INTERACTION OF METHEMOGLOBIN WITH SOME DERIVATIVES OF BENZENE

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Absorbancy changes induced in the Soret band region were used for calculating apparent spectroscopic stability constants (K_s) characterizing the affinity of several mono- and disubstituted derivatives of benzene to methemoglobin molecule. The determined stability constants K_s of the compounds studied were found to correlate with their solubility in water as well as with their partitioning between 1-octanol and water.

Studies investigating quantitative relations between the biological activity of a substance and its physico-chemical properties indicate that the decisive step in the biological action was most often of physical nature[1,2]. It is, however, not very clear, whether it is so on the cellular and organ level only, or whether a rate-limiting step in foreign compound — biomacromolecule interactions is of physical nature, too. It was found that the way in which sets of compounds bound with bovine serum albumin, bovine hemoglobin, ribonuclease, and mitochondrial protein as well[3], rather closely followed their mode of partitioning between 1-octanol and water. To complete these data, interaction of methemoglobin with a set of benzene derivatives has been followed.

The ligand interaction with methemoglobin molecule was accompanied by changes in spectral and magnetochemical properties[4]. The spectral changes were used to study the interaction by differential spectroscopic method in the similar way as in the case of P-450 cytochrome interaction with its substrates as suggested by Estabrook and coworkers[5,6].

In this report, spectroscopic stability constants characterizing an affinity of small neutral aromatic compounds to methemoglobin were calculated using an equation similar to that of Hildebrand–Benesi[7]. Correlations of the apparent spectroscopic stability constants found with some physico-chemical properties of the organic compounds studied were used to find such a property as would satisfactorily describe the affinity of the compounds to methemoglobin.

EXPERIMENTAL

Materials. Crystalline bovine methemoglobin (MHb) was solved in 0·15M phosphate buffer (pH 7·4) in refrigerator using slow dissolution without stirring. In all cases, the iron concentration in the solutions used was $3{\cdot}2-4{\cdot}9 \cdot 10^{-5}$M. The content of iron was determined by the atomic absorption spectrophotometer of Varian-Techtron, model AA-4, equipped with a Fe hollow cathode lamp and ultraviolet-sensitive HTV-106 photomultiplier. The analytical line at 2483·3 Å

was used. The purity of mono- and disubstituted derivatives of benzene (Table I) was checked by gas chromatography. The maximum content of impurities was 1%.

Difference spectrum measurements. The difference spectra in the Soret band region of MHb were recorded at room temperature with Unicam SP-700 and Optica-Milano CF 4 NI spectrophotometers in carefully closed 10 mm quartz cells. In the sample cell, 3 ml of the MHb solution containing 2·2—2·3 μg Fe/1 ml was mixed with 50 μl of concentrated ethanolic solution of a given compound. An equal volume of ethanol was added to the MHb solution in the reference cell. The base line was recorded with the MHb solution in both cells being without any admixture.

Partition coefficients. To determine partition coefficients between 1-octanol ($P_{o/w}$) or heptane $P_{h/w}$) and water, the concentration of a compound was determined spectrophotometrically in either organic or aqueous layer. The system was shaken at room temperature for 1 h and than centrifuged for 1·5 h to eliminate microscopic emulsion formed by the organic compound in carefully closed test-tubes with ground-in glass stoppers. Corrections for evaporization of compounds into the air phase above the system during partitioning was found to be insignificant. In the similar way, solubilities of compounds (S_w, mol/l) in water at 30°C (±0·5) were determined.

Apparent spectroscopic stability constants. To estimate apparent spectroscopic stability constants (K_s, 1/mmol) under the conditions of measurements, three assumptions were made:

a) the interaction was considered as a simple process and the total equilibrium stability constant (K) was calculated as follows:

$$K = \frac{[HL_n]}{[H]\,[L]^n} . \qquad (1)$$

In the formula [H] represents molar concentration of free MHb, [L] molar concentration of a free ligand, $[HL_n]$ molar concentration of the complex formed and n is the number of bound molecules of a ligand;

b) in all ranges studied, concentrations of MHb were much lower than those of added compounds;

c) molar extinction coefficients of both MHb and its complex were of the same value.

Using the assumptions mentioned above, the Eq. (2) was derived:

$$\frac{2A_0}{\Delta A} = \frac{n^n}{K_s}\left(\frac{1}{c}\right)^n + 1 , \qquad (2)$$

where A_0 means optical density of the original MHb solution, ΔA optical density changes measured from difference spectra, c the total concentration of an organic compound added (mmol/l), and K_s apparent spectroscopic stability constant (1/mmol).

A suitable form for the estimation of n and K_s is the log transform:

$$\log \frac{2A_0 - \Delta A}{\Delta A} = n \log \frac{1}{c} + \log \frac{n^n}{K_s} . \qquad (3)$$

RESULTS

After adding aromatic compounds to MHb solutions, a difference spectrum characterized by a peak at 419 to 421 nm and by a trough at 402 to 403 nm (Fig. 1) appeared in all studies cases. Moreover, a broad band with low intensity was observed in the

TABLE I

Spectroscopic Apparent Stability Constants with Methemoglobin (K_s), Solubilities in Water at 30°C (S_w), Partition Coefficients in the 1-Octanol–Water ($P_{o/w}$) and Heptane–Water ($P_{h/w}$) Systems of Several Mono- and Disubstituted Derivatives of Benzene[a]

No	Compound	n	log K_s	log S_w	log $P_{o/w}$	log $P_{h/w}$
1	benzene	1·00	−2·50	−1·62[b]	2·15	2·22
2	toluene	0·92	−2·11	−2·20[c]	2·80	2·75
3	chlorobenzene	1·05	−1·99	−2·42	2·46	2·91
4	nitrobenzene	1·02	−2·30	−1·80	1·88	1·46
5	aniline	1·00	−2·99	−0·37[c]	0·98	−0·15
6	*p*-xylene	0·92	−1·95	−2·63[c]	3·15	2·72
7	*m*-xylene	0·81	−1·93	−2·75[b]	3·20	3·04
8	*o*-xylene[d]	0·89	−1·84	−2·46	2·77	3·12
9	*p*-dichlorobenzene	1·02	−1·59	−3·26[c]	3·35	3·43
10	*m*-dichlorobenzene	0·98	−1·46	−3·18	3·38	3·24
11	*p*-chlorotoluene	0·85	−1·65	−2·74	3·33	3·30
12	*p*-toluidine	0·82	−2·52	−1·00	1·41	0·42
13	*p*-chloroaniline	1·08	−2·48	−1·61	1·81	0·62
14	hexobarbital	1·23	−2·54	—	—	—

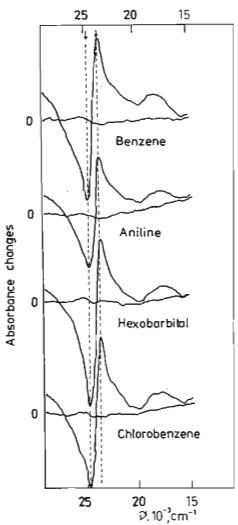
[a] The standard errors of the data are available at the author. [b] Literature data. [c] The mean values from literature data and values determined in our laboratory. [d] Calculation of n and K_s was made on hand of one concentration dependence only.

TABLE II

Comparison of Spectroscopic Apparent Stability Constants of Complexes of Benzene, Chlorobenzene, Aniline, and *p*-Chloroaniline with Methemoglobin (K_s) in Aqueous Solutions and in the Presence of 3% of Ethanol

Compound	log K_s	
	water	3% ethanol[a]
Aniline	−2·95 ± 0·12	−2·99 ± 0·14
p-Chloroaniline	−2·45 ± 0·24	−2·48 ± 0·10
Benzene	−2·34 ± 0·24	−2·50 ± 0·10
Chlorobenzene	−1·92 ± 0·18	−1·99 ± 0·22

[a] Data from Table I.



range of α-bands from 525 to 625 nm. Ratios between optical densities of troughs and peaks of difference spectra were found to be 0·8 to 1·4. In the majority of cases, they were about 1·0. After 10 to 15 minutes mixing the sample, the intensity of the difference spectra remained constant. Volatility effect of the substances was found to be negligible for 1 h under conditions of the experiments. After 25 minutes of slow continuous bubbling with pure oxygen, the difference spectra disappeared.

FIG. 1

Typical Example of Difference Spectra at Room Temperature Showing Spectral Changes Caused by Adding Benzene, Aniline, Hexobarbital, and Chlorobenzene to MHb Solution

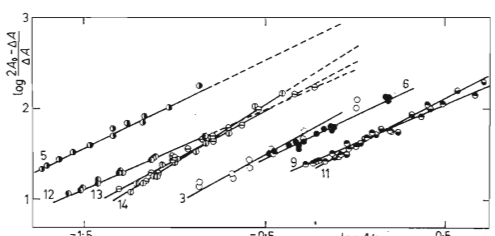


FIG. 2

Several Cases of Double-log Plots According to Eq. (*3*)

The numbers indicate corresponding compounds added (*cf.* Table I): *5* aniline (◑), *3* chlorobenzene (○), *6* *p*-xylene (●), *9* *p*-dichlorobenzene (◒), *11* *p*-chlorotoluene (◓), *12* *p*-toluidine (◐), *13* *p*-chloroaniline (⊖), *14* hexobarbital (⊙). The best location of the lines was found by the least square method.

The plot according to Eq. (*3*) resulted in straight lines in the used range of concentrations (Fig. 2). The determined K_s of the compounds studied were of the magnitude of about $3 \cdot 10^{-2}$ to about 10^{-3} l/mmol (Table I). The values of n constants ranged from 0·8 to 1·2, in the majority of cases they were around 1·0 (Table I). Under the

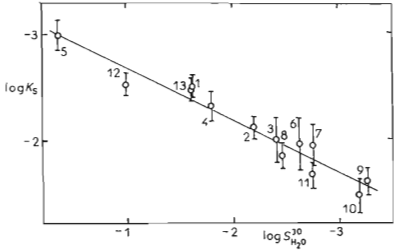


FIG. 3

The Double-log Plot of Apparent Spectroscopic Stability Constants (K_s) against Solubilities in Water at 30°C of Benzene Derivatives Used ($S^{30}_{H_2O}$)

The solid line is drawn to vizualize the equation (*4*); it has no other physical interpretation. The vertical lines represent the 95% confidence limits of the values of K_s. The compounds are numbered as in Table I.

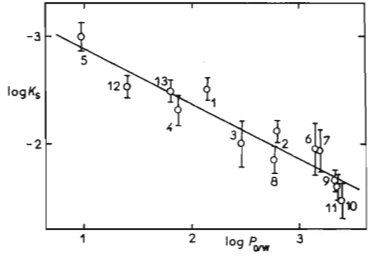


FIG. 4

The Double-log Plot of Apparent Spectroscopic Stability Constants (K_s) against 1-Octanol/Water Partition Coefficients of Benzene Derivatives ($P_{o/w}$)

For the legend see Fig. 3. The solid line is drawn to vizualize the equation (*5*).

conditions used, values of the stability constants of four chosen compounds were not significantly influenced up to 3% ethanol concentration (Table II), which was the highest concentration used in the experiments.

Statistically significant correlations (on 99% level of significance) of $\log K_s$ with $\log S_w$, $\log P_{o/w}$, and $\log P_{h/w}$ are shown on Figs 3 and 4, and in Eqs (*4*), (*5*), and (*6*):

$$\log K_s = -0{\cdot}504 \log S_w - 3{\cdot}188 ; \quad N = 13 ; \quad r = -0{\cdot}971 ; \quad s = 0{\cdot}110 , \qquad (4)$$

$$\log K_s = 0{\cdot}515 \log P_{o/w} - 3{\cdot}395 ; \quad N = 13 ; \quad r = 0{\cdot}941 ; \quad s = 0{\cdot}155 , \qquad (5)$$

$$\log K_s = 0{\cdot}325 \log P_{h/w} - 2{\cdot}828 ; \quad N = 13 ; \quad r = 0{\cdot}91 ; \quad s = 0{\cdot}20 , \qquad (6)$$

where s is the residual standard deviation along the fitted lines, N is the number of experimental data and r the correlation coefficient.

DISCUSSION

The correlations found between $\log K_s$ and both $\log S_w$ (Eq. (*4*) and Fig. 3) and $\log P_{o/w}$ (Eq. (*5*) and Fig. 4) show the existence of relationships between affinities of the compounds to MHb molecule and their solubility and partition between 1-octanol and water in the whole set of the compounds. The relations indicate that the more hydrophobic is the nature of a compound, the higher is its affinity to MHb. It means that the limiting process is more of physical nature than of chemical one.

The comparison of the Eq. (*5*) of this report with the Eq. (*49*) in the Hansch paper[3]

$$\log 1/C = 0{\cdot}71 \log P_{o/w} + 1{\cdot}51 ; \quad N = 17 ; \quad r = 0{\cdot}950 ; \quad s = 0{\cdot}160 ,$$

where C is a molar concentration of various aromatic compounds necessary to produce a 1 : 1 complex of a compound with oxyhemoglobin, shows that the two processes are similar in their character. The slope found in the case of ferrohemoglobin[3] does not differ much from that of ferrihemoglobin, the respective values being 0·71 and 0·51. Thus, a change of the heme iron from that of ferroheme to that of ferriheme does not influenced significantly affinities of the aromatic compounds studied to the macromolecules. The same results were obtained in studies of binding properties with organic and inorganic phosphates[8].

The values of the n constants (Table I) for the set of the compounds are near to 1·0. Although it is necessary to have in mind that the situation is probably more complex this could mean formation of a 1 : 1 complex. Disappearance of the spectral changes after bubbling the solution with oxygen suggests that the complex formation is of reversible nature. The increasing intensity of band in the range of 525 to 625 nm indicate a possibility of the formation of a charge-transfer complex[7].

It is necessary to underline that the spectroscopic method used has limitations and only the changes occurring in the vicinity of the heme could be observed. The inter-

action of molecules of the compounds with other parts of the MHb molecule does not interfere with the observations unless it has influenced the surroundings of the heme. Therefore out of the results obtained it is difficult to say whether the spectral changes are caused by adsorption of aromatic molecules in the vicinity of ferriheme or whether adsorption on protein moiety results in changes in the ferriheme environment. Nevertheless, it is of interest to present some of possible explanations.

Different anion ligands were found to change the ratio between the high and low spin states[4] of Fe(III). Thus aquo complexes of MHb with predominantly high spin configuration of Fe(III) could be changed after addition of the aromatic compounds studied in complexes with predominantly low spin configuration. Correlation between position of the Soret band and magnetic susceptibility of the central Fe(III) of various MHb and metmyoglobin derivatives with different ligands was pointed out[4,9–12].

Using ferriheme solutions as a model system, it was shown that the spectral changes of ferricytochrome P-450 might be induced by alternation of the electronegativity or polarity of any ligand of the heme[13]. This spectral changes were interpreted as changing the ferriheme ligand from water to OH^- ions. According to this model spectral changes observed with MHb could be induced by polarity decrease in the vicinity of ferriheme.

A disruption of hydrophobic bonding in heme-heme polymers of protoferroheme-ethyl isocyanide complexes caused by lipophilic compounds[14] could offer another explanation. Disruption of heme-heme interaction or of the interaction between heme and a residue of the hemoprotein was suggested as the reason of the unusual spectral properties of reduced cytochrom P-450.

In hemoglobin, a part of the heme group is surrounded by non-polar side chains of the globin, with a large concentration of aromatic residues available for π-bonding with the porphyrin ring or adjoint vinyl groups similarly as in the myoglobin molecule[15]. The linkage between the aromatic parts of the heme and aromatic residues of the globin might be disrupted as was suggested in the case of ferrimyoglobin[16] (*e.g.* phenylalanine residues in the positions α 43, β 42, γ 42, α 46, β 45, γ 55, α 98, β 203, γ 103 of the α-, β-, and γ-chains of hemoglobin[15]). The formation of new charge-transfer complexes would correspond to the changes observed in the range of α-bands of MHb. This suggestion is also consistent with the polarity changes around the heme moiety mentioned above. At the same time is is necessary to bear in mind that some of the compounds might disrupt the linkage between the iron of the heme and the imidazole group of the histidyl residue in proximal position 92, as Cann[17] suggested for the interaction of Zn^{2+} with ferrimyoglobin.

For the time being, an interpretation of the observed shift of the Soret band of MHb, caused by addition of substances of different metabolic fates is difficult because of possible different ways of interaction between MHb molecule and the compounds.

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