BBA 75556

IONIC TRANSPORT THROUGH MODEL MEMBRANES

I. EFFECTS OF ACCELERATORS OF CARCINOGENESIS

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(Received August 3rd, 1970)

SUMMARY

1. Egg phosphatidyl choline adsorbed at an hydrocarbon–water interface was used as a physical model of a membrane–cytoplasm interface. The rate of transport of an anionic dyestuff could be measured across the interface from the aqueous to the organic phase under conditions of emulsification. The process simulates carrier transport in that the lecithin is an essential requirement, forming a tightly bound complex with the dye that becomes solubilized in the phospholipid micelles in the bulk organic phase.

2. The standard organic phase was then modified by inclusion of *n*-alkanes from C_7 to C_{24}. Most of the alkanes that have biological activity as accelerators of carcinogenesis, C_{10}–C_{20}, produced a significant reduction of the rate of transport of the dyestuff into the organic phase.

3. Correlation was inferred between these observations and those of investigators of intercellular ionic transport in epithelial tissue cultures, who found a marked reduction of transport rates in certain malignant epithelia in contrast to the corresponding normal tissues. A membrane-mediated mechanism of control of initiated neoplastic cells is suggested. The hypothetical control depends upon a specific surface charge distribution at the lipoprotein–cytoplasm interface that is altered by the absorption of C_{10}–C_{20} *n*-alkanes.

INTRODUCTION

Some years ago it was discovered that long-chain saturated, as well as alkylaromatic, hydrocarbons could have a profound effect on the rate of induction of skin tumors by polycyclic aromatic compounds[1]. These findings led to the consideration that intermolecular forces (chain–chain interactions) might play a rate-controlling role in carcinogenesis mechanisms at the cellular level. The probability was high that such hydrocarbons, absorbed by epidermal cells, would be localized in the lipoprotein membranes. There, interaction with the alkyl chains of phospholipids could result in significant changes in the conformation of the lipids and structural proteins at the aqueous interfaces.

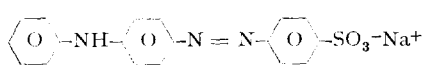
We were impressed by the differences in intercellular transport found by

LOEWENSTEIN AND KANNO[2,3] and BOREK *et al.*[4] between normal and malignant epithelia. The junctional membranes of the malignant cells proved much less permeable (between adjoining cells) to ions. The possibility seemed worth investigation that interactions between promoting hydrocarbons and membrane phospholipids might result in changes in surface charge distribution leading to alteration in normal ionic transport properties of the membranes.

The report by HIRT AND BERCHTOLD[5] that alcohols of different chain lengths affected anionic transport by phospholipids across a simple organic–aqueous interface to differing extents suggested a model system for investigation of our question. It was our intention then to study the transport of a suitable anionic dye from an aqueous phase into an organic phase in the presence of a phospholipid.

As our organic phase, we selected a solution of egg phosphatidyl choline in the condensed-ring saturated hydrocarbon, decalin. Previous biological experiments on decalin had led to its selection as a standard non-accelerating vehicle for carcinogenesis tests[1,6].

The anionic dye, Orange IV, has proved satisfactory for our transport study



as long as the ionic strength of the aqueous phase is greater than 0.1. To simulate the aqueous phase of the cell, we have maintained a pH of approx. 7.1 using phosphate buffer and sufficient KCl to bring the ionic strength up to 0.14.

The rate of interaction of the dye with the phospholipid is measured as the rate of transfer of the dye from the aqueous to the organic phase. Achievement of an easily measured and reproducible rate ($\pm$ 10 %) depends upon scrupulous care in removing surface active contaminants from the mixing chambers, and standardization of the procedure for making and breaking the necessary emulsion of the two phases. We have adopted a procedure in which about 35 % of the dye is transferred to the organic phase in 10 min mixing.

It will be shown that this system is selectively susceptible to the modification of the organic phase by addition of those *n*-alkanes that have previously been found to accelerate benzopyrene carcinogenesis, *i.e.* C_{10}–C_{20} (ref. 6). Paraffins of shorter or longer chain length are ineffective in either situation. Correlation between the molecular length of the various fatty acid chains of membrane phospholipids and the chain length of the effective *n*-paraffins will be noted.

It may be more than coincidence that the transport rate is markedly retarded by the carcinogenesis accelerators. Coupled with Loewenstein's observation of reduced ionic permeability through junctional cell membranes of malignant epithelia, a tissue-mediated control of the behavior of the benzopyrene-initiated cell is suggested, which may be broken down by repeated absorption of C_{10}–C_{20} alkanes.

MATERIALS AND METHODS

Egg phosphatidyl choline, General Biochemicals, chromatographically pure. Orange IV, sodium *p*-(*p*-anilinophenylazo)benzene sulfonate, Matheson, Coleman and Bell. Aqueous phase, 134 mM KCl, 4 mM phosphate buffer (Beckman No. 3581, pH 7 concentrate) diluted in doubly distilled (glass) water. Organic solvents: *cis*-

and *trans*-decalin, Matheson, Coleman and Bell, Practical grade. *n*-Alkanes, C_7–C_{24}, Matheson, Coleman and Bell or Humphrey Chemical, 99%. All hydrocarbons chromatographed on activated silica gel to remove ultraviolet absorbers (at 262 mμ).

Procedure

Round-bottomed flasks (50 ml) were cleaned by soaking overnight in hot, concentrated HNO_3 in 94% H_2SO_4, and rinsed thoroughly with doubly distilled (glass) water. The organic phase was prepared as a 1 mM solution of egg phosphatidyl choline (mol. wt. 750 est.) in a hydrocarbon solvent (either decalin or a mixture of decalin and a *n*-alkane). For the aqueous phase a solution of Orange IV, usually 40 μM, was prepared in the buffered KCl solution.

Equal volumes of the organic and aqueous phases (6 ml of each) were placed in the 50-ml flasks, the flasks stoppered with corks washed in nanograde hexane, and shaken on a Burrell wrist-action shaker (at a maximum setting) for specified times ranging from 2 min to 3 h. As soon as it was removed from the shaker, each set of emulsions was transferred to centrifuge tubes (including rinsing of the flasks with measured equal volumes of the aqueous buffer and the original organic phase) and spun for 10 min at 7500 rev./min (Servall), and then for 10 min in a clinical centrifuge.

The clarified phases were transferred to spectrophotometer cells by pipetting and the absorbance maximum near 440 mμ (425 mμ in the organic phase) measured in a Beckman DB spectrophotometer. Frequently an interfacial emulsion persisted after centrifugation. To estimate the dye trapped in this emulsion, it was broken by using *n*-butanol at an estimated 5% of the volume (this concentration had no effect on dye distribution) and heating 5 min on a steam bath. The resulting two phases were carefully diluted and their absorbance at 440 mμ measured (425 mμ in the organic phase).

When this procedure of working up the interfacial emulsion was followed and the amounts of dye found in the aqueous and organic parts of the emulsion added to those found in the clarified phases, the total recovery of the dye usually exceeded 95% (calculations based on the assumption that the absorptivity of the dye in the organic phase is approximately equal to that in the aqueous phase). Further the ratio of dye concentration in the organic and aqueous parts of the emulsion was the same as it was in the clarified organic and aqueous phases. Therefore, this work-up of the interfacial emulsion was generally omitted in the experimental program except for spot checks to insure the continued validity of this assumption. Therefore, in calculations of the percent of the dye transported to the organic phase in the experiments in which the interfacial emulsion was not treated, the percent transport was based upon the total dye recovered in the two clarified phases.

No detailed investigation has been made to date of the effect of temperature. Preliminary indications are that temperatures above 28° diminish the observed differences in transport rates.

EXPERIMENTAL RESULTS

The effect of variation in the concentration of phosphatidyl choline in the organic phase was determined over the range 0.2–3.0 mM. The 1 mM concentration

was chosen as the standard for the rest of the experiments since maximal rates were achieved with this concentration and higher concentrations provided excessive emulsion problems.

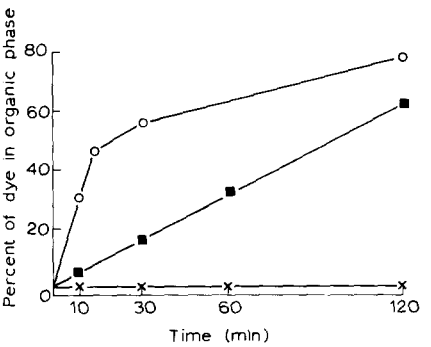
In Fig. 1 is shown the rate of approach to equilibrium using a 1 mM solution of egg phosphatidyl choline as the carrier in decalin as the organic phase and 40 μM Orange IV as the transported anion in the buffered aqueous phase. For further comparisons the estimate of the initial rate of transport provided by the percent of recovered dye found in the organic phase after 8 min mixing was chosen as a standard.

The lack of transport shown by the system without lecithin in the organic phase (lower curve of Fig. 1.) indicates the partition coefficient for Orange IV in the organic *versus* the aqueous phase is close to 0. This raised the question about the nature of the hypothetical complex which appeared to be required between the phosphatidyl choline and the dyestuff in the organic phase.

The reversibility of the transport was tested by using an organic phase in which the concentration of Orange IV was 20 μM (from a transport experiment involving 1 mM lecithin in decalin) together with an equal volume of buffer solution not containing Orange IV. Only 15 % of the dye was recovered in the aqueous phase after 2 h mixing. The results indicate the very slow reversibility of the process under these conditions. Apparently a simple physical distribution is not involved but rather a strong interaction of the phospholipid and the anionic dyestuff.

A limited investigation has been made of conditions for removal of the dye from the lecithin micelles in the organic phase. Apparently the "complex" is fairly stable in the presence of 0.01 M HCl or 0.15 M $CaCl_2$ but the dye may be displaced much more rapidly by anions such as hydroxyl or laurate.

Finally the effects of the homogolous series of *n*-alkanes used as components of the organic phase were investigated. Mixtures of equal volumes of *n*-alkane and decalin were substituted for the decalin solvent and the fraction of the dye recovered in the organic phase after 8 min mixing was determined. In the case of the less soluble alkanes ($>C_{18}$), 20 % solutions in decalin were used. The results are shown in Figs. 1



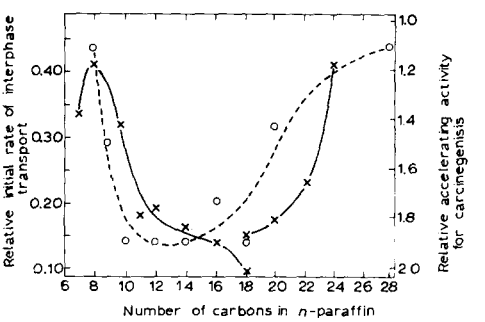


Fig. 1. Rate of interphase transport of Orange IV by egg lecitin. ○—○, transport with 1 mM lecithin in decalin; ×—×, transport without lecithin; ■—■, Transport with 1 mM lecithin in *n*-$C_{18}H_{38}$–decalin (50:50, by vol.).

Fig. 2. Comparison of effects of *n*-paraffins on anionic transport with their effects on benzopyrene carcinogenesis. ×—×, transport rates using *n*-alkane–decalin (50:50, by vol.) for C_7–C_{18}, *n*-alkane–decalin (20:80, by vol.) for C_{18}–C_{24}; ○—○, relative accelerating activity of *n*-alkane–decalin (50:50, by vol.) for skin carcinogenesis.

and 2. In Fig. 2 they are compared with the carcinogenesis accelerating activity of mixtures of equal volumes of *n*-alkanes and decalin (biological data shown by the broken line).

The molecular lengths of the alkanes associated with strong acceleration of benzopyrene carcinogenesis, C_{10}–C_{20}, proved in the main to be the ones that retard the phospholipid carrier transport of the anionic dyestuff across the model interface. The transport system is significantly affected by 10% of the C_{16}–C_{22} paraffins in decalin whereas greater than 20% of $C_{12}H_{26}$ or $C_{14}H_{30}$ was found to be required. To be effective as a carcinogenesis accelerator in the earlier biological experiments[7], greater that 20% *n*-$C_{12}H_{26}$ had similarly proved necessary. To check this correlation further, a new mouse test has been started to determine the accelerating activity of 10–20% of the C_{16}–C_{20} paraffins.

DISCUSSION

The requirement that lecithin be present in the organic phase to effect any transfer of the dyestuff into that phase indicates that ion pair formation is probably an essential requirement to produce the organic solubility of the complex. Once the complex is formed and taken into the micellar aggregates in the bulk organic phase, one would expect the sulfonate group to be buried in the interior of the micelles. Thus it would be rather completely removed from exposure to any aqueous interface the micelle might encounter. This would explain the apparent slow reversibility of the transport process in this model system.

Since the phospholipid micelles in the bulk organic phase would be expected to have the head groups centrally oriented, the larger the micelles the greater the extent of removal of the ionic head groups from the micellar surface. Thus the decreasing rate of interaction of lecithin with the dyestuff as alkanes of longer than C_{10} chain length are added to the organic phase could be explained if the molecular aggregation of the phospholipids into micelles is thereby increased. Studies of the micellar solutions of egg lecithin in these hydrocarbons are being made by light scattering techniques to investigate this question.

Thus the retarding effects of *n*-alkanes of appropriate chain length on the interphase transport of an organic anion is probably due to the reduced effective concentration of phospholipid charge groups exposed at the aqueous interface. It is interesting to speculate that a similar mechanism may operate at tight junctions between epithelial cells under conditions in which intercellular ionic transport is retarded. LOEWENSTEIN *et al.*[8] have noted that any condition that results in a significant increase in the cytoplasmic Ca^{2+} concentration at the junctional membranes leads to retarded transport rates. Since it is generally considered that the surface conformation of phosphatidyl choline at the membrane–cytoplasm interface is affected by interaction with Ca^{2+} (ref. 9), it is an attractive speculation that the charge distribution required for this interaction could be altered by interdigitation of appropriate *n*-alkane molecules in the liquid crystalline phase of the membrane adjacent to the aqueous interface.

Maximum strength of interaction of *n*-alkanes with interfacial phospholipids would be expected if the molecular chain lengths were compatible, geometrically. This might well explain why those *n*-alkanes whose molecular chain lengths are

comparable to the alkyl chains of the phospholipids are the ones that effectively retard transport in our model system and affect the rate of carcinogenesis in epithelial situations.

Since in the final analysis the tissue control of a latent cancer cell probably depends upon a mechanism as yet unknown involving the effector end of the normal immune response, one can go no further in expanding this hypothesis. However, it is attractive to think about the possibility that some of the final stages in complement-mediated cytolysis will be inhibited when the latent cancer cell membrane has its surface charge distribution sufficiently altered by absorption of a *n*-alkane of one of these effective chain lengths.

ACKNOWLEDGMENTS

This research program was supported by Public Health Service Research Grant CA-08788 and Contract PH-43-67-675 (National Institutes of Health Division of Biologics Standards), and by Chesebrough-Pond's Inc.

The following members of our research group made substantial contributions to this experimental investigation: Joseph L. Alvarez, Patricia E. Goings, Kenneth A. Hegge, James C. Haley, and Stephen R. Wiener.

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