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Physico-chemical Aspects of Serum Nitrite Expression by Iron and Glutathione

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In the present study the effect of $NaNO_2$ and sodium nitrorpusside (Na-Np) on binding parameters of some iron complexes as well as reduction effects of glutathione and cysteine and their pH equilibrium has been reported.

Serum nitrite levels of patients with iron related diseases and *in vitro* effects of addition of iron containing compounds and glutathione and cysteine on these are also presented. Significant variation in these levels appears to suggest some kind of correlation of NO levels to binding of iron complexes and glutathione. Physico-chemical parameters appear to be significant and relevant to serum nitrite level regulation.

Key Words: Nitric oxide–Fe^{2+}, Fe^{3+} binding, glutathione, Cysteine, Antioxidant effects, Serum NO_2^- levels.

INTRODUCTION

Cell signalling is an important aspect of current research in biology. Nitric oxide (NO) is a well known signalling molecule and also a neurotransmitter, which appears to regulate almost evergy physiological activity[1]. NO is essential for proper functioning of several physiological processes and acts as a friend. However, in excessive quantities it turns into a foe and causes many diseases. Newer stategies are being considered to down regulate over-production of NO as modern means of clinical medicine[2, 3].

There are several reports which suggest involvement of compounds of iron and sulphur in the pathological, cellular malfunctions and mutagenic effects of NO. Effects of NO on oxidative stress in relation to iron and sulphur; destruction of Fe-S clusters; NO as ligand in Fe-heme complexes; reaction with transitional elements; spin changes in heme proteins, etc. have been discussed by several workers which establishes the role of compounds of iron in effects of NO[4–8].

Similarly several reports indicate the influence of cysteine and glutathione on the biological complications caused by NO such as peroxynitrite formation, apoptosis, anti-platelet effects. tissue injury nitrosation of protein thiols[9–12]. Cardioprotective effects[13] are also known. We have therefore attempted in our present study to see the effects of Na-Np (NO donor) and $NaNO_2$ (as NO_2 is end product of NO in body) on binding interactions of complexes of Fe(II) and Fe(III) and redox titrations of iodine and ascorbic acid with glutathione and cysteine and H^+ ion equilibra using pH titration curves.

To examine if nitrite levels in blood serum are affected by compounds of iron and thiols we have determined the serum nitrite levels of about 50 patients suffering from iron metablolism disorders by cadmium reduction-colorimetry method. Further changes have been observed in these levels on *in vitro* addition of hemoglobin (Hb); $K_3Fe(CN)_6$; glutathione (GS) and cysteine. Physico-chemical characters seem to have an important role in blood serum nitrite expression.

EXPERIMENTAL

All chemicals used in the study are of analytical grade procured from reputed companines like Qualigen, E. Merck etc. Deionized water passed through Ion-Exchange India Ltd. resins was used throughout.

Glutathione, cysteine and sodium nitroprusside were procured from S.D. Fine Chemicals. N_1'-napthylene diamine dichloride (NADA), cadmium granules and sulphanilamide were obtained from Loba Chemie.

Equiptronics model EQ-61 pH-meter of Electronic Corporation of India was used for all pH measurements and absorbance values were measured on Spectronics-21 spectrophotometer.

Ferrous ammonium sulphate (0.005 M); *o*-phenanthroline (0.25 mg/100 mL) in acetate buffer pH = 5.0, 0.005 M and hydroxylamine 5% were used for preparing Fe(II)-*o*-phenanthroline complexes. Absorbance values at 410 nm were used for the calculation. For $[K_3Fe(CN)_6]$ complexes (500 mg/100 mL) aqueous solutions and Fe(II)-ammonim sulphate 0.0005 M were used and absorbance at 610 nm was used for calculation.

For the Fe(III) complexes, ferric ammonium sulphate (0.005 M) and potassium thiocyanate (0.005 M) aqueous solutions were used. The absorbance at 475 nm was used for calculation. For salicylic acid complexes 0.002 M aqueous solution of Fe(III) ammonium sulphate and 0.005 M salyicylic acid aqueous solution was used. The absorbance values at 520 nm were used.

All the complexes were prepared by the Peacock and Skerette method[14, 15] and the binding parameters (n the number of binding sites and K′ the association constant) were calculated from the Scatchard plots[16].

The H^+ equilibrium was observed by plotting H^+-pH titration curves (not shown) as per Tanford[17]. The redox titrations of iodine with $Na_2S_2O_3$ and ascorbic acid in presence of glutathione and its constituent amino acids were performed by the common undergraduate laboratory methods[18]. The concentrations of $NaNO_2$ and Na-Np were maintained at 10 mg/mL and 2.5 mg/mL respectively.

The Green's method[19] based on cadmium reduction and colorimetry described by Bapat and Patil[20] was used for the NO_2 level measurements in blood serum, after suitable modifications to suit our laboratory environment and standardization of the protocol.

Blood samples of patients (50 in number) with iron metabolism disorders were supplied by Dr. Pangam, Hematology Department, Topiwalla National Medical College, Mumbai.

RESULTS AND DISCUSSION

The binding interaction between iron and *o*-phenanthroline have been studied plotting the Scatchard's plots (Fig. 1). The nature of the curve is in agreement with the discussion of Muller and Crothers[21].

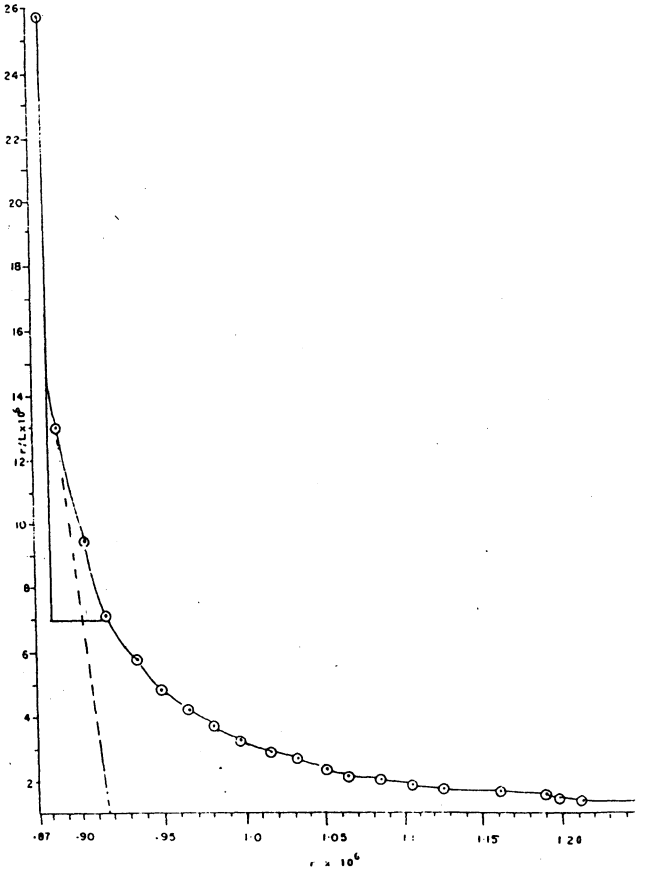

Fig. 1 Absorbance values, r and r/L values at 410 nm of Fe^{2+} and *o*-phenonthroline complex

$$\text{Slope} = \frac{16.5 - 7}{0.89 - 0.91} = \frac{9.5}{0.2} = 47.5$$

The values of n and 'k'' calculated from the graphs (after correction by the least square method) for various complexes of Fe^{2+}, Fe^{3+} and effect of addition of $NaNO_2$ and Na-Np on these parameters are presented in Table-1.

These results indicate that both $NaNO_2$ and Na-Np cause, in general, reduction in the binding sites available for the ligands and also induce change in the binding affinities to a variable extent. It can be inferred from these observations that NO could interfere with the interactions of iron in biomolecules such as iron containing enzymes heme proteins and 2Fe-2S clusters.

TABLE-1*
BINDING PARAMETERS OF Fe^{2+} AND Fe^{3+} COMPLEXES
EFFECT OF $NaNO_2$ AND Na–Np

Experiment	n	k′
Fe^{2+} + *o*-phenanthroline	0.918	475
Fe^{2+} + *o*-phenanthroline + 2 mL $NaNO_2$	0.656	240
Fe^{2+} + *o*-phenanthroline + 2 mL Na-Np	1.180	344
Fe^{2+} + $K_3[Fe(CN)_6]$	0.182	500
Fe^{2+} + $K_3[Fe(CN)_6]$+ 2 mL $NaNO_2$	0.172	1500
Fe^{2+} + $K_3[Fe(CN)_6]$+ 2 mL Na-Np	0.144	625
Fe^{3+} + KCNS	0.930	760
Fe^{3+} + KCNS + 1 mL $NaNO_2$	0.710	720
Fe^{3+} + KCNS 1 mL Na-Np	0.840	260
Fe^{3+} + salicylic acid	3.920	1750
Fe^{3+} + salicylic acid + 1 mL $NaNO_2$	2.980	4000
Fe^{3+} + salicylic acid+ 1 mL Na-Np	3.480	1333

* These values are calculated for primary strong reaction.

The redox titration curves of glutathione and its constituent amino acid with $Na_2S_2O_3$ and ascorbic acid (Fig. 2) indicate the strong antioxidant properties of

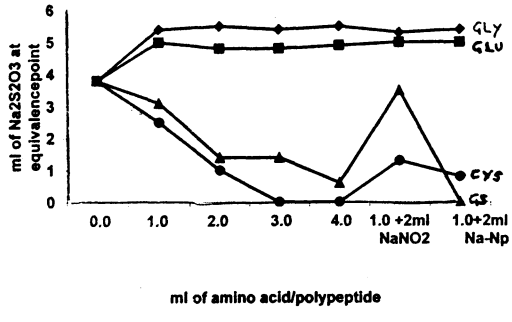

Fig. 2. (a) I_2 (0.05 N) *vs.* $Na_2S_2O_3$ (0.05 N) titration with Gs, Cys, Gly and Glu effects of $NaNO_2$ and NaNp

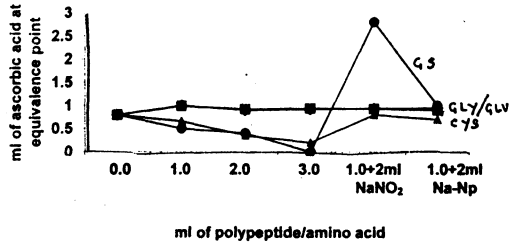

Fig. 2. (b) I_2 (0.05 N) *vs.* ascorbic acid (25 mg/mL) titration with Gs, Cys, Gly and Glu effects of $NaNO_2$ and NaNp

glutathione, particularly of cysteine and its variation in presence of $NaNO_2$ and Na-Np. The titration values are shown in terms of mL of $Na_2S_2O_3$ or ascorbic acid required for the neutralization of iodine solution in presence of various constituents. These observations show that NO cuases considerable interference in the antioxidant function of glutathione and cysteine. This may be possibly due to the effect of NO on disulphide bridge formation by thiols in aqueous biological media as reported earlier[22].

The values of pK_a and K_a calculated from the pH titration curves of glutathiones are shown in Table-2. Both $NaNO_2$ and Na-Np show a very strong effect on dissociation constant of glutathione suggesting a modification of glutathione solution structure by the two nitrogen donors. This also can possibly influence the effects of NO on the behaviour of glutathione.

TABLE–2
K_a of GS (CALCLULATED FROM pH TITRATION CURVE OF GLUTATHIONE AND EFFECT OF $NaNO_2$ and Na-Np)

Experiment	pka	K_a
Glutathione in D/W	6.22	6.025×10^{-7}
Glutathione in D/W + 2 mL $NaNO_2$	6.60	2.511×10^{-7}
Glutathione in D/W + 2 mL Na-Np	6.57	2.691×10^{-7}

The $NO_2^{'}$ levels in patients with iron metabolic disorders show wide variation (spread) in values as compared to the control values. There are several reports which support these observations that $NO_2^{'}$ levels in serum samples of patients having certain disorders as well in mutagenic developments are quite high or low as compared to normal values. This observation can be of good help in early diagnosis of certain metabolic disorders.

We have also observed the in vitro changes in the levels of $NO_2^{'}$ in these serum samples on addition of $K_3Fe(CN)_6$ and haemglobin and also cysteine and glutathione. It is observed that compounds of iron show elevation in these levels while cysteine and glutathione show a marked tendency towards reduction in these levels. This is quite significant and interesting and needs further investigation.

The physico-chemical factors mentioned above involving NO interference in complexes of iron and their solution behaviour have relevance to effects of NO′ on physiological and biological functions.

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