Biochimica et Biophysica Acta, 640 (1981) 595—608

BBA 79068

DIFFERENTIAL PERTURBATION OF ERYTHROCYTE MEMBRANE FUNCTION BY STRUCTURALLY RELATED POLYCYCLIC AROMATIC HYDROCARBONS

MARY E. ABERLIN * and GARY W. LITMAN

Sloan-Kettering Institute for Cancer Research, Donald S. Walker Laboratories, 145 Boston Post Rd., Rye, NY 10580 (U.S.A.)

(Received September 25th, 1980)

Key words: Polycyclic aromatic hydrocarbon; Acetylcholinesterase; Glucose exchange; Antihemolysis; (Erythrocyte membrane)

Summary

Examination of the interaction of a number of structurally related polycyclic aromatic hydrocarbons with the erythrocyte plasma membrane indicated that the presence and position of methyl groups on the lipophilic hydrocarbon nucleus determined whether the compound acted as an inhibitor of membrane function. 7,12-Dimethylbenz(*a*)anthracene, a potent carcinogen, acted as a noncompetitive inhibitor of membrane acetylcholinesterase. The inhibition depended on the anion composition of the buffer at the time of exposure of the cells to inhibitor, i.e., it was only manifest in the presence of an anion gradient. The temperature dependence of the intact cell enzyme in the presence of inhibitor was influenced by the temperature at which the compound was added prior to assay and may involve the perturbation of tightly associated lipids. Glucose exchange across the membrane was inhibited by the same compounds which inhibit acetylcholinesterase. The temperature dependence of the exchange was not grossly altered by the presence of 7,12-dimethylbenz(*a*)anthracene.

The observed inhibition of two membrane functions by the polycyclic aromatic hydrocarbons does not correlate simply with their theoretical octanol/water partition coefficients, water solubilities, or ability to confer membrane stabilization against osmotic hemolysis. This demonstration of differential inhibition by compounds having the same overall hydrophobicity was unexpected and suggests a more complex mode of interaction with the cell membrane.

* Present address: The Rockefeller University, New York, NY 10021, U.S.A.

Introduction

The functional perturbation of biological membranes by small hydrophobic molecules leads to such pharmacological events as anesthesia, tranquilization, and narcosis by a mechanism(s) which is poorly understood at present. Upon interaction with red blood cell membranes, such lipophilic molecules usually protect erythrocytes from hypotonic lysis [1—3]. Quantitative studies indicate that the protective ability of hydrophobic compounds is related to the molecule's overall hydrophobicity rather than to its structure, suggesting that after partition into the membrane, alteration of membrane function proceeds by a common mechanism [3]. For a family of structurally related compounds, the concentration at which individual members protect erythrocytes against osmotic hemolysis should depend only on the change in overall hydrophobicity afforded by various substitutions in the molecule. Concomitant changes in functional properties of the membrane should be similarly affected.

In the course of characterizing the interaction of plasma membranes with lipophilic carcinogens, Litman et al. [4,5] examined the stabilization against osmotic hemolysis afforded by a series of azo dye and polycyclic aromatic hydrocarbon carcinogens. Methyl-substituted derivatives of the azo dye carcinogen, dimethylaminoazobenzene, exhibited protection which correlated with the octanol/water partition coefficients of the compounds. The polycyclic aromatic hydrocarbons, however, conferred little, if any, protection even though these compounds have much higher partition coefficients and demonstrated considerably higher absolute binding to the red blood cells. The lack of a correlation between the partition behavior and erythrocyte stabilization led to the suggestion that polycyclic aromatic hydrocarbon carcinogens interacted with the membrane in a different fashion than the azo dyes [5].

An investigation of the perturbation of erythrocyte membrane function by this family of structurally similar antihemolytic azo dyes indicated that functional perturbation did not correspond with the ability of the dyes to protect erythrocytes against osmotic hemolysis [6], a property known to be correlated with the pharmacological potency of a variety of lipophilic compounds [3]. This unexpected behavior is apparently the result of a complex interaction with the membrane and suggested that further investigation of functional perturbation by other families of structurally related lipophilic compounds was necessary in order to evaluate the generality of this finding.

This communication reports the functional perturbation which accompanies the binding of polycyclic aromatic hydrocarbons to the erythrocyte membrane. It was found that inhibition of two erythrocyte membrane functions, acetylcholine hydrolysis and glucose exchange, depended on the presence and position of methyl substituents in a number of families of structurally related polycyclic aromatic hydrocarbons, some of which have been used as probes of membrane dynamics and many of which are carcinogenic. A preliminary report of this work has appeared [7].

Materials and Methods

Materials. Benz(*a*)anthracene, 7,12-dimethylbenz(*a*)anthracene, 3-methylcholanthrene, benzo(*a*)pyrene, pyrene and phenanthrene were obtained from

Eastman and recrystallized from ethanol or benzene/ethanol. Anthracene, 2-methylanthracene, 9-methylanthracene and 1-methylphenanthrene were purchased from Aldrich Chemical Co. 2-, 3-, 5-, 6-, 8-, 9- and 11-methylbenz(*a*)anthracene, as well as 4-, 2,7- and 4,5-dimethylphenanthrene were the generous gift of Dr. M.S. Newman (Ohio State University). 4-, 10- and 3,9-dimethylbenz(*a*)anthracene were kindly donated by Dr. R.G. Harvey (University of Chicago) and 7- and 12-methylbenz(*a*)anthracene by Dr. H. Marquardt (Sloan-Kettering Institute). All compounds were assayed for purity by thin-layer chromatography and ultraviolet spectroscopy and purified by recrystallization or thin-layer chromatography when necessary. 1 mM stock solutions were prepared by dissolving the compounds in absolute ethanol and were stored at −20°C after flushing with N_2.

Methods. Human red cell suspensions were prepared and assayed for acetylcholinesterase activity as described previously [6] using a modification of the spectroscopic method of Ellman et al. [8]. Temperature-activity studies were also performed as reported earlier [6]. The effect of the different incubation procedures on the binding of 7,12-dimethylbenz(*a*)anthracene (DMBA) to intact erythrocytes was assayed using ^{3}H-labeled DMBA.

The isolation of human erythrocyte acetylcholinesterase by affinity chromatography was performed as outlined by Niday et al. [9]. The high-salt method of Mitchell and Hanahan [10] was used to extract acetylcholinesterase from washed erythrocyte ghosts.

Ghosts were prepared by the method of Dodge et al. [11] using hypotonic phosphate buffer. Protein was measured according to the method of Lowry et al. [12] using bovine serum albumin as a standard.

Glucose exchange was measured exactly as described previously [6].

Results

Fig. 1 illustrates the compounds which were tested for their ability to inhibit both erythrocyte membrane acetylcholinesterase and glucose exchange across the membrane. The results of the functional studies are summarized in Table I.

Acetylcholinesterase studies

In the benz(*a*)anthracene molecule, those positions marked 'I' in Fig. 1 indicate that monomethyl substitution at this position resulted in a compound which inhibited both membrane functions, while those marked 'NI' as well as the nonmethylated parent compound, benz(*a*)anthracene, exhibited no inhibition when tested at the same final concentration. 3,9-Dimethylbenz(*a*)anthracene was a non-inhibitor, whereas dimethyl substitution in the 7- and 12-positions resulted in a compound which was inhibitory.

In the anthracene series, 9-methylanthracene was inhibitory, while anthracene and 2-methylanthracene were non-inhibitory. Also shown in Fig. 1 are the structures of three additional non-inhibitory polycyclic aromatic hydrocarbons: pyrene, benzo(*a*)pyrene and 3-methylcholanthrene.

When tested at the same final concentration for their ability to inhibit red cell acetylcholine hydrolysis, the 4- and 4,5-methyl-substituted phenanthrenes were twice as inhibitory as the 1- and 2,7-methyl compounds. Phenanthrene

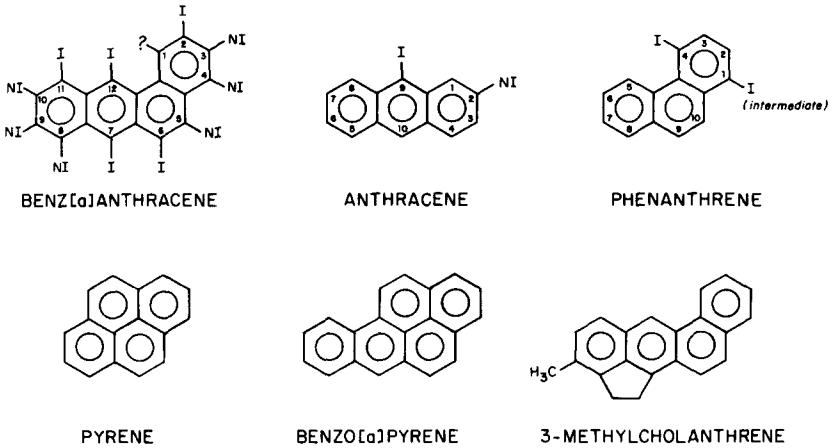


Fig. 1. Structures of polycyclic aromatic hydrocarbons tested as inhibitors of membrane function. Positions marked 'I' indicate that monomethyl substitution of the nonmethylated parent molecule resulted in a compound which inhibited both erythrocyte membrane acetylcholinesterase and glucose exchange across the membrane. Those marked 'NI' exhibited no inhibition when tested at the same final concentration.

was non-inhibitory at this concentration, although it was inhibitory when tested at 5- and 10-fold higher concentrations.

The concentration dependence of the inhibition of acetylcholinesterase by DMBA and 9-methylanthracene is shown in Fig. 2. 2-, 7- and 12-methylbenz(*a*)-anthracene exhibited the same concentration dependence as DMBA (not shown). The shift of the 9-methylanthracene inhibition curve to higher concentrations is consistent with the lower theoretical octanol/water partition coefficient of this compound [13,14]. The increase in the theoretical partition coefficient (*P*) caused by dimethyl substitution of benz(*a*) anthracene is apparently not reflected by a shift in its inhibition curve relative to those of the monomethylbenz(*a*)anthracenes, probably because the difference is much smaller than the difference between DMBA and 9-methylanthracene (Δlog *P* (octanol/water) = 0.5 and 1.27, respectively).

The inhibition of acetylcholinesterase by DMBA, a potent carcinogen, was characterized in greater detail. An examination of the dependence of inhibition on the length of time for which the cells were incubated with DMBA prior to assay showed that maximum inhibition was reached at exposures as short as 1 min. Although the standard incubation time for the assay of acetylcholinesterase was 15 min, doubling this time had no effect on the amount of inhibition monitored.

DMBA appears to act as a noncompetitive inhibitor of acetylcholinesterase (Fig. 3). The kinetic parameters derived from a Lineweaver-Burk plot of the activity as a function of substrate concentration in the presence (2.0 μM) and absence of inhibitor are: K_m = 108 μM; $V = 1.0 \cdot 10^{-15}$ mol/min per cell; K_i = 4.2 μM.

The temperature behavior of acetylcholinesterase in the presence of DMBA is illustrated in Figs. 4 and 5. There was no obvious discontinuity in the tem-

TABLE I

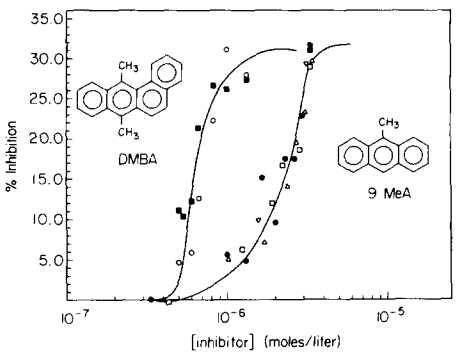
SUMMARY OF INHIBITION DATA

— , indicates that the compound conferred little or no protection against osmotic hemolysis. n.t., not tested.

Compound	Acetylcholinesterase			Glucose exchange	
	AH_{50} (μM)	% inhibition ± S.D. (3.3 μM) **	Concentration range	% increase in exit time ± S.D. (24.5 μM) **	Concentration range
Benz(*a*)anthracene	—	0	$3.3 \cdot 10^{-9}$—$3.3 \cdot 10^{-5}$	0	$2.5 \cdot 10^{-6}$—$2.5 \cdot 10^{-7}$
1-Me	n.t.	n.t.	n.t.	n.t.	n.t.
3-Me	4.3	0	$3.3 \cdot 10^{-6}$	0	$2.4 \cdot 10^{-5}$
4-Me	24.0	0	$3.3 \cdot 10^{-6}$	n.t.	n.t.
5-Me	13.0	0	$3.3 \cdot 10^{-6}$	0	$2.4 \cdot 10^{-5}$
8-Me	25.0	0	$3.3 \cdot 10^{-6}$	0	$2.4 \cdot 10^{-5}$
9-Me	4.7	0	$3.3 \cdot 10^{-6}$	0	$2.4 \cdot 10^{-5}$
10-Me	4.0	0	$2.8 \cdot 10^{-6}$	n.t.	n.t.
2-Me	2.8	27.5 ± 6.1	$3.3 \cdot 10^{-7}$—$3.3 \cdot 10^{-6}$	55.8 ± 9.7	$2.4 \cdot 10^{-5}$
6-Me	1.2	27.4 ± 4.7	$3.3 \cdot 10^{-6}$	55.9 ± 18.5	$2.4 \cdot 10^{-5}$
7-Me	n.t.	25.9 ± 1.4	$3.3 \cdot 10^{-7}$—$3.3 \cdot 10^{-6}$	n.t.	n.t.
11-Me	1.0	29.6 ± 1.5	$3.3 \cdot 10^{-6}$	84.3 ± 17.0	$2.4 \cdot 10^{-5}$
12-Me	1.0	31.6 ± 5.2	$3.3 \cdot 10^{-6}$	100.6 ± 11.3	$2.4 \cdot 10^{-5}$
3,9-Me	—	0	$6.6 \cdot 10^{-7}$—$3.3 \cdot 10^{-6}$	n.t.	n.t.
7,12-Me	13.0	28.8 ± 3.5	$3.3 \cdot 10^{-8}$—$1.6 \cdot 10^{-5}$	71.0 ± 11.8	$6.1 \cdot 10^{-7}$—$2.0 \cdot 10^{-5}$
Anthracene	—	0	$3.3 \cdot 10^{-6}$—$3.3 \cdot 10^{-5}$	0	$2.4 \cdot 10^{-5}$
2-Me	—	0	$3.3 \cdot 10^{-6}$	0	$2.4 \cdot 10^{-5}$
9-Me	2.7	29.9 ± 5.5	$3.3 \cdot 10^{-7}$—$1.6 \cdot 10^{-5}$	>200	$2.4 \cdot 10^{-5}$
Phenanthrene	5.3	0	$3.3 \cdot 10^{-6}$—$3.3 \cdot 10^{-5}$	n.t.	n.t.
1-Me	3.8	12.8 ± 2.8	$3.3 \cdot 10^{-6}$	46.1	$2.4 \cdot 10^{-5}$
4-Me	3.8	34.7 ± 2.2	$3.3 \cdot 10^{-6}$	>200	$2.4 \cdot 10^{-5}$
2,7-Me	3.1	14.4 ± 1.9	$3.3 \cdot 10^{-6}$	67.8	$2.4 \cdot 10^{-5}$
4,5-Me	10.0	35.8 ± 2.3	$3.3 \cdot 10^{-6}$	>200	$2.4 \cdot 10^{-5}$
Pyrene	18.0 *	0	$1.6 \cdot 10^{-5}$—$3.3 \cdot 10^{-6}$	n.t.	n.t.
Benzo(*a*)pyrene	n.t.	0	$3.5 \cdot 10^{-6}$	0	$2.4 \cdot 10^{-5}$
3-Methylcholanthrene	—	0	$1.7 \cdot 10^{-7}$—$3.3 \cdot 10^{-6}$	0	$2.4 \cdot 10^{-5}$

* The value for pyrene never reached 50%, therefore, the concentration at which pyrene conferred a 40% reduction in hemolysis is indicated.

** Concentration for which % inhibition or % increase in exit time is reported. The results show a statistical significance of $P \leq 0.05$ when analyzed by the Student's *t*-test.



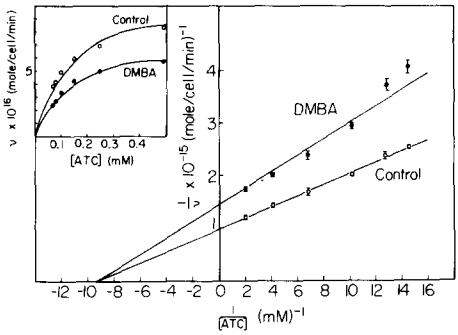


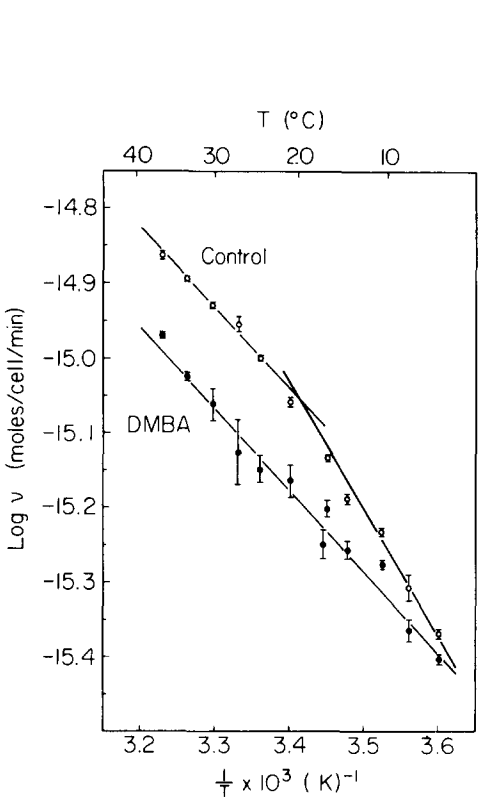
Fig. 2. Concentration dependence of erythrocyte membrane acetylcholinesterase inhibition by polycyclic aromatic hydrocarbons. The enzyme activity of intact cells was assayed at 22°C. Inhibition is expressed as a percentage of the rate of hydrolysis of an ethanol control (final concentration, 0.33%, v/v). Each point represents a triplicate determination of the rate at that concentration. The various symbols represent separate experiments. 9 MeA, 9-methylanthracene.

Fig. 3. Kinetics of erythrocyte membrane acetylcholinesterase activity. The enzyme was assayed at 21.7°C using an acetylcholine (ATC) substrate. Each point represents the average value of a triplicate determination ± S.D. The control contained ethanol at a final concentration of 0.33% (v/v). DMBA was present at a final concentration of 1.98 μM.

perature dependence of the activity seen in either figure when compared with intact cells in the absence of inhibitor. However, the slope of the line depended on the temperature at which the intact cells were incubated with buffer prior to assay. When the cells were added to a solution of buffer precooled to the assay temperature, the enzyme inhibition decreased as the temperature was lowered (Fig. 4). When the cells were exposed to inhibitor at room temperature and then equilibrated at the assay temperature prior to the addition of substrate, the increased amounts of inhibition at lower temperatures resulted in a slope increase, i.e., the dependence of inhibition on the assay temperature was no longer evident. This temperature behavior of the enzyme in the presence of DMBA was qualitatively similar to that previously reported for the azo dye inhibitor, 2-methyldimethylaminoazobenzene [6].

The temperature dependence of DMBA inhibition was not due to differential binding as a result of the incubation procedure. When binding as a function of incubation was measured using ^{3}H-labeled DMBA, 38.4 ± 1.7% of the added radioactivity (in cpm) remained in the supernatant when cells were added to precooled buffer containing 3.3 μM DMBA, while 38.0 ± 2.1% of the added radioactivity (cpm) remained in the supernatant when cells were incubated with inhibitor at room temperature prior to cooling. This agrees with the finding of Harris et al. [15] who reported that DMBA binding to the lipid envelope of oncornaviruses was the same at 4°C as it was at 37°C.

It is unlikely that DMBA solubilized the enzyme upon interaction with intact erythrocytes, since assay of the supernatant remaining after removal of treated cells showed that it contained no acetylcholinesterase activity. Furthermore, DMBA did not inhibit the enzyme activity when added to preparations of washed erythrocyte ghosts prepared by hypotonic lysis, nor when added to extracts of the enzyme isolated by high-salt treatment of the ghosts or by affinity chromatography.



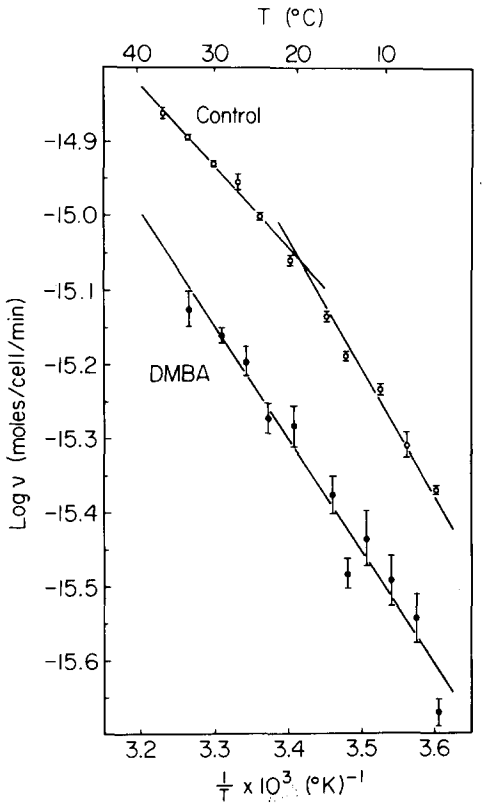


Fig. 4. Effect of DMBA on the temperature dependence of erythrocyte acetylcholinesterase activity. Incubation at assay temperature. Intact red blood cells were added to a buffer solution containing ethanol (0.33%, v/v) or 1.98 μM DMBA which had been pre-equilibrated to the temperature at which the assay was performed. The red cells were incubated at this temperature for 15 min before the reaction was initiated by the addition of substrate. Each point in the plot represents the average of a triplicate determination ± S.D.

Fig. 5. Effect of DMBA on the temperature dependence of erythrocyte acetylcholinesterase activity. Preincubation at room temperature. Cells were incubated with DMBA (1.98 μM) for 10 min at room temperature and then equilibrated to the assay temperature for 15 min prior to the addition of substrate. Under these conditions, inhibition is increased at low temperatures when compared with the inhibition under conditions described in the preceding figure.

As previously reported for the azo dye inhibitor [6], acetylcholinesterase inhibition by DMBA was found to depend on the anion composition of the buffer (Table IIA). No inhibition by DMBA was observed when the effect of inhibitor was assayed in buffers containing anions which are rapidly transported across the red cell membrane, viz., HCO_3^- and Cl^-. When cells were incubated with DMBA in a buffer which allowed inhibition, and were pelleted, and then assayed in a buffer in which inhibition was not usually seen, the compound still acted as an inhibitor. When the incubation buffer did not support inhibition, inhibition was not seen when assayed in buffer which did support inhibition (see Table IIB). These results indicate that the transmembrane potential at the time of exposure to inhibitor is the determining factor. A similar

TABLE II

INHIBITION OF ACETYLCHOLINESTERASE: (A) DEPENDENCE OF INHIBITION ON BUFFER COMPOSITION; (B) DEPENDENCE OF INHIBITION ON COMPOSITION OF INCUBATION BUFFER VERSUS ASSAY BUFFER; (C) DEPENDENCE OF INHIBITION ON CELL INTACTNESS

(A) Assay temperature: 22° C. All buffers contained 5 mM phosphate, pH 8. Controls contained ethanol at a final concentration of 0.33% (v/v). Enzyme activity is expressed in fmol/min per cell. Values are reported as the average of a triplicate determination ± S.D. The statistical significance for % inhibition is indicated. (B) Cells were added to incubation buffer containing ethanol (0.33%, v/v) or DMBA. After a 15 min incubation, the cells were pelleted, resuspended in assay buffer, and the rates determined. All procedures were conducted at room temperature. Inhibition is expressed as a percent of the control values. The statistical significance for % inhibition is indicated. (C) Buffer: 100 mM sodium phosphate, pH 8. Assay temperature: room temperature. The controls contained ethanol at a final concentration of 0.33% (v/v). The enzyme activity for the intact and lysed conditions is expressed in fmol/min per cell. Ghost activity is expressed in μmol/min per mg membrane protein. Cells were lysed after exposure to inhibitor, but prior to assay. Ghosts were prepared by hypotonic lysis and added to assay from a dilution of the ghost pellet in hypotonic buffer. The statistical significance for % inhibition is indicated.

(A) Assay buffer	Control activity	+DMBA (1.98 μM)	% inhibition
100 mM sodium phosphate	0.88 ± 0.04	0.60 ± 0.07	31.8 ($P < 0.025$)
100 mM Na_2SO_4	0.81 ± 0.01	0.60 ± 0.07	25.9 ($P < 0.050$)
150 mM NaCl	0.84 ± 0.07	0.82 ± 0.02	—
150 mM $NaHCO_3$	0.90 ± 0.10	0.93 ± 0.04	—

(B) Incubation buffer	% inhibition by DMBA (3.30 μM)	
	Assay buffer: 100 mM sodium phosphate	150 mM NaCl
100 mM sodium phosphate	33.5 ($P < 0.025$)	36.7 ($P < 0.025$)
150 mM NaCl	0	0

(C) State of cells when exposed to inhibitor	Control activity	+DMBA (3.30 μM)	% inhibition
Intact	0.85 ± 0.02	0.56 ± 0.07	33.6 ($P < 0.025$)
Lysed	1.07 ± 0.01	0.67 ± 0.08	37.1 ($P < 0.025$)
Ghosts	1.59 ± 0.04	1.59 ± 0.03	0

result using linolenoyl sorbitol as an inhibitor of acetylcholinesterase was first reported by Livne and Bar-Yaakov [16], who also found that inhibition could be measured in lysed cells if they were lysed after exposure to inhibitor in the presence of a Cl^- gradient. Table IIC shows that this, too, was the case for DMBA inhibition of acetylcholinesterase.

Glucose exchange studies

The families of polycyclic hydrocarbon, for which the inhibition of acetylcholinesterase was reported above, were also tested for their effect on glucose exchange across the cell membrane (inhibition generally assayed at a final concentration of $2.4 \cdot 10^{-5}$ M). There was a direct correlation with acetylcholinesterase inhibition: all compounds which inhibited acetylcholine hydrolysis also inhibited glucose exchange (50—100% increase in exit time), while those which were non-inhibitors of the enzyme also failed to inhibit glucose exchange.

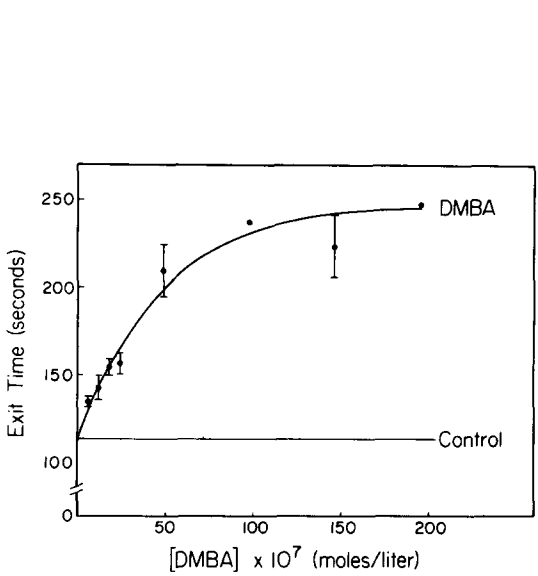
The concentration dependence of DMBA inhibition of glucose exchange is

shown in Fig. 6. An Arrhenius plot of the rate of glucose exchange as a function of temperature is shown in Fig. 7. There was a change in the slope of the plot at approx. 23°C seen both in the control and in the presence of DMBA ($1.5 \cdot 10^{-5}$ M). As was the case with the temperature dependence of the azo dye inhibition [6], DMBA does not appear to alter grossly the temperature characteristics of glucose exchange across the membrane, nor does the inhibition appear to be affected by the incubation temperature conditions.

Antihemolysis studies

The degree of protection against osmotic hemolysis provided by the various families of polycyclic hydrocarbons, of which the effect on acetylcholine hydrolysis and glucose exchange were reported above, was examined over a concentration range which included the highest concentrations used to monitor inhibition of both functions. The object was to ascertain whether a correlation existed between inhibition of membrane function and membrane stabilization against osmotic hemolysis.

The concentration at which a compound provided a 50% reduction in osmotic lysis when compared with a solvent control (AH_{50}) is shown in Table I. An examination of these values indicates that there was not an absolute correlation between inhibition and protection in the benz(*a*)anthracene series. Stabilization did correlate with inhibition for the substituted anthracenes which were



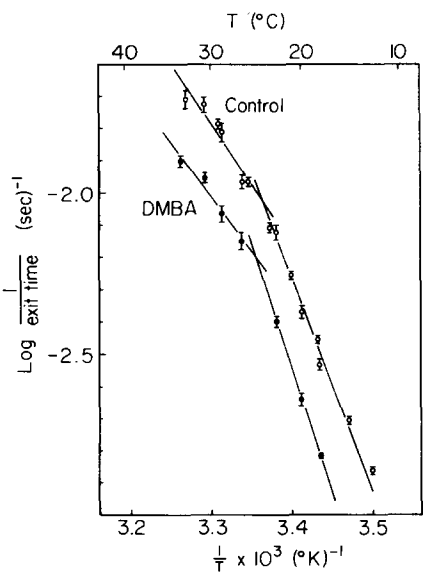


Fig. 6. Concentration dependence of the inhibition of glucose exchange by DMBA. The exit time (± S.D.) is plotted as a function of the final concentration of DMBA and compared with a control containing the solvent ethanol at a final concentration of 2.4% (v/v).

Fig. 7. Effect of DMBA on the temperature dependence of glucose exchange across the erythrocyte membrane. Since the rate of glucose exchange across the membrane is inversely proportional to the exit time, this quantity was used directly for comparison of the rate of exchange in the presence of DMBA (final concentration, 14.7 μM) with the rate of exchange in the presence of an ethanol control (final concentration, 2.4%, v/v). Each point represents a triplicate determination ± S.D.

examined. For the phenanthrenes, however, it is evident that the degree of inhibition did not correlate well with the amount of osmotic protection afforded by these compounds.

Binding

Litman et al. [5] have shown that lack of protection against osmotic hemolysis was not the result of lack of binding of the polycyclic aromatic hydrocarbons to the red cell. Additional qualitative evidence of membrane binding by both inhibitory and non-inhibitory benz(*a*)anthracenes was the enhanced fluorescence exhibited by all the compounds in the presence of red cell ghosts (data not shown). Furthermore, the main fluorescence peak of the compounds in the membrane was blue-shifted when compared with its position in the buffer spectra. Such shifts have been used to quantitate the transfer of benz(*a*)anthracene from particulates into phospholipid vesicles [17].

Discussion

Systematic examination of the effect of polycyclic aromatic hydrocarbons on the function of two membrane proteins resulted in the unexpected finding that inhibition of these functions depended on the presence and position of methyl substituents. These results suggest that inhibition of membrane function may depend on the shape of the hydrocarbon molecule. All substituted compounds which did not inhibit membrane function share the common structural features: (1) three aromatic rings fused linearly, and (2) methyl substituents located at the long axis ends of the molecule. 3-Methylcholanthrene appears to be an exception to this generalization and this may be the result of the different orientation of the cyclopentyl group compared with that of a methyl substituent.

Although the phenanthrene molecule does not contain three linearly fused aromatic rings, it is interesting to note that the substituted phenanthrenes, though all inhibitors, showed a quantitative dependence on the substituent position. Intermediate inhibitors in the phenanthrene series were those with methyl groups at the ends of the parent molecule's long axis.

A possible explanation for the dependence of the inhibition of membrane function on substituent position could be that the location of the methyl group(s) determines how the compound interacts with membrane lipids. Specificity in the interaction of polycyclic aromatic hydrocarbons with lipid monolayers has been reported [18—24], and while the interaction of these compounds with a biological membrane is obviously more complex than that seen with simple monolayers, it is noteworthy that the position of alkyl substituents affects the behavior of polycyclic aromatic hydrocarbons with steroid films [18—20].

Using a cholestane spin label, Sanioto and Schreier [25] examined the effect of a number of polycyclic compounds on the physical state of artificial phospholipid bilayers. Dipalmitoyl phosphatidylcholine bilayers were fluidized by the added hydrocarbon as a function of size and angularity (pyrene $<$ anthracene $<$ benz(*a*)anthracene $\simeq$ phenanthrene $<$ DMBA $<$ 9-methylanthracene $\simeq$ benzo(*a*)pyrene $<$ 3-methylcholanthrene). These authors postulated that the

unexpectedly great fluidizing capability of 9-methylanthracene was due to the position of the methyl substituent in the anthracene molecule which allowed this compound to cause greater disruption of phospholipid-phospholipid side-chain interactions upon intercalation of the compound.

It is interesting to note that the inhibition of erythrocyte membrane function reported herein does not correlate with the ability of these compounds to alter the fluidity of phospholipid bilayers as measured by the spin resonance technique reported above. This suggests that functional perturbation of the erythrocyte membrane by the hydrocarbons is more complex and may involve a direct interaction of the compounds with membrane protein. The possibility of direct interaction of polycyclic aromatic hydrocarbons with hydrophobic regions of membrane protein is suggested by the albumin binding observed for a series of carcinogenic hydrocarbons [26,27].

The complexity of the membrane interaction was also underscored by a detailed examination of DMBA inhibition of two red cell membrane functions. DMBA is a potent carcinogen and is known to interact with both cholesterol and phospholipid [18—20,25] as well as to confer a certain degree of membrane stabilization against osmotic hemolysis [5].

DMBA inhibited human erythrocyte membrane acetylcholinesterase in a noncompetitive manner (Fig. 3), but did not inhibit the enzyme in unsealed erythrocyte ghosts or a high-salt-extracted form of the enzyme. The nature of the acetylcholinesterase inhibition is complicated by the necessity for the presence of an anion gradient in order for the inhibition to be manifest (Table II). It is interesting that even if the cells were lysed after exposure to inhibitor but prior to assay (Table IIC), which essentially results in a preparation of unsealed erythrocyte ghosts, the inhibition was manifest. Thus, the presence of a transmembrane potential was required only at the time of exposure to DMBA. Several recent reports indicate that ion gradients affect the position of proteins in the membrane [28,29], and it is possible that the presence of the anion gradient across the membrane affects the interaction of DMBA with either the enzyme itself or some other membrane component(s) involved in its regulation.

In the presence of DMBA the shape of the Arrhenius plot was found to depend on the method of incubation of the cells with inhibitor prior to assay (see Figs. 4 and 5). The difference in the plots for DMBA is not due to a temperature-dependent difference in the amount of DMBA bound to the cells under the two incubation conditions, in agreement with the report by Harris et al. [15] for DMBA binding to oncornaviruses. It must, therefore, reflect some temperature-dependent regulation of the site where DMBA exerts its inhibitory effect. If the nonlinear Arrhenius plot of the enzyme in the absence of inhibitor is due to a temperature-dependent change in tightly associated lipids (suggested by Beauregard and Roufogalis [30]), then DMBA may be unable to perturb these lipids to the same degree when they are gelled (i.e., below 20°C) as it can when added to cells where the lipids are in a more fluid state (above 20°C).

All polycyclic aromatic hydrocarbons that were tested for inhibition of acetylcholinesterase also inhibited glucose exchange across the membrane, which at first suggested that the inhibition of both functions might have a common basis. However, glucose exchange was measured in a buffer in which

no inhibition of acetylcholine hydrolysis was detectable (phosphate-buffered saline) and the conditions for assaying the temperature dependence of glucose exchange were those which showed diminished inhibition at lower tempertures. There is no evidence for this in the glucose temperature plot (Fig. 7). Glucose efflux from red cells is inhibited by a variety of small hydrophobic molecules [31,32] but the basis for such inhibition is unknown.

As previously reported for three azo dyes [6], the ability of a large number of polycyclic aromatic hydrocarbons to inhibit membrane function does not correlate well with their ability to protect erythrocytes against osmotic hemolysis (Table I) and neither inhibitory ability nor membrane stabilization correlates with binding. These results were unexpected, since the octanol/water partition coefficients for a large number of lipophilic molecules correlated very well with the membrane stabilization afforded by the compounds [3]. Because the membrane interaction depended on overall hydrophobicity rather than structure, polycyclic aromatic hydrocarbons with identical theoretical octanol/water partition coefficients [13,14] were expected to alter membrane function

TABLE III

SOME PHYSICAL PROPERTIES OF TESTED COMPOUNDS

Inhibition — when all compounds were tested at the same final concentration: ++, most inhibitory; +, intermediate inhibition; —, non-inhibitory. Water solubility values (T = 29°C) are those reported by Davis et al. [34]. The values in parentheses were determined by May et al. [35].

Compound	Inhibition	Melting point (°C)	log P (octanol/water) (Refs. 13,14)	Water solubility (μg/l)
Benz(*a*)anthracene	—	160—161	5.91	11 (12.2)
1-Me	n.t.	138—139	6.41	55
3-Me	—	163—164	6.41	
4-Me	—	197—198	6.41	
5-Me	—	156—157	6.41	
8-Me	—	158—159	6.41	
9-Me	—	151—152	6.41	
10-Me	—	183—184	6.41	
2-Me	++	151—152	6.41	
6-Me	++	126—127	6.41	
7-Me	++	140—141	6.41	55
11-Me	++	118—119	6.41	
12-Me	++	139—140	6.41	66
3,9-Me	—	186—187	6.91	
7,12-Me	++	123—124	6.91	43
Anthracene	—	216	4.64	75 (57)
2-Me	—	204—206	5.14	28 *
9-Me	++	79—81	5.14	
Phenanthrene	—	99—101	4.64	1650 (1220)
1-Me	+	118—120	5.14	338 *
4-Me	++	50—51	5.14	
2,7-Me	+	100—101	5.64	
4,5-Me	++	76—77	5.64	
Pyrene	—	156	5.66	165 (162)
Benzo(*a*)pyrene	—	179	7.21	4.0
3-Methylcholanthrene	—	179—180	7.36	1.5

* Values calculated as described in Ref. 35.

in a predictable fashion. A number of physical properties of the polycyclic hydrocarbons are listed in Table III along with a summary of their effect on membrane function. For the limited data available, neither water solubility nor melting point was correlated with a compound's ability to inhibit membrane function.

The data presented for several families of structurally related polycyclic aromatic hydrocarbons indicate that there is an unexpected dependence of the ability to perturb membrane function on the presence and position of methyl substituents. As a group, the polycyclic aromatic hydrocarbons have been widely used as fluorescent probes of the dynamic state of lipids in both artificial lipid bilayers and biomembranes [33]. The results of the present study suggest that such molecules should be carefully evaluated for their ability to perturb membrane function. On the other hand, the unique shape dependence of the membrane functional perturbation caused by the hydrocarbons suggests that these molecules may be interesting probes of the interaction between membrane proteins and lipids.

Acknowledgements

This work was supported in part by Grants from NCI CA-24861, 08748, and 16889, and was submitted by M.E.A. to Cornell University in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

References

1 Seeman, P., Kwant, W.O., Sauks, T. and Argent, W. (1969) Biochim. Biophys. Acta 183, 490—498
2 Seeman, P., Kwant, W.O. and Sauks, T. (1969) Biochim. Biophys. Acta 183, 499—511
3 Seeman, P. (1972) Pharmacol. Rev. 24, 583—655
4 Litman, G.W. and Litman, R.T. (1974) Biochem. Biophys. Res. Commun. 60, 865—871
5 Litman, G.W., Litman, R.T. and Henry, C.J. (1976) Cancer Res. 36, 438—444
6 Aberlin, M.E. and Litman, G.W. (1979) Biochim. Biophys. Acta 553, 96—106
7 Aberlin, M.E. and Litman, G.W. (1978) Biophys. J. 21, 202a
8 Ellman, G.L., Courtney, K.D., Andres, V. and Featherstone, R.M. (1961) Biochem. Pharmacol. 7, 88—95
9 Niday, E;, Wang, C.S. and Alaupovic, P. (1977) Biochim. Biophys. Acta 469, 180—193
10 Mitchell, C.D. and Hanahan, D.J. (1966) Biochemistry 5, 51—57
11 Dodge, J.T., Mitchell, C.D. and Hanahan, D.J. (1963) Arch. Biochem. Biophys. 100, 119—130
12 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
13 Hansch, C. and Fujita, T. (1964) J. Am. Chem. Soc. 86, 1616—1626
14 Dunn, W.J. and Hansch, C. (1974) Chem. Biol. Interactions 9, 75—95
15 Harris, R., Kupfer, D. and Luftig, R.B. (1978) Fed. Proc. 37, 1475
16 Livne, A. and Bar-Yaakov, O. (1976) Biochim. Biophys. Acta 419, 358—364
17 Lackowicz, J.R., Englund, F. and Hidmark, A. (1978) Biochim. Biophys. Acta 543, 202—216
18 Clowes, G.H.A., Davis, W.W. and Krahl, M.E. (1939) Am. J. Cancer 36, 98—109
19 Clowes, G.H.A., Davis, W.W. and Krahl, M.E. (1939) Am. J. Cancer 37, 453—454
20 Davis, W.W., Krahl, M.E. and Clowes, G.H.A. (1940) J. Am. Chem. Soc. 62, 3080—3098
21 Weiner, N.D., Chawdry, I. and Felmeister, A. (1971) J. Pharm, Sci. 60, 425—428
22 Felmeister, A., Tsai, D. and Weiner, N.D. (1972) J. Pharm. Sci. 61, 1065—1068
23 Belmonte, A.A. and Swarbrick, J. (1973) J. Pharm. Sci. 62, 481—483
24 Belmonte, A.A. and Swarbrick, J. (1973) Biochim. Biophys. Acta 323, 647—652
25 Sanioto, D.L. and Schreier, S. (1975) Biochem. Biophys. Res. Commun. 67, 530—537
26 Franke, R. (1968) Biochim. Biophys. Acta 160, 378—395
27 Franke, R. (1969) Mol. Pharmacol. 5, 640—657
28 Amar, A., Rottem, S. and Razin, S. (1978) Biochem. Biophys. Res. Commun. 84, 306—312
29 Mikkelsen, R., Verma, S.P. and Wallach, D.F.H. (1978) Proc. Natl. Acad. Sci. U.S.A. 75, 5478—5482

30 Beauregard, G. and Roufogalis, B.D. (1977) Biochem. Biophys. Res. Commun. 77, 211—219
31 Lacko, L. and Wittke, B. (1977) Experientia 33, 191—192
32 Lacko, L., Wittke, B. and Geck, P. (1975) J. Cell. Physiol. 86, 673—680
33 Radda, G.K. (1975) Methods Membrane Biol. 4, 97—188
34 Davis, W.W., Krahl, M.E. and Clowes, G.H.A. (1942) J. Am. Chem. Soc. 64, 108—110
35 May, W.E., Wasik, S.P. and Freeman, D.H. (1978) Anal. Chem. 50, 997—1000