Kinetics. The water content of the acetic acid used in the acetolysis studies was determined using the method of Bruckenstein.[17] Enough anhydrous sodium carbonate was added to make the solution of 0.035 *M* in sodium acetate and enough acetic anhydride was added to remove the water and leave the solution 0.01 *M* in acetic anhydride.

The rate of acetolysis of spiro[2.3]hexyl-4 tosylate was determined by preparing a 0.0300 *M* solution in acetic acid at the desired temperature. The flask was placed in a thermostat, and 3-ml aliquots were removed at appropriate intervals. The aliquots were titrated with 0.0300 *M* *p*-toluenesulfonic acid to a bromphenol blue end point. The rates of acetolysis of the other compounds were determined by preparing the solution at room temperature, placing 3.2-ml aliquots in ampoules, and sealing the ampoules. They were placed in a thermostat and removed and cooled at appropriate times. The ampoules were opened; 3.0 ml of solution was removed and titrated as above. In each case, two determinations were made and the average is reported in Table I. The average deviation was generally ±2% or less.

(17) S. Bruckenstein, Ph.D. Thesis, University of Minnesota, 1954, pp 9–11.

Biosynthesis of Ergot Alkaloids. Evidence for Two Isomerizations in the Isoprenoid Moiety during the Formation of Tetracyclic Ergolines

H. G. Floss,[1a,b] U. Hornemann,[1b] N. Schilling,[1c] K. Kelley,[1b] D. Groeger,[1d] and D. Erge[1d]

Contribution from the Department of Medicinal Chemistry, School of Pharmacy and Pharmacal Sciences, Purdue University, Lafayette, Indiana 47907, the Organisch-Chemisches Institut, Technische Hochschule, München, West Germany, and Institut für Biochemie der Pflanzen, Halle (Saale), East Germany. *Received April 22, 1968*

Abstract: Chanoclavine-I (**1**), but not chanoclavine-II (**5**) or isochanoclavine-I (**6**), is an efficient precursor of tetracyclic ergot alkaloids. Its cyclization to give agroclavine (**2**) is accompanied by a *cis–trans* isomerization at the double bond of the isoprenoid moiety. Experiments with mevalonic acids stereospecifically tritiated at C-4 indicate that another such *cis–trans* isomerization occurs earlier in the pathway. Thus the apparently "normal" labeling of tetracyclic ergot alkaloids from mevalonic-2-^{14}C acid in the *trans*-carbon atom of the isoprenoid moiety is an accidental result caused by two isomerizations. The cyclization of chanoclavine-I proceeds with complete retention of the hydrogen at C-10, but with only 70% retention of the hydrogen at C-9. The latter result is discussed in view of possible mechanisms of the reaction.

Ergot alkaloids are formed from L-tryptophan, mevalonic acid, and the methyl group of methionine.[2] There is evidence that 4-dimethylallyltryptophan is an early intermediate in the biosynthetic pathway, and studies on the biogenetic interrelationships of these alkaloids have established the sequence agroclavine (**2**) → elymoclavine (**3**) → lysergic acid derivatives (**4**).[3] Recently, we[4] reported that chanoclavine-I (**1**) is an efficient precursor of tetracyclic ergot alkaloids of the clavine and lysergic acid amide type, and the same observation was made independently by two other groups.[5,6] Chanoclavine-I has a methyl group in the *cis* position[7] and a hydroxymethyl group in the *trans* position at the Δ^8 double bond,[8,9] and could thus give rise to elymoclavine in two ways: by oxidative cyclization between the methyl group and the nitrogen, or by reaction between the hydroxymethyl group and the nitrogen, followed by a hydroxylation at the methyl group (Scheme I). The latter reaction would involve an isomerization at the Δ^8 double bond, and our finding[4] that chanoclavine-I was not only converted to elymoclavine but also to agroclavine suggested that this pathway is operative. The possibility of such an isomerization was also indicated by work from Arigoni's group, who found[5,10] that mevalonic-2-^{14}C acid predominantly (>90%) labels the C-methyl group of chanoclavine-I (**1**), isochanoclavine-I (**6**), and chanoclavine-II (**5**), whereas the tetracyclic ergolines are all predominantly labeled in carbon 17.[11,12]

In this paper we report on the mechanism of the conversion of chanoclavine-I into tetracyclic ergot alkaloids. Evidence will be given which indicates that two *cis–trans* isomerizations at the double bond of the isoprenoid portion take place in the course of ergot alkaloid biosynthesis. Preliminary reports of some of this work have appeared.[13,14]

(1) (a) To whom inquiries should be directed. (b) Purdue University. (c) Organisch-Chemisches Institut, Technische Hochschule, München, West Germany. (d) Institut für Biochemie der Pflanzen, Halle, East Germany.

(2) *Cf.* F. Weygand and H. G. Floss, *Angew. Chem. Intern. Ed. Engl.*, 2, 243 (1963).

(3) *Cf.* S. Agurell, *Acta Pharm. Suecica*, 3, 71 (1966).

(4) D. Groeger, D. Erge, and H. G. Floss, *Z. Naturforsch.*, *B*, 21, 827 (1966).

(5) T. Fehr, W. Acklin, and D. Arigoni, *Chem. Commun.*, 801 (1966).

(6) R. Voigt, M. Bornschein, and G. Rabitzsch, *Pharmazie*, 22, 326 (1967).

(7) *cis* and *trans* refer to the position relative to the benzylic carbon atom.

(8) D. Stauffacher and H. Tscherter, *Helv. Chim. Acta*, 47, 2186 (1964).

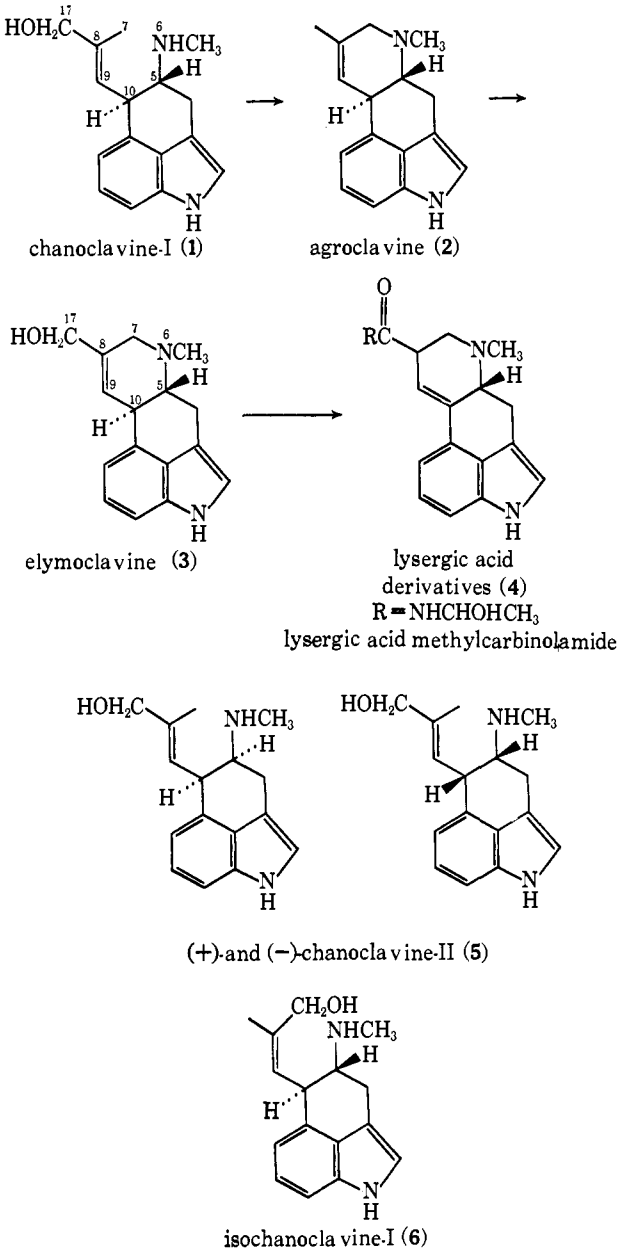
(9) W. Acklin, T. Fehr, and D. Arigoni, *Chem. Commun.*, 799 (1966).

(10) D. Arigoni, private communication.

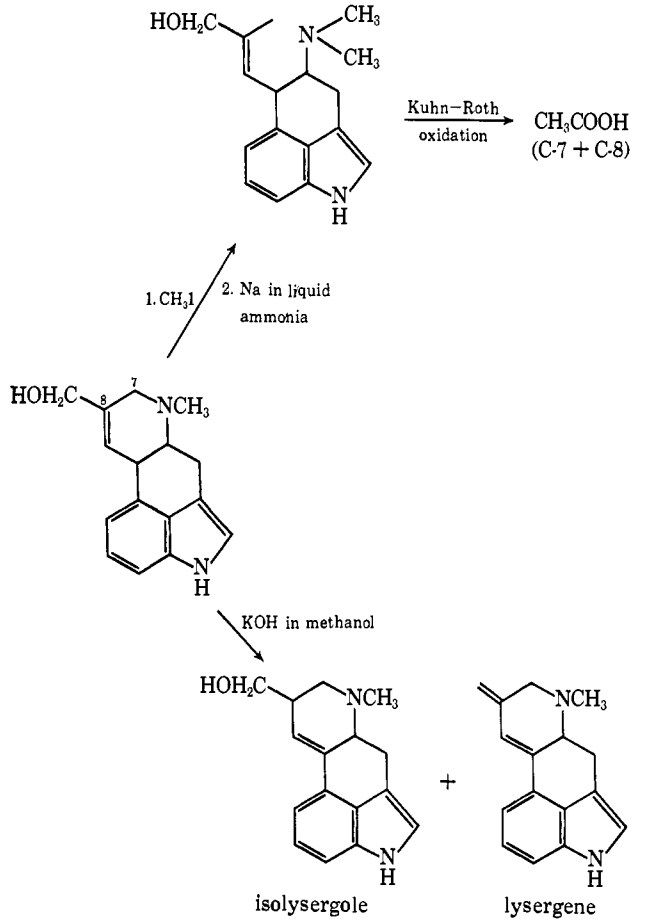
(11) S. Bhattacharji, A. J. Birch, A. Brack, A. Hofmann, H. Kobel, D. C. C. Smith, H. Smith, and J. Winter, *J. Chem. Soc.*, 421 (1962).

(12) R. M. Baxter, S. I. Kandel, and A. Okany, *Tetrahedron Lett.*, 596 (1961); *J. Amer. Chem. Soc.*, 84, 2997 (1962).

Scheme I



Scheme II



Results

In order to demonstrate directly that the conversion of chanoclavine-I into elymoclavine involves a *cis–trans* isomerization, (*R,S*)-mevalonic-2-^{14}C acid was fed to cultures of a *Claviceps paspali* strain which produces mainly chanoclavine-I. After purification to constant specific radioactivity, an aliquot of the labeled chanoclavine-I was degraded by Kuhn–Roth oxidation to give acetic acid (C-7 + C-8), and the rest was fed to *Claviceps* strain SD 58, which mainly produces elymoclavine. The latter, after purification, was subjected to an Emde–Birch fission[11] followed by Kuhn–Roth oxidation to give acetic acid (C-7 + C-8) (Scheme II). For comparison, a sample of elymoclavine obtained by feeding mevalonic-2-^{14}C acid directly to *Claviceps* strain SD 58 was degraded in the same way. As shown in Table I, chanoclavine-I in agreement with the results from Arigoni's group carried about 90% of the label at C-7,[15] whereas the elymoclavines obtained biosynthetically from this chanoclavine-I and directly from mevalonic-2-^{14}C acid both had only about 10% of the label at C-7. Thus, chanoclavine-I labeled at C-7 gives rise to elymoclavine labeled at C-17.[16]

Table I. Degradation of Chanoclavine-I and Elymoclavine for Location of ^{14}C from Mevalonic-2-^{14}C Acid

Compound	Sp radioact, dpm/mmol	% of radioact
Chanoclavine-I from mevalonic-2-^{14}C acid	6.82×10^4	100
Acetic acid (C-7 and C-8 of chanoclavine-I)	6.30×10^4	92
Elymoclavine from chanoclavine-I	1.3×10^4	100
Acetic acid (C-7 and C-8 of elymoclavine)	1.6×10^3	12
Elymoclavine from mevalonic-2-^{14}C acid	7.02×10^4	100
Acetic acid (C-7 and C-8 of elymoclavine)	5.85×10^3	8

(13) H. G. Floss, U. Hornemann, N. Schilling, D. Groeger, and D. Erge, *Chem. Commun.*, 105 (1967).
(14) H. G. Floss, *ibid.*, 804 (1967).

(15) The acetic acids were not further degraded to distinguish between C-7 and C-8; however, earlier work[11] has shown that C-8 is essentially unlabeled.
(16) The radioactivity of C-17 was not determined directly, but the possibility that chanoclavine-I is extensively degraded by the fungus prior to the incorporation of its radioactivity into elymoclavine is excluded by the double-labeling experiments mentioned below. The samples used in the degradations were actually double-labeled specimens obtained in experiments 2 and 3, Table II.

It was tempting, at this point, to speculate that chanoclavine-I, although efficiently converted into tetracyclic ergolines, is not a natural intermediate in their biosynthesis. Rather, isochanoclavine-I, from its stereochemistry, seemed to be the most suitable intermediate, and chanoclavine-I might enter the pathway *via* this compound. The conversion of chanoclavine-I to isochanoclavine-I could be visualized as occurring by (a) reversible 1,4 dehydration involving a conjugated diene as an intermediate or (b) reversible shift of the double bond into the 9,10 position. Both these mechanisms would involve loss of the hydrogen from C-10. Alternatively, a suitable agent (*e.g.*, water or the SH group of an enzyme) might reversibly add across the double bond as in the isopentenyl pyrophosphate isomerase reaction[17] (c). In this case, the original hydrogen from C-9 would be lost, assuming that addition and elimination take the same steric course, *e.g.*, both *trans*. A modification of this mechanism (d) would be the addition and elimination of an anionic group (*e.g.*, enzyme-S^-) at C-8, giving rise to an enzyme-bound carbanion intermediate. This would be analogous to the glutathione-stimulated *cis–trans* isomerization of maleylpyruvate, which proceeds without incorporation of deuterium from the medium.[18] In this case, the hydrogen at C-9 would be retained.

To examine these possibilities, we prepared chanoclavine-I labeled with ^{14}C as a reference and with tritium at carbons 9 and 10, respectively, by feeding (*R,S*)-mevalonic-2-^{14}C-4-T and -2-^{14}C-5-T acids to *Claviceps paspali*. These samples of chanoclavine-I, after purification, were administered to cultures of *Claviceps* strain SD 58. For comparison, the same labeled mevalonic acids were also fed directly to strain SD 58. The results are summarized in Table IV. It was already known[3,19] that chanoclavine-I, agroclavine, elymoclavine, festuclavine, pyroclavine, and 6-methyl-Δ^8-ergolene-8-carboxylic acid all retained one of the two labeled hydrogens of mevalonic-5-T acid. A loss of the hydrogen from C-10 of chanoclavine-I during its conversion to tetracyclic ergolines could thus only be expected if this reaction was not a part of the natural pathway from mevalonate to the tetracyclic compounds. The data given in Table IV (expt 3 and 4) show that this hydrogen is not affected in the conversion since both the elymoclavine produced from chanoclavine-I and the reisolated chanoclavine-I had the same T/^{14}C ratio as the starting material. By alkaline isomerization[20] (Scheme II), it was confirmed that essentially all of the tritium in elymoclavine from expt 2 and 3 was confined to position 10 (Table II).[21] It was noticed that chanoclavine-I and elymoclavine formed directly from mevalonic-2-^{14}C-5-T acid retained somewhat more tritium than the theoretical 50%. This was also observed in the conversion of mevalonic-2-^{14}C-4-T acid to chanoclavine-I, and subsequent chromatographic examination of the mevalonic-2-^{14}C acid used in these experiments indicated the presence of two radioactive contaminants, which amounted to about 15% of the radioactivity of this preparation. The T/^{14}C ratios of the mevalonic acids used in expt 1 and 2 therefore should be corrected by this factor.

Table II. Degradation of Elymoclavine for Location of Tritium from Mevalonic-2-^{14}C-5-T Acid

Compound	T/^{14}C	T retn, %
Elymoclavine from expt 2[a]	3.08	100
Isolysergole	0.02	0.7
Lysergene	0.02	0.7
Elymoclavine from expt 3[a]	2.78	100
Isolysergole	0.13	4.7
Lysergene	0.14	5.0

[a] See Table IV.

Partly due to unfavorable experimental conditions (low specific radioactivity of the tritiated mevalonate, unfavorable T/^{14}C ratio) the results obtained with mevalonic-2-^{14}C-4-T acid were less clear. They did show, however, that chanoclavine-I, as expected, retained one of the two hydrogens from C-4 of mevalonate. In the conversion of this chanoclavine-I to elymoclavine, most (84%, 92%), but not all of the tritium was retained.[22] The experiments thus demonstrated that the conversion of chanoclavine-I to elymoclavine occurs without complete loss of the hydrogens at C-9 and C-10.

More direct evidence concerning the role of isochanoclavine-I and also chanoclavine-II was obtained by preparing labeled samples of these compounds biosynthetically from L-tryptophan-indole-T, using a *Pennisetum*-type strain of ergot,[23] and comparing their incorporation into elymoclavine by resting cells of strain SD 58 with that of chanoclavine-I and agroclavine. The results (Table III) indicate that chano-

Table III. Incorporation of Agroclavine and Chanoclavine Isomers into Elymoclavine by Replacement Cultures of *Claviceps* Strain SD 58[a]

Precursor	Radioact introduced, dpm	Total alkaloid found, μg	Sp act. of elymoclavine, dpm/μmol	Incorporation,[b] %
Isochanoclavine-I	4.36×10^5	316	1.36×10^4	1.9
Chanoclavine-II	3.46×10^5	294	3.77×10^3	0.6
Chanoclavine-I	5.99×10^5	344	8.04×10^4	9.0
Agroclavine	4.42×10^4	458	4.72×10^3	9.6

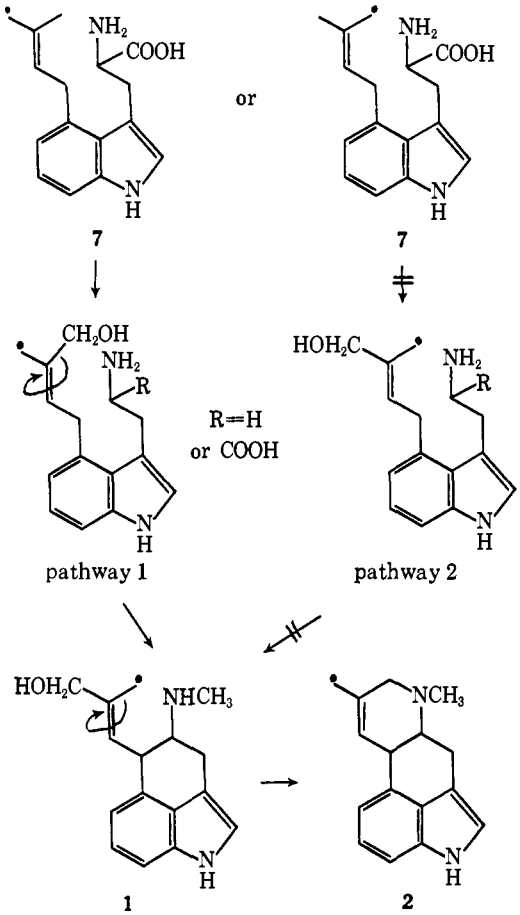
[a] Each precursor was tritium labeled in the indole moiety and was fed in a quantity of 100 μg. [b] Based on 50% elymoclavine in the total alkaloid mixture, as estimated from the chromatograms.

(17) B. W. Agranoff, H. Eggerer, U. Henning, and F. Lynen, *J. Amer. Chem. Soc.*, **81**, 1254 (1959).
(18) L. Lack, *J. Biol. Chem.*, **236**, 2835 (1961).
(19) R. M. Baxter, S. I. Kandel, A. Okany, and K. L. Tam, *J. Amer. Chem. Soc.*, **84**, 4350 (1962).
(20) E. Schreier, *Helv. Chim. Acta*, **41**, 1984 (1958).
(21) Attempts to isomerize chanoclavine-I in the same way failed. Besides unchanged starting material, the reaction only produced traces of chanoclavine-II.
(22) In order to avoid the large dilution of radioactivity encountered in feeding experiments with normal submerged or surface cultures, the conversion experiments with chanoclavine-I-7-^{14}C-9-T had to be carried out with resting cells to limit the *de novo* synthesis of alkaloids. The physiological conditions in these experiments thus were different from those in the others. In both these experiments, a significant quantity of isolysergole was isolated from the culture filtrate. This alkaloid, which is not present in normal submerged cultures of strain SD 58 to any appreciable extent, showed a tritium retention of 168 and 202%, respectively, *i.e.*, an increase over the tritium content of the starting material. Taken together with the slightly too low tritium content of the elymoclavine, this seemed to indicate the operation of yet unknown complex reactions in the further conversions of elymoclavine, which affect the hydrogen at C-9 and involve large isotope effects.[13,14] As shown later, however, this explanation proved to be wrong.
(23) A. Stoll, A. Brack, H. Kobel, A. Hofmann, and R. Brunner, *Helv. Chim. Acta*, 37, 1815 (1954).

clavine-I and agroclavine are equally efficient precursors of elymoclavine, whereas isochanoclavine-I and chanoclavine-II gave much lower incorporations. These two compounds are therefore unlikely to be intermediates in the formation of tetracyclic ergolines. For isochanoclavine-I this was shown even more convincingly by Arigoni's group.[5] The still relatively high incorporation rate of isochanoclavine-I, although probably due to contamination of the isolated elymoclavine,[24] left open the possibility that some of the compound was converted to elymoclavine *via* chanoclavine-I. This possibility was excluded by another experiment, in which isochanoclavine-I-^{14}C, prepared by light-catalyzed isomerization of chanoclavine-I-^{14}C,[9,25] was fed to *Claviceps paspali*. The radioactivity in the purified crystalline chanoclavine-I from this experiment corresponded to less than 0.006% incorporation, ruling out any conversion of isochanoclavine-I to chanoclavine-I.

The evidence presented demonstrates that mevalonic-2-^{14}C acid gives rise to chanoclavine-I labeled in the C-methyl group which occupies the *cis* position at the Δ^8 double bond, and this in turn is converted into elymoclavine labeled in the *trans* position. This raises the question as to whether in the original 4-dimethylallyltryptophan (**7**) the *cis*- or the *trans*-methyl group is derived from C-2 of mevalonate. Chanoclavine-I labeled in the way observed could arise from **7** labeled in the *cis*-methyl group by hydroxylation at the *trans*-methyl group or from **7** labeled in the *trans*-methyl group by hydroxylation at the *cis*-methyl group followed by *cis–trans* isomerization (Scheme III). Work by Arigoni[26] and by Birch, *et al.*,[27] indicates that in the isopentenyl pyrophosphate isomerase reaction, the methylene group, which arises from C-2 of mevalonate, becomes the *trans*-methyl group of dimethylallyl pyrophosphate. In this reaction, the hydrogen originating from the 4*S* position of mevalonate is removed while the 4*R* hydrogen is retained.[28] The same stereospecificity is also observed in the polycondensation of isoprene residues leading to the formation of *trans* double bonds,[28,29] while in the biosynthesis of rubber the 4*R* hydrogen is eliminated, resulting in the formation of *cis* double bonds.[30] On the basis of these results, elimination of the 4*S* hydrogen of mevalonate in ergot alkaloid formation would indicate that C-2 of mevalonate labels the *trans*-methyl group of the original dimethylallyl substituent, and elimination of the 4*R* hydrogen would indicate labeling of the *cis* position.

Scheme III



The results given in Table V show that the 4*R* hydrogen of mevalonate is completely or partly retained during the conversion into chanoclavine-I and elymoclavine (expt 1 and 3). The samples obtained from mevalonate tritiated in the 4*S* position on the other hand were essentially devoid of tritium (expt 2 and 4). The mevalonic acids used in these experiments were racemic mixtures with respect to both the configuration at C-3 and the position of the tritium at C-4 in the sense that "(4*R*)-mevalonic-2-^{14}C-4-T acid" was actually a 1:1 mixture of (3*R*,4*R*)mevalonate-2-^{14}C-4-T and (3*S*,4*S*)-mevalonate-2-^{14}C-4-T and "(4*S*)-mevalonic-2-^{14}C-4-T acid" was a 1:1 mixture of (3*R*,-4*S*)-mevalonate-2-^{14}C-4-T and (3*S*,4*R*)-mevalonate-2-^{14}C-4-T.[28] The previous use of these racemic mixtures for determining the steric course of the hydrogen elimination from C-4 was based on the observation that liver mevalonic kinase shows absolute specificity for the natural 3*R* isomer of mevalonate.[31] It was not known, however, whether it is also the 3*R* isomer of mevalonate which is used in ergot alkaloid biosynthesis. Since the conclusion about the stereochemistry would be just contrary if the 3*S* isomer were the alkaloid precursor,[32] it was important to examine this question. (3*R*,*S*)-Mevalonic-2-^{14}C acid was incubated with a partially purified enzyme fraction from pig liver,[33] which converted all the 3*R* isomer into isopentenyl pyrophosphate and traces of mevalonate 5-phosphate and -pyrophosphate. The unreacted 3*S* isomer was isolated and fed to a culture of *Claviceps* strain SD 58.

(24) All samples were chromatographed twice and, except the one from agroclavine, which was only analyzed after the second purification step, did not lose radioactivity on rechromatography (constant specific radioactivity within ±10%). However, isochanoclavine-I is closest in R_f to elymoclavine and may not have been removed completely by this purification.

(25) T. Fehr, Ph.D. Dissertation, Eidgenössische Technische Hochschule, Zürich, 1967.

(26) D. Arigoni, *Experientia*, **14**, 153 (1958).

(27) A. J. Birch, M. Kocor, N. Sheppard, and J. Winter, *J. Chem. Soc.*, 1502 (1962).

(28) J. W. Cornforth, R. H. Cornforth, C. Donninger, and G. Popjak, *Proc. Roy. Soc., Ser. B*, **163**, 492 (1966).

(29) T. W. Goodwin and R. J. H. Williams, *ibid.*, **163**, 515 (1966).

(30) B. L. Archer, D. Barnard, E. G. Cockbain, J. W. Cornforth, R. H. Cornforth, and G. Popjak, *ibid.*, **163**, 519 (1966).

(31) R. H. Cornforth, J. W. Cornforth, and G. Popjak, *Tetrahedron*, **18**, 1351 (1962).

(32) We are indebted to Professor D. Arigoni, Zürich, for bringing this point to our attention.

(33) D. H. Shah, W. W. Cleland, and J. W. Porter, *J. Biol. Chem.*, **240**, 1946 (1965).

Table IV. Feeding Experiment with Mevalonic-2-^{14}C-5-T Acid and Chanoclavine-I Obtained from It

Expt	Feeding conditions: Precursor, μmol	Feeding conditions: Radioact, dpm	Feeding conditions: Organism[c]	Chanoclavine-I obtained: Quantity, mg	Chanoclavine-I obtained: Sp radioact, dpm/mmol	Chanoclavine-I obtained: T retn,[a] %	Elymoclavine obtained: Quantity, mg	Elymoclavine obtained: Sp radioact, dpm/mmol	Elymoclavine obtained: T retn,[a] %
1	(*R,S*)-Mevalonic-2-^{14}C-5-T acid, 40	^{14}C: 1.20×10^8 T: 5.70×10^8 (T/^{14}C = 4.63)	*Claviceps paspali*, I	90	^{14}C: 7.96×10^6 T: 2.19×10^7 (T/^{14}C = 2.75)	60			
2	(*R,S*)-Mevalonic-2-^{14}C-5-T acid, 9	^{14}C: 2.6×10^7 T: 1.2×10^8 (T/^{14}C = 4.63)	*Claviceps* strain SD 58, II				86	^{14}C: 1.36×10^7 T: 4.13×10^7 (T/^{14}C = 3.08)	66
3	Chanoclavine-I-7-^{14}C-10-T,[b] 40	^{14}C: 3.2×10^6 T: 8.8×10^5 (T/^{14}C = 2.75)	*Claviceps* strain SD 58, I		^{14}C: 5.8×10^6 T: 1.6×10^7 (T/^{14}C = 2.75)	100	113	^{14}C: 1.5×10^5 T: 4.1×10^5 (T/^{14}C = 2.78)	101
4	Chanoclavine-I-7-^{14}C-10-T,[b] 30	^{14}C: 2.4×10^5 T: 6.6×10^5 (T/^{14}C = 2.75)	*Claviceps* strain SD 58, II		^{14}C: 5.2×10^6 T: 1.4×10^7 (T/^{14}C = 2.72)	99	74	^{14}C: 1.0×10^5 T: 2.7×10^5 (T/^{14}C = 2.70)	98

[a] Tritium retention is defined as: [(T/^{14}C of product)/(T/^{14}C of precursor)]100 (%). [b] From expt 1. [c] I = submerged culture; II = surface culture.

Table V. Feeding Experiments with (3*R*,4*R*)- and (3*R*,4*S*)-Mevalonic-2-^{14}C-4-T Acid and Compounds Derived Thereof

Expt	Precursor, μmol	Radioactivity fed, dpm	Strain[c]	Total alkaloid, mg	Chanoclavine-I isolated: Sp radioact, dpm/mmol	Chanoclavine-I isolated: T retn, %	Elymoclavine isolated: Sp radioact, dpm/mmol	Elymoclavine isolated: T retn, %	LAMC[e] obtained: Sp radioact, dpm/mmol	LAMC[e] obtained: T retn, %
1	(3*R*,4*R*)-Mevalonic-2-^{14}C-4-T acid lactone,[a] 0.54	^{14}C: 4.93×10^6 T: 1.94×10^7 (T/^{14}C = 3.94)	*Claviceps* strain SD 58	98			^{14}C: 2.19×10^6 T: 6.0×10^6 (T/^{14}C = 2.74)	70		
2	(3*R*,4*S*)-Mevalonic-2-^{14}C-4-T acid lactone,[b] 0.65	^{14}C: 5.56×10^6 T: 2.46×10^7 (T/^{14}C = 4.42)	*Claviceps* strain SD 58	133			^{14}C: 1.87×10^6 T: 3.00×10^3 (T/^{14}C = 0.002)	<0.1		
3	(3*R*,4*R*)-Mevalonic-2-^{14}C-4-T acid lactone,[a] 0.72	^{14}C: 6.55×10^6 T: 2.60×10^7 (T/^{14}C = 3.96)	*Claviceps paspali*	123	^{14}C: 7.90×10^5 T: 3.16×10^6 (T/^{14}C = 4.00)	101				
4	(3*R*,4*S*)-Mevalonic-2-^{14}C-4-T acid lactone,[b] 0.64	^{14}C: 5.40×10^6 T: 2.70×10^7 (T/^{14}C = 4.78)	*Claviceps paspali*	120	^{14}C: 7.72×10^5 T: 4.5×10^4 (T/^{14}C = 0.058)	1.2				
5	(3*R*,4*R*)-Mevalonic-2-^{14}C-4-T acid lactone,[a] 0.72	^{14}C: 6.55×10^6 T: 2.60×10^7 (T/^{14}C = 3.96)	*Claviceps paspali*	171					^{14}C: 5.36×10^5 T: 1.48×10^6 (T/^{14}C = 2.76)	70
6	Elymoclavine-17-^{14}C-9-T, 22.4	^{14}C: 4.9×10^4 T: 1.35×10^5 (T/^{14}C = 2.74)	*Claviceps* strain SD 58	45–50			^{14}C: 2.62×10^5 T: 7.4×10^5 (T/^{14}C = 2.82)	103		
7	Chanoclavine-I-7-^{14}C-9-T, 67	^{14}C: 5.29×10^4 T: 2.12×10^5 (T/^{14}C = 4.00)	*Claviceps* strain SD 58	45–50	^{14}C: 3.37×10^3 [d] T: 1.33×10^4 [d] (T/^{14}C = 3.94)	98	^{14}C: 8.15×10^4 T: 2.25×10^5 (T/^{14}C = 2.76)	69	Agroclavine ^{14}C: 160[d] T: 332[d] (T/^{14}C = 2.1)	~55

[a] The preparation fed also contained an equal amount of (3*S*,4*S*)-mevalonic-2-^{14}C-4-T acid. Radioactivity given is that of 3*R* isomer. [b] The preparation fed also contained an equal amount of (3*S*,4*R*)-mevalonic-2-^{14}C-4-T acid. Radioactivity given is that of 3*R* isomer. [c] Submerged culture. [d] Dpm total. [e] LAMC, lysergic acid methylcarbinolamide.

A parallel culture was incubated with (3*R*,*S*)-mevalonate. As shown in Table VI, (3*R*,*S*)-mevalonate was incorporated into elymoclavine 50 times better than the 3*S* specimen, indicating that the 3*R* isomer is an at least 100 times better alkaloid precursor than its antipode. It is clear then that the assumption made in the above experiments is correct and that the formation of ergot alkaloids involves loss of the 4*S* hydrogen of mevalonate.

Table VI. Incorporation of (*S*)- and (*R*,*S*)-Mevalonic-2-^{14}C Acid into Elymoclavine by Submerged Cultures of *Claviceps* Strain SD 58

	Precursor, μmol	
	(*S*)-Mevalonic 2-^{14}C acid, 0.33	(*R*,*S*)-Mevalonic 2-^{14}C acid, 0.30
Radioact fed, dpm	1.07×10^6	9.8×10^5
Alkaloid formed, mg	69	46
Sp act. of isolated elymoclavine, dpm/mmol	5.3×10^3	3.5×10^5
Total act. incorporated, dpm	1.4×10^3	6.3×10^4
Incorporation, %	0.13	6.5

The availability of (4*R*)-mevalonate-4-T of high specific activity enabled us to reinvestigate the partial loss of tritium during the conversion of chanoclavine-I to elymoclavine observed in the earlier experiments using mevalonate tritiated nonstereospecifically at C-4. The results of a number of experiments with (4*R*)-mevalonate-2-^{14}C-4-T are summarized in Table V. While chanoclavine-I had the same T/^{14}C ratio as the mevalonic acid from which it was formed (expt 3), elymoclavine retained only 70% of the tritium from the 4*R* position of mevalonate (expt 1); 70% retention of tritium from this position was also observed in the conversion of mevalonate into an amide derivative of lysergic acid (expt 5), indicating that the hydrogen of the 9 position of elymoclavine is not affected during the conversion into lysergic acid derivatives. Elymoclavine-17-^{14}C-9-T (isolated from expt 1) on incubation with the ergot fungus under the normal conditions of a feeding experiment and reisolation did not lose tritium (expt 6), ruling out the earlier assumption[22] of an isotope effect in some reaction involved in further conversions or in the degradation of elymoclavine. Finally, chanoclavine-I-7-^{14}C-9-T (isolated from expt 3) on incubation with *Claviceps* strain SD 58 gave elymoclavine with 69% tritium retention, while chanoclavine reisolated from the culture had the same T/^{14}C ratio as the starting material (expt 7). A very small quantity of agroclavine was also obtained in this experiment. Its T/^{14}C ratio also indicated the partial loss of tritium (55% retention), but this measurement only rests on a small number of counts. These experiments show that a 30% loss of tritium from position 9 occurs in the conversion of chanoclavine-I to elymoclavine, presumably between chanoclavine-I and agroclavine.

Discussion

The results obtained in this study provide conclusive evidence that the conversion of chanoclavine-I into tetracyclic ergot alkaloids involves a *cis–trans* isomerization at the Δ^8 double bond. The same conclusion was also reached by Arigoni and his coworkers.[5] Since the 4*S* hydrogen of mevalonic acid is eliminated in the biosynthesis, the original dimethylallyl residue (*e.g.*, in **7**) apparently carries the label from C-2 of mevalonate in the *trans*-methyl group. The conversion of **7** to chanoclavine-I therefore seems to involve hydroxylation at the *cis*-methyl group followed by another *cis–trans* isomerization at the allylic double bond (Scheme III, pathway 1). Thus, the apparently "normal" labeling of the tetracyclic ergot alkaloids in the *trans* carbon atom following administration of mevalonate-2-^{14}C seems to be only the accidental result of a more complex series of reactions involving two *cis–trans* isomerizations.

One might ask whether the natural pathway of ergot alkaloid biosynthesis really follows this course, or, put in a different way, whether chanoclavine-I is a natural intermediate in this biosynthesis. As in most biosynthetic investigations it is very difficult to decide this question beyond doubt. There is, however, a considerable amount of evidence indicating that chanoclavine-I is indeed a natural intermediate in the pathway. (a) The efficiency of chanoclavine-I as a precursor of elymoclavine is very high[34] and comparable to that of agroclavine. (b) As shown by Fehr, *et al.*,[5] and in this study, isochanoclavine-I is not a precursor of tetracyclic ergot alkaloids. (c) Chanoclavine-I is formed from the same precursors as the tetracyclic ergolines, and the labeling patterns obtained in carrying out the transformation mevalonate → tetracyclic ergolines directly is qualitatively and quantitatively the same as that obtained in carrying out this transformation in the two steps mevalonate → chanoclavine-I → tetracyclic ergolines. All these findings are in agreement with and support the view that the biosynthesis of ergot alkaloids does in fact proceed *via* chanoclavine-I as an intermediate.

A rather interesting finding is the observed decrease of the T/^{14}C ratio in the conversion of chanoclavine-I-7-^{14}C-9-T to elymoclavine. The 30% decrease of the T/^{14}C ratio seems to be associated with this reaction sequence itself, presumably, although the one measurement on the small amount of agroclavine obtained in expt 7, Table V, is not sufficient to prove this, with the cyclization of chanoclavine-I to agroclavine.

Any mechanism that is considered for the conversion of chanoclavine-I to agroclavine will have to explain this decrease in the T/^{14}C ratio. In our preliminary communication,[13] we proposed a mechanism which involves formation of an allylic carbonium ion at C-17 by elimination of the OH group (probably as phosphate after phosphorylation). This carbonium ion could then cyclize directly to give agroclavine. This mechanism, which was also put forward by Agurell,[3] would account for the retention of both the hydrogens at C-9 and C-10 and for the fact that isochanoclavine-I is not an intermediate in the conversion. The more recent data render this mechanism less likely, since it is somewhat difficult to visualize how it could involve a partial loss of the tritium from C-9. The 30% decrease of the T/^{14}C ratio could be due to an isotope effect or to exchange. The latter might simply involve labilization of the hydrogen at C-9 at some intermediary stage.[35]

(34) Incorporations of up to 40% were obtained[4] in the conversion of chanoclavine-I to elymoclavine.

Alternatively, a second hydrogen may be added to C-9 and the original one eliminated as a proton, which is accepted by an enzyme (similar to mechanism c mentioned above). The observed result would be accounted for if the proton on an enzyme catalyzing both these steps underwent only partial exchange with the protons of the medium and were mainly transferred to a new molecule of substrate, resulting in a partial intermolecular hydrogen transfer. Isotope effects could account for the result in several ways. A consideration of the various possibilities indicates that the reaction in this case would also have to involve the addition of a second hydrogen at C-9, followed by removal of one hydrogen, one of which steps would be nonstereospecific, and/or that there should be at least one free intermediate between chanoclavine-I and the step involving the isotope effect, which carries the excess tritium since chanoclavine-I reisolated from the experiment is not enriched in tritium.[36] Further experimental data will be required to explain this observation and to clarify the mechanism of this conversion.

Experimental Section

The following strains of the ergot fungus were used: *Claviceps paspali* Li 342/SE 156[4] for the production of chanoclavine-I, *Claviceps paspali* MAR 413/SE 126[4] for lysergic acid methylcarbinolamide, *Claviceps* strain SD 58[37,38] for elymoclavine, and a *Claviceps* strain from *Pennisetum* grass described by Stoll, *et al.*,[23] for agroclavine, chanoclavine-II, and isochanoclavine-I. The culture conditions and the methods of feeding labeled precursors were the same as in earlier experiments.[4] The total alkaloid content of culture filtrates was determined colorimetrically with *p*-dimethylaminobenzaldehyde.[39,40] The isolation and purification of crystalline chanoclavine-I,[4] elymoclavine,[38] and lysergic acid methylcarbinolamide[41] were carried out as described previously. In experiments with resting cells, 7-day-old mycelia from submerged cultures of strain SD 58 were filtered, washed with water, and transferred under sterile conditions into 25 ml of $^1/_{15}$ *M* phosphate buffer (Soerensen), pH 7.3, containing the labeled precursors. The ratio mycelial weight:volume of medium was the same as in the original cultures. The incubations were carried out with shaking for 1 day. After filtration and addition of ammonia, the alkaloid mixtures were extracted from the culture filtrates with chloroform and were resolved by thin layer chromatography on silica gel G in the solvent system ethyl acetate–acetone–dimethylformamide 5:5:1, followed by rechromatography in chloroform–*t*-butyl alcohol, 3:1, in an atmosphere saturated with ammonia. The single alkaloids were chromatographically homogeneous after this purification. The alkaloids were detected by spraying a small part of the thin-layer plate with Ehrlich's reagent.

Labeled Precursors. (*R,S*)-Mevalonic-2-^{14}C and -5-T acids (as dibenzylethylenediammonium salts) were obtained from New England Nuclear Corp. and were fed to the cultures as the base salts. The ^{14}C preparation was several years old and contained, at the time of use, two radioactive impurities accounting for about 15% of its activity. (*R,S*)-Mevalonic-4-T acid has been synthesized earlier[42] by the method of Cornforth, *et al.*[43] (*R,S*)-Mevalonolactone-2-^{14}C, (3*R*,4*R*)-mevalonolactone-4-T, and (3*R*,4*S*)-mevalonolactone-4-T were products of the Radiochemical Center, Amersham, obtained through Nuclear Chicago, Inc. L-Tryptophan-indole-T was prepared by acid-catalyzed exchange of the nonlabeled material with tritiated water.[44] Agroclavine-indole-T has been obtained biosynthetically from L-tryptophan-indole-T in earlier work.[45]

Chanoclavine-II and Isochanoclavine-I. L-Tryptophan-indole-T (39.1 mg, specific activity 12.3 mCi/mmol) was added to ten 100-ml surface cultures of the *Pennisetum* type ergot strain[23] on the 25th day after inoculation. Twenty days later the cultures were homogenized in a blender and centrifuged to remove the cell debris. This sediment was washed with water, and the supernatants, after addition of ammonia, were extracted with a 1:3 mixture of isopropyl alcohol and chloroform. After evaporation of the extract, the residue was dissolved in 2% tartaric acid, and the solution was successively extracted with methylene chloride at acidic pH, pH 7.2, and pH 11–12. The last extract (48 mg) which contained mainly the chanoclavine fraction of the alkaloids was chromatographed on a small column of silica gel (10 g) with ethyl acetate–acetone–dimethylformamide, 5:5:1, as solvent. The fraction containing the chanoclavine isomers (10 mg) was further resolved by tlc on silica gel G in chloroform–*t*-butyl alcohol, 3:1, saturated with 15% ammonia. Isochanoclavine-I (R_f 0.27) was rechromatographed on silica gel G in chloroform–methanol, 9:1, saturated with 25% ammonia, in an atmosphere saturated with ammonia. Chanoclavine-II (R_f 0.18) and the band of lowest R_f (0.13) were further purified by tlc on silica gel G with the solvent chloroform–*t*-butyl alcohol, 3:1, in an atmosphere of ammonia. All three compounds were chromatographically pure after this step as shown by tlc in another solvent. The two chanoclavine isomers cochromatographed exactly with authentic materials in at least two different tlc systems. The material from the band of R_f 0.13 could not be correlated with any of the known alkaloids. Since a feeding experiment showed it not to be incorporated into elymoclavine it was not investigated further.

(*S*)-Mevalonic Acid-2-^{14}C. The method of Shah, *et al.*,[33] for conversion of mevalonic acid into isopentenyl pyrophosphate was followed. The enzyme fraction from pig liver was purified through the calcium phosphate gel step. (*R,S*)-Mevalonic-2-^{14}C acid sodium salt (2 μmol, 3.6 μCi) was incubated with the enzyme preparation and cofactors as described.[33] After denaturation and removal of the protein, aliquots of the reaction mixture were chromatographed on strips of Whatman No. 1 paper in 1-propanol–concentrated ammonia–water, 6:3:1, and the chromatograms were scanned for radioactivity. The two major radioactive peaks corresponded to isopentenyl pyrophosphate, containing a trace of 5-phosphomevalonic acid, and mevalonic acid. Integration of the radioactivity of chromatogram scans from three different incubations by cutting out the areas under the radioactive peaks and weighing the paper showed that unreacted mevalonic acid accounted for 50.2, 48.1, and 46.2%, respectively, of the total activity, indicating quantitative conversion of the *R* isomer. The remainder of an incubation mixture was chromatographed on a sheet of Whatman No. 3 paper in the above solvent. Elution of the mevalonate zone gave (*S*)-mevalonate-2-^{14}C (0.9 μCi), which was radiochemically homogeneous in two other paper chromatography systems.[46]

Degradations of Chanoclavine-I and Elymoclavine. Emde–Birch cleavage of elymoclavine as described by Bhattacharji, *et al.*,[11] gave N-methyl-6,7-secoelymoclavine (chromatographically homogeneous, mp 149–150° after recrystallization from ether) in 60% yield. Kuhn–Roth oxidations of this material and of chanoclavine-I were carried out by the standard procedure,[47] using a Wiesenberger apparatus[48] for the distillation. The acetic acid was titrated to pH 8.5 with 0.02 *N* NaOH to determine the yield, and, after concentration, the sodium acetate was counted for radioactivity. Yields of acetic acid were 56% from chanoclavine-I and 23 and 46%, respectively, from N-methylsecoelymoclavine.

(35) This labilization would have to occur with some free or bound intermediate, which is not in equilibrium with chanoclavine-I, since the latter retains all its tritium.

(36) It might be suspected that the chanoclavine-I in the medium does not equilibrate with the material in the cells. However, the reisolated chanoclavine-I has a lower specific radioactivity than the starting material (Table IV, expt 3 and 4), suggesting at least some exchange between the two pools.

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Determination of Radioactivity. In the experiments with nonstereospecifically tritiated mevalonic acids the determination of tritium and ^{14}C was carried out by a dry combustion method, which involves physical separation of the two isotopes, followed by gas phase proportional counting.[49] All other isotope determinations were carried out directly in a Packard Tricarb or Beckman LS 100 liquid scintillation counter, using Bray's solution[50] or PPO and POPOP in toluene as scintillators. Counting efficiencies were determined by adding internal standard to every sample. It was noted that ergot alkaloids gave a temperature-dependent chemoluminescence reaction with the peroxide present even in reagent grade, stabilized dioxane, which contributed considerably to the tritium counts, when these samples were counted in Bray's solution in the ambient-temperature Beckman instrument, but not in the refrigerated Tricarb. Thus, a nonlabeled sample of elymoclavine dissolved in dioxane without scintillator gave several thousand counts in the tritium channel. The dioxane-containing scintillation mixture was therefore not used with the Beckman counter. Radioactivity on paper and thin-layer chromatograms was detected using a Packard chromatogram scanner.

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Acknowledgments. We are greatly indebted to Drs. A. Brack, A. Hofmann, and D. Stauffacher of Sandoz Research Laboratories, Basel, Switzerland, who generously provided us with reference alkaloids and a strain of ergot. Our thanks are also due to Drs. D. Arigoni and W. Acklin, Eidgen. Technische Hochschüle, Zürich, Switzerland, for communicating to us experimental details (*e.g.*, some of the tlc systems for the separation of chanoclavine isomers) and results of their work prior to publication. Financial support of this work by Deutsche Forschungsgemeinschaft, Eli Lilly and Co., and the National Institutes of Health (Grant No. AM 11662-01) is gratefully acknowledged.

Mechanism of the Photodephosphorylation of Menadiol Diphosphate. A Model for Bioquantum Conversion[1]

Pill-Soon Song[1] and Thomas A. Moore

Contribution from the Department of Chemistry, Texas Technological College, Lubbock, Texas 79409. *Received May 27, 1968*

Abstract: Photooxidation and photodephosphorylation of menadiol diphosphate by riboflavin and oxygen have been investigated as a model for biological quantum conversion. The details of the mechanism for this process in water have been studied experimentally. The results obtained suggest that the singlet oxygen generated by the triplet energy transfer from riboflavin is the photochemically reactive species which oxidizes and dephosphorylates menadiol diphosphate. The implication of this photoprocess as a bioquantum conversion model has been discussed.

Recently, we made a preliminary attempt at understanding the biological quantum conversion process in terms of a model system.[2] The model consists of a flavin and menadiol diphosphate. These compounds occur in chloroplasts in the form of FMN and menadiones, respectively. In our preliminary investigation, it was found that oxygen is required to oxidize and dephosphorylate menadiol diphosphate in the flavin-sensitized photoreaction. It was, however, not possible to elucidate the mechanism of the photoprocess due to a lack of understanding of the nature of the flavin triplets and insufficient kinetic data. We now report a detailed mechanistic elucidation of the photodephosphorylation of menadiol diphosphate in the light of additional kinetic data as well as theoretical and spectroscopic results on flavin triplets.[3-5]

Experimental Section

Materials. Riboflavin (6,7-dimethyl-9-ribitylisoalloxazine), Sigma Grade (recrystallized from acetic acid), was obtained from Sigma Chemical Co. Menadione (2-methyl-1,4-naphthoquinone, vitamin K, M=O), of spf grade, was obtained from Sigma Chemical Co. and was used after purification by vacuum sublimation. Menadiol sodium diphosphate (tetrasodium 2-methyl-1,4-naphthalenediol bis(dihydrogen phosphate), synkayvite, M) was a gift from Dr. W. E. Scott of Hoffmann-La Roche Ind. *p*-Benzoquinone was obtained from Eastman Organic Chemicals and was used after purification by vacuum sublimation. Potassium ferrioxalate for the chemical actinometry was purchased from City Chemical Co. (New York) and was recrystallized from warm water before use. A reagent grade potassium iodide (J. T. Baker Co.) was used without further purification. Water was first deionized, followed by distillation. Acetic acid was purified and dehydrated for use as solvent. Matheson Research Grade N_2 and O_2 were used without further purification.

Photolysis and Actinometry. Photolysis of the reaction mixture was done with a Bausch and Lomb xenon light source with a high-intensity monochromator with grating (catalog no. 33-86-07). This unit was fitted with a water-cooled aluminum cell holder (23 ± 1°) for Beckman 46005 10-mm path cells or equivalent. The entrance slit width was 3.56 mm and exit slit width 2.00 mm for all photolysis experiments. This gives a bandpass of 14.8 nm. The absorbance measurements were made on a Beckman DB spectrophotometer. The quantum yields were determined on the basis of orthophosphate produced and the chemical actinometry using potassium ferrioxalate as described by Parker and Hatchard.[6] The analytical method for determination of orthophosphate was that of Murakami and Martell.[7]

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