malate in this organism, then the simplest interpretation of the results above is that an intramolecular hydride shift is involved in the interconversion of these compounds.

The series of reactions leading to the synthesis of α-ketoglutarate and α-ketoisocaproate involves the conversion of a α-keto acid to a homologous α-keto acid containing one more methylene group. An identical pathway has been proposed for the synthesis of α-ketoadipate from α-ketoglutarate (Strassman and Weinhouse, 1953) and for the synthesis of α-ketobutyrate, α-keto-*n*-valerate, and α-keto-*n*-caproate from pyruvate, α-ketobutyrate, and α-keto-*n*-valerate, respectively (Ingraham *et al.*, 1961). It will be interesting to see whether further studies bear out the generality of this pathway.

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The Biological Transformation of Xanthopterin by a Bacterium Isolated from Soil*

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The utilization of the naturally occurring pteridine, xanthopterin, by a bacterium isolated from soil, has been studied in the hope that some of the intermediates produced might be related to intermediates in the normal biogenesis of pteridines. The following products have been isolated from cultures of this organism and identified by ultraviolet absorption spectra, infrared absorption spectra, and paper chromatography: 6-oxylumazine, 6,7-dioxylumazine, and leucopterin. Enzymes have been obtained from this organism which catalyze (1) the deamination of xanthopterin and isoxanthopterin to 6-oxylumazine and 7-oxylumazine respectively, (2) the oxidation of 6-oxylumazine, but not 7-oxylumazine, to 6,7-dioxylumazine, and (3) the transformation of 6,7-dioxylumazine to an unidentified compound.

The relationship between purines and pteridines has been the subject of a number of investigations during the past several years. Plaut (1954), in his studies with the yeast *Ashbya gossypii*, found that the metabolites glycine, CO_2, and formic acid were incorporated into riboflavin in a pattern similar to that of their incorporation into purines. Somewhat later, a radioactive lumazine was isolated from the mycelia of this organism during further studies of the incorporation of formate C^{14} into riboflavin (Maley and Plaut, 1959a). These authors believe that the lumazine is, in fact, an intermediate in the biogenesis of riboflavin (Maley and Plaut, 1959b). Evidence to the contrary, however, has been presented by others (Korte and Aldag, 1959). In another yeast, *Eremothecium ashbyii*, McNutt (1956, 1961) demonstrated that all of the atoms of the purine ring except the carbon atom in position 8 were incorporated as a unit into the pteridine portion of riboflavin. A lumazine derivative, also present in this organism, was similarly labeled (McNutt

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TABLE I

THE EFFECTIVENESS OF VARIOUS COMPOUNDS AS THE NITROGEN SOURCE FOR THE ORGANISM

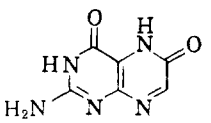
Nitrogen Source	% Transmission (640 mμ)
O	95
NH_4NO_3	40
Adenine	38
Hypoxanthine	38
Guanine	40
Xanthine	41
Uric acid	41
Isoguanine	51
Caffeine	97
6,7-Dioxylumazine	45
Xanthopterin	61
6-Oxylumazine	63
7-Oxylumazine	67
2-Amino-4-hydroxypteridine	70
2-Amino-4-hydroxypteridine-6-carboxylic acid	98
Cytosine	89
Orotic acid	89
Uracil	94
Thymine	97
Alloxan	90
Diaminouracil	98
4-Amino-2,6-dioxypyrimidine	97

and Forrest, 1958; McNutt, 1959). More recently, adenine was shown to be incorporated into pteroylglutamic acid in *Cornybacterium* (Vieira and Shaw, 1961). Higher organisms, the larvae of the amphibian Xenopus, after being fed guanine-2-C^{14}, produced a labeled compound which could be oxidized to 2-amino 4-hydroxypteridine 6-carboxylic acid (Zeigler-Gunder *et al.*, 1956). When, however, labeled purines were fed to butterflies, Weygand and Waldschmidt (1955) could find only feeble activity in the pteridines isolated from the insects.

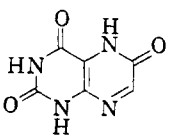
RESULTS AND DISCUSSION

This article represents an attempt to study further the relationship between pteridines and purines. A bacterium which utilizes xanthopterin as its sole source of carbon and nitrogen has been isolated in pure culture from soil, and individual steps in the utilization of the pteridine by this organism have been studied. It was hoped that an understanding of the mechanism by which this organism utilizes the pteridine might give an insight into the normal course of biosynthesis of pteridines.

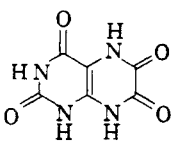
Compounds other than xanthopterin serve equally well as the nitrogen source for this organism. Naturally occurring purines are more



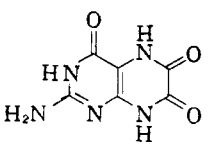
Xanthopterin



6-Oxylumazine



6,7-Dioxylumazine



Leucopterin

effective sources of nitrogen than xanthopterin itself, whereas naturally occurring pyrimidines are not effectively used (Table I).

Three products have been isolated from cultures of this organism grown in response to xanthopterin. They have been characterized as: 6-oxylumazine; 6,7-dioxylumazine; and leucopterin. Of these products, we believe 6-oxylumazine and 6,7-dioxylumazine are actual biological intermediates in the utilization of xanthopterin by this organism. A deaminase which catalyzes the conversion of xanthopterin to 6-oxylumazine is present in the cells of this organism. The enzyme is extracellular, as a few colonies on an agar plate containing xanthopterin are capable of clearing the xanthopterin from ever-widening areas surrounding the colonies until the entire plate loses its green color and assumes a bluish

TABLE II

THE R_F VALUES OF PRODUCTS ISOLATED FROM THE CULTURE AND AUTHENTIC COMPOUNDS

Substance	1-Propanol (60 ml)–Water (30 ml)–15 N NH_4OH (1.5 ml) R_F	1-Butanol (120 ml)–Acetic Acid (50 ml)–Water (30 ml) R_F	3% NH_4Cl Solution R_F	5% Acetic Acid Solvent R_F
Isolated 6-oxylumazine	0.25	0.34	0.61	0.60
6-Oxylumazine	0.24	0.32	0.62	0.59
Isolated 6,7-dioxylumazine	0.04	0.05	0.05	0.09
	0.13	0.11	0.42	0.50
6,7-Dioxylumazine	0.04	0.05	0.06	0.11
	0.13	0.11	0.41	0.50
Isolated leucopterin	0.12	0.14	0.26	0.34
Leucopterin	0.11	0.13	0.27	0.34

fluorescence characteristic of 6-oxylumazine. The organism also contains a hydroxylating enzyme which catalyzes the conversion of 6-oxylumazine to 6,7-dioxylumazine. Since the hydroxylase has not been separated from the deaminase we do not have enzymatic evidence for the conversion of xanthopterin to leucopterin. All of these products were isolated in a yield of only a small per cent of the xanthopterin fed, and the possibility that leucopterin may arise as a chemical artifact has not been ruled out with certainty.

Three kinds of enzymatic activity have been demonstrated in crude extracts of these bacteria: (1) deamination, (2) hydroxylation, and (3) "isomerization" of 6,7-dioxylumazine to a product having ultraviolet absorption maxima at 292 mμ and 263 mμ at *p*H 7.5.

TABLE III
SPECTROPHOTOMETRIC ASSAY OF THE DEAMINATION OF XANTHOPTERIN

Wave Length (mμ)	Optical Density Change	Xanthopterin Which Disappeared (mμmoles)	6-Oxylumazine Produced (mμmoles)
275	−0.440	59.1	
380	+0.092		55.6

Deamination, in the absence of the other enzymatic activities, was accomplished with an acetone powder of cells. This preparation had deaminase activity without interfering amounts of the other enzymes. Since crude enzyme preparations showed deaminase activity which far exceeded the other kinds of enzymatic activity, the same result was accomplished by merely using small amounts of crude enzyme. For each mole of xanthopterin which disappeared one mole of 6-oxylumazine and one mole of ammonia were produced (Tables III and IV). The preparations catalyzed the deamination of other pteridines and purines also. For each mole of isoxanthopterin which disappeared one mole of 7-oxylumazine and one mole of ammonia were formed (Tables V and VI). Adenine was converted to hypoxanthine, and guanine was converted to xanthine (Table VII).

The deaminase activity in crude extracts of this organism differs from that of the pterin deaminase in *Alcaligines metacaligines* (Levenberg and Hayaishi, 1959), since xanthopterin was not deaminated by the latter preparation. The deaminase activity dealt with here differs also from that present in another soil microorganism (Cotton and Forrest, 1961, unpublished). The preparation from that microorganism catalyzed the deamination of xanthopterin but did not act on isoxanthopterin or guanine.

TABLE IV
THE STOICHIOMETRY OF THE ENZYMATIC DEAMINATION OF XANTHOPTERIN

Experiment	Xanthopterin in the Control Tube (mμmoles)	Xanthopterin in the Experimental Tube (mμmoles)	Xanthopterin Which Disappeared	6-Oxylumazine in the Experimental Tube (mμmoles)	Ammonia Produced (mμmoles)
I	528	283	245	243	239
II	520	275	245	251	230

TABLE V
SPECTROPHOTOMETRIC ASSAY OF THE DEAMINATION OF ISOXANTHOPTERIN

Time of Incubation (hr.)	Wave Length (mμ)	Optical Density Change	Isoxanthopterin Which Disappeared (mμmoles)	7-Oxylumazine Produced (mμmoles)
3	249	−0.560	150	
	273	+0.600		151
4	249	−0.603	161	
	273	+0.656		165

TABLE VI
STOICHIOMETRY OF THE ENZYMATIC DEAMINATION OF ISOXANTHOPTERIN

Isoxanthopterin Added (μmoles)	Isoxanthopterin in the Control Tube (μmoles)	Isoxanthopterin in the Experimental Tube (μmoles)	Isoxanthopterin Which Disappeared (μmoles)	7-Oxylumazine in the Experimental Tube (μmoles)	NH_3 Produced (μmoles)
2.0	1.85	0.03	1.8	1.86	2.06

TABLE VII
THE ENZYMATIC DEAMINATION OF ADENINE AND GUANINE

Compound	Amount Added (mμmoles)	Hypoxanthine Produced (mμmoles) at: 15 min.	Hypoxanthine Produced (mμmoles) at: 40 min.	Xanthine Produced (mμmoles) at: 15 min.	Xanthine Produced (mμmoles) at: 40 min.
Adenine	40.0	37.4	38.0		
Guanine	38.8			23.4	36.2

The hydroxylating enzyme was prepared from cells grown in response to 6-oxylumazine. It is not yet clear to us why this preparation did not have interfering amounts of the enzyme which catalyzes the further transformation of 6,7-dioxylumazine.

The conversion of 6-oxylumazine to 6,7-dioxylumazine was followed spectrophotometrically (Fig. 4). For each mole of 6-oxylumazine which disappeared one mole of 6,7-dioxylumazine was formed (Table VIII). The enzyme also catalyzed the conversion of lumazine to 7-oxylumazine (Fig. 5); for each mole of lumazine which disappeared one mole of 7-oxylumazine was formed (Table IX). The hydroxylating enzyme from this organism thus resembles xanthine oxidase both in its ability to hydroxylate the 7-position of pteridines and in its inability to hydroxylate the 6-position of pteridines (Bergmann and Kwietny, 1958, 1959).

The enzyme which catalyzes the conversion of 6,7-dioxylumazine to a product having ultraviolet absorption maxima at 292 mμ and 263 mμ (*p*H 7.5) was prepared from cells which were grown in response to 6,7-dioxylumazine. The spectrophotometric changes associated with this reaction are shown in Figure 6. The presence of two isosbestic points at 247 mμ and at 308 mμ suggests that a single product is produced. The molecular extinction coefficients, calculated from the amount of 6,7-dioxylumazine converted, were ϵ 263 mμ, 9,470 and ϵ 292 mμ, 12,980 at *p*H 7.5. Evidence will be presented in a later article to the effect that this substance is xanthine-8-carboxylic acid and that, in the enzymatically catalyzed "isomerization," position 6 of the pteridine becomes the 8-position in the contracted ring system and the carbon atom in position 7 of the pteridine is moved outside the ring where it appears in the carboxyl group.

Enzymes capable of converting this last substance to simpler products are also present in crude extracts of this organism. The intermediate steps in the further metabolism of this substance have yet to be investigated.

It seems likely that this organism is able, in only a few steps, to divert pteridines into the general pathway of purine metabolism.

EXPERIMENTAL

Materials.—Xanthopterin and leucopterin were obtained from the Mann Research Laboratories, Inc. The compounds below were synthesized by methods described in the listed references: 2-amino-4-hydroxypteridine-6-carboxylic acid (Weygand and Schaefer, 1952); 2-amino-4-hydroxypteridine (Mowat *et al.*, 1948); lumazine (Pfleiderer, 1957); 6-oxylumazine (Albert *et al.*, 1956); isoxanthopterin (Purrmann, 1941); 7-oxylumazine (Tschesche and Korte, 1951); 6,7-dioxylumazine (Bertho and Bentler, 1951).

The molecular extinction coefficients at *p*H 7.5 of the following compounds were determined: isoxanthopterin $\epsilon^{max}_{228\ m\mu}$, 25,040; $\epsilon^{max}_{283\ m\mu}$, 6,580; $\epsilon^{max}_{332\ m\mu}$, 12,540; 7-oxylumazine, $\epsilon^{max}_{273\ m\mu}$, 9,590; $\epsilon^{max}_{327\ m\mu}$, 16,600; 6,7-dioxylumazine $\epsilon^{max}_{228\ m\mu}$, 16,600; $\epsilon^{max}_{289\ m\mu}$, 8,820; $\epsilon^{max}_{331\ m\mu}$, 12,350; $\epsilon^{max}_{345\ m\mu}$, 12,400.

METHODS

Protein was determined by the method of Lowry *et al.* (1951).

The Isolation and Culture of the Organism.—The inorganic medium consisted of KH_2PO_4 (0.75 g); Na_2HPO_4 (0.05 g); $MgSO_4 \cdot 7H_2O$ (0.2 g); $CaCl_2$ (0.02 g); $FeCl_3 \cdot 6H_2O$ (0.005 g); $MnCl_2$ (0.002 g); $NaMoO_4 \cdot 2H_2O$ (0.001 g) per liter. The medium used in the isolation of the pure culture from soil consisted of the inorganic medium plus xanthopterin at a concentration of 0.1 g per liter of medium. Cultures were incubated aerobically at 37° in the dark.

For the growth of cells to be used in the preparation of enzyme solutions the medium consisted of inorganic medium supplemented with 0.1 g of xanthopterin and 10 g of sodium succinate $\cdot 6H_2O$ per liter. About 8 g of wet cells were obtained per 15 liters of 24-hour culture. Stock cultures were carried on slants of this last medium supplemented with agar. The organism has yet to be classified.

The Utilization of Various Compounds as the Nitrogen Source.—Various compounds were assayed microbiologically for their ability to satisfy the nitrogen requirement of the organism. Each compound was added in amount sufficient to supply 1 mg of nitrogen per 20 ml of inorganic medium supplemented with 1% sodium succinate $\cdot 6H_2O$. The inoculum was prepared from 20 ml of a 20-hour culture grown in inorganic medium plus xanthopterin. The cells were washed twice with water and resuspended in 5 ml of minimal medium. A 0.1-ml aliquot of this suspension was introduced into each 125-ml flask containing 20 ml of medium. The flasks were placed on a shaker, and the cultures were incubated aerobically for 20 hours at 37°.

The measurements of turbidity in Table I are averages of duplicate determinations. A per cent transmission reading of 95 represents essentially no cell growth.

Isolation and Characterization of 6-Oxylumazine.—Ten liters of inorganic medium containing 1 g of xanthopterin and 100 g of sodium succinate·$6H_2P$ were inoculated with 2 liters of culture which had been grown for 16 hours in the same medium. After 20 hours of aerobic incubation at 37°, the cells were removed and the filtrate was adjusted to *p*H 4 with acetic acid and treated with 200 g of Norite (Grade A Fisher Scientific). The Norite was washed with 2 liters of water, and the pteridines were eluted with 5 liters of 30% pyridine solution. The eluate was evaporated in vacuum and chromatographed on 40 sheets of Whatman No. 3 MM in 5% acetic acid solution. The principal band (R_F 0.59) had a bright blue fluorescence in ultraviolet light (365 mμ). The substance was eluted from the paper and rechromatographed on 16 sheets of paper in 3% NH_4Cl solution. The material from the main fluorescent band (R_F 0.61) was eluted, and the solution was adjusted to *p*H 5 with acetic acid and absorbed on a "Filtrol" column (4.5 × 7 cm) (Filtrol Catalyst-Filtrol Corporation). The substance was eluted with 50% acetone. The eluate was evaporated to dryness and the residue dissolved in 20 ml of N NaOH. This solution was treated with a small amount of Norite, heated to boiling, and filtered. Upon the addition of solid CO_2 to the filtrate, crystals of the sodium salt of 6-oxylumazine came out of solution. After 4 hours, the crystals were collected by centrifugation, washed with 1% bicarbonate solution, and dissolved in 15 ml of hot water. The acid, precipitated from this solution by the addition of 5 ml of 4 N HCl, was converted to the sodium salt by dissolving it in 20 ml of N NaOH and adding solid CO_2. The crystalline salt was again converted to the acid as described. After washing with ethanol followed by ether, the acid was dried in vacuum over H_2SO_4 and NaOH. Yield, 27.3 mg.

This product had the R_F values of 6-oxylumazine in four solvents (Table II). It co-chromatographed with 6-oxylumazine in 1-butanol–acid–water to give a single spot, R_F 0.35. The molecular extinction coefficients of the substance in 0.1 N NaOH, ϵ_{234}^{max}, 12,650; ϵ_{274}^{max}, 11,380; ϵ_{394}^{max}, 6,270, agreed with the values found for authentic 6-oxylumazine, ϵ_{234}^{max}, 12,710; ϵ_{274}^{max}, 11,400; ϵ_{394}^{max}, 6,320. Maxima in 0.1 N HCl were at 223, 248, 300, and 363 mμ. 6-Oxylumazine showed maxima at these same wave lengths ±1 mμ. The infrared absorption spectra of the two substances agree (Fig. 1).

Isolation and Characterization of 6,7-Dioxylumazine.—The cells were removed from 15 liters of culture which had grown for 20 hours in inorganic medium supplemented with xanthopterin and sodium succinate. The supernatant solution was reduced to 500 ml, and the solid which separated was filtered off. The filtrate was taken to dryness at 55°; the residue was taken up in 3% NH_4OH and chromatographed on 64 sheets of paper in the propanol solvent (Table II). The lower edges of the sheets were serrated to permit even dripping from the solvent front, and the development of the chromatogram was permitted to go on for 46 hours. The bands showing a blue fluorescence in ultraviolet light at 4 cm from the origin were cut from the sheets. The substance was eluted from the paper with 3% NH_4OH, and after the solution was evaporated to dryness the residue was taken up in 5 ml of N NaOH. The solution was treated with a small amount of Norite and filtered. Upon the addition of excess CO_2 to the solution crystals of the sodium salt of 6,7-dioxylumazine separated out. These were washed with 1% $NaHCO_3$ solution, redissolved in 10 ml of hot water, and converted to the acid with 5 ml of 4 N HCl. Conversion to the sodium salt and reconversion to the acid were repeated. It was dried over P_2O_5 in vacuum at 140°. Yield, 9.7 mg. 6,7-Dioxylumazine moves as two distinct bands on paper chromatograms (Table II). In the isolation of the substance from the culture only that portion of the material in the lower band was isolated. Under the usual conditions of paper chromatography in the propanol-ammonia-water system only one third or less of the 6,7-dioxylumazine chromatographed appears in the lower band (see below). Thus,

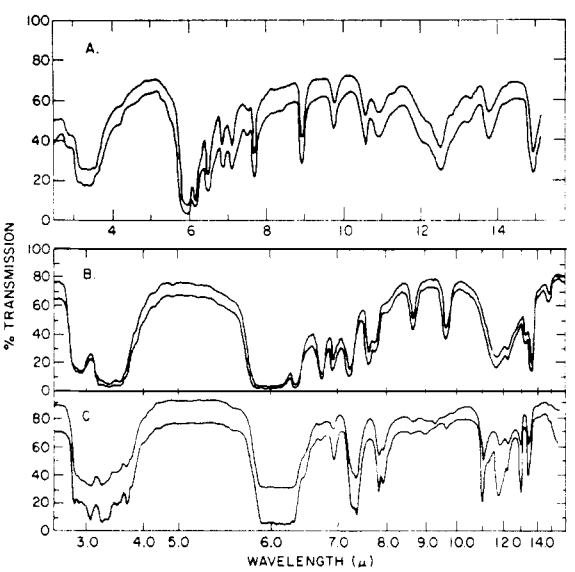


FIG. 1.—Infrared absorption spectra of synthetic pteridines compared with isolated fermentation products; 0.2% in KBr, 1–2 mm thick uncorrected for the absorption of KBr. *A*, 6-oxylumazine (lower curve) and the fermentation product; *B*, 6,7-dioxylumazine (lower curve) and the fermentation product; *C*, leucopterin (lower curve) and the fermentation product.

the amount of 6,7-dioxylumazine present in this culture perhaps exceeded 29 mg.

The R_F values of the isolated substance agree with those of 6,7-dioxylumazine (Table II). In 0.1 N HCl molecular extinction coefficients of the substance were: ϵ_{288}^{max}, 9,410; ϵ_{327}^{max}, 8,430, and those of 6,7-dioxylumazine were: ϵ_{288}^{max} 9,880, ϵ_{327}^{max}, 8,880. In 0.1 N NaOH the isolated substance showed ultraviolet absorption maxima at 233, 290, and 347 mμ, as did 6,7-dioxylumazine. The infrared absorption spectra of the two substances agree (Fig. 1).

6,7-Dioxylumazine was prepared by the method of Bertho and Bentler (1951). We did not encounter the low yield nor the difficulty of purification reported by Albert *et al.* (1956).

The Chromatographic Behavior of 6,7-Dioxylumazine.—Albert *et al.* (1956) observed that certain 6-oxypteridines move as two separate bands on paper chromatograms. This peculiarity is very striking in the case of 6,7-dioxylumazine whether it is chromatographed in acidic or in alkaline solvents (Table II). 6,7-Dioxylumazine was chromatographed in the propanol-ammonia-water system. Both the upper and the lower bands showed the ultraviolet absorption spectrum at *p*H 7.5 characteristic of 6,7-dioxylumazine. Although the lower band showed a brighter bluish fluorescence, it contained only one third (or less) as much substance as that present in the upper band. Upon elution of the substance with dilute NH_4OH from the upper and lower bands and chromatography a second time, each did not again separate into two bands in that proportion found on the first chromatogram. In fact, the lower band upon rechromatography failed to give rise to a detectable amount of substance having the higher R_F value. The upper and lower bands appearing on the second chromatogram each showed the ultraviolet absorption spectrum at *p*H 7.5 characteristic of 6,7-dioxylumazine.

6,7-Dioxylumazine migrated as two bands on paper which had been washed with a 1% solution of ethylenediaminetetraacetic acid in order to remove heavy cations from the paper.

Isolation and Characterization of Leucopterin.—Leucopterin was present in the same culture from which 6,7-dioxylumazine was obtained. It appeared on the above chromatograms as a dull gray fluorescent band at about 5 cm from the origin. These bands were cut from the sheets, and the leucopterin was isolated and purified as described above. It was dried over H_2SO_4 and NaOH. Yield, 14.8 mg.

This substance had R_F values characteristic of leucopterin (Table II). In 0.1 N NaOH its molecular extinction coefficients were: ϵ_{240}^{max}, 14,730; ϵ_{285}^{max}, 6,240; ϵ_{340}^{max}, 9,730; those of leucopterin were ϵ_{240}^{max}, 15,580; ϵ_{285}^{max}, 6,630; ϵ_{340}^{max}, 10,350. In 0.1 N HCl it showed ultraviolet absorption maxima at 223, 296, and 335 mμ, as did leucopterin. The infrared absorption spectra of the two compounds are compared in Figure 1.

Preparation of an Acetone Powder of Cells.—Forty-five liters of inorganic medium supplemented with xanthopterin and succinate were inoculated with 3 liters of culture grown for 20 hours in the same medium. After 22 hours of aerobic growth at 37°, the cells were separated and washed with water. The cells (24 g wet weight) were suspended in 100 ml of water and poured into 4 liters of acetone at −10°. The mixture was shaken four times at 5-minute intervals, and the residue was collected, washed well with acetone, and dried in vacuum over H_2SO_4. Yield, 2.2 g. The acetone powder was stored at −10°. Over a period of 3 months, it underwent no apparent loss in deaminase activity.

Preparation of Deaminase Solution.—The acetone powder (1 g) was stirred with 20 ml of 0.05 M Tris buffer, *p*H 7.5, at 1°. The mixture was centrifuged at 10,000 × *g* (Servall) for 25 minutes. The supernatant solution was used as the source of deaminase. It did not catalyze the hydroxylation of 6-oxylumazine or the disappearance of 6,7-dioxylumazine.

Preparation of Crude Hydroxylating Enzyme.—Inorganic medium (15 liters) containing 1.5 g of 6-oxylumazine and 150 g of sodium succinate·$6H_2O$ was inoculated with 2 liters of culture grown in the same medium for 20 hours. After 20 hours of growth aerobically the cells (8 g wet weight) were collected, washed with water, suspended in 40 ml of 0.05 M Tris buffer, *p*H 7.5, and disrupted in a sonic oscillator (Raytheon 10 KC) for 15 minutes at 4°. The mixture was centrifuged at 10,000 × *g* for 40 minutes. The supernatant was distributed in 5-ml aliquots and stored at −10°. Portions were thawed and used as required. This preparation did not catalyze the disappearance of 6,7-dioxylumazine when added in amount sufficient to catalyze the hydroxylation of 6-oxylumazine (Table VIII).

Preparation of Crude 6,7-Dioxylumazine "Isomerase."—Inorganic medium (15 liters) containing 1.5 g of 6,7-dioxylumazine and 150 g of sodium succinate·$6H_2O$ was inoculated with 2 liters of culture grown for 18 hours in inorganic medium supplemented with 200 mg of xanthopterin and 20 g of sodium succinate·$6H_2O$. After 20 hours of growth, the cells (10 g wet weight) were collected and suspended in 45 ml of 0.05 M Tris buffer, *p*H 7.5. An enzyme solution was prepared from these cells by sonic oscillation and centrifugation as described above. The solution was stored in the frozen state. This enzyme contained, in addition to "isomerase," deaminase and slight hydroxylase activity.

The Enzymatic Conversion of Xanthopterin to 6-Oxylumazine: The Spectrophotometric Method.—At *p*H 7.5, 6-oxylumazine has a molecular extinction coefficient greater than that of xanthopterin at 380 mμ and a molecular extinction coefficient less than that of xanthopterin at 275 mμ. These theoretical values for the conversion of xanthopterin to 6-oxylumazine are: $\Delta\epsilon$ 380 mμ = +1,653

TABLE VIII
THE ENZYMATIC OXIDATION OF 6-OXYLUMAZINE TO 6,7-DIOXYLUMAZINE

Wave Length (mμ)	Theoretical Values at pH 7.5 for Conversion of 6-Oxylumazine to 6,7-Dioxylumazine	Difference in Optical Density	6-Oxylumazine Which Disappeared (mμmoles)	6,7-Dioxylumazine Produced (mμmoles)
390	$\Delta\epsilon = -5,256$	−0.150	28.5	
345	$\Delta\epsilon = +9,378$	+0.270		28.8

and $\Delta\epsilon$ 275 mμ = −7,440. The deamination of xanthopterin was followed spectrophotometrically, with the decrease in optical density at 275 mμ representing the disappearance of xanthopterin and the increase in optical density at 380 mμ representing the appearance of 6-oxylumazine. To 86.6 mμmoles of xanthopterin in 1 ml of 0.05 M Tris buffer, pH 7.5, in a cuvet having a 1-cm light path, 1 μl of crude enzyme (25 μg of protein) was added, and the spectrum was recorded periodically (Fig. 2). The reference cuvet contained enzyme and buffer but no substrate. The results at the end of 2 hours (Table III) show that for each mole of xanthopterin which disappeared 0.92 mole of 6-oxylumazine was formed. The sum of xanthopterin and 6-oxylumazine, calculated from the optical density (0.412) at the isosbestic point (396 mμ) divided by the molecular extinction coefficient of 6-oxylumazine at this wave length (ε, 4,980) equals 82.7 mμmoles. Theory, 86.6.

Stoichiometry of the Enzymatic Deamination of Xanthopterin.—To a tube containing 540 mμmoles of xanthopterin (0.4 ml) and 0.1 ml of 0.05 M Tris buffer, pH 7.5, 0.5 ml of acetone powder extract (15 mg of protein) was added, and the contents were incubated at 21° for 1 hour. The reaction was stopped by adding 0.1 ml of 12% trichloroacetic acid solution. The supernatant solution was used for the analysis of NH_3, xanthopterin, and 6-oxylumazine. The control consisted of boiled enzyme, xanthopterin solution, and buffer in the same proportions. The ammonia in single 0.4 ml aliquots of experimental and control samples was determined. Excess K_2CO_3 solution was added to the aliquots, the aspirated NH_3 was trapped in dilute acid, and the ammonia was analyzed with Nessler's reagent. Aliquots (0.4 ml) of experimental and control samples were chromatographed on paper in propanol-water-NH_4OH. Two fluorescent bands appeared on the experimental chromatogram, a green band of R_F value 0.19 (xanthopterin) and a blue band of R_F value 0.24 (6-oxylumazine). Only the band of xanthopterin appeared on the control chromatogram. The substances eluted from the paper had ultraviolet absorption spectra characteristic of xanthopterin and 6-oxylumazine in 0.1 N NaOH and 0.1 N HCl. The amount of each was estimated spectrophotometrically. The results of two experiments are summarized in Table IV.

The Conversion of Isoxanthopterin to 7-Oxylumazine: The Spectrophotometric Method.—At pH 7.5, 7-oxylumazine has a molecular extinction coefficient greater than that of isoxanthopterin at 273 mμ and a molecular extinction coefficient less than that of isoxanthopterin at 249 mμ. These theoretical values for the conversion of isoxanthopterin to 7-oxylumazine are: $\Delta\epsilon$ 273 mμ = +4,050 and $\Delta\epsilon$ 249 mμ = −3,820. The deamination of isoxanthopterin was followed spectrophotometrically, the decrease in optical density at 249 mμ representing the disappearance of isoxanthopterin and the increase in optical density at 273 mμ representing the appearance of 7-oxylumazine. To 200 mμmoles of isoxanthopterin in 1 ml of 0.01 M Tris buffer, pH 7.5, in a cuvet at 20°, acetone powder extract (20 μl, 1.4 mg of protein) was added, and the spectrum was recorded periodically (Fig. 3). The reference cuvet contained buffer and enzyme in the same proportions. The results (Table V) show that for each mole of isoxanthopterin which disappeared 1 mole of 7-oxylumazine appeared. The presence of two isosbestic points (Fig. 3) throughout the 4-hour period suggests that a single product was formed. The sum of isoxanthopterin and 7-oxylumazine, calculated from the optical density (1.051 × 1.02) at the isosbestic point (261 mμ) divided by the molecular extinction coefficient of 7-oxylumazine at this wave length (ε, 5,630), equals 190 mμmoles. Theory, 200.

Stoichiometry of the Enzymatic Deamination of Isoxanthopterin.—Isoxanthopterin (2 μmoles) in 10 ml of 0.01 M Tris buffer, pH 7.5, was incubated at 21° with 0.2 ml of acetone powder extract (15 mg of protein) for 4 hours. The reaction was stopped by adding 1 ml of 12% trichloroacetic acid solution. The control tube consisted of enzyme and buffer, as above, similarly incubated. After addition of trichloroacetic acid to the control tube, isoxanthopterin solution (2 μmoles) was added, and the supernatant solutions from both the control and experimental tubes were analyzed for ammonia, isoxanthopterin, and 7-oxylumazine. Ammonia was determined as previously described in triplicate. Isoxanthopterin and 7-oxylumazine were estimated after chromatographic separation on paper in the propanol solvent (Table II). The chromatogram of the contents from the experimental tube showed one principal band of R_F 0.34 (7-oxylumazine, R_F 0.31) and a very weak band of R_F 0.21 (isoxan-

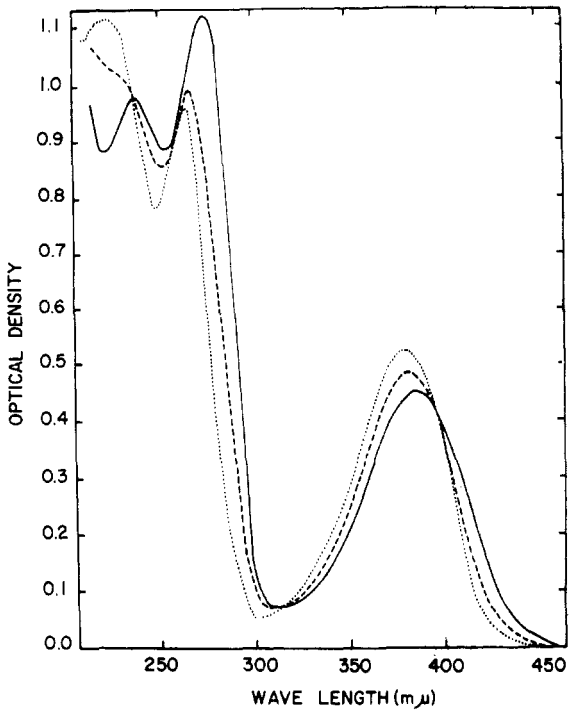


FIG. 2.—Spectrophotometric assay of the deamination of xanthopterin. The reaction was initiated by the addition of enzyme to a cuvet containing substrate and buffer at 23°. The spectra were recorded at zero time (xanthopterin) ———; after 1 hour – – – – –; and after 2 hours .....

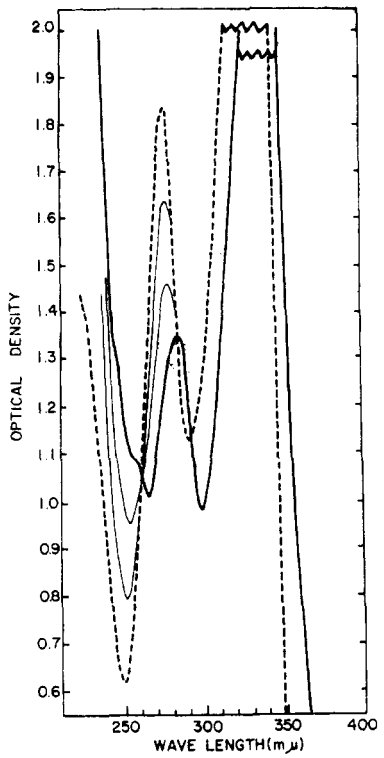


FIG. 3.—Spectrophotometric assay of the deamination of isoxanthopterin. The heavy dark line is the spectrum at zero time (isoxanthopterin). The lighter lines are the spectra taken at 80-minute intervals. The dashed line is the spectrum at the end of 4 hours (mostly 7-oxylumazine).

thopterin). The chromatogram of the contents from the control tube showed isoxanthopterin (R_F 0.22) and no 7-oxylumazine. The substances, eluted from the paper and corrected for the background absorption of the paper, showed spectra characteristic of isoxanthopterin and 7-oxylumazine in 0.1 N HCl and 0.1 N NaOH. The amounts present were thus determined. (The residual isoxanthopterin from the experimental tube was present in such small amount that a good spectrum of this sample was not obtained.) The results are shown in Table VI.

The Deamination of Adenine and Guanine.—The reaction mixtures consisted of 40 mμmoles of adenine or 38.8 mμmoles of guanine in 1 ml of 0.05 M Tris buffer, *p*H 7.5, containing 25 μg of protein (acetone powder extract). The deamination of adenine (Heppel *et al.*, 1957) and guanine (Roush and Norris, 1950) at 22° by this enzyme preparation are shown in Table VII.

The Oxidation of 6-Oxylumazine to 6,7-Dioxylumazine: The Spectrophotometric Method.—6-Oxylumazine (50.2 mμmoles; 0.2 ml) was added to a cuvet containing 0.6 ml of 0.05 M Tris buffer, *p*H 7.5, and 0.2 ml of hydroxylating enzyme (0.72 mg of protein). The reference cuvet contained the same amount of enzyme and buffer in 1 ml. The spectral changes associated with this reaction are shown in Figure 4. The changes in optical density at the end of 2 hours' incubation at 23° (Table VIII) show that for each mole of 6-oxylumazine which disappeared (as measured by the decrease in optical density at 390 mμ) 1 mole of 6,7-dioxylumazine was formed (as measured by the increase in optical density at 345 mμ).

The presence of an isosbestic point at 368 mμ (Fig. 4) during the 2-hour period suggests that a single product was formed. The sum of 6-oxylumazine plus 6,7-dioxylumazine, calculated from the density (0.299) at 368 mμ divided by the molecular extinction coefficient of 6,7-dioxylumazine at this wave length (ε, 5,880), equals 50.8 mμmoles. Theory, 50.2.

Proof that the Product of Enzymatic Oxidation of 6-Oxylumazine is 6,7-Dioxylumazine.—6-Oxylumazine (50.2 mμmoles) buffer and enzyme as described above were incubated at 22° for 2 hours. The control tube differed from the experimental tube only in having boiled enzyme rather than active enzyme. Both mixtures were chromatographed on paper in the propanol solvent (Table II). Two fluorescent bands were present on the experimental chromatogram: 6-oxylumazine (R_F 0.24) and 6,7-dioxylumazine (R_F 0.05). The control chromatogram had 6-dioxylumazine only.

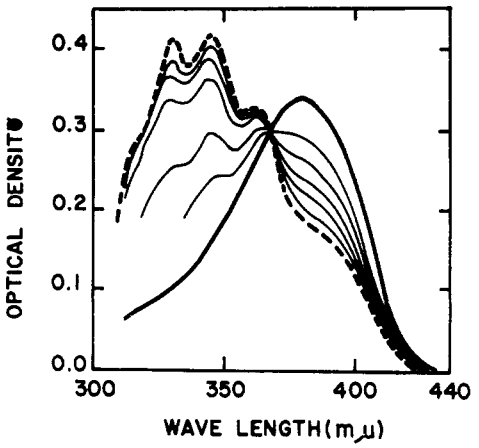


FIG. 4.—The enzymatic oxidation of 6-oxylumazine to 6,7-dioxylumazine. The reaction was initiated by the addition of 6-oxylumazine to a cuvet containing enzyme and buffer. The heavy dark line is the spectrum at zero time (6-oxylumazine). The lighter lines are the spectra at intervals of 20 minutes. The dashed line is the spectrum after 2 hours.

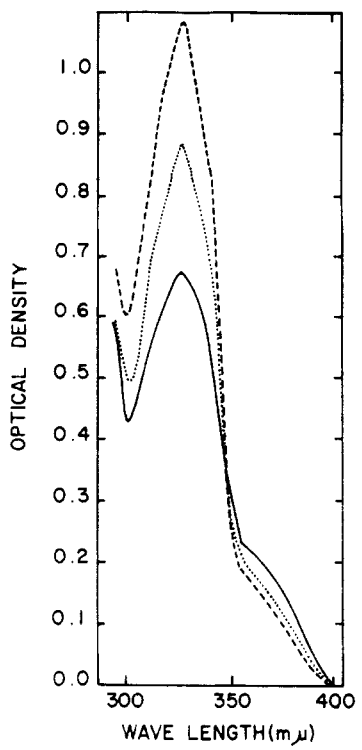


FIG. 5.—The enzymatic oxidation of lumazine to 7-oxylumazine. The spectrum at zero time (lumazine) ———; after 1 hour . . . . . ; and after 2 hours – – – – – –.

The compound in the lower band was eluted and showed the ultraviolet absorption spectra of 6,7-dioxylumazine in 0.1 N HCl and in 0.1 N and in 0.1 N NaOH. The substance was rechromatographed on paper in 5% acetic acid solution and had an R_F value of 0.11; 6,7-dioxylumazine, 0.10.

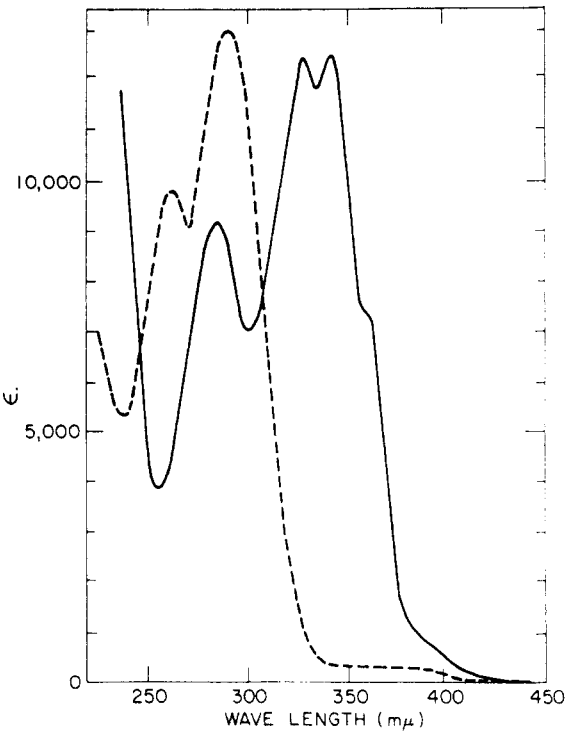


FIG. 6.—The enzymatic conversion of 6,7-dioxylumazine to an unidentified compound. The heavy line is the spectrum at zero time (6,7-dioxylumazine). The dashed line is the spectrum of an unknown compound after 14 hours.

The Oxidation of Lumazine to 7-Oxylumazine.—Lumazine (114 mμmoles; 0.4 ml) was added to a cuvet containing 0.5 ml of 0.05 M Tris buffer, *p*H 7.5, and 0.1 ml of hydroxylating enzyme (0.38 mg of protein) was added. The reference cuvet contained the same quantities of enzyme and buffer in 1 ml. The spectral changes associated with the oxidation of lumazine to 7-oxylumazine are seen in Figure 5. The results at the end of 2 hours (Table IX) show that the changes in the optical density at 300 mμ and 327 mμ are in agreement with the theoretical differences in molecular extinction coefficients of lumazine and 7-oxylumazine at these two wave lengths.

Proof that the Product of Oxidation of Lumazine is 7-Oxylumazine.—Lumazine (114 mμmoles), buffer, and enzyme, as above, were incubated at 21° for 4 hours. The control tube differed from the experimental tube in that it had boiled enzyme. Both the experimental and control incubation mixtures were chromatographed on paper in the propanol solvent (Table II). The experimental chromatogram had two fluorescent bands: Lumazine (R_F 0.49) and 7-oxylumazine (R_F 0.34). Only lumazine appeared on the control chromatogram. The compound was eluted from the lower band and rechromatographed in the butanol solvent (Table II), R_F 0.32. Au-

TABLE IX
THE ENZYMATIC CONVERSION OF LUMAZINE TO 7-OXYLUMAZINE

Wave Length (mμ)	Theoretical Values at *p*H 7.5 for Conversion of Lumazine to 7-Oxylumazine	Ratio $\frac{\Delta\epsilon \text{ 327 m}\mu}{\Delta\epsilon \text{ 300 m}\mu}$	Difference in Optical Density	Ratio $\frac{\Delta \text{ OD at 327 m}\mu}{\Delta \text{ OD at 300 m}\mu}$
327	$\Delta\epsilon = +8,500$	2.24	+0.407	2.39
300	$\Delta\epsilon = +3,800$		+0.170	

thentic 7-oxylumazine, R_F 0.31. Authentic 7-oxylumazine did not serve as substrate in this system. No spectral changes were observed even after 6 hours of incubation, and chromatograms of these incubation mixtures did not show the appearance of any other fluorescent or ultraviolet absorbing compound.

The Enzymatic Conversion of 6,7-Dioxylumazine to an Unknown Compound.—To 17.3 μg of 6,7-dioxylumazine in 1 ml of 0.05 M Tris buffer, *p*H 7.5, at 21°, 20 μl of crude "isomerase" enzyme (141 μg of protein) was added. The reference cuvet contained equal amounts of buffer and enzyme. The spectral changes over a 14-hour period are shown in Figure 6. Two isosbestic points (247 mμ and 308 mμ) suggest that a single product was produced, having ultraviolet absorption maxima at 263 mμ and 292 mμ (Fig. 6). The optical density of the unknown compound at 292 mμ at the completion of the reaction was 1.11, and the optical density of 6,7-dioxylumazine at 345 mμ at zero time was 1.06. This fraction (1.11/1.06) multiplied by the molecular extinction coefficient of 6,7-dioxylumazine at 345 mμ (ε, 12,400) gives the value; ε 292 mμ, 12,980 for the unknown compound at *p*H 7.5. At 263 mμ the optical density was 0.833, hence the value of ε 263 mμ was 9,740.

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