[CONTRIBUTION FROM THE KERCKHOFF LABORATORIES OF BIOLOGY, CALIFORNIA INSTITUTE OF TECHNOLOGY]

The Incorporation of the C^{14} of Adenine into *a* Pteridine Derivative by *Eremothecium ashbyii*[1]

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The yeast *Eremothecium ashbyii* incorporates the pyrimidine ring of adenine into the pyrimidine-portion of the isoalloxazine ring of riboflavin. In the search for intermediates between adenine and riboflavin a substance (compound A) showing a purple fluorescence in ultraviolet light has been isolated from cultures of this organism. Riboflavin and compound A, isolated from the same cultures which were fed C^{14}-adenine, had very similar molar specific activities, a fact which suggests a close biochemical relationship between these two substances. Though attempts have been made to show that compound A serves as an intermediate in the biogenesis of riboflavin we have been unable to obtain any evidence in support of this view. The most which may be made of the evidence presented here is that riboflavin and compound A arise from a common biochemical intermediate.

Introduction

Adenine has been shown to serve as a precursor of riboflavin, in the metabolism of the yeast *Eremothecium ashbyii*, through the contribution of an intact pyrimidine ring to the isoalloxazine ring of riboflavin.[2] As no intermediate steps between adenine and riboflavin are known, we have investigated cultures of this yeast, grown in the presence of C^{14}-adenine, for compounds other than riboflavin which are labeled to a comparable extent. A compound which shows a purple fluorescence in ultraviolet (compound A) has been isolated from cultures of this organism. Though present in an amount much smaller than that of the riboflavin, the compound has a molar specific activity almost identical to that of the riboflavin. The compound appears to bear a formal relationship to riboflavin in that it seems to have a 2,4-dihydroxypteridine structure instead of the 2-amino-4-hydroxypteridine structure so characteristic of naturally occurring pteridines. Yet we have been unable to prove by direct experiment that the compound is an intermediate in the biogenesis of riboflavin.

That this pteridine derivative attains a high specific activity following the administration of C^{14}-adenine is itself an interesting fact, for such extensive labeling of the molecule suggests a rather direct utilization of the purine in the biogenesis of pteridines.

In this article certain experiments having to do with the biogenesis of this substance are described. The isolation of the substance in crystalline form, its physical properties and the chemical evidence in support of a structure having a 2,4-dihydroxypteridine configuration are reported in a separate article.[3]

Experimental Part

Materials.—Randomly labeled C^{14}-adenine·$3H_2O$ (0.82 μc. per mg.) was a Schwarz product.[4] Authentic compound A was isolated as described elsewhere[3]; m.p. 256–258°; neut. equiv., 348; $[\alpha]^{25}D$ +8.7 ± 0.5° (2.8% solution in 0.1 *N* NaOH; *l* = 1 dcm.).

Procedures and Results.—Radioactive counting was carried out as reported earlier.[2] Cultures of *E. ashbyii* were grown in a medium which differed from that previously reported[5] in having twice the amount of phosphate buffer. To the two day-old cultures the C^{14}-labeled compound to be tested was added in 1–2 ml. of sterile solution per 20 ml. of culture, and the incubation was continued for five more days.

Incorporation of C^{14} of Adenine into the Pteridine Derivative and into Riboflavin.—A total of 5.1 mg. of C^{14}-adenine (1,530,000 c.p.m.) was added to four cultures of the yeast (20 ml. of medium per 250 ml. flask). After incubation the mycelia from the pooled cultures were separated and washed once with water. Forty ml. of 4% acetic acid solution was added to the mycelia, and the mixture was permitted to stand in the refrigerator for two hours. Upon filtering off the mycelia and washing twice with cold water the residue was practically devoid of fluorescence. It was discarded. The yellow solution was passed through a "Filtrol" column (2 × 15 cm.) after which the column was washed with 175 ml. of 2% acetic acid solution, discarding the filtrate and washings. The pteridine derivative was eluted with 30% acetone–water (75 ml.). It passed down the column as a purple fluorescent band, in ultraviolet light, just above the solvent front. The riboflavin was eluted subsequently with 30% acetone–water (400 ml.). The riboflavin, obtained in crystalline form following chromatography on paper,[2] was taken up in hot water (6 ml.); 0.24 ml. of this solution contained by spectrophotometric analysis (0.1 *N* HCl): 47.1 μg. at 223 mμ; 45.6 μg. at 267 mμ; 46.7 μg. at 375 mμ; and 46.6 μg. at 445 mμ—an average of 46.5 μg. It had an average count of 3,490 c.p.m. or 282 c.p.m. per 10^{-8} mole of riboflavin.

The solution of compound A from the column, contaminated with riboflavin phosphate and flavin adenine dinucleotide, was purified by paper chromatography in the butanol–acetic acid–water system of Whitby[6]; R_f 0.27 and in (*n*-propyl alcohol, 80 ml.; water, 40 ml.; concd. NH_4OH, 2 ml.) R_f 0.44. The resulting band was eluted with water as was also a corresponding area of paper from the chromatogram. Each solution was reduced to 2 ml. For spectrophotometric analysis an aliquot of eluate from the blank paper was placed in the reference cuvette, while a like aliquot of the eluate containing compound A was placed in the experimental cuvette. The solution in 0.1 *N* HCl showed the maxima and minima characteristic of the authentic compound (3). One and one-half ml. showed 61.7 μg. at 282 mμ and 60.3 μg. at 326 mμ, an average of 61.0 μg. It had an average count of 4,830 c.p.m. or 273 c.p.m. per 10^{-8} mole (mol. wt. = 344). The specific activities of the riboflavin and compound A in the culture filtrate were similarly determined. The results (Table I) show that the two substances were labeled to an almost identical extent.

A similar experiment was carried out in which C^{14}-guanosine (labeled equally in the purine and in the ribosyl group) was administered. The resulting riboflavin was degraded and found to have essentially no C^{14} in the ribityl group of riboflavin (that is, more than 90% of the C^{14} of the riboflavin was restricted to the dimethyl isoalloxazine portion of the molecule). When a comparison was made of the specific activities of the riboflavin and compound A from the

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TABLE I

INCORPORATION OF C^{14}-ADENINE INTO RIBOFLAVIN AND COMPOUND A

Specific activity of the administered adenine	Counts per minute per 10^{-8} mole: Specific activity of the riboflavin		Specificity activity of compd. A	
	Filtrate	Mycelium	Filtrate[a]	Mycelium[a]
567	243	282	256	273

[a] Though not isolated quantitatively the amount of compound A obtained in each case was: from the filtrate, 80.6 μg.; from the mycelium, 81.3 μg.

mycelia of these cultures, it was found that the agreement was again within the experimental error of the method. In view of the very similar labeling of the two substances it seems reasonable to suppose that both arise from a common intermediate.

Is the Pteridine Derivative an Actual Intermediate in the Biogenesis of Riboflavin?—The direct experiment of administering the C^{14}-pteridine derivative to a culture of the yeast and determining the incorporation of C^{14} into riboflavin was next carried out. C^{14}-compound A (56.3 μg.; 4,470 c.p.m., 273 c.p.m. per 10^{-8} mole) was added to a 20-ml. culture. At the end of the incubation period the total amount of riboflavin in the culture was isolated by chromatography on "Filtrol" and on paper. The riboflavin band was eluted from the paper and the total radioactivity in the eluate was determined. Found: 32 c.p.m. Therefore, only 0.7% of the radioactivity of the pteridine derivative was recovered in the riboflavin—a value much smaller than that found for the incorporation of radioactive adenine into riboflavin.[5] Hence, there is no evidence from direct experiment that the pteridine derivative is a precursor of riboflavin.

Does the Pteridine Derivative Arise from the Biochemical Degradation of Riboflavin?—3.15 mg. (204,000 c.p.m.) of C^{14}-riboflavin (243 c.p.m. per 10^{-8} mole) was added to 80 ml. of culture (4 cultures). At the end of the incubation period the mycelia from the pooled cultures were filtered off, washed and sucked dry. The riboflavin and compound A were isolated and their specific activities were determined. The results (Table II) show that no significant activity is found in the pteridine derivative. Hence, it may be concluded that the pteridine derivative does not arise from riboflavin.

TABLE II

EVIDENCE THAT COMPOUND A DOES NOT ARISE FROM RIBOFLAVIN

Amount of riboflavin present (mg.)		Specific activity of the riboflavin c.p.m./10^{-8} mole		Amount of compd. A present (μg.)		Specific activity of compd. A c.p.m./10^{-8} mole	
Filtrate	Mycelium	Filtrate	Mycelium	Filtrate	Mycelium	Filtrate	Mycelium
5.69	0.66	121	8.8	35.4	9.1	0.3	0.1

The Inability of the Pteridine Derivative to Support the Growth of Certain Riboflavin Requiring Mutants of *Neurospora* and *Aspergillus*.—The pteridine derivative was tested for its ability to support the growth of certain riboflavin requiring mutants. Three of them were mutants of *Neurospora crassa:* Y-30539 (from Dr. E. L. Tatum); 76R5 (from Dr. Robert L. Metzenberg); 107 (isolated by us from ultraviolet irradiated conidia, using the method of Woodward, DeZeeuw and Srb.[7] A mutant of *Aspergillus nidulans* was given to us by Dr. A. Roper. The first two mutants require about 0.75 μg. of riboflavin per ml. for $^1/_2$ maximum growth, while the latter two require about a third this concentration. All of these organisms were grown in the usual minimal medium for the culture of *Neurospora*.[8]

None of the mutants responded, even at levels of the compound thirty times that at which riboflavin produces $^1/_2$ maximum growth.

Discussion

Several considerations led us to suppose that this pteridine derivative is involved in the biogenesis of riboflavin by *E. ashbyii*. It differs from the naturally occurring pteridines in having not the 2-amino-4-hydroxy configuration but probably the 2,4-dihydroxy configuration characteristic of riboflavin. Also, C^{14}-adenine when fed, gives rise to extensive labeling of the riboflavin, and the pteridine derivative becomes labeled as well to an equal extent. Inasmuch as we have been unable to show by direct feeding of the C^{14}-pteridine derivative to the yeast or by feeding the substance to certain riboflavin-requiring mutants that it functions as an intermediate in the biogenesis of riboflavin, the evidence permits only the supposition that both this substance and the riboflavin originate from the same intermediate. As it has been shown that C^{14}-adenine gives rise in this yeast to C^{14}-riboflavin which is labeled specifically in the four carbon atoms constituting the pyrimidine portion of the riboflavin ring system[2] it seems likely that this substance is correspondingly labeled in the pyrimidine ring of the pteridine. A decision on this point cannot be made until the structure of the compound is established and its chemical degradation has been worked out.

The inference has been drawn by various investigators that purines, pteridines and riboflavin are biochemically related. Koschara and Haug found a parallel relationship in the amount of riboflavin and xanthopterin present in the tissues and organs of various animals and assumed that the two substances are functionally dependent.[9] In the metabolism of the yeast, *E. ashbyii*, we have found, also, that under conditions such that riboflavin production is poor the amount of the pteridine derivative is much reduced. The amount of riboflavin has been shown to decrease in the latter stages of development of the grasshopper egg as the amount of pteridine increased. This change was attributed to the biochemical conversion of riboflavin into pteridines.[10,11] (That the pteridine derivative in *E. ashbyii* does not arise from riboflavin was proved by the use of C^{14}-riboflavin.) Also, Kikkawa has concluded from studies upon the genetics of the silk worm that a close biochemical relationship exists between uric acid, riboflavin and pteridines,[12] while Ziegler-Günder, *et al.*,[13] find that labeling appears in a pteridine following the injection of guanine-2-C^{14} into larvae of the amphibian *Xenopus laevis*. Certain purines may be converted to pteridines also by simple chemical means.[14]

Though the findings reported here are disappointing insofar as they relate to the biogenesis of riboflavin, there are perhaps other products of adenine metabolism in cultures of this organism which may be implicated as intermediates in the biosynthesis of riboflavin. A systematic search for such intermediates is under way.

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