrophotometer or a Beckman DU spectrophotometer. The chemicals and their commercial sources were: sodium salt of 2,6-dichloroindophenol, dihydrate (Eastman Kodak Co.); L-cysteine hydrochloride monohydrate (Mann Research Laboratories); potassium borohydride, 97% pure (Metal Hydrides); ether, analytical grade in metal cans (Mallinckrodt). The cysteine and dye were nominally accepted as 100% pure. The water was purified by distillation, followed by passage through a mixed bed resin (Amberlite MB3) and re-distillation. Before use the water was degassed by boiling, and cooled in a completely stoppered vessel. All extractions were done in open calibrated 15-ml. centrifuge tubes whose contents were mixed and layers separated by means of Pasteur pipets fitted with a rubber bulb.

[CONTRIBUTION FROM THE SCHOOL OF CHEMISTRY, UNIVERSITY OF MINNESOTA, MINNEAPOLIS]

Biosynthesis of Gramine: Feeding Experiments with Tryptophan-β-[H^3,C^{14}][1]

BY DANIEL O'DONOVAN[2] AND EDWARD LEETE

RECEIVED AUGUST 6, 1962

A mixture of DL-tryptophan-β-C^{14} and DL-tryptophan-β-H^3 was fed to barley seedlings and resulted in the formation of radioactive gramine which was labeled solely on the methylene group of the side chain with carbon-14 and tritium. The ratio of carbon-14 to tritium in the gramine was the same as in the administered tryptophans. This result strongly suggests that the methylene group of the tryptophan side chain maintains its integrity during the conversion of tryptophan to gramine.

Gramine (XIV) which is formed in germinating barley[3] was one of the first alkaloids to be studied using radioactive tracers,[4,5] and it was established that tryptophan is a precursor of this simple indole alkaloid. However, despite considerable effort by several groups of workers the mechanism of this biosynthesis has not been determined. When a mixture of tryptophan-2-C^{14} [6] and tryptophan-β-C^{14} was administered to barley[7] the resultant radioactive gramine was labeled solely at C-2 of the indole nucleus and on the methylene group of the side chain. The ratio of activity at these two positions was the same as in the administered tryptophans indicating that the bond between the 3-position of the indole nucleus and the side chain remained intact during the biosynthesis of gramine.

Many radioactive compounds have been fed to barley in the hope of discovering intermediates between tryptophan and gramine. These are listed in Fig. 1, the positions which were labeled with C^{14} being indicated with asterisks. Breccia and Marion[8] using intact barley seedlings found that radioactive indole-3-acetic acid (I), indole-3-aldehyde (II), indole-3-acetamide (III), and indole-3-glyoxylic acid (IV) failed to yield gramine containing significant radioactivity. They considered that lack of incorporation in the experiment involving

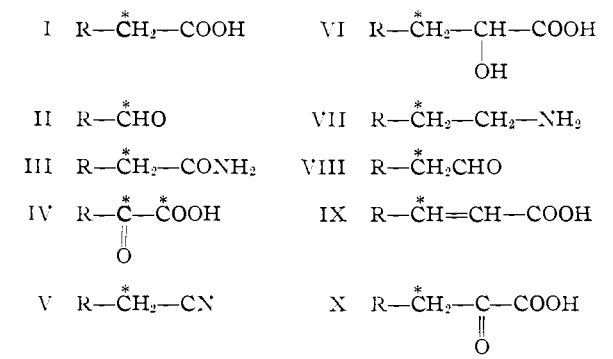


Fig. 1.—Radioactive compounds which have been administered to barley.

(1) This investigation was supported by research grant MY-2662 from the National Institute of Mental Health, U. S. Public Health Service.

(2) On leave of absence from the University College, Cork, Ireland.

(3) Gramine (= Donaxine) has also been isolated from *Arundo donax* (A. Orekhov and S. Norkina, *Ber.*, **68**, 436 (1935)), *Acer saccharinum* (I. J. Pachter, D. E. Zacharias and O. Ribeiro, *J. Org. Chem.*, **24**, 1285 (1959)), and *Acer rubrum* (I. J. Pachter, *J. Am. Pharm. Assoc., Sci. Ed.*, **48**, 670 (1959)).

(4) K. Bowden and L. Marion, *Can. J. Chem.*, **29**, 1037 (1951).

(5) K. Bowden and L. Marion, *ibid.*, **29**, 1043 (1951).

(6) Label on C-2 of the indole nucleus.

(7) E. Leete and L. Marion, *Can. J. Chem.*, **31**, 1195 (1953).

(8) A. Breccia and L. Marion, *ibid.*, **37**, 1066 (1959).