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THE ROLE OF THIAMINE, LEUCINE AND CO_2 IN THE BIOSYNTHESIS OF CAROTENES BY THE MOLD *PHYCOMYCES BLAKESLEEANUS*

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Abstract—It has been demonstrated that the mold *Phycomyces blakesleeanus* can metabolize thiamine ([2-^{14}C]thiazole) to $^{14}CO_2$. In addition, the $^{14}CO_2$ produced can be incorporated into mevalonic acid and β-carotene when the mold was incubated in the presence of non-radioactive leucine. The role of leucine in the fixation of $^{14}CO_2$ has been implicated by a study of the labeling patterns of mevalonic acid and β-carotene; thus, mevalonic acid was labeled primarily in the C-1 carboxyl group, with the remaining 11 per cent of the total ^{14}C in the C-5 carbon atom. This labeling pattern suggests that $^{14}CO_2$ was fixed to β-methylcrotonyl CoA, a leucine metabolite, which could then give rise to [1-^{14}C]β-hydroxy-β-methylglutaryl CoA. [1-^{14}C]β-Hydroxy-β-methylglutaryl CoA could then be reduced directly to [1-^{14}C]mevalonic acid, the major reaction, or it could be split by the cleavage enzyme 3-hydroxy-3-methyl glutaryl CoA-acetoacetate lyase (4.1.3.4) to [1-^{14}C]acetoacetate and acetyl CoA. These small molecules, by a process involving activation and condensation, could then randomize the ^{14}C to give [5-^{14}C]β-hydroxy-β-methylglutaryl CoA which would be reduced to [5-^{14}C]mevalonic acid. The loss of the C-1 carbon of mevalonic acid at the formation of isopentenylpyrophosphate would account for the low incorporation of $^{14}CO_2$ into β-carotene.

INTRODUCTION

THE MOLD *Phycomyces blakesleeanus* has long been used as an organism for the study of carotene biosynthesis. Friend and Goodwin[1] reported that the organism required thiamine for both growth and carotenogenesis when grown in a medium containing asparagine and glucose.

Studies by Goodwin and Lijinsky[2] and Mackinney *et al.*[3] demonstrated that carotene synthesis was greatly stimulated when leucine was substituted for one-half of the asparagine in the medium. More recent studies by Chichester *et al.*[4] have explained the reason for leucine stimulation of carotene synthesis. They found that various labeled ^{14}C leucines could be broken down into small units and that these units could be incorporated into β-carotene presumably by way of a six-carbon intermediate such as β-hydroxy-β-methylglutaric acid or mevalonic acid.

The thiamine requirement of the mold *P. blakesleeanus* has been well established[1] but

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[1] J. FRIEND and T. W. GOODWIN, *Biochem. J.* **57**, 534 (1954).
[2] T. W. GOODWIN and W. LIJINSKI, *Biochem. J.* **50**, 268 (1952).
[3] G. MACKINNEY, T. O. M. NAKAYAMA, C. D. BUSS and C. O. CHICHESTER, *J. Am. Chem. Soc.* **74**, 3456 (1952).
[4] C. O. CHICHESTER, H. YOKOYAMA, T. O. M. NAKAYAMA, A. LUKTON and G. MACKINNEY, *J. Biol. Chem.* **234**, 598 (1959).

thiamine metabolism has not been studied in this mold. Thiamine metabolism, however, has been extensively studied in the rat by Pearson *et al.*[5] They have shown that the thiazole ring of thiamine undergoes extensive degradation yielding $SO_4^=$, CO_2, and some unidentified products. The pyrimidine component of thiamine is metabolized to pyrimidine carboxylic acid.

Thiamine metabolism in *P. blakesleeanus* presented an interesting problem. If thiamine ([2-^{14}C]thiazole) was metabolized to $^{14}CO_2$ as in the rat,[5] would the mold utilize this $^{14}CO_2$ for β-carotene synthesis? If this $^{14}CO_2$ fixation occurred, how would $^{14}CO_2$ be incorporated into β-carotene and what role would leucine have in this fixation? The present investigation was undertaken to answer these questions.

RESULTS

Thiamine requirements. The levels of thiamine required for optimum growth and carotenogenesis in *P. blakesleeanus* grown in an asparagine–glucose medium have been established at 2·0 μg thiamine/100 ml of medium.[1] We have shown, using this method, that the same amount of thiamine was required when the mold was grown on a leucine–asparagine–glucose medium.

TABLE 1. DESTRUCTION OF THIAMINE ([2-^{14}C]THIAZOLE) BY *Phycomyces blakesleeanus*

Traps changed after	Total radioactivity in $BaCO_3$ (dis/min)	Specific activity (dis/min/mg/$BaCO_3$)
Asparagine–glucose medium		
3 Days	266	981
5 Days	10,456	2361
8 Days	12,487	3396
11 Days	12,307	2473
Leucine–asparagine–glucose medium		
2 Days	158	53·0
4 Days	2619	1223
8 Days	11,401	2373

Asparagine–glucose medium. Eighteen petri dishes, each containing 20 ml of asparagine–glucose medium and 0·4 μg thiamine ([2-^{14}C]thiazole) (total radioactivity added in the experiment: $4{\cdot}21 \times 10^6$ dis/min) were incubated in the vacuum system described in the text. The cultures were swept with CO_2-free air for 6 hr in the daytime and kept in the closed desiccator overnight. The total time of incubation was 11 days. The outlet traps were changed at intervals, and the CO_2 trapped was counted as $BaCO_3$–Cab-O-Sil suspensions.

Leucine–asparagine–glucose medium. This experiment was conducted the same way as the above described experiment with the following exception: the cultures were grown in a leucine–asparagine–glucose medium containing a total of $5{\cdot}49 \times 10^6$ dis/min of thiamine ([2-^{14}C] thiazole). The cultures were swept with CO_2-free air for 6 hr, and kept in a closed desiccator for 42 hr. The total time of incubation was 8 days. The outlet traps were changed at intervals and the $BaCO_3$ counted as indicated above.

Metabolism of thiamine ([2-^{14}C]*thiazole*). The production of $^{14}CO_2$ by *P. blakesleeanus* incubated with thiamine ([2-^{14}C]thiazole) in asparagine–glucose medium and leucine–asparagine–glucose medium is shown in Table 1. The results show that thiamine was metabolized to $^{14}CO_2$ when the mold was grown in either of the two media. The low yield of $^{14}CO_2$, trapped as $BaCO_3$, can be explained by the fact that the $^{14}CO_2$ produced was trapped

[5] M. BALAGHI and W. N. PEARSON, *J. Nutr.* **91**, 9 (1957).

for only a short time and that the $^{14}CO_2$ which did build up in the desiccators could have been utilized for carotene or other CO_2 fixation reactions.

The lower total activity and specific activity of $Ba^{14}CO_3$ found in cultures grown in the leucine–asparagine–glucose medium can be explained by the fact that the $^{14}CO_2$ was trapped for only 6 hr out of every 48 hr, as opposed to 6 hr out of 24 hr with cultures grown in the asparagine–glucose medium.

At the end of the incubation period, the cultures were harvested according to procedures described in Experimental. The results, shown in Table 2, show that no significant radioactivity was incorporated into the carotenes when the mold was grown in the asparagine–glucose medium. However, a small but significant amount of radioactivity was incorporated

TABLE 2. RADIOACTIVITY INCORPORATED INTO β-CAROTENE BY *Phycomyces blakesleeanus* CULTURES INCUBATED INCUBATED WITH THIAMINE ([2-^{14}C]THIAZOLE)

Fraction	Total activity (dis/min)	Specific activity (dis/min/mg pigment)
Asparagine–glucose medium		
Phytoene	0	—
Phytofluene	28	—
β-Carotene (crude)	77	100
β-Carotene (1st crystals)	15	36
Medium	667,000	—
Leucine–asparagine–glucose medium		
Phytoene	1168	—
Phytofluene	97	—
β-Carotene (1st crystals)	308	248
β-Carotene (2nd crystals)	185	243
Medium	$1{\cdot}63 \times 10^6$	—

Asparagine–glucose medium. The conditions used are described in Table 1. The carotene isolation and chromatography procedures are described in Experimental. β-Carotene was crystallized once; the other fractions were not crystallized. A portion of the culture medium was also counted.

Leucine–asparagine–glucose medium. The conditions used are as described above except for the following: β-carotene was crystallized twice; the other fractions were not crystallized.

into β-carotene when the mold was grown in the leucine–asparagine–glucose medium. Although the amount of ^{14}C incorporated into β-carotene was small, the specific activity of β-carotene remained constant after two recrystallizations.

In both media, however, the majority of the radioactivity was found in the culture media. If the levels of ^{14}C found in the leucine–asparagine–glucose medium ($1{\cdot}63 \times 10^6$ dis/min) are compared with the level of ^{14}C found in the asparagine–glucose medium ($0{\cdot}667 \times 10^6$ dis/min), it can be seen that the leucine–asparagine–glucose medium is about $2\frac{1}{2}$ times more radioactive than the asparagine–glucose medium, even though the initial level of thiamine ([2-^{14}C]thiazole) was only about 20 per cent higher ($5{\cdot}49 \times 10^6$ vs. $4{\cdot}21 \times 10^6$ dis/min). The identity of the radioactive components of the media was not determined.

To test the validity of thiamine destruction by the mold, a control experiment was run in which 422,100 dis/min of thiamine ([2-^{14}C]thiazole) was incubated without *P. blakesleeanus*. The experiment was conducted like the asparagine–glucose experiment described in

Table 1. At the end of 11 days, only 85 dis/min was found in the $BaCO_3$. Therefore it can be concluded that the production of $^{14}CO_2$ was the result of a biochemical reaction, not just a photodecomposition of thiamine by light and air.

Incorporation of $^{14}CO_2$ into carotene intermediates and β-carotene. Growing cultures that had just begun to form carotene were exposed to a $^{14}CO_2$ atmosphere for 24–48 hr and the carotenes extracted and counted. Table 3 shows that although the amount of radioactivity incorporated into β-carotene was small, a significant amount of radioactivity remained after crystallization.

Identification of the [^{14}C]β-carotene intermediates. The possibility that the medium from cells grown in $^{14}CO_2$ contained [^{14}C]β-carotene intermediates was investigated by fractionation of the medium from $Ba^{14}CO_3$ fed cultures. Cultures grown in the presence of 100×10^6 dis/min of $Ba^{14}CO_3$ (see Table 3) showed $5{\cdot}31 \times 10^6$ dis/min of radioactivity

TABLE 3. INCORPORATION OF $^{14}CO_2$ INTO β-CAROTENE BY *Phycomyces blakesleeanus*

Fraction	Total activity (dis./min)	Specific activity (dis./min/mg carotene)
Acetone extract (lipids)	897,000	—
Non-saponifiable	582,000	—
β-Carotene (crude)	33,500	224,800
β-Carotene (crystals)	18,800	160,600
Medium	$5{\cdot}31 \times 10^6$	—

Eighteen petri dishes each containing 20 ml of leucine–asparagine–glucose medium, were incubated under Gro-lux illumination for 48–56 hr. The carotene producing cultures were then placed in an atmosphere containing 0·3% CO_2 and 100×10^6 dis/min of $^{14}CO_2$ for 24–48 hr. The carotenes were extracted and chromatographed as described in the text.

in the combined medium–water–extract. After steam distillation of the concentrated extract, the steam-volatile fraction contained 1·04% of the radioactivity and the non-volatile fraction 98·96%.

Paper chromatography of the steam-volatile fraction in ethanol–ammonia–water (80:5:15) showed a single radioactive spot with R_f 0·47. The possible identity of this spot with acetic acid was dispelled when a mixture of [2-^{14}C]acetic acid and the steam-volatile fraction were co-chromatographed. Two peaks appeared, one at R_f 0·58, corresponding to acetic acid, and the other at R_f 0·48, corresponding to the steam-volatile spot. Attempts were made to identify the steam-volatile fraction by gas chromatography on a Perkin–Elmer model 226 gas chromatograph equipped with a flame ionization detector. Under conditions which would separate acetic acid and isobutyric acid (15 per cent BDS on 80–100 mesh Chromosorb W at 115°, flow rate 60 cm^3/min), no peak appeared with the steam-volatile fraction. It is possible that a small amount of non-volatile material passed over during the steam distillation.

The non-volatile fraction, containing the majority of the radioactivity ($2{\cdot}57 \times 10^6$ dis/min) was subjected to a group separation procedure to separate the non-volatile water-soluble fraction into acidic, basic, and neutral components. After fractionation, the organic acid fraction contained $2{\cdot}06 \times 10^6$ dis/min of radioactivity.

The organic acid fraction was identified by three different criteria. Paper chromatography in ethanol–ammonia–water (80:5:15) showed one radioactive spot at R_f 0·62. The

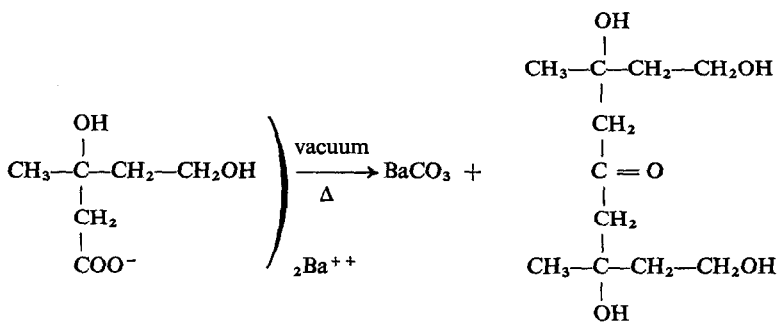
acid was then chromatographed with [2-^{14}C]mevalonic acid and compared with chromatograms of [3-^{14}C]3-hydroxy-3-methylglutaric acid and [2-^{14}C]mevalonic acid standards. The results showed that the unknown acid co-chromatographed with [2-^{14}C] mevalonic acid (R_f 0·64) as compared to R_fs of 0·61 for [2-^{14}C] mevalonic acid and 0·45 for [3-^{14}C]3-hydroxy-3-methylglutaric acid.

Chromatography of the unknown acid on Dowex-1-formate impregnated paper developed with 0·01N formic acid showed a single radioactive spot (R_f 0·66). Chromatography of the acid with [2-^{14}C]mevalonic acid showed a single radioactive spot at R_f 0·65. Chromatography of [3-^{14}C]3-hydroxy-3-methylglutaric acid and [2-^{14}C]mevalonic acid showed R_fs of 0·11 and 0·88 respectively. It was noted that the R_fs of mevalonic acid depended on the salts present in the sample; thus mevalonic acid free of salts had R_f 0·88, but after addition of a small amount of KCl or NaCl, the R_f dropped to 0·65.

The organic acid fraction was lactonized and the i.r. spectrum compared with commercial mevalonolactone. The i.r. spectra were identical in form, although the unknown sample showed some evidence of accompanying impurities. Nevertheless, the lactone bands, carbonyl bands, and C-H bands were identical.

On the basis of these three criteria, the unknown acid was identified as mevalonic acid.

TABLE 4. THE DECARBOXYLATION OF MEVALONIC ACID



Weight of barium mevalonate (mg)	307
Radioactivity of barium mevalonate (dis/min)	60,400
Theoretical yield of $BaCO_3$ (mg)	143
Actual yield of $BaCO_3$ (mg)	45·8
Percent yield of $BaCO_3$	31·5
Radioactivity found in $BaCO_3$ (dis/min)	8430
Radioactivity theoretically present in $BaCO_3$ (dis/min)	26,680
Radioactivity theoretically present in the carboxyl group of mevalonic acid ($BaCO_3$ × 2) (dis/min)	53,360
Per cent of label in the carboxyl group	88·3

The conditions used for the decarboxylation are described in Experimental.

Localization of the radioactivity in mevalonic acid. Cultures fed $^{14}CO_2$ produced the carotene intermediate [^{14}C]mevalonate. Mevalonolactone isolated from the culture medium was converted to barium mevalonate and pyrolyzed in a combustion tube. Table 4 shows the stoichiometry of the reaction and the results obtained. Barium carbonate contained 8403 dis/min and the yellow oil, deposited on the walls of the combustion tube, contained

7746 dis/min of radioactivity. Calculations in Table 4 show that the carboxyl group of mevalonic acid contained 88·3 per cent of the ^{14}C.

Rather than isolate the decarboxylated product from the pyrolytic degradation of mevalonic acid, and do further degradations on it, advantage was taken of the known labeling patterns between mevalonic acid and β-carotene.[6] A simple partial degradation of β-carotene would then give information pertaining to the distribution of label in mevalonic acid.

To locate the label in β-carotene, a partial chromic acid oxidation was performed on the pigment. The results, shown in Table 5, indicate less than 3% of the total radioactivity present in β-carotene was present in the acetic acid. The acetic acid liberated came from the 3′-methyl and 3-carbon positions of mevalonic acid.

TABLE 5. PARTIAL DEGRADATION OF β-CAROTENE

Theoretical yield of acetic acid (mequiv.)	0·269
Actual yield of acetic acid (mequiv.)	0·178
Per cent yield of acetic acid	66·4
Radioactivity found in acetic acid (dis/min)	578
Theoretical radioactivity in 0·269 mequiv. of acetic acid (dis/min)	870
Radioactivity of degraded β-carotene (dis/min)	29,880
Per cent of the radioactivity in acetic acid	2·91

β-Carotene was degraded by a chromic acid oxidation as described in Experimental.

DISCUSSION

The results of these experiments have shown that thiamine ([2-^{14}C]thiazole) can be metabolized to $^{14}CO_2$ and that this metabolic CO_2 can be incorporated into β-carotene. The incorporation of $^{14}CO_2$ into β-carotene is dependent on leucine since there is essentially no incorporation in the absence of this amino acid.

Radioactive mevalonic acid has been isolated from the medium of *P. blakesleeanus* cultured in leucine–asparagine–glucose medium grown in the presence of $^{14}CO_2$. Pyrolytic decarboxylation of mevalonic acid yielded $^{14}CO_2$ which represented 88·3 per cent of the radioactivity present in mevalonic acid. This $^{14}CO_2$ came from the C-1 carboxyl group of mevalonic acid. The amount of radioactivity found in the other product indicated that a small amount of radioactivity was present in the other carbons of mevalonic acid.

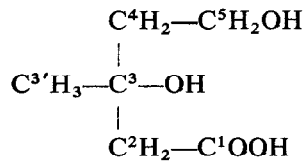
The pathway between mevalonic acid and β-carotene involves a decarboxylation reaction at the formation of isopentenyl pyrophosphate. The CO_2 lost at this step is the same carboxyl group which we have found to contain the majority of the radioactivity of mevalonic acid. This loss of labeled CO_2 then indicates that the label found in β-carotene must come from the remaining 5 carbon fragment.

Experimental results indicate that the pyrolytic decarboxylation product of mevalonic acid and presumably isopentenyl pyrophosphate contain only about 12% of the label initially present in mevalonic acid. Degradation studies on β-carotene yield indirect evidence that less than 3% of the radioactivity remaining in the decarboxylated mevalonic acid was present in the 3′-methyl and 3-carbon positions.

[6] C. O. CHICHESTER and T. O. M. NAKAYAMA, in *Biogenesis of Natural Compounds* (edited by P. BERNFELD), p. 641, Pergamon Press, 2nd. Ed., London (1968).

The only other logical place for radioactivity to be found would be the C-5 position of mevalonic acid. One would not expect to see any ^{14}C at the C-2 and C-4 methylene positions of mevalonic acid, since these carbons have no close relationship to CO_2 fixation. Studies on the formation of HMG (3-hydroxy-3-methylglutaric acid or β-hydroxy-β-methylglutaric acid) from acetate[7], and on the formation of HMG from the catabolism of leucine[8], and its subsequent conversion into mevalonic acid[9], would substantiate the C-5 labeling since HMG is a symmetrical molecule and can be cleaved into acetoacetic acid and acetic acid in either of two places. Equilibration of either or both acids, with pools present in *P. blakesleeanus*, would result in different labeling after resynthesis of HMG from these acids.

We have therefore concluded that mevalonic acid is labeled as shown:



Carbon	*% Of total radioactivity*
1	88·3
2	0
3	0·35
3′	
4	0
5	11·3

The labeling pattern indicated above explains the very low incorporation of $^{14}CO_2$ into β-carotene, since almost 90 per cent of the label in mevalonic acid is lost as $^{14}CO_2$ at the decarboxylation step leading to isopentenyl pyrophosphate. In contrast, the incorporation of ^{14}C from mevalonic acid labeled in the 2 position can be as high as 8·2%,[10] as opposed to the observed incorporation of 0·021% from $^{14}CO_2$.

The questions to be answered now are: how is CO_2 incorporated into mevalonic acid; and what role, if any, does leucine play in CO_2 fixation? As was mentioned before, previous studies have shown that $^{14}CO_2$ is incorporated into β-carotene much more readily in the presence of leucine than its absence.[4] Obviously, leucine and CO_2 are related in some manner since leucine stimulates carotenegenesis from $^{14}CO_2$.

The most obvious solution to this problem would be for $^{14}CO_2$ to be incorporated into leucine or some leucine metabolite. This intermediate could then presumably be converted into mevalonic acid, which in turn would be incorporated into β-carotene. Such a CO_2 fixation reaction with a leucine metabolite has been demonstrated in a liver system by Lynen.[8] Leucine is transaminated to α-ketoisocaproic acid, which in turn is decarboxylated and esterified with coenzyme A, giving isovaleryl CoA. Isovaleryl CoA is then dehydro-dehydrogenated to β-methylcrotonyl CoA, which reacts with CO_2 in a biotin reaction,

[7] H. Rudney, P. R. Stewart, P. W. Majerus and P. R. Vagelos, *J. Biol. Chem.* **241**, 1266 (1966).
[8] F. Lynen, *J. Cellular Comp. Physiol.* **54**, Supplement No. 1, p. 33 (1959).
[9] I. F. Durr and H. J. Rudney, *J. Biol. Chem.* **235**, 2572 (1960).
[10] H. Yokoyama, T. O. M. Nakayama and C. O. Chichester, *J. Biol. Chem.* **237**, 681 (1962).

yielding β-methylglutaconyl CoA. β-Methylglutaconyl CoA is then hydrated, giving HMG CoA, which in this liver system is split into acetoacetic acid and acetyl CoA.

HMGCoA, an intermediate in the catabolism of leucine, however, is a direct precursor of mevalonic acid and can be converted directly to mevalonic acid with NADPH and the enzyme, mevalonate:NADP oxidoreductase (1.1.1.34).[9] In addition to being a precursor of the sterols in yeast, [3-^{14}C]HMG has been shown to be incorporated into β-carotene in *P. blakesleeanus*.[11] One problem that remains is that if $^{14}CO_2$ was incorporated into β-methylglutaconyl CoA, the label would end up in the carboxyl group of mevalonic acid. Our experiments indicate that 88·3% of the label is in the carboxyl carbon and that the rest of the label is predominantly in the C-5 carbon. This can be explained by assuming that *P. blakesleeanus* contains an HMG CoA cleavage enzyme which would lead to a randomization of the label, a reasonable assumption since this cleavage enzyme has been found in bacteria.[12] Figure 1 shows the labeling patterns expected between $^{14}CO_2$ and mevalonic acid, along with possible pathways that would explain the randomization observed.

Experimentally, we find that the carboxyl carbon of mevalonic acid contains 88·3% of the ^{14}C, so the major fate of [1-^{14}C]HMG would be a direct enzymatic reduction to mevalonic acid. To explain the randomization of the label in mevalonic acid, we can visualize the cleavage of the remaining [1-^{14}C]HMG (11·7%) into [1-^{14}C]acetoacetate and acetyl CoA. [1-^{14}C]Acetoacetate could then be activated with coenzyme A to give [1-^{14}C]acetoacetyl CoA. At this point, the [1-^{14}C]acetoacetyl CoA could react in one of two ways:

(1) The major pathway, based on experimental findings, would be a simple condensation with acetyl CoA to give [5-^{14}C]HMG, which in turn would be emzymatically reduced to [5-^{14}C]mevalonic acid.

(2) A small amount of [1-^{14}C]acetoacetyl CoA could also be broken down by β-ketothiolase into [1-^{14}C]acetyl CoA and acetyl CoA. The acetyl CoA's produced could then be condensed in a different manner by β-ketothiolase, yielding [3-^{14}C]acetoacetyl CoA. Whether this cleavage and resynthesis would result in a randomization of label is doubtful, though quite possible. Nevertheless, if this randomization did occur, condensation of acetyl CoA with [3-^{14}C]acetoacetyl CoA would yield [3-^{14}C]HMG, which would be reduced to [3-^{14}C]mevalonic acid. This series of reactions would account for the labeling observed.

It must be emphasized that these enzymes have not been demonstrated in *P. blakesleeanus*, and that the actual pathway of CO_2 to mevalonic acid might be by some as yet unknown pathway. Nevertheless, the explanation given is reasonable since all of these enzymes have been isolated and characterized to some extent in other organisms. Attempts to prepare cell-free homogenates of *P. blakesleeanus* that will carry out these reactions have not been successful as yet.

EXPERIMENTAL

Culture conditions. Phycomyces blakesleeanus, NRRL 1554,(-) strain was grown from a spore inoculum in either a glucose–leucine–asparagine medium or a glucose–asparagine medium previously described.[4] Thiamine was included at a concentration of $1·0 \times 10^{-5}$%.

Gas system for CO_2 experiments. In experiments where CO_2 was trapped, a desiccator gas flow system was utilized. This system consisted of a desiccator with inlet and outlet stopcocks. The inlet was connected to

[11] H. YOKOYAMA, C. O. CHICHESTER and G. MACKINNEY, *Nature* **185**, 687 (1960).
[12] M. A. SIDDIQI and V. W. RODWELL, *J. Bacteria.* **93**, 207 (1967).

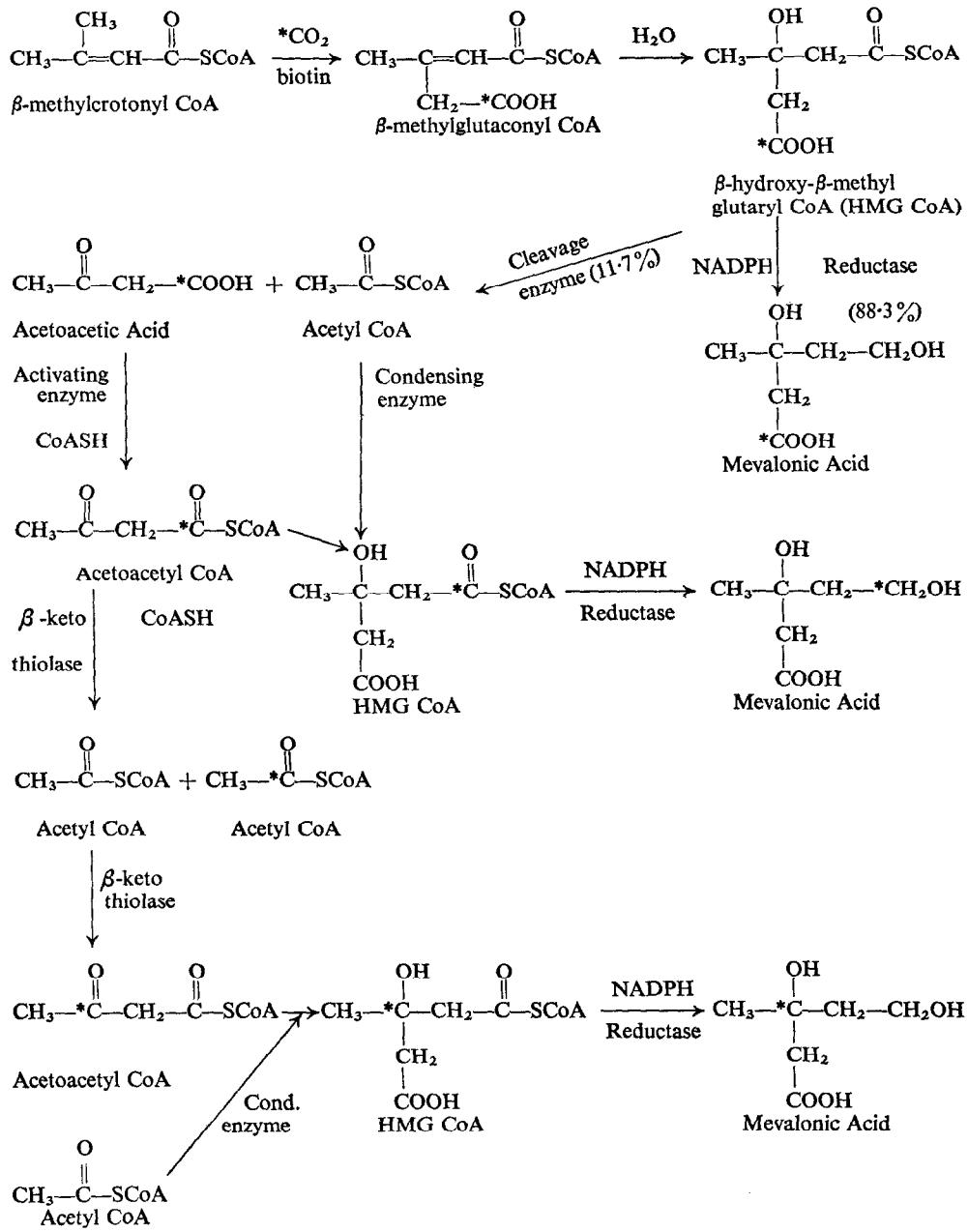


FIG. 1. GENERAL PATHWAY BETWEEN $C^{14}O_2$ AND MEVALONIC ACID.

two 2-l. Erlenmeyer CO_2 traps containing 10% NaOH. Petri dishes, containing the inoculated medium, were placed in the desiccator and the outlet connected to a series of four 10% NaOH CO_2 traps. The entire system was operated under a slight vacuum so that the inlet traps removed atmospheric CO_2 but still allowed oxygen to pass through into the desiccator. The expired CO_2 was trapped in the outlet traps and precipitated as $BaCO_3$. The entire apparatus was illuminated by Gro-lux lamps of 6×10^3 ergs/cm^{-2}sec intensity as measured with a YSI-Kettering Radiometer, model 65, with a 6551 probe. In some cases, the desiccator lid was alternately closed and opened over an interval of time to allow expired $^{14}CO_2$ to build up.

In experiments where $^{14}CO_2$ was fed to cultures, the desiccator containing the cultures was used as a CO_2 generator. A porcelain extraction thimble, containing Ba $^{14}CO_3$ was dropped into a beaker of excess 10% H_3PO_4 and the desiccator immediately closed.

Extraction, purification and determination of carotenes. The mycelial mats were collected on a Buchner funnel, minced with scissors and extracted with acetone and saponified as previously described.[13] The dried nonsaponifiable fraction was then chromatographed on a 1-mm thick layer of aluminium oxide G (Merck) developed with 50% Et_2O in hexane. This procedure separated the carotenes, which ran at the solvent front, from the sterols, which trailed behind. The carotene bands were removed from the plate and chromatographed on a column of MgO–Hyflo Super-Cel (1:2, w/w).[14]

The β-carotene band was eluted and recrystallized at least once after the addition of non-labeled carrier β-carotene. β-carotene and the other polyenes were determined spectrophotometrically.[15]

Isolation, separation and identification of mevalonic acid. Mevalonic acid was isolated from the media of *P. blakesleeanus* by a procedure involving extraction, steam distillation, ion exchange chromatography, and paper chromatography. The cultures were filtered and the mycelial mats washed with water. The water extract was combined with the filtrate, adjusted to pH 10 with NH_4OH, clarified by filtration, concentrated, adjusted to pH 3 with H_3PO_4 and steam distilled.

The steam volatile fraction was adjusted to pH 10 with NH_4OH, concentrated, and chromatographed on acid and base washed Whatman No. 1 paper using 95% ethanol–conc. NH_4OH–water (80:5:15). The nonvolatile fraction was made alkaline with NH_4OH, concentrated, and shaken with acid-washed Darco for 15 min to remove aromatic substances. The suspension was filtered and the filtrate, after adjustment of the pH to 10 was absorbed onto a large excess of Dowex 50W-X-4, H^+ form. After 30 min, the resin was filtered, and the filtrate containing the organic acids and neutral substances applied to a Biorex AG-1 column (Dowex-1-X8, 200–400 mesh, formate form) and washed with water until the neutral eluates showed a negative $KMnO_4$ reducing spot test. The organic acids were eluted with a 0–6 N formic acid linear gradient. All of the organic acid eluates were combined and evaporated to dryness repeatedly to remove formic acid.

The organic acids were then chromatographed alone and with standards on acid and base washed Whatman no. 1 paper using the solvent system described above. The spots were visualized with 0·04% Bromcresol Green in 95% ethanol. Radioactivity of the chromatograms was determined by cutting the papers into strips and scanning them in a Vangard chromatogram scanner, model 800. The unknown acid was then chromatographed on S & S No. 2493 paper (Dowex-1-chloride impregnated paper converted to the formate form) in 0·01 N formic acid, pH 3·2.

For i.r. spectroscopy, the unknown was converted to the lactone and extracted with Et_2O according to Guchhait and Porter.[16] After drying, the Et_2O extract was concentrated and the resulting oil spread between two NaCl windows and the i.r. spectrum run on a Beckman IR-5 against an air reference.

Degradation of mevalonic acid and β-carotene. Mevalonic acid was decarboxylated by formation of the barium salt and pyrolysis of this salt at 500° in a vacuum of 20 μ.[17] Pyrolysis was done in a Sargent Micro-Combustion apparatus using porcelain boats. After obtaining a vacuum of 10–20 μ, the heater was turned on and heated to 500°. A yellow oil condensed on the cool parts of the combustion tube. After cooling, the $^{14}CO_2$ was freed from the charred reactants with hot concentrated phosphoric acid. The CO_2 generated was trapped in $Ba(OH)_2$. The material deposited on the combustion tube was rinsed out and counted.

β-Carotene was partially degraded with chromic acid by a slight modification of the procedure previously described.[18] 6 mole of acetic acid are produced per mole of β-carotene in this reaction. 25 mg of [^{14}C]β-carotene was mixed with 1·30 g CrO_3, 0·33 g $K_2Cr_2O_7$ 3·23 ml distilled water and 2·15 ml conc. H_3PO_4 in a 20 ml two-neck pear-shaped flask. The flask was equipped with a reflux condenser and a tube inlet. The inlet was not used during the initial reaction. The reaction vessel was placed in a boiling water bath for 3·5–

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4 hr. After cooling, 3 ml of distilled water was added and the inlet opening fitted with a capillary tube. N_2 was allowed to pass through the capillary and bubble into the reaction mixture. The reflux condenser was removed and replaced by a distillation head. A micro steam distillation was run until the mixture began to froth; water was added and a total of 12 ml of water distilled. The distillate containing the acetic acid was concentrated and counted.

Scintillation counting techniques. All measurements of radioactivity were made with a Packard Tri Carb liquid scintillation spectrometer. The total volume of solution in the vials was 10 ml. Internal standards were used on selected samples to monitor quenching. The measurement of radioactivity in β-carotene samples was done following recrystallization and bleaching.[19] Water soluble samples were counted in the same solution. Barium carbonate was counted in Cab-O-Sil.[20] Radioactivity on paper chromatograms was first located in a scanner and then cut out and counted in the scintillation counter.[20]

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