Regular paper

Interaction of anthracene and its oxidative derivatives with human serum albumin[*]

Katarzyna Skupińska[1], Monika Zylm[1], Irena Misiewicz[1] and
Teresa Kasprzycka-Guttman[1,2]✉

[1]*Confocal Microscopy Laboratory, National Institute of Public Health, Warszawa Poland; [2]Department of Chemistry, Warsaw University, Warszawa, Poland; ✉e-mail: guttman@il.waw.pl*

Binding affinities of ten polycyclic aromatic hydrocarbons to albumin were determined: anthracene, its eight oxy-derivatives: anthraquinone, 9-anthracenemethanol, 9-anthraldehyde, 9-anthracenecarboxylic acid, 1,4-dihydroxyanthraquinone, 1,5-dihydroxyanthraquinone, 1,8-dihydroxyanthraquinone, 2,6-dihydroxyanthraquinone and benzo[a]pyrene. The quenching of albumin fluorescence was used to measure the PAH — protein interaction. The theoretical curve of calculated fluorescence was fitted to experimental data after necessary corrections regarding PAHs fluorescence and inner filter effect. From the numerical fitting the final association constants were calculated. Anthracene and anthraquinone failed to quench the albumin fluorescence. 9-anthracenecarboxylic acid showed the highest, while 9-anthracenemethanol the weakest albumin binding affinity. The affinity constants determined for 9-anthraldehyde and benzo[a]pyrene were of the same magnitude and indicated low-affinity binding to albumin. The constants obtained for the four dihydroxyanthraquinones were higher, but dissimilar, which suggests that the position of the functional group in anthracene molecule influences the binding constant. Moreover, this study suggests that the type of substituent plays a significant role in PAH-albumin complex formation. The carboxylic group increases the binding affinity of the anthracene molecule the most rather than the presence of both carbonyl and hydroxyl groups. The lowest affinity constants were obtained for aldehyde, methyl and carbonyl substituents.

Anthracene is a three-ring compound belonging to polycyclic aromatic hydrocarbons (PAHs). PAHs are environmental pollutants and some of them are considered carcinogenic to humans. At least several PAHs have been shown to induce tumors in experimental animals (Wang *et al.*, 1995; Pashko & Schwartz, 1996; Kumar *et al.*, 2001; Flesher *et al.*, 2002). Epidemiological studies on humans revealed also more frequent incidence of skin cancer in chimney sweepers exposed to coal tar. People are exposed to PAHs mainly at the workplace, however, many PAHs are found in tobacco smoke or diet (Philips, 1999).

The PAHs present in the environment are considered as non active and unable to cause carcinogenesis directly. To become reactive, they must be firstly metabolized. PAHs are metabolized by many enzymes, primarily by cytochrome P450 enzymes: CYP1A1, CYP1A2 and CYP1B1 and by epoxide hydrolase. As products of these reactions many oxy-derivatives can be obtained, e.g.: epoxides, dihydrodiols, diol-epoxides, phenols and quinones (Prodi *et al.*, 1984; Lecoq *et al.*, 1991; Otto *et al.*, 1992; Honey *et al.*, 2000; Willett *et al.*, 2000; Hecht *et al.*, 2003; Lee *et al.*, 2003; Pangrekar *et al.*, 2003). The diolepoxides have been shown to bind to biological molecules like DNA or proteins. Such PAH derivatives-DNA adducts can cause mutations and in consequence carcinogenesis and tumor development (Garner, 1998; Boysen & Hecht, 2003).

Abbreviations: HSA, human serum albumin; PAH(s), polycyclic aromatic hydrocarbon(s).

PAHs adducts with biomolecules are used as biomarkers in order to assess human exposure to PAHs. A number of studies have been conducted to evaluate the usability of these biomarkers (Nielsen *et al.*, 1996; Sram & Binkova, 2000; Jacob & Seidel, 2002; Ruchirawat *et al.*, 2002). PAH-albumin adducts were found to be very useful biomarkers since albumin is the most abundant protein in the blood. Moreover, no repair occurs in protein so the adduct level is a total sum of human exposure to PAHs which took place during the lifetime of the protein. It was shown also that the level of PAH-albumin adducts depends on the duration of exposure (Lee *et al.*, 1991; Stella *et al.*, 1995; Ehrenberg *et al.*, 1996).

Albumin is a predominantly α-helical protein consisting of 585 amino acids and is built of three domains. This protein has an ability to bind covalently or reversibly a number of various endo- and exogenous compounds (Kragh-Hansen, 1990). Up to now various classes of binding sites have been determined. It was shown that albumin has one specific binding site for PAHs — either histidine-146 or lysine-137 located in subdomain IB near the single tryptophan-214 which is a fluorescent residue of albumin. Lysine-385 located in subdomain IIIA was also proposed to be a binding site (Tannenbaum *et al.*, 1993; Brunmark *et al.*, 1997). Fabriciova *et al.* (2004) showed that the binding sites for dihydroxyanthraquinones are localized in subdomains IIA and IIIA of albumin. These two subdomains are believed to be a general binding site for small organic molecules. These subdomains are also characteristic for binding drugs like warfarin (IIA), ibuprofen (IIIA) or oxazepan (IIIA) which consist of benzene rings (Epps *et al.*, 1995).

It has been shown that energy transfer is possible from the single tryptophan residue to the PAHs bound, which can result in albumin fluorescence quenching (Ma *et al.*, 1977). Albumin fluorescence quenching as a result of ligand binding was also described for albumin interactions with drugs and proteins (Epps *et al.*, 1995; Gou *et al.*, 2004; Qg *et al.*, 2005). In our work this phenomenon was applied to determine the binding constants of albumin and PAHs and their oxo-derivatives. Ten different compounds of these groups were selected, varying in the aromatic ring number and structure as well as in the type of oxy-substituents (carbonyl, carboxyl and hydroxyl) and their position in the anthracene molecule chosen by us as a representative. Our results are the first approach in calculations of anthracene derivatives-albumin dissociation constant as, according to our knowledge, there is no literature data on the affinity of these compounds toward albumin. The aim of this study was to establish if PAHs unsubstituted and substituted by oxygen-containing substituents can bind to albumin, and how the type, number and position of the substituents influence the binding affinity.

MATERIALS AND METHODS

Chemicals. All PAHs derivatives (abbreviated further as PAH): anthracene, anthraquinone, 9-anthracenemethanol, 9-anthraldehyde, 9-anthracenecarboxylic acid, 1,4-dihydroxyanthraquinone, 1,5-dihydroxyanthraquinone, 1,8-dihydroxyanthraquinone, 2,6-dihydroxyanthraquinone, benzo[a]pyrene and human serum albumin (Fraction V) were purchased from Sigma Chemical Co. The purity of all compounds were in the range 99.6–99.9%. All PAHs were dissolved in ethanol and albumin was dissolved in phosphate buffered saline (PBS) (0.05 M, pH 7.4). The solvents were purchased from POCh (Poland).

Spectroscopic measurements. Absorbance measurement was carried out with a UV-VIS Shimadzu Uvmini1240 spectrometer in a 1 × 1 cm cuvette.

A Shimadzu RF-5000 spectrofluorimeter was used to measure the fluorescence of albumin-PAH solutions. The mixture in a 1 × 1 cm quartz cuvette was stirred continuously during measurement and the temperature was kept constant at 25°C. The albumin fluorescence was measured at the excitation wavelength of 278 nm and the signal was observed at 340 nm (the excitation slit width was set at 3 nm and emission slit width at 10 nm). These conditions were chosen to ensure strongest albumin fluorescence signal.

Albumin stock solutions in PBS were prepared prior to experiment and the final concentration of 0.5 μM was confirmed by absorbance measurement (ε_{278} = 37000 $M^{-1}cm^{-1}$). This concentration was chosen as addition of increasing amounts of PAHs resulted in a fluorescence decrease down to a minimal value. The albumin solution was prewarmed to 25°C in the cuvette for 15 min and then fluorescence was measured until stabilized. PAHs were dissolved in ethanol until saturation and several dilutions of such solutions were prepared. Their concentrations were checked by means of spectroscopic measurements. Titration with PAHs was performed by adding aliquots of PAHs solutions to 2900 μL of albumin dissolved in PBS so as to achieve uniformly increasing PAHs concentrations in the cuvette. The final PAHs concentrations were as follows: anthracene 0.003–2.793 μM, anthraquinone 0.005–7.07 μM, 9-anthracenemethanol 0.002–44.665 μM, 9-anthraldehyde 0.002–25.402 μM, 9-anthracenecarboxylic acid 0.034–8.583 μM, 1,4-dihydroxyanthraquinone 0.0003–4.971 μM, 1,5-dihydroxyanthraquinone 0.003–1.873 μM, 1,8-dihydroxyanthraquinone 0.03–25.248 μM,

2,6-dihydroxyanthraquinone 0.002–7.638 μM and benzo[a]pyrene 0.002–25.193 μM. After addition of each PAH sample the fluorescence was measured according to literature data (Niedzwiecka *et al.*, 2002; Zuberek *et al.*, 2003): fluorescence signal was registered 4 times during 40 s and mean ± S.D. was calculated. Then, there was a 20 s break for the next sample addition. Each titration was repeated independently 5 times. The total amount of PAHs in ethanol never exceeded 100 μl (3.3%, v/v). Such concentration of ethanol was shown not to disturb albumin fluorescence.

Additional titrations were performed to determine the fluorescence of increasing amounts of ethanol in PBS and PBS was titrated with PAHs to verify if the fluorescence of any PAH occurs. All these measurements were performed under the same conditions (including the volume of aliquots added) as the albumin titrations. The absorbance of increasing concentrations of PAHs was also determined for the excitation and emission wavelength of albumin in order to calculate the inner filter effect.

Data analysis. In order to determine the dissociation constants, two methods are usually applied: the protein concentration is kept constant while ligand concentration is increased from a concentration lower than that of albumin to a higher one. Another option is to keep the ligand concentration constant and to increase the protein concentration, however, this one has not been well studied yet (Epps *et al.*, 1995; 1999). In this study PAHs were added to a constant concentration of albumin and the fluorescence of the solutions was measured.

Binding of PAHs to n identical sites in albumin can be described by the Scatchard equation:

$$n = \frac{nL}{K+L} \tag{1}$$

where
v – average number of ligand molecules bound per protein molecule,
n – number of binding sites,
L – free (unbound) ligand concentration,
K – dissociation constant of ligand–albumin complex.

However, v can be written by an other equation:

$$n = \frac{L_0 - L}{A_0} \tag{2}$$

where
L_0 – total ligand concentration,
A_0 – total albumin concentration.

The fluorescence of albumin is quenched by ligand binding and is the sum of free albumin and albumin–ligand complex fluorescences:

$$F = f_A (A_0 - AL) + f_{AL} AL = F_0 - (f_A - f_{AL}) AL \tag{3}$$

where
AL – concentration of complexed albumin,
f_A and f_{AL} are molar emissivity of free and complexed albumin.

The AL concentration can be calculated from the formula:

$$AL = \frac{L_0 - L}{n} \tag{4}$$

It follows from calculations (Eqns. 1, 2 and 4) arises that the complex concentration (AL) is described by the following equation:

$$AL = \frac{(n \cdot A_o + L_o + K) - \sqrt{(n \cdot A_o + L_o + K)^2 - 4 \cdot n \cdot A_o \cdot L_o}}{2 \cdot n} \tag{5}$$

Hence, substituting the calculated value of AL (Eqn. 5) to Eqn. 3, the fluorescence of the mixture can be expressed as (Guo *et al.*, 2004; Zuberek *et al.*, 2003):

$$F = F_o - (f_A - f_{AL}) \cdot \frac{(n \cdot A_o + L_o + K) - \sqrt{(n \cdot A_o + L_o + K)^2 - 4 \cdot n \cdot A_o \cdot L_o}}{2 \cdot n} \tag{6}$$

This equation yields information about the dissociation constant and also about the binding stoichiometry.

The above equation is correct only if the binding sites specific for PAHs in albumin are identical to each other and act independently. Moreover, this formula is correct only for strong binding sites. In the case of low affinity ligands, a modified equation describes the fluorescence of the mixture (the case of ligand excess with respect to albumin). Unfortunately this method does not supply information on the stoichiometry of the binding. However, when fitting Eqn. 6 gives no results, a low affinity of a compound toward albumin is to be assumed and the following equation is fitted:

$$F = F_o - (f_A - F_{AL}) \frac{A_0 \cdot L_o}{K + L_o} \tag{7}$$

Using the above equations the experimental dependence of F on L_o was analyzed by non-linear least-squares regression. From the best fit, the *K* value was calculated. The fittings were performed with SigmaPlot 2000 SPSS Inc.

Corrections

PAHs fluorescence. Some of the compounds studied exhibit fluorescence in the excitation/emission wavelengths specific for albumin. In this case the fluorescence of the mixture consists of free albumin fluorescence, fluorescence of the complex and fluorescence of PAH. After an initial fluorescence drop, a total fluorescence signal increase can be observed for high concentration of PAHs as a result of

predominant ligand fluorescence. Then corrections must be done. The modified equations describing solution fluorescence in dependence on ligand concentration will be applied:

$$F = F_o + f_L \cdot L_o - f \cdot \frac{(n \cdot A_o + L_o + K) - \sqrt{(n \cdot A_o + L_o + K)^2 - 4 \cdot n \cdot A_o \cdot L_o}}{2 \cdot n}$$

$$f = f_L + f_A - f_{AL}$$

(8)

where f_L – molar emissivity of free (unbound) ligand, f_A – molar emissivity of free albumin, and f_{AL} – molar emissivity of albumin–PAH complex.

For low-affinity ligands:

$$F = F_o + f_L \cdot L_o - f \cdot A_o \frac{L_o}{K + L_o}$$

(9)

Inner filter effect. The absorbance spectra of all compounds studied overlap the excitation and emission wavelengths of albumin. Therefore, the compounds absorb the light used to excite albumin as well as the light emitted by albumin. All data were corrected and the experimental fluorescence values were multiplied by factor $10^{\frac{(\varepsilon_{exc} + \varepsilon_{em})L_o l}{2}}$ where ε_{exc} – ligand molar extinction coefficient at the albumin excitation wavelength, ε_{em} – ligand molar extinction coefficient at the albumin emission wavelength, L_o – ligand concentration, l – optical length. The coefficients were determined before the main experiments by fitting a line to experimentally determined absorbance values for increasing concentrations of PAHs.

Other corrections. Other corrections include the albumin dilution during titration and the background fluorescence of PBS and ethanol, which were subtracted from experimental values.

RESULTS

Ten PAHs were studied regarding the influence of structure differences on HSA affinity. These PAHs were: anthracene and its eight oxy-derivatives: anthraquinone, 9-anthracenemethanol, 9-anthraldehyde, 9-anthracenecarboxylic acid, 1,4-dihydroxyanthraquinone, 1,5-dihydroxyanthraquinone, 1,8-dihydroxyanthraquinone, 2,6-dihydroxyanthraquinone as well as benzo[a]pyrene, which is considered carcinogenic to people. Anthracene is not recognized as a carcinogen, however, it is toxic toward people and hence specified by US EPA as a hazardous substance. Quenching of albumin intrinsic fluorescence was the method used in order to determine the dissociation constant of the PAH – albumin complexes. In the study fraction V human albumin was used in order to mimic the conditions of human blood plasma.

All PAHs were dissolved to achieve the highest concentrations possible and series of dilutions

were prepared. Aliquots of these PAHs dilutions were added to albumin solution in order to achieve increasing concentrations of PAH in the measured samples and the mixture fluorescence was recorded. The background fluorescence (buffer and ethanol) was subtracted from the experimental value. Then all the data obtained were corrected for inner filter effect as all compounds studied showed the absorbance in both the albumin excitation and emission wavelengths (not shown). The spectral data of all compounds studied are presented in Table 1. The excitation and emission wavelengths (278/340 nm) were chosen as to provide the best fluorescence of albumin. The wavelength of 278 nm excites both tryptophan and tyrosine, however, tyrosine fluorescence is observed at 305 nm. The emission registered at 340 nm responds to tryptophan fluorescence.

The PAHs are hardly soluble in water and for the highest concentration we observed their precipitation. To establish the maximal concentration of PAHs which is soluble in PBS, additional measurements of PAHs fluorescence spectrum were performed. An increase in background fluorescence indicates that the precipitation process has started as the light is scattered on the precipitated particles. In this way the maximal PAHs quantities soluble in PBS were determined.

When albumin was titrated with anthracene no fluorescence decrease was observed (Fig. 1a), however, for higher (above 1 µM) anthracene concentrations the fluorescence signal increased. The lack of albumin fluorescence quenching can suggest that there are no interactions between albumin and anthracene. Nevertheless, models 8 and 9 were applied because of the fluorescent properties of anthracene. No good fitting was achieved which indicates an absence of interactions. Hence, anthracene is assumed as non-binding to albumin.

For anthraquinone a slight drop in fluorescence intensity and no increase despite the fluorescent properties of anthraquinone were observed (Fig. 1b). All four equations considering low and high affinity as well as fluorescence and lack of fluorescence of the compound were fitted but no good fitting was achieved. This can indicate that either no interaction occurs or the models used in the study do not describe the anthraquinone–albumin interaction.

Two oxy-anthracene derivates, 9-anthracenemethanol and 9-anthraldehyde, exhibit similarly low affinity towards albumin. The obtained dissociation constants are 13.040 ± 1.187 and 7.252 ± 0.781 µM, respectively (Table 2). The albumin fluorescence was quenched by these compounds at concentrations higher than 0.5 µM for 9-anthracenemethanol and 0.1 µM for 9-anthraldehyde, as seen in Fig. 1c and Fig. 1d. For 9-anthraldehyde a distinct rise of fluo-
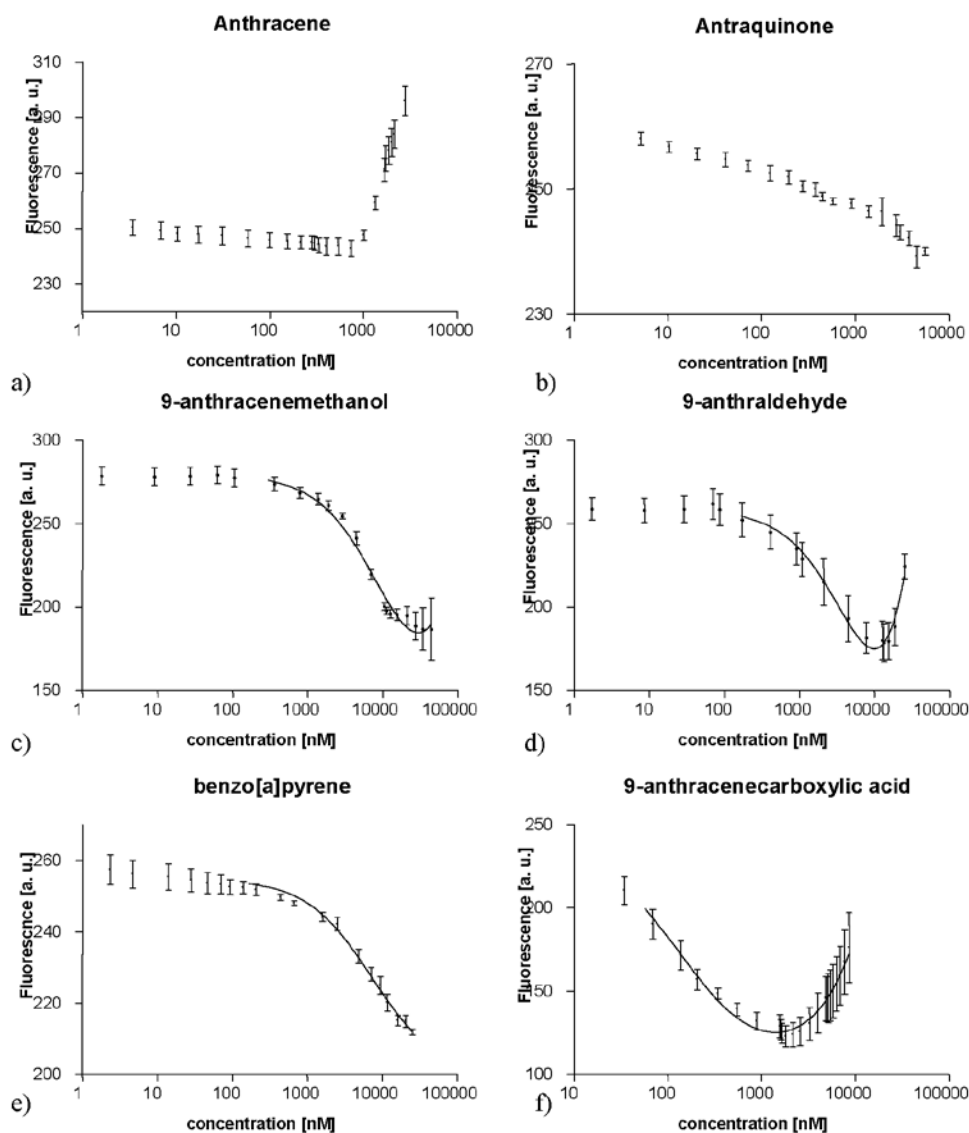
Figure 1. Albumin fluorescence when titrated with:
a, anthracene; b, anthraquinone; c, 9-anthracenemethanol; d, 9-anthraldehyde; e, benzo[a]pyrene; f, 9-anthracenecarboxylic acid. Albumin at 0.5 μM was titrated at 25°C in 0.05 M PBS, pH 7.4. Albumin was excited at 278 nm and emission was detected at 340 nm. Each experimental point is a mean of 5 titrations. The solid line is a best-fit theoretical curve.

rescence was observed for the concentrations above 10 μM, hence models assuming fluorescence of the compound were used. Model 9, assuming low affinity for albumin, was applied as it yielded a better value of $R^2 = 0.92$, $P > 0.9$. For 9-anthracenemethanol no distinct rise of fluorescence was observed, nevertheless fitting of various equations to experimental data yielded the best fit for the model assuming ligand fluorescence and a low affinity binding to albumin ($R^2 = 0.95$, $P > 0.9$). The lower goodness of fit obtained for 9-antrhaldehyde can arise from the fact that the experimental points which are a mean of several measured values have relatively large standard deviations (Fig. 1d). Because a model assuming low-affinity interactions was applied, no data on stoichiometry was obtained.

When albumin was titrated with benzo[a]pyrene a weak fluorescence decrease was observed for concentrations higher than 0.1 μM. No fluorescence increase was observed even for the highest benzo[a]pyrene concentration (Fig. 1e). Since benzo[a]pyrene fluorescence was several times weaker than that of the other PAHs studied, equation 7, assuming no fluorescence of the compound, was used to calculate the dissociation constant. The curve fitting was good yielding the $R^2 - 0.98$ and the dissociation constant was calculated to be 7.202 ± 0.332 μM (Table 2). This value indicates that benzo[a]pyrene binds to albumin with low affinity.

When albumin was titrated with dihydroxyanthraquinones fluorescence quenching was observed for all concentrations of 1,8-, 1,5- and 2,6-dihydroxy-

Table 1. Literature and experimental spectral properties of PAHs

PAH	UV-VIS λ_{max} (ε [$M^{-1}cm^{-1}$])	Fl λ_{max}	UV-VIS exp λ_{max}	Fl exp λ_{max}
Anthracene	252 (220000)[1] 323 (2800)[1] 339 (5500)[1] 356 (8500)[1] 374 (8500)[1]	378[2] 399[2] 423[2] 449[2]	258 327 343 361 380	389 410 433
Anthraquinone	251 (54000)[1] 321 (4800)[1]	–	257 326	–
9-Anthracenemethanol	256 (14664)[3] 332 (505)[3] 348 (1125)[3] 366 (1767)[3] 386 (1577)[3]	450 (0.04)[3]	259 333 349 368 388	420 398 444
9-Anthraldehyde	–	463[7]	264 372 403	499
Benzo[a]pyrene	297 (63000)[1] 347 (12200)[1] 365 (25000)[1] 385 (29500)[1]	403[2] 408.5[2] 427[2]	299 351 369 389	412 436 463
1,4-Dihydroxyanthraquinone	248 (33000)[1] 279 (10800)[1] 479 (8200)[1]	526[4]	255 475	550
2,6-Dihydroxyanthraquinone	–	–	275 303 351	532
1,8-Dihydroxyanthraquinone	430 (2200)[5] 284 (2136)[5] 272 (2092)[5] 253 (1720)[5]	586[4]	259 428	582
1,5-Dihydroxyanthraquinone	436 (–)[4] 414 (–)[4]	582[4]	259 420	583
9-Anthracenecarboxylic acid	330 (28840)[6] 346 (58884)[6] 363 (69183)[6] 383 (27542)[6]	470[6]	259 348 365 385	468

[1]Atlas of Organic Compounds, Butterworths, Verlag Chemie, 1974; [2]Dabestani & Ivanov, (1999); [3]Toulokhonova (2003); [4]Morzocchi *et al.* (1998); [5]Absorption Spectra in the Ultraviolet and Visible Region, Lang L. ed, Publishing House Hungarian Academy of Science, Budapest, Hungary (1963); [6]Abdel-Mottaleb *et al.* (2000); [7]Lin *et al.* (2000).

anthraquinones and for concentrations higher than 0.025 μM of 1,4-dihydroxyanthraquinone (Figs 2a, b, c, d). All dihydroxyanthraquinones exhibit fluorescence when excited at 278 nm, therefore models assuming ligand fluorescence were used in order to calculate dissociation constants. Indeed, for the highest PAHs concentrations (> 5 μM for 1,8-dihydroxyanthraquinone, > 3 μM for 1,4-dihydroxyantaquinone and > 2.5 μM for 2,6-dihydroxyanthraqui-none) a PAH-albumin solution fluorescence increase is observed with the exception of 1,5-dihydroxyanthraquinone.

The lack of fluorescence increase is probably caused by the fact that the concentration of 1,5-dihydroxyanthraquinone did not achieve 2 μM and was too low to increase the fluorescence of solution. However, due to the spectral properties of this compound, models 8 and 9 were applied. For

Table 2. Association constants and stoichiometry for albumin–PAHs complexes

PAH	K_{ass} [μM^{-1}]	n	Model
Anthracene	–	–	8, 9
Anthraquinone	–	–	6, 7, 8, 9
9-Anthracenemethanol	0.077 ± 0.007	–	9
9-Anthraldehyde	0.138 ± 0.015	–	9
Benzo[a]pyrene	0.139 ± 0.006	–	7
1,4-Dihydroxyanthraquinone	0.347 ± 0.021	–	9
2,6-Dihydroxyanthraquinone	0.372 ± 0.045	–	9
1,8-Dihydroxyanthraquinone	0.526 ± 0.034	1.17	8
1,5-Dihydroxyanthraquinone	2.353 ± 0.161	1.08	8
9-Anthracenecarboxylic acid	7.519 ± 0.678	0.03	8

all dihydroxyanthraquinones two models were fitted (8 and 9) and better fit was chosen. For 1,4- and 2,6-dihydroxyanthraquinones the equations describing low-affinity interactions gave better results of fitting, whereas for the 1,8- and 1,5-dihydroxyanthraquinones Eqn. 8, describing high-affinity interactions, was more appropriate. The equations describe well the experimental points ($R^2 > 0.97$) for

three dihdroxyanthraquinones except for 2,6-dihydroxyanthraquinone ($R^2 = 0.9$). This lower value of R^2 indicates that the goodness of fit is lower than for the other dihydroxyanthraquinones. Similarly to 9-antraldehyde this is possibly caused by the relatively large standard deviations of the experimental data obtained for 2,6-dihydroxyantraquinine. The obtained values of dissociation constants are: 2.883
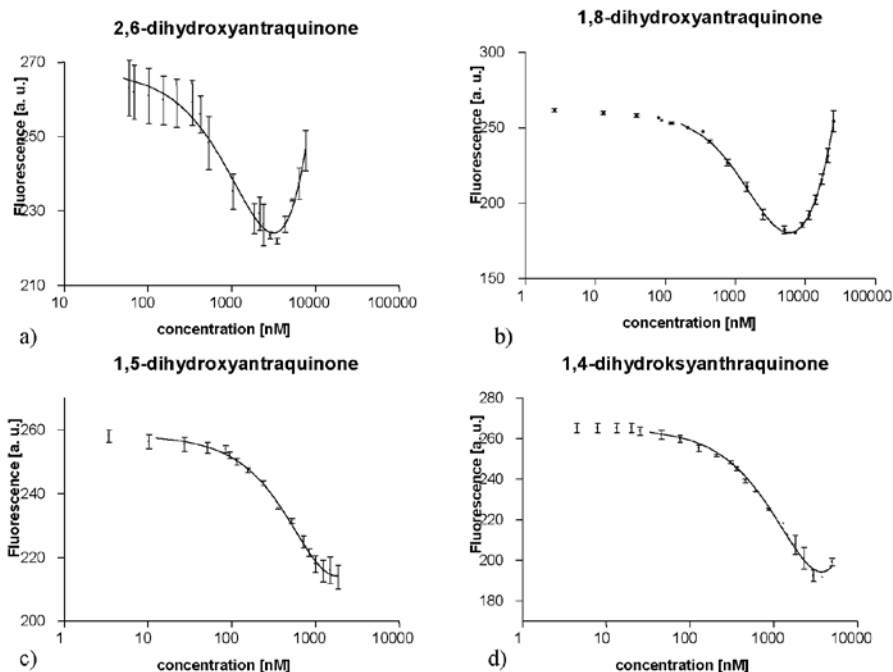

Figure 2. Titration curves for:
a, 2,6-Dihydroxyanthraquinone; b, 1,8-dihydroxyanthraquinone; c, 1,5-dihydroxyanthraquinone; d, 1,4-dihydroxyanthraquinone. Albumin, 0.5 μM, was titrated at 25°C in 0.05 M PBS at pH 7.0. Albumin was excited at 278 nm and emission was detected at 340 nm. Each experimental point is a mean of 5 titrations. The solid line is a best-fit theoretical curve.

± 0.171 µM for 1,4-dihydroxyanthraquinone, 2.688 ± 0.325 µM for 2,6-dihydroxyanthraquinone, 1.902 ± 0.123 µM for 1,8-dihydroxyanthraquinone and 0.425 ± 0.029 µM for 1,5-dihydroxyanthraquinone (Table 2). For 1,8-dihydroxyanthraquinone and 1,5-dihydroxyanthraquinone model 7 was applied and the stoichiometry of binding was calculated: n = 1.17 and n = 1.08, respectively.

9-Anthracenecarboxylic acid is the compound of the highest affinity toward albumin. Titration of albumin with this compound yielded a significant and rapid drop of albumin fluorescence for all concentrations used (Fig. 1f). Subsequent fluorescence rise for the highest 9-anthracenecarboxylic acid concentrations > 2 µM was observed. Hence, models assuming ligand fluorescence were applied. The fitting of model 8 to experimental data with R^2 = 0.7 yielded the dissociation constant 0.133 ± 0.012 µM and n = 0.03 (Table 2).

For all PAHs, the dissociation constants are collected in Table 2. The models used to calculate the constants and stoichiometry are also given there.

DISCUSSION

The binding affinities of 10 PAHs (structures presented in Fig. 3) for albumin were determined in this study in order to investigate the influence of the type and position of oxy-substituents in the PAH molecule. The method applied was albumin titration with the titrants containing increasing concentrations of the compound studied. Theoretical models assuming different types of interactions and different spectral properties of the compound were applied. Additionally, corrections for inner filter effect and albumin dilution were introduced to obtain accurate values of dissociation constants.

Up to now, the binding constants to albumin have been established for drugs or endogenous products of metabolism (e.g. bilirubin, free fatty acids) as it is an important aspect of the activity of these substances. There is no literature data available on PAHs and other pollutants' affinity toward albumin.

Several methods have been used to determine the ligand–protein binding constants, fluorescence quenching being one of them.

The association constant describing the ligand–protein bond strength was commonly calculated from curve fitting to experimental points. Some researchers fit the equation described by Scatchard to experimentally obtained values of albumin fluorescence and from this determine the affinity constant and capacity (Levine, 1977; Guo et al., 2004). Calculation of the constants from the Scatchard equation was also a method used by Paal et al. (2001), however, the fluorescence intensity was not measured, but the concentrations of unbound ligand which were determined from RP-HPLC measurements.

In our work the quenching of albumin fluorescence due to increasing concentrations of ligand in the titrant, was measured and equations describing the fluorescence dependence upon concentration of the ligand were applied.

Epps et al. (1995; 1999) used this method with success to determine the affinity of drugs to albumin. This method was also applied by Niedzwiecka et al. (2002) and Zuberek et al. (2003) to determine the interaction between the cap-binding protein eIF4E with mRNA 5′ end and its analogs. This method allows us to calculate the dissociation constants of the studied compound–albumin complexes.

Since our calculation gave dissociation constants and literature data present mostly association ones, we recalculated the dissociation constants to the association ones to make comparison of our results easier. They are shown in Table 2. The values of the dissociation constants were in the range 13.040–0.111 10^{-6} M, which gives the association constants in the range 0.077–9.009 10^6 M^{-1}.

The association constants obtained by us indicate that the affinity of PAHs toward albumin is similar to the affinity of drugs and endogenous substances, e.g. the highest binding constants were observed for bilirubin and long-chain fatty acids (K_{ass} ≈ 10^7–10^8 M^{-1}) (Levine, 1977; Paal et al., 2001). The association constants obtained for Furosemid was 13 × 10^6 M^{-1} (Levine, 1977), for Warfarin 0.29 × 10^6 M^{-1} and for Dansylsulfonamide 0.13 × 10^6 M^{-1} (Epps et al., 1995).

Epps et al. (1995) determined also the constants for other nonfluorescent drugs by the method of displacement of fluorescent probe, such as Ibuprofen which the constant 0.25 × 10^6 M^{-1} and for a low-affinity drug – a nonpeptidic HIV protease inhibitor U-105,665 it is 0.018 × 10^6 M^{-1}.
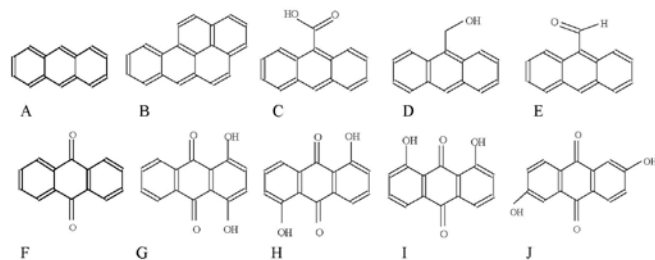
Figure 3. Structures of PAHs:
A, anthracene; B, benzo[a]pyrene; C, 9-anthracenecarboxylic acid; D, 9-anthracenemethanol; E, 9-anthraldehyde; F, anthraquinone; G, 1,4-dihydroxyanthraquinone; H, 1,5-dihydroxyanthraquinone; I, 1,8-dihydroxyanthraquinone; J, 2,6-dihydroxyanthraquinone

In a recent paper Epps *et al.* (1999) studied the association constants and found the following values: for high affinity drugs: 0.45 and 2.08×10^6 M^{-1} and for low-affinity 0.054×10^6 M^{-1}. The studies were also conducted with bovine serum albumin and the following values of the constants were obtained for hemin – 50×10^6 M^{-1} (Silva *et al.*, 2004) and for the antibiotic Gatifloxacin 0.1×10^6 M^{-1} (Guo *et al.*, 2004).

Paclitaxel, an antitumor drug, was shown to bind to albumin at one site with a high affinity of 2.4×10^6 M^{-1} and at a second one with a moderate affinity of 0.1×10^6 M^{-1} (Paal *et al.*, 2001).

Among the PAHs studied, 9-antracenocarboxylic acid (9.0×10^6 M^{-1}) and all dihydroxyanthraquinones ($0.3 - 2.4 \times 10^6$ M^{-1}) demonstrate a high affinity toward albumin. Benzo[a]pyrene and 9-antraldehyde (0.14×10^6 M^{-1}) are recognized to bind to albumin with moderate affinity and 9-anthracenemethanol (0.08×10^6 M^{-1}) with low affinity. These results indicate that some of PAHs derivates can bind as strongly as drugs and endogenous substances often very important for organism functioning.

Anthracene and anthraquinone were determined in this study as non-binding to albumin. The presence of aldehyde or methyl substituents only slightly increases the affinity of anthracene derivatives for albumin.

A very interesting result was obtained for benzo[a]pyrene. It is believed that PAHs must be metabolised to oxy-derivatives which in this form only are able to interact with biological molecules. We obtained good fitting of a theoretical curve calculated for weak ligand affinity for experimental points. This suggests that the quenching of albumin fluorescence is due to weak albumin–benzo[a]pyrene interaction.

Dihydroxyanthraquinones exhibit relatively high values of affinity constants. It is probably the result of albumin interaction with these PAHs through hydrophobic stacking and H-bonds involving =O and –OH groups (Fabriciova *et al.*, 2004).

The highest affinity was determined for anthracenecarboxylic acid (7.519 ± 0.678 μM) which indicates that carboxyl group is the most reactive toward albumin. This result is in agreement with previous findings that fatty acids and bilirubin (Levine, 1977) or Ibuprofen (Epps *et al.*, 1995) exhibit very high affinity toward albumin. All these molecules contain –COOH groups, however, the structure of these compounds is complex and it is difficult to decide if the high affinity toward albumin is caused only by the presence of the –COOH groups.

The studied compounds can be divided into two groups. The first: anthracene substituted with groups containing carbon on gradually increasing oxidated state: carbohydroxyl (C–OH), carbonyl (C=O) and carboxylic (COOH); this means anthracenemethanol, aldehyde and acid ones. In this case the strength of PAHs affinity toward albumin increases together with the carbon oxidated state.

The second group are anthracene derivatives containing both hydroxyl and carbonyl groups attached in various position in the molecule — dihydroxyanthraquinones. The addition of two carbonyl groups (C=O) to the anthracene molecule (anthraquinone) does dot increase the binding affinity for albumin; only the presence of both carbonyl and hydroxyl groups distinctly increases the affinity. The highest albumin affinity was shown by 1,5-dihydroxyanthraquinone, then by 1,8- and the least affinity was shown by 2,6- and 1,4-dihydroxyanthraquinone. Up to 5-fold differences in affinity constants were observed, which indicates that the position of the hydroxyl substituents in the anthracene molecules determines its binding affinity.

High affinity constants were achieved when both hydroxyl and carbonyl groups were located next to each other. The strongest effect is when hydroxyl groups are located on two opposite sides of the molecule (in positions 1 and 5). The configuration of the =O and –OH groups in 1,5-dihydroxyanthraquinone is very similar to the –COOH group in anthracenecarboxylic acid. Moreover, the presence of =O and –OH substituents on the opposite sides of the anthracene skeleton gives more possibilities to interact with albumin as this molecule can bind at either side.

The presence of two –OH groups close to the =O group (1,8-dihydroxyanthraquinone) also is beneficial, however, the binding strength is lower due to a steric barrier caused by the close proximity of the =O group in position 9 and the –OH groups in positions 1 and 8.

This observation shows that the affinity toward biological molecules increases with the increase in the electrophilic properties of PAH molecule caused by hydroxyl groups. Hence, PAH metabolic transformation seems to be a crucial step in the PAH distribution into tissues and cells.

All compounds studied by us can be a model of oxygen-containing PAHs obtained after the metabolism of parent polyaromatic compounds and also after their subsequently deeper oxidation in living organism. They can be also examples of products obtained by polyaromatics combustion and hence could be present in, e.g., cigarette smoke.

Similarly like drugs and endogenous substances, PAHs bound to albumin can be distributed all over the body; hence PAHs can reach the organs which are not directly exposed to PAHs. Then they can disturb this organ's metabolism, eventually leading to the carcinogenesis.

On the other hand, as it is described for drugs, binding of ligands with albumin can decrease their (here PAHs) concentration in the blood, hence it can reduce the harmful effect of exposure to PAHs. With respect to this, the albumin capacity could be a very important factor leading to scavenging of PAHs biological effect on humans exposed to this pollutant. If a single molecule of albumin is able to bind more than one PAH molecule (as it has been shown in the literature) it can reduce the level of pollutants and its metabolites in the blood even more effectively.

We were able to determine the binding stoichiometry of three compounds, which showed high affinity toward albumin: 1,5-dihydroxyanthraquinone (n = 1.08), 1,8-dihydroxyanthraquinone (n = 1.17) and 9-anthracenecarboxylic acid (n = 0.03). The results obtained for dihydroxyanthraquinones suggest that only one PAH molecule can bind to one molecule of albumin, which agrees with previous findings of Tannenbaum and Brunmark (Tannenbaum et al., 1993; Brunmark et al., 1997) suggesting that albumin has one specific binding site for PAHs — either histidine 146 or lysine 137.

The stoichiometry of binding of anthracenecarboxylic acid to albumin (n = 0.03) found by us reveals low capacity of HSA. This indicates that although anthracenecarboxylic acid binds to albumin the most strong of all PAHs studied, a relatively small number of molecules interact with albumin. However, this can be explained by the fact that the carboxylic group of anthracenecarboxylic acid is likely to be dissociated at pH 7.4. Hence, some anthracenecarboxylic acid molecules, a relatively weak acid exist in anionic form. These molecules are able to form strong ionic bonds with albumin. The molecules which are not dissociated are able to interact with albumin through the much weaker hydrogen bonds. Thus only a fraction of the anthracenecarboxylic acid molecules introduced to albumin solution participate in strong albumin binding.

Another aspect of albumin–PAHs interaction is the site of binding. Albumin is a protein which plays a significant role in transporting many substances and has many binding sites.

Up to now only few studies have been performed to determine albumin binding sites for PAHs. Binding of PAHs diolepoxides was studied and histidine-146, lysine-137 (subdomain IB) and lysine-385 (subdomain IIIA) were proposed as the binding sites.

Fabriciova et al. (2004), studied binding of two dihydroxyanthraquinones: 1,8-dihydroxyanthraquinone and 1,4-dihydroxyanthraquinone to human serum albumin. They proposed albumin subdomains IIIA and IIA to be the dihydroxyanthraquinone binding region. However, in the presence of fatty acids, in the case of fraction V albumin, only subdomain IIA is the binding region for these PAHs because subdomain IIIA is a specific primary binding site of fatty acids.

The binding sites for the PAHs studied by us can be proposed also on the basis of the binding sites characteristic for drugs which contain aromatic rings and oxy-substituents in the structure. There are literature data on a number of such drugs, e.g.: Chloropromazine (three aromatic rings), Ibuprofen (one ring, –COOH), Oxazepan (two rings, =O, –OH), Warfarin (three rings, =O, –OH), Tolmetin (one ring, –COOH) as well as Ochratoxin (two rings –OH, =O) and its derivatives, hydroquinone and on O-methylated derivative. All these drugs were shown to bind either in subdomain IIA or IIIA (Epps et al, 1995; Qiu et al., 1998; Perry et al., 2003; Silva et al., 2004).

Thus, while summarizing the above information, we can conclude that albumin subdomains IB, IIA or IIIA are specific for the PAHs studied. However, in the presence of fatty acids in albumin fraction V only IB and IIA should be considered as binding regions for PAHs.

The albumin fluorescence quenching by PAHs studied can indicate that the compounds interact with albumin in the surrounding of the albumin fluorescent residue tryptophan 214 localized in subdomain IIA. The binding of a PAH molecule to albumin can affect its fluorescence by acting as a tryptophan quencher via collisional or energy transfer. It is also possible that the PAH molecule physically interacts with the tryptophan, by which changes the polarity of its environment or prevents solvent accessibility, which complexation also influences the fluorescence. This could suggest also that subdomains IB and IIA are specific binding regions of anthracene derivatives.

However, fluorescence quenching can be also caused by binding of the ligand to a site distant from the tryptophan. Such interaction can change the protein conformation which can influence the tryptophan environment and in this way change the fluorescence intensity (Mayers, 2000).

For all compounds studied a fluorescence drop was observed – the most rapid one for anthracenecarboxylic acid, which can indicate that this compound binds to albumin very close to the tryptophan. We can suppose also that anthraquinone binds to albumin weakly and probably far from tryptophan 214, since despite the observed fluorescence intensity quenching, we were not able to fit a theoretical equation to the experimental data. This indicates that this compound does not quench tryptophan fluorescence directly but, possibly by binding to a remote site, it changes the albumin conformation.

Although the results achieved by us are not decisive, they are in accordance with the literature

data on drug or PAHs binding to albumin. They indicate that subdomains IB or IIA are the albumin specific region for anthracene derivatives binding.

In order to determine the exact binding site of the compounds studied displacement measurements should be conducted. Also simultaneous measurement of tyrosine and tryptophan fluorescence, extended also to fatty-acid-free albumin or even bovine albumin, could determine the binding sites. These investigations are in progress.

Summarizing, since anthracene was shown not to interact with albumin, its metabolic transformations to oxy-derivatives is an essential condition to produce such PAHs forms which are able to interact with biological molecules. Our results indicated that oxy-PAHs present in the environment can immediately create adducts with albumin and the affinity of these oxy-derivatives for albumin, depends considerably on the type and position of substituents in the PAH molecule.

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