Epigenetic Effectiveness of Complete Carcinogens: Specific Interactions of Polycyclic Aromatic Hydrocarbons and Aminoazo Dyes with Cholesterol and Apolipoprotein A–I

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During a co-precipitation of cholesterol (Chol) and slight amounts of polycyclic aromatic hydrocarbons (PAHs) or aminoazo dyes (AZOs) in aqueous albumin solution, complex particles are formed; on their surfaces apolipoproteins with an amphipathic α-helix (*e.g.* apoA-I) are more or less firmly adsorbed. An efficacy index can be calculated from the strength of the hydrophobic interactions between apoA-I and the [Chol/PAH]- or [Chol/AZO]-complex, and the solubility of the PAH or AZO in an aqueous medium, which correlates to the carcinogenicity of these compounds. A short-term test for PAHs and AZOs is described, in which the efficacy index can be determined in the simplest manner without any great expenditure on equipment. The previous results suggest that the parent compounds of the PAHs and AZOs can be involved in a specific interaction with cholesterol-domains of the plasma membrane of a cell. The changes in membrane fluidity and architecture caused by these specific interactions could modulate the distribution and/or activity of membrane proteins which are critical to the regulation of cellular proliferation.

Key words: Polycyclic Aromatic Hydrocarbons, Aminoazo Dyes, Cholesterol

Introduction

Carcinogenesis is a multi-staged process (initiation → promotion → progression) in which genes that control normal cellular growth (proto-oncogenes, tumor suppressor genes) are altered by sequential mutational events, with subsequent clonal growth of the resulting precancerous or cancerous cells (Kadlubar *et al.*, 1976; Miller, 1978; Farber, 1984; Cavalieri and Rogan, 1985; Shields and Harris, 1993; Schwarz, 1995; Marks and Fürstenberger, 1995). Initiator and promoter can be different agents, but a "complete carcinogen" is both initiator and promoter in one (Miller, 1978). According to conventional wisdom, many complete carcinogens, such as for instance polycyclic aromatic hydrocarbons (PAHs) and aminoazo dyes (AZOs), have no direct impact on this process, but rather must be metabolized to "ultimate carcinogens" in the target cell.

Earlier results (Contag, 1991), however, indicated that the parent compounds of PAHs and AZOs could directly participate in the multistaged process of carcinogenesis. The notion that the parent compounds of PAHs and AZOs, whose molecules as "singles" are generally considered completely inert, could develop a specific impact in the form of organized groupings in the complicated process of carcinogenesis, is already relatively old (Hendry *et al.*, 1951; Contag, 1975, 1978). However, it wasn't until 1991 that cholesterol was recognized as a possible reactive partner for an epitaxy with PAH (Contag, 1991). It was hypothesized that cholesterol domains of the plasma membrane could adsorb crystal nuclei of a barely soluble complete carcinogen such as PAH or AZO, in a structure-dependent, very specific way due to the geometry of their molecular arrangement. Our earlier studies demonstrated that PAHs and AZOs more or less strongly influence the adsorption of apolipoprotein A-I (apoA-I) on cholesterol crystals. The present paper attempts to make a quantitative statement about the adsorption of apoA-I on crystalline [Chol/PAH]- and [Chol/AZO]-complexes. It seemed that the most sensible approach would be to represent the methods in the form of

Abbreviations: PAHs, polycyclic aromatic hydrocarbons; AZOs, aminoazo dyes; DAB, *N*,*N*-dimethyl-4-aminoazobenzene; apoA-I, apolipoprotein A-I; Alb*, special kinds of albumin (see Materials and Methods); Chol, cholesterol; EPE, epigenetic effect.

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short-term tests, which can be carried out in the simplest manner without any great expenditure on equipment.

Materials and Methods

Equipment

Photometer, ce 1010 (Cecil Instruments, Cambridge); Centrifuge (Christ).

Chemicals

Acetone *p.a.*; ethanol, min. 99.5%; phosphate buffer, pH 7.2 (per liter: 2.49 g KH_2PO_4 and 8.62 g $Na_2HPO_4 \cdot 2H_2O$); cholesterol cryst. pure (Merck, Darmstadt, Germany) or cholesterol min. 99% (Sigma-Aldrich, Germany). All PAHs were purchased from PAH Research Institute, Dr. W. Schmidt, Greifenberg, Germany. *N*,*N*-Dimethyl-4-aminoazobenzene (DAB) and 3′-methyl-DAB were purchased from Schuchard, Munich, Germany, and 2-methyl-DAB from EGA-Chemie, Steinheim, Germany. All other AZOs mentioned in this paper were produced in our laboratory in accordance with bibliographic references (Miller and Milller, 1948; Miller *et al.*, 1949).

ApoA-I and albumin: In principle all preparations can be used which contain bovine albumin and apoA-I in a non-denatured form and which are free of fatty acids. Due to cost considerations as well as the simple handling, a special kind of albumin was used, which is contaminated with a slight amount of apolipoproteins, mainly apoA-I, on account of its method of manufacture (Spector, 1975), *i.e.* initial fractionation by cold alcohol precipitation (Cohn fraction V). The ingredient in question is "albumin, bovine, essentially fatty acid free, No. A 6003, Sigma-Chemie, Deisenhofen, Germany". These special kinds of albumin will be referred to as Alb* in the text below. Other kinds of albumin which were either pasteurized or contained free fatty acids turned out to be unsuitable for colloid stabilization.

Solution A: $1.5 \cdot 10^{-2}$ M cholesterol in ethanol.

Solution B1: $2.5 \cdot 10^{-4}$ M PAH in ethanol/acetone (v/v 1:1).

Solution B2: $5 \cdot 10^{-4}$ M AZO in ethanol/acetone (v/v 1:1) with the "basic concentration S_{AZO}". Regarding the determination of S_{AZO} see later on.

Solution C: Alb* in phosphate buffer (pH 7.2). The solutions C were produced in various concentrations (0.01–1.00 mg/ml).

Attention: a) Solution C is sensitive to light. The solution must be produced and stored in a dark-brown bottle. All experiments must be performed under weak or yellow lights. b) Solution C is sensitive to oxygen: Any reaction with atmospheric oxygen is prevented by having the closed bottle brim-full during the dissolving process. Each newly produced batch of the solution should be processed within a few minutes.

Short-term tests

Colloid particles in aqueous albumin solution have proven to be a suitable *in vitro* model for those structures which presumably can also arise in the plasma membrane of a cell.

The colloids can be manufactured by co-precipitation of cholesterol (Chol) with a slight amount of PAH or AZO (mol/mol 30:1) in the presence of apoA-I (see Fig. 1).

In their present form these tests are only suited for polynuclear hydrocarbons without polar substituents and for simple derivatives of *N*,*N*-dimethyl-4-aminoazobenzene (DAB). The applicability of the test for PAHs has its limit in those cases in which the PAH is insufficiently soluble in the solvent mixture used. For example, it was not possible so far to determine the indices of picene, pentacene and ovalene.

Preparation of the colloids

20 ml of solution C are put into a 50 ml beaker. A mixture of 1 ml solution A and 2 ml solution B1 or 1 ml of solution A and 1 ml of solution B2 are injected into this stirred solution C by means of a flask pipette. The suspension is centrifuged (1000 × *g*) immediately afterwards for 10 min and the

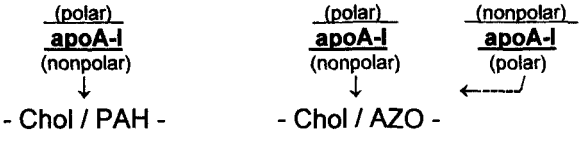

Fig. 1. Stabilization of the colloid particles by adsorption of apolipoprotein A-I (apoA-I). Working hypothesis: "The apoA-I adsorbs on the [Chol/PAH]-complex preferably with the nonpolar side of the amphipathic α-helix. In the case of AZOs a competitive adsorption of the polar side of the helix is involved in the process. The apoA-I (nonpolar) is adsorbed by [Chol/AZO] in appreciable amounts only when the adsorption of apoA-I (polar) is slight. Only the nonpolar (hydrophobic) interactions seem to be of any considerable significance for the epigenetic effectiveness of the PAHs and AZOs."

turbidity, *e.g.* the extinction of the colloid, is measured immediately against water in a 10 mm cuvette (623 nm).

Determination of the relative solubility S_{PAH} in aqueous medium

Mixtures consisting of 2 ml solution B1 and 1 ml ethanol are diluted with varying quantities of water. After 5 min the extinction values of these mixtures are measured against water in a 10 mm-cuvette at 623 nm. S_{PAH} is used to designate that volume (ml) of water at which the onset of crystallization of PAH can be observed through weak turbidity or an extinction rate > 0.020 in the test mixture.

Determination of the relative solubility S_{AZO} in aqueous albumin solution

1 ml ethanol is mixed with 1 ml of a solution of AZO (different concentrations in ethanol/acetone, v/v 1:1) in a 25 ml beaker. 20 ml of an albumin solution (*i.e.* solution C with 20 mg Alb* in 20 ml buffer) from a second beaker are poured "in one go" into this mixture. After 5 min the turbidity of this mixture, *i.e.* the extinction at 623 nm, is measured in a 10 mm-cuvette. A relative gauge of the solubility of the azo dye in the test mixture and simultaneously the "basic concentration S_{AZO}" for preparing solution B2 in saturated solution is provided by the concentration of the AZO solution, which creates a weak turbidity or an extinction of 0.020 in the test mixture due to beginning crystallization.

Evaluation

Step 1: Determination of C^{max}-values

Plotting the measured extinction values of the colloids as a function of the concentration (mg/ml) of solution C (logarithmic scale) provides a curve with a maximum for each PAH or AZO. C^{max} is the concentration of solution C by means of which – under the experimental conditions described – maximum turbidity is produced. Above this concentration, the entire quantity of suspended [Chol/PAH]- or [Chol/AZO]-complex is present in the sample as a colloid. $^{\circ}C^{max}$ is the C^{max} value for cholesterol alone, *i.e.* without PAH or AZO.

Step 2: Transformation of C^{max} values in activity factors F_{PAH} or F_{AZO}

As the production lots of Alb* differ with respect to their activity, however, it is absolutely essential when carrying out comparative investigations to transform the registered C^{max} value of a PAH or AZO into an activity factor F_{PAH} or F_{AZO} which is independent of the activity of the respective lot of Alb*. The transformation can be depicted graphically, namely by a projection of the C^{max} values on a F_{PAH} or F_{AZO} scale from 0 to 100. The different courses of the calibration curves for PAHs and AZOs are a consequence of the different polarities of the molecules. In the case of the PAHs, stronger hydrophobic interactions result in lower C^{max} values, *i.e.* less apoA-I or Alb* is required to stabilize the colloids. In contrast, with AZOs the low C^{max} values express the strong polar interactions between the amphipathic helix of apoA-I and the [Chol/AZO]-complexes. Hydrophobic interactions with the AZOs only then become relevant when the polar interactions are weak, *i.e.* when C^{max} attains high values. The linear functions make it possible to calculate the activity factors F_{PAH} and F_{AZO} from the measured values C^{max} and $^{\circ}C^{max}$:

$$F_{PAH} = 100 - (100\ C^{max}/^{\circ}C^{max}), \quad (1)$$

$$F_{AZO} = (100\ C^{max}/^{\circ}C^{max}). \quad (2)$$

The activity factor F_{PAH} or F_{AZO} is consequently a relative measurement for the strength of the hydrophobic interaction of apoA-I with the [Chol/PAH]- or [Chol/AZO]-complexes in the colloid.

Step 3: Determination of efficacy indices I^*_{PAH} or I_{AZO}

The activity factor F_{PAH} or F_{AZO} is not a definitive measurement of the suspected epigenetic effect of a PAH or AZO on a cell membrane *in vivo*. The effectiveness of a crystalline substance as adsorbent is restricted by its solubility in an aqueous medium. The solubility S_{PAH} or S_{AZO} must therefore be taken into consideration in its assessment. An efficacy index I^*_{PAH} or I_{AZO} can be calculated from the activity factor F_{PAH} or F_{AZO} and the solubility S_{PAH} or S_{AZO} with the following equations (for a derivation of the equations, see appendix in Results):

$$I^*_{PAH} = F_{PAH} - 10\ S_{PAH} + 35, \quad (3)$$

$$I_{AZO} = F_{AZO} - S_{AZO}. \quad (4)$$

I^*_{PAH} or I_{AZO} is an efficacy index which experience to date has shown is proportional to the carcinogenic effect of a PAH in 67% or AZO in nearly 100% of cases. We may therefore assume that the efficacy index actually mirrors the suspected epigenetic effect (EPE) of a PAH or AZO and that a realistic evaluation of the test results according to the criteria in Tables I and II is possible.

To avoid any misunderstandings we emphasize that due to the different assay conditions the efficacy indices I^*_{PAH} are not directly comparable with I_{AZO}. The test results permit separate rankings of the epigenetic efficacy for PAHs and AZOs.

Results

To date, 25 different AZOs and 58 different PAHs have been subjected to this test.

AZOs

A comparison between the determined efficacy index I_{AZO} of the suspected epigenetic effect (EPE) and complete carcinogenicity (CA) of simple derivatives of the aminoazo dye DAB reveals compliance of effective strengths in nearly 100% of cases (Table III). Hitherto no other characteristics of these compounds are known which correlate so strikingly with the carcinogenic activity. The correlation suggests that the parent compounds are effectively involved in the complicated process of tumorigenesis in the liver.

Table I. Evaluation of test results for PAHs.

Efficacy Index I^*_{PAH}	Strength of expected epigenetic effect	
> 50	strong	+ + +
41–50	moderate or strong	+ +/+ + +
31–40	weak or moderate	+/+ +
0–30	inactive or weak	0/+
< 0	inactive	0

Table II. Evaluation of test results for AZOs.

Efficacy Index I^*_{AZO}	Strength of expected epigenetic effect	
> 40	strong	+++
31–40	moderate	++
10–30	weak	+
< 10	inactive	0

Albumin, a main component of the serum, can exert a direct influence on the course of carcinogenesis in a very specific way by raising the solubility S_{AZO} of some AZOs (Table IV), thereby lowering their epigenetic effectiveness.

It can be presumed that 4′-trifluoromethyl-DAB as well as 4′-methoxy-DAB would be highly epigenetically effective if they were not retained in solution to such an extent by albumin. However it is noteworthy that the albumin in fact has no influence on the solubility of the methyl, ethyl and fluoro derivatives of DAB, so that their solubility remains less than the unsubstituted compound.

PAHs

All PAHs with a strong carcinogenic effect have without exception an efficacy index I^*_{PAH} larger than 40 (see Table V). All PAHs with an $I^*_{PAH} < 0$ are generally non-carcinogenic. Since only possibly "false positive", but in not a single case "false negative" results could be attained, we can also speak of a correlation in this connection, namely a correlation between the I^*_{PAH} values with the carcinogenicity of the PAHs in 67% of the cases. The relatively high proportion (~ 33%) of "false positive" results (Table VI) is not surprising, since a PAH can be weak or inactive despite a strong epigenetic effectiveness, when *in vivo* the reactivity or mutagenicity of its metabolites is too slight. The "false positive" results refer to PAHs, which, when at all, are only weak initiators. In combination experiments with stronger mutagens, the epigenetic effectiveness of the "false positive" substances would have to be discernible. This concept requires further experimental investigations.

Appendix: Derivation of equations (3) and (4) using the test results

Equation (3)

Fig. 2 shows the carcinogenicities of a large number of known PAHs (see Table V) as a function of the activity factor F_{PAH} and of their relative solubility S_{PAH}. Areas of different carcinogenicities could be separated by parallel lines in the diagram.

The selected slope of the parallels with exactly 10.0 is naturally somewhat arbitrary, but it exactly fulfills the prerequisite that PAHs with strong carcinogenic potential are found without exception above the upper line, whereas exclusively inactive PAHs are found below the lower line. If we take

Table III. Test results for AZOs (derivatives of *N,N*-dimethyl-4-aminoazobenzene, DAB). The complete carcinogenicity (CA) (Miller and Miller, 1953; Badger, 1954; Hansch and Fujita, 1964; Garner *et al.*, 1984) correlates with the efficacy index I_{AZO} or with the strength of the suspected epigenetic effect (EPE) (see Table II). S_{AZO}, relative solubility in albumin/buffer; F_{AZO}, activity factor (hydrophobic interactions of apoA-I with [Chol/AZO]-complexes). Results are the mean ± S.D. of two or three separate determinations.

Derivatives of DAB	S_{AZO}	F_{AZO}	I_{AZO}	EPE	CA	
4′-Ethyl-	2 ± 0.5	94 ± 4	92 ± 4.5	+ + +	+ + +	10
4′-Fluoro-	6 ± 0.5	92 ± 2	86 ± 2.5	+ + +	+ + +	10–12
2′,5′-Difluoro-	8 ± 2.0	76 ± 3	68 ± 5.0	+ + +	+ + +	> 10
2′,4′-Difluoro-	8 ± 0.5	74 ± 4	66 ± 4.5	+ + +	+ + +	> 10
3′-Methyl-	6 ± 1.5	72 ± 3	66 ± 4.5	+ + +	+ + +	10–12
2′,4′,6′-Trifluoro-	14 ± 2.0	70 ± 2	56 ± 4.0	+ + +	+ + +	> 10
3′-Fluoro-	12 ± 0.5	66 ± 3	54 ± 3.5	+ + +	+ + +	10–12
3′-Methoxy-	20 ± 2.0	64 ± 3	44 ± 5.0	+ + +	+ + +	10–12
3′-Chloro-	14 ± 1.0	52 ± 3	38 ± 4.0	+ +	+ +	5–6
DAB	16 ± 1.5	52 ± 3	36 ± 4.5	+ +	+ +	6
2′-Fluoro-	22 ± 2.0	56 ± 2	34 ± 4.0	+ +	+ +	7
2′-Chloro-	16 ± 2.0	42 ± 3	26 ± 5.0	+	+	2
2′-Methyl-	14 ± 1.5	34 ± 3	20 ± 4.5	+	+	2–3
4′-Methyl-	12 ± 1.5	28 ± 2	16 ± 3.5	+	+	1
4′-Methoxy-	58 ± 3.0	74 ± 4	16 ± 7.0	+	+	3
4′-Chloro-	40 ± 1.5	56 ± 3	16 ± 4.5	+	+	1–2
2′-Methoxy-	50 ± 3.0	64 ± 2	13 ± 5.0	+	+	2
2-Methyl-	8 ± 1.0	20 ± 2	12 ± 3.0	+	0	1
2′,4′-Dimethyl-	12 ± 1.5	24 ± 3	12 ± 4.5	+	0	0
2′-Trifluoromethyl-	20 ± 2.0	28 ± 2	8 ± 4.0	0	0	0
4′-Trifluoromethyl-	82 ± 3.0	90 ± 2	8 ± 5.0	0	0	0
2′,5′-Dimethyl-	16 ± 1.5	22 ± 3	6 ± 4.5	0	0	0
3′-Trifluoromethyl-	40 ± 3.0	44 ± 3	4 ± 6.0	0	0	0
2′,4′,6′-Trichloro-	30 ± 2.5	31 ± 4	1 ± 6.5	0	0	0
2′,5′-Dichloro-	36 ± 2.0	24 ± 2	< 0	0	0	0

Table IV. Albumin increases the solubility of a few AZOs by the amount ΔS_{AZO}. $S°_{AZO}$, relative solubility in phosphate buffer, pH 7.2; without albumin; S_{AZO}, relative solubility in phosphate buffer, pH 7.2; with albumin (γ = 1 mg/ml).

	$S°_{AZO}$	S_{AZO}	ΔS_{AZO}
4′-Trifluoromethyl-DAB	12	82	70
4′-Methoxy-DAB	2	58	56
3′-Trifluoromethyl-DAB	8	40	32
2′,5′-Dichloro-DAB	8	36	28
4′-Chloro-DAB	20	40	20
2′,4′,6′-Trichloro-DAB	10	30	20
2′-Methoxy-DAB	36	50	14
2′-Trifluoromethyl-DAB	8	20	12
3′-Methoxy-DAB	14	20	6

the point of intersection between one of these lines and the ordinate as a measurement to define the epigenetic effectiveness, then one can calculate with the measurements F_{PAH} and S_{PAH} an "efficacy index I_{PAH}" with the following equation for the dashed lines in Fig. 2:

$$F_{PAH} = 10\ S_{PAH} + I_{PAH}$$
$$\text{or } I_{PAH} = F_{PAH} - 10\ S_{PAH}.$$

In order that negative I_{PAH} values are exclusively reserved for inactive PAHs, the scale of the ordinates must be arbitrarily shifted by 35 units. This way a modified efficacy Index I^*_{PAH} can be defined by calculation:

$$I^*_{PAH} = I_{PAH} + 35$$
$$\text{or } I^*_{PAH} = F_{PAH} - 10\ S_{PAH} + 35. \quad (3)$$

Equation (4)

Fig. 3 shows the carcinogenicities of 25 AZOs (see Table III) as a function of activity factor F_{AZO} and of their relative solubility S_{AZO} in an aqueous albumin solution.

We can proceed in exactly the same manner as with the PAHs, *i.e.* areas of different effectiveness can be separated by parallel lines. Parallels with a slope of 1.0 fulfill these conditions. We can calculate an "efficacy index I_{AZO}" with the measure-

Table V. Test results for PAHs. The efficacy index I^*_{PAH} or the strength of the epigenetic effect (EPE) (see Table I) correlates with the carcinogenicity (CA) (Dipple *et al.*, 1984; Yang and Silverman, 1988). S_{PAH}, relative solubility in aqueous medium; F_{PAH}, activity factor (hydrophobic interactions of apoA-I with [Chol/PAH]-complexes). Results are the mean ± S.D. of two or three separate determinations.

	S_{PAH}	F_{PAH}	I^*_{PAH}	EPE	CA
Dibenzo[a.l]pyrene	2.8 ± 0.4	75 ± 2	82 ± 6	+ + +	+ + +
Dibenzo[a.e]pyrene	1.4 ± 0.2	59 ± 3	80 ± 5	+ + +	+ + +
7,12-Dimethylbenz[a]anthracene	5.6 ± 0.2	92 ± 2	72 ± 4	+ + +	+ + +
6-Methylbenz[a]anthracene	6.6 ± 0.3	94 ± 2	63 ± 5	+ + +	+ + +
3-Methylcholanthrene	3.2 ± 0.2	60 ± 2	63 ± 4	+ + +	+ + +
7-Methylbenz[a]anthracene	6.1 ± 0.2	87 ± 5	61 ± 7	+ + +	+ + +
8-Methylbenz[a]anthracene	4.5 ± 0.2	71 ± 3	61 ± 5	+ + +	+ + +
Dibenzo[a.h]pyrene	0.4 ± 0.1	27 ± 2	58 ± 3	+ + +	+ + +
12-Methylbenz[a]anthracene	6.4 ± 0.2	84 ± 7	55 ± 9	+ + +	+ + +
Benzo[b]fluoranthene	4.6 ± 0.3	61 ± 4	51 ± 7	+ + +	+ + +
Dibenzo[a.i]pyrene	1.0 ± 0.3	26 ± 3	51 ± 6	+ + +	+ + +
Benzo[a]pyrene	4.4 ± 0.2	59 ± 2	50 ± 4	+ + +	+ + +
5-Methylchrysene	7.2 ± 0.2	83 ± 3	46 ± 5	+ +/+ + +	+ + +
Benzo[j]fluoranthene	5.0 ± 0.3	61 ± 2	46 ± 5	+ +/+ + +	+ + +
Dibenz[a.h]anthracene	1.3 ± 0.2	22 ± 2	44 ± 4	+ +/+ + +	+ +
7-Methylbenzo[a]pyrene	5.2 ± 0.2	58 ± 2	41 ± 4	+ +/+ + +	+ +
2-Methylchrysene	3.6 ± 0.4	37 ± 1	36 ± 5	+/+ +	+
Benzo[e]pyrene	4.4 ± 0.3	40 ± 2	31 ± 5	+/+ +	+
Naphtho[2,3-a]pyrene	1.1 ± 0.2	7 ± 2	31 ± 4	+/+ +	+ +
Chrysene	2.5 ± 0.2	17 ± 3	27 ± 5	0/+	+
2,7-Dimethylanthracene	1.8 ± 0.2	10 ± 3	27 ± 5	0/+	0
Benz[a]anthracene	4.6 ± 0.3	38 ± 2	27 ± 5	0/+	+
Benzo[c]phenanthrene	10.3 ± 0.5	90 ± 2	22 ± 7	0/+	+
2,3-Dimethylanthracene	2.3 ± 0.2	10 ± 4	22 ± 6	0/+	0
Benzo[b]fluorene	4.2 ± 0.2	26 ± 2	19 ± 4	0/+	0
2-Methylanthracene	4.4 ± 0.2	28 ± 3	19 ± 6	0/+	0
Benzo[k]fluoranthene	3.1 ± 0.3	14 ± 3	18 ± 6	0/+	+
Benzo[a]fluorene	5.1 ± 0.3	33 ± 5	17 ± 8	0/+	0
Pyrene	9.2 ± 0.4	73 ± 4	16 ± 8	0/+	0
9,10-Dimethylanthracene	8.0 ± 0.5	53 ± 2	8 ± 7	0/+	+
Anthracene	4.8 ± 0.3	12 ± 2	– 1 ± 5	0	0
1,5-Dimethylanthracene	14 ± 0.3	94 ± 4	– 11 ± 7	0	0
1,4-Dimethylanthracene	> 30	93 ± 5	< – 65	0	0
1,3-Dimethylanthracene	> 30	93 ± 3	< – 65	0	0
1,2-Dimethylanthracene	> 30	93 ± 4	< – 65	0	0
1-Methylanthracene	> 30	87 ± 3	< – 65	0	0
9-Methylanthracene	> 30	87 ± 4	< – 65	0	0
Phenanthrene	> 15	23 ± 6	< – 65	0	0
Naphthalene	> 15	8 ± 2	< – 65	0	0

ments F_{AZO} and S_{AZO} using the following equation for the dashed lines in Fig. 3:

$$F_{AZO} = S_{AZO} + I_{AZO}$$
$$\text{or } I_{AZO} = F_{AZO} - S_{AZO}. \quad (4)$$

Discussion

The parent compounds of AZOs and PAHs, increasingly viewed as "inert" in the context of cancer research, apparently seem indeed to be able to participate in the process of carcinogenesis. They can intervene epigenetically when their hydrophobic interactions with cholesterol in the cell membrane are not prevented by too high solubility in water or serum. The correlations found in this study hardly allow for any other conclusion. The specific interactions of the parent compounds with cholesterol allow to presume that these compounds can trigger an *epigenetic* event in a cell membrane, most probably in the plasma membrane. We still do not know what functions are affected by the formation of the hydrophobic [Chol/AZO]- or [Chol/PAH]-complexes in cholesterol-domains of a cell membrane and which play a major role in the multi-staged process of tumorigenesis. At present we can only speculate on the

Table VI. "False positive" test results for PAHs. The complete carcinogenicity (CA) lower than the efficacy index I^*_{PAH} (see Table I) leads us to expected EPE. Results are the mean ± S.D. of two or three separate determinations.

	S_{PAH}	F_{PAH}	I^*_{PAH}	EPE	CA
Strongly divergent results					
1-Methylbenz[a]anthracene	5.4 ± 0.4	85 ± 9	66 ± 13	+ + +	+
3,9-Dimethylbenz[a]anthracene	2.2 ± 0.3	59 ± 2	72 ± 5	+ + +	0
11-Methylbenz[a]anthracene	5.7 ± 0.2	76 ± 6	54 ± 8	+ + +	+
10-Methylbenz[a]anthracene	3.1 ± 0.2	49 ± 2	53 ± 4	+ + +	+
Dibenz[a.c]anthracene	3.2 ± 0.1	50 ± 2	53 ± 3	+ + +	+
Triphenylene	4.7 ± 0.2	61 ± 4	49 ± 6	+ +/+ + +	0
Perylene	2.0 ± 0.1	28 ± 4	43 ± 5	+ +/+ + +	0
1-Methylchrysene	2.3 ± 0.2	30 ± 2	42 ± 4	+ +/+ + +	0
Benzo[g.h.i]perylene	2.4 ± 0.4	28 ± 5	39 ± 9	+ +/+ + +	0
Moderately divergent results					
Dibenz[a.j]anthracene	3.4 ± 0.2	81 ± 3	82 ± 5	+ + +	+ +
5-Methylbenz[a]anthracene	3.6 ± 0.4	61 ± 2	60 ± 6	+ + +	+ +
6-Methylchrysene	7.1 ± 0.2	83 ± 3	47 ± 5	+ +/+ + +	+
3-Methylchrysene	4.6 ± 0.3	57 ± 2	46 ± 5	+ +/+ + +	+
4-Methylchrysene	4.8 ± 0.3	57 ± 2	44 ± 5	+ +/+ + +	+
Coronene	1.2 ± 0.2	14 ± 3	38 ± 5	+ /+ +	0
Benzo[g.h.i]fluoranthene	8.2 ± 0.4	82 ± 3	35 ± 7	+ /+ +	0
Pentaphene	1.4 ± 0.2	14 ± 4	35 ± 6	+ /+ +	0
Benzo[a]tetracene	0.7 ± 0.2	3 ± 3	31 ± 5	+ /+ +	0
Benzo[b]chrysene	0.8 ± 0.2	3 ± 2	30 ± 4	+ /+ +	0

subject. Cholesterol is known to broaden the gel-to-liquid crystalline phase transition of phospholipid membranes and to affect membrane permeability and membrane fluidity. The fluidity of the membrane or their lipid domains has a great influence on the activities of membrane-bonded proteins, enzymes, and receptors (Barnett *et al.*, 1974; Chen *et al.*, 1978; Cavenee *et al.*, 1981; Chen, 1984; Yeagle, 1989; Schroeder *et al.*, 1991, 1995). The changes in membrane fluidity or microviscosity caused by the interaction of AZO or PAH with cholesterol could modulate the distribution and activity of membrane proteins which are critical to regulation of cellular proliferation. It is tempting to speculate that the "epigenetic event" – perhaps in collaboration with the products of activated "ras-like" oncogens – could have an influence on the organization and bonding of actin filaments in the plasma membrane, so that a direct influence on cytokinesis and cell division (with mitotic abnormalities resulting in aneuploidy?) appears possible. Such mechanisms remain to be investigated.

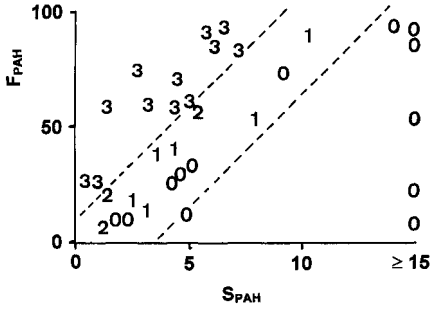

Fig. 2. Carcinogenic activity of PAHs (see Table V) as a function of activity factor F_{PAH} and of relative solubility S_{PAH} in aqueous medium. Numbers are in accordance with the complete carcinogenicity: 0, inactive; 1, weak; 2, moderate; 3, strong. The dashed lines indicate the fields of equal activity. False-positives (Table VI) are not marked.

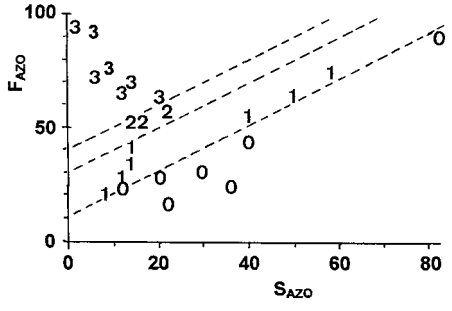

Fig. 3. Carcinogenic activity of AZOs (see Table IV) as a function of activity factor F_{AZO} and of relative solubility S_{AZO} in a phosphate buffer (pH 7.2) with 1.0 mg Alb*/ml. Numbers are in accordance with the carcinogenicity: 0, inactive; 1, weak; 2, moderate; 3, strong. The dashed lines indicate the fields of equal activity.

The question whether the apoA-I, which we use as indicator for the hydrophobicity of the [Chol/AZO]- or [Chol/PAH]-complexes, also plays a direct role *in vivo* in the processes in the plasma membrane by stabilizing the complexes as in the *in vitro* tests, cannot yet be conclusively answered at this time.

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