On the origin of pristane in marine organisms

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ABSTRACT Phytol-U-^{14}C was adsorbed on algae and ingested in this form by zooplanktonic copepods (two species of *Calanus*). The lipids of these animals were analyzed after 48 hr and found to contain radioactive pristane and radioactive phytanic acid. The conversion of phytol to pristane by the copepods is interpreted as a likely biological source of pristane in nature.

KEY WORDS pristane · hydrocarbon biosynthesis · isoprenoid fatty acids · decarboxylation of fatty acids · phytol · *Calanus* · marine organisms · intestinal bacteria

THE SATURATED NORDITERPANE, pristane, occurs in various geological sediments and crude oils (1) and its presence has been generally considered to be evidence for a biological contribution to the formation of hydrocarbons in nature. A likely precursor of pristane is the monounsaturated diterpenyl alcohol, phytol, which occurs as an ester in chlorophyll and is therefore ubiquitously present in the flora of the past and the present. Pristane has been found in many marine organisms (2), the most striking example being the planktonovorous basking shark (*Cetorhinus maximus* Gunnerus), the liver of which contains substantial amounts of pristane in its lipids (3, 4). The hydrocarbon was found in relatively large quantity in copepods of the genus *Calanus* (5), and these zooplanktonic organisms were regarded as one of the primary sources of pristane in the marine biosphere. Pristane also occurs in trace amounts in marine algae (6), and its presence together with phytane in terrestrial animals was recently described (7).

The results of the present study with phytol-U-^{14}C indicate that calanid copepods are capable of converting to pristane the phytol normally present in their phytoplankton diet.

METHODS

Copepods (*Calanus finmarchicus* and *Calanus hyperboreus*) were collected in August in a No. 000 net at a depth of 180–200 ft over the Atlantic shelf east of Provincetown, Mass.

For the feeding experiment with phytol-^{14}C, 120 animals were selected and put into 175 ml of unfiltered seawater at 0°C. Phytol-U-^{14}C was prepared from a commercially obtained nonsaponifiable lipid fraction of *Chlorella* grown on $^{14}CO_2$ (Volk Radiochemical Co., Burbank, Calif.). 1 mg of carrier phytol was added to about 50 μc of ^{14}C-labeled nonsaponifiable lipid fraction (specific radioactivity approximately 600 μc/mg) and chromatographed on thin layers of silica gel in benzene–ethyl acetate 4:1. The phytol zone was eluted and an aliquot was subjected to GLC on a column containing 10% EGS on Gas-Chrom P (Applied Science Laboratories Inc., State College, Pa.) at 180°C. The chromatographic fractions were collected and their radioactivity was determined. The radiopurity of the phytol preparation exceeded 93%, and none of the impurity was identifiable as radioactive pristane. The specific radioactivity was approximately 50 μc/mg. The phytol-U-^{14}C was dissolved in 0.05 ml of acetone, 0.5 ml of a concentrated algal suspension (*Thalassiosira fluviatilis*) was added to the solution, and the mixture was introduced to the copepod culture. A slow stream of air was passed through the medium and then through 50 ml of 20% NaOH for absorption of any $^{14}CO_2$ formed. The animals were kept near 0°C during the first 24 hr and at 10°C during an additional 24 hr period. After the initial 24 hr, survival was nearly 100%; the presence of fecal pellets suggested normal feeding. By the end of the experiment on the 2nd day, about 30% of the animals had died. An attempt to do the experiment under sterile

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Abbreviations: EGS, ethylene glycol succinate polyester; GLC, gas–liquid chromatography.

conditions was unsuccessful: another batch of copepods was incubated in the presence of streptomycin, but the animals died soon after the addition of the antibiotic.

After the incubation, the animals were manually removed from the medium and their lipids were extracted into chloroform–methanol 2:1. The total lipids were saponified in ethanolic NaOH and the nonsaponifiable and saponifiable fractions were extracted into hexane and diethyl ether, respectively. The nonsaponifiable fraction was subjected to GLC on 15% EGS on Gas-Chrom P at 140°C. Several chromatographic fractions, including the ones with retention times corresponding to those of pristane and phytane, were collected and their radioactivity was determined.

The saponifiable fraction was esterified with methanol in H_2SO_4 to give the methyl esters; methyl phytanate and methyl pristanate were added as carriers and the fraction was subjected to GLC on a mixed liquid phase of 1.5% SE-30–7% EGS on Gas-Chrom P at 180°C. Several GLC fractions, including methyl pristanate and methyl phytanate, were collected and their radioactivities determined.

Pristane was supplied by K & K Laboratories, Inc., Plainview, N. Y. Phytane was synthesized from phytol, as described earlier (7). The preparation of phytanic acid was described previously (8). Pristanic acid was synthesized from methyl phytanate by Barbier–Wieland degradation.

RESULTS AND DISCUSSION

The results in Table 1 show the high lipid content of the animals. Pristane was present in the *Calanus* lipids in concentrations consistent with previously published findings (5). As seen in Table 2, a significant part, namely 13.5% of the substrate ingested, was metabolized to CO_2. Most of the radioactivity in the nonsaponifiable fraction could not be recovered from the GLC column at the relatively low temperature at which it was operated (Table 3). Almost all of the radioactivity eluted from the column (97.5%) was in pristane and very little, or possibly none at all, in phytane. (No detectable mass peak corresponding to phytane emerged from the gas chromatograph.) The missing material was not identified, but it very likely consisted chiefly of the unchanged phytol-^{14}C.

TABLE 1 LIPID COMPOSITION OF CALANUS

	% of Dry Wt	% of Total Lipids
Total lipids	49.1	100
Nonsaponifiable lipids recovered	23.3	47.5
Saponifiable lipids recovered	21.5	43.7
Pristane	0.77	1.56

TABLE 2 RADIOACTIVITY IN LIPIDS OF CALANUS INCUBATED WITH PHYTOL-U-^{14}C

	Radioactivity
	dpm
Total radioactivity incubated	1.1×10^8
Total lipid in animals	3.1×10^6
Nonsaponifiable fraction	1.36×10^6
Saponifiable fraction	1.67×10^6
CO_2	4.1×10^5

TABLE 3 DISTRIBUTION OF RADIOACTIVITY IN GLC FRACTIONS OF NONSAPONIFIABLE AND SAPONIFIABLE LIPIDS

	% of Total Injected
Nonsaponifiable lipids	(100)
Preceding pristane	0.0
Pristane	3.9
Between pristane and phytane	0.03
Phytane	0.01
After phytane (elution time equal to that of the combined previous fractions)	0.06
Saponifiable lipids	(100)
Preceding pristanic acid	2.6
Pristanic acid	1.4
Between pristanic acid and phytanic acid	1.2
Phytanic acid	41.7
After phytanic acid (elution time equal to that of the combined previous fractions)	27.0

A control experiment proved beyond doubt that the copepods and not the algae are responsible for the conversion of phytol to pristane. Another batch of *T. fluviatilis* was incubated with phytol-U-^{14}C without the copepods, but in the same medium and under the same conditions as for the incubation with copepods. Lipids were extracted from the medium and the algae, and a small amount of nonradioactive pristane was added to the extract. The pristane was subsequently isolated by GLC of the nonsaponifiable fraction and found to contain no radioactivity. Pristane isolated from a sample of copepods by the above GLC method was further identified by its parent mass peak in an MS-9 mass spectrometer (Associated Electronic Industries, Ltd., Manchester, England). The radioactive pristane, however, was identified solely on the basis of its gas-chromatographic behavior. An error in identification would seem to be extremely unlikely in view of the limitations imposed by the nature of the radioactive precursor.

The greatest part (73%) of the radioactivity in the fatty acid fraction was recovered from GLC in a number of fractions, the most prominent of which was phytanic acid. Very little radioactivity was recovered with the 19-carbon pristanic acid, a known metabolite of phytanic acid in mammalian tissues (9). The fraction that preceded pristanic acid on GLC could be expected to contain a number of shorter-chain isoprenoid fatty acids

that normally are formed in the course of oxidative metabolism of phytanic acid (10). The fraction that followed phytanic acid included stearic and oleic acids. These abundant fatty acids may be expected to be formed in a de novo synthesis from labeled 2-carbon fragments produced by degradation of the isoprenoid structure. A similar incorporation of phytanic acid-U-^{14}C radioactivity into stearic and oleic acids was recently observed in a mammalian system in which the phytanic acid was broken down (11). The radioactive fraction eluted from the GLC column after phytanic acid is likely to also contain one or more isomers of phytenic acid, a compound that has been shown to appear in animals fed phytol (12). No attempt, however, was made to isolate phytenic acid in the present study.

The phytanic acid produced from phytol represented the predominant labeled fatty acid component and was probably directly converted through a decarboxylation process to the labeled pristane found in the tissues of the animals. Although pristanic acid could conceivably give rise to pristane through a series of reductive steps, such a process is highly unlikely under aerobic conditions. The small amount of radioactivity recovered in pristanic acid in the present study and the absence of labeled phytane in spite of the presence of large amounts of radioactivity in phytanic acid would tend to rule out pristanic acid as a precursor of pristane. A very small, rapidly turning over pool of pristanic acid would, of course, be consistent with its possible role in the biosynthesis of pristane, but such conditions are rather unlikely.

The various *Calanus* species accumulate significant concentrations of pristane, and it is probable that the production of this hydrocarbon is accelerated in the organism's natural habitat during the spring plankton bloom. These metabolic conditions might not have been reproduced in the laboratory. However, the conversion of phytol to pristane in these marine animals has been shown, at least qualitatively, to take place. Because calanid copepods serve as important links in the marine food chain, their synthesis of pristane may very well be the most significant source of the hydrocarbon, both in animal tissues and in geological formations. Whether enzymes of *Calanus* or of the copepods' intestinal bacteria are responsible for the production of pristane is not answered by the present study, since these very sensitive animals could not be kept alive in the presence of an antibiotic. It is entirely possible, therefore, that symbiotic microorganisms are converting the ingested phytol or the phytanic acid produced from it into pristane and that the pristane is absorbed and stored in the *Calanus* tissues.

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