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*THE METABOLISM OF ISOPRENOID PRECURSORS IN A PLANT SYSTEM**

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Introduction.—It has been shown earlier[1, 2] that the synthesis of the isoprenoid, rubber, by a rubber-bearing plant, guayule (*Parthenium argentatum*), involves acetate as an intermediate. Not only does the administration of acetate to this plant result in a net increase in the amount of rubber formed, but, in addition, rubber synthesized by plants supplied with doubly C^{14}-labeled acetate possesses the same specific activity as the acetate given, indicating that all the carbon atoms of the rubber derive from and channel through acetate.[3] Rubber, like other isoprenoids, is made up of repeating units of isoprene, which has the five-carbon branched-chain structure shown below. This structure appears, then, to be derived in the plant from acetate. It has also been shown in earlier work[1, 2] that the five-carbon acid β-methyl crotonate (BMC) is able to replace acetate in supporting rubber formation and is, in fact, more effective than acetate in this function. BMC, which is known as a naturally occurring plant constituent,[4] has been shown to become radioactive in plant tissues supplied with C^{14}-labeled acetate,[3] and it has been suggested that BMC arises from acetate. This paper is concerned with the pathway by which BMC is synthesized from acetate by a plant enzyme system. The data to be presented suggest that the reactions involved follow the general course outlined below.

Route of Synthesis of β-Methyl Crotonate in a Plant System

$$\underset{\text{Acetate}}{CH_3COOH} + CoA \leftrightharpoons \underset{\text{Acetyl-CoA}}{CH_3CO\text{-}CoA} \tag{1}$$

$$\underset{\text{Acetyl-CoA}}{2\ CH_3CO\text{-}CoA} \leftrightharpoons \underset{\text{Acetoacetyl-CoA}}{CH_3COCH_2CO\text{-}CoAO} + CoA \tag{2}$$

$$CH_3COCH_2CO\text{-}CoA + H_2O \leftrightharpoons CH_3COCH_2COOH + CoA \tag{3}$$

$$\underset{\text{Acetoacetate}}{CH_3COCH_2COOH} + \underset{\text{Acetyl-CoA}}{CH_3CO\text{-}CoA} \leftrightharpoons \underset{\beta\text{-OH, }\beta\text{-Methyl glutaryl-CoA}}{CH_3(HO)C(CH_2COOH)(CH_2CO\text{-}CoA)} \tag{4}$$

$$\underset{\beta\text{-OH, }\beta\text{-Methyl glutaryl-CoA}}{CH_3(HO)C(CH_2COOH)(CH_2CO\text{-}CoA)} \leftrightharpoons \underset{\beta\text{-Methyl crotonyl-CoA}}{(CH_3)_2C{=}CHCO\text{-}CoA} + CO_2 + H_2O \tag{5}$$

The key intermediate in this series of reactions is the substance β-hydroxy-β-methyl glutarate (BOG). This substance was first suspected as an intermediate in BMC synthesis when it was found by Bandurski *et al.*[5] to be a product of the metabolism of acetate by a leaf enzyme preparation. Bloch has suggested BOG as a possible intermediate in the synthesis of the isovaleryl carbon skeleton from acetate,[6] and Rabinowitz and Gurin have demonstrated the ability of a liver preparation to synthesize BOG from acetate.[7]

Materials and Methods.—Flax seedlings were the source of the enzyme system used in this work. It has been shown that flax seeds contain considerable amounts of BOG,[8] and flax was found to be more active in synthesizing BOG than other plants investigated in the present work. The plant material was grown from seed in vermiculite supplied with Hoagland's nutrient solution. The material was harvested after three weeks of growth under conditions of a 16-hour day (26° C.) and an 8-hour night (20° C.).

For the preparation of the enzyme, 40 gm. (fresh) of the apical inch were ground for 5 minutes in a mortar containing 5 gm. of washed sea sand and 80 ml. of a grinding medium modified from Rabinowitz and Gurin[9] and made up as follows: K_2HPO_4, 0.067 *M*; KH_2PO_4, 0.042 *M*; $MgCl_2$, 0.006 *M*; nicotinamide, 0.03 *M*; sucrose, 0.30 *M*. The resultant brei was strained through cheesecloth and the filtrate centrifuged at 500 × g for 10 minutes to remove debris. The greenish-yellow supernatant was then centrifuged at 30,000 × g for 40 minutes. The resultant pellet was triturated with four volumes of water and allowed to stand for 1 hour, after which the suspension was recombined with 40 ml. of the supernatant. The activity of the system appears to depend upon the presence of enzymes from the lysed particulate matter as well as upon components of the soluble fraction. The preparation of the enzyme was carried out at 1° C.

Reaction mixtures were incubated in stoppered 250-ml. Erlenmeyer flasks, in general, for 16 hours at 23° C. The standard reaction mixture included, in addition to substrates, ATP, GSH, and 5 ml. of enzyme (containing 4–5 mg. of protein per milliliter) in a total volume of 7 ml. BOG, BMC, and acetoacetate were added in the amount of 0.1 *M* to the reaction mixture. Radioactive substrates (1-C^{14}-acetate, 3-C^{14}-BMC, and $KHC^{14}O_3$) were used in the amount of 5 μM per reaction vessel. At the end of the experimental period, 2 ml. of reaction mixture were removed and added to an equal volume of saturated 2,4-dinitrophenylhydrazine in 2 *N* HCl for removal of carbonyl-containing compounds. To the remainder of the reaction mixture, 2 ml. of 1 *N* KOH were added and the solution allowed to remain for 1 hour at room temperature for hydrolysis of acyl-CoA derivatives. The solution was then adjusted to pH 2 and centrifuged, and the supernatant was extracted continuously with ether for 24 hours for removal of organic acids.

BOG was isolated from the ether extract of the reaction mixture by use of a silicic acid column prepared according to Bulen *et al.*,[10] with 10 per cent butanol in benzene as the eluant. The organic acids could also be separated by two-dimensional ascending paper chromatography, using 95 per cent ethanol, ammonia, and water (80:5:15), and tertiary amyl alcohol, formic acid, and water (65:30:5) as the solvents. Tentative identification of BOG was based upon coincidence of radioactivity in the column eluate or on the paper with authentic carrier material. The radioactive material eluted from chromatograms was also mixed with authen-

tic BOG and crystallized from a mixture of ether and ligroin. Repeated recrystallization gave no significant change in specific activity. BMC was isolated on one-dimensional chromatograms, using the alkaline solvent. To confirm the identity of the material, the eluted substance together with carrier BMC was dried *in vacuo*, sublimed, and then rechromatographed. The activity was confined to the BMC area of the chromatogram.

The 2,4-dinitrophenylhydrazones were extracted from the reaction mixture with chloroform and separated on paper using as a solvent 1.5 *M* $(NH_4)_2CO_3$ and 1.5 *M* NH_4OH (1:1).

Experimental Results.—The flax system is capable of converting the carbon of C^{14}-labeled acetate into citrate, acetoacetate, BOG, and BMC, as shown in Table 1.

TABLE 1

INCORPORATION OF 1-C^{14}-ACETATE INTO BOG, BMC, AND RELATED MATERIALS BY AN ENZYME SYSTEM FROM FLAX SEEDLINGS*

SYSTEM	COUNTS/MIN PER REACTION MIXTURE			
	BOG	BMC	Citrate	ACETOACETATE
Complete	1,000	3,500	9,000	8,400
No ATP	400	2,000	900	4,100
No CoA	800	2,500	10,300	3,600

* Reaction mixture contains 5 ml. enzyme; 40 μM DPN; 60 μM GSH; K phosphate buffer, pH 7.0, 0.05 *M*; 5 μM acetate containing 2.5 × 10^5 counts/min; and with or without 60 μM each of ATP and CoA, as indicated. Total volume, 7.0 cc.

These conversions are dependent upon the presence of added ATP in the reaction mixture and are, in general, increased in amount by the presence of added CoA. The unpurified system must be expected to contain both these substances to some degree. The synthesis of both citrate and acetoacetate in plant and other systems has already been extensively investigated and is relatively well understood.[11, 12, 13] Both reactions depend upon the formation of acetyl-CoA from acetate. The present system possesses the ability to activate acetate in the presence of CoA and ATP, as is shown in Table 2. For this experiment the reaction mixture was in-

TABLE 2

ACTIVATION OF ACETATE, BOG, AND BMC BY AN ENZYME SYSTEM FROM FLAX SEEDLINGS*

SYSTEM	μM ACTIVATED PER REACTION MIXTURE PER 3 HOURS		
	Acetate	BOG	BMC
Complete	0.32	0.77	0.77
No ATP	.50	.37	.31
No CoA	0.07	0.50	0.07

* Reaction mixture contains 5 ml. enzyme; 40 μM DPN; 60 μM GSH; K phosphate buffer, pH 7.0, 0.05 *M*; 5 μM substrate; and with or without 60 μM each of ATP and CoA, as indicated. Total volume, 7.0 cc.

cubated with substrate for 3 hours, and hydroxylamine was then added to convert the acetyl-CoA formed to acetyl hydroxamate, which was then determined colorimetrically.[14] The flax enzyme system contains an endogenous source of oxalacetate (malate) formed by a carboxylation reaction in the presence of ATP, and citrate is synthesized by union of acetyl-CoA with oxalacetate in the presence of condensing enzyme.[11] The synthesis of acetoacetate by the flax system indicates the presence of an acetoacetate cleavage enzyme capable of synthesizing acetoacetyl-CoA from acetyl-CoA.[13] The acetoacetate recovered in the reaction mixture accumu-

lates, however, as the free material rather than as the CoA derivative, indicating the presence in the reaction mixture of an acetoacetyl-CoA deacylase.

The BOG and BMC formed by the flax system do not accumulate as the respective free acids or salts but rather as derivatives which may be converted to the free acids by alkaline hydrolysis under conditions suitable for the hydrolysis of acyl-CoA derivatives (0.28 *N* KOH for 1 hour). The evidence suggests strongly that it is the CoA derivatives of BOG and BMC which are involved in the present reaction. The same conclusion has been arrived at on other grounds for the metabolism of BMC by liver (Robinson *et al.*[15]) and for the metabolism of BOG by a heart preparation by Bachhawat *et al.*[16]

The data of Table 1 show that with this system the accumulation of carbon from acetate in BMC is greater than in BOG. The accumulation of activity in BMC exceeded that in BOG by two to five times in separate experiments under varying experimental conditions.

That acetoacetate participates as an intermediate in the synthesis of BOG and BMC is indicated by the data of Table 3. In this experiment the enzyme

TABLE 3

PARTICIPATION OF ACETOACETATE IN BOG AND BMC SYNTHESIS AS INDICATED BY DILUTION OF 1-C^{14}-ACETATE INCORPORATION INTO THESE MATERIALS BY ACETOACETATE*

	COUNTS/MIN PER REACTION MIXTURE		
SYSTEM	BOG	BMC	Citrate
1-C^{14}-acetate	1,000	3,500	9,000
1-C^{14}-acetate + acetoacetate	200	1,900	7,000

* Reaction mixture contains 5 ml. enzyme; 40 μM DNP; 60 μM ATP; 60 μM CoA; 60 μM GSH; K phosphate buffer, pH 7.0, 0.05 *M*; 5 μM acetate containing 2.5×10^5 counts/min; and with or without 50 μM acetoacetate. Total volume, 7.0 cc.

system was supplied with acetoacetate as well as with C^{14}-labeled acetate. The appearance of C^{14} in BOG and in BMC was depressed by the presence of the acetoacetate. This is not due to any considerable conversion of acetoacetate (through acetoacetyl-CoA) to acetyl-CoA, since the conversion of acetate carbon to citrate is depressed but little by the presence of acetoacetate. Incorporation of acetate carbon into BOG, on the contrary, was depressed two- to fivefold by acetoacetate and that in the BMC by about 50 per cent. The results are in qualitative agreement with those to be expected on the basis of reaction (4) above.

The sequence of reactions (4) and (5) above can be approached in the reverse direction, that is, from the BMC side. Thus, as shown in Table 4, the flax system synthesizes BOG from BMC and C^{14}-labeled CO_2. This synthesis is dependent upon the presence of added ATP and CoA (Table 4). The labeled BOG as well as the labeled BMC formed are present in the reaction mixture as alkali hydrolyzable derivatives, presumably the corresponding acyl-CoA compounds. It has been shown earlier that BMC is activated to form β-methyl crotonyl-CoA by plant systems,[13] and the data of Table 2 show that the present system activates both BOG and BMC in the presence of ATP and CoA.

When flax enzyme BMC and C^{14}-labeled CO_2 are incubated in a complete reaction mixture, labeled BMC appears as well as labeled BOG. This is not explicable in terms of reaction (5) alone. It may indicate that the free carboxyl group of

BOG becomes equilibrated with the acyl-CoA group, perhaps by a side reaction involving the cleavage and resynthesis of BOG-CoA. Alternatively, the carboxylation reaction may be more complex. That this is possible is indicated by the requirement of the carboxylation reaction for ATP reported by Bachhawat *et al.*[16]

The data of Table 5 show that the reverse of reaction (5), synthesis of BOG-CoA from BMC-CoA and CO_2, may be coupled to the reverse of reaction (4) to yield acetoacetate. This may be done either with unlabeled BMC and C^{14}-labeled CO_2 or with labeled BMC (3-C^{14}-BMC) and unlabeled CO_2. In both cases labeled acetoacetate appears in an amount considerably exceeding that of the BOG formed. In both types of experiment, the reactions formulated in (4) and (5) above would be expected to result in labeled acetoacetate, as was found, and in unlabeled acetyl-CoA. The data of Table 5 show, however, that a significant amount of labeled acetyl-CoA was recovered in the form of citrate. It is difficult to account for the presence of the labeled citrate in the reaction mixtures which contains 3-C^{14}-labeled

TABLE 4

SYNTHESIS OF BOG FROM BMC AND $C^{14}O_2$ BY AN ENZYME SYSTEM FROM FLAX SEEDLINGS AS INFLUENCED BY ADDED ATP AND CoA*

	Counts/Min per Reaction Mixture	
System	BOG	BMC
Complete	1,400	2,000
No ATP	735	200
No CoA	130	67

* Reaction mixture contains 5 ml. enzyme; 40 μM DPN; 60 μM ATP and 60 μM CoA; 60 μM GSH; K phosphate buffer, pH 7.0, 0.05 *M*; 5 μM BMC; 5 μM $C^{14}O_2$ containing 3.0×10^5 counts/min; and with or without ATP and CoA, as indicated. Total volume, 7.0 cc.

TABLE 5

DEGRADATION OF BMC BY AN ENZYME SYSTEM FROM FLAX SEEDLINGS IN THE PRESENCE OF $C^{14}O_2$*

	Counts/Min per Reaction Mixture		
System	BOG	Acetoacetate	Citrate
BMC + $C^{14}O_2$	1,400	4,200	600
3-C^{14}-BMC + CO_2	1,610	3,100	460

* Reaction mixture contains 5 ml. enzyme; 40 μM DPN; 60 μM ATP; 60 μM CoA; 60 μM GSH; K phosphate buffer, pH 7.0, 0.05 *M*; 5 μM substrate; and 5 μM CO_2. Total volume, 7.0 cc. Total activity of $C^{14}O_2$, 3×10^5 counts/min. Total activity of 3-C^{14}-BMC, 2.5×10^5 counts/min.

BMC and CO_2 as substrates on grounds other than the activation and cleavage of a small portion of the acetoacetate to yield labeled acetyl-CoA.

Discussion.—It was suggested some years ago[2] that the synthesis of BMC might take place by the union of acetate with acetone. This was based upon the observation that both acetoacetate and acetone are effective in inducing net synthesis of rubber in intact rubber plants. These observations are, however, not incompatible with the pathway of BMC synthesis suggested by the present work, in which acetoacetate is also a metabolite. The role of acetone in the synthesis of isoprenoids has not yet been further elucidated in a plant system. It is possible that this substance may be recarboxylated to acetoacetate in the plant as it is in liver.[17]

The reactions involved in the synthesis of BOG and BMC by the flax enzyme system are very small-scale reactions as compared, for example, to those of the respiratory metabolism. The reaction mixture which synthesizes as much as 0.2 μM of citrate from acetyl-CoA and oxalacetate in a given time accumulates but one-tenth this amount of BOG in this same period. The same is true of other plant

systems, including guayule and lemon grass (*Cymbopogon citratus*), which have been found to synthesize labeled BOG from C^{14}-labeled acetate.

It has been shown above that the flax seedling, as well as a variety of other plant preparations, can activate and metabolize BMC. The flax system, unlike other plant systems investigated (spinach, guayule, bean, and lemon grass), is also able to activate BOG. It may well be, therefore, that free BOG as such is not an important metabolite in plant tissues in general but that it is rather the active BOG-CoA which is of significance.

The fact that the carbon atoms of steroids, such as cholesterol and ergosterol, are derived from labeled acetate[6] has led to studies of the manner in which acetate is converted to these isoprenoid-like compounds by animal tissues. In this case, too, BMC appears to be an intermediate and is synthesized from acetate.[18] In liver, also, BOG has been shown to arise from acetate.[19] For the liver as for plant systems the mechanism by which β-methyl crotonyl units are further converted to the higher isoprenoids has still to be worked out.

Summary.—

1. The mechanism of synthesis of branched-chain compounds from acetate has been investigated with the aid of an enzyme system prepared from flax seedlings.

2. It has been shown that this enzyme system is capable of activating acetate to acetyl-CoA and that the acetyl-CoA thus formed is converted to derivatives of β-hydroxy-β-methyl glutarate and β-methyl crotonate. The synthesis of β-hydroxy-β-methyl glutarate appears to take place through the acetylation of acetoacetate.

3. β-methyl crotonate is activated by the enzyme preparation in the presence of ATP and CoA and is converted with the uptake of CO_2 to β-hydroxy-β-methyl glutarate. The latter can be further degraded to acetoacetate and acetyl-CoA.

4. The sequence of reactions studied is of interest in relation to the role of β-methyl crotonate in the biosynthesis of rubber, steroids, and other isoprenoid compounds.

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*THE PRODUCTION OF MUTATIONS IN DROSOPHILA BY TERTIARY-BUTYL HYDROPEROXIDE**

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The mutagenic action of certain organic peroxides was first shown in work of Wyss, Stone, *et al.*[1] in 1947–1948 on *Micrococcus pyogenes* (*Staphylococcus aureus*) and, soon afterward, in work of Dickey, Cleland, and Lotz[2] in 1948 on *Neurospora crassa*. Because of the importance of peroxides in the production of mutations in microörganisms and as intermediates in the production of mutations by ultraviolet light, it seemed worth while to expand further the data concerning the mutagenic effectiveness of peroxides by exposing *Drosophila* to one of the more stable organic peroxides (*tertiary*-butyl hydroperoxide) and testing the offspring for the presence of mutations. The difficulty of exposing directly the reproductive cells of higher organisms to chemical mutagens has considerably delayed the study of mutagenesis. Of the higher forms, *Drosophila* is a particularly valuable organism because of its well-known genetic background and the important role it has hitherto played in the study of radiation mutagenesis. Chemical agents have in the past been applied to *Drosophila* by several methods: mixing the chemical with the food, spraying the flies, using the chemical as an aerosol, injecting or inserting the chemical into the hemocoele, applying it as a vaginal douche before copulation[3] or immediately after as a "sperm bath,"[4] and the laborious method of treating the entire gonad after its removal from the larva. Of these, the sperm-bath method is the most direct. Another reasonably practical method, accomplishing almost direct contact of the chemical agent with the germ cells, without operative procedure, involves treatment of early embryos after the fashion used by Edgar Altenburg[5] for ultraviolet light. The result of the experiments here described has been to demonstrate that, when *tert.*-butyl hydroperoxide is applied by this method, it produces a significant increase over the spontaneous lethal mutation rate in the second pair of autosomes in *Drosophila melanogaster*, although efforts to induce lethal mutations in the X chromosome by exposure of the adult flies to the peroxide failed.

Methods.—In the first series of experiments lightly etherized newly hatched adult *y v* males were placed in a glass jar lined with moist blotting paper and containing a dish filled with 5 ml. of a 50 molar per cent solution of *tert.*-butyl hydroperoxide in dibutyl phthalate covered with cotton gauze. The jar was glass-stoppered, and the flies were exposed to the peroxide vapor for varying lengths of time. The surviving males were mated individually to females of a balanced stock constructed