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COMPARISON OF THE PROMOTING ACTIVITY OF PRISTANE AND *n*-ALKANES IN SKIN CARCINOGENESIS WITH THEIR PHYSICAL EFFECTS ON MICELLAR MODELS OF BIOLOGICAL MEMBRANES

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In 1976 (Horton, A.W., Butts, C.K. and Schuff, A.R. (1976) Colloid Interface Sci. 5, 159–168) we assayed pristance (2,6,10,14-tetramethylpentadecane) in a model interfacial system that has given excellent correlation with cocarcinogenic activity among *n*-alkanes, as tested in cycloalkane diluents. It was predicted that this branched-chain derivative of the diterpenes would have higher activity than *n*-$C_{18}H_{38}$, one of the most cocarcinogenic of the *n*-alkanes in such diluents. Pristane was compared with *n*-$C_{18}H_{38}$ and two other *n*-alkanes for their promoting activities in cyclohexane for C3H male mice after a single application of 7,12-dimethylbenz[*a*]anthracene. The branched-chain alkane proved to be more active. 20% *n*-tetracosane in cyclohexane was inactive, which correlated with its effects in this diluent in the physical assay system. The promoting activity of 75% *n*-octane in cyclohexane, predicted by the physical assay, was confirmed by tests on mice. The combined by-products of the synthesis of tetracosane, including C_{12} alkanes and alkenes, C_{19} and C_{20} alkylbenzenes, and C_{24} alkenes, proved to be a very active promoter. However, a mixture in cyclohexane of purified tetracosane with this composite of potential impurities was inactive. From the alkanes' behavior in physical systems involving vatious membrane phospholipids and steroids, it is hypothesized that the primary step in their biological activity is a chain-chain interaction with membrane lipids that alters the properties of liquid-crystalline phases at aqueous interfaces. Resulting changes in the microfluidity of the lipid phase and the lateral mobility of critical hormone receptors and enzyme systems, such as the nucleotidyl cyclases, would perturb control systems that maintain the normal behavior of the initiated cell. Thus, its progression to a proliferating neoplasm may be favored.

Introduction

The saturated isoprenoid hydrocarbon, pristane (2,6,10,14-tetramethylpentadecane), is an important consistuent of the unsaponifiable fraction of shark liver oil together with the unsaturated triterpene, squalene ($C_{30}H_{50}$) [1]. It is an almost ubiquitous component of the lipids or hydrocarbons of nature. The many materials in which it has been detected include plankton, bovine brain and liver, bituminous coal, crude petroleum, oil shale, human sebum and tobacco. Pristane became of particular interest to us when Potter and co-workers [2,3] reported that, after intraperitoneal injection in Balb/c mice, it blocked the effectivity of established immunity and caused plasmacytoma cells to grow more efficiently.

With the exception of the demonstration of Potter et al. [4] of its action as a 'primer' in the viral induction of plasmacytomas, pristane had not been tested for its cocarcinogenic activity. We have developed a physical assay system to correlate the cocarcinogenic activity of hydrocarbons with their effects on the physical properties of mixed phospholipid/cholesterol micelles, estimated from the kinetics of penetration of the micelles by a selected dyestuff probe [5]. In this physical system, pristane retarded the kinetics

in a manner comparable to those of the *n*-alkane promoters. Quantitatively, it was predicted that pristane should have higher cocarcinogenic activity than the straight-chain alkanes previously tested [6]. Biological tests to compare the promoting activity of pristane with n-C_8H_{18}, n-$C_{18}H_{38}$ and n-$C_{24}H_{50}$ in cyclohexane are presented in this paper. Based on the physical assay, octane had been predicted to be promoting at high concentrations, tetracosane to be inactive at its solubility limit, 20%. Earlier tests on C_{18}, C_{20}, C_{24} and C_{28} *n*-alkanes in decahydronapthalene demonstrated that all were active promoters except n-$C_{24}H_{50}$ [7]. The latter sample had failed to retard the kinetics of transfer of the probe in the physical assay. We have investigated the possibility that impurities in the sample tested were responsible for the lack of promoting activity.

Materials and Methods

The initiating carcinogen was 7,12-dimethylbenz-[*a*]anthracene (Sigma Chemical Co., St. Louis, MO), used as received. *N*-Octadecane and *n*-tetracosane (Humphrey Chemical Co., North Haven, CN), and *n*-octane and pristane (Aldrich Chemical Co., Milwaukee, WI) were purified by chromatography on activated silica gel. This procedure eliminated any hydroperoxide or aromatic impurities. Reagent grade benzene (General Chemical, Morristown, NJ) was used as the solvent for the initiating applications of 7,12-dimethylbenz[*a*]anthracene. Cyclohexane, spectro ACS (Eastman, Rochester, NY) was used as received. The mother liquor from the commercial recrystallization of *n*-tetracosane was obtained through the cooperation of Humphrey Chemical Co. The solvent, iso-octane, was removed by flash evaporation. The residue was analyzed by gas chromatography-mass spectrometry on a Finnegan instrument.

Egg phosphatidylcholine and lysophosphatidylcholine (99%, lyophilized; Grand Island Biologicals, Santa Clara, CA) were freed of residual solvent under vacuum before use. Cholesterol (reagent grade; Merck, Rahway, NJ) was recrystallized three times from ethanol. Oleic acid (Sigma grade, 99%; Sigma Chemical Co., St. Louis, MO) was used as received.

The promoting activity of the four alkanes and the by-products of tetracosane synthesis was determined at various dilutions in cyclohexane. An initiating application of 60 µl of 0.3% 7,12-dimethylbenz[*a*]-anthracene in benzene was given to male C3H mice (average weight 22 g; Jackson Memorial Laboratory, Bar Harbor, ME). After a 2 week interval, solutions of the alkanes in cyclohexane were applied (60 µl) twice each week for 50 weeks or until the appearance of a 1 mm^3 nonregressing papilloma. Cyclohexane was selected as the solvent because it has the advantage of being much less irritating than decahydronaphthalene. In using the more volatile vehicle, it should be kept in mind that the concentration of alkane in the solution being absorbed into the epidermis is much higher than that prepared, probably at least double.

Tumors were classified grossly as malignant only when they had developed a rolled border and firm attachment (by palpation) to underlying connective tissue. At necropsy (52 weeks or sooner if gross evidence of malignancy developed) malignancy was diagnosed by microscopic examination by Dr. Richard Moore, Professor of Pathology, of the tissue fixed in formalin and stained with hematoxylin and eosin. Only tumors that invaded the dermis were classified as malignant.

The physical model for comparing the effects of various alkanes on membrane lipids has been described previously [6]. Modification of the lipid composition in the following manner improves the sensitivity of the organic phase micelles at 21°C to promoting concentrations of those alkanes that are active.

An intimate mixture of 71% egg phosphatidylcholine and 29% egg lysophosphatidylcholine (1.75 : 1 molar ratio) is prepared by coprecipitating them from solution in chloroform by evaporation of the solvent under dry N_2. The residue, dried under vacuum, is dissolved in cyclohexane at 0.225 mg/ml (0.2 mM in phosphatidylcholine). Cholesterol is added at a concentration of 30 mM and oleic acid at 55 mM (2% of the organic phase).

Results

The physical assay involves measurement of the rate of transfer of a water-soluble, hydrocarbon-insoluble, anionic dyestuff probe from an aqueous phase into micelles of membrane phospholipids dissolved in a cycloalkane phase [6]. The kinetics are controlled by suitable combinations of cholesterol

TABLE I

COMPARISON OF THE PROMOTING ACTIVITY OF PRISTANE AND *n*-ALKANES

All mice were initiated with one 60 μl application of 0.3% (by weight) DMBA in benzene. After 2 weeks, solutions of the alkanes (60 μl per application) in cyclohexane were applied twice a week for 50 weeks or until appearance of a 1 mm^3 papilloma. Effective number of mice; original number less number that died without tumor prior to median time of appearance of tumors. $I_{52} = 1 + \left(\frac{\text{No. of mice with papillomas} + 2\times \text{No. of mice with carcinomas in 52 weeks}}{\text{Effective no. of mice}}\right)$. *P*, significance of difference in tumor incidence from cyclohexane control, by Fisher's exact probability test; n.s., not significant.

Hydrocarbon tested as a promoter	Concentration in cyclohexane (% by volume)	Mole fraction in cyclohexane	Original number of mice	Effective number of mice	Number of nonregressing tumors in 52 weeks		Relative promoting activity (I_{52})	Probability (*P*)
					Benign	Malignant		
n-Octane (C_8H_{18})	75	0.69	17	11	2	4	1.9	0.002
n-Octadecane ($C_{18}H_{38}$)	20	0.08	15	13	2	2	1.5	0.04
	40	0.18	15	14	3	7	2.2	0.001
Pristane ($C_{19}H_{40}$)	20	0.07	15	13	3	7	2.3	0.001
	40	0.16	36	33	8	21	2.5	0.001
n-Tetracosane ($C_{24}H_{50}$)	20	0.06	16	14	1	1	1.2	n.s.
(by-products)	20	–	15	14	3	8	2.4	0.001
Mixture ($C_{24}H_{50}$)	17	–	16	16	0	2	1.2	n.s.
(by-products)	5	–						
Cyclohexane	–	–	35	32	0	2	1.1	–
Nil (control)	–	–	35	33	1	2	1.15	n.s.

and oleic acid with a relatively unsaturated egg phosphatidylcholine and a saturated lysophosphatidylcholine (see Materials and Methods). Thus, control levels provided 25–30% transfer of the dyestuff probe to the organic phase micelles in 1 h under conditions of emulsification.

In Fig. 1 is seen the retardation of the transfer of the probe that occurs when cocarcinogenic *n*-alkanes are introduced into the cycloalkane phase. Higher concentrations of the alkanes are required for significant effects when cyclohexane is substituted for the decalin previously used [6] as the bulk organic diluent.

The effect of pristane on the kinetics is seen to be greater than that of the *n*-alkanes at any given concentration. Hence, it was predicted that pristane should be a more active promoter than *n*-octadecane in cyclohexane. At high concentrations even *n*-octane retarded the kinetics.

Pristane was also compared with purified *n*-tetracosane at its solubility limit, 20%, in cyclohexane and with a mixture of 17% tetracosane with 5% of the mixed by-products formed in its synthesis from 1-bromododecane. A lipid mixture described earlier [6] was used for this transport experiment. Pristane retarded the transfer of the dyestuff probe to the organic phase as usual but, in contrast, $C_{24}H_{50}$ almost doubled the rate. The mixture with the by-products increased the rate 60%.

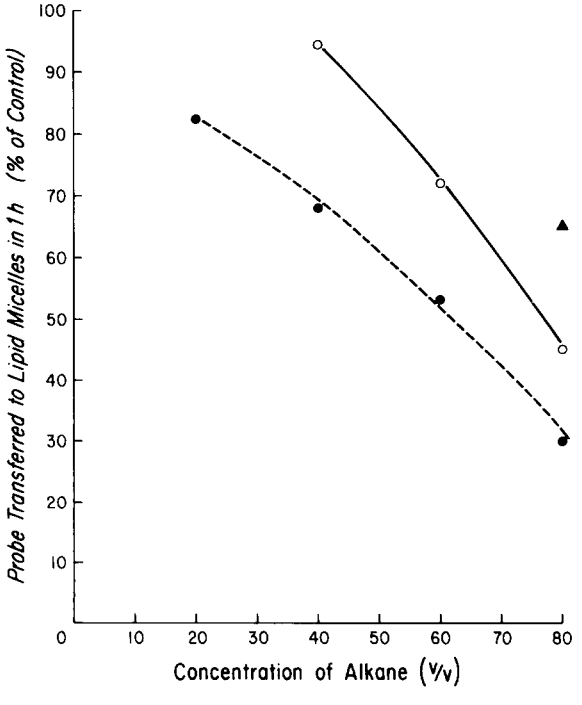

Fig. 1. Effects of alkanes on lipid micelles in cyclohexane. ●- - - - -○, pristane; ○———○, *n*-octadecane; ▲, *n*-octane.

In Table I are shown the results of initiation-promotion experiments on male C3H mice to compare the promoting activities of pristane, *n*-octane, *n*-octadecane, *n*-tetracosane and its by-products, in cyclohexane. By 52 weeks, most of the induced tumors had progressed to malignancy, microscopically invasive squamous cell carcinomas. Comparison of the physical data of Fig. 1 with these biological results shows that the more effective the alkane is in retarding the transfer of the probe into the lipid micelles, the greater its promoting activity.

Thus, pristane in cyclohexane proved to be a more active promoter than octadecane in this diluent. In contrast, not only did highly purified tetracosane prove, as predicted, to be inactive as a promoter when tested at 20%, its solubility limit in cyclohexane, but it was still inactive when contaminated by the very promoting by-products of its synthesis. Gas chromatographic-mass spectrometric analysis of the latter showed that the major components were probable promoters: dodecane, dodecenes, and C_{19} and C_{20} alkylbenzenes (from alkylation of an aromatic solvent employed in the synthesis). Unreacted dodecyl bromide and dimer tetracosenes were also present.

n-Octane proved to have significant promoting activity ($P < 0.01$) when tested as a 75% solution in cyclohexane.

Discussion

Depending upon their chain length, alkanes may either enhance or retard the rate of solubilization of an ionic dyestuff into mixed phospholipid/cholesterol micelles in cyclohexane. Those that retard this physical process have proved capable of enhancing the progression of latent skin tumors in male C3H mice initiated by 7,12-dimethylbenz[*a*]anthracene. It is of interest to note that most of the hydrocarbons that have shown significant promoting activity in cycloalkane diluents contain 8–20 carbon atoms in their structure, i.e., equal to or less than the number of

carbon atoms in the initiators 7,12-dimethylbenz[*a*]-anthracene, $C_{20}H_{16}$, or benzopyrene $C_{20}H_{12}$. The promoters include not only alkanes, but also alkenes, alkylcyclohexanes, alkylbenzenes and alkylnaphthalene [8].

Containing only four more C atoms than the active promoter, eicosane, $C_{20}H_{42}$ [7], is the nonpromoter, tetracosane, $C_{24}H_{50}$. Unfortunately, adequate testing of the higher *n*-alkanes is inhibited by their very limited solubility in hydrocarbon solvents and even lower solubility in acetone. Branched-chain alkanes are lower melting compounds and generally more soluble in cyclohexane and should prove more useful in an expanded investigation of the properties of hydrocarbons containing more than 20 carbon atoms.

The lack of promoting activity of the mixture of tetracosane with the very active by-products of its synthesis has a parallel in our earlier work with non-cocarcinogenic mineral oils. In that research we found that as much as 50% of the active cocarcinogen, *tert*-dodecylbenzene, could be incorporated into highly refined white mineral oils without causing them to develop significant cocarcinogenic activity [9].

The report that *n*-hexadecane in acetone was a partial inhibitor of benzopyrene carcinogenesis [10] may have been complicated by the fact that this alkane is only about 15% soluble in acetone at 21°C and crystallizes out of solution almost completely below 15°C. Storage of the solutions in amber bottles in the refrigerator, an admirable experimental precaution against oxidation and evaporation, would in this case have aggravated the problem of phase separation, making its observation difficult and rendering uncertain what composition was actually applied to the mice.

These problems of low solubility in acetone would be worse with higher melting *n*-alkanes, such as eicosane ($C_{20}H_{42}$) or octacosane ($C_{28}H_{58}$), but should not be present in testing the liquid polyalkene, squalene.

We have previously noted that, in view of their very high oil/water partition coefficients, hydrocarbons containing 10 or more carbon atoms/molecule would be expected to localize in lipoprotein membranes upon absorption into the epidermis [7]. X-ray diffraction data indicate that the long-chain alkanes align parallel to the phospholipid acyl chains, the wide-angle diffraction pattern being characteristic of the liquid-crystalline state [11]. The developing correlation between their biological effects on cells previously initiated by 7,12-dimethylbenz[*a*]anthracene and their physical effects on the rate of transfer of an anionic organic probe across lipid/water interfaces strongly suggests that their primary effect in the epidermis is to alter critical phase relationships at water/cell membrane interfaces. It is known, for example, that introduction of *n*-hexadecane into colloidal systems of water/surface-active substance/amphophilic substance can dramatically alter the structure of the micellar and liquid-crystalline phase involved, sometimes stabilizing microemulsions that change the microfluidity of the system grossly [12].

The importance of the amphiphile cholesterol to the success of our physical model system in predicting promoting activity suggests that the cellular membrane of importance for hydrocarbon promotion is one in which this sterol is critical, e.g., the surface or nuclear membranes, but not those of the mitochondria. The structure and microfluidity of the surface membrane in the interfacial region would be expected to influence the penetration of integral proteins into the hydrophobic region of the membrane, which in turn influences their lateral mobility. Seemingly paradoxically, it was found by Shinitizky et al. [13] that increase in membrane microviscosity were accompanied by an increase in the rotational mobility of concanavalin A receptors. The vertical displacement of the protein receptors (normal to the plane of the water/membrane interface), so as to decrease the penetration into a more viscous membrane, permitted greater mobility.

We have initiated an investigation of the effects of promoting hydrocarbons on the microfluidity of various lipid compositions, particularly those that have proved useful in providing the correlations between physical effects and biological activity presented in this and earlier papers. The technique of Shinitizky et al. [13], utilizing the polarization of the fluorescence of aromatic probes, is showing promise.

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