BIOSYNTHESIS OF 2-METHYLALKANES IN THE CRICKETS *Nemobius fasciatus* and *Gryllus pennsylvanicus*[1]

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Received December 6,1975

Summary: The biosynthesis of 2-methylalkanes was studied in the crickets *Nemobius fasciatus* and *Gryllus pennsylvanicus*. Labelled acetate, valine, and isobutyric acid were incorporated into the cuticular hydrocarbon of *N. fasciatus* at levels of 6.0 ± 1, 6.5 ± 2, and 1.5 ± 0.7 percent respectively. The hydrocarbons of this insect are 20 percent 2-methylalkanes, primarily of even numbered carbon chain lengths, and 80% n-alkanes. Of the label incorporated into the hydrocarbon fraction, 28 ± 2 percent of sodium [1-^{14}C] acetate, 98 ± 1 percent of L-[G-^{3}H] valine, and 75 ± 10 percent of [1-^{14}C] isobutyric acid were incorporated into the 2-methylalkanes. This suggests that valine is converted to isobutyric acid and is incorporated into the even numbered carbon chain length 2-methylalkanes during the initial stages of chain elongation. Similar data obtained in *G. pennsylvanicus* suggests that leucine is converted to isovaleric acid which is then incorporated into the odd numbered carbon chain length 2-methylalkanes.

Normal and methyl branched hydrocarbons are common constituents of the cuticular lipids of plants (1) and insects (2). The biosynthesis of the cuticular components has received considerable attention in plants, with convincing evidence on the pathways leading to the major components reported (1). n-Alkanes are formed by an elongation-decarboxylation pathway in which fatty acids are elongated and decarboxylated to the alkane one carbon unit shorter (3,4). The branching methyl group of the 2-methylalkanes in plants has been shown to arise from the branched amino acids valine and leucine (5), and that of the 3-methylalkanes from isoleucine (6) The internally branched monomethylalkanes in algae are formed by the

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addition of the active methyl group of methionine to a preformed carbon chain (7,8,9).

Until recently, little work had been done on the biosynthetic pathways leading to insect cuticular hydrocarbons. Acetate had been shown to be incorporated into the hydrocarbons of live insects (2,10), excised cuticles (2), and oenocyte rich fat body preparations (11, 12). Indirect evidence of n-alkane biosynthesis obtained from studying the formation of secondary alcohols in insects (13, 14, 15) suggests that an elongation-decarboxylation pathway is operative, although definitive work has not been done.

Recent work has demonstrated that the 3-methyl- and internally branched monomethylalkanes in insects are formed by a pathway different from that reported in plants and algae. Propionate, incorporated during the penultimate biosynthetic step, has been shown to serve as the methyl branch donor for the 3-methylalkanes in the cockroach Periplaneta americana (16). Similarly, propionate incorporated as a methylmalonyl derivative during chain elongation has been indicated to serve as the branching methyl donor for the internally branched monomethylalkanes in the cockroach Periplaneta fuliginosa (17). Since insects appear to utilize different metabolic pathways than plants or microorganisms for the biosynthesis of some of the major hydrocarbon components, the biosynthesis of the 2-methylalkanes in insects was studied in N. fasciatus and G. pennsylvanicus. No work has been previously reported on the biosynthesis of this type of hydrocarbon in insects.

The hydrocarbons of N. fasciatus contain 20 percent 2-methylalkanes, primarily of even numbered carbon chain lengths in addition to n-alkanes (18). 2-Methylalkanes of both odd and even carbon number chain lengths comprise 26 percent of the cuticular hydrocarbons in G. pennsylvanicus with internally branched monomethyl-, dimethyl-, and n-alkanes also present (18).

EXPERIMENTAL PROCEDURES

Sodium [1-^{14}C] acetate (55 mCi/mM) was purchased from New England

Nuclear. L-[G-^{3}H] valine (6.8 Ci/mM), L-[G-^{3}H] leucine (47.6 Ci/mM), [1-^{14}C] isobutyric acid (10 mCi/mM) and [1-^{14}C] isovaleric acid (2.0 mCi/mM) were purchased from ICN. All solvents were redistilled from glass prior to use.

Insects were collected in Southwestern Mississippi. Adult specimens were used in all studies.

In Vivo Studies: Between 0.1 and 0.5 µCi of labelled substrate in 2 µl of water was injected just beneath the cuticle between the eighth and ninth abdominal segments. The insects were kept at room temperature and sacrificed after 10 hours. The cuticular lipids were extracted by immersion of the insects in hexane for 10 minutes and then the solvent removed under nitrogen. Three to five insects were used in each experimental group.

Separation of Lipid Components: The lipid samples were transferred to a pasteur pipette containing 50 mg BioSil A in 1 ml hexane. The hydrocarbons were eluted into a vial with 8 ml hexane. The hydrocarbon sample was divided into two equal fractions, and all fractions were taken to dryness under nitrogen. The branched hydrocarbons were isolated by adding molecular sieve 5 Å (19) to one fraction, followed by shaking for six hours in 1 ml of 2,2,4-trimethylpentane. The branched hydrocarbons in 2,2,4-trimethylpentane were transferred to another vial, the molecular sieve washed twice with an additional 1 ml aliquot of 2,2,4-trimethylpentane and the washes combined and taken to dryness under nitrogen.

Determination of Radioactivity: Samples in hexane were transferred to counting vials, the solvent removed under nitrogen, and 10 ml of a fluor solution (0.4% PPO in toluene) added. Samples were counted for 10 minutes on a Packard Tri Carb liquid scintillation counter. All data points are the average of 3 to 6 experiments.

Gas-Liquid-Chromatography: The same procedure described above was used to isolate total and branched hydrocarbon fractions from insects not injected with labelled substrates. GLC was performed on a 6 ft X 1/8 in column containing 3% SE-30 on gas chrom Q programmed from 150 to 280°C at 8° per minute. Integration was obtained by disc integration.

RESULTS

Figure 1 shows the GLC trace of the total and branched hydrocarbons of N. fasciatus. The only components not included in molecular sieve 5 Å are 2-methyldocosane (2 percent), 2-methyltricosane (trace) and 2-methyltetracosane (18 percent) (18).

Labelled acetate was readily incorporated into the cuticular lipids of the crickets N. fasciatus and G. pennsylvanicus. Time studies showed a linear increase in the amount of label incorporated into hydrocarbon for at least 10 hours, the time used for all further studies.

Sodium [1-^{14}C] acetate, L-[G-^{3}H] valine and [1-^{14}C] isobutyric acid were incorporated into the cuticular hydrocarbons of N. fasciatus

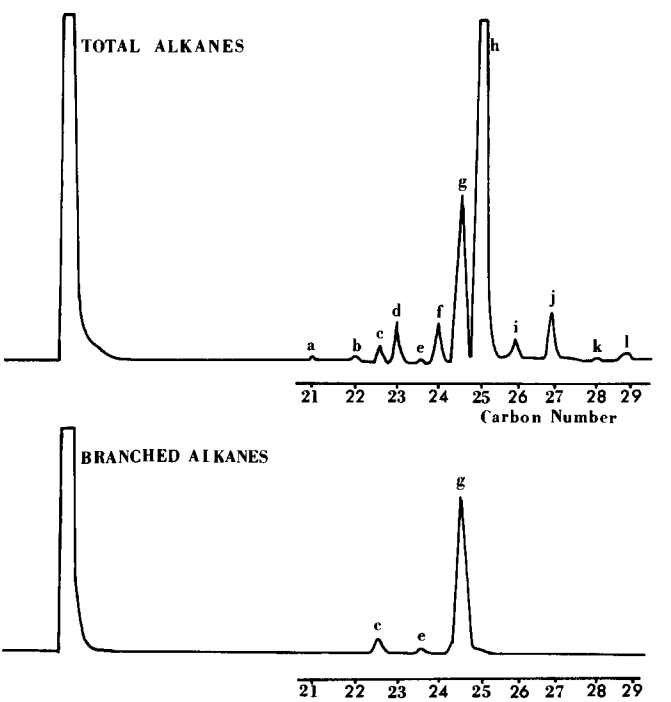

Figure 1. GLC traces of total (top) and branched (bottom) hydrocarbon of N. fasciatus. Peaks are identified as (a) n-heneicosane, (b) n-docosane, (c) 2-methyldocosane, (d) n-tricosane, (e) 2-methyltricosane, (f) n-tetracosane, (g) 2-methyltetracosane, (h) n-pentacosane, (i) n-hexacosane, (j) n-heptacosane, (k) n-octacosane, and (l) n-nonacosane (18).

at levels of 6.0 ± 1, 6.5 ± 2, and 1.5 ± 0.7 percent respectively (Table I). Labelled L-leucine and isovaleric acid were incorporated at levels of less than 1 percent. Of the label incorporated into hydrocarbon, 28 ± 2 percent of the labelled acetate was incorporated into the 2-methyl-alkanes, compared to 98 ± 1 percent for valine and 75 ± 10 percent for labelled isobutyric acid. Sixty ± 10 and 22 ± 2 percent of the label incorporated into hydrocarbon from injected labelled L-leucine and isovaleric acid was found in the branched alkanes.

Table II shows the incorporation of labelled substrates into the total and branched alkanes of G. pennsylvanicus. The hydrocarbons of this insect consist of 31 percent n-alkanes, 8 percent internally branched monomethyl-alkanes, 34 percent dimethylalkanes, and 28 percent 2-methylalkanes, with nearly equal amounts of odd and even carbon chain length 2-methylalkanes present (18). Five ± 1 percent of the injected labelled L-valine was incorporated into cuticular hydrocarbon, followed by labelled isovaleric acid (2.0 ± 1 percent), acetate (0.9 ± 0.3 percent), L-leucine (0.6 ± 0.2 percent)

TABLE I

Incorporation of labelled acetate, L-valine, L-leucine, isobutyric acid and isovaleric acid into the cuticular hydrocarbons of Nemobius fasciatus.

Substrate	Percent Incorporated into cuticular Hydrocarbon	Label incorporated into Hydrocarbon	
		Percent in 2-Methylalkane	Percent in n-alkane
Sodium (1-^{14}C)acetate	6.0 ± 1	28 ± 2	72 ± 2
L-(G-^{3}H)valine	6.5 ± 2	98 ± 1	2 ± 1
(1-^{14}C)isobutyric acid	1.5 ± 0.7	75 ± 10	25 ± 10
L-(G-^{3}H)leucine	0.4 ± 0.3	60 ± 10	40 ± 10
(1-^{14}C)isovaleric acid	0.7 ± 0.2	22 ± 2	78 ± 2

TABLE II

Incorporation of labelled acetate, L-valine, L-leucine, isobutyric acid and isovaleric acid into the cuticular hydrocarbons of Gryllus pennsylvanicus.

Substrate	Percent Incorporated into cuticular Hydrocarbon	Label incorporated into Hydrocarbon	
		Percent in branched	Percent in n-alkane
Sodium (1-^{14}C)acetate	0.9 ± 0.3	24 ± 5	76 ± 5
L-(G-^{3}H)valine	5.0 ± 1	78 ± 6	22 ± 6
(1-^{14}C)isobutyric acid	0.2 ± 0.05	89 ± 4	11 ± 4
L-(G-^{3}H)leucine	0.6 ± 0.2	90 ± 3	10 ± 3
(1-^{14}C)isovaleric acid	2.0 ± 1	46 ± 10	54 ± 10

and isobutyric acid (0.2 ± 0.05 percent). The branched amino acids valine and leucine, as well as isobutyric acid and isovaleric acid were preferentially incorporated into the branched alkanes compared to labelled acetate. Of the label incorporated into hydrocarbon, 90 ± 3 percent from L-leucine, 89 ± 4 percent from isobutyric acid, 78 ± 6 percent from L-valine, 46 ± 10 percent from isovaleric acid and 24 ± 5 percent from acetate was incorporated into the branched fraction.

DISCUSSION

Results of these studies suggest that the 2-methylalkanes in insects arise from appropriate branched amino acids. Valine is apparently converted to isobutyric acid which is then incorporated into the even numbered carbon chain length 2-methylalkanes and leucine is converted to isovaleric acid which is incorporated into the odd numbered carbon chain length 2-methylalkanes (Figure 2). The preferential incorporation of labelled L-valine and isobutyric acid into the 2-methylalkanes in N. fasciatus and the additional incorporation of labelled

L-leucine and isovaleric acid into the branched alkanes of G. pennsylvanicus over that of labelled acetate support the pathways suggested. In contrast to the situation for the 3-methylalkanes and the internally branched monomethylalkanes, insects appear to utilize the same biosynthetic pathways for the formation of the 2-methylalkanes as do plants and microorganisms.

With the concept that valine is converted to isobutyric acid and then incorporated into even numbered carbon chain length 2-methylalkanes, it is interesting to note that valine is incorporated to a greater extent into hydrocarbons and a greater extent into branched hydrocarbons than is isobutyric acid. This observation is indicative of a cellular hydrocarbon biosynthesis system that utilizes the branched amino acid as the natural substrate which it more readily transports than the intermediate isobutyric acid. In the whole insect, isobutyric acid may be more susceptible to degradation than is valine

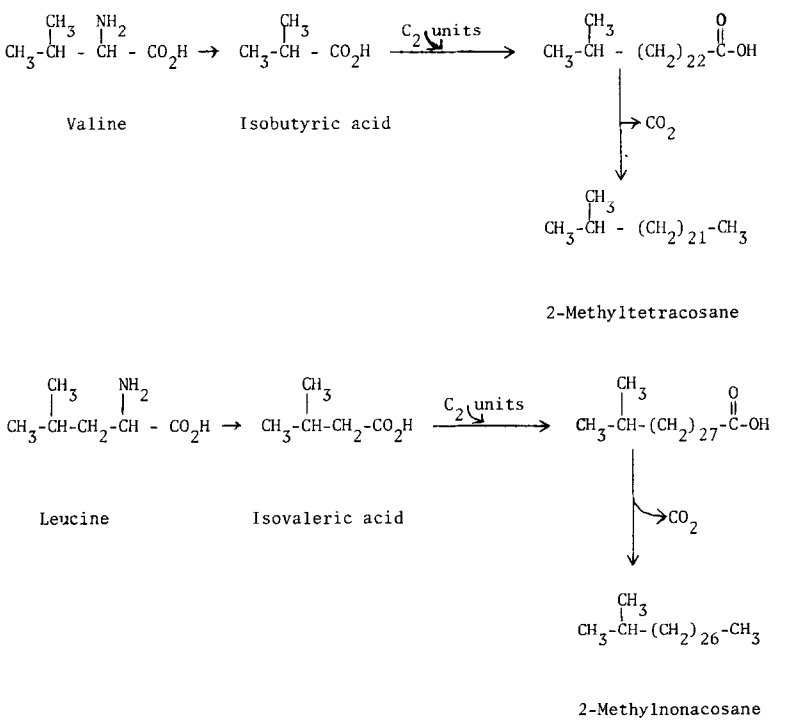

Figure 2. Proposed biosynthetic pathways for 2-methylalkanes in Insects.

which would result in less incorporation of isobutyric acid and also a greater incorporation of label into normal hydrocarbons coming from degradation products of isobutyric acid. The same argument could be made for relative incorporation of leucine and isovaleric acid. The results of this paper support such an argument in all cases but one, where the percent incorporation of isobutyric acid into branched hydrocarbons of G. pennsylvanicus is greater than the percent incorporation of valine into branched hydrocarbons. This can be explained by the fact that isobutyric acid can be metabolized to propionate which is a substrate for internally branched monomethylalkanes (17) that are present in G. pennsylvanicus and absent in N. fasciatus hydrocarbons.

The percent incorporation of isovaleric acid and acetate into the branched hydrocarbons of N. fasciatus is nearly equal and suggests that isovaleric acid might be degraded to acetate prior to incorporation into branched hydrocarbons in insects that do not have odd numbered carbon chain length 2-methylalkanes.

Very high levels of both acetate and valine were incorporated into the cuticular hydrocarbons of N. fasciatus. This, and the simple hydrocarbon composition observed suggest that this may be an excellent experimental animal for additional studies on the biochemistry of hydrocarbon synthesis in insects.

ACKNOWLEDGEMENTS

This work was supported in part by a Cottrell Grant from Research Corporation and a Sigma Xi Grant-in-Aid of Research to Gary J. Blomquist. Contribution from Agricultural Experiment Station, Montana State University, Bozeman and published as Journal Series No. 640.

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