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Effect of 2,3,7.8- Tetra Chlorodibenzo-P-Dioxin on the Structure and the Function of Hemoglobin in Rats

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Abstract: Dioxins are a group of 210 chemicals that have similar structures and chemical properties and are not intentionally produced.. Not all dioxins have the same toxicity, the most toxic chemical in this group is 2,3,7,8-Tetrachlorodibenzo-para-dioxin (2,3,7,8-TCDD). This work aimed to study the effect of dioxin as a toxic compound on the structure and function of hemoglobin as a macromolecule (conformational changes) in exposed rats. This study was conducted on 30 female rats of 4-6 weeks old, divided into 2 groups G_1 (10 rats) served as normal control and G_2 (20 rats received, 0.25% of the lethal dose (LD50) of dioxins compounds (17 congers) orally. Results revealed decrease in the oxygen tension at which Hb is half saturated (P_{50}) in rats received dioxin which indicates a drop in its affinity to oxygen. All forms of abnormal Hb derivatives increased concomitant with dramatic decrease in oxy-Hb in exposed rats as a result of the oxidation process of the normal oxy-Hb as well as the increment of heat content ΔH and molecular disorder ΔS confirmed the unstabilzation of Hb molecule through the elevation of hydrogen bonds, maintaining the tertiary structure of Hb molecule. There was a rise in the percentage of dielectric increment for exposed rats than control group as a result of its unfolding and/or increase in the molecular size (quaternary structure of Hb).

Key words: Dioxins · Hb derivatives · Thermodynamic · Dielectric constant

INTRODUCTION

Dioxin is the name given to a group of persistent, very toxic organic chemicals that consists of a pair of benzene ring, two oxygen atoms and four chlorine atoms, the most toxic form of which is 2,3,7,8- Tetra chlorodibenzo-p-dioxin (TCDD)[1,2].

Dioxin builds up in the living tissue (biaccummulate) over time, so even exposures to small doses may accumulate to dangerous levels [3].

Today, concentrations of dioxins are found in all humans, as they are constantly exposed to dioxins through their diet. Dioxins are persistent and once exposure occurs, these compounds remain in human tissues, particularly fatty tissues, for extended time period [4]. Dioxin enters the general population almost exclusively from ingestion of food, specially, through the consumption of fish, meat and dairy products since dioxins are fat soluble readily climb [5].

Atmospheric dispersion, deposition and subsequent accumulation in the food chain seen to be the major pathways of exposure to the general population. Residues of these chemicals have been detected in soil, sediment, fish, meat, cow's milk, human adipose tissue and mother milks. The elimination half life of TCDD in humans is approximately 7-11 years [6].

One of the earliest findings of dioxins toxicity in animals was that, it caused birth defects in mice at very low levels [7]. This finding led to dioxin- being characterized as one of the most potent teratogenic environmental agents [8].

Once dioxin enters the body, the blood stream readily distributes it to all organs [9], As dioxin does not dissolve well in the blood, it stays there for only a short time and tends to accumulate in fatty tissues, where is does dissolve and in liver which becomes very fatty. Its distribution to the various internal organs depends on blood flow to a given organ, relative organ size and the exposure dose [10].

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Dioxins produce a toxicity syndrome, characterized by progressive weight loss and delayed lethality in rats, mice, rabbits, guinea pigs, hamsters and non human primates [11]. Acute toxicity has not been observed among highly exposed humans despite the occurrence of high accidental exposures that exceed the doses known to produce acute toxicity in guinea pig [12].

MATERIALS AND METHODS

Experiment Animals: Female rats of 4-6 weeks old were obtained from the Animal House of the National Research Centre. Animals were maintained under a control condition one week before initiation of the experiment, passes several times of light/dark cycles, under average ambient temperature of 22±1°C, humidity of 40-60% and allowed free access to foods and water.. Experimental design was modulated in 30 rats divided into two groups: G_1 included 10 rats served as the control group and G_2 included 20 rats received 0.25% of the lethal dose (LD_{50}) of dioxins orally [13,14].

Dioxin Standard: A seventeen congeners labeled with C^{13} and ^{17} native congeners at equal proportion was delivered from Chemishes und Verterinaruntersuchungsant reiburg, Germany, The stock standard solution containing pg WHO-TEO (TCDDs/PCDFs) of 17 congeners labeled with C^{13} and ^{17} native congeners at equal proportion, each μL of Dioxin congeners contains 10 pg of 17 congeners labeled with C^{13} and ^{17} native congeners at equal proportion.

Treatment of rats: Fifty μl from dioxin standard was orally administrated to female rats using a stomach tube daily for one week. This dose equals to 0.25 of LD_{50} of dioxin standard for rats [13,14]. After one week of treatment, blood samples were collected and rats were sacrificed.

Blood samples that collected for the biochemical and biophysical parameters study were collected in tubes containing heparin, blood was centrifuged at 800 rpm for 10 min at 4°C, then the plasma was removed and packed cells was washed with 5 volume saline at 20°C, this step was repeated three times. Packed cells were lysed with two volume of deionized water and then mixture was centrifuged at 5000 rpm for 30 min at 4°C to obtain the hemoglobin solution on which experiments were carried out.

Hemoglobin of different ligand derivatives: The millimolar extinction coefficients were put into four linear equations with the four unknown concentrations of hemoglobin pigments (C_{HbO2}, C_{HbCO}, C_{MetHb} and C_{SHb}).

$$A^{500} = 5.05\ C_{HbO_2} + 5.35\ C_{HbCO} + 9.04\ C_{Met.Hb} + 7.2\ C_{SHb} \quad (1)$$

$$A^{569} = 11.27\ C_{HbO_2} + 14.27\ C_{HbCO} + 4.1\ C_{Met.Hb} + 8.1\ C_{SHb} \quad (2)$$

$$A^{577} = 15.37\ C_{HbO_2} + 10.0\ C_{HbCO} + 4.1\ C_{Met.Hb} + 8.1\ C_{SHb} \quad (3)$$

$$A^{620} = 0.24\ C_{HbO_2} + 0.33\ C_{HbCO} + 3.35\ C_{Met.Hb} + 20.8\ C_{SHb} \quad (4)$$

Where the absorption bands at wavelengths 500, 569, 577 and 620 nm represent the absorption maxima of Met-Hb, HbCO, HbO_2 and SHb, respectively.

The above linear system of equations can be represented in the matrix form as:

$$\begin{bmatrix} 5.05 & 5.35 & 9.04 & 7.2 \\ 11.27 & 14.27 & 4.10 & 8.1 \\ 15.37 & 10.0 & 4.10 & 8.1 \\ 0.24 & 0.33 & 3.35 & 20.8 \end{bmatrix} \cdot \begin{bmatrix} C_{HbO_2} \\ C_{HbCO} \\ C_{Met.Hb} \\ C_{SHb} \end{bmatrix} = \begin{bmatrix} A^{500} \\ A^{569} \\ A^{577} \\ A_{620} \end{bmatrix} \quad (5)$$

This linear system of equations was solved by mathematical manipulation, using the Gaussian elimination method. For matrix calculation [15] to yield the following equations:

$$C_{SHb} = \frac{A^{620} - 0.442293A^{500} + 0.1065519A^{569} + 0.0515769A^{577}}{18.895404} \quad (6)$$

$$C_{Met.Hb} = \frac{9.0602343A^{500} - A^{577} - 2.6960235A^{569} - 35.295898\ C_{SHb}}{66.750821} \quad (7)$$

$$C_{HbCO} = \frac{A^{569} - 2.2316831A^{500} + 16.074415\ C_{Met.Hb} + 7.9681188\ C_{SHb}}{2.330495} \quad (8)$$

$$C_{HbO_2} = \frac{A^{500} - 5.35\ C_{HbCO} - 9.04\ C_{Met.Hb} - 7.2\ C_{SHb}}{5.05} \quad (9)$$

Where A^{500}, A^{569}, A^{577} and A^{620} are the absorbances of hemoglobin solution at the wavelengths 500, 569, 577 and 620 nm, respectively.

Blood gas measurements: Determination of oxygen in blood samples has been achieved through the use

of pH blood Gas Analyser Model Corning 166 manufactured in England within 15 minutes from taking samples.

The partial pressure In mmHg of blood oxygen was measured through the use of chrktype staw Severinghious electrodes simultaneously. P_{50} represents the partial pressure of blood oxygen at half concentration.

Thermodynamic parameters: In this experiment, the absorbance of hemoglobin solution of concentration (3.4×10^{-5} M in heme), at the spin or spin heme-heme interaction band (A^{578}) were measured at various temperature in the range 25-40°C, 5°C intervals, using temperature controlled spectrophotometer. The spin state constant (K), at each temperature, was calculated by using the following equation [16,17]:

$$K = \frac{A^{578}}{(1-A^{578})} \tag{10}$$

Dielectric Measurements: The dielectric dispersion for 5% aqueous solution of Hb was measured at 25°C in frequency range 0.1 and 10 MHz for rats received dioxin compared to the normal control group through the use of a Loss Factor meter type 1033,RFT., Funkwerk Erfurt. Germany. The Hb samples were measured by the cell type pw 9510/60, manufactured by Philips. The sample cell has two squared platinum black electrodes each having an area of 0.8x0.8 cm^2 with an intermediate distance of 1 cm. The cell with the sample is kept at 25°C±0.1 in a temperature controlled incubator Kotterman type 2771 Germany. The value of ε' (Relative permittivity of the sample in the cell) was calculated at each frequency from the constant K (the cell constant that depend on the cell dimensions) and C_o (The residual capacitance) and the measured values of C, also the loss tangent (tan δ) was obtained from the measured values of the resistance R and C in farad as

$$\tan\delta = \frac{1}{2\pi f RC} = \frac{\varepsilon''}{\varepsilon'} \tag{11}$$

The dielectric loss ε'' was calculated from the relation

$$\varepsilon'' = \varepsilon' \tan\delta \tag{12}$$

The conductivity (σ) was then calculated from the relation

$$\sigma = 2\pi f \varepsilon'' \varepsilon_o = C/K \tag{13}$$

For spherical macromolecules the dielectric relaxation time depends on the viscosity of the liquid η and its absolute temperature T. Viscosity measurements of each Hb solution was carried out with an ostwald viscometer at concentration of 5% and 25°C bidistilled water was used first at fixed volume to pass through certain height of the Ostwald's capillary, then the efflux of water t_2 is determined three times and an average value was taken also the averaged efflux times t_1 for both the Hb of rats intake dioxin and the control group were determined, then the viscosity coefficient $\eta_1$for each sample was calculated as

$$\frac{\eta_1}{\eta_2} = \frac{(f_1 t_1)}{(f_2 t_2)} \tag{14}$$

Where η_2 is the viscosity coefficient of water, f_1 and f_2 are the densities of water and solute molecules respectively.

Statistical analysis: Data were computed in form of means and standard error (SE) and statistically analyzed.

RESULTS

The obtained data in Table 1 reveal that, the non functional hemoglobin (Met-Hb, S-Hb and Hb-co) increased ($P<0.01$) in G_2 as compared to the control group. Also, the oxy-Hb (functional Hb) decreased ($P<0.01$) in G_2 group.

Oxygen tension at half concentration and at pH 7.4 of Hb of rats received dioxin are shown in Table 2. Mean value of P_{50} *in vivo* as well as P_{50}. 7.4 decreased ($P<0.01$) in G_2 as compared to normal rats, as well as the spin state band of Hb A_{578}/A_{540}

Spin state constant (K) and the free energy ΔF were calculated from the measured absorbance A_{578} at temperature T= 298K through Arrhinus equation:

Table 1: Hb of different ligand derivatives of rats received dioxin (Mean±SE)

Group	Met-Hb	S-Hb	Hb-co	HB-O_2
Control G_1	1.45±0.61	0.623±0.034	3.01±0.089	91.06±0.32
Experimental G_2	2.34±0.53**	0.862±0.031**	4.23±0.096**	84.69±0.38**

*P<0.05 **P<0.01

Table 2: Oxygen tension at half saturation and spin state band of Hb of rats received dioxin (Mean±SE)

Group	$P_{50}O_2$ mmHg	P_{50} at 7.4	A_{578}/A_{540}
Control G_1	36.2±0.4	40.62±1.6	1.056
Experimental G_2	20.03±0.2**	23.26±1.41**	1.032**

*P < 0.05 **P<0.01

Table 3: Spin constant and thermodynamic of Hb of experimental rats received dioxin (Mean±SE)

Group	Spin state constant K	ΔF J/mole	ΔH J/mole	ΔS J/mole K
Control G1	0.812±0.000325	4.853x102±0.432	-7.56x103±0.185	-26.658±0.00765
Experimental G2	0.601±0.00026**	9.16x102±0.221**	-5.67x103±0.2162**	-20.325±0.068**

*P<0.05 **P<0.01

Table 4: Values of the static ε_S and infinite ε_∞ dielectric constant, dielectric increment per g per 1000 ml, cole-cole parameter α, relaxation time τ_β in *M* sec., viscosity coefficient η in poise and molecular radius in nm (Mean±SE)

Group	ε_∞^*	ε_s^*	Δ_β	α^*	τ_β^*	η	r
Control G_1	50.14±0.53	95.55±0.46	0.44±0.005	0.01252±0-000035	0.248±0.00062	0.0252+0.00029	2.974±0.013
Experimental G_2	47.15±0.69	104.73±0.55	0.678±0.003	0.10142±0.00028	0.439±0.00057	0.0142±0.00042	3.7030±.0004

*Fitted parameters from the computer program

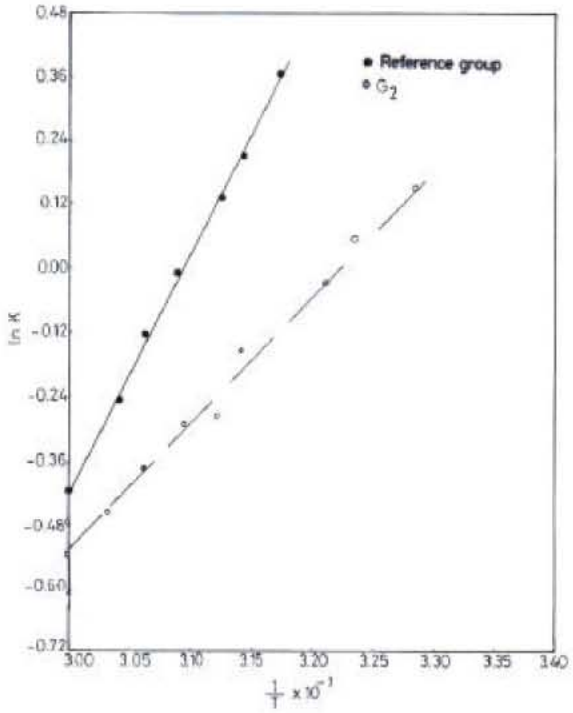

Fig. 1: Relationship between natural logarithm of spin state constant (K) of iron heme and $\frac{1}{T}$

$$K = \frac{A_{578}}{(1\text{-}A_{578})}$$

The values of ln K were plotted as a function of 1/T as shown in figure 1. From the slope of the lines the enthalpy (ΔH°) was calculated through the use of equation:

$$\Delta H = \text{-R}\left[\frac{\partial \ln K}{\partial \frac{1}{T}}\right]$$

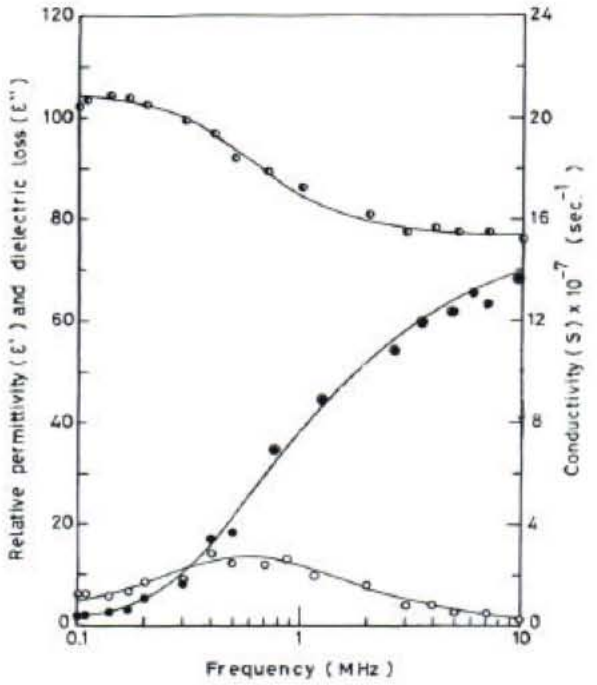

Fig. 2: The variation of relative permittivity (ε'), (°); dielectric loss (ε''), (0); conductivity (S), (•) with frequency for 5% aqueous normal control Hb solution at 25°C

It is clear that the slope of this relation is remarkable shifted away from the control group

From Table 3, spin state constant (K) of heme iron decreased (P<0.01) in G_2, compared to control group. An opposite behavior was observed in free energy (ΔF) and (ΔS) in (G_2) regarding reference group.

Figures 2,3 illustrate the results of the relative permittivity έ, the dielectric loss ε̋ and conductivity S were measured in the frequency range o.1 to 10 MHz for normal control rats and G_2 respectively. These figures indicate

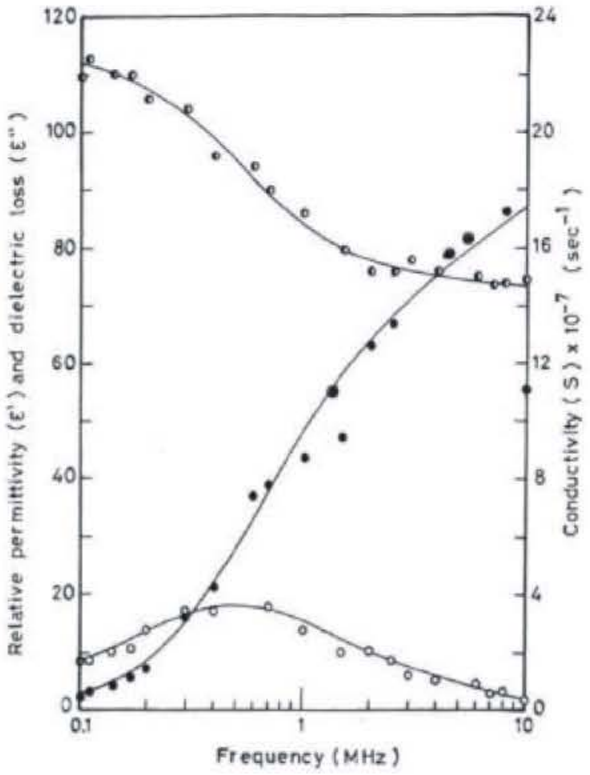

Fig. 3: The variation of relative permittivity (ε'), (°); dielectric loss (ε''), (◦); conductivity (S), (•) with frequency for 5% aqueous (G_2) Hb solution at 25°C

that Hb has a critical frequency f_c ranging from 0.5 to 0.6 MHz at 25°C & 5% aqueous solution of hemoglobin.

Table 4 illustrates the values of the static ε_s and infinite ε_∞ dielectric constant, dielectric increment per g per 1000 ml, cole-cole, the relaxation time τ_β, viscosity coefficient η in poise and molecular radius in nm for G_2 compared to reference group.

The molecular radius of Hb (r) was calculated from the data of relaxation time τ_β through $\tau_\beta = \frac{4\pi r^3 \eta}{k\tau}$

The results indicated that the radius of Hb molecule increased as well as the relaxation time in G_2 compared to normal control

The dielectric increment (Δ_i) per g per liter was calculated from $\tau_\beta = \frac{\varepsilon_s - \varepsilon_\infty}{C}$ where C is the concentration of Hb solution in g/liter

The results of the dielectric increment indicated a higher value in G_2 compared to normal control.

Figures 4,5 show cole- cole plot (ε'' vs ε') for normal control and G_2 respectively. From these figures, the values of the cole-cole parameter (α) for all samples are deduced and given in Table 4, these results revealed that there is a wide distribution of the relaxation times of Hb molecules of G_2 compared to reference group. The curve fitting analysis has shown that, the cole –cole model gave a better fit for the dielectric data.

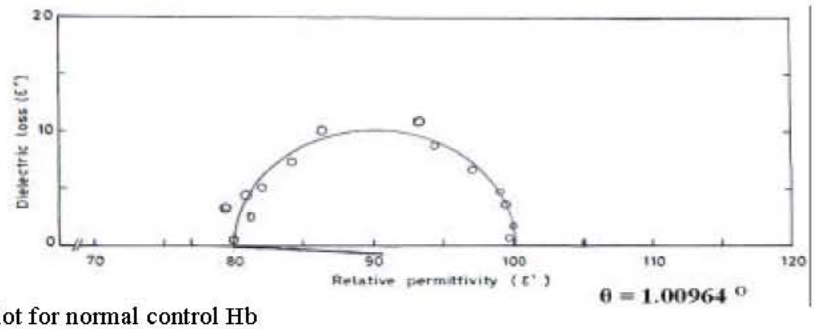

Fig. 4: Cole-Cole plot for normal control Hb

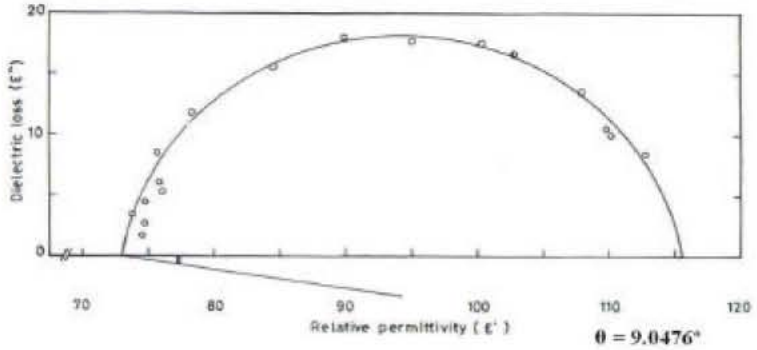

Fig. 5: Cole-Cole plot for a sample of (G_2) Hb

DISCUSSION

The goal of this study was to evaluate the effect of dioxin as the prototype for the toxicity of this chemical class on the structure and the function of Hb. It is well known that proteins are dynamic systems that wriggle and breathes (their motion is essential to their function [18].

Hb $-O_2$ affinity depends mainly on the heme- heme interaction and the spin state of heme iron [19] A_{578}/A_{540}. It is clear that, the markedly decrease in P_{50} in exposed rats goes hand by hand with the increase in met-Hb. S-Hb and Hb-C0 (non functional groups or the number of autooxidized ferric Hb subunits. The dependence of HbO_2 affinity on the met-Hb level and the amount of partially autooxidized (Ferric) Hb subunits was reported [20,21]. This finding could be explained according to the shift of Hb towards T- quaternary or tertiary structure, with high oxygen affinity i.e low P_{50} concomitant with significant decrease in spin state band, draw a line of evidence about the unstabilization of Hb macromolecule.

The obviously increase in the concentration of (Met-Hb,S-Hb and Hb-co in exposed rats represents an evidence of the inactivation of the couple enzymes related to elevated of oxidizes Hb. Moreover. It is safely to suggest that in rats received dioxin, the tendency of reduced enzyme activity, could extend to the enzymes involved the Met-Hb reductase system within the red cell, this suggestion comes from the fact that, when there is oxidation- reduction disorder namely Met-Hb reductase system, a higher Met-Hb should be found, It could be due to such toxins from dioxin [22,23].

Formation of free radical in toxicity situation is confirmed by many reports [24]. Oxidative stress is considered a possible molecular involved toxicity. The rate of normal dissociation of oxyhemoglobin to methemoglobin is highly dependent on the tertiary and quaternary structure of hemoglobin molecule [25].

S-Hb has not been completely characterized, it is unable to carry oxygen and unlike Met-Hb it can't be converted back to Hb. The highly clearly increase in S-Hb is attributed to disorder in oxidation – reduction potential [26].

Free energy of any reaction tells in which direction and how far a reaction will go, in order to reach equilibrium. When it occurs under standard condition, ΔF° of Hb proceeds toward equilibrium its value is negative. Thus, in the present work, as ΔF° increases in G_2, compared to normal control which signify the high affinity of Hb to oxygen.

The enthalpy ΔH° (heat content) of the molecule indicates that the reacting system releases to or absorbs heat from its surrounding at constant temperature and pressure. When the reacting system loses heat the sign of ΔH° is negative. The marked increment of heat content in G_2 means that the system loses heat to the surrounding, which signify unfolding of Hb as a globular protein [27,28]

Entropy or molecular disorders increases during chemical such as oxidation (conversion to Met Hb or physical process such as oxygenation). The clearly increase in molecular disorder or entropy in G_2 compared to reference group is resulting from the oxidation potential which appeared as hybrid of Hb and Met-Hb.(unstabilization of Hb probably though the elevation of hydrogen bonds) [29-31].

Dielectric relaxation technique, gives more useful information about some biophysical properties of the molecule such as the relaxation time τ_β, the shape of the molecule and the viscosity coefficient η. The variation of the conductivity S as a function of frequency can be considered as another view point for treating the dispersion data in the â region. It was shown that at high frequency end, the conductivity curve is still elevated for control group while flattening appeared in G_2

It is clear from the dielectric relaxation data in this study that both the relaxation time and radius of the Hb molecules increased for G_2 as compared to normal control. The behavior of the relaxation time and molecular radius(r) is similar, the shift towards lower or higher frequencies fc, as indicated from the â dispersion is attributed to changes in molecular radius. Since smaller molecules have shorter relaxation times and hence larger critical frequencies [32-35].

There is a marked increase in the dielectric increment for G_2, as compared to the control group. Theoretical treatment of the dielectric relaxation data to calculate the cole –cole parameter α for the Hb of G_2 as compared to normal control, illustrates another form of the conformational changes in the hemoglobin. The values of α show a very wide distribution of relaxation time. The shape of Hb molecule in the reference group is not completely spherical, so Cole-Cole plot (ε'' vs ε') is nearly semi circle. The shape of Hb molecule tends to deviate from the spherical form (Fig; 4-5) i.e an increase in the unfolding.

It could be concluded that, the different degree of unfolding of Hb as a globular protein coincides with the change in hydrophobic/ hydrophilic ratio, the change in the tertiary structure of Hb molecule results in a change in

its molecular shape from nearly spherical to non spherical form with different values of the parameter α. This finding is attributed to an increase in the surface charges of Hb molecule, with high affinity of Hb to oxygen and a decrease in the delivery of oxygen to tissue and/or the increase in the molecular size in Hb of rats received dioxin, give an evidence for the failure of Hb in running its metabolic activity.

REFERENCES

1. Aylward, L. and S. Hayes, 2002. Temporal trends in human TCDD body burden: Decreases over three decades and implications for exposure levels. J. Exposure Anal. and Environm. Epidemol., 12: 319-328.
2. Yong, A.L., 1983. Human and environmental risks of chlorinated dioxins and related compounds. Plenumarly press: New York., pp: 173-190.
3. Theelen, R.M., 1991. Biological basis for risk assessment of dioxins and related compounds; cold spring harbor Laboratory press; New York, pp: 277-290.
4. Schecter, A., P. Cramer and K. Boygess, 2001. Intake of dioxins and related compounds from food in the US, population. Journal of Toxicological Environment. Health part A, 63: 18-27.
5. Van den Berg and M. BirnbaunLS, 2006. The 2005world health organization re evaluation of human and mammalian toxic equivalency factors for dioxins and dioxin- like compounds. Toxicol. Sci., 93: 223-241.
6. Debdas Mukerjee, 1977. Health impact of polychoinated dibenzo-p-dioxins: A critical review. J. Air Managem. Assoc., 48: 157-165.
7. Courtney, K.D. and J.A. Moore, 1971. Teratology studies with 2,4,5- T and 2,3,7,8-.TCDD. Toxicol. and Appl. Pharmacol., 20: 396-403.
8. Pratt, R.M., C.S. Kim, L. Decker and V.M. Diewert, 1984. Mechanism of TCDD- induced cleft palate in the mouse. In Biological Mechanisms of Dioxin Action, Poland, A. and Kimbrough, R.D. (Eds). Banburg report 18, Cold Spring. Harbor, Laboratory, New York, pp: 61-71.
9. Vanden Berg, M., D e Jongh, H. Poiger and J.R. Olson, 1994. The toxica kinetics and metabolism of [olychlorinated dibenzo-p- dioxins (PCDDs) and dibenzofurans and their relevance for toxicity. Environm. Health Perspectives, 106: 776-792.
10. Olson, J.R., 1994. Pharmacokinetics of dioxins related compounds. In: Dioxins and Health, Schecter, A., (Ed) New York: Plenum Press, pp: 163-197.
11. Poland, A and J.C. Knutson, 1982. 2.3.7,6 tetra choloro dibenzo- p- dioxin and related halogenated aromatic hydrocarbons: examination of the mechanism of toxicity. Ann. Rev. Pharmacol. Toxicol., 22: 517-554.
12. Mocarelli, P., L.L. Needham, A. Marocchi, D.G. Patterson, P. Brambilla, P.M. Gerthowx, L. Meazza and V. Carreri, 1992. Serum conc. of 2,3,7.8-Tetra cholorodibenzo-p- dioxin and test results from selected residents of sevese, Italy. J. Toxicol. Environm. Health, 33: 357-366.
13. Poland, A. and J.C. Knutson, 1982. 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and related halogenated aromatic hydrocarbons: Examination of the mechanism of toxicity. Ann. Rev. Pharmacol. Toxicol., 22: 517-554.
14. Lin, P., S.W. Hu and T.H. Chang, 2003. Correlation between gene expression of aryl hydrocarbon receptor (AhR), hydrocarbon receptor nuclear translocator (Arnt), cytochromes P4501A1 (CYP1A1) and 1B1 (CYP1B1) and inducibility of CYP1A1 and CYP1B1 in human lymphocytes. Toxicol. Sci., 71: 20-26.
15. Blun, E.K, 1972. Numerical analysis and computation theory and practice. Addison Weseley Publishing company, Massachusetts,Menlo,Park,California. London. Don Mills, Ontrio, 104.
16. Perepechko, I.I., 1981. An introduction to polymer physics, Mir Publishers, Moscow, pp: 152-172.
17. Atkns, Peter and deparala, 2002. Physical chemistry. 7th edi., W.H. Freeman and company.
18. Clementi, E. and R. Sarma, 1983. Structure and dynamic of nucleic acids and patients. Academic Press, New York.
19. Mawatari, K., Matsukawa and Y. Yoneyama, 1983. Spectral changes upon submit association in valancy hybrid Hb. Biochemical Biophysical acta, 748: 381.
20. Mawatari, M., S. Yoneyama, 1987. Valency hybrid Hb with special attention to submit organization. Biochemical Acta, 36: 5320.
21. Sprokholt, R., S. Van Ooik, R.A.M. Van den kamp, B.N. Bownn, W.G. Zijlstra and A. Maas, 1986. Preparing a quality control material for blood gases, PH and hemoglobinomotery using stroma- free Hb solution. Second. J. Clinic. Lab. Invest., Suppl. (pub.1987). 47:93.
22. Poland, A. and J.C. Knutson, 1982. 2,3,7,8- Tetra chlorodibenzo-p- dioxin and related halogenated aromatic hydrocarbons: Examination of the mechanism of toxicity. Ann. Rev. Pharmacol. Toxicol., 22: 517-557.

23. Gray, A., R. Rockwood and I. Steven, 2003. Species comparison of Met Hemoglobin reductase. Baskin Experimental Biology and Medicine. Jan, 228: 79-83.
24. Machartava, V., J. Racek, V. Kohout, Holecek, I. Krejcova and V. Senft, 1993. Indication of effects of free radicals in worker at risk of lead exposure. Vntrlek, 44: 835.
25. Moreira, E.G., G.J. Rosa, S.B. Barros, V.S. Vaqssilieff and I. Vassilieff, 2001. Autooxidation defense in rat brain regims after developmental lead exposure Toxicology, 14: 145-151.
26. Mawatari, K., W.A. Matsuka. and Y. Yoneyama, 1983. Spectral changes upon subunit association in valency hybrid Hb. Biochemical Biophysical Acta, 748: 381.
27. Chiew, Y., C. Kuehnen, D.H.W. Blanch and J.M. Prausnitz, 1995. Molecular thermodynamics for salt induced protein precipitation.ALCHE J., 41: 2150-2159.
28. Smith, T.F., 1995. Models of protein folding. Sci., 507: 959-961.
29. Creighton, T.E., 1990. Review Article on protein folding. Biochem. J., 270: 1-16.
30. Jia-tihandshukao, 1990. The thermodynamic properties of protein. J. Physi. Appl. Phys., 23: 967-980.
31. Haddad, W. And C. Vijay, 2005. Thermodynamics. A dynamical systems Approach. Princeton University Press.
32. Debye, P., 1929. Polar molecules chemistry. Catalog co., New York.
33. Bishay, S.T., 2000. Numerical Methods for the calculation of the cole-cole parameters. Egyptian Journal of Solid. 23: 2.
34. Lide. D.R., 2004. Handbook of Chemistry and Physics, 8th ed.. CRC Press Boca Raton. pp: 8-141.
35. Braslavsky, S.E., 2007. Glossary of terms used in photochemistry. (IUPAC) Recommendations 2006. Pure and Applied Chemistry, 79: 293-465.