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Biosynthesis of the Dimethylbenzene Moiety of Riboflavin and Dimethylbenzimidazole: Evidence for the Involvement of C-1 of a Pentose as a Precursor†

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ABSTRACT: The relative incorporations of specifically labeled pyruvate, lactate, erythritol, D-erythrose, D-ribose, and D-glucose precursors into the dimethylbenzene carbon atoms of the 5,6-dimethylbenzimidazole unit of vitamin B_{12} by *Propionibacterium shermanii* have been determined. The incorporation data provide information regarding the putative four-carbon biosynthetic unit which is involved in the formation of 6,7-dimethyl-8-ribityllumazine and which is the source of the eight dimethylbenzene carbon atoms of both 5,6-dimethylbenzimidazole and riboflavin. The relative incorporations of the labeled lactate and pyruvate precursors are not consistent with either acetoin or 2,3-butanedione functioning as the four-carbon biosynthetic unit. The relative incorporations of the labeled hexose, pentose, and tetrose precursors indicate that the observed incorporation of C-1 of the pentose into the dimethylbenzene carbon atoms does not involve metabolism to a tetrose intermediate, but occurs more directly. It is concluded that the C-1 position of a pentose precursor is involved in the formation of the putative four-carbon biosynthetic unit.

The biosynthesis of riboflavin begins with guanosine triphosphate and proceeds via a series of pyrimidine intermediates (II–IV) to 6,7-dimethyl-8-ribityllumazine (V). Riboflavin synthetase then catalyzes a condensation between two molecules of 6,7-dimethyl-8-ribityllumazine (V) to yield riboflavin and to regenerate a molecule of the 4-ribitylamino-5-amino-2,6-dihydroxypyrimidine intermediate (IV). The biosynthetic pathway in Figure 1 incorporates the recent finding of Mailänder and Bacher (1976) that in *Salmonella typhimurium* riboflavin biosynthesis begins with GTP and that the ribityl side chain of riboflavin is derived from the ribose unit of the guanosine nucleotide. Additional evidence supporting this pathway has been summarized in reviews by Demain (1972) and by Plaut et al. (1974). It is to be emphasized that the eight carbons of the dimethylbenzene moiety of riboflavin are derived from a putative four-carbon precursor that is added to IV to yield V. In the riboflavin synthetase reaction, one lumazine molecule serves as a "donor" of this four-carbon unit and is converted into another molecule of IV, while the other lumazine molecule serves as an "acceptor" for the four-carbon unit and is converted into a molecule of riboflavin. Many details of this riboflavin synthetase reaction have been elucidated, including the fact that the methyl groups attached to C-6 in V ultimately become C-6 and the methyl group attached to C-8 in the riboflavin product, as outlined (Plaut et al., 1974, and references therein).

Investigations carried out in our laboratory (Alworth et al., 1969, 1971; Lu and Alworth, 1972) and those of Renz (Renz and Reinhold, 1967; Renz, 1970; Kühnle and Renz, 1971; Renz and Weyhenmeyer, 1972) have established a biosynthetic relationship between riboflavin and the 5,6-dimethylbenzimidazole (DBI)[1] component of vitamin B_{12}. Indeed, evidence suggests that riboflavin is an obligatory intermediate in the biosynthesis of DBI (Renz, 1970; Renz and Weyhenmeyer, 1972; see also discussion in Plaut et al., 1974). The biosynthetic relationship between riboflavin and DBI permits us to interpret results obtained from investigations of DBI biosynthesis in terms of the biosynthesis of the related groups of riboflavin. In particular, the incorporation of various precursors into the dimethylbenzene moiety of DBI can be interpreted in terms of their potential to serve as biosynthetic precursors of the four-carbon unit involved in the formation of V from IV. In this paper evidence is presented which leads us to conclude that this biological four-carbon unit is derived via reactions that involve the C-1 carbon atom of a pentose precursor.

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[1] Abbreviation used is DBI, 5,6-dimethylbenzimidazole.

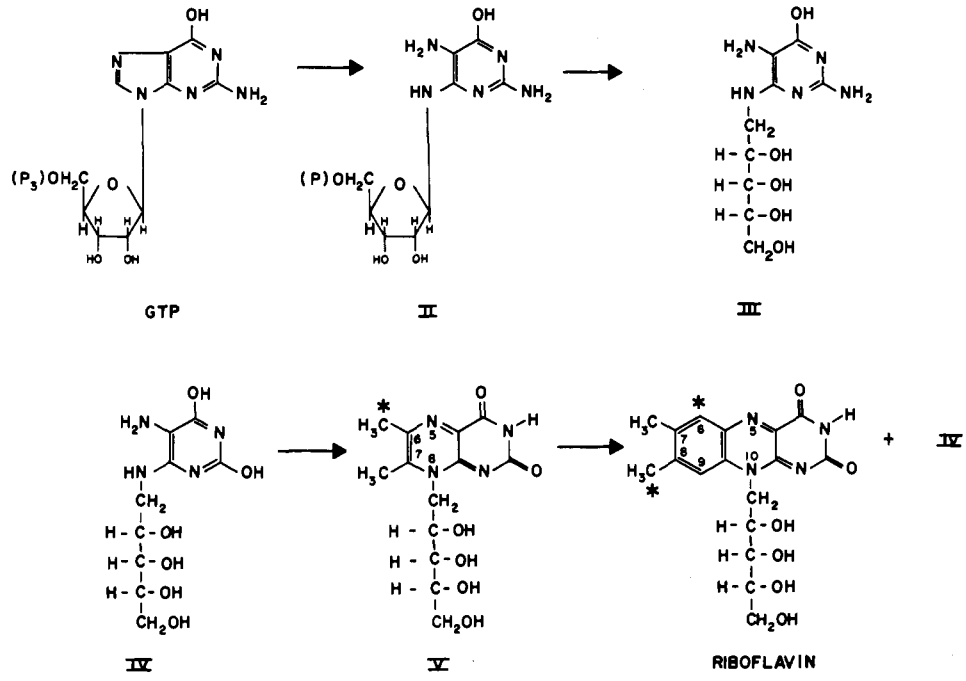

FIGURE 1: Riboflavin biosynthesis pathway.

Materials and Methods

Melting points were determined on a calibrated Hoover capillary melting point apparatus. Elemental analyses were performed by Sprang Microanalytical Laboratory, Ann Arbor, Mich. The specific activities were determined by liquid scintillation counting in a Beckman Series 200 instrument using external standardization. Radiochromatograms were analyzed on a Packard Model 7201 radiochromatogram scanner.

[6-^{14}C]-4,6-O-Ethylidene-D-glucose was prepared by the method of Hockett et al. (1951) as modified by Barker and MacDonald (1960) from [6-^{14}C]-D-glucose (New England Nuclear). A 60% yield of white crystalline solid, mp 176–181 °C (lit. 179–181 °C), was obtained. Anal. Calcd for $C_8H_{14}O_6$: C, 49.31; H, 6.90; O, 43.79. Found: C, 49.11; H, 6.92; O, 43.51.

[4-^{14}C]-2,4-O-Ethylideneerythrose Benzylamine Schiff's Base. The [4-^{14}C]-2,4-*O*-ethylideneerythrose was prepared and isolated as the crystalline benzylamine Schiff's base derivative according to the procedure of Ziderman and Dimant (1966). The Schiff's base was recrystallized from petroleum ether (40–60 °C) and the white, needle-like crystals, mp 88–91 °C (lit. 88–91 °C), obtained in 52% yield from 4,6-*O*-ethylidene-D-glucose, were stored in a vacuum desiccator over P_2O_5 at 0 °C. Anal. Calcd for $C_{13}H_{17}O_3$: C, 66.36, H, 7.28; N, 5.95. Found: C, 66.32; H, 7.30; N, 6.09. The specific activity of the recrystallized Schiff's base was 4.88×10^7 dpm/mmol (0.0220 Ci/mol).

[4-^{14}C]-D-Erythrose. The Schiff's base was first hydrolyzed under mild conditions with dilute H_2SO_4, the benzylamine removed with a weakly acidic ion exchange resin, and the acetal then hydrolyzed in dilute acid at 60 °C for 24 h as described by Ballou (1960). The solution containing the hydrolysis product was then deionized with ion-exchange resins according to the procedure of Rappoport and Hassid (1951) and lyophilized to dryness. The [4-^{14}C]-D-erythrose product was a colorless syrup (71% yield) which was chemically and radiochemically pure as determined by paper chromatography (propanol–ethyl acetate–water; 7:1:2) and radiochromatographic scanning.

[4-^{14}C]Erythritol. The mother liquors from recrystallizations of the benzylamine Schiff's base of [4-^{14}C]-2,4-*O*-ethylidene-D-erythrose were evaporated to dryness and the Schiff's base and the ethylidene group hydrolyzed as described above. Lyophilization of the solution of [4-^{14}C]-D-erythrose yielded a light amber syrup which was dissolved in water and reduced with potassium borohydride. After 4 h, the excess borohydride was destroyed with cation exchange resin (H^+ form), most of the water was removed by lyophilization, and then three 150-ml portions of absolute methanol were added successively and evaporated in vacuo. The resulting [4-^{14}C] erythritol product was crystallized from absolute ethanol–pentane, diluted with nonradioactive erythritol, and recrystallized three times from absolute ethanol. White crystals, mp 118–120 °C (lit. 121.5 °C) were obtained with a specific activity of 3.91 mCi/mol.

Growth of Cultures. Stock cultures of *Propionibacterium shermanii* ATCC 13673 were maintained at 4 °C in agar slabs prepared by adding 2% agar to a sodium lactate medium [1% yeast extract, 1% sodium L-lactate, 0.15% ammonium sulfate, 0.1% 1 M magnesium sulfate, and 2% 1 M potassium phosphate, pH 7.1]. For each experiment stock cultures were used to inoculate 5 ml of lactate medium and the cells cultured serially for successive 5 day periods at 24 °C without agitation in 5, 50, and 500 ml of the lactate medium.

The experiments reported in Table I were carried out in two 12-l. flasks, each containing 5 l. of a rich growth medium (Bernhauer et al. 1959, modified by Friedmann and Harris, 1962). The flasks were inoculated with 500 ml of the lactate-grown culture and incubated at 28 °C. During the first 4 days of growth, a slow stream of nitrogen was bubbled through the medium; during the last 4 days of growth, air was bubbled through the medium. The gases were sterilized by passage through a Millipore filter. The pH of the cultures was adjusted to 7.0 daily by the addition of 50% NH_4OH solution. When the cultures ceased producing acid additional 50% glucose solution (100 ml/day) was added. Foaming was controlled by the manual addition of Antifoam B Emulsion (Sigma). The

TABLE I: Incorporation of Labeled Precursors into the Dimethylbenzene Unit of Vitamin B_{12}.

Precursor[a]	Amount Added (mmol)	Total B_{12} Act. in DBI (%)	Incorp of Spec Act. into DBI (%)[b]	Total DBI Act. in Dimethylbenzene Unit (%)[c]	Incorp. of Spec Act. into Dimethylbenzene Unit (%)[d]
Sodium [1-^{14}C]-DL-lactate, 5.36 Ci/mol	0.093	30	0.0016	96	0.0015
Sodium [1-^{14}C]pyruvate, 3.16 Ci/mol	0.048	22	0.0014	82	0.0011
Sodium [2-^{14}C]pyruvate, 3.26 Ci/mol	0.046	31	0.002	91	0.0019
[1-^{14}C]-D-Glucose, 5.15 Ci/mol	0.02	30	0.006	76	0.005
[1-^{14}C]-D-Ribose, 50.04 Ci/mol	0.003	36	0.003	60	0.002
[U-^{14}C]Erythritol, 2.26 Ci/mol	0.04	62	0.02	94	0.02

[a] The labeled erythritol was purchased from Nuclear Chicago; all other precursors were purchased from New England Nuclear. [b] Specific activity of DBI ÷ specific activity of precursor × 100. [c] (DBI activity less C-2 label) ÷ DBI activity × 100. [d] Specific activity of dimethylbenzene ÷ specific activity of precursor × 100.

TABLE II: Incorporation of Specifically Labeled Tetrose, Pentose, and Hexose Precursors into the Dimethylbenzene Unit of Vitamin B_{12}.

Precursor[a]	Amount Added (mmol)	Total B_{12} Act. in DBI (%)	Incorp of Spec Act. into DBI (%)[b]	Total DBI Act. in Dimethylbenzene Unit (%)[c]	Incorp of Spec Act. into Dimethylbenzene Unit (%)[d]
[1-^{14}C]-D-Ribose, 15.5 mCi/mol	6.46	56	1.74	53	0.92
[4-^{14}C]-D-Erythrose, 22 mCi/mol	6.43	22	0.35	100	0.35
[4-^{14}C]Erythritol, 3.91 mCi/mol	5.92	48	0.62	64	0.40
[1-^{14}C]-D-Glucose, 32 mCi/mol	6.44	22	0.55	61	0.34

[a] The labeled erythrose and erythritol samples were prepared from [6-^{14}C]-D-glucose purchased from New England Nuclear (see Materials and Methods); the ribose and glucose precursors were purchased from ICN. [b] Specific activity of DBI ÷ specific activity of precursor × 100. [c] (DBI activity less C-2 label) ÷ DBI activity × 100. [d] Specific activity of dimethylbenzene ÷ specific activity of precursor × 100.

radioactive precursors listed in Table I were added to the incubations after the 4 days of anaerobic growth. After 4 days of aerobic growth in the presence of the labeled compounds, the cells were harvested by centrifugation.

The experiments reported in Table II were carried out under similar conditions but in a Virtis Model 40-112 fermenter with the pH of the incubation maintained at 7.0 by the automatic addition of 50% NH_4OH and foaming controlled by automatic addition of Antifoam B Emulsion. Six liters of the modified Bernhauer medium was inoculated for each experiment reported in Table II. The growth conditions for each experiment in Table II were duplicated as closely as possible. The first addition of the 50% glucose solution was made 24 h after inoculation and subsequent additions were made at 12-h intervals. During the 100 h of the anaerobic growth phase, a total of 475 ml of 50% glucose solution was added. The ^{14}C-labeled precursors were added after 100 h of anaerobic growth and aeration was begun. No glucose was added for a period of 6 h prior to the addition of the labeled precursors; 6 h after addition of the labeled precursors addition of the glucose solution was resumed and continued at the rate of 100 ml three times a day. A total of 1 l. of 50% glucose solution was added during the aerobic growth phase. One hundred hours after addition of the labeled precursors, the cells were harvested.

Isolation and Degradation Procedures. Isolation of vitamin B_{12} was carried out according to methods previously reported (Alworth and Baker, 1968; Alworth et al., 1969). The ^{14}C-labeled DBI was obtained by hydrolysis of the vitamin B_{12} (Brink and Folkers, 1950) and diluted with unlabeled DBI in order to accurately determine the specific activity. The diluted, recrystallized ^{14}C-labeled DBI was subjected to degradation with benzoyl chloride in KOH to determine the distribution of the label between the dimethylbenzene carbon atoms and the C-2 carbon. The carbon-by-carbon degradation procedure used to determine the labeling pattern in the ^{14}C-labeled DBI isolated after feeding [1-^{14}C]-D-ribose has been previously described (Alworth et al., 1970).

Results

The results of a series of experiments which tested the relative effectiveness of [1-^{14}C]pyruvate, [2-^{14}C]pyruvate, [1-^{14}C]-D-glucose, [6-^{14}C]-D-glucose, [1-^{14}C]-DL-lactate, [1-^{14}C]-D-ribose, and [U-^{14}C]erythritol as precursors of the DBI unit of vitamin B_{12} are summarized in Table I. The results of a second series of experiments designed to test the relative effectiveness of specifically labeled hexose, pentose, and tetrose molecules as potential precursors of the dimethylbenzene moiety of DBI are summarized in Table II. Unlabeled glucose was supplied to the cultures as a carbon and energy source during the course of both sets of experiments (see experimental details); the values in Tables I and II thus represent the incorporation of labeled precursors into DBI in the presence of significant amounts of unlabeled glucose and glucose metabolites.

The labeling pattern in the DBI portion of vitamin B_{12} resulting from the incorporation of [1-^{14}C]-D-ribose (experiment from Table I) as determined by a carbon-by-carbon degradation is summarized in Figure 2.

Discussion

Acetoin or its oxidation product 2,3-butanedione has been frequently proposed as the four-carbon biosynthetic unit involved in the formation of 6,7-dimethyl-8-ribityllumazine (V) from IV. (See, for example, Weimar and Neims, 1975.) Goodwin and Treble (1958) have described the specific incorporation of [2-^{14}C]acetylmethylcarbinol (methyl-labeled

acetoin) into the dimethylbenzene ring of riboflavin by *Eremothecium ashbyii*. These workers also reported that the acetoin was more efficiently incorporated into ring A of riboflavin than was [2-^{14}C]acetate. Bryn and Størmer (1976) have recently found that *Aerobacter aerogenes* mutants deficient in the ability to form acetoin and 2,3-butanedione also exhibit decreased riboflavin production relative to wild type cultures. Using cell-free extracts of *E. ashbyii*, Kishi et al. (1959) and Katagiri et al. (1959) detected conversion of IV to V when incubations were carried out in the presence of acetoin or pyruvate and thiamine pyrophosphate, respectively. The reported conversions, however, were slight (1.4%, 2.5%), and Goodwin and Horton (1961) were unable to detect these conversions in cell-free extracts of *Candida flaveri*.

Although acetoin does not condense with IV to form V at 37 °C under neutral conditions, the condensation of 2,3-butanedione with IV to yield V proceeds smoothly under these conditions (Kishi et al., 1959). This observation makes the observations of Goodwin and Treble (1958), Kishi et al. (1959), and Katagiri et al. (1959) ambiguous. Since V is an established riboflavin precursor and as IV is known to be present as a normal intermediate (and product) of the riboflavin biosynthetic pathway, the observed incorporation of acetoin or 2,3-butanedione into riboflavin by these workers is not definitive proof that either of these four-carbon compounds functions as the true in vivo four-carbon precursor. If a modest amount of the acetoin added to cell cultures or to cell-free incubations were to be oxidized to 2,3-butanedione, V could be produced from IV nonenzymatically. For example, Lingens et al. (1967) found that two riboflavin deficient mutants of *Saccharomyces cerevisiae*, that were unable to synthesize V and that thus accumulated IV, were able to grow and to produce large amounts of riboflavin if they were cultured in a medium supplemented with 2,3-butanedione.

Ali and Al-Khalidi (1966) examined the incorporation of a series of compounds into the dimethylbenzene ring of riboflavin by *E. ashbyii* cultures. Based upon the observation that labeled acetoin was an inefficient precursor of riboflavin and upon the relative efficiencies of labeled acetate and labeled pyruvate as precursors of acetoin and of riboflavin, these workers concluded that acetoin was not the precursor of the four-carbon unit which ultimately forms the dimethylbenzene moiety of riboflavin.

The results of the experiments that are summarized in Table I also lead to the conclusion that neither acetoin nor 2,3-butanedione functions as the precursor of the four-carbon biosynthetic unit involved in the formation of the dimethylbenzene unit of riboflavin and of DBI. All known pathways for acetoin and 2,3-butanedione formation (Juni, 1952a, 1952b; Juni and Heym, 1955; Speckman and Collins, 1968; Chuang and Collins, 1968) involve the loss of the carboxyl group (C-1) of a pyruvate precursor.[2] If, therefore, acetoin or 2,3-butanedione were involved in the formation of the dimethylbenzene portion of DBI, the label from [1-^{14}C]-DL-lactate and [1-^{14}C]pyruvate should be lost in the biosynthetic sequence while the label from [2-^{14}C]pyruvate should be retained. Since we find that [1-^{14}C]-DL-lactate, [1-^{14}C]pyruvate, and [2-^{14}C]pyruvate are of comparable effectiveness as precursors of labeled dimethylbenzene carbons in DBI, we conclude that the condensation–decarboxylation of a three-carbon unit to form acetoin or 2,3-butanedione is not involved in the biosynthesis of the dimethylbenzene moiety of DBI or, therefore, of riboflavin.

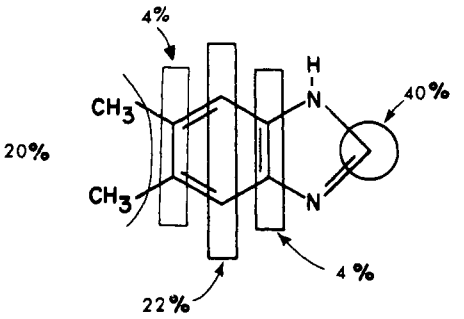

FIGURE 2: Distribution of ^{14}C label in DBI resulting from incorporation of added [1-^{14}C]-D-ribose.

Our studies on the biosynthesis of the dimethylbenzene portion of DBI by *P. shermanii* cultures are also in accord with the conclusion of Ali and Al-Khalidi (1966) and the earlier conclusion of Plaut (1954) that acetate is not directly involved as a precursor of the dimethylbenzene unit of riboflavin. Experiments involving [1-^{14}C]acetate and [2-^{14}C]acetate, previously reported (Alworth et al., 1969), established that acetate was an order of magnitude less efficient as a DBI precursor than the labeled pyruvate and lactate precursors listed in Table I.

From their studies of riboflavin biosynthesis Ali and Al-Khalidi (1966) concluded that an intermediate of the pentose phosphate pathway was the precursor of the dimethylbenzene unit of the riboflavin molecule. This conclusion was based, first upon the effectiveness of labeled pentoses as precursors of the lumichrome portion of riboflavin and, second, upon the similar distribution of incorporated label between the ribityl side chain and the dimethylbenzene moiety of riboflavin from a series of precursors. Plaut (1954) had previously noted a similar distribution of incorporated label between these two portions of the riboflavin molecule. The ribityl side chain of riboflavin was recently shown to derive from the ribose unit of the GTP nucleotide precursor (Mailänder and Bacher, 1976) (cf. Figure 1). The comparable incorporation of various precursors into the ribityl side chain and into the dimethylbenzene unit, therefore, supports the view that an intermediate of the pentose phosphate pathway is involved in those metabolic steps that yield the four-carbon biosynthetic unit.

More recently, however, Jabasini and Al-Khalidi (1975) have concluded that neither acetate, nor acetoin, nor an intermediate of the pentose phosphate pathway functions as the precursor of the dimethylbenzene unit of riboflavin in *E. ashbyii*. Biosynthetic riboflavin, labeled by the incorporation of a series of glucose and pentose precursors, was degraded via a new, selective scheme. It was concluded that the observed labeling patterns were not consistent with the involvement of a pentose cycle intermediate in the biosynthesis, that the C-1 and C-2 positions of glucose were not incorporated into the dimethylbenzene ring as a unit, and that the incorporation of label from glucose precursors occurred without passing through pyruvate, through a pentose, or through a component of the citric acid cycle. The labeling results of Jabasini and Al-Khalidi are difficult to interpret since the observed patterns, at least in the case of [1-^{14}C]glucose and [6-^{14}C]glucose, are incompatible with the established regiospecificity of the riboflavin synthetase reaction (Plaut et al., 1974).

[2] It has been reported that 2,3-butanedione formation in *Propionibacteria* proceeds via α-acetolactate (Antila, 1956/1957).

[3] The catabolic pathway established by Wawszkiewicz and Barker (1968) in *Propionibacterium pentosaceum*, if present in *P. shermanii*, would convert the added [4-^{14}C]erythritol into [1-^{14}C]-D-glyceraldehyde 3-phosphate.

Based upon the data summarized in Table I, upon the ^{14}C-labeling pattern in DBI resulting from [1-^{14}C]-D-ribose incorporation (Figure 2), and upon Ali and Al-Khalidi's (1966) observations, we concluded (Alworth et al., 1970) that a tetrose, formed via the pentose phosphate pathway, served as the precursor of the four-carbon biosynthetic unit involved in dimethylbenzene formation. In order to test this proposal biosynthetic experiments with comparable amounts of [4-^{14}C]-D-erythrose, [4-^{14}C]erythritol, [4-^{14}C]-D-ribose, and [1-^{14}C]D-glucose were carried out under comparable conditions.

The relative incorporation efficiencies of [1-^{14}C]-D-ribose into various portions of the B_{12} molecule as summarized in Tables I and II emphasize the fact that this specifically labeled pentose is a close in vivo precursor of the dimethylbenzene carbons of DBI. The vitamin B_{12} molecule contains an intact ribose unit in the form of the ribofuranosyl ring, found in the unusual *N*-α-glycosidic 3′-nucleotide structural element (Hodgkin, 1965). Furthermore, it has been established that the DBI is added at the same time as the ribofuranosyl ring in the B_{12} biosynthetic pathway. That is, the DBI base is converted into a nucleotide containing a DBI-ribofuranosyl linkage and then attached to a preformed cobinamide (Plaut et al., 1974; Friedmann, 1975). Naturally, ribose is anticipated to be an efficient precursor of the ribose unit of the ribofuranosyl nucleotide portion of vitamin B_{12}. As shown in Tables I and II, however, when [1-^{14}C]-D-ribose is incorporated into B_{12} by *P. shermanii* cultures, from 36 to 56% of the incorporated label is found in the DBI moiety. Thus, that remaining portion of the B_{12} molecule, which contains an intact ribofuranosyl ring, contains only about half of the incorporated [1-^{14}C]-D-ribose label.

It has been established that the C-2 carbon atom of DBI is derived biosynthetically from the 1′-C atom of the ribityl side chain of V (Alworth et al., 1971; Lu and Alworth, 1972) and of riboflavin (Renz and Weyhenmeyer, 1972) (cf. Figure 1). Since recent evidence establishes that this ribityl side chain is derived from the ribose unit of a guanosine triphosphate precursor (Mailänder and Bacher, 1976), [1-^{14}C]-D-ribose is also anticipated to be an effective precursor of the C-2 carbon atom of DBI. As shown in Tables I and II, however, when [1-^{14}C]-D-ribose is incorporated into DBI by *P. shermanii* cultures, from 53 to 60% of the DBI label is found in the dimethylbenzene carbons. Thus, the C-2 carbon of DBI, derived from a 1′-carbon of the ribityl group of riboflavin, contains less than half of the total label incorporated into DBI.

Based upon this analysis we conclude the [1-^{14}C]-D-ribose is an effective precursor of at least *three portions of the vitamin B_{12} molecule*—the cobinamide + ribofuranosyl portion (about half the total incorporated label), the C-2 carbon of DBI derived from a 1′-C of a ribityl group (about one-fourth of the total incorporated label), *and the dimethylbenzene carbons of DBI* (about one-fourth of the total incorporated label). Furthermore, while the incorporation of [1-^{14}C]-D-ribose into the dimethylbenzene moiety of DBI involves the labeling of more than one carbon, the incorporation pattern is quite specific (Figure 2). We therefore conclude that the C-1 of a pentose intermediate must be involved in the formation of the four-carbon biosynthetic unit from which the eight dimethylbenzene carbons are ultimately derived.

The experimental data summarized in Table II do not support our previous proposal that the four-carbon biosynthetic unit is derived from a tetrose (Alworth et al., 1970). According to this proposal, the incorporation of [1-^{14}C]-D-ribose into the dimethylbenzene carbons of DBI would proceed via a [3-^{14}C]fructose intermediate and would thus involve the passage through the hexose pool, while added [4-^{14}C]-D-erythrose and [4-^{14}C]erythritol precursors should be converted more directly into the four-carbon biosynthetic unit. The observed incorporations of specific activities of the precursors into the dimethylbenzene carbons (column 6 of Table II), however, establish that [1-^{14}C]-D-ribose incorporation into this portion of the DBI molecule occurs with less dilution than does the incorporation of either [4-^{14}C]-D-erythrose or [4-^{14}C]erythritol. The data are consistent with the view that the incorporation of [4-^{14}C]-D-erythrose and [4-^{14}C]erythritol, but not that of [1-^{14}C]-D-ribose, occurs via the hexose pool.

We conclude that a biosynthetic pathway exists for the relatively direct conversion of C-1 of a pentose intermediate into a portion of the four-carbon biosynthetic unit involved in dimethylbenzene formation. The data summarized in Tables I and II and in Figure 2 suggest that, if 2,3-butanedione or acetoin is the true four-carbon biosynthetic unit, then this butanedione or acetoin must be derived in a process that involves C-1 of a pentose intermediate rather than via the known processes that involve the joining and decarboxylation of pyruvate molecules.

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Anthroylouabain: A Specific Fluorescent Probe for the Cardiac Glycoside Receptor of the Na-K ATPase†

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ABSTRACT: Anthroylouabain (AO) was synthesized by reaction of anthracene-9-carboxylic chloride with ouabain. Nuclear magnetic resonance spectroscopy of AO suggests that the anthracene is esterified to the rhamnose in the glycoside. AO inhibits Na-K ATPase from human red cells, eel electroplax and rabbit and dog kidney with a $K_I < 1$ μM. AO bound to rabbit or dog kidney Na-K ATPase shows enhanced fluorescence and characteristic spectral shifts. AO binding requires Mg and is optimum in the presence of Mg + P_i or MgATP + Na; ouabain prevents AO binding and fluorescence enhancement if added before AO or reverses it if added after AO is bound. Na inhibits AO binding in the presence of Mg + P_i and K inhibits it in the presence of MgATP + Na. AO binding and dissociation rate constants measured by fluorescence agree qualitatively with reported measurements for ouabain, using other methods, although AO shows faster kinetics than ouabain. Dissociation constants obtained from kinetic measurements are 1.5×10^{-7} and 1.8×10^{-7} M for the MgATP + Na complex and Mg + P_i complex, respectively. K_D from fluorescence titrations is 2.3×10^{-7} M for the latter. The enzyme has 2-2.5 nmol of AO binding sites/mg of protein. No differences in the fluorescence parameters of the Mg + P_i or MgATP + Na complexes were observed, suggesting that the same enzyme conformation binds AO under both ligand conditions. Comparison of the AO fluorescence parameters in the enzyme with those of model systems suggests that the binding site is hydrophobic and/or viscous and shielded from H_2O. The results indicate that AO is a specific fluorescent probe of the cardiac glycoside receptor of the Na-K ATPase. Possible applications are discussed.

The mechanism of the sodium-potassium adenosine triphosphatase (Na-K ATPase)[1], which is the primary component of the pump responsible for the active transport of Na and K across plasma membranes, is not known. Under physiological conditions the enzyme catalyzes the transport of 3 Na ions out of the cell, 2 K ions into the cell, and the hydrolysis of 1 ATP molecule in a single cycle (Sen and Post, 1964; Garrahan and Glynn, 1967). The ion movements are thought to be obligatorily coupled to ATP hydrolysis in a series of intermediate steps which involve the formation of a phosphoenzyme, changes in conformation, and cleavage of the phosphoenzyme (for recent reviews, see Dahl and Hokin, 1974; Skou, 1974; Glynn and Karlish, 1975; Schwartz et al., 1975). The Na-K ATPase has been purified from a variety of sources (Kyte, 1971a; Jørgensen, 1974a; Hokin et al., 1973). Two polypeptides are found in all the purified preparations (Jørgensen, 1974b): a large chain of about 90 000 daltons containing the phosphorylation site (Kyte, 1971b) and a smaller glycopeptide.

Although conformation changes of the enzyme are probably related to the actual transport of Na and K, at present they can only be studied indirectly. Fluorescence spectroscopy is a possible approach to obtain information on the structure and molecular dynamics of the enzyme during different functional states owing to the sensitivity of the fluorescence parameters

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[1] Abbreviations used: Na-K ATPase, sodium-potassium adenosine triphosphatase; ATP and ADP, adenosine triphosphate and adenosine diphosphate, respectively; AO, anthroylouabain; ANS, 8-anilino-1-naphthalenesulfonate; EDTA, ethylenediaminetetraacetic acid; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; Cl_3AcOH, trichloroacetic acid; P_i, inorganic phosphate; NMR, nuclear magnetic resonance.