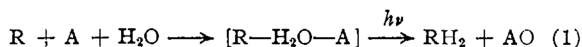
Search for an activator possessing an easily identified oxidation product led to methionine, the sulfoxide of which can be recognized by its differential chromatographic R_f value. Illumination of a deoxygenated neutral solution of riboflavin and methionine, with simultaneous electrolytic reoxidation of the reduced riboflavin, yielded methionine sulfoxide in an amount twelve times greater than the riboflavin present (Table I). When lumiflavin is used in place of riboflavin, reduced lumiflavin and methionine sulfoxide are obtained. Complete recovery of the lumiflavin is achieved upon subsequent aeration of the solution. This photochemical reaction is neither dependent upon the presence, nor a consequence of the loss of the ribityl side chain, as has been suggested.[6]

The chromatographic demonstration of sulfoxide obviously entails admission of oxygen subsequent to the anaerobic photoreaction. However, methionine is not converted to its sulfoxide by oxygen,[10] by equimolar concentrations of hydrogen peroxide at neutrality, or by electrolytic oxidation. Thus, water must be the source of oxygen for sulfoxide formation and the source of hydrogen for reduction of riboflavin. The mechanism of the photo-reduction of riboflavin (R) in the presence of an activator (A) may therefore be described by (1)



$$R + A + H_2O \longrightarrow [R\text{—}H_2O\text{—}A] \xrightarrow{h\nu} RH_2 + AO \quad (1)$$

The water may be regarded as being "suspended" between riboflavin and activator and as being "pulled apart" as the complex dissociates upon absorption of radiant energy. Energy considerations also dictate the water to be bound in a complex between riboflavin and activator: The energy absorbed per mole of light quanta at 440 mμ (a wave length effective for the reaction) is 65 kcal., which is less than the energy requirement calculated from the oxidation-reduction potentials of the reacting substances, considered separately, and assuming the entropy change to be small. Conductance studies indicate that riboflavin forms complexes, in the dark, with methionine and with Na_2EDTA.

TABLE I

PHOTOCHEMICAL CONVERSION OF METHIONINE TO ITS SULFOXIDE[a]

		Concentrations in moles × 10⁵/liter			
		[Flavin]	[Dihydro flavin]	[Meth.]	[Sulfoxide]
Expt. I	Initial	5.0	0	50	0
	Final	2.1	2.9	47 ± 1	2.8 ± 0.4
Expt. II[b]	Initial	2.0	0	100	0
	Final	1.8	0.2	72 ± 2	25 ± 2

[a] Methionine and methionine sulfoxide estimated by quantitative chromatographic procedure,[13] riboflavin by spectrophotometry. All solutions deoxygenated by repeated boiling *in vacuo* at room temperature and flushing with purified nitrogen. [b] Riboflavin continuously reoxidized electrolytically.

The photoöxidation of methionine to methionine sulfoxide in the presence of methylene blue has been demonstrated.[11] Supposedly, a "dehydrogenated methionine" was formed by the direct transfer of two hydrogens from methionine to the dye; this "dehydro compound" was assumed to be converted to methionine sulfoxide by its subsequent slow reaction with water. A "dehydromethionine"[12] reputedly is characterized by liberation of iodine from potassium iodide in hydrochloric acid solution. When an air-free solution of riboflavin was photoreduced in the presence of methionine, then treated anaerobically with 1.0 *M* KI and with 0.5 *M* HCl, no iodine could be detected by a spectrophotometric method capable of measuring iodine equivalent to as little as 10% of the riboflavin reduced. Therefore, "dehydromethionine" is not produced in this reaction. Substances that serve as activators for the photochemical reduction of riboflavin (and, presumably, for dyes such as methylene blue) function as acceptors of hydroxyl radicals derived from the photochemical cleavage of water by riboflavin.

(10) G. Toennies and J. Kolb, *J. Biol. Chem.*, **128**, 399 (1939).

(11) L. Weil, W. G. Gordon and A. R. Buchert, *Arch. Biochem. Biophys.*, **33**, 90 (1951).

(12) T. F. Lavine, *J. Biol. Chem.*, **169**, 477 (1947).

(13) L. Naftalin, *Nature*, **161**, 763 (1948).

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THE COMBINATION OF ENZYMATIC RESOLUTION WITH ASYMMETRIC TRANSFORMATION OF THE OPTICALLY LABILE 3,4-DEHYDROPROLINE AMIDE

Sir:

An optically labile racemic amide of an optically more stable amino acid should be cleaved by an amidase to the L-acid to an extent exceeding the theoretically possible 50% of L-isomer, provided the rate of asymmetric transformation[1] is comparable to the rate of enzymatic hydrolysis. This combination has now been realized with DL-dehydroproline amide (*cf.* II), accessible in 60% yield from pyrrole-2-carboxamide (I) by a modification of E. Fischer's reduction[2] using phosphonium iodide in fuming hydriodic acid (saturated at −20°) for 2 hours and ion exchange resin for separation from 10–20% of the DL-acid (*cf.* III) formed in the process.

The proof of structure for II and III was: (i) the optical lability of the chemically resolved free D-amide II which had a half life time of 48 hours in water, indicating easy abstraction of an allylic proton from C_2 next to the amide carbonyl. This lability is not shown by the L-acid III[3]; (ii) the smooth addition of hydrogen, deuterium or tritium[4] leads to L-proline IV of high optical purity; (iii) the n.m.r. data assigned to II, III and IV[5] expressed in τ-values[5] at 60 Mc./s. (reference internal tetramethylsilane; solvent trifluoroacetic acid) show two vinylic protons, at 3.88, excluding Δ_1-, Δ_2- and Δ_5-structures, and a peak at 4.66 of the proton at C_2 eliminating the Δ_4-structure which would have a peak at 5.2 like proline.[5]

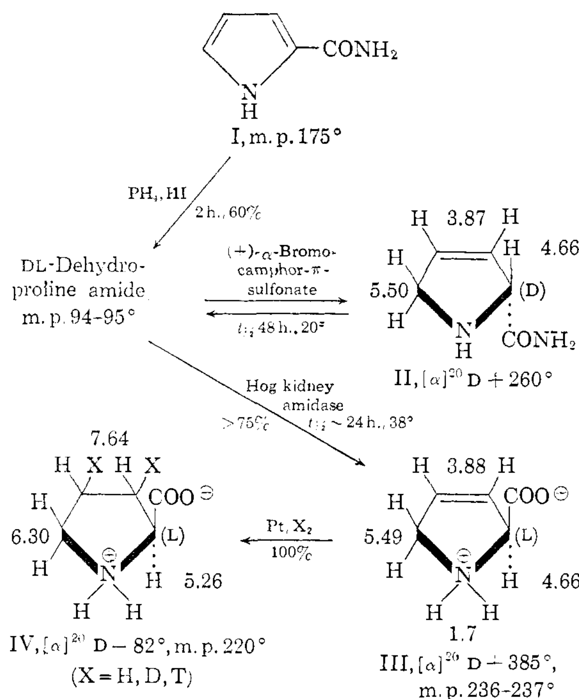
(1) *Cf.* M. M. Harris, "Progress in Stereochemistry," Academic Press, Inc., New York, N. Y., Vol. 2, p. 157 (1958).

(2) E. Fischer and F. Gerlach, *Ber.*, **45**, 2453 (1912).

(3) Solutions of III in 2.0 *N* ammonia lose half of their rotation at 20° after 17 days possibly through base-catalyzed racemization as well as secondary reactions.

(4) L-Proline-3,4-H^3 has been made available for metabolic studies by this procedure through New England Nuclear Corporation, Boston 18, Mass.

(5) F. A. Bovey and G. V. D. Tiers, THIS JOURNAL, **81**, 2870 (1959)



Hog kidney amidase, activated by magnesium ions,[6] hydrolyzed the racemic amide 50% after 24 hours, 75% after 4 days at 38° and addition of fresh enzyme on the third day. Preparations of the L-acid showing rotations as high as $[\alpha]^{20}D$ 385° were 97% optically pure as assayed by D-amino acid oxidase.[7] This leads to a calculated value of $[\alpha]^{20}D \sim -400°$ for pure III, *i.e.*, close to five times that of L-proline, $[\alpha]^{20}D$ −86°.[8] Yields of 100% of III were not obtained because of the formation of gummy side products during the long reaction time. All analyses were satisfactory.

Acknowledgment.—We are greatly indebted to Dr. S. M. Birnbaum for his valuable assistance and coöperation in the enzymatic resolution and assays.

(6) S. M. Birnbaum, "Methods in Enzymology," Academic Press, Inc., New York, N. Y., 1955, Vol. II, p. 397.

(7) L-Amino acid oxidase from snake venom converts III quantitatively to pyrrole-2-carboxylic acid, a reaction which has been used for a new spectrophotometric assay: H. H. Weissbach, A. V. Robertson, B. Witkop and S. Udenfriend, *Anal. Biochem.*, in press.

(8) D. Hamer and J. P. Greenstein, *J. Biol. Chem.*, **193**, 81 (1951).

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A ROLE FOR THYMIDINE NUCLEOTIDES IN THE BIOSYNTHESIS OF L-RHAMNOSE[1]

Sir:

The synthesis of thymidine diphosphate glucose and its conversion to thymidine diphosphate rhamnose has been observed in reaction mixtures of thymidine triphosphate, α-D-glucose-1-phosphate and an enzyme preparation from *Streptococcus faecalis*. The enzyme extract was obtained by Mickle disintegration of *S. faecalis* cells in 0.1 *M* phosphate buffer of *p*H 7.2 containing 0.05 *M* magnesium chloride. The reaction mixtures were prepared by dissolving 20 micromoles of thymidine triphosphate and 30 micromoles of α-D-glucose-1-phosphate in 0.5 ml. of enzyme solution and incubating at room temperature for 1 and 5 hour periods. Examination of these samples by paper chromatography in a solvent system of ethyl alcohol and 1 *M* ammonium acetate of *p*H 7.5 (7:3 by volume) revealed the presence of three new ultraviolet absorbing compounds with R_f values of 0.35, 0.25 and 0.20. A comparison of these values with those for reference compounds in Table I indicates that the compounds with R_f values of 0.25 and 0.20 are probably thymidine monophosphate and thymidine diphosphate, respectively. The compound at R_f value of 0.35 yielded ultraviolet spectra typical of thymidine derivatives and on acid hydrolysis was converted to thymidine monophosphate, inorganic phosphate and reducing sugars. When thymidine triphosphate was incubated with the enzyme extract in the absence of glucose-1-phosphate, small amounts of thymidine monophosphate and thymidine diphosphate were produced but the compound with R_f value of 0.35 was not produced in this reaction mixture.

TABLE I

THE R_f VALUES OF REFERENCE COMPOUNDS IN SOLVENT SYSTEM OF ETHYL ALCOHOL–1 *M* AMMONIUM ACETATE OF *p*H 7.5 (7:3 BY VOLUME)

Compound	R_f value
Thymidine triphosphate	0.11
Thymidine diphosphate	.20
Thymidine monophosphate	.26
Thymidine	.76
Thymine	.70
Uridine diphosphate glucose	.22
Uridine triphosphate	.07
α-D-Glucose-1-phosphate	.18
D-Glucose	.60
L-Rhamnose	.68

The ultraviolet absorbing material at R_f value of 0.35 was isolated by a paper chromatographic method in yields approximating 60% of the original thymidine triphosphate. Hydrolysis in 0.02 *N* hydrochloric acid for 1 hour at 100° converted the product from the 1-hour incubation period to thymidine monophosphate, inorganic phosphate and D-glucose and the product from the 5-hour sample to thymidine monophosphate, inorganic phosphate, D-glucose and L-rhamnose. Quantitative values for these hydrolytic products were obtained by ultraviolet absorption for thymidine monophosphate, by a modified Fiske–SubbaRow method for inorganic phosphate[2] and by the cysteine–sulfuric acid reaction for reducing sugars.[3] Molar ratios of these products are recorded in Table II. The presence of D-glucose and L-rhamnose in the acid hydrolysates was confirmed by R_f values in three different solvent systems, by enzymatic assay with

(1) Published with the approval of the Director as Paper No. 1047 Journal Series, Nebraska Agricultural Experiment Station. Supported in part by a grant from the National Science Foundation.

(2) C. H. Fiske and Y. SubbaRow, *J. Biol. Chem.*, **66**, 375 (1925).

(3) Z. Dische, "Methods of Biochemical Analysis," Vol. II, edited by D. Glick, Interscience Publishers, New York, N. Y., 1955, pp. 313–358.