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.

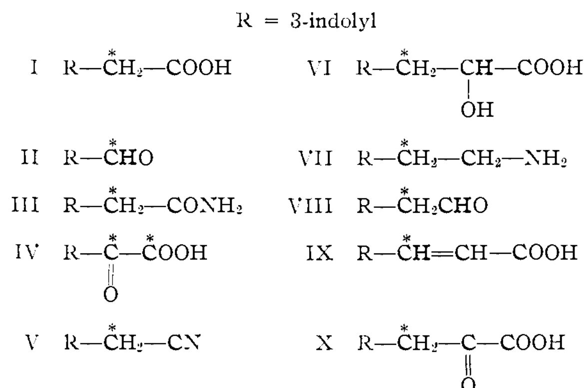


Fig. 1.—Radioactive compounds which have been administered to barley.

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

(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).

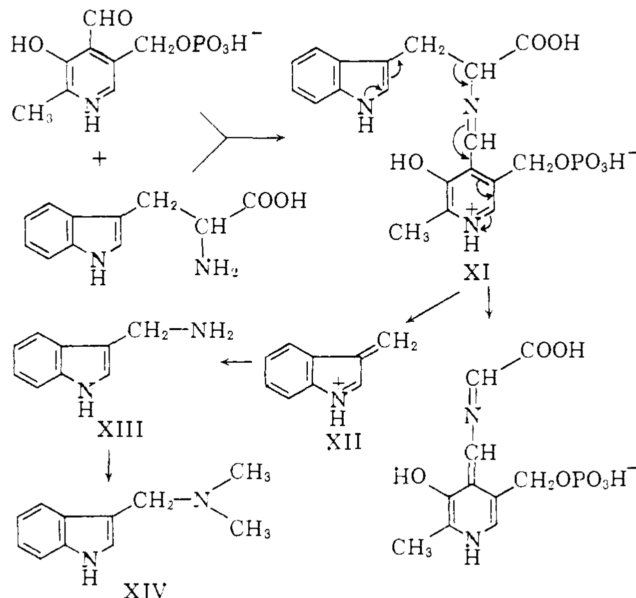
(8) A. Breccia and L. Marion, *ibid.*, **37**, 1066 (1959).

indole-3-acetic acid may have been due to destruction of this compound before it reached the site of gramine synthesis, namely, the shoots.[9] We[10] also found that indole-3-acetic acid-α-C^{14} did not yield radioactive gramine when it was administered to the roots of intact seedlings. On the other hand, Wightman, *et al.*,[11] who fed a variety of compounds to excised barley shoots, found that indole-3-acetic acid-α-C^{14} yielded radioactive gramine (0.48 % incorporation[12]). The incorporations of the other compounds which were fed to barley shoots at the same time of year were: indole-3-acetonitrile (V) 0.43%, indole-3-lactic acid (VI) 1.21%, tryptamine (VII) 0.50%, indole-3-acetaldehyde (VIII) 0.5%, and indole-3-acrylic acid (IX) 0.02%. However, since none of these compounds was as efficient a precursor of gramine as tryptophan-β-C^{14} (2.67% incorporation), they did not consider them to be intermediates in the biosynthesis of gramine. Breccia and Marion[8] found that the administration of indole-3-pyruvic acid (X) and indole-3-acrylic acid (IX) to barley yielded radioactive gramine (0.66 and 0.8% incorporation, respectively). The significant difference in the incorporation of indole-3-acrylic acid obtained by these two groups of workers may indicate that the intact plants contain an enzyme which catalyses the amination of indole-3-acrylic acid to tryptophan. Such a reaction would be analogous to the formation of aspartic acid from fumaric acid.[13]

In the present work we fed a mixture of DL-tryptophan-β-C^{14} and DL-tryptophan-β-H^3 to intact barley seedlings. The tritium labeled tryptophan was synthesized from paraformaldehyde-H^3 using the method of Snyder and Smith.[14] Gramine labeled with tritium on the methylene group of its side chain was an intermediate in this synthesis. Using some of this material we established that the tritium located at this position in the gramine was not labile in the various solvents which were used for extraction of gramine from barley shoots. There was also no loss of tritium from tryptophan-β-H^3 when it was dissolved in aqueous acetic acid. The gramine which was subsequently isolated from the barley was found to contain the same ratio of C^{14} to H^3 as in the administered tryptophans. Systematic degradation of this radioactive gramine by previously described methods[4] established that all the C^{14} and H^3 was located in the methylene group of the side chain (*cf.* Table I).

This result enables us to eliminate indole-3-carboxylic acid, indole-3-glyoxylic acid, indole-3-aldehyde, and indole-3-acrylic acid as precursors of gramine, since the formation of these compounds would involve the loss of part or all the tritium located on the β-carbon of the tryptophan side chain.[15] Indole-3-acetic acid, indole-3-pyruvic acid, and indole-3-acetaldehyde are also unlikely intermediates since in these compounds the tritium would be located on a carbon adjacent to a carbonyl group and would be labile.

Our results are consistent with the attractive hypothesis proposed by Wenkert[16] illustrated in Fig. 2. Tryptophan and pyridoxal phosphate react to form the Schiff base XI. A reverse Michael degradation of this compound affords the protonated 3-methyleneindolenine (XII) which yields 3-aminomethylindole (XIII) by reaction with ammonia. Methylation then gives gramine (XIV). The hydrogen located on the carbon attached to the 3-position of the indole nucleus would not be labile in any of the intermediates in this biogenetic scheme. Mudd[17] recently has isolated 3-aminomethylindole and 3-methylaminomethylindole from the shoots of 4-day old barley seedlings and it seems likely that these compounds are precursors of gramine.



Experimental

DL-**Tryptophan-β-H^3**.—Gramine labeled on its methylene group with H^3 was obtained by reaction between tritium labeled paraformaldehyde,[18] dimethylamine, and indole in aqueous acetic acid, using the procedure of Kühn and Stein.[19] This gramine

TABLE I

ACTIVITIES OF THE TRYPTOPHAN FED TO THE BARLEY AND OF GRAMINE AND ITS DEGRADATION PRODUCTS

	Amount fed, mg.	Activity, d.p.m.	Ratio C^{14}/H^3
DL-Tryptophan-β-C^{14} [20]	2.9	8.86×10^7	1.14
DL-Tryptophan-β-H^3 diacetate	100	7.76×10^7	

	Activities (d.p.m. $\times 10^{-5}$/m*M*) C^{14} assay Flow counter	C^{14} assay Scintillation counter	H^3 assay Scintillation counter
Gramine	2.1	2.0	1.8
3-Ethoxymethylindole	2.2	2.0	1.9
Ethyldimethylamine picrate	0	..	..
Indole-3-carboxylic acid	2.2	1.9	0
Indole	..	0	0
Indole picrate	0	..	..
Barium carbonate[21]	2.1	..	..

— Mean value of C^{14} activity in methylene group of gramine/mean value of H^3 activity in methylene group of gramine = 2.1/1.85 = 1.13

(9) V. E. Tyler, *J. Am. Pharm. Assoc. Sci. Ed.*, **47**, 97 (1958).
(10) E. Leete and D. W. Henry, unpublished work.
(11) F. Wightman, M. D. Chisholm and A. C. Neish, *Phytochemistry*, **1**, 30 (1961).
(12) Incorporation is defined as the total amount of radioactivity found in the isolated natural product divided by the total activity administered to the plant.
(13) A. I. Virtanen and J. Tarnanen, *Biochem. Z.*, **250**, 193 (1932).
(14) H. R. Snyder and C. W. Smith, *J. Am. Chem. Soc.*, **66**, 350 (1944).
(15) The tritium labeled paraformaldehyde used in the tryptophan synthesis was produced by the Wilzbach technique and calculations based on its specific activity indicated that more than 99% of the labeled formaldehyde molecules obtained from this paraformaldehyde would contain only *one* tritium atom. The tritium labeled tryptophan molecules would therefore only have *one* tritium atom attached to the β-carbon of the side chain. One could thus argue that a compound such as indole-3-aldehyde could be formed from the tryptophan-β-H^3 and retain more than half the tritium since enzymatic reactions might be expected to favor cleavage of a carbon-hydrogen bond rather than a carbon-tritium bond. However, it seems unlikely that the isotope effect would be of such magnitude that all the carbon-tritium bonds would survive.
(16) E. Wenkert, *J. Am. Chem. Soc.*, **84**, 98 (1962).
(17) S. H. Mudd, *Nature*, **189**, 489 (1961).
(18) Purchased from Volk Radiochemical Company, Skokie, Illinois.
(19) H. Kühn and O. Stein, *Ber.*, **70**, 567 (1937).
(20) Purchased from Tracer Lab, Waltham, Mass.
(21) Derived from the carboxyl group of the indole-3-carboxylic acid.

was converted to DL-tryptophan-β-H^3 diacetate by the procedure of Snyder and Smith.[14]

Assay of the Radioactive Samples.—Samples containing only C^{14} and those containing both C^{14} and H^3 were assayed for C^{14} using a Nuclear-Chicago Model C-115 low background Q gas flow counter. Determinations were carried out on samples of finite thickness making corrections for efficiency and self-absorption. Compounds containing C^{14} and H^3 were assayed for both isotopes in a Tri-Carb liquid scintillation counter, Model 314 E (Packard Instrument Co.).[22] The solvent used for assay of the tryptophan-β-H^3 consisted of 70% toluene and 30% ethanol and contained 0.5% 2,5-diphenyloxazole (PPO) as a scintillator. Gramine and its degradation products were assayed in toluene containing 0.5% PPO as a primary scintillator and 0.05% 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene as a secondary scintillator.

(22) We thank Dr. Frank Ungar of the Department of Physiological Chemistry for the use of his scintillation counter. We are also indebted to Drs. Maura E. Beary and Robert L. Conner for invaluable help in the preparation of samples and use of the counter.

Administration of the Tracers to the Plants and Isolation of the Gramine.—Charlottetown No. 80 barley (*Hordeum distichum*)[23] (720 g.) was germinated by placing in distilled water in Pyrex dishes as previously described.[4] It was found advantageous to spread out the barley on a piece of cotton cloth, almost complete germination thus being obtained. An aqueous solution of the labeled tryptophans was added to the roots of the barley seedlings 5 days after germination. The shoots (wet wt. 1065 g.) were cut off 6 days later and extracted as previously described[4] yielding gramine (201 mg.). The incorporation of tracers was 0.27%.

Degradation of Gramine.—A sample of the synthetic gramine labeled with H^3 on the methylene group was treated with ethyl iodide and sodium ethoxide yielding 3-ethoxymethylindole[4] which had the same specific activity as the gramine. Fusion of the tritium labeled gramine with potassium hydroxide yielded inactive indole-3-carboxylic acid. The radioactive gramine isolated from the barley was subjected to the same degradations and the activities of the degradation products are recorded in Table I.

(23) We thank Mr. R. B. MacLaren of the Experiment Station, Charlottetown, Prince Edward Island, Canada, for a generous supply of barley.

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, POLYTECHNIC INSTITUTE OF BROOKLYN, BROOKLYN 1, N. Y.]

Kinetics and Equilibria of Amide Formation in Aqueous Media[1,2]

BY H. MORAWETZ AND P. S. OTAKI

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Rates of amide formation were measured in aqueous solutions containing various combinations of a 0.2 *N* carboxylic acid (formic, acetic, propionic, butyric, isobutyric and succinic) and 1 *N* amine (ammonia, methylamine, ethylamine, isopropylamine, ethanolamine and dimethylamine) at one or several temperatures. The rate is first order in the anion of the acid and the basic form of the amine, the second-order rate constant being independent of the acidity of the medium. At 44.4°, formate reacts with methylamine 140 times more rapidly than acetate; for the higher fatty acids the rate decreases only slightly. Methylamine reacts with propionate and isobutyrate at 75.8° eleven and six times, respectively, as fast as with ammonia; the activation energy for the methylamine reaction is on the average lower by 5 kcal./mole. Equilibrium constants for amide formation (formulated as $R_1COO^- + R_2R_3NH \rightleftarrows R_1CONR_2R_3 + OH^-$) in dilute aqueous systems were calculated at one temperature for six systems and at a number of temperatures for seven systems from rate constants for amide formation and hydrolysis. Values for N-methylpropionamide and N-methylisobutyramide at 75.8° were larger by factors of about one hundred than those for the primary amides; the value for N,N-dimethylpropionamide was intermediate. For primary amides, ΔH of amide formation is 6–9 kcal./mole, for N-methylamides it is slightly negative. While N-methylformamide formation has ΔF^0 more negative than N-methylacetamide, the order is reversed with N-isopropylformamide and N-isopropylacetamide due, possibly, to an interaction of the hydrophobic residues in N-isopropylacetamide. Thermodynamic data for primary amide formation obtained in this study are similar to those reported for enzymatically catalyzed systems, but the formation of N-methylamides has ΔF^0 more negative by 4–5 kcal./mole and ΔH more negative by 8 kcal./mole than for the formation of peptide bonds. Amide formation from propionate and ammonia or methylamine is accelerated by addition of 1-propanol, the maximum rate (three times as high as in water) occurring at 70 weight % of the alcohol. No acceleration is observed on addition of 2-propanol.

Introduction

Very little is known about equilibria of organic reactions in dilute aqueous systems. This is particularly unfortunate in view of the fact that the life process proceeds in a medium of very high water concentration. The living cell is a dynamic system in which the concentrations of reagents and reaction products are generally not related by the conditions of chemical equilibrium, but a knowledge of the free energy relations of important biochemical processes is important to an understanding of the energy balance of the cell. In the absence of the required free energy data, biochemists have frequently used reaction heats as a rough measure of the driving force, but such a procedure has obvious shortcomings.

One of the most important biochemical processes is the synthesis of the amide bond, required for the production of protein molecules. It has long been known that this process is characterized, under physiological conditions, by a positive free energy change and that it must, therefore, be coupled to an exergonic reaction. Lipmann was first to suggest[3] that the energy required for the synthesis of an amide bond is derived from the splitting of the "high energy phosphate bond" of adenosine triphosphate and this mechanism has been demonstrated convincingly in the enzymatically catalyzed syntheses of peptides and proteins.[4]

The magnitude of the free energy requirement in the synthesis of a dipeptide from two amino acids was estimated by Huffman[5] for processes formulated as:

$$H_3\overset{+}{N}CHR_1COO^-(s) + H_3\overset{+}{N}CHR_2COO^-(s) \rightleftarrows H_3\overset{+}{N}CHR_1CONHCHR_2COO^-(s) + H_2O(l) \quad (1)$$

The $\Delta F_{293}{}^0$ varied from +1.4 to +3.7 kcal./mole. Attempts to obtain similar data for reactions proceeding in aqueous solution were first made by Borsook and Dubnoff,[6] who estimated ΔF^0 for the condensation of benzoate and glycine to hippurate from heats of combustion, heat capacity and solubility data. Later studies of the condensation of benzoyltyrosine with glycinamide[7a] and of glutamate or aspartate with ammonia[7b] utilized enzymatic catalysis to attain rapid chemical equilibrium. Since very little amide was

(1) Based in part on a Ph.D. thesis to be submitted by P. S. Otaki to the Graduate School, Polytechnic Institute of Brooklyn, in June, 1963.

(2) This investigation was supported by a grant of the National Institutes of Health.

(3) F. Lipmann, *Advan. Enzymology*, **1**, 154 (1941); *Federation Proc.*, **8**, 597 (1949).

(4) H. Chantrenne, "The Biosynthesis of Proteins," Pergamon Press, New York, N. Y., 1961, pp. 92–112.

(5) H. M. Huffman, *J. Phys. Chem.*, **46**, 885 (1942).

(6) H. Borsook and J. W. Dubnoff, *J. Biol. Chem.*, **132**, 307 (1942).

(7) (a) A. Dobry, J. S. Fruton and J. M. Sturtevant, *ibid.*, **195**, 149 (1952); (b) T. Benziger, C. Kitzinger, R. Hems and K. Burton, *Biochem. J.*, **71**, 400 (1959).