PRECURSORS OF THE XYLENE RING OF RIBOFLAVIN

ALI

THE ULTIMATE PRECURSORS OF THE XYLENE RING IN THE BIOSYNTHESIS OF RIBOFLAVIN

By

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THESIS ABSTRACT

THE ULTIMATE PRECURSORS OF THE XYLENE RING OF RIBOFLAVIN

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The nature of the ultimate precursors of the xylene ring of riboflavin was reinvestigated in the organism E. Ashbyii. Plaut (1) had shown that both carboxyl and methyl labelled acetate was incorporated into that ring. However, Klungsøyr (2) obtained negative results with the carboxyl labelled acetate. The suggestion of acetoin being the direct precursor of the xylene ring of riboflavin, originated from the work of Masuda (3) who isolated a green flourescent compound from the cultures of E. Ashbyii. Masuda postulated that the green compound could condense with acetoin to give riboflavin. This view was supported by the work of Goodwin (4) who claimed that acetoin was better incorporated into riboflavin than acetate-2-C^{14}.

In this study, the incorporation of acetate-1-C^{14}, acetate-2-C^{14}, and pyruvate-2-C^{14} was examined in riboflavin and acetoin, acetoin-1-C^{14} was synthesized by a modification of the procedure of Brady et al. (5) and its incorporation into riboflavin and its photodegradation product, lumichrome was examined.

The radioactive precursors were incubated with either the growing cells or standing cells suspended in a buffer. The various methods of estimation and purification of acetoin, riboflavin and lumichrome were improved.

In the first experiment the incorporation of pyruvate-2-C^{14}, and acetate-2-C^{14} into acetoin was measured. Following this in the next experiment, the incorporation of acetate-1-C^{14}, acetate-2-C^{14} and pyruvate-2-C^{14} was measured in both acetoin and riboflavin. The results of these experiments showed that incorporation of pyruvate into acetoin was much greater than acetate, however, incorporation of pyruvate into riboflavin was less than acetate-2-C^{14}.

Acetate-1-C^{14}, acetate-2-C^{14}, pyruvate-2-C^{14}, and acetoin-1-C^{14} was incubated with standing cells of E. Ashbyii. Riboflavin was extracted and crystallized to constant specific activity and finally degraded to lumichrome in sunlight. The lumichrome was purified by chromatography. The order of decreasing specific activity of riboflavin was, acetate-2-C^{14} acetate-1-C^{14}, pyruvate-2-C^{14} acetoin-1-C^{14}.

These results are evidence against involvement of acetoin in riboflavin biosynthesis. The overall incorporation of acetate was below 0.2 percent. This suggests that in all probability acetate is not an immediate precursor of riboflavin. The ultimate precursors of the xylene ring of riboflavin are still unknown.

1. Plaut, G. W. E., J. biol. Chem., 211, 111 (1954).

2. Klungsøyr, L., Acta Chem. Scand., 8, 723 (1954).

3. Masuda, T., Pharm. Bull., 5, 136 (1957).

4. Goodwin, T. W., and Treble, D. H., Biochem. J., 70, 14P (1958).

5. Brady, O. Roscoe, Rubinowitz, J., Van Baalen, J., and Gurin, S., J. biol. Chem., 193, 138 (1951).

My soul is full of longing
For the secret of the sea,
And the heart of the great ocean
Sends a thrilling pulse through me.

Longfellow, The Secret of the Sea.

A C K N O W L E D G M E N T

The author is very grateful to Dr. Usama al-Khalidi for suggesting the problem and for his valuable assistance and advice throughout the course of this work and during the preparation of the manuscript.

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CHAPTER I

INTRODUCTION

Higher animals and a large number of bacteria show a dietary requirement for riboflavin. The synthesis of riboflavin, however, occurs in many bacteria, protozoa, fungi, algae and higher plants. Intestinal bacterial synthesis of riboflavin supplies some of the vitamin for ruminant and non-ruminant mammals, in insects riboflavin formation is probably also due to the symbiotic activity of microorganism which they harbour. The subject has been reviewed by Goodwin (1) in detail.

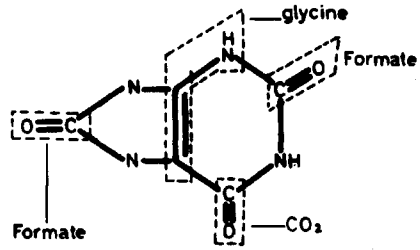
Exaggerated synthesis of riboflavin occurs in certain ascomycetes notably Eremothecium ashbyii and Ashbya gossypii. These two organisms have been used for the commercial production of riboflavin and their nutritional requirements and growth conditions have been extensively studied (2-16). For this reason we have used Eremothecium ashbyii throughout this study.

The structure of riboflavin was established by Kuhn and co-workers (17-18) and Karrer and co-workers (19, 20), who independently synthesized the vitamin in 1935. Later studies established its role as a co-factor in many specific enzyme reactions.

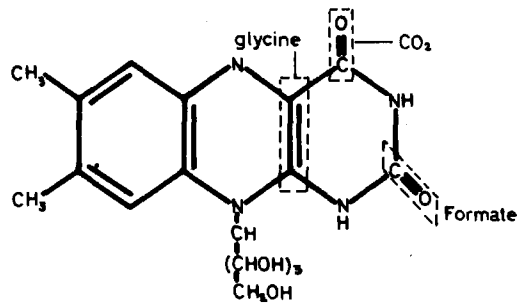
The first suggestion as to the mechanism of biosynthesis of riboflavin comes from the work of Woolley in 1951 (21) who postulated on structural basis that 1,2-diamino-4-5-dimethyl-benzene is a common intermediate in the biosynthesis of both riboflavin and vitamin-B_{12}. In a study of 26 different species of microorganisms, selected from widely divergent classes, he could demonstrate that 1:2-diamino-4 5-dichloro benzene inhibited growth of these organisms which had no metabolic requirement for riboflavin and vitamin B_{12}; but was without effect on those which required both these vitamins. Diamino-dimethyl-benzene and to a lesser extent O-phenelene diamine competitively overcame this inhibition.

Subsequently Woolley and Pringle (22) synthesized 35 different analogues of 1:2-diamino-4 5-dimethyl benzene and studied their growth inhibitory activity against different organisms. Some of these compounds suppressed synthesis of vitamin B_{12} in Bacillus megatherium without affecting growth. Foster and Pittillo (23) have arrived at similar results with B. subtillis; where 1:2-diamino-4 5-dichlorobenzene decreases riboflavin production but does not affect growth.

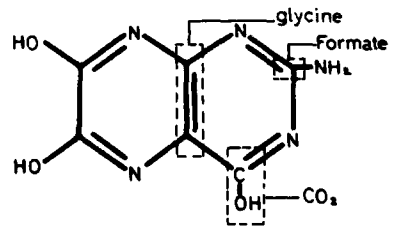
Later studies with labelled precursors of small molecular weight revealed a remarkable similarity in the pattern of labelling of purines, pteridines and riboflavin.



PURINE_URIC ACID



RIBOFLAVIN



PTERIDINE_LEUCOPTERIN

FIGURE I. INCORPORATION OF VARIOUS LABELLED PRECURSORS INTO RIBOFLAVIN PURINE AND PTERIDINE NUCLEUS (54, 55).

The prominent features of these studies are presented in Figure I. The similarity of labelling pattern was strongly suggestive of a common origin of these groups of compound (54, 55).

Direct evidence for purines as precursor of riboflavin came from the work of McNutt (14) who showed that in E. ashbyii, considerable labelling of isoalloxazine portion of riboflavin ring took place when uniformly labelled adenine was used as substrate, while only a trace of activity was incorporated from adenine-8-C^{14}. McNutt (24, 25, 26) further demonstrated that with adenine labelled with N^{15}, and xanthine labelled with N^{15}, and C^{14} the nitrogens from both the pyrimidine and imidazole portions of the purine ring was transferred into riboflavin.

The fact that riboflavin molecule incorporates purines does not prove that a purine is an obligatory intermediate in riboflavin biosynthesis. Although the work of Goodwin and Jones (27) lends strong support to such a hypothesis. In their experiment addition of small amount of unlabelled adenine to a culture of E. ashbyii greatly diluted the incorporation of label from $2C^{14}$ serine into riboflavin.

Masuda (28) in 1956 was the first to isolate a green fluorescent compound from the cultures of E. ashbyii. He called this compound Compound-G, because of its green fluorescence in ultraviolet light. The compound was synthe-

sized by Maley and Plaut (29, 30) in 1958, and by Masuda (31) in 1959, and its structure established as 6:7-dimethyl-ribityl-lumazine. Compound-G was postulated by Masuda as a possible intermediate in riboflavin biosynthesis.

Compound-G is reported to stimulate flavogenesis in cell free systems of a number of different organisms (32-39) which do not require riboflavin for growth. Lacto-bacillus Casei which requires riboflavin as a growth factor cannot convert compound-G to riboflavin (24, 34, 40).

Maley and Plaut (30) reported that compound-G becomes labelled by formate in a position analogous to riboflavin nucleus and in A. gossypii the incorporation of label from different substrate (formate, glycine, adenine), was higher than riboflavin in short incubation periods. These results make Compound-G a strong candidate as an intermediate in riboflavin biosynthesis.

A possible mechanism of riboflavin formation involving Compound-G, was suggested by Masuda (41), Figure II. Here a purine Compound I, gives rise to II, and II condenses with a 4 carbon fragment, probably acetoin to give Compound-G (III). That Compound-G may condense with another 4 carbon fragment similar to acetoin was also implicit in the argument. Conversion of I to Compound-G and of Compound-G to riboflavin takes place nonenzymatically in the presence of diacetyl and under more drastic conditions acetoin may react similarly

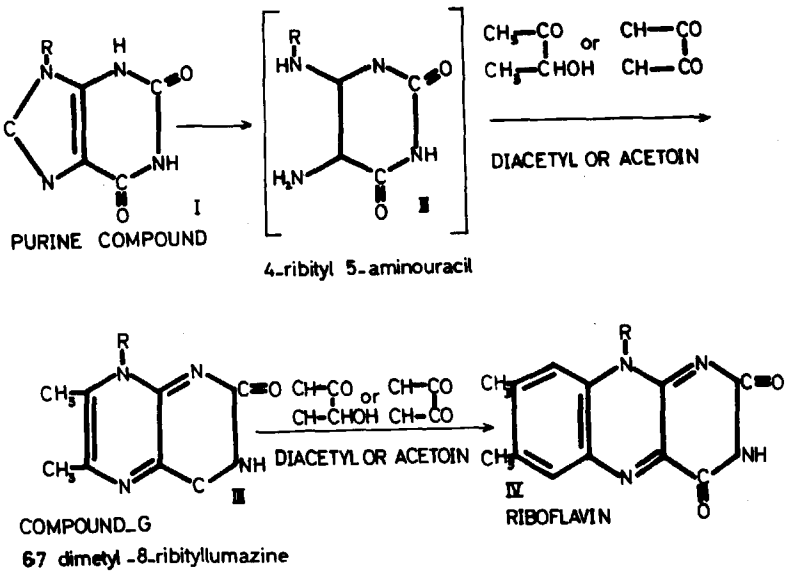


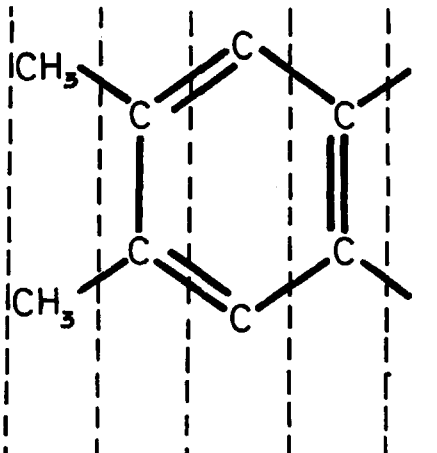
FIGURE II. POSSIBLE MECHANISM OF RIBOFLAVIN FORMATION AS SUGGESTED BY MASUDA (41).

to a lesser extent. It should be kept in mind that acetoin in aqueous solutions, is very susceptible to oxidation.

The role of acetoin as an intermediate is disputable and the experimental observation of different workers has led to controversial findings. For example Kishi et al. (42) claimed that cell free extracts of E. ashbyii converted 4-ribityl-5-aminouracil to Compound-G in presence of acetoin. However Plaut's result with this compound were inconclusive (37). Experiments of Goodwin and Horten (37) did not support the results of Kategiri et al.

The possibility of acetoin being an intermediate, in riboflavin biogenesis is suggested by Goodwin and Treble (43) who reported in a preliminary communication that, $1C^{14}$-3-hydroxy-2 butanone (acetoin) was more effectively incorporated into riboflavin than acetate $2\text{-}C^{14}$. The label was exclusively located in the xylene moiety of the riboflavin. Half of the incorporated radioactivity was found in the two methyl groups and the remainder could be accounted for in the aromatic ring. In contradistinction to this, Plaut (44) demonstrated that both acetate-$1C^{14}$ and acetate $2\text{-}C^{14}$ labelled the aromatic nucleus of riboflavin, Figure III.

Plaut's findings are best explained on the assumption that, acetoin is not an intermediate in the biosynthesis of riboflavin. In view of the known pathways for the



$CH_3\overset{*}{C}OOH$	8	77	8	73
$\overset{\bullet}{C}H_3COOH$	71	36	63	33

FIGURE III. THE INCORPORATION OF C^{14} ACETATE INTO THE XYLENE RING OF RIBOFLAVIN AFTER PLAUT (44). THE FIGURES REFER TO COUNTS PER MINUTE PER MICROMOLE x 10^{-3}.

biosynthesis of acetoin (Figure IV) this compound is produced from either pyruvate or acetaldehyde and more readily when both are present. The enzymes catalyzing this condensation, are widely distributed in yeast bacteria and animal tissue (45). Although according to the established mechanism of acetoin synthesis only the methylene carbon of acetate can enter into its formation; the possibility that direct reduction of acetate to acetaldehyde may take place in some microorganisms, cannot be completely excluded.

The results of Klungsøyr (46) contradicted those of Plaut's in that he could not detect, in standing cultures, any incorporation of label from acetate-1-C^{14} into the aromatic ring of riboflavin.

Recently Plaut (47) has studied the conversion of Compound-G into riboflavin in purified enzyme preparations from A. gossypii and Baker's yeast. The enzyme from A. gossypii was purified 157 fold. Various forms of labelled Compound-G were synthesized, and distribution of label in riboflavin estimated by established procedures (54), Figure V.

With 6:7-dimethyl-8-ribityllumazine-2C^{14} and 6:7-dimethyl-8-ribityllumazine 4a, 8aC^{14}, the molar activity of riboflavin produced was identical to that of the substrate. Moreover 6:7-dimethyl-8-ribityl C^{14}lumazine (ribose randomly labelled) was also converted to riboflavin

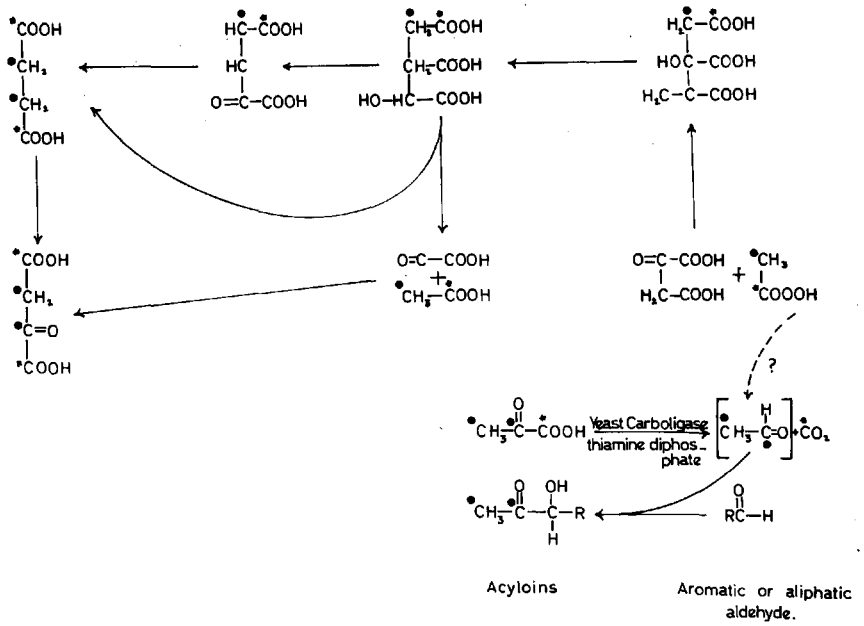


FIGURE IV. THEORETICAL CONVERSION OF ACETATE INTO ACYLOINS THROUGH ESTABLISHED PATHWAYS OF CARBOHYDRATE METABOLISM. THE DIRECT CONVERSION OF ACETATE TO ACETALDEHYDE (BROKEN ARROW), IS DOUBTFUL.

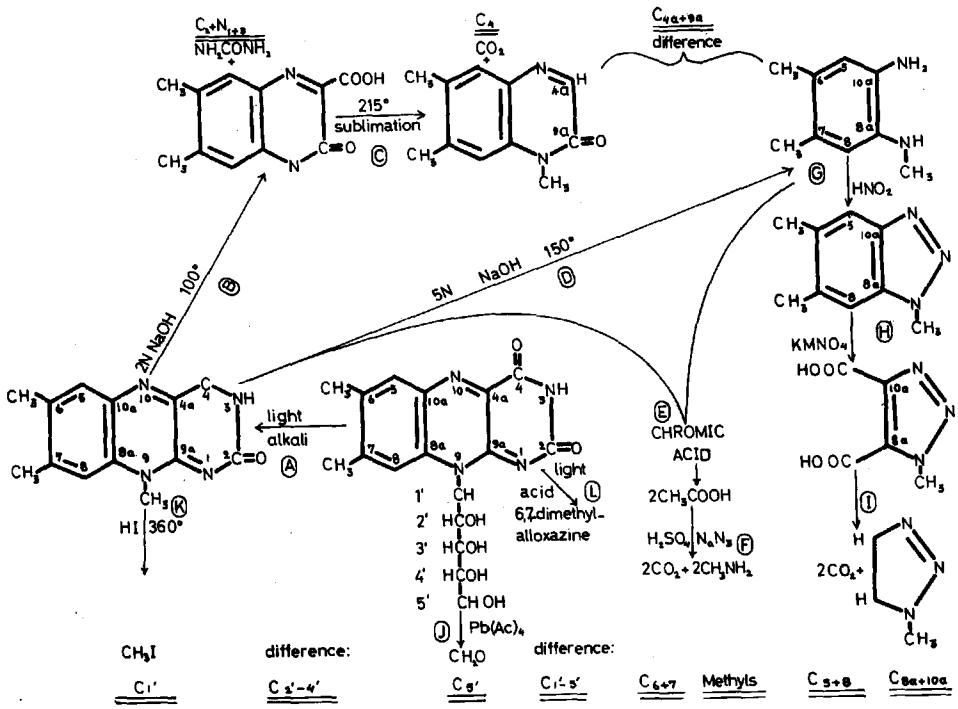


FIGURE V. METHOD OF DEGRADATION OF LABELLED RIBOFLAVIN AFTER PLAUT (54).

without dilution of the label. These results strongly indicate intact transfer of pyrimidine and ribitol moiety of the lumazine into riboflavin.

As mentioned earlier Compound-G requires 4 carbons for the completion of the xylene ring of riboflavin. The identity of these four carbons is still uncertain, both Plaut (48) and Goodwin and Horten (37) independently concluded the possibility that 4-carbon unit may arise from Compound-G itself.

To examine this possibility Plaut (47) studied the incorporation of radioactivity from 6:7-dimethyl C^{14}-8 ribityl-lumazine (both methyl groups labelled) into riboflavin. He found that riboflavin produced under these conditions had twice the specific radioactivity of the substrate compound used. When the methyl groups were converted to acetic acid by chromic acid oxidation, the acetate produced contained half the molar specific activity of the substrate compound. After a complete chemical degradation of riboflavin the label was exclusively located in methyl groups and carbon 5 and 8 of the xylene moiety of riboflavin molecule.

The conversion of Compound-G into riboflavin takes place by an unusual mechanism (Figure VI), where one molecule of this compound donates a 4-carbon unit to another molecule. The fate of the fragment of Compound-G after it has donated its 4-carbon, is not known and no degradation product has yet been identified.

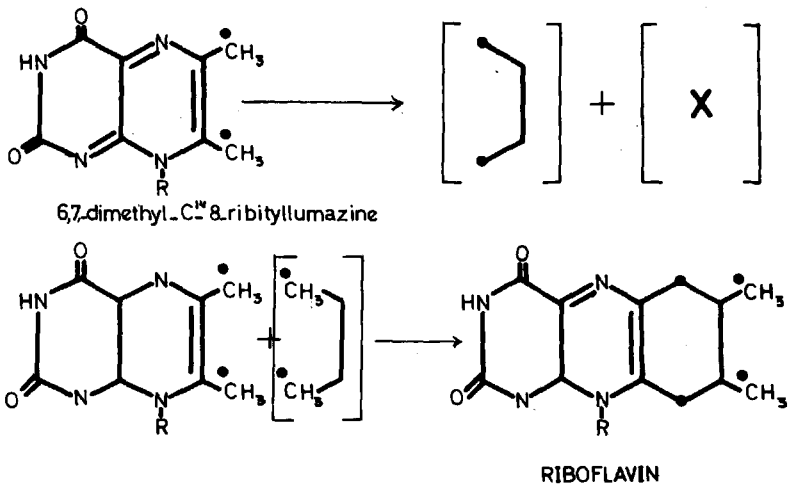


FIGURE VI. THE INCORPORATION OF LABEL FROM METHYL LABELLED COMPOUND-G INTO RIBOFLAVIN. THE FIGURES IN PARENTHESIS SHOW POSTULATED INTERMEDIATE AND DEGRADATION PRODUCT "X", AFTER GOODWIN (4) AND PLAUT (47).

It must also be mentioned that some investigators have challenged the authenticity of Compound-G as an intermediate in riboflavin biogenesis. Compound-G is not incorporated by intact cells of E. ashbyii (57) or A. gossypii (56). Korte et al. (49) made an extensive study of the metabolism of Compound-G in five riboflavin producing organisms. They found that while some of these organisms oxidized labelled Compound-G in no case there was any incorporation of label into riboflavin. Rowan and Wood (55) have reported a purely chemical conversion of Compound-G riboflavin in 55% yield, by reflexing for 15 hours, Compound-G dissolved in phosphate buffer.

The origin of the ribityl side chain of riboflavin was studied by Plaut and Broberg (51) in A. Gossypii. Their results are consistent with the assumption that the hexose monophosphate shunt and transaldolase-transketolase system probably contribute equal amounts of carbon towards the formation of this sugar.

The following reviews on chemistry (52), nutritional aspects (53) and biochemistry (51, 54, 55) of this vitamin are available.

CHAPTER II

MATERIALS AND METHODS

1. Biological material

E. ashbyii (Strain ATCC 6747) was maintained on agar plates of the medium used for growth and transferred periodically.

2. Growth conditions

Initially cells were grown with large inoculae (5 percent) in a medium that consisted of the following per liter:

Yeast extract	2 g.
Bacto peptone	5 g.
Corn steep liquor*	20 ml.
Glucose	40 g.

The final pH of the media was 5.6. Cells were grown in flasks, filled to 20 percent of their capacity with the medium with continuous shaking. The temperature was maintained at 30 ± 1° C.

3. Collection of mycelia

Twenty-four hours old cultures were filtered through muslin under suction and washed with cold saline. The

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* A centrifuged 25 percent w/w solution of corn steep liquor - A. E. Staley Manufacturing Co., Decator, Illinois.

resulting mycelial pad could then be peeled off from the cloth and weighed directly. The cells can be suspended in Kreb's Ringer phosphate-buffer for experiments with non-growing cells. The manner of incubation with radioactive samples is described elsewhere under results.

4. Extraction of riboflavin

The incubation was stopped by adding 1 ml. of conc. HCl per 30 ml. of medium. It was then autoclaved for twenty minutes under 15 lb. pressure. To the resulting hydrolysate enough sodium sulfate was added to saturate the solution. Riboflavin was twice extracted with 5-10 ml. of benzyl alcohol. At this stage benzyl alcohol forms a thick emulsion with the aqueous hydrolysate. The emulsion was easily broken by centrifugation and the upper benzyl alcohol layer was pipetted out. The benzyl alcohol extracts were pooled, diluted with 3 to 4 volumes of ether and riboflavin reextracted twice with 3 to 4 ml. of distilled water.

5. Column chromatography of riboflavin

Florisil* (60-100 mesh) was suspended in 2 percent acetic acid. When the earth settled, the slightly turbid supernatant was decanted. This earth was resuspended in the same liquid and packed as a slurry in columns 0.5 cm. in diameter and 10 cm. high.

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* Floridin Co., Warren, Pennsylvania.

The riboflavin extracted from benzyl alcohol was placed directly on the florisil column. The riboflavin was quantitatively adsorbed at the upper half of the column. The cclumn was washed with 300 ml. of 10 percent acetic acid and the yellow band eluted with a mixture of pyridine 1 volume and 0.3 percent acetic acid 4 volumes. The preliminary measurement of radioactivity and estimation of riboflavin was performed on a small aliquot of this eluate.

6. Estimation of riboflavin

The riboflavin was measured by the intensity of its yellow color at 445 mU. using the millimolar extinction co-efficient of 11.3. The extinction co-efficient of riboflavin was determined previously on an authentic sample of riboflavin (Eastman Kodak Co.) at pH 1.

7. Crystallization of riboflavin to constant specific activity

To the eluate obtained from florisil column, 25 mgm. of riboflavin (Eastman Kodak Co.) was added, and dissolved by heating in a water bath. The solution was evaporated *in vacuo* to almost complete dryness. The resulting riboflavin was taken up in 4-5 drops of conc. HCl and transferred to microcentrifuge tubes. It was then diluted with 0.5 ml. of water and left at 4° C. for 6 hours. The crystals of riboflavin could then be separated by

centrifugation and decanting the supernatant. The process of redissolution in HCl and recrystallization was repeated till the radioactivity per optical density unit became constant.

8. Preparation of lumichrome

Lumichrome was prepared by the procedure of Karrer et al. (56). A dilute solution of riboflavin - 20 mgm. per 100 ml. - was prepared in 50 percent methanol and exposed to direct sunlight, till the solution lost most of its yellow color and turned pale green. 15 ml. of the resulting lumichrome solution was evaporated to dryness in vacuo and the yellow material taken up in 3 ml. of 0.5 N sodium hydroxide. The alkaline solution was shaken once with 20 ml. of chloroform and the chloroform layer was discarded. The alkaline liquid was brought to pH 2 with conc. HCl and lumichrome extracted with 7 portions of 10 ml. of chloroform. The chloroform layer was pooled, shaken once more with 2 ml. of water and the aqueous layer discarded.

9. Chromatographic purification of lumichrome

10 gms. of celite 545* was triturated in a mortar with 4 ml. of distilled water and packed tightly in a column of 1.5 cm. in diameter. The combined chloroform

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* Light and Co. Ltd., Poyle Trading Estate Colnbrook Bucks, England.

extract was reduced to 1 ml. by evaporating it *in vacuo*, triturated with half a gram of celite and packed at the top of the column. The lumichrome was eluted with chloroform.

To test the purity of lumichrome, it was chromatographed on Whatman No. I filter paper in the different solvent developed by Whitby (57). In all cases it gave a single spot which corresponded in RF. to the 5 times crystallized lumichrome prepared on a large scale by the procedure of Karrer *et al.* (56).

However, under our conditions, the recrystallized material was never pure and upon chromatographing it on paper, it gave two spots. Spectral analysis of these spots showed that lumichrome was contaminated with traces of riboflavin. Therefore, the whole material was purified by passing it through celite columns and recrystallizing once more from glacial acetic acid.

10. Estimation of lumichrome

The chromatographically pure material was used for this purpose. Complete spectra were taken in 0.5 N sodium hydroxide and 0.1 N HCl. The shape of these spectra agreed with those reported by McNutt (14). The molar extinction coefficients were calculated at different wavelengths on this sample on the basis of the nitrogen content of the material, as determined by micro-Kjeldahl method. These values are reported in Table I.

TABLE I

Molar Extinction Coefficient of Lumichrome

Optical Property	In 0.1 N HCl		In 0.5 N NaOH		
Maxima (mu)	262	350	265	340	425
E_{max} ($Cm^2 \times mole^{-1} \times 10^3$)	33.3	8.81	40.3	6.50	6.95

11. Isolation of acetoin from mycelial cultures

Whenever incorporation of radioactivity into acetoin was to be determined, the following procedure was followed. To a suitable aliquot portion of incubation medium 50 mgm. of cold acetoin was added and final volume made up to 50 ml. with water. The mixture was distilled *in vacuo* to almost dryness. The clear distillate contained the acetoin.

12. Conversion of acetoin to 2:4-dinitrophenylosazone

1.5 g. of 2:4-dinitrophenylhydrazine dissolved in 50 ml. of 30 percent perchloric acid, was mixed with the distillate containing the acetoin. The mixture was refluxed for 6 hours on a water bath. The voluminous precipitate of the osazone was filtered on a Buchner funnel, washed with 10 ml. of water and dried at 50° C.

13. Crystallization of osazone to constant specific activity

The osazone was crystallized alternately from aniline and nitrobenzene till the radioactivity per mgm. of osazone became constant. In one case, after the osazone had been crystallized several times from the obove solvents, it was recrystallized from several litres of glacial acetic acid. The recrystallized material showed no decrease in radioactivity.

14. Organic synthesis of 1C^{14}-3hydroxy 2-butanone (acetoin).

Radioactive acetoin was synthesized by the procedure of Brady et al. (58). Considerable difficulty was encountered in carrying out the synthesis and the yield was poor, below 20 percent as determined on several non-radioactive runs.

The difficulties originated from scaling down the synthesis to a microscale of 10 millimole; 1/3rd. the quantity used by Brady et al. A greater source of trouble was the formation of a tough resinous precipitate, the double magnesium salt of lactonitrile, formed during the course of reaction, which was almost impossible to agitate by any conventional laboratory stirrer.

The purification by distillation of 0.5 ml. of acetoin also posed problems. Therefore, we had to design a special apparatus to carry out the synthetic reaction, Figure VII. We also designed a simple vacuum microdistillation apparatus, Figure VIII, in which 0.5 to 1 ml. of liquid could be conveniently distilled.

The apparatus for synthesis consists of a glass tube fitted with two side tubes, one of which is used to supply nitrogen and the other for introducing reactants. The reaction mixture is churned with a losely fitting glass piston, the shaft of which passes through a condensor and is connected at the top to an electrically driven motor.

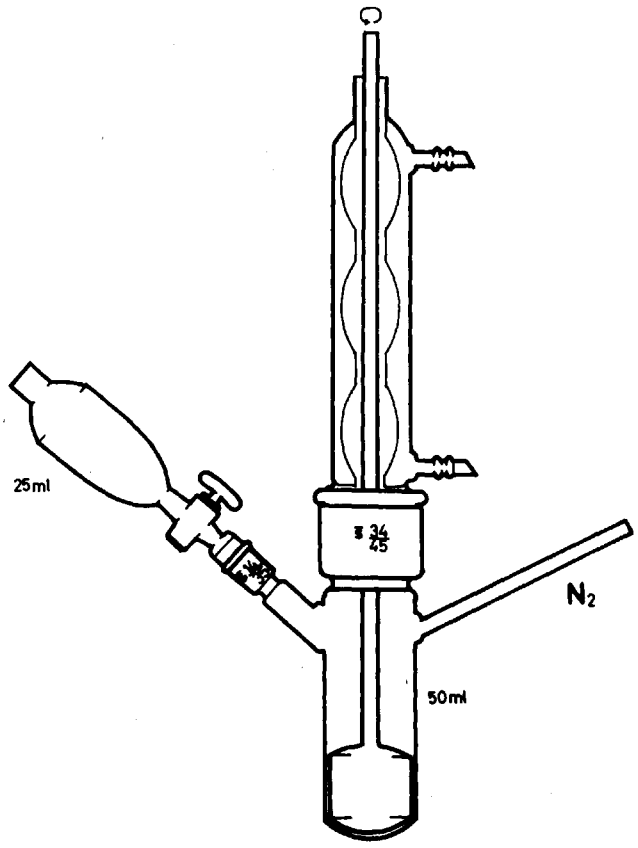


FIGURE VII. APPARATUS FOR THE SYNTHESIS OF RADIOACTIVE ACETOIN.

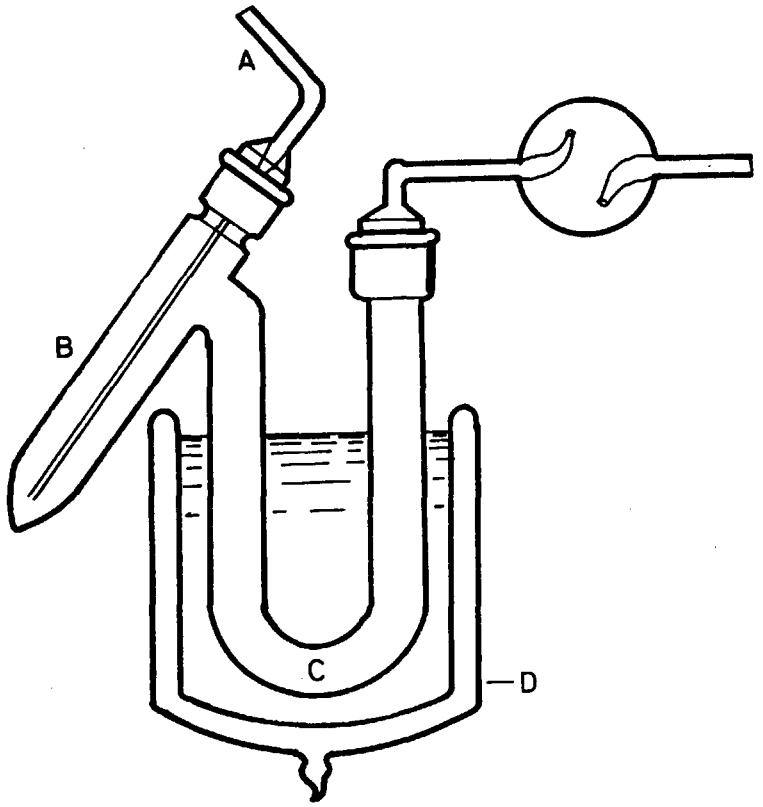


FIGURE VIII. MICRODISTILLATION APPARATUS FOR DISTILLATION OF ACETOIN. THE TUBE "B" CONTAINS THE LIQUID TO BE DISTILLED AND THE TUBE "C" IS CHILLED IN DRY ICE ALCOHOL MIXTURE. NITROGEN CAN BE BUBBLED THROUGH CAPILLARY "A."

The nitrogen was purified by passing through an assembly of tubes containing alkaline pyrogallol (0.25 percent pyrogallol in 400 ml. of 40 percent KOH), conc. H_2SO_4 and finally through anhydrous $MgSO_4$.

At the beginning of the reaction 10 millimole of lactonitrile was introduced into the glass tube. Through the side arm 11 millimole of methyl magnesium iodide dissolved in 25 ml. of anhydrous ether was slowly added.

When the evolution of hydrogen ceased, a similar amount of radioactive methylmagnesium iodide was introduced slowly over a half hour period. Vigorous stirring and a steady supply of nitrogen was maintained throughout the reaction. After the addition of radioactive methylmagnesium iodide, agitation with gentle warming was continued for an additional half hour. The reaction mixture was chilled in an ice bath and the double magnesium salt of the grignard reagent decomposed with minimal quantity of saturated sodium chloride. The pH was adjusted to 2 with conc. HCl and the whole reaction mixture was transferred to a liquid liquid extractor. Acetoin was continuously extracted in ether under nitrogen, and the ether extract was dried over anhydrous magnesium sulfate and a little anhydrous sodium carbonate. The ether was evaporated and the remaining oil was distilled under vacuum using a special microdistillation apparatus designed for this purpose.

The microdistillation apparatus consists of a glass tubing 6 x 1 cm., connected near its neck to a U shaped side arm. The liquid to be distilled is transferred by a pipette into the bottom of the glass tube and the side arm is immersed in a dry ice alcohol mixture. Vacuum can be applied through the open end of the side arm and a thin stream of nitrogen can be bubbled through the capillary to avoid bumping. The tube containing the distillate is heated by a microflame. From this apparatus 1-0.5 ml. of the liquid can be conveniently distilled. The overall yield of acetoin was 40 percent.

The acetoin distilled under these conditions had a boiling point of 144° as determined by the method of Emich (59) using a capillary for micro boiling point determination. This value agrees with the boiling point reported in literature.

15. Radioactive purity of acetoin

For determination of radioactive purity of acetoin as well as for further purification, the acetoin was chromatographed on a celite column. A similar column has been used by Neish *et al.* (60) to separate acetoin, diacetyl and 2:3-butane diol.

45 gms of celite 545 was wetted with 20 ml. of water and triturated thoroughly in a mortar. It was packed in a

column of 2 cm. diameter. The radioactive acetoin (0.4 gm. of total synthesized product) was taken up in 2 ml. of water and triturated with an additional 5 gm. of celite. It was packed on the top of the column. Acetoin was eluted with ethyl acetate saturated with water and several 10 ml. fraction were collected. Acetoin was determined colorimetrically and radioactivity estimated on the same sample. The profile obtained shows that acetoin comes out in the first few fractions of eluate and peak of radioactivity corresponds to the peak of acetoin concentration, Figure IX.

The acetoin was recovered from ethyl acetate by diluting it with two volumes of normal pentane, and reextracting with distilled water. The aqueous extractive was reshaken with normal pentane to get rid of most of the ethyl acetate. The aqueous acetoin was preserved at -12° C.

16. Colorimetric estimation of acetoin

For rapid determination of acetoin a modification of procedure of Happold and Spencer (61) was used. A sample containing 20-100 γ of acetoin, was mixed with 5 ml. of 0.1 percent 2:4-dinitrophenylhydrazine dissolved in 5 percent perchloric acid. It was placed in a tube tightly closed with a rubber stopper and boiled for one hour in a water bath. After cooling, a pinch of Kaolin was added to the tube and centrifuged. The supernatent was washed once with dilute

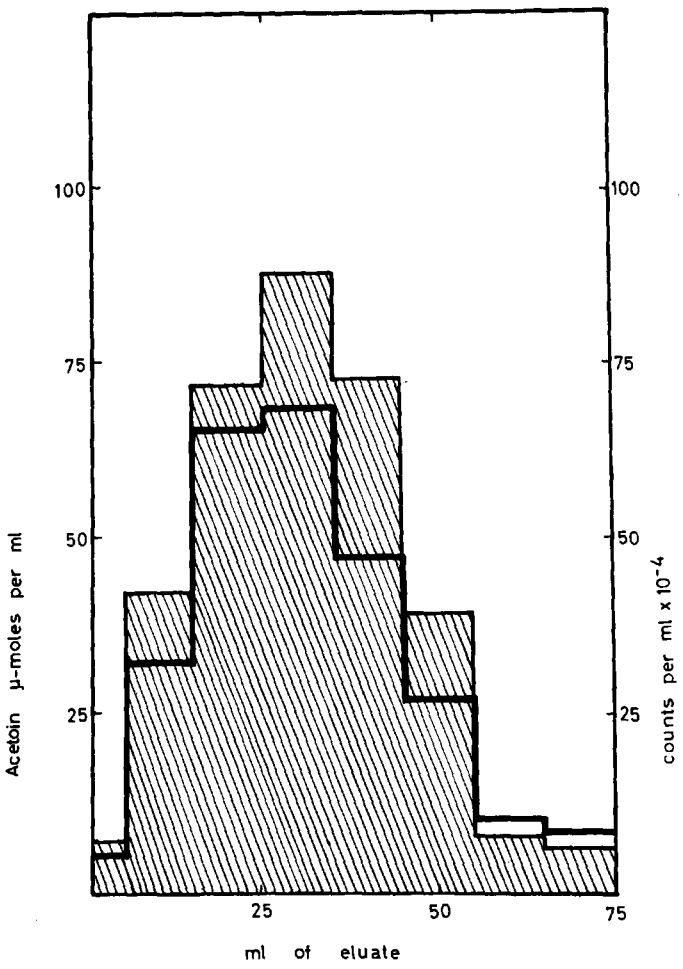


FIGURE IX. THE ELUTION PATTERN OF ACETOIN FROM CELITE COLUMN. HATCHED AREA DENOTES THE QUALITY OF ACETOIN IN µMOLES AND THE SOLID LINES REPRESENT C.P.M. PER µMOLE OF ACETOIN. FOR DETAILS SEE p. 26.

HCl and once with distilled water. The precipitate was extracted twice with 5 ml. of sodium ethylate. The optical density of violet color was read in a Klett photometer using a green filter. The value was compared against a standard containing 40 γ of acetoin. The color intensity is linear within this range. Sodium ethylate is prepared by dissolving 3 g. of clean sodium in 1000 ml. of absolute alcohol. Radioactivity measurement was performed on the same sample.

17. Measurements of radioactivity

Radioactivity measurements were done by plating the sample on glass planchets and correction for self-absorption (standard self-absorption tables) applied.

18. Miscellaneous determinations

Nitrogen was determined by the modified microkjeldahl procedure (62). Glucose was estimated enzymatically by the glucostat (63,64) method. Grignard reagent was assayed by titration with standard alkali using method of Gilman (65). Anhydrous ether was prepared by the method of Vogel (66).

Lactonitrile and iodomethane were purchased from Eastman Organic Chemicals, Acetate 1-C^{14}, Acetate 2-C^{14}, Pyruvate 2-C^{14} and methyl-iodide C^{14} were obtained from Radiochemical Centre Buckinghamshire, Amersham, England. Glucostat was procured from Worthington Biochemical Corporation, Freehold, New Jersey.

CHAPTER III

RESULTS

1. Time study of riboflavin production

The object of this study was to measure the riboflavin production by standing cultures (non-growing cells) of *E. ashbyii*. For this purpose, cells were grown for 24 hours, filtered, washed with cold saline and the excess saline removed by suction. The cells were then re-suspended in Kreb's ringer phosphate buffer pH 6.8, and the suspension was homogenized by passing it through a syringe several times. Each 100 ml. of this suspension contained 5 gm. of wet cells.

Four flasks each containing 25 ml. of the cell suspension were incubated on a Dubanoff Shaker (100 strokes per minute) at 30° C. For analysis, 5 ml. samples were aseptically withdrawn from the flasks at 6 hour intervals. The samples were acidified with 0.2 ml. of conc. HCl and preserved at -12° C. Finally the samples were autoclaved at 121° C for 15 minutes and the resulting mixutre saturated with sodium sulfate. Riboflavin was extracted from the mixture by shaking with 5 ml. of benzyl alcohol. The benzyl alcohol layer was separated by centrifugation.

Riboflavin was measured by its absorbancy at 445 mμ. The results are shown in Table II. This experiment shows that almost two-third of the riboflavin is synthesized during the first 12 hour period of incubation reaching a maximum value after 24 hours. Different flasks show a significant variation in the amount of riboflavin synthesized under the same conditions of incubation.

2. Effect of glucose on the incorporation of acetate into riboflavin

In one experiment cells were grown for 24 hours. Subsequently, acetate-1-C^{14}, acetate-2-C^{14} and pyruvate-2-C^{14} were added to the growth medium. After 12 hours of incubation riboflavin was isolated and purified as described before. No radioactivity was detectable in riboflavin.

Presence of glucose in the medium was thought to be a possible cause of the low radioactivity of riboflavin, since initially the medium contains 4 percent glucose (see Chapter II, p. 15).

The effect of glucose on the incorporation of radioactivity into riboflavin was investigated. Cells were grown as described previously. Intermittent samples of the culture were withdrawn aseptically for analysis. Glucose was measured enzymatically in these samples. After 48 hours of

Table II

Time Study of Riboflavin Production

Time in hours	µmoles of riboflavin produced per 5 ml. of cell suspension			
	Flask 1	Flask 2	Flask 3	Flask 4
6	0.075	0.160	-	-
12	-	-	0.18	0.19
18	0.27	0.38	-	-
24	-	-	0.25	0.39
48	0.34	0.43	-	-
72	-	-	0.22	0.40

incubation the concentration of glucose fell below 1.0 mg. per 100 ml. One-hundred ml. portions of the culture was transferred to two sterile flasks. One of these flasks contained 2 gm. of glucose. Both the samples were incubated with acetate-2-C^{14} (1.5×10^6 c.p.m. in 0.02 umole) for 12 hours. During incubation sterile, CO_2-free air was pumped through the flasks at a uniform rate, and the liberated CO_2 was trapped in 1 N NaOH.

The incubation was stopped by adding 2 ml. of conc. HCl to each flask. Riboflavin was extracted, purified and crystallized to constant specific activity as previously described in procedures. For the measurement of radioactivity in carbon dioxide, it was plated as sodium carbonate. Table III sums up the results.

Considerable dilution of radioactivity was caused by the presence of glucose. The ratio of specific activity (counts per minute per umole) of riboflavin synthesized in absence of glucose to that synthesized in presence of 2 percent glucose was 13:1. However, the acetate oxidized to CO_2 gave a ratio of 1.7:1 and a 30 percent excess riboflavin was synthesized in presence of added glucose. In view of the results shown in Table II, this increase may not be significant.

Table III

Effect of Glucose on the Incorporation of Acetate-2-C^{14}*

Flask	Riboflavin Synthesized umole	Riboflavin** c.p.m./ umole	Riboflavin total counts	Acetate percent incorporation in riboflavin	CO_2 Total activity
1. With 2 percent glucose	8.6	81.5	7.0×10^2	0.05 %	294×10^3
2. Without glucose	5.6	1570.0	8.8×10^3	0.88 %	515×10^3

* The activity of substrate added was 1.5×10^6 c.p.m./0.02 umole of the salt solution.

** These values refer to radioactivity measured after the addition of 25 mg. of carrier riboflavin, followed by four crystallization from conc. HCl.

3. The incorporation of acetate-1-C^{14} and pyruvate-2-C^{14} into acetoin in growing cells.

The only known metabolic route through which carboxyl labelled acetate can enter into acetoin involves its passage through the hexose monophosphate shunt resulting in the randomization of the label; the direct reduction of acetate to acetaldehyde is known to occur only in anaerobes. As acetate is known to be incorporated into riboflavin it was necessary to find how important is the incorporation of acetate and pyruvate into acetoin in E. ashbyii.

Cultures of E. ashbyii were grown for 24 hours in two flasks containing a total volume of 900 ml. of the medium after 24 hours the contents of the two flasks were mixed aseptically, and 400 ml. portions of the culture were incubated with acetate-1-C^{14} and pyruvate-2-C^{14} separately. After 12 hours of incubation 5 ml. conc. HCl was added to each flask and the whole mixture directly distilled in vacuo to almost complete dryness.

The distillate containing acetoin was treated with 2:4,dinitrophenylhydrazine to convert all the acetoin to acetoin 2:4, dinitrophenylosazone. The osazone was repeatedly crystallized to constant specific activity from different solvents. After the second recrystallization there was no appreciable drop in radioactivity.

The osazone is insoluble in common organic solvents but dissolves rapidly in hot nitrobenzene or aniline. The high solubility in these two solvents may cause co-precipitation of impurities. In order to confirm the purity of the osazone, it was dissolved in 3 liters of glacial acetic acid under reflux. The material recrystallized this way showed no change in radioactivity.

The ratio of specific activity of osazone obtained from pyruvate-2-C^{14} and acetate-1-C^{14} was 6.5:1. It also showed the total incorporation of pyruvate into acetoin to be 2.4 percent and of acetate into acetoin to be 0.4 percent. The values of specific activity of the osazone are summarized in Table IV.

4. Incorporation of acetate and pyruvate into riboflavin and acetoin in standing cultures

Our previous experiment showed that pyruvate is a more effective precursor of acetoin than acetate. To obtain a correlation between the labelling of riboflavin by acetate and pyruvate, the incorporation of these substrates into both acetoin and riboflavin was simultaneously assayed.

Cells were grown for 24 hours, filtered, washed and suspended as described under Section I. Each 100 ml. of suspension contained 10 gm. of wet cells. In each experiment

Table IV

Specific Activity of Acetoin-2:4-dinitrophenylosazone*

Nature of crystallization	Acetoin c.p.m./umole	
	Acetate-1-C^{14}	Pyruvate-2-C^{14}
Original	134	394
1. Aniline	57	260
2. Nitrobenzene	49	258
3. Aniline	40	273
4. Nitrobenzene	40	255
5. Glacial acetic acid	50	-

* Each flask contained a total of 1×10^6 c.p.m. per 1 umole of radioactive substrate.

50 ml. of the cell suspension was used for incubation with radioactive substrate. After the addition of substrate, cells were incubated for 12 hours at 30° C on a Dubanoff shaker incubator (100 strokes per minute).

At the end of this period 3 ml. of conc. HCl was added to each flask to stop the reaction. Acetoin was isolated by adding 50 mg. of unlabelled acetoin to each flask and distilling the mixture to almost complete dryness, in vacuum.

Acetoin present in the distillate, was converted to the osazone and crystallized to constant specific activity. Riboflavin was isolated from the thick suspension remaining in the flask, after distillation. This suspension was taken up from the flask in 10 ml. of distilled water and autoclaved at 121° C. Riboflavin was extracted from this mixture as described previously and crystallized to constant specific activity.

Table V sums up these results. Here unlike the experiments with growing cells (Section 3), the ratio of specific activity of acetoin from acetate-1-C^{14} and pyruvate-2-C^{14} is 1:60.

Table V

Incorporation of Acetate and Pyruvate in Riboflavin and Acetoin

Labelled* substrate	Unlabelled substrate	Riboflavin			Acetoin	
		µmole formed	c.p.m./µmole	Total c.p.m.	c.p.m./µmole	Total counts x10^3
Acetate-1-C^{14}	-	2.0	375.0	750.0	38.0	21.30
Acetate-1-C^{14}	Pyruvate 250 µmoles	2.1	280.0	600.0	31.0	22.40
Acetate-2-C^{14}	-	2.6	1450.0	2900.0	600.0	342.0
Acetate-2-C^{14}	Pyruvate 250 µmoles	2.5	1500.0	3000.0	630.0	363.0
Pyruvate-2-C^{14}	-	2.8	506.0	1420.0	2130.0	1200.0
Pyruvate-2-C^{14}	Acetate 250 µmoles	2.3	413.0	825.0	2700.0	1540.0

* 10.0×10^6 c.p.m. of total activity in 17 µmoles of salt solution.

5. Incorporation of acetate, pyruvate and acetoin in riboflavin in standing cultures

In this experiment several modifications were introduced into the original design of the experiments done so far. In addition to these modifications radioactive acetoin was added to the list of the substrates. Several changes were deemed necessary in order to arrive at a definite conclusion as to the role of acetoin in riboflavin synthesis. For this reason additional control experiments were performed using non-radioactive diluent. Labelled acetate was incubated alone and together with non-labelled acetoin, similarly labelled acetoin was incubated alone and in combination with either non-labelled acetate or non-labelled pyruvate.

For this experiment we used a cell suspension containing 5 gm. of wet cells per 100 ml. of suspending medium, prepared as described under Section I. A batch of 40 flasks, each containing 15 ml. of the cell suspension was divided into two groups.

To the first group all the substrate (labelled and unlabelled) was added in one portion. The flasks were incubated on a Dubanoff shaker at 30° C for 12 hours. At the end of this period 0.2 ml. of conc. HCl was added to each flask to stop the reaction.

The other group was first incubated with half the substrate as described above. After 12 hours of incubation the remaining half of the substrate was added and incubation continued for an additional 12 hours, thus bringing the total incubation period to 24 hours. The incubation was stopped by adding 0.2 ml. of conc. HCl to each flask. The riboflavin was isolated by extraction with benzyl alcohol and purified on a florisil column as described in the experimental section. Specific activity of riboflavin was determined on a 0.1 ml. aliquot of this eluate. The remaining riboflavin was converted to lumichrome as described under methods.

The specific activity was determined on the purified lumichrome and the results are summed up in Table VI.

This experiment was repeated once more with the following modification. Instead of incubating the cells on Dubanoff shaker incubator, CO_2 free air was bubbled through the flasks and the liberated CO_2 was trapped in carbonate free sodium hydroxide. The riboflavin was crystallized three times from concentrated HCl by the addition of 25 mg. of unlabelled riboflavin. The purified riboflavin was converted to lumichrome as described before. The results of this experiment appear in Table VII.

Table VI

Incorporation of Different Precursors into Riboflavin and Lumichrome*

Results of Experiment 5

	Riboflavin		Lumichrome	
Flask	c.p.m./μmole	Total c.p.m.	c.p.m./μmole	Total c.p.m.
Acetoin-1-C^{14}	4700	760	75	13
Acetoin-1-C^{14} + unlabelled acetate	4960	789	187	25
Acetoin-1-C^{14} + unlabelled pyruvate	2032	580	0	0
Pyruvate-2-C^{14}	5212	1261	42	8
Pyruvate-2-C^{14} + unlabelled acetoin	5950	1510	58	15
Acetate-2-C^{14}	17925	4554	710	173
Acetate-2-C^{14} + unlabelled acetoin	20125	4000	930	220
Acetate-1-C^{14}	4247	946	523	109
Acetate-1-C^{14} + unlabelled acetoin	6650	1694	350	85

The figures in the table represent each an average of 4-6 determinations.

1. Specific activity of acetoin was 5 x 10^5 c.p.m./40 μmole.
2. Specific activity of all radioactive substrate was 5 x 10^5 c.p.m./7.5 μmole of the salt solution.

Table VII

Incorporation of Different Precursors into Riboflavin and Lumichrome*
Results of Experiment 6

Flask	Riboflavin		Lumichrome		Carbon Dioxide	
	c.p.m./ µmole	Total c.p.m.	c.p.m./ µmole	Total c.p.m.	Percent oxidized	Total c.p.m.
Acetoin-1-C^{14}	38	19	19	21	0.24	1.2×10^3
Acetoin-1-C^{14} + acetate	78	25	25	8	0.24	1.2×10^3
Acetoin-1-C^{14} + pyruvate	43	13	11	6	0.7	3.5×10^3
Pyruvate-2-C^{14}	407	215	71	35	4.4	22.0×10^3
Pyruvate-2-C^{14} + acetoin	638	346	121	55	5.0	25×10^3
Pyruvate-2-C^{14} + acetate	130	35	89	24	1.20	6×10^3
Acetate-2-C^{14}	585	137	510	128	6.11	32×10^3
Acetate-2-C^{14} + acetoin	500	164	210	71	6.6	33×10^3
Acetate-2-C^{14} + pyruvate	700	176	452	110	5.4	27×10^3
Acetate-1-C^{14}	350	296	125	105	28.0	141×10^3
Acetate-1-C^{14} + acetoin	305	103	250	90	23.0	113×10^3
Acetate-1-C^{14} + pyruvate	350	63	300	80	10.0	51×10^3

* 1. The figures in the table represent each an average of 2 determinations.

2. Specific activity of acetoin was 5×10^5 c.p.m./40 µmole.

3. Specific activity of all radioactive substrate was 5×10^5 c.p.m./7.5 µmole of the salt solution.

CHAPTER IV

DISCUSSION

The riboflavin molecule can be divided into three metabolic subunits. A heterocylic purine like structure which is fused to a aromatic xylene ring comprises the isoalloxazine portion of the molecule to which is attached a ribitol side chain at position 9.

It has been shown that purines can be incorporated into riboflavin, with only the loss of carbon 8 (24, 25, 26). Further evidence suggests that the mechanism of biosynthesis of this portion of riboflavin is very similar to purines (27). Plaut (51) has obtained evidence suggesting that in the formation of ribityl side chain both glycolytic and hexose monophosphate shunt pathways are operative. However, very little is known about the mechanism of biosynthesis of aromatic xylene portion of the molecule.

Studies with simple precursors have given rise to controversial results. Plaut (44) has shown that both carboxyl labelled acetate and methyl labelled acetate enter into its formation while Klungsøyr (46) claims that carboxyl labelled acetate did not enter the xylene ring. The role of the four carbon intermediate, acetoin, was first suggested by Masuda (41). Later Kishi *et al.* (42) claimed that cell

free system of E. ashbyii could convert acetoin to a precursor of riboflavin i.e. compound G in presence of an acceptor compound. Further support of acetoin as an intermediate came from the work of Goodwin (43) who reported (in a preliminary communication) that acetoin-1-C^{14} was more effectively incorporated into riboflavin than acetate-2-C^{14}.

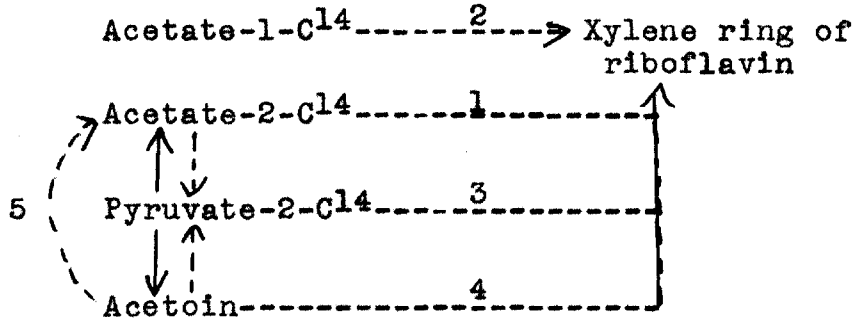
The incorporation of carboxyl labelled acetate into the xylene ring is evidence against the involvement of acetoin as an obligatory intermediate, as already discussed in the introduction.

We have reinvestigated the nature of the precursors of xylene ring using four different labelled precursors; namely, acetate-1-C^{14}, acetate-2-C^{14}, pyruvate-2-C^{14} and 1-C^{14}-3 hydroxy-2-butanone (acetoin). The precursors were incubated with either growing cells or standing cells suspended in a buffer.

Methods of quantitative assay and purification of riboflavin and its photodegradation product, lumichrome, were improved and modified. In several experiments riboflavin was further purified by addition of unlabelled riboflavin and crystallization to constant specific activity. Methods of purification and estimation of acetoin were also modified.

The simple precursors with the exception of acetoin are biologically very active and participate in a large

number of metabolic reactions, therefore, enough control experiments had to be designed to eliminate extraneous mechanisms. The various theoretical pathways through which these precursors can theoretically enter into riboflavin are illustrated below.



In our experiments using growing cultures of E. ashbyii we found that pyruvate-2-C^{14} is incorporated much more effectively into acetoin than acetate-1-C^{14}. The extent of incorporation being six-fold in growing cells (Table IV), and sixty-fold in standing cultures (Table V). The increased rate of incorporation of pyruvate in the case of standing cells could be due to differences in the quantitative importance of various metabolic pathways under the different conditions.

If we assume that acetoin is a precursor of riboflavin, then the incorporation of acetoin or any compound

that is directly converted to acetoin should yield riboflavin of relatively high specific activity. Since pyruvate is known to be a direct precursor of acetoin and has been shown to produce acetoin of high specific activity in our experiments, its incorporation into riboflavin should be considerable if acetoin were a direct precursor of riboflavin.

In experiment No. 4 (Table V), we have measured the relative incorporation of acetate and pyruvate simultaneously into acetoin and riboflavin. We found that the specific activity of riboflavin was maximal in case of acetate-2-C^{14} and the specific activity of acetoin was highest in case of pyruvate-2-C^{14}. The riboflavin had almost the same activity in case of acetate-1-C^{14} and pyruvate-2-C^{14}. It is of interest to note that known metabolic pathways can easily convert pyruvate-2-C^{14} to acetate-1-C^{14}. These results do not support the hypothesis that acetate is converted to acetoin before its incorporation in riboflavin.

The appearance of radioactivity in the isolated product is not a definite indication of a precursor product relationship. An added substrate may be degraded to simpler substances and enter into a common metabolic pool from which the desired product is synthesized. One way to test the precursor product relationship is to add a large unlabelled

excess of the suspected intermediate. This will tend to dilute the label and decrease the specific activity of the product, unless the intermediate is strongly bound to the enzyme in some modified form.

In experiments 4 and 5 we have added appropriate unlabelled diluents to all the radioactive precursors that were used. In no case was there any significant dilution effect. Acetoin did not decrease the labelling from pyruvate or acetate and vice versa.

Experiment No. 5 was designed to give more conclusive information about the nature of precursors of xylene ring. Radioactive acetoin was synthesized and added to the list of the substrates. Because the incorporation of added substrates was in general very low, we ran at least four duplicates for each experiment. Another possible source of complication was anticipated to be a rapid oxidation of the substrate. To examine this possibility radioactive substrate was added either all at once or in two divided portions at different intervals. This technique, however, did not improve the incorporation of substrate into riboflavin.

Riboflavin was isolated from florisil columns and assayed for specific activity. It was then converted to lumichrome by exposing it to sunlight. This is done because the added radioactive precursors can either enter into the

formation of ribitol side chain or the xylene ring of riboflavin, estimation of radioactivity in lumichrome was a measure of radioactivity in the xylene ring.

The results of this experiment appear in Table VI. Since in this experiment riboflavin was not crystallized to constant specific activity, the high specific activity of riboflavin is questionable. However, our method of purification of lumichrome yields the product of very high purity. It is seen from these results that only acetate-1-C^{14} or acetate-2-C^{14} retain significant activity in lumichrome, while acetoin hardly labelled the lumichrome at all.

The experiment was repeated once more and riboflavin was purified to constant specific activity prior to its degradation to lumichrome. The results are very similar except that the original riboflavin shows less activity. It is quite possible that an unknown compound very similar to riboflavin was present as contamination, which could not be resolved on florisil column but disappears upon crystallization.

These experiments give evidence against acetoin being an intermediate in the riboflavin formation. Incorporation of both radioactive acetoin and its immediate precursor pyruvate is relatively very poor. This could not be due to poor permeability of the cells to acetoin and pyruvate as in these experiments the cells metabolized both acetoin and pyruvate to CO_2.

The manner of incorporation of acetate into riboflavin indicates that both methyl and carboxyl group of this compound enter into its formation. Because the overall incorporation of acetate is less than 0.2 percent, it is doubtful that this compound enters directly into the formation of the xylene ring. The other possibility is that an unknown intermediate is involved which is still unidentified and into which acetate is incorporated probably in an activated form.

In support of this was the finding that glucose caused a marked dilution of the incorporation of acetate into riboflavin. This could not be a simple dilution of the specific activity of the acetate added by glucose degradation, as there was a much greater inhibition of acetate incorporation into riboflavin than acetate oxidation to CO_2.

The nature of the direct precursor of the xylene ring remains to be investigated further. There are not enough clues available at present to hypothesize about the nature of the possible intermediate with any certainty. More progress in this direction is expected when a cell free system carrying the individual enzymatic reactions is developed.

SUMMARY

The role of acetoin as an intermediate in riboflavin biosynthesis as well as the nature of the precursors of xylene ring has been investigated. Methods have been developed for the purification and quantitative estimation of riboflavin, lumichrome and acetoin. Method for the organic synthesis of acetoin has been modified. The experiments were carried on either growing cells or resting cells suspended in a buffer. Our studies did not give any indication of acetoin being a precursor of riboflavin as has been postulated by Masuda, Kishi and Goodwin. However, both acetate-1-C^{14} and acetate-2-C^{14} was incorporated into the aromatic ring of riboflavin. The overall incorporation of the substrate examined was below 0.2 percent.

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