# INTERACTION OF HUMAN HEMOGLOBIN WITH OXYGEN IN THE PRESENCE OF BENZENE AND USE OF COMPUTER FOR CONSTRUCTION OF SATURATION CURVES

Hana HÁJKOVÁ, Zdeněk PAVLÍČEK and Vítěz KALOUS

*Department of Physical Chemistry, Faculty of Natural Sciences, Charles University, 128 42 Prague 2*

Received July 17th, 1985

The time profile of changes in the oxygen saturation curves of human hemoglobin in the presence of benzene was investigated. The partial oxygen pressure $p_{50}$, necessary for a half saturation, decreased during the first 3·5 h of interaction of hemoglobin with benzene and did not change afterwards. The character of changes in Hill's coefficient was similar. The oxygen saturation curve was modeled both for hemoglobin alone and, using MWC as a model of allosteric interaction, also for hemoglobin and benzene in a computer.

Studies on hemoglobin (Hb)* have attracted the interest of numerous authors for a long time[1]. Attention has been directed mostly to the molecular mechanism of the cooperative effect and to changes in both the heme moiety and in the globin moiety of the molecule[2,3] accompanying this process. In this connection the interaction of Hb with allosteric effectors, such as 2,3-bisphosphoglycerate[2], inositol hexasulfate[4], inositol hexaphosphate, and adenosine triphosphate[5] has intensively been investigated. These compounds significantly decrease the affinity of Hb for oxygen while the heme–heme interaction remains unaltered. Low molecular weight hydrocarbons[6], mainly benzene, have been regarded as Hb ligands and recently studied. Among other reasons for such studies a role plays here probably the toxicity of benzene for the human organism and hence also occupational environmental problems. In the early studies the effect of aromatic hydrocarbons on Hb was examined predominantly *in vivo*[7–10]. The authors of these studies arrived at the conclusion that benzene, toluene, phenol, and the derivatives of these compounds significantly enhance the formation of metHb in blood. Benzene moreover acts on Hb at higher temperature and concentrations as an agent[6] increasing thermal denaturation[11] and denaturation by urea. Sedláček and coworkers[6] have observed that benzene acts as a marked allosteric effector which shifts the $R \leftrightarrows T$ equilibrium in Hb toward the *T*-state. This change takes place at concentrations of Hb $1{\cdot}4 \cdot 10^{-5}$ mol dm$^{-3}$ and of benzene $1 \cdot 10^{-2}$ mol dm$^{-3}$ during the first five hours of interaction; the structure of Hb returns to the *R*-state afterwards. Sedláček explains this finding by postulating that the benzene molecules readily bind first to the globin moiety and subsequently cause the transition of the Hb molecule to the *T*-form *via* an intramolecular mechanism. The binding of benzene to the heme moiety *via* $\pi$–$\pi$ interactions with the porphyrine ring or *via* hydrophobic interactions with the apolar amino acid termini of globin, directed toward the interior of the Hb molecule, takes place only after completion of the transition. The

* Abbreviations used: Hb — hemoglobin, $HbO_2$ — oxyhemoglobin, deoxyHb — deoxyhemoglobin, metHb — methemoglobin, IHP — inositolhexaphosphate.

Hb conformation is then transformed to the original *R*-state due to the effect of benzene additionally bound to the heme moiety.

Benzene has a marked effect on the binding of oxygen to Hb. Lampe[10] found that Hb saturated with benzene has a lower affinity for oxygen, the heme–heme interaction is decreased and the bond between the heme and the protein moiety is weakened. The binding of oxygen to Hb with dextran[13] or CM Sephadex[14] C-50 attached has also been investigated. A higher affinity for oxygen and a decreased heme–heme interaction were observed in both cases. The results obtained explained the authors[13] by postulating that dextran blocks the transition between various Hb conformations during its interaction with oxygen.

The aim of this study has been to study the interaction of hemoglobin with oxygen in the presence of benzene and to try to model this interaction in a computer.

## EXPERIMENTAL

*Isolation of Hb.* Human Hb was isolated[15] in the Institute of Hematology and Blood Transfusion in Prague 2. The exact concentration of Hb was determined spectrophotometrically. The stock Hb solution was kept at +4°C in a refrigerator for the whole period of the measurement.

*Measurement of saturation curves.* The interaction of Hb with benzene was assayed by the method of measurement of oxygen saturation curves described in detail in the preceding paper[16]. The measurement of one saturation curve takes approximately 2·5 h. The Hb solution in a closed-off vessel — the tonometer — connected to a spectrophotometric cell was deoxygenated 4 min at reduced pressure (oil pump) and then shaken 5 min in a mechanical shaker. The procedure was repeated once more. Benzene was then added to deoxyHb in the evacuated tonometer through a special stopper equipped with a silicone lining and the solution was again shaken for 5 min. The tonometer volume was 274 ml, the measured volume of Hb solution 5 ml and the volume of benzene added 20 μl. It was impossible to add benzene directly to $HbO_2$ since it would evaporate from the solution during the evacuation of the tonometer. Measured portions of air were added stepwise to the tonometer through a buret with a mercury drop as a marker; the solution was shaken after each addition and the absorption spectrum of Hb was recorded over the range 500–625 nm. The partial oxygen pressure $p$ was determined from the equation described by Vodrážka and coworkers[17]. The whole procedure was carried out at 20°C. The absorption spectra were measured in an UV VIS spectrophotometer (Carl Zeiss, Jena) with adapted cell compartment.

The saturation degree was calculated from the equation[17]

$$Y = \frac{(A_{540} - A^{d}_{540}) + (A^{d}_{555} - A_{555})}{(A^{0}_{540} - A^{d}_{540}) + (A^{d}_{555} - A^{0}_{555})}, \qquad (1)$$

where $A_{540}$ and $A_{555}$ stand for the absorption of incompletely saturated Hb at wavelengths of 540 and 555 nm, respectively, and $A^{d}_{540}$, $A^{d}_{555}$, $A^{0}_{540}$, and $A^{0}_{555}$ denote the absorbance of deoxy-Hb (at the beginning of the measurement) and of $HbO_2$ (at the end of the measurement) at the given wavelengths, respectively.

*Calculation of microscopic dissociation constants* of oxygen and benzene during binding to Hb and construction of saturation curves in computer. The saturation curves were constructed by using the equations which follow from the MWC model[18] of the allosteric phenomenon:

$$Y = \frac{\alpha(1 + \alpha)^3}{L + (1 + \alpha)^4} \tag{2}$$

$$Y = \frac{\alpha(1 + \alpha)^3}{L(1 + \beta)^4/(1 + \gamma)^4 + (1 + \alpha)^4}, \tag{3}$$

where $\alpha$ is the ratio of partial oxygen pressure $p$ to the microscopic dissociation constant[18] $k$, $\alpha = p/k$; $\beta$ is the ratio of concentration of allosteric inhibitor (such as, *e.g.* IHP), decreasing the affinity for oxygen, to the microscopic dissociation constant of the inhibitor, $\beta = C_I/k_I$, and $\gamma$ is the ratio of activator concentration, in our case of benzene, to its microscopic dissociation constant[18], $\gamma = C_b/k_b$. $L = [T]/[R]$ denotes the equilibrium constant for the $R \rightleftarrows T$ transition of Hb alone. The expression $L(1 + \beta)^4/(1 + \gamma)^4$ is referred to as $L'$ and expresses the equilibrium constant of the $R \rightleftarrows T$ transition of Hb interacting with allosteric effectors.

The construction of the saturation curves was performed in a HP 98-45 computer. To calculate the curve of Hb alone the values of partial pressure $p$ and of the saturation degree $Y$ experimentally determined with Hb not containing benzene were fed into the computer. The microscopic dissociation constant $k$ was determined by nonlinear regression analysis using the iterative procedure and the curve was constructed satisfying best function $Y(p)$ following from Eq. (*2*).

The dissociation constant of benzene and the saturation curve for Hb with benzene were calculated by means of Eq. (*3*). Similarly to the latter case the corresponding values of $p$ and $Y$ and also the value of $k$, determined in the preceding calculations, and the concentration of benzene, $c_b$, were fed into the computer. In experiments carried out in this study the system did not contain an allosteric effector and the value of $\beta$ was therefore zero.

Using the program described above the microscopic dissociation constant of benzene $k$ was calculated by the iterative procedure and the saturation curve described by Eq. (*3*) was constructed. The dissociation constant $k_b$ calculated permitted the saturation curves of Hb to be generated also in the presence of other, experimentally not measured concentrations of benzene.

## RESULTS

The time profile of the changes in the saturation curves of Hb was investigated in the presence of benzene. The saturation curve was measured at time $t = 0$, 1, 2·5, 5, and 15 h after the components had been mixed together. The pressure $p_{50}$ was achieved approximately one hour after the beginning of the individual measurement in all experiments. Fig.1 shows the time profile of $p_{50}$ values for the Hb solution with benzene including the $p_{50}$ value for benzene alone. The $p_{50}$ value decreases first, does not practically change, however, after 3·5 h. A similar profile show also the changes of Hill's coefficient $n$, decreasing from 2·5 to 1·4.

The effect of benzene on the saturation curve of Hb containing IHP, an allosteric effector, was also examined. A solution of $HbO_2$ and IHP ($c = 2 \cdot 10^{-3}$ mol dm$^{-3}$) was prepared, deoxygenated and benzene was added as described above. The saturation curve measured 2·5 h after the addition of benzene is shown in Fig. 2. The value of $p_{50}$ was 2·9 kPa.

Fig. 3 shows the saturation curve of Hb alone constructed in the computer. The points marked are experimental points obtained by measurement in the tonometer.

The value obtained for the microscopic dissociation constant was $k = 73{\cdot}4$ Pa.

The theoretical saturation curves of Hb–benzene systems for various benzene concentrations are shown in Fig. 4. The points obtained experimentally for the system in which the benzene volume was 20 µl are also marked on the curve. The value of the microscopic dissociation constant of benzene for this benzene quantity is $k_b = 23{\cdot}5$ µl. This value was used also for construction of curves in experiments with other quantities of benzene.

## DISCUSSION

Studies on the interaction of benzene with hemoglobin are important not only for the elucidation of the character of the bond between hydrocarbons and proteins but also from the viewpoint of the effect of ecologically important toxic products on the living organism. The effect of benzene on hemoglobin, an oxygen carrier in the organism, can be determined by the measurement of oxygen saturation curves. In this study the saturation curve of Hb alone was measured first and the saturation curves of Hb containing benzene were determined afterwards. The latter were measured at different time intervals after the addition of benzene to hemoglobin. The value of $p_{50}$ was then read off from these saturation curves (Fig. 1). The time

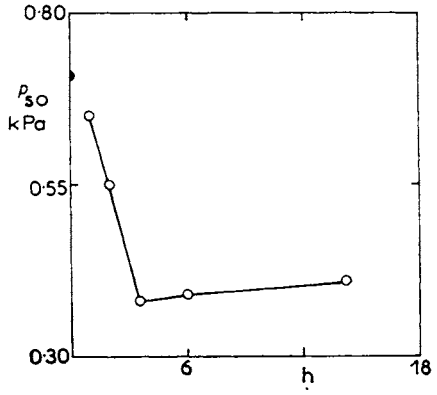


FIG. 1

Dependence of $p_{50}$ (kPa) read off from saturation curves of Hb in presence of benzene on time (time of mixing + 1 h of measurement). Phosphate buffer, pH 7·2; Hb concentration $1{\cdot}25 \cdot 10^{-5}$ mol dm$^{-3}$, experimental point (●) corresponds to Hb alone (without benzene)

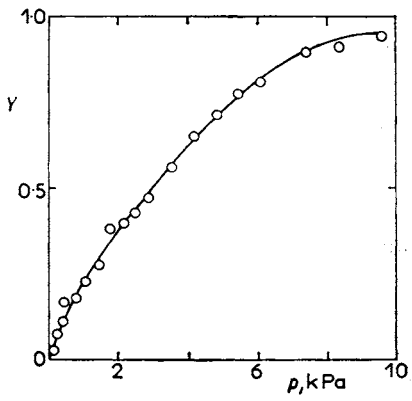


FIG. 2

Saturation curve of Hb in presence of IHP and benzene. Phosphate buffer pH 7·2; Hb concentration $1{\cdot}25 \cdot 10^{-5}$ mol dm$^{-3}$, IHP $2 \cdot 10^{-3}$ mol dm$^{-3}$; benzene volume 20 µl

profile of $p_{50}$ shows that benzene increased the affinity of Hb for oxygen with time yet decreased the heme–heme interaction as evidenced by a drop in Hill's constant $n$ with time. Both the increasing affinity for oxygen and the decreasing heme–heme interaction reached the extreme value after 3·5 h of action of benzene on Hb and did not practically change afterwards. These findings indicate that either benzene binds relatively slowly to Hb or that the conformational change brought about by benzene binding is relatively slow. Our finding that the quantity of benzene used increases the affinity for oxygen is in disagreement with the observation made by Lampe[10] who found a higher $p_{50}$ for Hb solutions saturated with benzene than for solutions of Hb alone. In our case benzene became distributed between the gas and liquid phase in the tonometer. The determination of benzene concentration in the liquid phase was carried out spectrophotometrically over the range of 250–300 nm. It was observed that there was only one liquid phase with a benzene concentration 0·03 mol . . $dm^{-3}$ after the equilibrium had established in the tonometer. We assume that probably a higher concentration was used in the experiments of Lampe[10] and that probably a partial denaturation of Hb and thus a decrease of its affinity tor oxygen might have occurred. The decrease of Hill's constant $n$ observed by Lampe[10] is in agreement with our results.

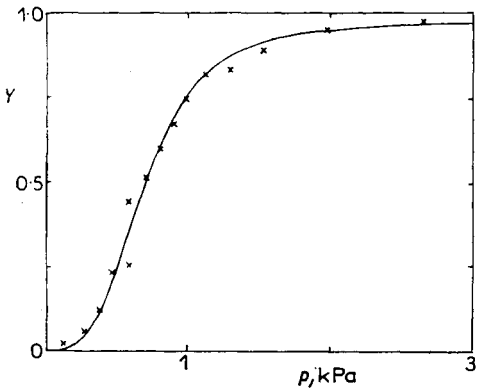


FIG. 3

Saturation curve of Hb calculated by means of Eq. (*2*), $k = 74{\cdot}28$ Pa, and constructed in computer. $x$ — experimentally obtained points of saturation curve of Hb, phosphate buffer pH 7·2; Hb concentration $1{\cdot}25 \cdot 10^{-5}$ mol . $dm^{-3}$

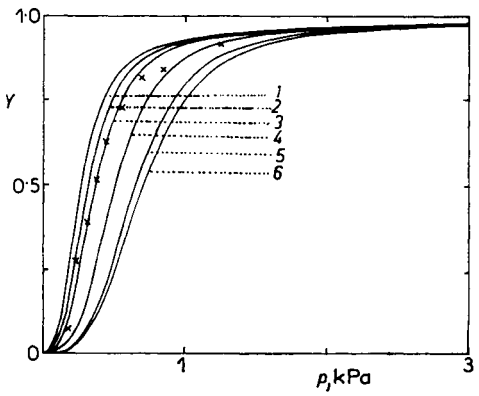


FIG. 4

Saturation curves of system Hb–benzene calculated by means of Eq. (*3*), $k = 74{\cdot}28$ Pa, $k_b = 23{\cdot}5$ µl, $\beta = 0$ and constructed in computer. Benzene volume in µl: *1* 35·5, *2* 26·6, *3* 20·0, *4* 8·9, *5* 2·2, *6* 0·0 (the experimentally obtained points of the saturation curve measured 2·5 h after the addition of benzene are marked on curve *3*), Hb concentration $1{\cdot}25 \cdot 10^{-5}$ mol $dm^{-3}$; phosphate buffer pH 7·2

The time profile of $p_{50}$ and $n$ reached in our experiments a minimum after several hours of interaction of Hb with benzene. It may be concluded from an analogous behaviour of the system Hb–haptoglobin[16] that the decreased allosteric interaction in the Hb molecule in the presence of benzene is caused by the binding of benzene to the globin moiety of the Hb molecule. Benzene obviously affects here the interchain contacts and thus modifies the transfer of the allosteric signal between the Hb subunits.

The saturation curve of Hb in the presence of IHP and benzene (Fig. 2) shows that both IHP and benzene can bind simultaneously to the Hb molecule. The binding of IHP considerably decreases the affinity of Hb for oxygen and benzene causes a loss of the heme–heme interaction.

Using the equations of the MWC model[18] the saturation curves for hemoglobin alone were constructed in a computer. The MWC model is very suitable for a phenomenological description of allosteric interactions in Hb yet does not explain their molecular mechanism. The character of the changes in tertiary and quaternary structure of the Hb molecule during the allosteric phenomenon and its causes are explained by the model of Perutz[3].

As can be seen in Fig. 3 the experimentally determined data of the saturation curve of hemoglobin alone are satisfactorily represented by the curve calculated and constructed by means of Eq. (*2*). It should be stressed though that parameter $k$ after whose insertion into Eq. (*2*) the curve can be calculated is relevant only to the MWC model. According to this model the saturation curves of Hb interacting with benzene can be mathematically described (Fig. 4). The value of $L'$ is lower than $10^4$ in this case and indicates that deoxyHb contains in the presence of benzene a higher amount of the *R*-state than Hb itself. It should be born in mind though that the meaning of symbols $T$ and $R$ is different in the MWC model and different in Perutz's model. These symbols describe two Hb states in the MWC model[18] of which one, the *R*-state, can bind oxygen. In Perutz's model[3] symbols $T$ and $R$ denote two states of the Hb molecule of different tertiary and quaternary structure and in this case it is not correct to conclude that the molecule in the *R*-state only can bind oxygen.

An effort has been made to apply the method described also to the calculation of the curve shown in Fig. 2 and characterizing the system of Hb + IHP + benzene. Since the saturation curve shown has a hyperbolic character whereas the curve calculated by means of Eq. (*3*) must be sigmoidal, the result is not satisfactory. The conformational changes of Hb which occur during its interaction with benzene in the presence of IHP are obviously deeper than in the case of benzene alone. The MWC model[18] which was satisfactory for the description of the interaction of Hb with benzene alone obviously failed in the presence of the allosteric effector.

*We thank Dr J. Kedršt for the calculation in the HP 98-45 computer.*

REFERENCES

1. Antonini E., Brunori M.: *Haemoglobin and Myoglobin in Their Reactions with Ligands.* Vol. 21. North-Holland Research Monographs 1971.
2. Benesch R., Benesch R. E.: Nature *221*, 618 (1969).
3. Perutz M. F.: Nature *228*, 726 (1970).
4. Benesch R., Edalji R., Benesch R. E.: Biochemistry *15*, 3396 (1976).
5. Adams M. L., Schuster T. M.: Biochem. Biophys. Res. Commun. *58*, 525 (1974).
6. Sedláček P., Pavlíček Z., Kalous V.: This Journal *49*, 1827 (1984).
7. Ishihara N., Kanaya A., Ikeda M.: Int. Arch. Occup. Environ. Health *36*, 161 (1976).
8. Watanabe T., Ishihara N., Ikeda M.: Int. Arch. Occup. Environ. Health *37*, 157 (1976).
9. Jenkins L. J., Jones R. A., Siegel J.: Toxicol. Appl. Pharmacol. *16*, 818 (1970).
10. Lampe J., Behlhe J., Graf W., Müller K., Scheler W.: Acta Biol. Med. Ger. *26*, 911 (1971).
11. Sojka J., Hrkal Z., Vodrážka Z.: This Journal *39*, 509 (1974).
12. Cann J. R.: Biochemistry *6*, 3427 (1967).
13. Dellacherie E., Bonneaux F., Labrude P., Vigneron C.: Biochim. Biophys. Acta *749*, 106 (1983).
14. Lampe J., Pommerening K.: Abh. Akad. Wiss. DDR 1973 (Publ. 1975); Int. Symp. Strukt. Funkt. Erythrozyten, 7th (1973), 187—9.
15. Kramlová M., Přistoupil T. J., Ulrych S., Hrkal Z.: Hematologia *10*, 365 (1976).
16. Hájková H., Pavlíček Z., Kalous V.: This Journal *51*, 1789 (1986).
17. Vodrážka Z., Jandová D., Pristach J., Balíková-Byčková V.: Cesk. Fysiol. *24*, 159 (1975).
18. Monod J., Wyman J., Changeux J. P.: J. Mol. Biol. *12*, 88 (1965).

Translated by V. Kostka.