Specific Crystal Chemical Interactions between Carcinogenic Aromatic Compounds and Cholesterol

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Carcinogenic Aromatic Compounds, Epitaxial Adsorption, Cholesterol, Plasma Membrane

Polycyclic aromatic hydrocarbons and aromatic amines exercise a highly specific influence on the crystallization of cholesterol. The strength of these non-covalent, presumably epitaxial interactions correlates with the carcinogenic activity of these substances. The presented results are in support of the assumption that a specific process of adsorption and crystallization with cholesterol of the plasma membrane takes place during the initial phase of the carcinogenesis by aromatic compounds.

Introduction

Polycyclic aromatic hydrocarbons (PAH) and aromatic amines (AAM) are the most thoroughly analyzed substances with carcinogenic features. It has generally been accepted for a long time, that these compounds have to be metabolically activated to highly reactive electrophilic intermediate products, the "ultimate carcinogens".

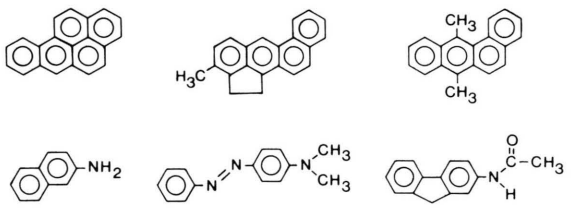

Fig. 1. Examples of the best-known carcinogenic aromatic compounds. PAH (above): benzo[a]pyrene, 3-methylcholanthrene, 7,12-dimethylbenz[a]anthracene. AAM (below): 2-naphthylamine, N,N-dimethyl-4-aminoazobenzene, 2-acetylaminofluorene.

In the cell the PAH are metabolized for detoxification to various different derivatives mainly phenols, dihydrodiols, glutathione conjugates and most interestingly diolepoxides. Some of the latter are highly reactive compounds with covalent binding to the bases of the DNA [1–4].

Abbreviations: PAH, polycyclic aromatic hydrocarbons; AAM, aromatic amines.

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The initial step in the activation of AAM is their oxidation to N-hydroxy derivatives. The N-hydroxy metabolites can be activated to the electrophilic "ultimate carcinogens" by (i) protonation of the nitrogen atom of the hydroxyamine with subsequent loss of water to yield a nitrenium ion, (ii) oxidation of N-acyl-N-hydroxy derivatives to free radicals and (iii) esterification of the N-hydroxy group generating reactive esters [4].

The electrophilicity of "ultimate carcinogens" results in reactions with nucleophilic sites within the eukaryotic cell. It is assumed that covalent modification of a cellular informational macromolecule, *i.e.* DNA, RNA or protein, is a necessary early event in the initiation phase of the carcinogenic process.

This study describes an activity of carcinogenic compounds which has not been properly evaluated to date in the published analysis of carcinogenic mechanisms. In aqueous media carcinogenic aromatic compounds have only a limited solubility. In tissue this could probably result in crystalline structures of these compounds playing an essential role during the initiation of carcinogenesis. The crystalline structures could adsorb biogenic molecules in a structure-dependent very specific way due to the geometry of their molecular arrangement. Structure-dependent adsorption of this kind has been described for the so-called "epitaxy", an oriented crystal nucleation and crystal intergrowth [5].

H. Seifert [6] has put special emphasis on the significance of structure-dependent adsorption in biological systems. However, in the literature on cancer research only two studies have dealt with

this aspect of the question. Hendry *et al.* [7] suggested that the molecules of the carcinogenic aromatic compounds could integrate into regular micells and thus could specifically bind linear macromolecules. Druckrey and Schmähl [8] referred to these ideas explaining the carcinogenic features of foreign-body implants made of plastic material or silicate. They assumed that linear cell proteins were very specifically bound by similar linear structures of the plastics and silicates initiating cellular degeneration.

In this study the question of the structure-dependent adsorption was further analyzed to gain more information with regard to the mechanism of high specificity of carcinogenic aromatic compounds.

The "Epitaxy"; a specific intermolecular interaction

Any structure-dependent intergrowth (overgrowth) of two chemical and/or structural different crystalline or subcrystalline phases is called "epitaxy" [5].

An epitaxial system consists of two partners: the host crystal (substrate) and the guest phase (deposit). An oriented adsorption and crystal intergrowth demands a contact interface of both phases corresponding with each other, *i.e.* molecules of both phases come in contact on the surface in a position generating noncovalent binding forces between the phases in an orientating way. A polymer-analogous conformity of the molecular distances is sufficient for orientation, *i.e.* an elementary period of the substrate is in general only analogous to a small integral multiple of the epitaxial intergrowth partner period (Fig. 2). Even though a one-dimensional conformity of the molecular distances can be sufficient for an epitaxy, a two-dimensional analogy would essentially be better. The precision of the analogy required for an epitaxy depends on the nature of the intergrowth partners [9].

These studies are based on the working hypothesis, that the carcinogenic aromatic compounds form a specific substrate by their crystal germs. An important component of the plasma membrane of a cell can be epitaxially adsorbed by this substrate. The basic idea is, that the geometry of the molecule arrangement could be decisive for the specificity of carcinogenic action.

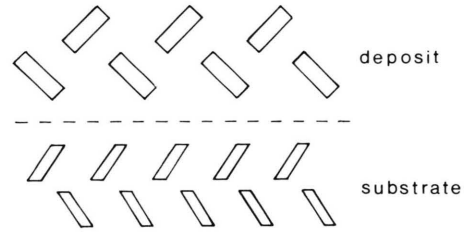


Fig. 2. Example of a one-dimensional structural analogy. A polymer-analogous conformity of the molecular distances is sufficient for the orientation of the deposit on the substrate. In this example: 2 periods of the deposit are analogous to 3 periods of the substrate.

If the epitaxy is actually of significance for the mode of action of carcinogenic aromatic compounds, the crystal structures of all carcinogenic aromatic compounds should show one common feature, to which the orientation of adsorbed biogenic molecules can direct itself. In fact, such a structural pattern had previously been determined by means of structural comparisons of more than 50 different PAH [10].

The specific structural pattern in the crystals of the PAH was assumed to be present, if

a) at least two of the three lattice constants corresponded with the period $n \cdot 7.45 \pm 0.55$ Å ($n = 2$ or 3) or

b) one lattice constant corresponded with approx. 9.2 Å and one further lattice constant corresponded with the period given in a).

Subsequently, knowledge of these lattice parameters enabled the promising search for the membrane component, the structure of which corresponds to this pattern and which therefore comes into consideration as a deposit in the putative epitaxy system. Moreover, the discovery was soon made, that only cholesterol met all requirements which were expected of a deposit in such an epitaxy system.

Assumed Epitaxy system: Aromatic compound/cholesterol

Cholesterol (cholest-5-en-3β-ol) is an essential component of the plasma membrane of each eucaryotic cell, mainly in the outer layer of the phospholipid bilayer. Both crystal structures of cholesterol – "anhydrate" [11, 12] and "monohydrate" [13, 14] – contain lattice planes of molecules piled in parallel.

The crystal structures of cholesterol should be compared with the structures of the lattice planes in the crystals of carcinogenic aromatic compounds, providing the information concerning the orientation of a putative epitaxy aromatic compound/cholesterol.

Table I shows the corresponding lattice parameters of the putative epitaxy partners as well as the laws of orientation of the possible crystal intergrowths. Only the energy-rich α-modifications were taken into account from among the polymorphous PAH, because only these corresponded to

Table I. Crystallographic data of deposit and substrate [10–14, 28, 29]. The periods d_x and d_y in two orthogonal directions of the matrices I and II are contrasted with the corresponding values d_1 and d_2 of the closest packed lattice plane (0 1 0) and/or (0 0 1) of the deposit. Carcinogenicity: 0 = inactive to + + + = very active [30–33, 36].

Deposit Unit cell parameter [Å] a_α b_β c_γ	Lattice plane (0 1 0) d_1 [Å]	 d_2 [Å]	Lattice plane (0 0 1) d_1 [Å]	 d_2 [Å]
I. Cholesterol, anhydrous 14.172; 34.209; 10.481 94.64°; 90.67°; 96.32°	$c = 2 \times 5.24$	$a = 14.17$		
II. Cholesterol, monohydrate 12.39; 12.41; 34.36 91.9°; 98.1°; 100.8° Pseudocell: [1 1 0] = $a' = 7.92$ [1 $\bar{1}$ 0] = $b' = 9.57$ $\gamma' = 90.1°$			$a' = 7.92$	$b' = 9.57$

Substrate Unit cell parameter [Å] a b c β	Matrix type I d_x [Å]	 d_y [Å]	Matrix type II d_x [Å]	 d_y [Å]	Carcinogenic activity
PAH with two-dimensional matrix					
1. Dibenzo[a,h]pyrene 15.07; 9.79; 10.71; 103.5°			$a = 2 \times 7.54$	$b = 9.79$	+ + +
2. Benzo[a]pyrene 7.59; 7.69; 22.38; –	$2b = 3 \times 5.13$	$2a = 15.18$			+ + +
3. 3-Methylcholanthrene 4.90; 11.36; 25.26; 95.3°	$b = 2 \times 5.68$	$3a = 14.70$	$2b = 3 \times 7.45$	$2a = 9.80$	+ + +
4. 7,12-Dimethylbenz[a]anthracene 7.62; 8.62; 21.11; –			$a = 7.62$	$b = 8.62$	+ + +
5. 7- Methylbenz[a]anthracene 23.60; 5.77; 19.00; 97.5°			$a = 3 \times 7.69$	$c' = 9.50$	+ + +
6. 12-Methylbenz[a]anthracene 9.27; 7.44; 9.18; 91.8°			$b = 7.44$	$a = 9.27$	+ + +
7. Dibenz[a,h]anthracene 6.59; 7.84; 14.17; 103.5°	$2b = 3 \times 5.23$	$c = 14.17$			+ +
8. Benzo[g,h,i]perylene 11.72; 11.88; 9.89; 98.5°			$2b = 3 \times 7.92$	$c = 9.89$	+ +
9. Benzo[c]phenanthrene 14.67; 14.16; 5.78; –	$c = 5.78$	$b = 14.16$			+ +
10. Dibenzo[a,h]acridine 12.87; 3.86; 13.91; 105.5°	$4b = 3 \times 5.15$	$c = 13.91$			+ +
11. Dibenzo[a,j]acridine 5.00; 18.10; 15.25; –			$= 2 \times 7.63$	$2a = 10.00$	+
12. Dibenz[a,c]anthracene 15.67; 5.09; 18.33; 103.9°	$b = 5.09$	$a = 15.67$	$a = 2 \times 7.83$	$2b = 10.18$	+
13. Coronene 16.10; 4.69; 10.15; 110.8°	$a = 3 \times 5.37$	$3b = 14.07$	$a = 2 \times 8.05$	$2b = 9.38$	0
14. Naphthalene 8.23; 6.00; 8.66; 122.9°			$4b = 3 \times 8.00$	$c = 8.66$	0

Table I (continued).

Substrate Unit cell parameter [Å]				Matrix type I		Matrix type II		Carcinogenic activity
a	*b*	*c*	β	d_x [Å]	d_y [Å]	d_x [Å]	d_y [Å]	
PAH with one-dimensional matrix								
15. Dibenzo[def,mno]chrysene								
12.10;	10.34;	10.72;	92.2°	$b = 2 \times 5.17$				++
16. Benz[a]anthracene								
7.95;	6.50;	12.12;	100.5°	$2a = 3 \times 5.30$		$a = 7.95$		+
17. Benzo[e]pyrene								
11.85;	12.15;	18.67;	106.9°			$2a = 3 \times 7.90$		+
18. Chrysene								
8.34;	6.18;	25.0;	115.8°	$2a = 3 \times 5.56$				+
19. Pyrene								
13.65;	9.26;	8.47;	108.5°	$2c = 3 \times 5.65$			$b = 9.26$	0
20. Phenanthrene								
8.46;	6.16;	9.47;	97.7°	$2a = 3 \times 5.64$			$c = 9.47$	0
21. Perylene								
10.3;	10.8;	13.6;	126.5°	$a = 2 \times 5.15$				0
22. Anthracene								
8.56;	6.04;	11.16;	124.7°	$c = 2 \times 5.58$		$2c = 3 \times 7.44$		0
23. Benzo[a]fluorene								
8.41;	6.11;	23.36;	–	$2a = 3 \times 5.61$				0
24. Benzo[c]acridine								
12.27;	5.19;	18.52;	–	$b = 5.19$		$3b = 2 \times 7.89$		0

the structures of the crystal nuclei. These structural comparisons suggested that an epitaxy between cholesterol and aromatic compounds could be possible, if the aromatic compound as a substrate could offer the cholesterol one of the following "matrices" for epitaxial adsorption:

a) *Matrix-type I* (two-dimensional)
(see Table I: Nos. 2; 3; 7; 9; 10; 12; 13)
In two orthogonal directions x and y, the spacing intervals of d_x and d_y amount to:

$d_x = n \cdot (5.10 \text{ to } 5.80 \text{ Å})$ with $n = 1, 2, 3$ or 4;
$d_y = 13.70$ to 15.70 Å.

The cholesterol could crystallize on this matrix with the lattice plane (010) of the anhydrous form having

$d_{[001]} = 2 \cdot 5.24$ Å and $d_{[100]} = 14.17$ Å.

b) *Matrix-Type II* (two-dimensional)
(see Table I: Nos. 1; 3–6; 8, 11–14)

$d_x = n \cdot (7.05 \text{ to } 8.20 \text{ Å})$ with $n = 1, 2$ or 3;
$d_y = 8.60$ to 10.20 Å.

The cholesterol could crystallize on this matrix with the lattice plane (001) of the hydrated form having

$d_{[110]} = 7.92$ Å and $d_{[1\bar{1}0]} = 9.57$ Å.

c) *Matrices with low specificity* (one-dimensional)
(see Table I: Nos. 15–24)
The spacing intervals corresponded to one of the values given under a) and b) for d_x or d_y.

Assay of crystal chemical interactions between cholesterol and the carcinogenic aromatic compounds

Two phases, which can epitaxially intergrow with one another, are of mutual influence with regard to their mode of crystallization. This was the basis of the experiments described below quantitatively measuring the influence of the aromatic compound on the crystallization of the cholesterol. The cholesterol was spontaneously coprecipitated with the aromatic compound in an albumin-containing phosphat buffer. The strength of the interaction was measured by means of a turbidometric assay.

It was indicated by the quantity of albumin needed to stabilize the resulting colloids. It was later realized that it was not the albumin itself but the α-globulin still present in it in small quantities that was responsible for stabilizing the colloids. Very few of the commercially available types of albumin were therefore suitable for the tests described below.

It was possible to realize the experiments with α-globulins only; but the contaminated albumin could be measured out more accurately. The activity of 24 mg albumin (bovine; essentially fatty acid free; No. A 6003, Sigma Chemical Co.) was equal to 1 mg globulins (bovine; Cohn Fraction IV-1; predominantly α-globulins; No. G. 8512; Sigma Chemical Co.).

49 PAH as well as 26 aromatic amines were tested. For the purpose of comparing studies with aromatic amines, the derivatives of the azo dye "dimethyl yellow" (N,N-dimethyl-4-aminoazobenzene) appeared to be the most suitable, since they all have the same organotropy. They exclusively cause hepatoma in feed experiments with rats, and their carcinogenic efficiency has been analyzed quantitatively [17, 18].

Materials

Cholesterol cryst. pure (Merck, Darmstadt, F.R.G.): Albumin, bovine, essentially fatty acid free, No. A-6003 (Sigma Chemie, Deisenhofen, F.R.G.); Acetone, p.a.; Ethanol, min. 99.5%; Phosphate buffer, pH 7.2 (per liter: 2.49 g KH_2PO_4 and 8.62 g $Na_2HPO_4 \cdot 2\,H_2O$).

The applied PAH are listed in Table II. Nos. 22 and 23 were purchased from EGA-Chemie, Steinheim (F.R.G.); Nos. 6, 7, 10, 12, 14, 17, 19 from Fluka AG, Buchs (Switzerland); Nos. 9, 16, 20, 21 from Merck-Schuchardt, Hohenbrunn (F.R.G.); all other PAH were obtained from Dr. W. Schmidt, PAH Research Institute, 8919 Greifenberg (F.R.G.).

The applied azo dyes are listed in Table III. Nos. 1 and 8 were purchased from Schuchardt, München (F.R.G.); No. 2 from EGA-Chemie, Steinheim (F.R.G.). All other azo dyes were produced in our own laboratory in accordance with bibliographic references [15, 16].

Solution A: 1.5×10^{-2} mol·l^{-1} cholesterol in ethanol.

Solution B_1: 2.5×10^{-4} mol·l^{-1} PAH in ethanol/acetone (1 : 1).

Solution B_2: Azo dye in ethanol/acetone (1 : 1). 2.5×10^{-5} mol azo dye in 50 ml of a solution with the "basic concentration C_B". Regarding the determination of C_B: see methods.

Solution C: Albumin in phosphate buffer, pH 7.2. The solutions were freshly prepared each time (in brown glass bottles) in different concentrations between 1 and 50 mg albumin in 20 ml buffer. Every charge was processed within 30 min.

Methods

All experiments were carried out under weak yellow light. The temperature was 23 ± 2 °C.

Relative solubility in aqueous medium

PAH. 2 parts by volume of solution B_1 were diluted with 1 part by volume of ethanol. 3 ml respectively of this solution were mixed with varying quantities of water. After 5 min the extinction values of these mixtures were measured against water in a 10 mm cuvette at 623 nm. V_w (see Table II) was used to designate that volume of water at which the onset of crystallization of PAH can be observed through a weak turbidity or an extinction of > 0.020 in the test mixture.

Azo dyes

1 ml ethanol was mixed with 1 ml of a solution of the azo dye (different concentrations in ethanol/acetone (1 : 1)) in a 25 ml beaker. 20 ml of an albumin solution (*i.e.* solution C with 20 mg albumin in 20 ml buffer) from a second beaker were poured "in one go" into this mixture. After 5 min the turbidity of this mixture, *i.e.* the extinction at 623 nm was measured in a 10 mm cuvette.

A relative gauge of the solubility of the azo dyes in the test mixture (see Table III) and simultaneously the "basic concentration C_B" for preparing solution B_2 (see chapter: Materials) is provided by the concentration of the azo dye solution, which created a weak turbidity or an extinction of 0.020 in the text mixture due to a crystallization beginning.

Crystal chemical interaction between aromatic compounds and cholesterol

Within one series of measurements, precipitation reactions were carried out with a constant concentration of aromatic compound and cholesterol, but with varying concentrations of albumin, and the remaining colloid turbidity after centrifuging was measured. The albumin or the α-globulin still present in it in small quantities, served as a competing adsorbate and to stabilize the colloid.

20 ml of the solution C were given into a 50 ml beaker. A mixture of 1 ml of the solution A and 2 ml of the solution B_1 (PAH) – or 1 ml of the solution B_2 (azo dye) – were injected into this stirred solution C by means of a flask pipette. The suspension was centrifuged (~ 1100 × *g*) immediately afterwards for 10 min and the turbidity, *e.g.* the extinction of the colloid was measured immediately against water in a 10 mm cuvette (623 nm).

Results

Plotting the measured extinction values (turbidity) as a function of the albumin concentration of the solution C one obtains a curve with a maximum (Fig. 3) for each aromatic compound.

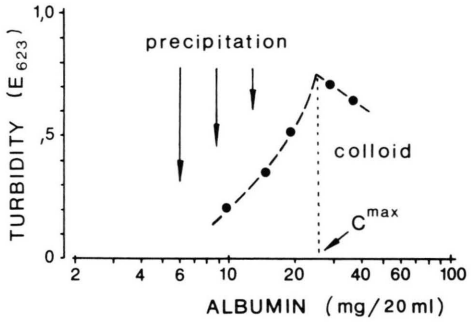


Fig. 3. The turbidity or the extinction of the colloid system aromatic compound/cholesterol/albumin as a function of the albumin concentration. C^{max} is the albumin concentration of solution C, by means of which – under the experimental conditions described – a maximum of turbidity is produced. In this example: C^{max} = 26 mg/20 ml.

The albumin concentration C^{max} of the solution C, which produced a maximum turbidity under the experimental conditions described proved to be characteristic of the strength of specific interaction between aromatic compounds and cholesterol during the mutual crystallization.

The values determined by this method for C^{max} and for the relative solubility of all 75 compounds investigated are listed in Table II (PAH) and Table III (azo dyes).

PAH

In the event of a strong specific crystal chemical interaction between PAH and cholesterol an increased nucleation took place and a colloidal suspension of finest crystallites developed.

The stronger the interactions between PAH and cholesterol, the less albumin – or α-globulin still present in it in small quantities – was needed to stabilize the colloid. The most important result of these experiments is the finding that the strength of the crystal chemical interactions correlates with the carcinogenic activity of PAH (Fig. 4). The solubility of PAH in aqueous media must also be taken into account, however, as a higher solubility weakens the nucleation.

Only seven of the 49 PAH investigated (*i.e.* approx. 15%) form an exception to the correlation (see Table II). Their behaviour can be designated false-positive, as their reactions with cholesterol are stronger in the tests than the intensity of their carcinogenic activity would suggest. The deviations are particularly great in the non-carcinogenic compounds triphenylene and 3,9-dimethylbenz[a]-anthracene and in the slightly carcinogenic 6-methylchrysene.

The existence of such false-positive exceptions indicates that the interaction with the cholesterol can be only one of a number of significant stages in the process of cell transformation.

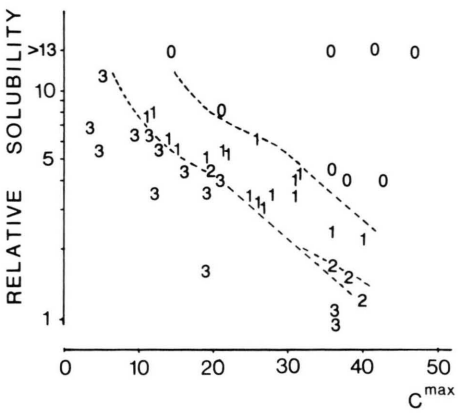


Fig. 4. Carcinogenic activity of PAH as a function of the relative solubility in aqueous medium (see V_w in chapter Methods) and C^{max} (see Fig. 3). Numbers are in accordance with the carcinogenicity: 0 = inactive, 1 = slight, 2 = moderate, 3 = strong. The broken lines indicate the fields of equal activity. False-positives are not marked.

Azo dyes

Whereas the *specific* crystal chemical interactions in the PAH/cholesterol system result in in-

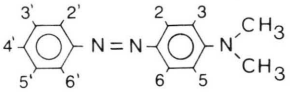
Table II. The results of the experiments with PAH. V_w = relative solubility in water; see chapter Methods. C^{max}: see Fig. 3 (f.p.: "false positive").

Compound	V_w	C^{max}	Carcinogenic activity [30–33, 36]
1. Dibenzo[a,e]pyrene	1.0	36	+++
2. Dibenzo[a,h]pyrene	1.1	36	+++
3. Dibenzo[a,i]pyrene	1.6	19	+++
4. Dibenzo[a,l]pyrene	3.5	12	+++
5. Benzo[a]pyrene	4.0	21	+++
6. 3-Methylcholanthrene	3.5	19	+++
7. Dibenz[a,h]anthracene	1.2	40	++
8. Dibenz[a,j]anthracene	3.5	8 f.p. (+++)	++
9. Benzo[c]phenanthren	8.5	6 f.p. (+++)	++
10. Benzo[g,h,i]perylene	1.5	38	++
11. Anthanthrene	1.7	36	++
12. Benz[a]anthracene	4.0	31	+
13. Chrysene	2.2	40	+
14. Dibenz[a,c]anthracene	3.2	26	+
15. Benzo[e]pyrene	3.4	31	+
16. Pyrene	8.0	12 f.p. (+)	0
17. Perylene	1.8	38 f.p. (+)	0
18. Triphenylene	4.0	17 f.p. (+++)	0
19. Phenanthrene	>13	36	0
20. Anthracene	4.0	43	0
21. Naphthalene	>13	45	0
22. Benzo[a]fluorene	4.5	36	0
23. Benzo[b]fluorene	4.0	38	0
24. 7H-Dibenzo[c,g]carbazole	11.5	5	+++
25. 13H-Dibenzo[a,i]carbazole	7.5	11	+
26. Dibenz[a,c]acridine	5.0	19	+
27. Dibenz[a,h]acridine	3.5	28	+
28. Dibenz[a,i]acridine	6.0	14	+
29. Dibenz[a,j]acridine	6.0	26	+
30. Benz[a]acridine	>13	47	0
31. Benz[c]acridine	>13	14	0
32. 9-Methylanthracene	8.5	21	0
33. 9,10-Dimethylanthracene	3.5	25	+
34. 1-Methylchrysene	2.5	36	+
35. 2-Methylchrysene	4.5	31	+
36. 3-Methylchrysene	5.5	21	+
37. 4-Methylchrysene	5.5	21	+
38. 5-Methylchrysene	7.0	10	+++
39. 6-Methylchrysene	7.0	10 f.p. (+++)	+
40. 1-Methylbenz[a]anthracene	8.0	12	+
41. 5-Methylbenz[a]anthracene	4.5	19	++
42. 6-Methylbenz[a]anthracene	7.0	4	+++
43. 7-Methylbenz[a]anthracene	6.5	9	+++
44. 8-Methylbenz[a]anthracene	4.5	16	+++
45. 10-Methylbenz[a]anthracene	3.0	26	+
46. 11-Methylbenz[a]anthracene	5.5	15	+
47. 12-Methylbenz[a]anthracene	6.5	12	+++
48. 3,9-Dimethylbenz[a]anthracene	2.5	21 f.p. (+++)	0
49. 7,12-Dimethylbenz[a]anthracene	5.5	5	+++
Cholesterol (without PAH)	–	50	–

tensified colloid formation, they are seen to result in weakened colloid formation in the azo dye/cholesterol system. The different polarity of these substances is certainly the cause of these contradictory reactions.

Reaction during precipitation	PAH (non-polar)	Azo dye (polar)
Crystallization rate of aromatic compound	high	low
Non-specific adsorption of aromatic compound to cholesterol	slight	strong

The *non-polar* PAH intensify the colloid formation by increased nucleus formation due to epitactic interactions with the cholesterol. The *polar* molecules of the azo dyes, on the other hand, are presumably adsorbed completely non-specifically onto the crystal nuclei of the cholesterol, inhibiting their growth and enhancing colloid formation. The epitactic, *i.e. specific* adsorption that is of special interest to us competes with this *non-specific* adsorption, with the result that colloid formation is prevented to the degree to which the *specific* crystalchemical interactions, *i.e.* the epitactic crystallization of the azo dyes with the cholesterol, are intensified. The nucleation of the cholesterol is un-

Table III. The results of the experiments with derivatives of N,N-dimethyl-4-aminoazobenzene (DMAB) 

C_B = relative solubility in albumin/buffer; see chapter Methods. C^{max}: see Fig. 3.

Compound	C_B	C^{max}	Carcinogenic activity [17, 18, 23, 37]	
1. DMAB	8	26	++	6
2. 2-Methyl-	4	10	0	<1
3. 2′-Methyl-	7	17	+	2–3
4. 2.′-Trifluormethyl-	~10	14	0	0
5. 2′-Methoxy-	29	32	+	2
6. 2′-Fluoro-	11	28	++	7
7. 2′-Chloro-	7	21	+	2
8. 3′-Methyl-	3	36	+++	10–12
9. 3′-Trifluormethyl-	~20	22	0	0
10. 3′-Methoxy-	10	32	+++	10–12
11. 3′-Fluoro-	6	33	+++	10–12
12. 3′-Chloro-	7	26	++	5–6
13. 4′-Methyl-	6	14	+/0	<1
14. 4′-Trifluormethyl-	41	45	0	0
15. 4′-Methoxy-	29	37	+	3
16. 4′-Fluoro-	3	46	+++	10–12
17. 4′-Chloro-	~20	28	+	1–2
18. 4′-Ethyl-	1	47	+++	10
19. 4′-Amino-	51	18	+/0	0
20. 2′,4′-Dimethyl-	6	12	0	0
21. 2′,4′-Difluoro-	4	37	+++	>10
22. 2′,5′-Dimethyl-	8	11	0	0
23. 2′,5′-Difluoro-	4	38	+++	>10
24. 2′,5′-Dichloro-	18	12	0	0
25. 2′,4′,6′-Trifluoro-	7	35	+++	>10
26. 2′,4′,6′-Trichloro-	15	15	0	0
Cholesterol (without azo dye)	–	50	–	–

likely to be influenced, as the polar azo dyes crystallize relatively slowly from the aqueous solution.

If the solubility is taken into consideration there is an evident correlation between the carcinogenic potency and the measured C^{max} as shown in Fig. 5. The higher the solubility the more rapidly the carcinogenic potency of azo dyes decreases. Neither false-positive nor false-negative exceptions have been observed to date.

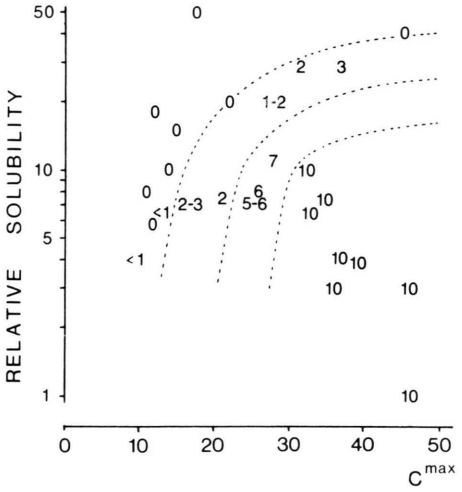


Fig. 5. Carcinogenic activity of 26 azo dyes as a function of the relative solubility in albumin/buffer (see C_b in chapter Methods) and the measured C^{max} (see Fig. 3). Numbers are in accordance with the indices of carcinogenicity according to Miller [17, 18] for 26 dimethyl-yellow derivatives of Table III. The broken lines indicate the fields of equal activity.

Discussion

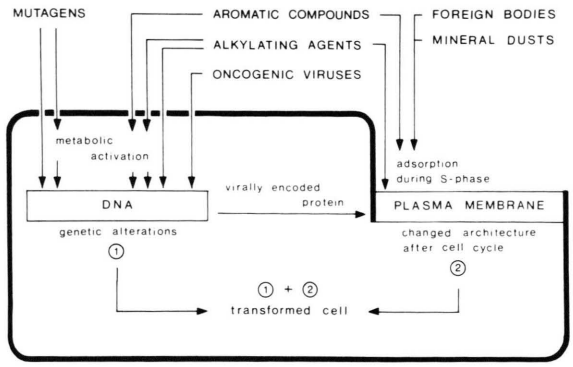
Although there is still a lack of information on the structure and composition of the colloids, it is possible to state with certainty that specific interactions take place during the mutual precipitation or crystallization of the cholesterol with the aromatic compounds. The strength of these interactions correlates with the carcinogenicity of the aromatic compounds involved (see Fig. 4 and 5). Since the molar ratio of crystallizing aromatic compound to cholesterol was only 1:30 in all experiments, one can speak of a "catalytic potential", by means of which the aromatic compounds are able to influence the nucleation and crystallization of cholesterol and thus the development of colloids.

None of the previously known properties of the parent aromatic compounds correlates so strongly with the carcinogenic behavior as these crystal chemical interactions. Such interactions can therefore reasonably be assumed to play a significant role in the first step of carcinogenesis. For example, crystal nuclei of a carcinogenic aromatic compound might form a specific substrate adsorbing cholesterol bonded to the plasma membrane of a cell as directed by its structure. In this case, the mobile liquid-crystalline phase of the membran-bonded cholesterol will be transformed by being fixed to the substrate into a crystalline immobile phase. The crystallization of the cholesterol can result both in the changing of the conformation and reactivity of neighboring molecules as well as in the preventing of lateral movement in other membrane components [20].

Since the *initiation* takes place during the transition from the G_1- to the S-phase in the cell cycle [21, 22], exactly at the moment when the microviscosity of the cell membrane lipid layer reaches a minimum [19], it can be supposed that at this time important membrane structures are usually duplicated. The specific disturbance of the membrane synthesis by the crystallization of the cholesterol probably results in the daughter cells inheriting an anomalous membrane architecture. The highly interesting observation made by Beisson and Sonneborn [24] that purely phenotypic features of a cell membrane can be inherited independently of the genom provided repeated impetus for the view that specific membrane structures may act as their own templates in further membrane synthesis [25–27]. Altered structures would then be inherited in subsequent generations.

We are faced with the remarkable fact that both the reactivities of the metabolites and crystal chemical activities of the parent compounds – which are completely independent of the metabolism – correlate with the carcinogenic behavior. From this known fact we can conjecture that two reactions in different locations are needed for the transformation of a cell, one in the interior of the cell and one on the plasma membrane.

The same conclusion was recently drawn by Yano *et al.* [34, 35] when they found a positive correlation between degree of membrane changes and tumorigenic potencies while investigating the reaction of alkylating agents with membrane compo-



nents. Since carcinogenic chemicals are *promoters* as well as *initiators*, attention should be given to the possible promoting activity of a changed membrane architecture.

Fig. 6. Simplified scheme of possible interactions during carcinogenesis. It seems quite conceivable that irreversible alterations in two different locations are needed for the transformation of a cell, one in the interior of the cell (DNA) and one in the plasma membrane.

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