Saturated Hydrocarbons in Bovine Liver

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(Received 14 April 1969)

A homologous series of *n*-alkanes (C₁₄–C₃₃) and two isoprenoid hydrocarbons, 2,6,10,14-tetramethylhexadecane (phytane) and 2,6,10,14-tetramethylpentadecane (pristane) have been identified in bovine liver. Another branched but non-isoprenoid alkane and three isomers of molecular formula C₂₀H₄₀ were partially identified. Phytane and the C₁₈–C₂₂ and C₂₉–C₃₃ *n*-alkanes were found to be the major components in liver, suggesting that at least the main hydrocarbon components were derived from various plants in the diet. The hydrocarbons were separated and identified by a series of steps involving solvent extraction, saponification, elution chromatography on alumina and silica gel columns, molecular sieving and by infrared and ultraviolet spectroscopy, followed by combined capillary gas chromatography–mass spectrometry.

Hydrocarbons are ubiquitous in Nature. They occur not only in large concentrations in petroleum but also as trace constituents of a large portion of the earth's sedimentary rocks and marine sediments, even in rocks more than 2 billion years old (Burlingame, Haug, Belsky & Calvin, 1965; Oro, Nooner, Zlatkis, Wikstrom & Barghoorn, 1965). Hydrocarbons in rocks and sediments are usually considered to be of biological origin. Hydrocarbons have been found in a large variety of plants and animals: for example in the cuticle of apple (*Pyrus malus*) fruits (Chibnall, Piper, Pollard, Smith & Williams, 1931), in the leaf cells of cabbage and Brussels sprouts (*Brassica oleracea*) (Sahai & Chibnall, 1932), and in tobacco (*Nicotiana tabacum*) (Chibnall, Piper, Pollard, Williams & Sahai, 1934).

With the advent of modern microtechniques, hydrocarbons have been detected in many other organisms: for example, in the bacteria *Saprospira grandis* (Oro, Tornabene, Nooner & Gelpi, 1967), *Chromatium* sp. (Nagy *et al.* 1964) and *Sarcina lutea* (Tornabene, Gelpi & Oro, 1967); in a number of species of blue-green algae (Winters, Parker & Van Baalen, 1969; Han, McCarthy, Calvin & Benn, 1968), in the multicellular brown alga *Pelagophycus porra* (Nagy *et al.* 1964), in the alga *Botryococcus braunii* (Maxwell, Douglas, Eglinton & McCormick, 1968), in the moss *Polytrichum commune*, in the fungus *Pholiota praecox* (Nagy *et al.* 1964), in the fungal spores of *Ustilago maydis* (Laseter, Weete & Weber, 1968), *U. nuda* and *Sphacelotheca reiliana* (Oro, Laseter & Weber, 1966); in various plant cuticular waxes, *Aloe* (Liliaceae), *Agave* (Agavaceae), *Kalanchoe*, *Echeveria*, *Crassula* and

Sedum (Crassulaceae), and in *Eucalyptus* (Myrtaceae (Herbin & Robins, 1968*a,b*), in banana leaf (*Musa sapientum*) (Nagy, Modzeleski & Murphy, 1965), in string-bean (*Phaseolus aureus*) wax (Wanless, King & Ritter, 1955), in marigold (*Calendula officinalis*) flowers (Stevenson, 1961), in the grass *Leptochloa digitata* (Kranz, Lamberton, Murray & Redcliffe, 1961), in Grenache grape (*Vitis vinifera*) (Stevens, Bomben & McFadden, 1967), in tangerines (*Citrus reticulata* Blanco) (Ashoor & Bernhard, 1967), in black pepper (*Piper nigrum*) (Muller, Creveling & Jennings, 1968), in cotton (*Gossypium hirsutum*) buds (Struck, Frye & Shealy, 1968) etc. It has been suggested that hydrocarbons may be used for the chemical taxonomy of plants (Eglinton, Hamilton, Raphael & Gonzalez, 1962).

Hydrocarbons have also been found in animals: for example, in the tissues, excluding the digestive tract, of the anthozoan, sea anemone *Bunodes rigidus* (Nagy *et al.* 1964), in beeswax from *Apis mellifica* (Downing, Kranz, Lamberton, Murray & Redcliffe, 1961), in rat liver (Skipski, Smolowe, Sullivan & Barclay, 1965), mouse liver (Nelson, 1962), sheep wool (Simmonds, Nooner, Zlatkis & Oro, 1968), shark liver oil (Gelpi & Oro, 1968), rancid herring oil (Kiesvaara, Nikkilä & Westergren, 1967), cooked chicken meat (Nonaka, Black & Pippen, 1967) etc. Hydrocarbons have also been detected in human tissues. Gazzarrini & Nagy (1966) found that femoral artery walls and plaques contain saturated hydrocarbons and Skipski *et al.* (1967) reported *n*-alkanes in a human serum lipoprotein fraction. MacKenna, Wheatley & Wormall (1952) reported *n*-alkanes and squalene from surface

skin fat (i.e. human sebum), and Riley, Hokama & Kratz (1958) found hydrocarbons in human liver. There is the possibility that the hydrocarbons in human tissues are not endogenous but that they have been absorbed from the diet or that they are the post-mortem decomposition products of lipids. Oro, Nooner & Wikstrom (1965a,b) found that the distribution of *n*-alkanes in the whole plant and in the leaves of the spotted bur clover (*Medicago arabica*) resembled the *n*-alkane distribution in cattle manure, suggesting that these substances are not synthesized or metabolized in animal tissues.

This paper investigates further the problem of the biological function and role of hydrocarbons in animal tissues.

EXPERIMENTAL

Extraction of the hydrocarbons. Great caution was exercised throughout the experiments to avoid contaminations. All glassware was cleaned in hot conc. H_2SO_4 - HNO_3 (85:15, v/v). No stopcock grease was used in the laboratory and the samples never came in contact with plastic or rubber. All solvents were freshly distilled through 12in. columns packed with Raschig rings and their purity was checked before use. Frequent complete blank runs were made to detect any laboratory contaminations should such have occurred (none was detected).

Bovine liver, obtained from a freshly killed animal at the slaughterhouse, was wrapped immediately in aluminium foil that had been washed with distilled chloroform, chilled and transported to the laboratory. The outer 1 cm. layer and major arteries and veins were discarded. All dissection was performed with solvent-cleaned scalpels.

The remaining tissue (1675 g.) was homogenized at room temperature in a modified Waring Blendor, without lubricants and equipped with Teflon gaskets. The sample was extracted twice, first with 10 ml. of chloroform-methanol (2:1, v/v)/g. of liver and secondly with 5 ml. of solvent/g. of liver. The extract was filtered through a medium-porosity glass filter and the solvent removed by flash evaporation. A resinous, brown solid (133.6 g.) was obtained.

Four portions were saponified separately by refluxing for 1 hr. with 600 ml. of benzene-methanol (40:60, v/v; the methanol contained 27 g. of KOH and 15 ml. of water). The methanolic KOH and benzene formed two distinct layers. The benzene layer contained predominantly the non-saponifiable fraction. After separation of this layer and evaporation of the benzene, the yellow non-saponifiable residue was extracted with *n*-hexane. The *n*-hexane extract was washed twice with water, once with aq. 5% KOH and again twice with water. Emulsions were avoided by forming a slurry with prewashed alumina and eluting the slurry with *n*-hexane in a large Buchner funnel. The non-saponifiable portions were combined and filtered through a 0.45 μm . pore size Millipore filter. The *n*-hexane solution was evaporated under purified N_2 and 12.7 g. of a waxy, yellow residue was recovered.

The residue was chromatographed with *n*-hexane on a column containing 723.5 g. of prewashed alumina (Woelm, neutral grade). The eluate weighed 31 mg.; it was rechrom-

atographed on a column containing 93 g. of prewashed silica gel (Davison grade 923). The sample was eluted consecutively with *n*-hexane, carbon tetrachloride, benzene and methanol: 9 mg. of material was eluted with *n*-hexane, nothing with carbon tetrachloride and small amounts with benzene and methanol. Some non-hydrocarbon compounds were present in the methanol eluate.

Infrared and ultraviolet spectra of the *n*-hexane eluate showed the presence of saturated hydrocarbons only; the u.v. spectra had no absorption bands down to 180 m μ . The *n*-hexane eluate was a colourless, oily wax. This fraction was next separated into straight-chain and branched/cyclic fractions by refluxing in benzene solution with 50 times its weight of Linde 5A molecular sieve pellets, pre-activated at 200° for 2 weeks. After the removal of the benzene solution and repeated washing of the pellets with benzene, the molecular sieve was refluxed with *n*-hexane for 1 week to remove the *n*-alkanes. The branched fraction recovered from the benzene weighed 7.3 mg. and the *n*-alkanes 0.04 mg. A good separation was obtained of the normal hydrocarbons up to C_{27} . A duplicate study of the saturated hydrocarbons from the liver of another animal, although too small to permit molecular sieving, also showed prominent *n*-alkane components.

RESULTS

Analysis by combined capillary column gas-liquid chromatography and mass spectrometry. A Perkin-Elmer model 226 gas chromatograph, equipped with a hydrogen flame detector, Brooks Instrument model no. 8743 flow regulator and Apiezon L-coated capillary columns of varying length (up to 300 ft. long and 0.02 in. i.d.), was connected by a heated transfer line and a Watson-Biemann molecular separator to a Hitachi RMU-6E single-focusing mass spectrometer. The sample was introduced into the gas chromatograph either in solution or, after melting, with a calibrated, heated syringe. The effluent from the chromatographic column was divided: approximately 5% went to the hydrogen flame detector and the remaining 95% was introduced directly into the mass spectrometer (Modzeleski, MacLeod & Nagy, 1968). The mass scans were made in approximately 3 sec.

With a 50 ft. column programmed to 200° in 60 min. and run at 200° for a further 40 min. six peaks were identified on the gas chromatogram of the liver branched/cyclic hydrocarbons (Fig. 1). The main component was identified as the isoprenoid hydrocarbon, 2,6,10,14-tetramethylhexadecane (phytane) by comparison of the mass spectrum with that of the phytane standard. A smaller peak, 2, was found to be 2,6,10,14-tetramethylpentadecane (pristane). Three other peaks, 1, 4 and 5, were isomers, having the molecular formula $\text{C}_{20}\text{H}_{40}$. These compounds may have been alkyl-substituted cyclohexanes, since an $(M-84)^+$ ion was present in each spectrum, and in the mass spectrum of one of them a metastable ion was

Fig. 1. Gas chromatogram of the branched/cyclic saturated hydrocarbon fraction from bovine liver. Hydrogen flame-ionization detector record of a Perkin-Elmer model 226 gas chromatograph connected directly to a Hitachi RMU-6E mass spectrometer: 50 ft. long, 0.02 in. i.d., support coated open tubular (S.C.O.T.) Apiezon L column. The carrier gas was helium at a flow rate of 3 ml./min.

detected at m/e 36.5, corresponding to the transition $83^+ \rightarrow 55^+ + 28$. Since double bonds were absent, only one cyclic structure is necessary to satisfy the molecular formula $C_{20}H_{40}$.

The last peak, 6, appeared to be a saturated hydrocarbon of the molecular formula $C_{21}H_{44}$. The presence of a prominent group of peaks at C_{17} in the mass spectrum indicated a point of branching in the carbon skeleton of this compound, and the presence of a vicinal dimethyl group was implied from the intensity ratio of m/e 234 to m/e 235 > 1 (McCarthy, Han & Calvin, 1968).

The components of the straight-chain fraction from the molecular sieve separation all gave the characteristic n -alkane fragmentation pattern, i.e. ions of decreasing intensity at increasing m/e 14 increments. They were readily identified as a homologous series of n -alkanes.

DISCUSSION

Role of saturated hydrocarbons in liver. The biosynthesis of hydrocarbons is not fully understood. There are, however, certain observations that indicate possible synthetic pathways. The n -alkanes in tissues generally show an odd number of carbon atoms preference; on the other hand, fatty acids that contain even numbers of carbon atoms in their straight chains are far more abundant than the odd-number acids. This would suggest that n -alkanes are derived from fatty acids by decarboxylation. Similarly, steranes seem to have been derived from steroids by the loss of the OH group and by saturation.

In the bovine livers examined, saturated hydro-

carbons were present in trace quantities only. In spite of the several chemical steps involved in the extraction of these hydrocarbons, the two livers yielded approximately the same quantities of hydrocarbons; the amounts were $3 \mu\text{g./g.}$ for the smaller liver sample and $4 \mu\text{g./g.}$ for the larger one. Fat deposits in animals would also be expected to contain trace amounts of hydrocarbons, perhaps in higher concentrations than in liver. Phytane, and the C_{18} and C_{19} , C_{20} , C_{21} and C_{22} n -alkanes, were the most abundant components in both samples, followed by the C_{29} , C_{31} and C_{33} n -alkanes. In the hydrocarbon fraction that was fractionated by molecular sieving, the C_{15} and C_{17} n -alkanes were the major components; these were not observed in the other liver sample. It is also noteworthy that a bimodal distribution of the n -alkanes was observed, i.e. the odd number of carbon atoms preference was not uniform; it was only present in the C_{14} – C_{18} and C_{25} – C_{33} n -alkanes.

The great abundance of the isoprenoid hydrocarbon, pristane, in the copepods *Calanus finmarchius*, *C. glacialis* and *C. hyperboreus* has been noted by Blumer, Mullin & Thomas (1963). This isoprenoid hydrocarbon is also present in the liver oils of sharks and whales, which feed on these planktonic crustaceans. However, in an investigation (Gershbein & Singh, 1969) of the hydrocarbon content of the unsaponifiable fraction of pig liver, the absence of pristane was reported, despite a specific search for this compound. Ultimately the isoprenoids are thought to be derived from chlorophyll (Bendoraitis, Brown & Hepner, 1962); thus their presence in the livers of herbivorous animals, including ox liver, would not be unexpected.

Part of this investigation was performed at the University of California at San Diego. The authors would like to thank Mrs Mary Ellen Gowan and Dr William MacLeod for assistance in the experiment and the slaughterhouse at National City, California for providing the liver samples. This investigation was supported by the National Aeronautics and Space Administration.

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