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DEVELOPMENT OF A NEW STRATEGY FOR ANTIMALARIAL DRUGS

Principal Investigators : Prof. Shalom Sarel and Prof. Dan T. Spira
Grantee Institution : The Hebrew University of Jerusalem

Collaborators :
Dr Shelly Avramovici-Grisaru, The Hebrew University of Jerusalem
Dr. Nipon Wongvisetsirikul, King Mongut's Institute of Science and Technology, Bangkok,Thailand

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cc: BOSTID, CDIE

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Executive Summary

Some 70 chelating agents based on pyridoxal and salicylaldehyde were prepared according to standard methods, and their structures characterized and identified by elementary analysis, and chemical spectroscopy (IR, UV, NMR, MS).

About 30 chelators were tested against ribonucleotide diphosphate reductase. Depending on structure, the compounds ranged from essentially non-active, to significant inhibitors, relative to hydroxyurea as reference compound.Their relevance to biological properties is herein discussed.

Some 35 compounds were tested against *Plasmodium falciparum* (FCR-3) maintained in human A^+ erythrocites. Compounds (**13**) and (**29**) were outstanding in their ability to kill the drug-resistant malaria parasites at concentrations as low as 5-10 μM within 24 hrs., with IC_{50} values of 1.2 μM and 1.9 μM, respectively. This interesting discovery prompted a study of the mechanism of action.Towards this end we secured pertinent data as follows: i) Fe^{2+} and Fe^{3+} ions blocked the inhibitory effect of the chelators; ii) early removal of cheletable iron from the erythrocite-parasite complex renders *protection* to the parasite, indicating that iron is essential for the parasiticidal action, in other words, the antimalarial activity is *iron-dependent*..

Ligand (**29**) exhibit moderate affinity for Fe(II), and much stronger affinity for Fe(III). Study has shown that the Fe(II)-, and Fe(III)-complexes differ considerably in their sensitivity towards pH. Whereas the 1:1-ligand-Fe(II) is stable in the pH range 4-9, 1:1-Fe(III)-ligand is stable only at pH < 6. At pH > 6, it transforms into corresponding 1:2-Fe(III)-complex.

ESR measurements indicated that 1:1-Fe(II)-chelate of (**29**), produces C-centered free radicals, both in presence and in absence of living cells. The Fe(III)-complex produces free radicals *only* in the presence of actively metabolizing cells.

Most significantly, Fe(II)-(**29**) complex induces breaks both in the molecular λ phage DNA, and in a lower molecular pBR322 supercoiled plasmid DNA.This was reasonably extended to explain the antimalarial action *in vitro*. This offers an interesting venue to antimalarial drug design implicating intracellular generation of harmful free radicals by iron-dependent inducers. The rapid spread of multi-drug resistant strains of the malarial parasites prompts the use of new models for drug design to provide more effective antimalarials operating by hitherto unknown mechanisms. It is highly likely that (**13**) and/or (**29**), or another analogue could demonstrate other antiparasitic and/or antitumor activity of potential therapeutic utility.

Research Objectives

This project aimed at developing a new chemotherapeutic strategy against the drug-resistant strains of Plasmodium falciparum, the main causative parasite for human malaria. Already in the mid-'80 World Health Organization (WHO) has alarmed the international scientific community over the rapid spreading of a new species of Falciparum malaria into areas such as South America and South East Asia[1-4], which hitherto were considered not to be endemic for Falciparum malaria. It is unfortunate that to this date the world medical arsenal does not posses effective weapons to meet this health challenge.

Iron is an essential element to virtually all living organisms. An invading pathogen must compete with its host for iron. If the parasite cannot obtain sufficient iron, its proliferation may be retarded and the infection attenuated. Host defences may involve iron withholding. A decrease in serum iron has been observed in malarial infections in man. This drop in serum iron levels during parasitemia can be regarded as a mechanism of non-specific resistence. Iron chelators have, therefore, been shown to affect the growth and development of parasites both *in vitro* and *in vivo*[5-14].

It was not long ago that the antimalarial drug - chloroquine - has been one of the most successful antimicrobial agent ever produced by mankind. Its beneficial effects on public health have been enormous. Now, when chloroquine is not effective any more against the resistant parasite, novel chemical weapons are promptly needed to counter the menace of the dreadful disease.

The guiding idea was to devise a novel antimalarial strategy based on a new mechanism of action, targeted at protozoal processes implicating iron[14]. For good reasons we selected two keynote cell-targets of the parasite, controlling cell-proliferation and cell-survival. One, relates to the metalloenzyme which controls the synthesis of DNA, and the second, to the stability of the DNA as a bio-macromolecule.

The group of ribonucleotide diphosphate reductases (RdR)[15-17] play a central role in the DNA bio-syntheses, catalyzing the conversion of nucleotide diphosphates, NDPs, to deoxynucleotide diphophates, dNDPs. The metalloenzymes[17] posses two subunit structures, B_1 and B_2, in which the B_2 subunit posses an unusual bi-nuclear iron center, and an organic tyrosyl radical as cofactors. Inhibition of RdR could be effected by targeting either at the iron-center , *via* metal-chelation (mode a, see Chart 1), or, at the tyrosyl-radical co-factor, by interaction with an electron-transfer (ET) reductant (mode b, Chart 1).

Chart 1

POSSIBLE MODES FOR ANTIMALARIAL ACTION IMPLICATING Fe-CHEMISTRY

INHIBITION OF DNA SYNTHESIS via Inactivation of Ribonucleotide Diphosphate Reductase (RDPR)

Mode *a*

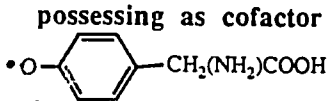
RDPR
B_2 subunit possess
Fe(III)-O-Fe(III) as cofactor
(active)

$\xrightarrow[\text{chelator}]{\text{removal of Fe-O-Fe}}$

APO-RDPR
(inactive)

Mode *b*

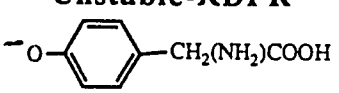
RDPR
possessing as cofactor



O-centered radical

$\xrightarrow[\text{electron-transfer}]{+\ e^-}$

Unstable-RDPR



(inactive)

DEGRADATION OF DNA MACROMOLECULE

Mode *c*

$$R^{\bullet} + DNA \xrightarrow[\text{electron-transfer}]{\text{Fe-mediated}} [R]^{+} + [DNA]^{-\bullet}$$

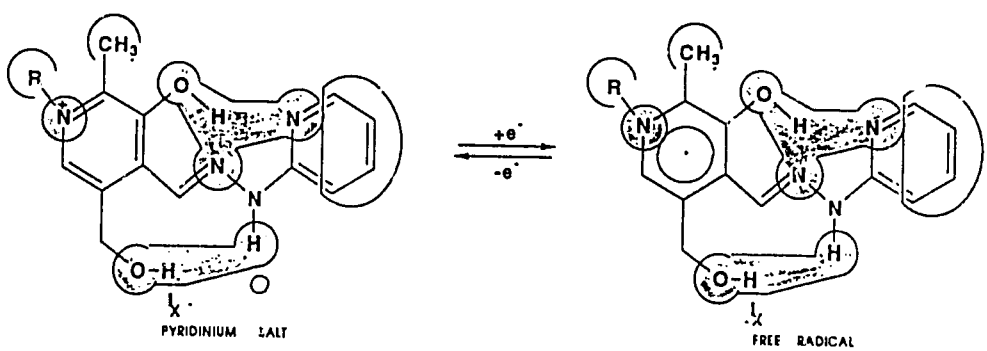
$$[DNA]^{-\bullet} \longrightarrow \text{Fragmentation}$$

Another possible mode for antimalarial action implicating iron chemistry is illustrated by mode c. In this mode, the parasite is killed due to a damage to its DNA, caused by a free radical. Through electron-transfer, the DNA macromolecule becomes an highly unstable anionic radical, which fragments as formed.

In the framework of this project, the israeli and the thai partners have produced some 70 structurally related candidates for antimalarial evaluation. They were related to vitamin B_6 (pyridoxal), a co-factor that Nature has invented for playing pivotal role as an electron-sink in numerous critical biochemical pathways (amino-acid metabolism)[18].

The drug design aimed at producing new pyridoxal-based chelators, comprised of four distinctly different domains (see, Chart 2), capable of penetrating into the parasite iron-pool, depriving it from its iron, and, in turn, inhibiting the synthesis of DNA in the parasite cell. Moreover, these molecules are capable of internal electron-trasferring, rendering damage to the parasite DNA. The four domains comprise: (a) an electrophoric domain, conferring electron-sink properties; (b) a transition-metal binding domain; (c) a hydrogen binding domain; and (d) a lipophilic domain, endowing facile trans-membrane transport capabilities to the molecule.

Chart II



›philic domain ⊙Hydrophilic domain ○ Metal-binding compartment ⊕ Electron-sink domain

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Methods and Results

Synthetical Methodologies

Structurally, the synthetical ligands comprise ten classes of hydrazones of 2-hydroxy aromatic-aldehydes, acyl hydrazones, thioacyl-hydrazones, guanidino-hydrazones, and aryl-hydrazones, outlined in Charts II - VIII. They comprise of two different aromatic rings which are connected by bridges made of hydrazonic moieties. Six of these contain bridges which are capable of transmitting electronic effects, as follows : (i) CH=N-NH (an hydrazone group), (ii) CH=N-NH-CO (an acyl hydrazone group), (iii) CH=N-NH-CO-NH-N=CH, (iv) CH=N-NH-CS-NH-N=CH,
(v) CH=N-NH-[aromatic ring]-NH-N=CH. The remaining four, contain chains of carbon and nitrogen which are uncapable of transmitting electronic effects. These are : (vi) CH=N-NH-CO-[CH2]x-CO-NH-N=CH, (vii) CH=N-[CH2]x-N=CH, (viii) CH=N-NHCO-CH2 , (ix) CH=N-NHCOO-CH2, and (x) CH=N--[CH2]x-N=CH.

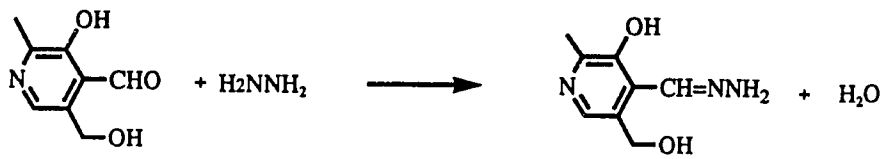
Most of the chelators presented in Charts III-VII were prepared in very good yields following the standard method outlined by equation (3)[19]. The two-stage pathway, implying sequences (equ. 1) and (equ. 2) were employed for the special cases of the symmetrical bis-hydrazones, compds. **8** (Chart IV) and **67** (Chart VII), and for the unsymmetrical azines, compds. **9** and **19** (Chart IV). The redox active pyridoxylidenium salts, ligands **22** - **31** (Chart V), were produced conveniently by N-alkylation of the respective aryl-, and acyl hydrazones, following equ. 4[20-22]. Depending on reaction conditions, the respective O^2-acetate (mono), **17** (Chart IV), and O^1,O^2-diacetates, **16** and **18**, could be produced according to equs. 5 & 6. Finally, the corresponding 1:1-, and 1:2-transition metal complexes were easily produced according to equ. 7.

Depending on properties (mainly solubility in H_2O) the chelators were selectively screened for activities against the metalloenzyme - ribonucleotide diphophate reductase (RdR) - and against the growth of the drug-resistant species of Plasmodium falciparum. The methodologies and results are given in the sequence.

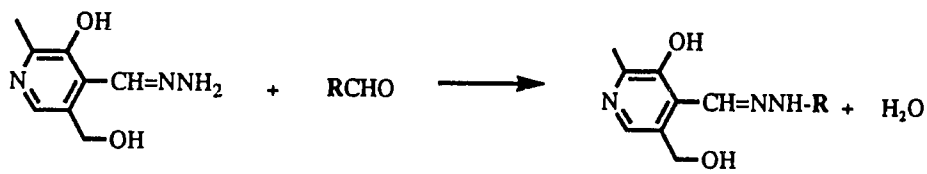
To shed some light on structure-activity-relationship, four representative chelators {PIH, (10), (25), and (29)} were selected for quantum mechanical and thermodynamical studies. These included, ab initio calculations, ionization constants - pKa values, species distribution in aqueous solutions, pH dependence of carbon-13 magnetic resonance spectra, formation constants of the Fe(II) and Fe(III) complexes, and redox potentials by cyclic voltametry .

Methods of Synthesis

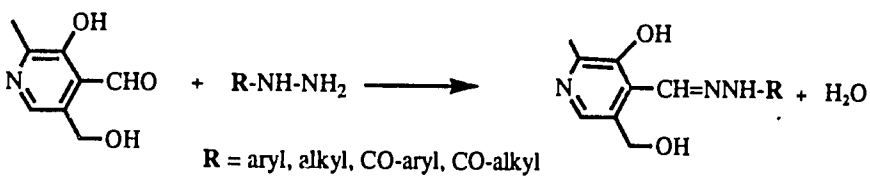
(Formation of hydrazones by condensation reaction. Alkylation at pyridinic ring-nitrogen. Acetylations at alcoholic and phenolic moieties of pyridoxal.Complex formation with metals)



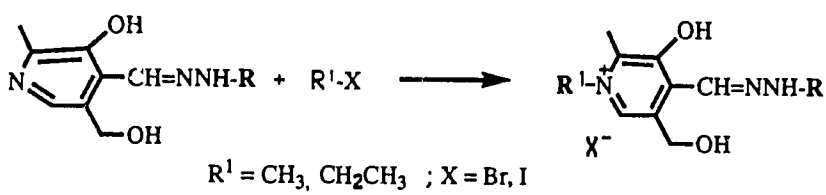
(equ. 1)



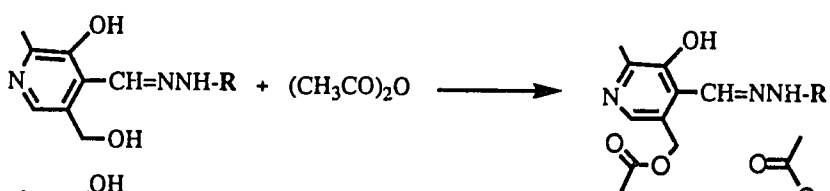
(equ. 2)



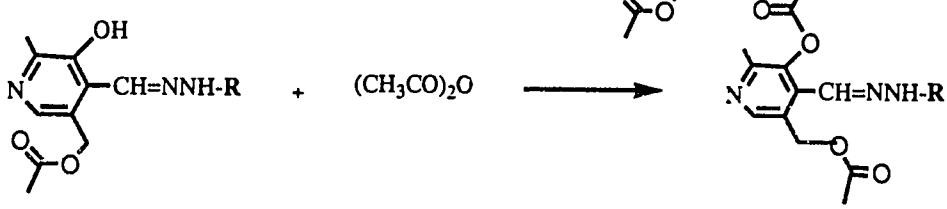
(equ. 3)



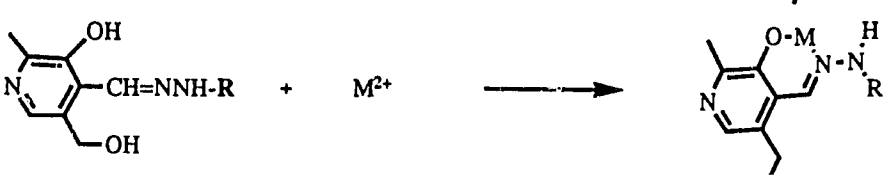
(equ. 4)



(equ. 5)



(equ. 6)



(equ. 7)

COMPOUNDS PREPARED ACCORDING TO METHODES OUTLINED IN CHARTS III - VII

CHART III
Halogenated Salicyl Aldehyde 2-Pyridyl Hydrazones

1-[3,5-Dichlorosalicylidene]-2-[2'-pyridyl]hydrazine (1)
1-[5'-Bromosalicylidene]-2-[2''-pyridyl]hydrazine (2)
1-[3,5-Dichlorosalicylidene]-2-[2'-N-methylpyridinium]hydrazine Iodide (3)

CHART IV
Pyridoxal Aryl Hydrazones

1-[Pyridoxylidene]-2-[phenyl]hydrazine (4).
1-[Pyridoxylidene]-2-[4'-methoxyphenyl]hydrazine Hydrochloride (5)
1-[Pyridoxylidene]-2-[4'-nitrophenyl]hydrazine (6).
1-[Pyridoxylidene]-2-[4'-carboxyphenyl]hydrazineHydrochloride (7).
1,1'-[Di-pyridoxylidene]-2,2'-[ortho-xylidene]di-hydrazin (8).
1-[Pyridoxylidene]-2-[5'-nitrofurfurylidene]hydrazine (9).
1-[Pyridoxylidene]-2-[2'-pyridyl]hydrazine (10).
1-[Pyridoxylidene]-2-[2'-pyrimidyl]hydrazine (11).
1-[Pyridoxylidene]-2-[2'-pyrazyl]hydrazine (12).
1-[Pyridoxylidene]-2-[1'-phthalazyl]hydrazine Dihydrochloride (13).
1-[Pyridoxylidene]-2-[6'-purinyl]hydrazine Hydrochloride (14).
1-[Pyridoxylidene]-2-[5'-1'H-tetraazolyl]hydrazine: (1) dihydrate (15-I);
(2) sulphate (15-II).
1-[Pyridoxylidene-O^1,O^2-diacetate]-2-[2'-pyridyl]hydrazine (16).
1-[Pyridoxylidene-O^2-acetate]-2-[2'-pyrimidyl]hydrazine (17).
1-[Pyridoxylidene-O^1,O^2-diacetate]-2-[2'-pyrimidyl]hydrazine (18)
1-[Pyridoxylidene]-2-[2'-oxo-3'-sulfonic acid naphthylidene]hydrazine (19).
Copper(II)-chelate of 1-[Pyridoxylidene]-2-[2'-pyridyl]hydrazine sulphate (20a)
Di-copper(II)-chelate of 1-[Pyridoxylidene]-2-[2'-pyridyl]hydrazine (20b).
Copper(II)-chelate of 1-[Pyridoxylidene]-2-[2'-pyrimidyl]hydrazine sulphate.
1:1-Ligand:Cu complex (21a).
Iron(II)-chelates of 1-[Pyridoxylidene-O^2-acetate]-2-[2'-pyrimidyl]hydrazine.
1:1-Ligand:Fe complex (21b). 1:2-Ligand:Fe complex (21c).

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CHART V

Pyridoxylidenium Salts of Aryl Hydrazones

1-[N-Methylpyridoxylidenium]-2-[phenyl]hydrazine iodide (22).
1-[N-Methylpyridoxylidenium]-2-[4'-methoxyphenyl]hydrazine iodide (23).
1-[N-Methylpyridoxylidenium]-2-[4'-nitrophenyl]hydrazine iodide (24
1-[N-Methylpyridoxylidenium]-2-[2'-pyridyl]hydrazine iodide (25).
The MeOH-Compound of 1-[N-Methylpyridoxylidenium]-2-[2'-pyridyl]hydrazine iodide
1-[N-Methylpyridoxylidenium]-2-[2'-(1'-methylpyridylidene]..ydrazine iodide(26)
1-[N-Methylpyridoxylidenium]-2-[2'-pyrimidyl]hydrazine iodide (27).
1-[N-Methylpyridoxylidenium]-2-[4'-methyl-2'-pyrazyl]hydrazine iodide (28).
1-[N-Ethoxycarbonylmetpyridoxylidenium]-2-[2'-pyridyl]hydrazine Bromide (29).
1-[N-Ethoxycarbonylmethyl pyridoxylidenium]-2-[2'-pyrimidyl]hydrazine Bromide (30).
Di-{1-[N-Ethylpyridoxylidenium]-2-[2'-pyridyl]hydrazine}-amine Di-chloride (31)
1-[Pyridoxylidene]-2-[3'-chloro-2-pyridyl]hydrazine Hydrochloride (32)
1-[Pyridoxylidene]-2-[5'-chloro-2-pyridyl]hydrazine Hydrochloride (33).
1-[Pyridoxylidene]-2-[3',5'-dichloro-2-pyridyl]hydrazine Hydrochloride (34).
1-[Pyridoxylidene]-2-[3',4',5',6'-tetrachloro-2-pyridyl]hydrazine HCl (35).
1-[Pyridoxylidene]-2-[6'-chloro-4'-pyrimidyl]hydrazine Hydrochloride (36)
1-[Pyridoxylidene]-2-[4'-hydroxy-6'-methyl-2'-pyrimidyl]hydrazine (37).
Iron(II)-chelate of 1-[N-ethoxycarbonylmethyl pyridoxylidenium]-2-[2'-pyridyl]hydrazine Bromide (38)
Iron(II)-chelate of 1-[N-ethoxycarbonylmethyl pyridoxylidenium]-2-[2'-pyrimidyl] hydrazine Bromide (39).
Copper(II)-chelate of 1-[N-ethoxycarbonylmethyl pyridoxylidenium]-2-[2'-pyrimidyl]hydrazine Bromide (40).

CHART VI

Hydrazidic Derivatives of Pyridoxal

Pyridoxal Semicarbazone Hydrochloride (41) .
Pyridoxal Thiosemicarbazone Hydrochloride (42)
Pyridoxal Guanidinohydrazone (43).
Pyridoxal Carbohydrazone Hydrochloride (45).

Pyridoxal Thiocarbohydrazone Hydrochloride (46).
Pyridoxal Oxamic Acid Hydrazone Hydrochloride (47).
Pyridoxal Oxalic Acid Hydrazone Hydrochloride (48).
Bis-Pyridoxal Carbohydrazone Hydrochloride (49).
Bis-Pyridoxal Thiocarbohydrazone Hydrochloride (5(
Bis-[N-Methylpyridoxylenium] Carbohydrazone Di-iodide (51).
Pyridoxal Adipic Acid Hydrazone Hydrochloride (52).
Bis-Pyridoxal Adipic Acid Hydrazone Di-Hydrochloride (53).

CHART VII

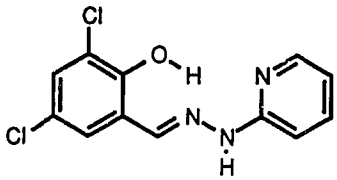
Pyridoxal Acyl Hydrazone

1-[Pyridoxylidene]-2-[2'-furoyl]hydrazine hydrochloride (54)
1-[Pyridoxylidene]-2-[isonicotinoyl]hydrazine.Pyridoxal Isonicotinoyl Hydrazone, (55)
1-[Pyridoxylidene]-2-[4'-quinolinoyl]hydrazine (56).
1-[Pyridoxylidene]-2-[$N^{1'}$-methylpyridiniumcarbonyl]hydrazine Iodide. Pyridoxal N-Methylisonicotinoyl Hydrazone Iodide (57).
1-[N-Methylpyridoxylidenium]-2-[$N^{1'}$-methylpyridiniumcarbonyl]hydrazine Diiodide. Pyridoxal Isonicotinoyl Hydrazone Dimethiodide (58).
5-Bromosalicyl Isonicotinoyl Hydrazone (59).
3,5-Dichlorosalicyl Isonicotinoyl Hydrazone Methiodide (60).
1-[5'-Chloro-salicylidene]-2-[N-ethoxycarbonylmethyl-nicotinoyl]hydrazine Bromide (61).
1-[5'-Bromo-salicylidene]-2-[N-ethoxycarbonylmethyl-nicotinoyl]hydrazine Bromide (62).
2:1-Ligand:copper(II) of 1-[5'-Bromo-salicylidene]-2-[N-ethoxycarbonylmethyl-nicotinoyl]hydrazine Sulphate (63).
2:1-Ligand:copper(II) of 1-[5'-Bromo-salicylidene]-2-[isonicotinoyl]hydrazine Formate (64).
The respective 1:1-ligand:copper(II) complex of 1-[pyridoxylidene]-2-[5'-chloro-2'-pyridyl]hydrazine sulphate (65)
1:1-Ligand:copper(II) complexes of 1-[pyridoxylidene]-2-[3',5'-dichloro-2'-pyridyl]hydrazine (66a) and (66b) .
1,1'-Bis-[pyridoxylidene]-2-2'-[1,4-phthalazyl]dihydrazine Trisulphate (67).
Bis-pyridoxal Ethylene-Diazine (68).
Pyridoxal 4-Methoxybenzyloxycarbonyl Hydrazone (69).
1-[Pyridoxylidene]-2-[N-pyridiniumacetyl]hydrazine Chloride (70).

PYRIDOXAL-BASED CHELATORS

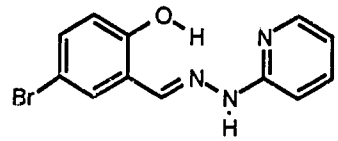
CHART III

Halogenated Salicyl Aldehyde 2-Pyridyl Hydrazones



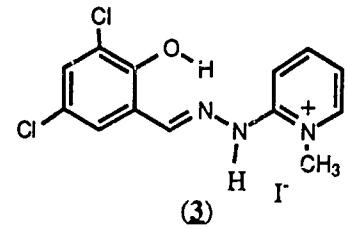
(1)

1-[3,5-Dichlorosalicylidene]-2-[2'-pyridyl]hydrazine



(2)

1-[5-Bromosalicylidene]-2-[2'-pyridyl]hydrazine

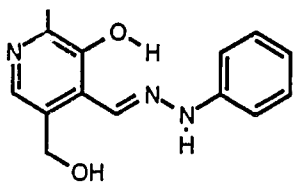


(3)

1-[3,5-Dichlorosalicylidene]-2-[2'-N-methylpyridylene]hydrazi

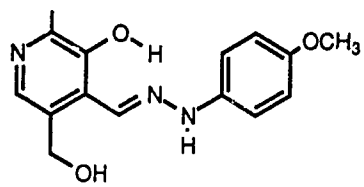
CHART IV

Pyridoxal Aryl Hydrazones



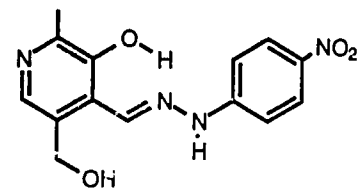
(4)

1-[Pyridoxylidene]-2-[phenyl] hydrazine



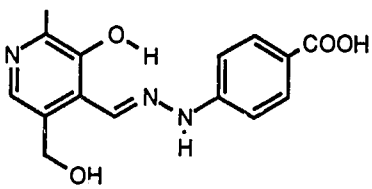
(5)

1-[Pyridoxylidene]-2-[4'-methoxy-phenyl]hydrazine



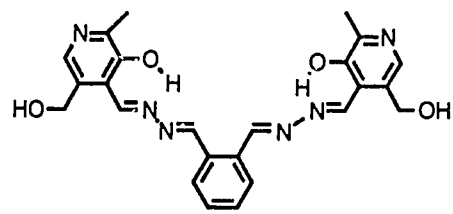
(6)

1-[Pyridoxylidene]-2-[4'-nitro-phenyl]hydrazine



(7)

1-[Pyridoxylidene]-2-[4'-benzene-carboxylic acid]hydrazine



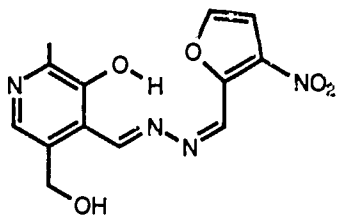
(8)

1,1'-[Dipyridoxylidene]-2,2'-[ortho-xylidene] di-hydrazine

13

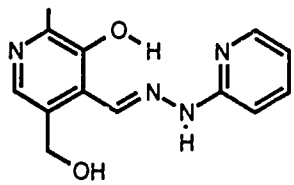
CHART IV

Pyridoxal Aryl Hydrazones



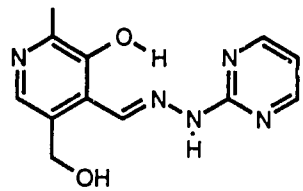
(9)

1-[Pyridoxylidene]-2-[5'-nitro-furfurylidene]hydrazine



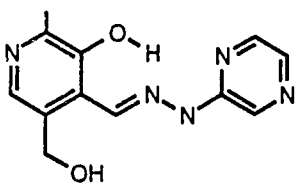
(10)

1-[Pyridoxylidene]-2-[2"-pyridyl]hydrazine



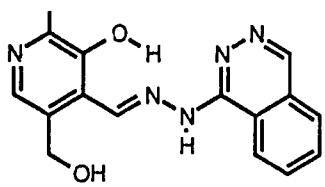
(11)

1-[Pyridoxylidene]-2-[2"-pyrimidyl]hydrazine



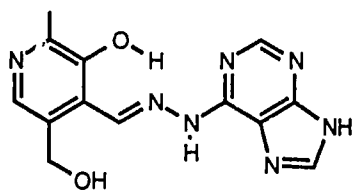
(12)

[Pyridoxylidene]-2-[2'-pyrazyl] hydrazine



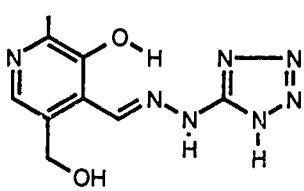
(13)

1-[Pyridoxylidene]-2-[1'-phthalayl]hydrazine



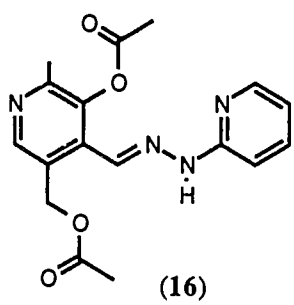
(14)

1-[Pyridoxylidene]-2-[6'-purinyl]hydrazine



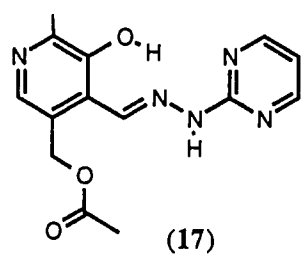
(15)

1-[Pyridoxylidene]-2-[2"-tetrazolyl]hydrazine



(16)

1-[Pyridoxylidene-O^1,O^2-di-acetate]-2- [2' pyridyl]hydrazine



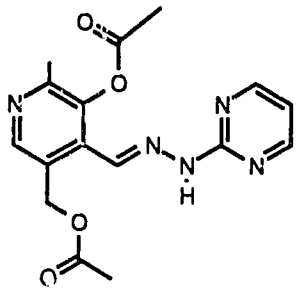
(17)

1-[Pyridoxylidene-O^2-acetate]-2- [2' pyrimidyl] hydrazine

14

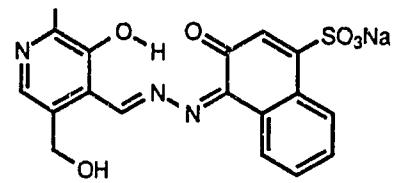
CHART IV

Pyridoxal Aryl Hydrazones



(18)

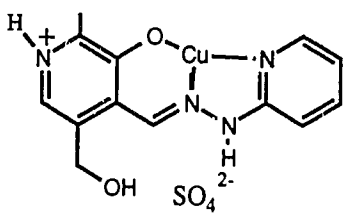
1-[Pyridoxylidene-O[1]-O-[2] diacetate]-2-[2'- pyrimidyl] hydrazine



(19)

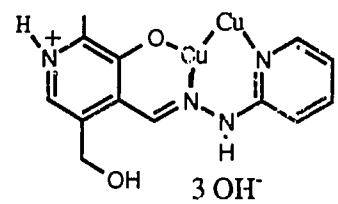
1-[Pyridoxylidene]-2-[2'-oxo-3'- sulfonic acid naphthylidene]hydrazine

II-3. Copper(II) and Iron(II)-chelates of 1-[Pyridoxylidene]-2-[2'-pyridyl] and [2'-pyrimidyl]hydrazine



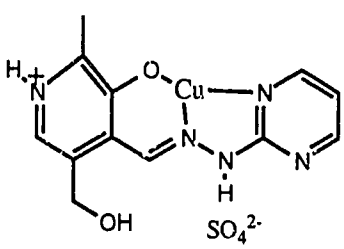
(20a)

Copper(II)-chelate of 1-[pyridoxylene] 2-[2'-pyridyl]hydrazine Sulphate



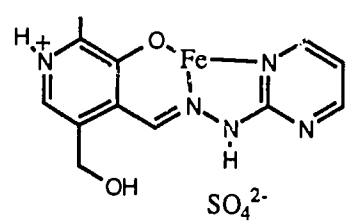
(20b)

Dicopper(II)-chelate of 1-[pyridoxylene] 2-[2'-pyridyl]hydrazine Dihydroxide



(21a)

Copper(II)-chelate of 1-[pyridoxylene] 2-[2'-pyrimidyl]hydrazine Sulphate



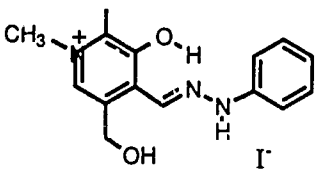
(21b)

Iron(II)-chelate of 1-[pyridoxylene] 2-[2'-pyrimidyl]hydrazine Sulphate

CHART V

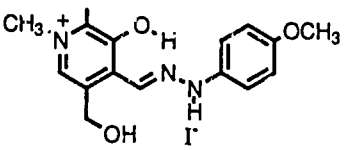
Pyridoxal-Betaine Salts of Aryl Hydrazones

1-[N^1-Alkylpyridoxylidenium]-2-[Aryl]hydrazine Halides



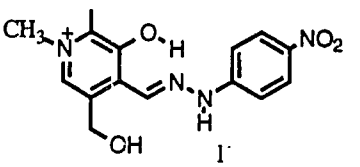
(22)

1-[N-Methylpyridoxylidenium]-2-[phenyl]hydrazine



(23)

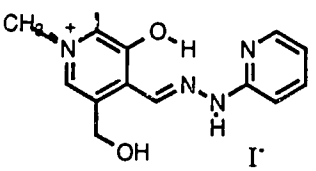
1-[N-Methylpyridoxylidenium]-2-[4'-methoxy-phenyl]hydrazine



(24)

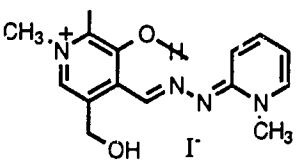
1-[N-Methylpyridoxylidenium]-2-[4'-nitrophenyl]hydrazine

1-[N^1-Alkylpyridoxylidenium]-2-[Heteroaryl]hydrazine Halides



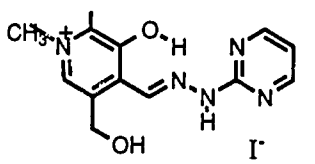
(25)

1-[N-Methylpyridoxylidenium] -2-[2'- pyridyl]hydrazine Iodide



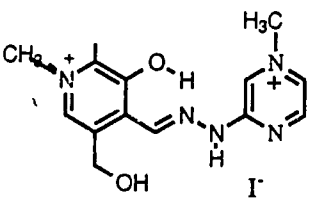
(26)

1-[[N-Methylpyridoxylidenium] -2-[2"-N-methyl pyridinium]hydrazine Iodide



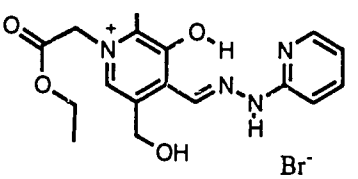
(27)

1-[N-Methylpyridoxylidenium] -2-[2"-pyrimidyl]hydrazine Iodide



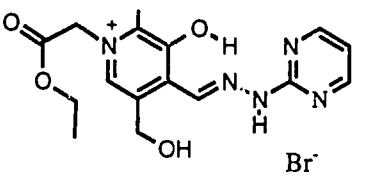
(28)

1-[N-Methylpyridoxylidenium]-2-[4'-methylpyrazyl]hydrazine Diiodide



(29)

1-[N-Methylpyridoxylidenium] -2-[2'- pyridyl]hydrazine Bromide

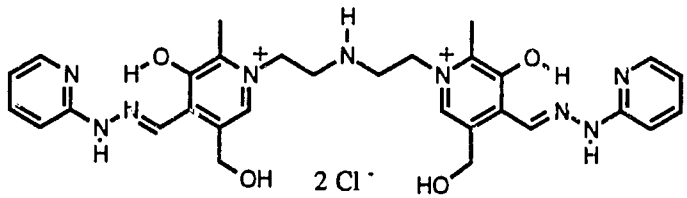


(30)

1-[N-Methylpyridoxylidenium] -2-[2'- pyrimidyl]hydrazine Bromide

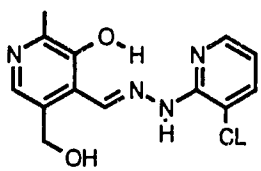
CHART V

.1-[N[1]-Alkylpyridoxylidenium]-2-[Heteroaryl]hydrazine Halides



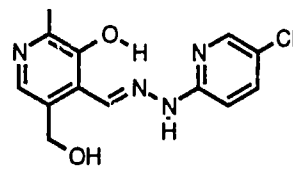
(31)

Di-{1-[N-Ethylpyridoxylidenium]-2-[2'-pyridyl]hydrazine}-amine Dichloride



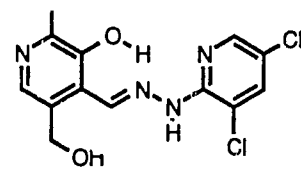
(32)

1-[Pyridoxylidene]-2-[3'-chloro-2'-pyridyl]hydrazine



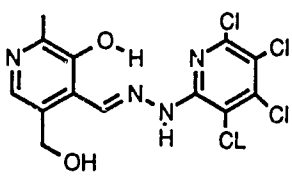
(33)

1-[Pyridoxylidene]-2-[5'-chloro-2'-pyridyl]hydrazine



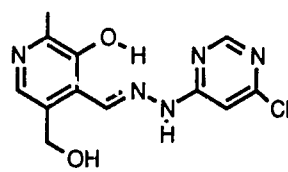
(34)

1-[Pyridoxylidene]-2-[3',5'-dichloro-2'-pyridyl]hydrazine



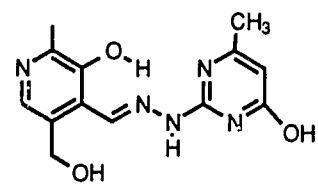
(35)

1-[Pyridoxylidene]-2-[3',4',5',6'-tetrachloro-2'-pyridyl]hydrazine



(36)

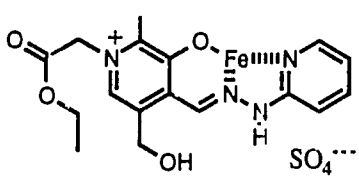
1-[Pyridoxylidene]-2-[6'-chloro-4'-pyrimidyl]hydrazine



(37)

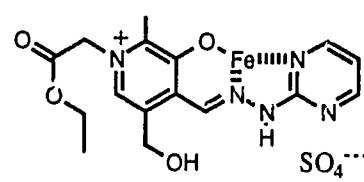
1-[Pyridoxylidene]-2-[4'hydroxy-6'-methyl-2'-pyrimidyl] hydrazine

Iron(II)=chelates of 1-[Pyridoxylidenium]-2-[2'-pyridyl] and [2'-pyrimidyl]hydrazine Sulphates



(38)

Iron(II)-chelate of (29)



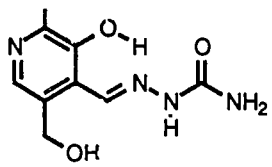
(39)

Iron(II)-chelate of (30)

CHART VI

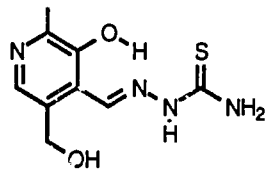
Hydrazidic Derivatives of Pyridoxal

Pyridoxal Semicarbazone and Thio-semicarbazone



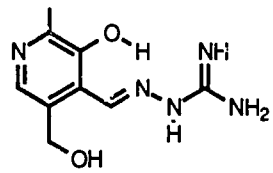
(41)

Pyridoxal Semicarbazone



(42)

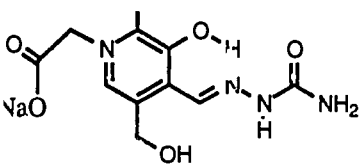
Pyridoxal Thiosemicarbazone



(43)

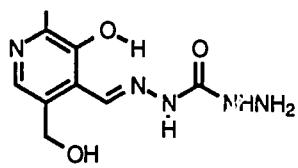
Pyridoxal Guanidinohyazone

Pyridoxal Carbhydrazone and Thiocarbhydrazone



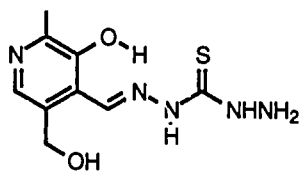
(44)

1^1-Sodiocarboxymethylpyridoxyl-denium Semicarbazone Chloride



(45)

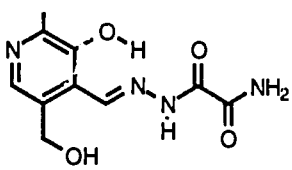
Pyridoxal Carbhydrazone



(46)

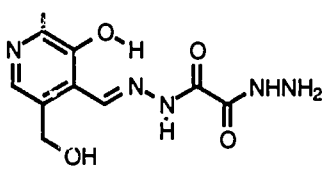
Pyridoxal Thiocarbhydrazone

Pyridoxal Semioxamazones



(47)

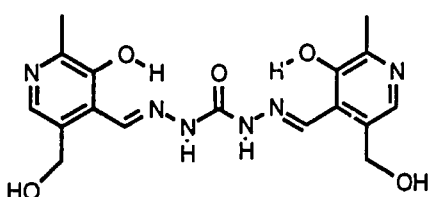
Pyridoxal Oxamic Acid Hydrazone



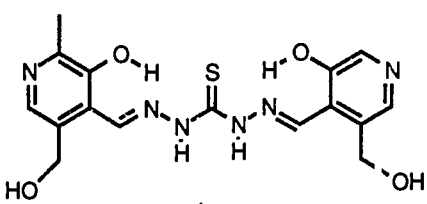
(48)

Pyridoxal Oxalic Acid Hydrazone

Bispyridoxal Carbohydrazone and Thiocarbohydrazone

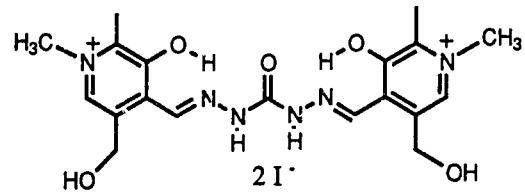


(49)



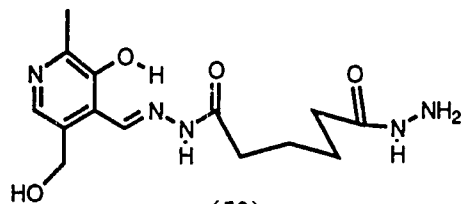
(50)

Bis-[N-Methylpyridoxylenium] Carbohydrazone Di-iodide



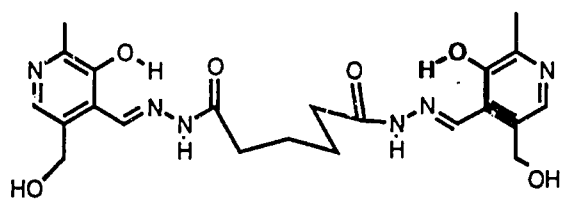
(51)

Mono- and Bis-Pyridoxal Adipic Acid Hydrazone



(52)

Pyridoxal Adipic Acid Hydrazone

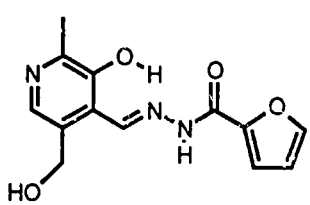


(53)

Bis-Pyridoxal Adipic Acid Hydrazone

CHART VII

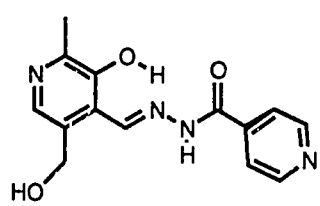
Pyridoxal Acyl Hydrazones



(54)

Pyridoxal 2-Furoyl Hydrazone

(PFH)

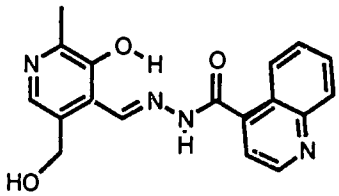


(55)

Pyridoxal Isonicotinoyl Hydrazone

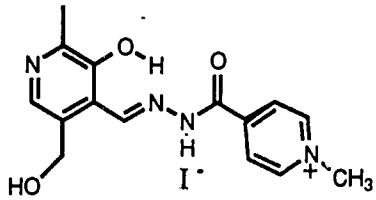
(PIH)

19



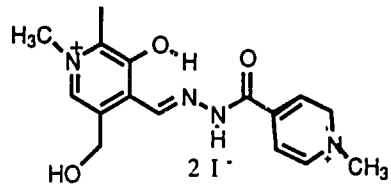
(56)

Pyridoxal 4-Quinoline Acyl Hydrazone



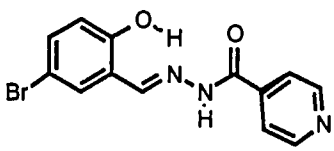
(57)

Pyridoxal N-Methylisonicotinoyl Hydrazone Iodide



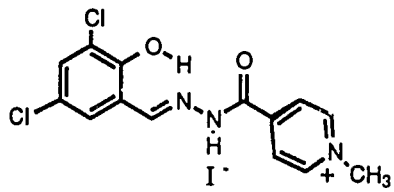
(58)

N-Methylpyridoxyledenium N-Methylisonicotinoyl Hydrazone Diodide



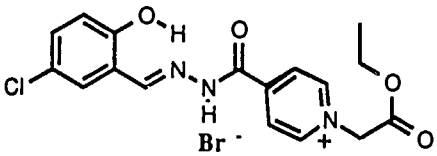
(59)

5-Bromosalicyl Isonicotinoyl Hydrazone



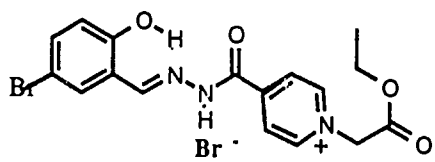
(60)

3,5-Dichloeosalicyl N-Methylisonicotinoyl Hydrazone Iodide



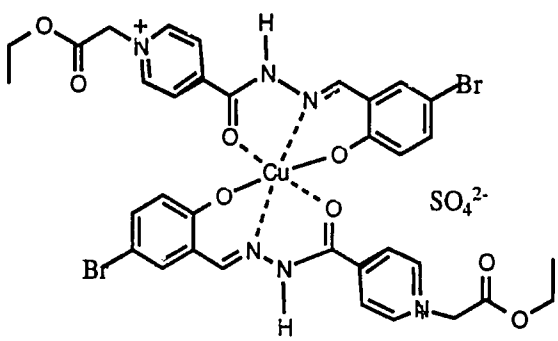
(61)

·Chlorosalicyl N-Ethoxycarbonylmethyl -isonicotinoyl Hydrazone Bromide



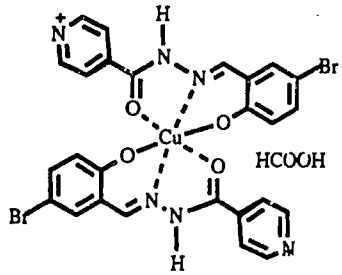
(62)

5-Bromorosalicyl N-Ethoxycarbonylmethyl -isonicotinoyl Hydrazone Bromide



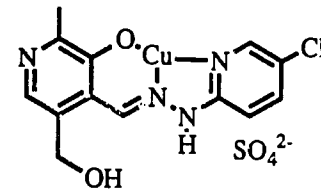
(63)

2:1-Ligand:Cu Complex of 5-Bromosalicyl N-Ethoxycarbonyl methylisonicoyinoyl Hydrazone Sulphate



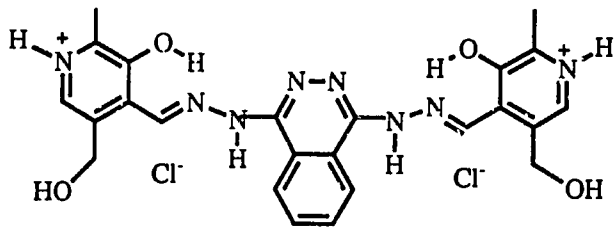
(64)

2:1-Ligand:Cu Complex of 5-Bromosalicyl Isonicoyinoyl Hydrazone Formate



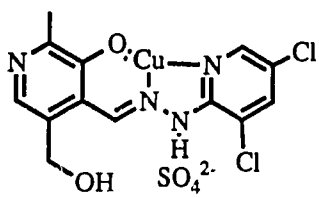
(65)

Copper(II)-chelate of (33)



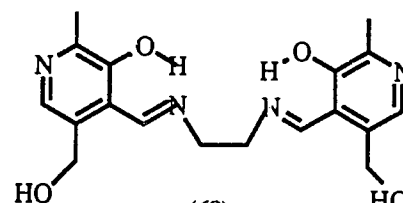
(67)

Bis-Pyridoxal 1,4-Phthalazinyl Dihydrazone Dihydrochloride



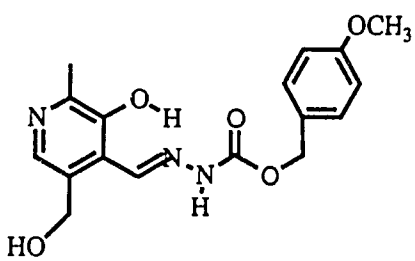
(66)

Copper(II)-chelate of (34)



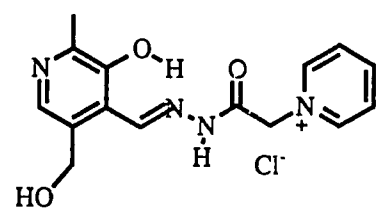
(68)

Bis-Pyridoxal Ethylene Diazine



(69)

Pyridoxal 4-Methoxy-benzyloxycarbonyl Hydrazone



(70)

1-[Pyridoxylidene]-2-[N-pyridinium-acetyl]hydrazine Chloride

Inhibition of Ribonucleotide Diphosphate Reductase

Some half of the chelators produced in this project underwent testings of activity against the metalloenzyme ribonucleotide diphosphate reductase (RdR). The enzyme was isolated from a subcutaneously growing murine tumor (sarcoma 180 implanted in $B_6D_2F_3$ male mice).

Tests of activity of the metalloenzyme, RdR, was followed by the method of Takeda and Weber, published in 1981[24]. Cytidine was separated from deoxycytidine by use of thin-layer-chromatography over a period of 4 hours on cellulose foil. Distribution of radioactivity was evaluated on an automatic thin-layer-chromatography linear analyzer. Hydroxyurea was used as a reference compound through all experiments. The results obtained are assembled in Tables 1-4, and illustrated by Fig. 1.

Biological Relevance of Results

From Tables **1-4,** and Fig. **1**, it can be seen that on the basis of their ability to inhibit the activity of RdR the chelating ligands could be divided into two distinctly different classes: 1) a group of very weak inhibitors of RdR, related to pyridoxal aroyl hydrazones, of which PIH is a representative; 2) a group of strong RdR inhibitors related to pyridoxal pyridyl hydrazone, represented by pyridoxal 2-pyridyl hydrazone (PPH, **10**). PIH is essentially non-toxic to the enzyme RdR. However, removal of the carbonyl group from the acyl-hydrazone bridge connecting the two pyridinic rings in PIH (PIH → PPH) confers anti-RdR activity to the molecule.Moreover, replacing the pyridyl ring in PPH by 2-pyrimidinic ring enhances activity against RdR. Likewise, chlorination of the pyridinic moiety in PPH (**10** → **34**) decreases effectively the activity of RdR. N-Alkylation of the pyridoxal ring-nitrogen manifests an opposite effect on the activity of the metalloenzyme.

Paradoxically, these trends do not parallel with those on growth of drug-resistant species of Plasmodium falciparum (CDR-3). Thus, whereas the non-active PPH becames highly active against P. falciparum as result of N^1-alkylation (**10** → **29**), the inhibitory effect of **29** drops below the level of hydroxyurea.Similarly, going from **29** to **30**, the antimalarial activity ***decreases*** whereas the anti-RdR activity ***increases.*** The same occurs on going from PPH to **34.**

The data produced here indicates that there is no direct correlation between the ability of the chelating ligands to inhibit the growth of **Plasmodium falciparum (CDR-3) in vitro,** ***and their ability to inhibit the action of*** **RdR.** ***The anti-RdR activity in the killing process of the parasite appears to be one factor among others.***

Table 1

. **Inhibition of Ribonucleotide Diphosphate Reductase by Chelators**

Chelator No.	Formula	Molecular Weight	Concentration (mM)	Inhibition of RdR (%) Chelator	Hydroxy urea
1	$C_{12}H_9N_3OCl_2$	282	1.0	-	82
			0.033	65	-
			0.0033	34	-
			0.00033	12	-
2	$C_{12}H_{10}N_3OBr$	292	0.01	0	--
3	$C_{13}H_{12}N_3O_{1.5}Cl_2I$	432	0.033	58	--
			0.0033	34	--
			0.00033	8	--
10	$C_{13}H_{15}N_4O_2Cl$	294.5	0.077	72	--
11	$C_{12}H_{13}N_5O_2$	259	1.0	78	73
			0.1	40	37
			0.01	30	15
14	$C_{13}H_{13}N_7O_2$	299	1.0	-	81
			0.033	72	---
			0.0033	21	--
			0.00033	18	--
16	$C_{17}H_{18}N_4O_4$	342	1.0	65	73
			0.1	68	37
			0.01	31	15

Table 2

INHIBITION OF RIBONUCLEOSIDE DIPHOSPHATE REDUCTASE BY CHELATORS

INHIBITION TEST

Chelator _No.	Formula	Molecular Weight	Concentration (mM)	Inhibition of RdR (%) Chelator	Hydroxy urea
17	$C_{14}H_{15}N_5O_3$	301	1.0	84	73
			0.1	45	37
			0.01	27	15
18	$C_{16}H_{17}N_5O_4$	343	1.0	79	73
			0.1	43	37
			0.01	24	15
20	$C_{13}H_{14}N_4O_6SCu$	417.5	1.0	90	73
			0.1	90	37
			0.01	35	15
21	$C_{12}H_{17}N_5O_8SCu$	454.5	1.0	86	73
			0.1	78	37
			0.01	38	15
26	$C_{15}H_{21}N_4O_3I$	432	1.0	-	81
			0.01	0	15
27	$C_{15}H_{19}N_4O_2I$	414	0.01	0	15
29	$C_{17}H_{20}N_4O_4Br$	423	0.01	0	15
30	$C_{16}H_{20}N_5O_4Br$	425	1.0	79	72
			0.1	76	--
			0.01	79	15

Table 3

ACTIVITY OF PYRIDOXAL-BASED CHELATORS IN THE RIBONUCLEOSIDE DIPHOSPHATE REDUCTASE (RdR) INHIBITION TEST

Chelator No.	Formula	Molecular Weight	Concentration (mM)	Inhibition of RdR (%) Chelator	Hydroxy urea
30	$C_{16}H_{20}N_5O_4Br$	425	1.0	79	72
			0.1	76	--
			0.01	79	15
34	$C_{13}H_{13}N_4O_2Cl_3$	362.5	1.0	83	72
			0.1	78	--
			0.01	79	15
35	$C_{13}H_{11}N_4O_2Cl_5$	432.5	1.0	43	72
			0.1	18	--
			0.01	25	15
37	$C_{13}H_{15}N_5O_3$	289	0.01	0	15
56	$C_{18}H_{17}N_4O_3Cl$	372.5	1.0	--	81
			0.027	63	--
			0.0027	32	--
			0.00027	11	--
57	$C_{15}H_{17}N_4O_3I$	428	0.01	0	15
58	$C_{16}H_{20}N_4O_3I$	570	0.01	0	15
60	$C_{14}H_{12}N_3O_2Cl_2I$	452	0.01	0	15

Table 4

ACTIVITY OF PYRIDOXAL-BASED CHELATORS IN THE RIBONUCLEOSIDE DIPHOSPHATE REDUCTASE (RdR) INHIBITION TEST

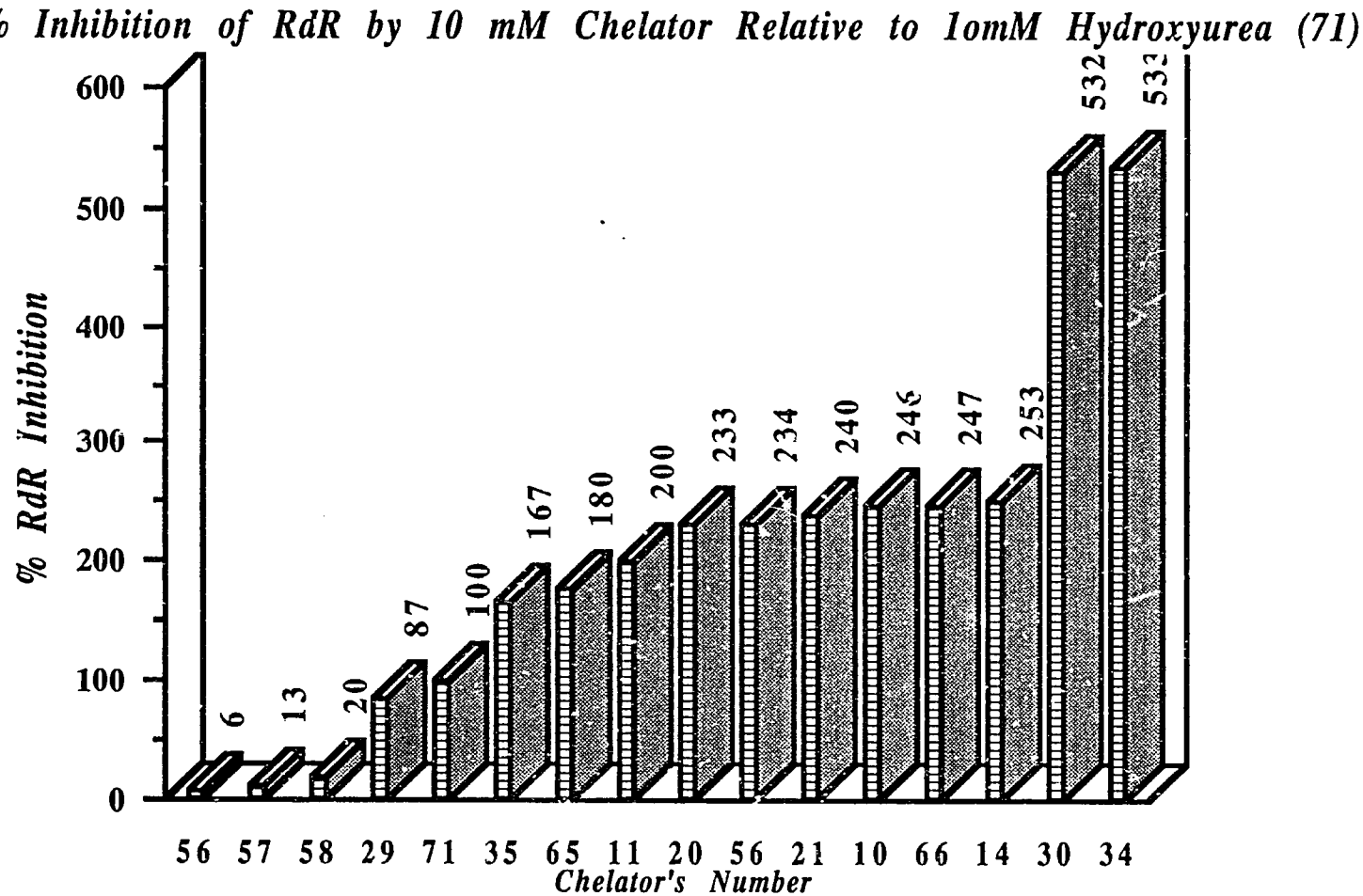
Chelator No.	Formula	Molecular Weight	Concentration (mM)	Inhibition of RdR (%) Chelator	Hydroxy urea
61	$C_{17}H_{17}N_3O_4BrCl$	441.5	1.0	--	81
			0.02	77	--
			0.002	10	--
			0.0002	18	--
62	$C_{17}H_{17}N_3O_4Br_2$	505	1.0	-	81
			0.01	0	15
63	$C_{34}H_{134}N_6O_{11}Br_4S_{0.5}Cu$	1103.5	1.0	71	72
			0.1	64	--
			0.01	35	15
64	$C_{27}H_{20}N_6O_6Br_2Cu$	749.5	1.0	59	73
			0.1	28	--
			0.01	22	15
65	$C_{13}H_{13}N_4O_6ClSCu$	452	1.0	82	81
			0.1	65	--
			0.01	27	15

26

ACTIVITY OF PYRIDOXAL-BASED CHELATORS IN THE RIBONUCLEOSIDE DIPHOSPHATE REDUCTASE (RdR) INHIBITION TEST

Chelator No.	Formula	Molecular Weight	Concentration (mM)	Inhibition of RdR (%) Chelator	Hydroxy urea
66	$C_{13}H_{12}N_4O_6Cl_2SCu$	486.5	1.0	85	81
			0.1	83	--
			0.01	37	15
67	$C_{24}H_{26}N_8O_4Cl_2$	561	0.01	0	15
68	$C_{18}H_{22}N_4O_4$	358	0.01	0	15
69	$C_{17}H_{18}N_3O_5$	344	0.01	0	15

Figure 1


% Inhibition of RdR by 10 mM Chelator Relative to 1omM Hydroxyurea (71)
% RdR Inhibition
600
500
400
300
200
100
0
6
13
20
87
100
167
180
200
233
234
240
246
247
253
532
532
56 57 58 29 71 35 65 11 20 56 21 10 66 14 30 34
Chelator's Number

Inhibition of Growth of *Plasmodium falciparum*

EFFECT OF AQUEOUS SOLUTIONS OF CHELATORS OF VARIOUS CONCENTRATIONS ON GROWTH OF DRUG-RESISTANT SPECIES OF Plasmodium falciparum AFTER 2 HRS. EXPOSURE TO 40 μM OF THE POTENTIAL DRUGS[14,26].

The new series of pyridoxal-based chelators **(2)** - **(70)** were tested for their inhibitory effect on the growth of drug-resistant species of Plasmodium falciparum during a period of 2 hours of incubation.

Parasites were synchronized by the gelatin sedimentation method and diluted with fresh red cells to 0.3% parasitemia and 4% haematocrit. They were dispensed into various six-wells macro plates and left to stand for about an hour. The medium was then replaced with 40 μM of the various chelators. The parasites were then incubated in the chelator medium for 2 hours at 37°C. After the 2 hrs. incubation period, the chelator medium was washed out and the parasites washed thrice with RPMI washing solution. They were then cultured in normal growth medium. The concentrations were maintained throughout the three days culture period. Parasite growth was determined by both microscopic slide counts and incorporation of ^{3}H-hypoxanthine[14,25,26].

The chelator solutions were prepared by dissolving the appropriate amount in a minimal volume of double distilled water to give a stock solution of 1 mM. Further serial dilutions were done in RPMI growth medium to give the required working concentrations of 2, 5, 10, and 20 μM.

EFFECT OF CHELATORS ON GROWTH OF DRUG-RESISTANT SPECIES OF Plasmodium falciparum AFTER 2 HRS. EXPOSURE TO 40 μM OF CHELATORS IN DIMETHYL SULFOXIDE (DMSO)

Parasites were synchronized by the gelatin sedimentation method and diluted with fresh red cells to 0.3% parasitemia and 4% haematocrit. They were dispensed into various six-wells macro plates and left to stand for about an hour. The medium was then replaced with 40 μM of the various chelators. The parasites were then incubated in the chelator medium for 2 hours at 37°C. After the 2 hrs. incubation period, the chelator medium was washed out and the parasites washed thrice with RPMI washing solution. They were then cultured in normal growth medium. The concentrations were maintained throughout the three days culture period. Parasite growth was determined by both microscopic slide counts and incorporation of ^{3}H-hypoxanthine.

Because of low solubility in water, chelators no. **4, 5, 9, 11, 17, 18, 24, 29, 38, 55,** and 57, were first dissolved in 1 molar aqu. DMSO. They were thereafter diluted with RPMI washing solution to the required working concentrations. The same concentration of DMSO was added to the RPMI washing solution used as the control. First concentration of DMSO in the test solutions was 0.01 M. A positive control was normal RPMI washing solution, namely, without DMSO.

Biological Importance of Results

Structure-Activity-Relationship study (see, Chart VIII) indicated strong electronic influence on antimalarial activity. Thus, the antimalarial activity **decreases** dramatically on **23** → **22** → **7**, and **25** → **27** → **22** transformations. But, it **increases** on **24** → 9, and 22 → 23 conversions. Electron-releasing substituents (methoxy group, OCH_3) on the aromatic ring **enhances** the antimalarial activity, whereas electron-withdrawing substituents (NO_2, COOH) exerts an opposite effect. Moreover, the π-electron densities, and the pKa values of the heterocyclic ring of the ligands profoundly affects the antimalarial activity.

From Tables 7-11, and Fig. 2, it can be seen that 1-[pyridoxylidene]-2-[1'-phthalazyl] hydrazine (**13**), 1-[N-ethoxycarbonylmethylpyridoxylidenium]-2-[2'-pyridyl]hydrazine (**29**), pyridoxal semicarbazone (**41**), and pyridoxal oxalic acid hydrazone (**48**) are outstanding in their ability to inhibit in vitro the growth of the chloroquine-resistant *Plasmodium falciparum* (FCR-3). The corresponding IC_{50} values (concentration causing 50% inhibition of growth) found were :

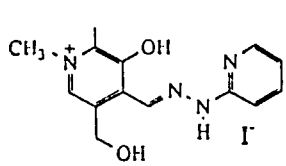
IC_{50} : (**13**) = 1.2 μM ; (**29**) = 1.9 μM; (**41**) = 14.2 μM;

It is worthy of note that chloroquine, which is effective *only* against the *sensitive* species of *Plasmodium falciparum* , has the value IC_{50} = **0.12** μM, and quinine has the value of IC_{50} = **0.30** μM[27].

The results produced here clearly indicate that pyridoxal hydrazones when properly substituted provide novel class of antimalarials operating on distinctly different mode of action as reported earlier in 1990 in a series of three articles [14,25,26].

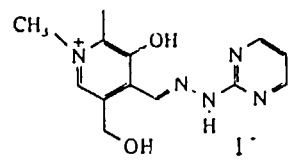
Substituent Effects on Growth Inhibition of Didentate and Tridentate Chelators Based on Pyridoxal

Inhibition (%) of [^{3}H] hypoxanthine Incorporation by Chelators at 40μM Concentration



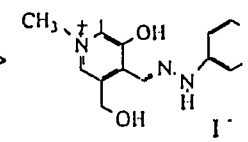
ridentate **88%**

>

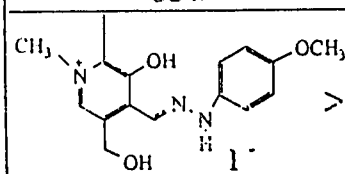


tridentate **55%**

>

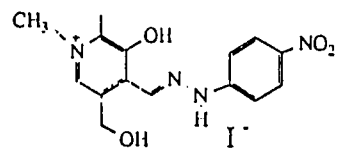


didentate **33%**

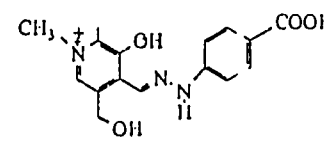


didentate . **61%**

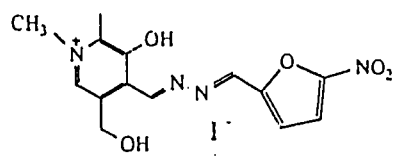
>



didentate , **28%**



inactive, (%



didentate , **33%**

Electron-Release Ordering : OCH_3 > H ;
Electron-Withdrawing Ordering : H < NO_2 ≥ COOH

π-Electron Density Ordering :

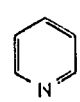
π-excessive

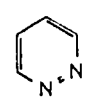
<

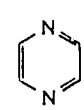
<

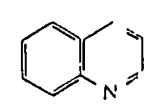
π–defic ent

Basicity









pK_a , 20°C	5.23	3.5	1.3	0.6	4.93

Table 5

INHIBITION OF GROWTH OF DRUG-RESISTANT SPECIES OF Plasmodium falciparum AFTER 2 HRS. EXPOSURE TO 40 μM OF CHELATORS

Chelato No.	FORMULA	MW	SLIDE COUNTS % PARTISEMIA DAY 1	DAY 2	DAY 3	^{3}H-HYPOXANTHINE INCORPORATION COUNT	% GROWTH
	control (norm. Med)		0.91	1. 6.91		44861	100
2	$C_{12}H_{10}N_3OBr$	292	0.83	1.91	4.08	33981	75
3	$C_{13}H_{12}N_3O_{1.5}Cl_2I$	432	0.58	1.41	3.25	19677	43
7	$C_{15}H_{16}N_3O_4Cl$	337.5	2.16	1.91	14.70	60714	100
10	$C_{13}H_{15}N_4O_2Cl$	294.5	1.08	1.75	3.25	45515	100
13	$C_{16}H_{18}N_5O_{2.5}Cl_2$	391	0.58	0.41	0.66	1960	5
19	$C_{18}H_{17}N_3O_{7.5}Na$	418	0.66	1.83	3.83	51311	100
25	$C_{14}H_{17}N_4O_2I$	400	0.41	0.58	1.41	5560	12
26	$C_{15}H_{21}N_4O_3I$	432	0.75	0.66	2.25	11952	26
27	$C_{15}H_{19}N_4O_2I$	414	0.83	1.58	2.91	20280	45
28	$C_{14}H_{20}N_5O_{2.5}I_2$	552	0.66	1.16	2.41	19158	42
29	$C_{17}H_{20}N_4O_4Br$	423	0.33	0.25	0.58	556	1
30	$C_{15}H_{20}N_5O_4Br$	425	0.83	1.33	4.5	22204	49
41	$C_9H_{13}N_4O_3Cl$	260.5	0.91	0.41	0.58	2032	3
42	$C_9H_{13}N_4O_2SCl$	276.5	1.58	2.08	15.70	59550	100
44	$C_{11}H_{14}N_4O_5ClNa$	330.5	1.91	2,08	16.1	50604	84

Table 6

EFFECT OF CHELATORS ON GROWTH OF DRUG-RESISTANT SPECIES OF Plasmodium falciparum AFTER 2 HRS. EXPOSURE TO 40 μM OF CHELATORS

Chelato No.	FORMULA	MW	SLIDE COUNTS % PARTISEMIA			^{3}H-HYPOXANTHINE INCORPORATION	
			DAY 1	DAY 2	DAY 3	COUNT	% GROWTH
	control (norm. Med)		0.91	1.83	6.91	44861	100
45	$C_9H_{14}N_5O_3Cl$	275.5	1.66	1.66	13.4	54067	90
46	$C_9H_{14}N_5O_2SCl$	291.5	1.50	1.25	11.6	64650	100
47	$C_{10}H_{13}N_4O_4Cl$	288.5	1.58	1.58	12.74	31414	83
48	$C_{10}H_{14}N_5O_4Cl$	303.5	0.33	0.25	0.58	886	2
49	$C_{17}H_{22}N_6O_5Cl_2$	461	1.58	2.16	14.3	55826	93
50	$C_{17}H_{22}N_6O_4SCl$	441.5	1.83	1.50	14.6	59765	100
51	$C_{19}H_{26}N_6O_5I_2$	672	1.50	1.91	11.25	22786	60
53	$C_{17}H_{22}N_6O_4SCl_2$	477	1.25	1.91	11.25	31165	83
54	$C_{13}H_{13}N_3O_4$	275	1.58	2.16	14.58	54227	90
55	$C_{14}H_{15}N_4O_3I$	322.5	2.08	1.58	10.75	34192	91
57	$C_{15}H_{17}N_4O_3I$	428	1.91	2.16	15.7	66296	100
61	$C_{17}H_{17}N_3O_4BrCl$	441.5	0.91	1.25	4.25	29203	65
70	$C_{15}H_{17}N_4O_3Cl$	336.5	2.16	2.08	12.75	39054	100

Figure 2

rowth Ihibition of P. falciparum After 2 hrs. Exposure to 40 mM of Chelator

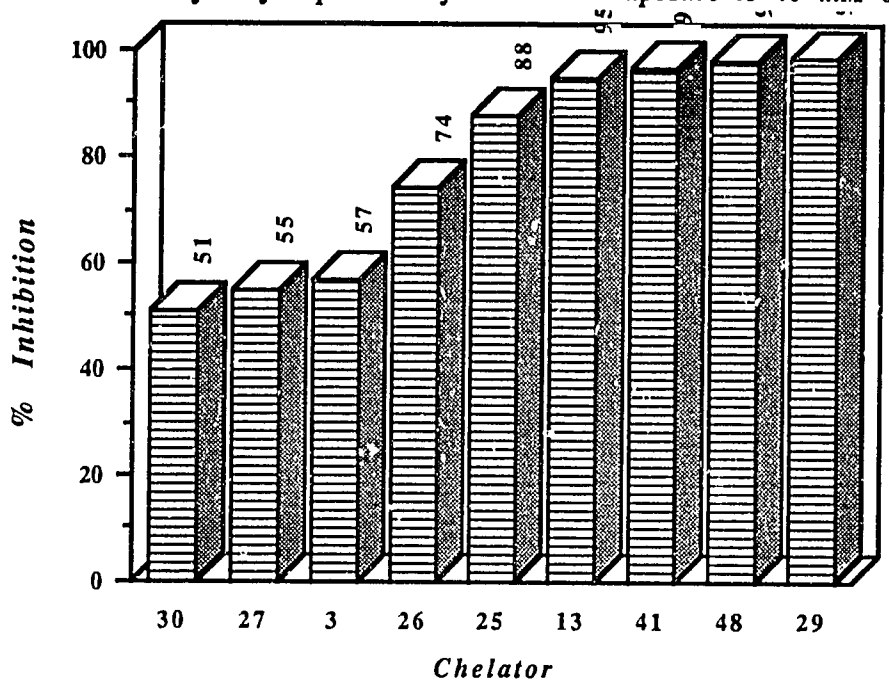


Table 7

EFFECT OF CHELATORS ON GROWTH OF DRUG-RESISTANT SPECIES OF Plasmodium falciparum AFTER 2 HRS. EXPOSURE TO 40 μM OF CHELATORS IN DIMETHYL SULFOXIDE (DMSO)

Chelato No.	FORMULA	MW	SLIDE COUNTS % PARTISEMIA			^{3}H-HYPOXANTHINE INCORPORATION	
			DAY 1	DAY 2	DAY 3	COUNT	% GROWTH
4	$C_{14}H_{15}N_3O_2$	257	2.53	3.75	17.25	115726	67
5	$C_{15}H_{18}N_3O_3Cl$	323.5	1.25	2.30	8.25	68214	39
9	$C_{13}H_{12}N_4O_5$	304	1.83	3.75	13.5	115715	67
11	$C_{12}H_{13}N_5O_2$	259	1.0	1.41	3.58	48939	28
17	$C_{14}H_{15}N_5O_3$	301	2.58	3.25	17.75	131861	76
18	$C_{16}H_{17}N_5O_4$	343	1.92	3.30	15.50	125781	72
24	$C_{15}H_{19}N_4O_5I$	462	2.41	3.58	16.50	124105	72
29	$C_{18}H_{24}N_4O_4Br$	440	0.40	0.58	1.16	15744	9
38	$C_{17}H_{22}N_4O_9BrSFe$	594	1.75	3.58	18.25	132132	76
55	$C_{14}H_{15}N_4O_3Cl$	322.5	2.83	3.75	18.25	137085	79
57	$C_{15}H_{17}N_4O_3I$	428	1.58	3.25	18.25	160562	93
Norm. Medium without DMSO			2.92	3.50	18.50	184826	100
Norm. Medium with 0.01 M DMSO			2.75	3.83	19.50	172951	100

35

Table 8

EFFECT OF CONTINOUS EXPOSURE OF Plasmodium falciparum TO VARIOUS CONCENTRATIONS OF CHELATORS

Inhibition Test

COMPOUND	FORMULA	CONCENTRATION (μM)	% INHIBITION DAY 1	DAY 2	DAY 3
4	$C_{14}H_{15}N_3O_2$	5	10	20	10
		10	--	28	21
		20	41	31	37
5	$C_{15}H_{18}N_3O_3Cl$	5	14	3	8
		10	28	9	32
		20	50	44	45
19	$C_{18}H_{17}N_3O_{7.5}Na$	5	23	22	33
		10	41	45	60
		20	50	56	79
25a	$C_{14}H_{17}N_4O_2I$	2	63	83	98
		5	74	83	98
		10	71	91	100
		20	74	100	100
25b	$C_{14}H_{21}N_4O_3I$	2	64	79	98
		5	64	83	99
		10	71	87	99
		20	76	100	100

36

Table 9

Inhibition Test

COMPOUND	FORMULA	CONCENTRATION (μM)	% INHIBITION DAY 1	DAY 2	DAY 3
27	$C_{15}H_{19}N_4O_2I$	2	3	33	45
		5	32	68	96
		10	58	87	98
		20	71	91	99
28	$C_{14}H_{20}N_5O_{2.5}I_2$	2	21	0	52
		5	28	33	96
		10	48	89	100
		20	57	89	100
29	$C_{18}H_{24}N_4O_4Br$	2	34	40	40
		5	58	75	97
		10	68	87	98
		20	76	100	100

Table 10

EFFECT OF CONTINOUS EXPOSURE OF Plasmodium falciparum TO VARIOUS CONCENTRATIONS OF CHELATORS

Inhibition Test

COMPOUND	CONCENTRATION (mM)	% PARASITEMIA DAY 1	DAY 2	DAY 3	% INHIBITION OF GROWTH ON DAY 3
13	5	57	50	33	67
	10	35	31	20	80
29	5	93	68	53	47
	10	71	43	33	67
41	5	93	93	97	3
	10	100	100	90	10
48	5	93	100	71	26
	10	93	68	67	33
55	5	78	68	58	42
	10	65	44	44	56
Artemisin	5	21	100	100	100
	10	21	100	100	100

38

Table 11

EFFECT OF 3 DAYS EXPOSURE OF Plasmodium falciparum TO VARIOUS CONCENTRATIONS OF CHELATORS

Inhibition Test

COMPOUND	CONCENTRATION (mM)	3H-HYPOXANTHINE INCORPORATION COUNT	INHIBITION %
13	5	4199	71
	10	2697	82
29	5	9390	34
	10	6425	55
41	5	16162	0
	10	17168	0
48	5	15877	0
	10	10621	26
55	5	9779	32
	10	7828	45
artemisin	5	0	100
	10	0	100

ab initio CALCULATIONS OF BINDING ENERGIES AND DISTRIBUTION OF ELECTRONIC CHARGES IN SOME LIGANDS

Structure-activity-relationship study indicated that the antimalarial activity of ligands included in this investigation appears to be controlled by electronic effects. In view of this we deemed it important to calculate distribution of electronic charges in (**29**), most promising new antimalarial candidate against chloroquine-resistant species, and in related active compounds. In addition, we undertook the determination of proton binding energies at four sites of ligand (10) for assignment of pKa values derived by potentiometric titrations

Proton Binding Energies at Ionisible Sites of 1-[Pyridoxylidene]-2-[Pyridyl]hydrazine (**10**)

SCF Calculations

Ab initio calculations were based on sto-3g set[28]. Full optimization was performed for the unprotonated species, L^-, of (**10**). For each computation of proton-binding, the optimization was confined to site position of binding. The results of Muliken population analysis[29] and its protonated species were calculated by CNDO / 2[30], were attested by comparison with data produced for (**10**) by use of *ab initio* calculations.The results summerized in Table **12** were obtained from two different modes of calculation. Qualitatively, they are identical. The distribution of electronic charges around the 19-21 atoms in (10), (25), (29), and PIH are presented in Table **13.** All calculations were performed by use of IBM RISC / 6000 computer of The Hebrew University of Jerusakem.

Table 12

The Total and the Relative Energies of Binding of Protons at Various Sites of Pyridoxal Pyridyl Hydrazone (10)

Binding Sites	Total Energy[a] (in atmoic units)	Binding Energy[b] (in kcal/mol)
L^-	- 854.33597	---
O^2	- 855.47231	713.05
$N^{3''}$	- 855.35986	642.49
$O^{1'}$	- 855.24823	572.44
$N^{1''}$	- 855.16845	522.38
$N^{4''}$	- 855.11898	491.34

a) *ab initio* STO-#G basis set calculations; b) The binding energy relative to the deprotonated species L^-

Table 13

Distribution of Electronic Charges in (10), (25), (29), and PIH

Atom	(10)	(25)	Δ^1	(29)	Δ^2	PIH	Δ^3
N(1)	−0.146	0.034	−0.180	0.032	−0.177	−0.142	−0.003
C(1)	0.047	0.098	−0.051	0.095	−0.048	0.046	0.001
C(2)	0.008	0.012	−0.004	0.014	−0.006	0.010	−0.002
C(3)	-0.026	0.052	-0.078	0.050	- 0.076	-0.028	0.002
C(3)	-0.026	0.052	-0.078	0.050	- 0.076	-0.028	0.002
C(4)	0.167	0.165	0.001	0.167	- 0.000	0.171	-0.004
C(5)	0.052	0.113	-0.060	0.111	- 0.059	0.051	0.001
O(1)	- 0.250	-0.231	-0.019	- 0.233	- 0.017	-0.249	-0.001
C(6)	- 0.019	-0.037	0.019	- 0.039	0.021	-0.019	0.000
C(7)	0.142	0.140	0.002	0.140	0.002	0.142	0.001
O(2)	- 0.267	-0.264	- 0.003	- 0.265	-0.003	- 0.266	- 0.001
N(3)	- 0.161	-0.143	- 0.018	- 0.143	- 0.018	- 0.157	- 0.004
C(9)	0.217	0.208	0.009	0.209	0.008	- 0,004	--
C(10)	- 0.083	- 0.070	- 0.012	- 0.071	- 0.012	- 0.010	--
C(11)	0.064	0.069	- 0.005	0.069	- 0.005	0.073	--
C(12)	- 0.055	- 0.042	- 0.013	-0.043	- 0.012	0.072	--
C(13)	0.102	0.106	- 0.004	0.106	- 0.004	0.001	--
N(4)	- 0.209	- 0.213	0.004	- 0.215	0.006	-0.136	- 0.073
C(8)	0.062	0.024	- 0.037	0.027	- 0.034	0.071	- 0.009
N(2)	- 0.051	0.002	- 0.053	0.000	- 0.052	- 0.047	-0.005
CC(1)	--	--	--	--	--	0.333	--
O(3)	--	--	--	--	--	- 0.336	--

Δ^1 – is the difference in charge between respective atoms of (**10**) and (**25**) ; Δ^2 - is the charge difference between respective atoms of (**10**) and (**29**), and Δ^3 relates to similar diffrences in charge between PIH and (**10**)

Biological Relevance of Results

From ab initio calculation data assembeled in Table 12 it can be seen that the ease of proton removal from the possible binding sites decreases in the ordering:

$$\text{PYR ring-}N^4 > \text{Pyridoxal-ring } N^1 > O^1H > N^3H > O^2H$$

Thus, the highest binding energy rests between H and alcoholic oxygen (O^2), whereas the binding-energy between H and phenolic oxygen (O^1) is 140.61 kcal/mol smaller.The least binding energy is between H and pyridinic ring-N^4, which is 31.04 kcal/mol smaller than the pyridoxyl-N^1, which in turn, is 50.06 kcal/mol smaller than that of the phenolic bond.

Proton Binding Constants and Species Distribution Over pH Range 1.5 - 12.0

Two of the most active antimalarials produced in this study, **(13)** and **(29)**, consist of two different rings, each of which exists in the aqueous media as several prototropic species. The behaviour of the pyridoxal moiety has been thoroughly analyzed, and this species was shown to be present in aqueous media under at least four pH-dependent structures.ab initio Calculations of the parent compound, **(10)**, have shown that it possesses 4 proton ionizable sites, and, depending on pH, it could exist in aqueous solution in 4 different structural species.We deemed it essential to identify the pH range in which the antimalarial compounds could largely exist in the neutral form adequate for iron chelation. Towards this end we undertook a study aimed at determining the pKa's, and species distribution of **(29)**, and of three related compounds, **(10)**, **(25)**, and **(55)**.

Protonation Constants

Stock solutions.

Nitric acid and sodium hydroxide solutions were obtained from "Merck" (Titrisol). The concentrations of these solutions were checked regularly by acid-base titrations in the course of electrode calibration[31]. Stock solutions of 0.2 mM of **(10)**, **(25)**, **(29)**, and PIH were freshly prepared before each titration by dissolving the respective ligand in in 0.15 M KNO_3 (Merck Analytical grade). Double-distilled water was used throughout the experiments.

40'

Potentiometric titrations

Potentiometric (pH) titrations were used to determine the protonation constants for ligands **(10)**,**(25)**, **(29)**, and **(55)** . Potentiometric titration data were obtained using a purpose-built automatic titrator of PHM62 Radiometer pH meter, with a 5 ml piston burette, a digital electrometer and stirrer, which all were under the control of a programable calculator.Potentiometric measurements were carried out in a capped titration vessel, fitted with a GK 2322C combine electrode and a fibre-tipped saturated calomel electrode.The titrations were performed under inert atmosphere by flowing pure N_2 to the titration vessel. The entire apparatus was kept in a thermostate at 37 ± 0.5°C. The electrode calibrations were made in a 0.15 M KNO_3 at 37°C by acid-base titration[31] under identical above conditions.

Analysis of the titration data for **(10)**, **(25)**, **(29)**, and PIH was generally performed using the SUPERQUARD computer program[32] under IBM RISC / 6000 computer machine of The Hebrew University of Jerusalem. The data were between 300 and 500 for the calculations.

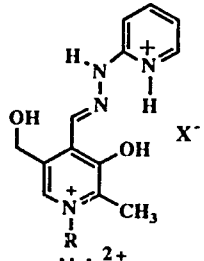
Species Distribution Plot as a Function of pH

Analysis of the titration curves for **(10)**, **(25)**, **(29)**, and **PIH** yielded four pKa values for the two unsubstituted ligands, **(10)** and **PIH,** and three each for the other two N^1-alkylated ligands, **(25)** and **(29)**. Plotting of species distribution in solution of PIH, (10), (25), and (29) *against* pH, in the range pH 1.5-12.5 (Figures **3-4a-c**, and Table 14), clearly indicates that at pH < 3, three to four sites on the molecules are protonated. From plots in Fig. 3-4a-c, it can be seen that at pH 5.2, the molecules of **PIH** and **(10)** exist in solution as 4:1-mixtures of the *cationic* species of $\{H_3L^+\}$, and of the neutral form of $\{H_2L\}$, respectively, and with the absence of the *anionic* species, HL^-. The molecules of (25) and (29), by contrast, do not contain at all the *cationic* $\{H_3L^+\}$ species, but rather contain to some extent the *anionic* $\{HL^-\}$ species, in a 10:1 ratio of $\{H_2L\}$: $\{HL^-\}$. Most interestingly, at physiological pH (7.2), molecules **(10)** and **PIH** in solution exist respectively, as 2:1 and 1:1-mixtures of the neutral (H_2L) and of the singly charged *anionic* species (HL^-), whereas the corresponding ratio for N^1-alkylated molecules, (25) and (29), at the same pH (7.2), is 1:2 (see, Table 14). Evidently, lowering the physiological pH by 2 pH-units, confers predominancy to the neutral forms of the N^1-alkylated molecules, (25) and (29), causing loss of this dominancy to parent molecules, in (10), and in PIH.These results would indicate that the chelating agents would be lipophilic, and furthermore, that (25) and (29), would be able to diffuse through cell membrane , allowing absorption from the stomach and access to intracellular iron pools. In **PIH** and (10), the presence of the neutral species would be maximal at pH 6 and hence maximal absorption would probably occur in the small intestine where the pH is in this range[33]. The

CHART XI

Pyridoxal Pyridyl Hydrazone
Protonated-Neutral-Deprotonated Species

Preferred Conformation : trans-transoid



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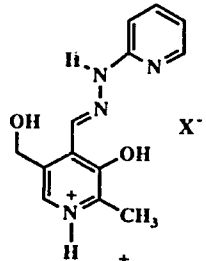

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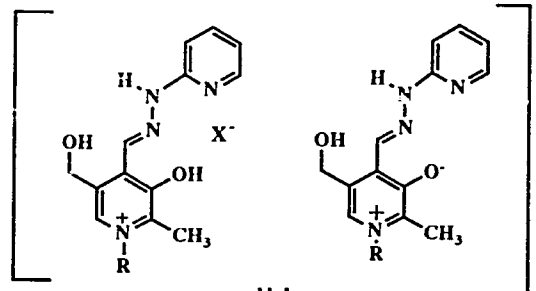

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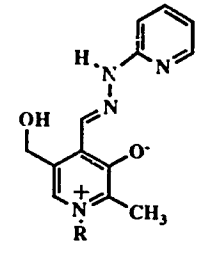

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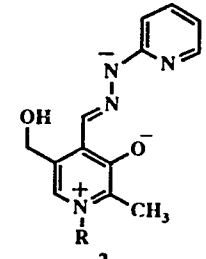
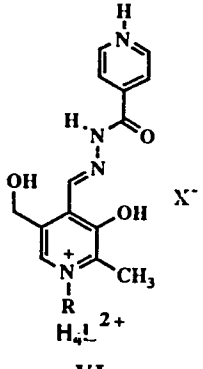


V

Pyridoxal Isonicotinoyl Hydrazone

PIH



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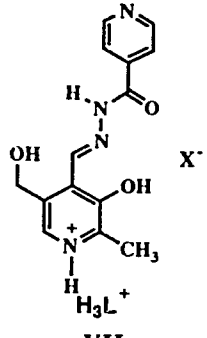

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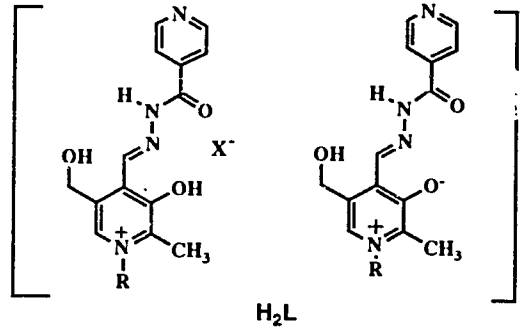

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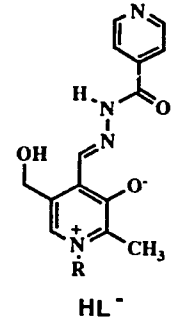

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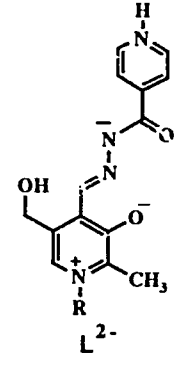


X

enhancement of percentage of H_2L species on pH decrease from 7.2 to 5.2 as result of N^1-alkylation (**10** → 29 conversion) could explain, at least partly, the observed remarkable enhancement in the *in vivo* antimalarial activity on **25** ← 10 → 29 conversions. All ligands are virtually fully deprotonated at pH > 11. The species distribution of (**10**), (**25**), (**29**), and **PIH** as a function of pH are shown in Figures 3 - 4a-c. They agree well with the data of related structures in the literature[34,35,36].

Table 14

Distribution (%) of H_3L^+, H_2L, and HL- Species in Solution of (10), (25), (29), and PIH at pH 5.2 and 7.2

Ligand	pH = 5.2		pH = 7.2
	$\{H_3L^+\}$: $\{H_2L\}$	$\{H_2L\}$: $\{HL^-\}$	$\{H_2L\}$:$\{HL^-\}$
(10)	*20 : 80*	------	*65 : 35*
(25)	-----	*87 : 13*	*33 : 67*
(29)	-----	*93 : 7*	*37 : 63*
PIH	*17 : 83*	-----	*55* : **45**

The Assignment of pKa Values to Ionisable Sites $O^{1'}$, $N^{1''}$, $N^{3''}$, and N^4

The assignment of pKa values to ionisable sites ($O^{1'}$, $N^{1''}$, $N^{3''}$, and $N^{4''}$) on the ligands could not be made with confidence by coparison with its components : pyridoxal, the acid hydrizde, and related compounds[34], nor with PIH[35,36]. Caution is necessary because theoretical calculations indicate that pyridoxal pyridyl hydrazone (10) is likely to lose its pyridoxal ring N^1- proton first, prior to its phenolic proton. To avoid possible ambiguities, a ^{13}C NMR study as a function of pH for **(10)**, **(25)**, **(29)**, and **PIH**, was undertaken, in spite of difficulties due to low solubilities of the ligands at pH > 9.

Carbon-13 Nuclear Magnetic Resonance Study

The sensitivity of carbon-13 nuclear magnetic resonance (^{13}C NMR) to electronic structure is well known[37]. Luckily, the complete studies of the ^{13}C NMR spectroscopy of the pyridine

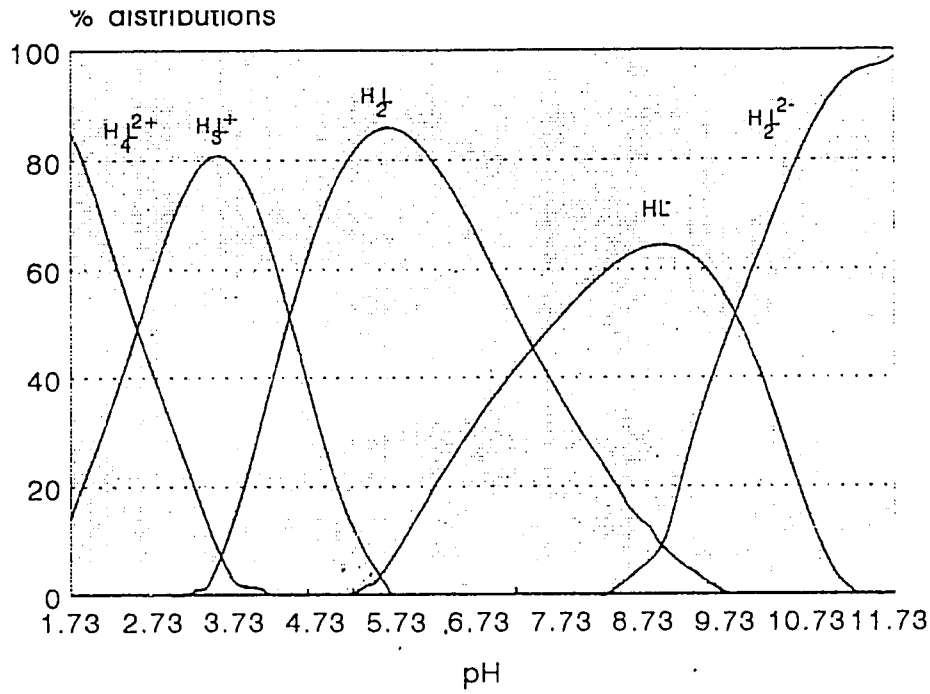
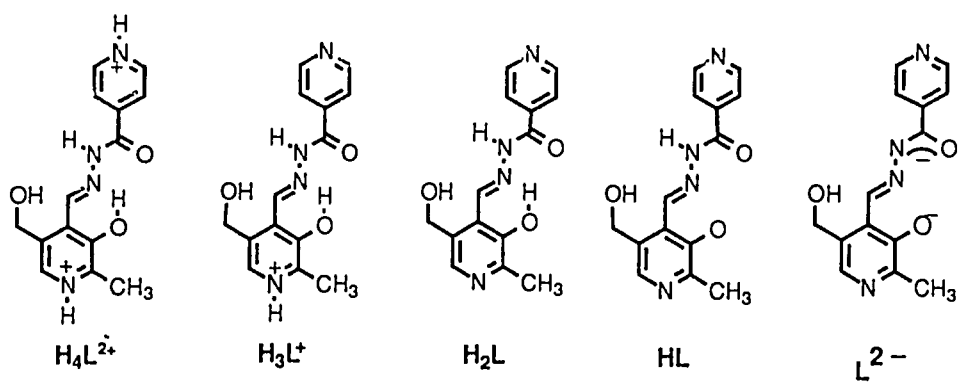


Figure 3. Species distribution plot over pH 1.5-12 for Pyridoxal Isonicotinoyl Hydrazone. H_4L^{2+} is the second protonated species at pyridinic N^4; H_3L^+ is the first protonated species at pyridoxal ring N^1; H_2L is neutral **PIH**; HL^- is the first deprotonated species; HL^{2-} is the second deprotonated species of **PIH**



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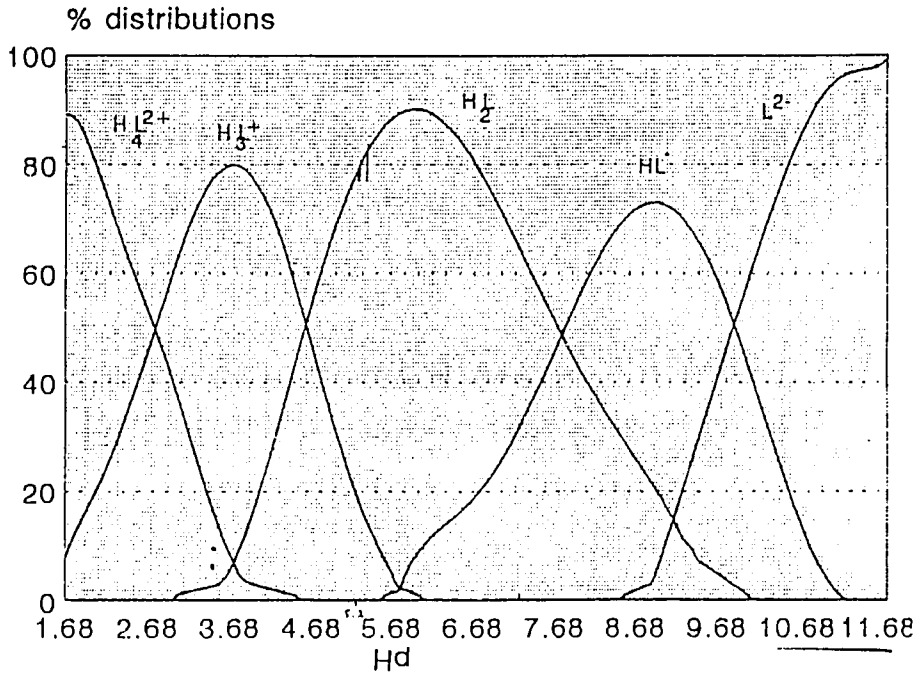
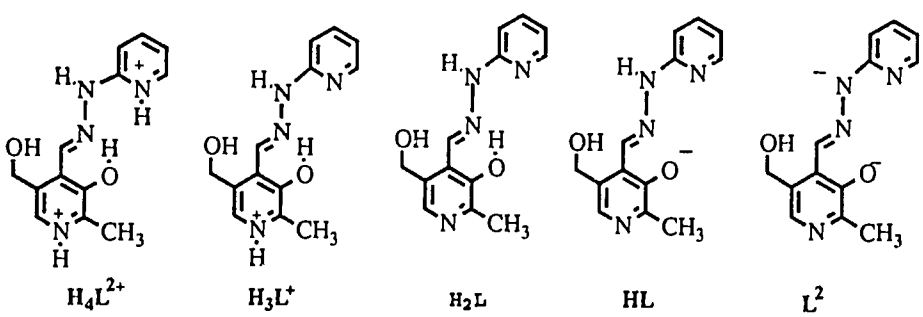


Figure 4a. Species distribution plot over 1.5 - 2 pH for Pyridoxal Piridyl Hydrazone (10); H4L2+ is the second protonated species at pyridinic ring N^4; $\mathbf{H_3L^+}$ is the first protonated pecies of pyridoxal ring N^1; $\mathbf{H_2L}$ is neutral species of of (10); $\mathbf{HL^-}$ is the first deprotonated species of (10); $\mathbf{L^{2-}}$ is the second de-protonated species of Pyridoxal Pyridyl Hydrazone.



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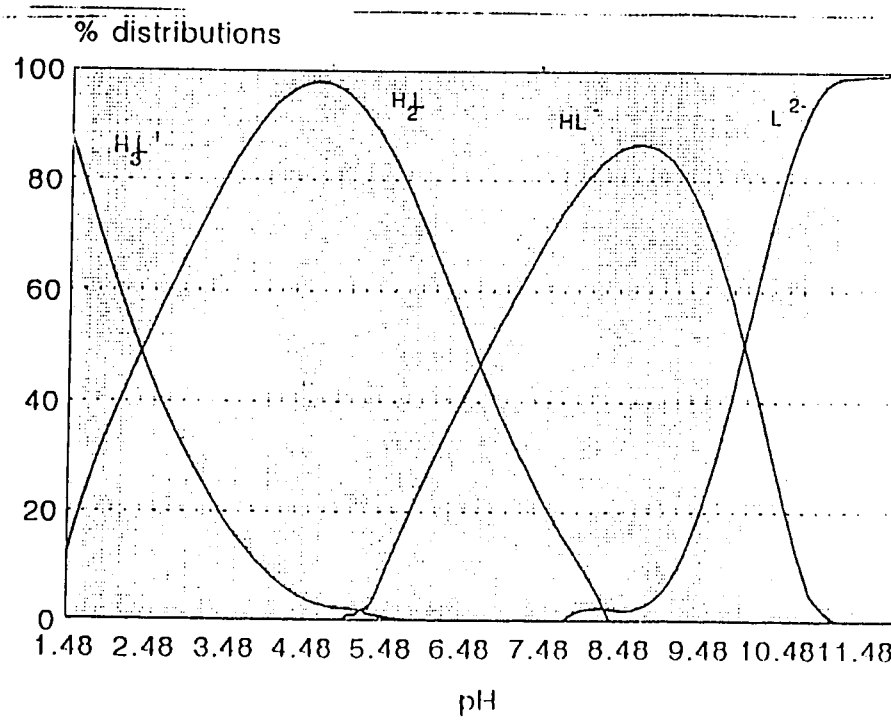
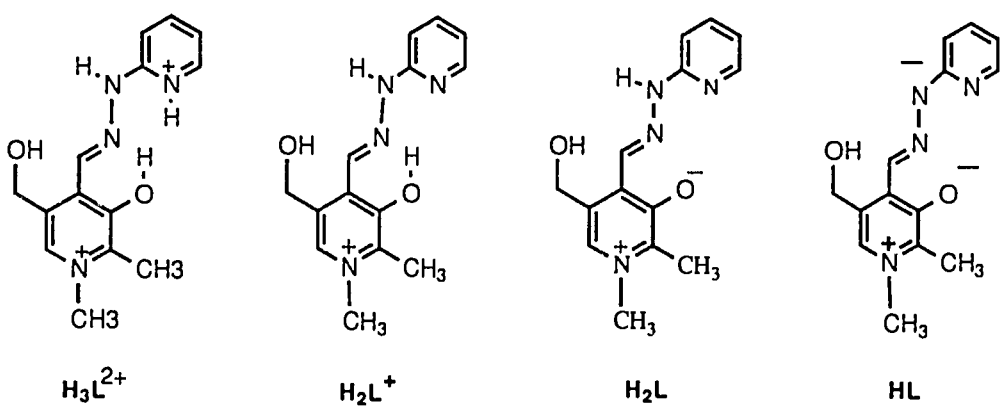


Figure 4b. Species distribution plot over pH 1.5 - 12.0 for 1-[N-methylpyridoxylidenium]-2-[2'-pyridyl]hydrazine iodide (**25**). H_3L^{2+} is the first protonated species of (**25**); H_2L^+ is the non-protonated species of (**25**); **HL** is the first deprotonated species of (**25**); **HL**$^-$ is the second deprotonated species of (**25**)



species dist 3 (saved) 2/1/94 16:13

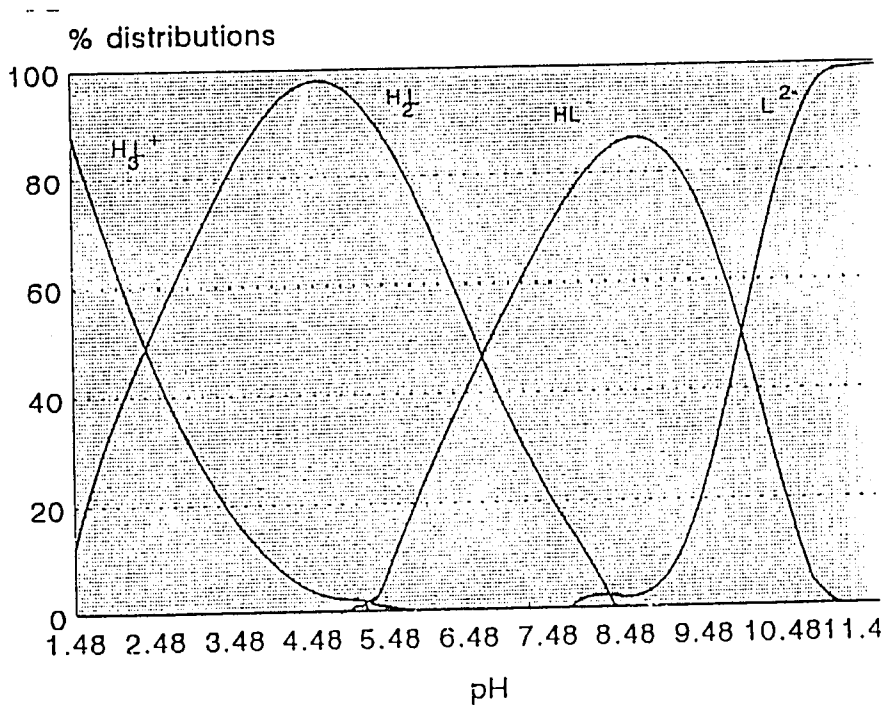
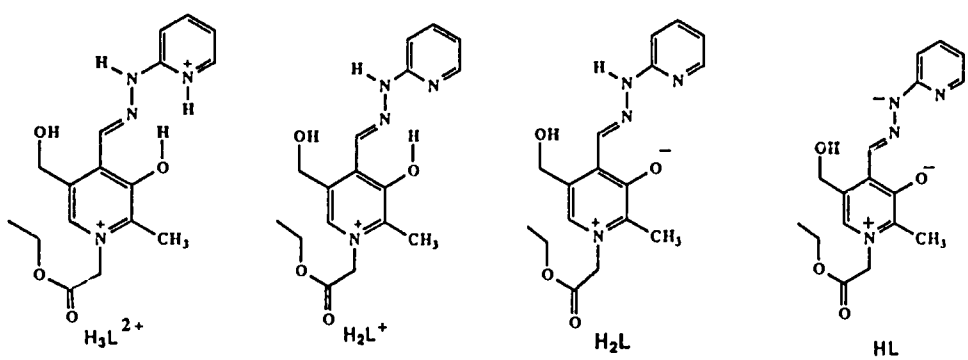


Figure 4c. Species distribution plot over pH 1.5 - 12.0 for 1-[N-ethoxycarbonylmethyl-pyridoxylidenium]-2-[2'-pyridyl]hydrazine bromide (29); H_3L^{2+} is the first protonation species; H_2L^+ is the non-protonated species; HL is the first deprotonated species ; HL is the second deprotonated species



species dist 4 2/1/94 16:16

ring, the vitamin B-6 Schiff bases, and their metal complexes, are well recorded in the literaure[37-41], enabling their use for assignment resonances of pyridoxal pyridyl hydrazone and its analogues. Indeed, the assignment of carbon chemical shifts was done by comparison with pyridoxal and pyridine. We have measured the ^{13}C NMR spectral assignments for **(10)**, **(25)**, **(29)**, and **PIH**, and their pH dependence.

Analysis of ^{13}C NMR data permits to assign most positively that the respective pK_a values of *2.39 ± 0.31* and *2.31± 0.15* , 7.30±0.42 and 7.43±0.35, obtained for ligands **(25)** and **(29)** (see **Table 15**), belong correspondingly to protons bound to pyridinic ring $N^{4''}$ and phenolic $O^{1'}$. Thus, it can be established that the ease of proton removal from the four binding sites in ligands included in this study *decrease* in the ordering:

$$\text{PYR ring-}N^4 > \text{Pyridoxal-ring } N^1 > O^1H > N^3H > O^2H$$

in excellent *agreement* with the theoretical calculations put forward previously, and consistent with the findings of Dubois and co-workers[35].

BIOLOGICAL RELEVANCE OF RESULTS

Compound **(10)** is inactive in vitro against the malaria parasite. However, putting a methyl substituent at the *electrophoric center* (at the pyridoxal ring-nitrogen) **(10** → 25) confer notable antimalarial activity to the molecule. Substituting methyl for ethoxycarbonylmethyl group (**25** → **29**) dramatically enhances the antimalarial activity of the molecule. This parallels the trends in species distribution with pH. At pH 5.2, the $\{H_2L\}:\{HL^-\}$ ratio in **(29)** is 13:1, but in **(10)** the $\{LH^-\}$ species is missing. Instead, a positively charged species is present, with $\{H_3L^+\}:\{H_2L\}$ ratio of 1:4. Significantly, at pH 7.2, the $\{H_2L\}:\{HL^-\}$ ratio is 1:2 in **(29)**, and 2:1 in **(10)**. This correlation between antimalarial activity with pH-dependent species distribution merits further investigations, since it could provide a clue for the mode of action of the new antimalarials.

Table 15

Prototautomerism pKa of PIH, (10), (25), and (29)

pK	PIH	(10)	(25)	(29)	PBH[9]	*Assignment*
pK_a 1	2.45 ± 0.10	2.62 ± 0.09	2.39 ± 0.31	2.31 ± 0.15	---	PYR ring N
pK_a 2	4.54 ± 0.07	4.63 ± 0.05	---	---	4.59 ± 0.04	PDX ring N
pK_a 3	7.44 ± 0.14	7.96 ± 0.07	7.30 ± 0.42	7.43 ± 0.35	8.47 ± 0.04	PDX OH
pK_a 4	9.94 ± 0.50	9.84 ± 0.07	9.76 ± 0.76	9.70 ± 0.51	11.40 ± 0.04	NH-N=
pK_a 5	13.35 ± 0.02	13.35 ± 0.01	13.33 ± 0.04	13.34 ± 0.04	---	

Complex Formation Constants With Fe(II) and Fe(III)

In the framework of this project we reported[25,26] that the remarkable *in vitro* antimalarial activities of (**13**), (**25**), (**29**) and related structures are iron-dependent[14]. The active antimalarial species was shown to be the Fe(II)-chelate which acts presumably as a carbon-centered radical capabale of damaging the parasite DNA[26]. This was verified by electron spin resonance (ESR)[25] studies.These prompted a study of the coordination chemistry underlying interactions between the redox-active ligands and iron(II), and/or Fe(III).Since such information is important for understanding the *in vivo* behaviour of these drugs, we have undertaken a study of *complex formation* between ions of $Fe^{2+}$and of Fe^{3+} and the active agents : (10), (25), (29), and PIH.

Iron Complex Formation

Stock Solutions.- The acid, HNO_3, and the base, NaOH, were purchased from Merck (Tritrisol).Double distilled water was used through all experiments. Solutions of $Fe(NO_3)_2$ were prepared by mixing solutions of $FeSO_4$ and $Ba(NO_3)_2$ in freshly distilled water . The supernatant was decanted and filtered under oxygen-free nitrogen, HNO_3 added to give a final acid concentration of 0.05 M and a final [Fe(II)] of approx. 0.1 M. The exact [Fe(II)] was determined by titration with standard $KMnO_4$.

Formation constants of the Fe(III) complexes were estimated by a combination of spectrophotometry and potentiometry. Spectrophotometry was utilized to measure initial complexation at pH 1.5-3.2. Potentiometric titration (H^+-titrant) were then employed to determine the acid dissociation constants of the Fe(III) complexes at higher pH. Dilute solutions with [Fe(III)] = 1-5 $x10^{-5}$ M were used to avoid precipitation. The more soluble complexes of Fe(II) were studied at [Fe(II)] = $2x10^{-4}$ M over pH range 2-10 by potentiometry under oxygen-free nitrogen. The titration apparatus and procedures for pH measurements are described above. The measurements were made at 37°C at an ionic strenght of 0.15 M KNO_3 .

As elaborated above, the pyridoxal-based series of synthetic ligands exist in aqueous solution as several pH-dependent species. It was assumed that H_2L was the most prtonated species of the ligandsthat can bind significantly to the metal ions. Hence, data fitting began with 8 parameters : the formation constant (β) and molar absorptivities (ε), of the four mono- and bis-complexes of H_2L : i) $M[H_2L]$, ii) $M[H_2L]_2$, and of HL^- : iii) $M[HL^-]$, iv) $M[HL^-]_2$, and their standard deviations providing a measure of goodness-of-fit using the non-linear algorithm. Various combinations of metal complexes were evaluated in this fashion to give the best standard deviation. Speciation plots were calculated from the mass balance for metal amd ligand at each pH value. Variables were the measured pH. [L]T and [M]T derived from the initial totals and the titration volumes, and the parameters were the acid dissociation constants of the complexes.

The pyridoxal-based sereies of synthetic ligands exist in aqueous media as several pH-dependent species. For the analysis of the experimental data, each experimental point was considered to represent four variables : i) initial total concentration of the ligand $[L]_T$; ii) initial total concentration of the metal $[M]_T$; iii) pH ; iv) molar absorptivities (ε), and/or potentiometric values. We assume that the neutral species H_2L is the most protonated species of the ligand that can bind significantly to the metal ions. Hence data fitting begins with 8 parameters : the formation constants and molar absorptivity of the four mono-, and bis-complexes of H_2L, namely, $M\{H_2L\}$ and $M\{H_2L\}_2$, and of HL^-, i.e., $M\{HL^-\}$ and $M\{HL^-\}_2$. Various combinations of metal complexes were evaluated in this fashion to give the best standard deviation. Specification plots were calculated from the mass balance for metal and ligand at each pH value.Variables were the measured pH, $[L]_T$ and $[M]_T$ derived from the initial totals and the titration volumes, whilst the parameters were the acid dissociation constants of the complexes.

The association of the ligand with the metal can be represented generally as follows:

$$H_2L + M \rightarrow M\{HL\} + H^+ \qquad \beta_1 = \{M(HL)\}\{H^+\} / \{M\}\{H_2L\} \qquad \text{(equ. 10)}$$

$$2\,H_2L + M \rightarrow M\{HL\}_2 + 2\,H^+ \qquad \beta_2 = \{M(HL)_2\}\{H^+\}^2 / \{M\}\{H_2L\}^2 \qquad \text{(equ. 11)}$$

$$H_2L + M + H_2O \rightarrow M\{HL\}(OH) + 2H^+$$

$$\beta=\{M(HL)(OH)\}\{H^+\}^2/\{M\}\{H_2L\} \qquad \text{(equ. 12)}$$

$$H_2O \rightarrow OH^- + H^+ \qquad \beta_4 = \{H^+\}\{OH^-\}/\{H_2O\} \text{ (equ. 13)}$$

where H_2L is free ligand, M is either Fe(II) or Fe(III), $M\{HL\}$ is 1:1-iron complex, $M\{HL\}_2$ is 1:2-iron:ligand complex, and $M\{HL\}(OH)$ is a 1:1:1-iron:ligand:hydroxyl complex. Table **16** summerizes the formation constants β_2 and β_3 for : (10), (25), (29), and PIH.

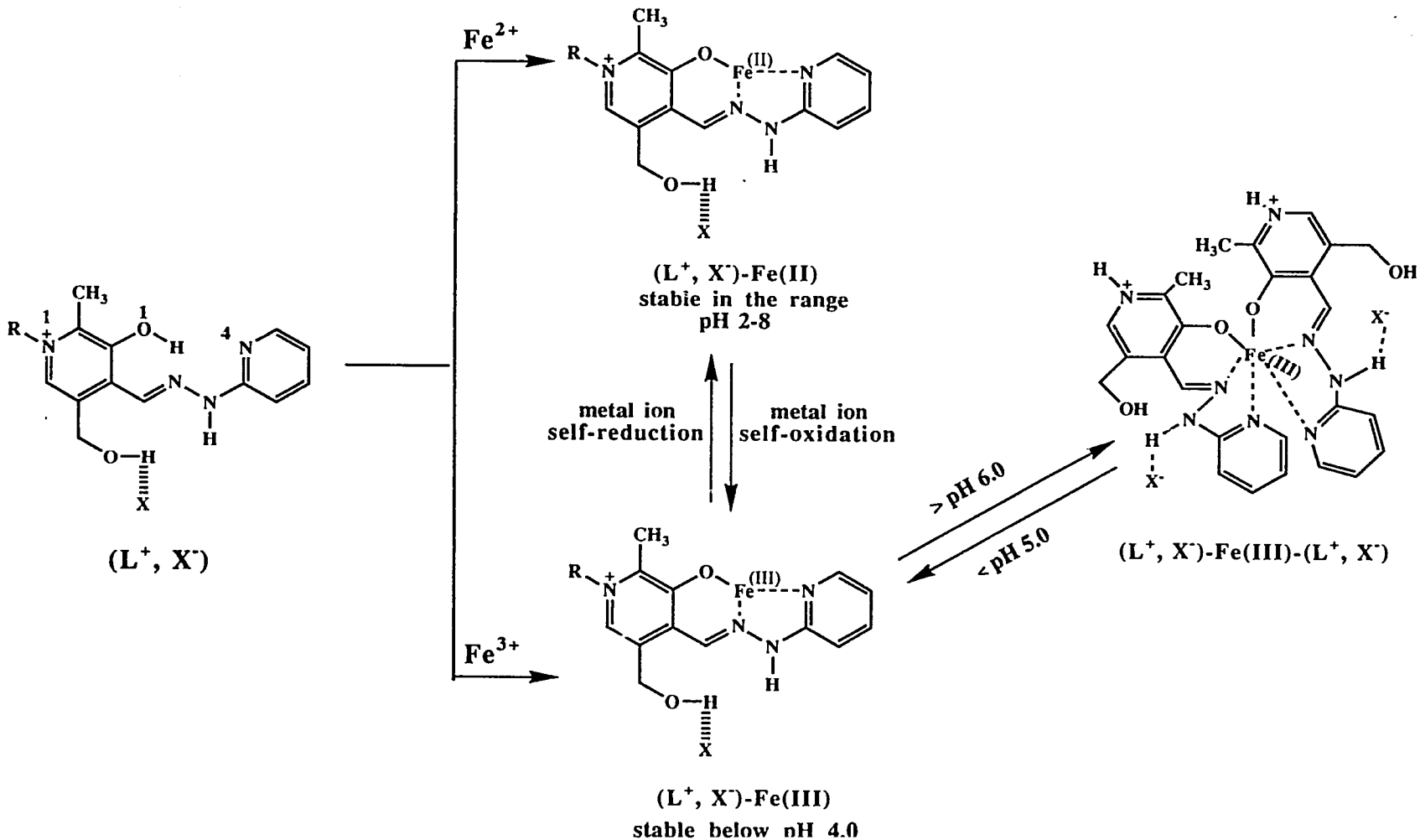
Table 16

*Formation Constants, (β_2) and (β_3), of (10), (25), (29), and **PIH** with Fe(II) and Fe(III)*

Ligand	$\log\beta_2^*$	$\log\beta_3^{**}$
(10)	11.01 ± 0.32	9.56 ± 0.52
(25)	11.86 ± 0.22	8.74 ± 0.22
(29)	11.80 ± 0.75	8.40 ± 0.44
PIH ---		8.67 ± 0.22

$*\beta_2 = \{L(OH)Fe^{II}\}\{H^+\}^2/\{Fe^{2+}\}\{H^+\}$; $** \beta_3 = \{L_2Fe^{III}\}\{H^+\}^2/\{Fe^{3+}\}\{HL\}^2$

Complexation of Pyridoxal-Based Chelators with Fe(II) and Fe(III) pH Dependence



Ultraviolet Spectra of Ligands, LH : (10), (25), (29), and their Respective 1:1-Fe(III)-Complexes, MLH, at pH 3.5

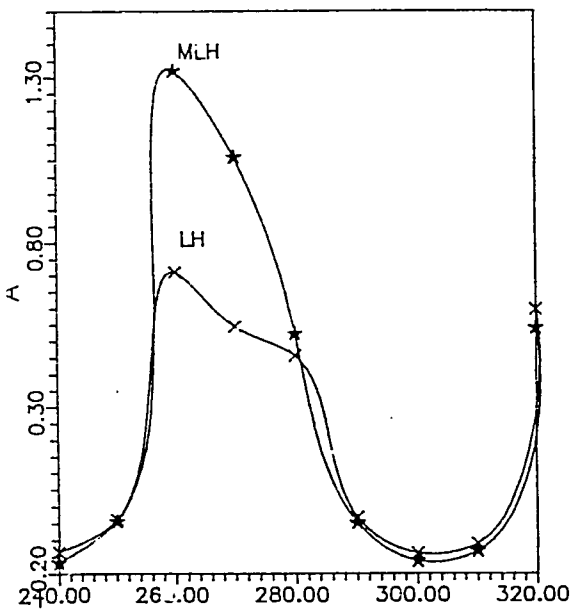


Fig. 3a. 1-[Pyridoxylidene]-2-[2'-pyridyl] hydrzine (10)

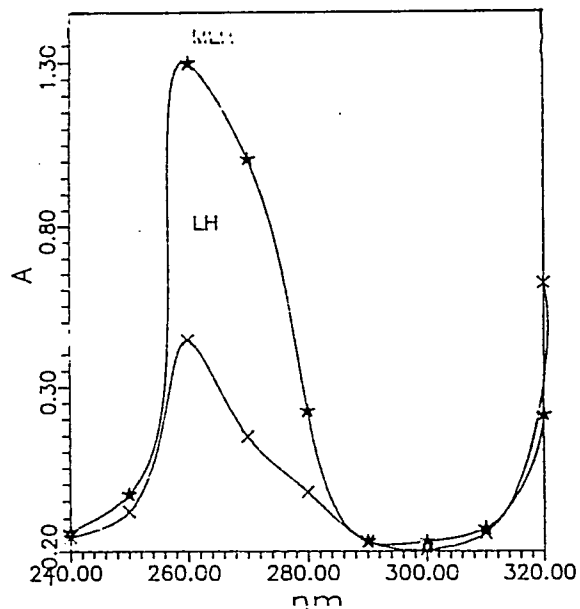


Fig. 3b. 1-[N-methylpyridoxylideniume]-2-[2'-pyridyl]hydrzine iodide (25)

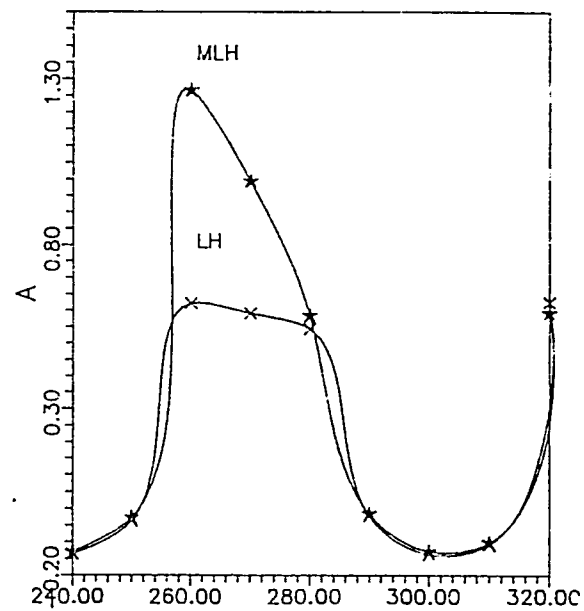


Fig. 3c. 1-[N-Ethoxycarbonylmethyl-pyridoxylidenium]-2-[2'-pyr idyl] hydrzine bromide (29)

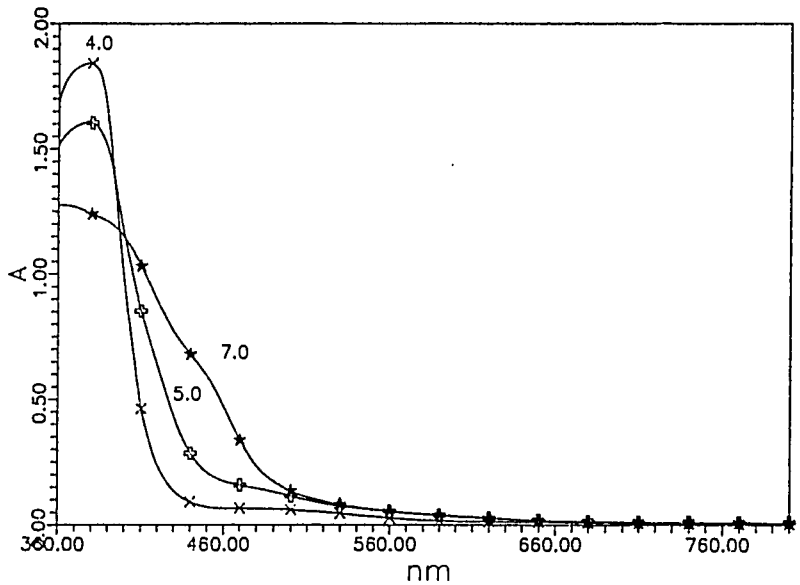


Figure 6a. Visible Spectra of 1:1-Fe(II)-chelate of 1-[pyridoxylidene]-2-[2'-pyridyl]hydrazine (**10**) at pH 4.0, 5.0, and 7.0

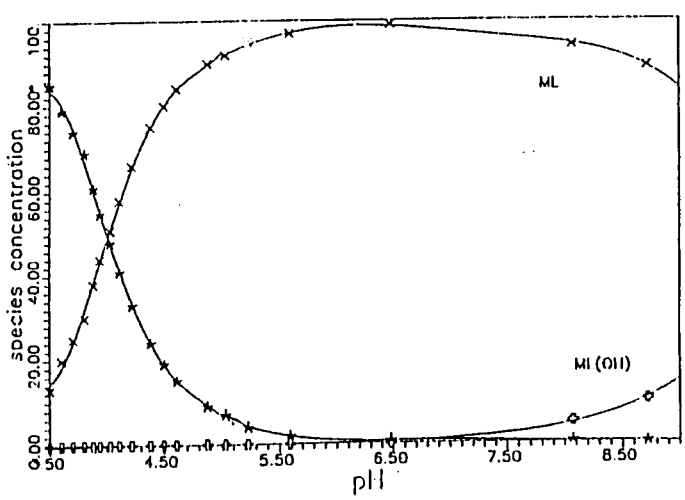


Figure 7a. Species distribution plot over pH 3.5 - 8.5 for 1:1-Fe(II)-chelate of 1-[pyridoxylidene]-2-[2'-pyridyl]hydrazine (**10**) at 37°C in 0.15 M KNO_3.

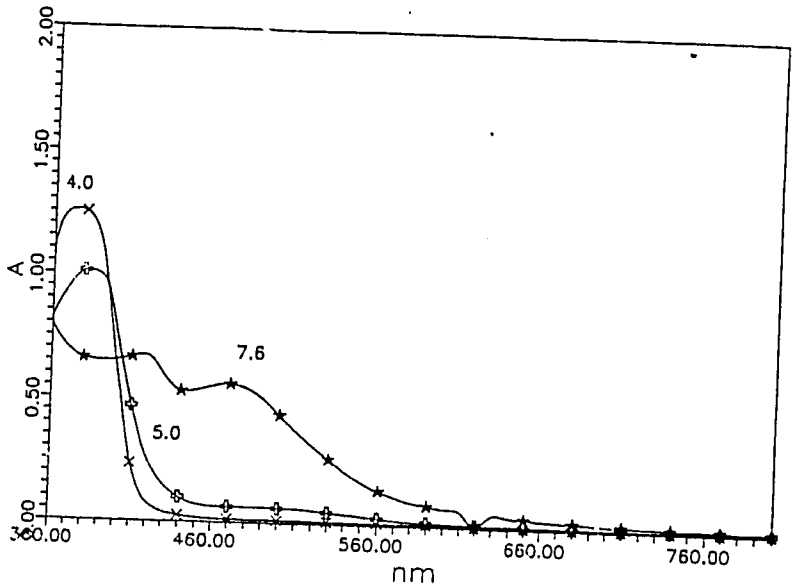


Figure 6b. Visible Spectra of 1:1-Fe(II)-chelate of 1-[N-methylpyridoxylidenium]-2-[2'-pyridyl]hydrazine iodide (**25**) at pH 4.0, 4.3, and 7.7.

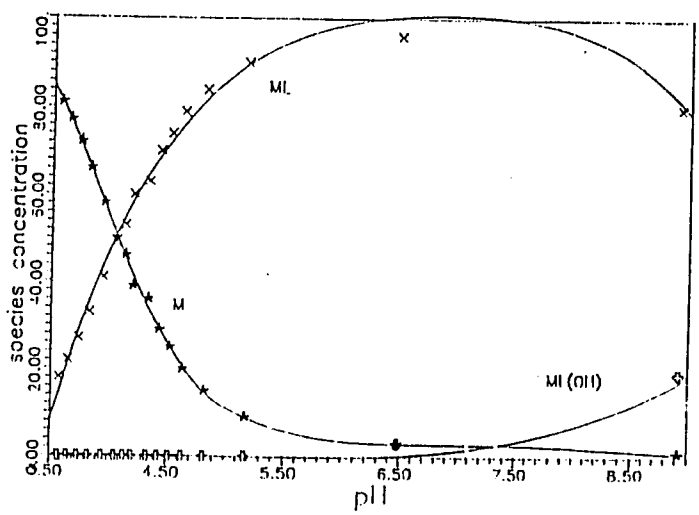


Figure 7b. Species distribution plot over pH 3.5 - 8.5 for 1:1-Fe(II)-chelate of 1-[N-methyl-pyridoxylidenium]-2-[2'-pyridyl]hydrazine iodide (**25**) at 37°C in 0.15 M KNO_3

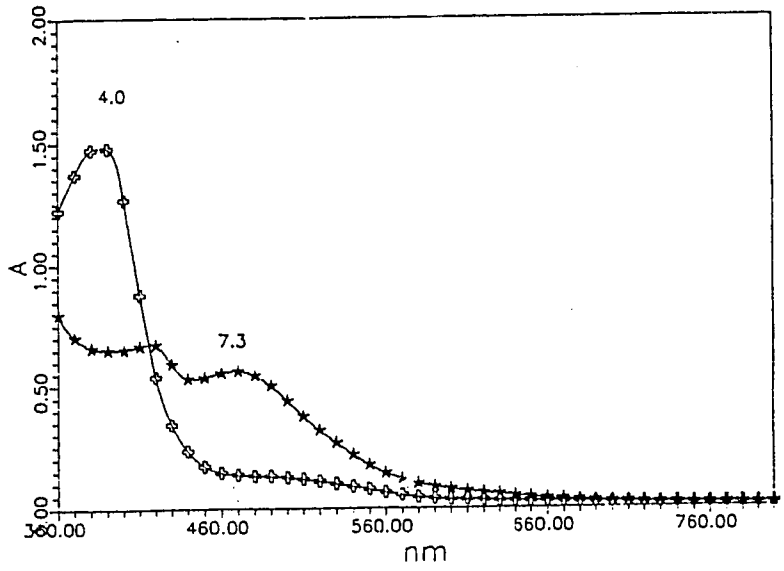


Figure 6c. Visible Spectra of 1:1-Fe(II)-chelate of 1-[N-ethoxycarbonylmethylpyrdoxyl idenium]-2-[2'-pyridyl]hydrazine bromide (**29**) at pH 4.0, and 7.3

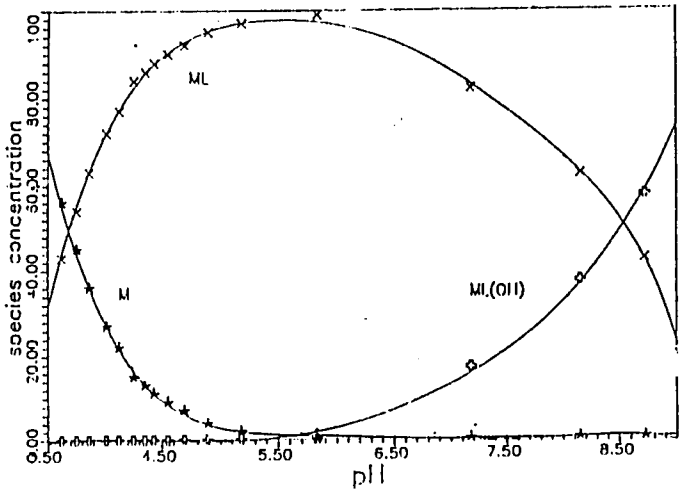


Figure 7c. Species distribution plot over pH 3.5 - 8.5 for 1:1-Fe(II)-chelate of 1-[N-ethoxy-carbonylmethylpyrdoxylidenium]-2-[2'-pyridyl]hydrazine bromide (**29**) at 37°C in 0.15 M KNO_3

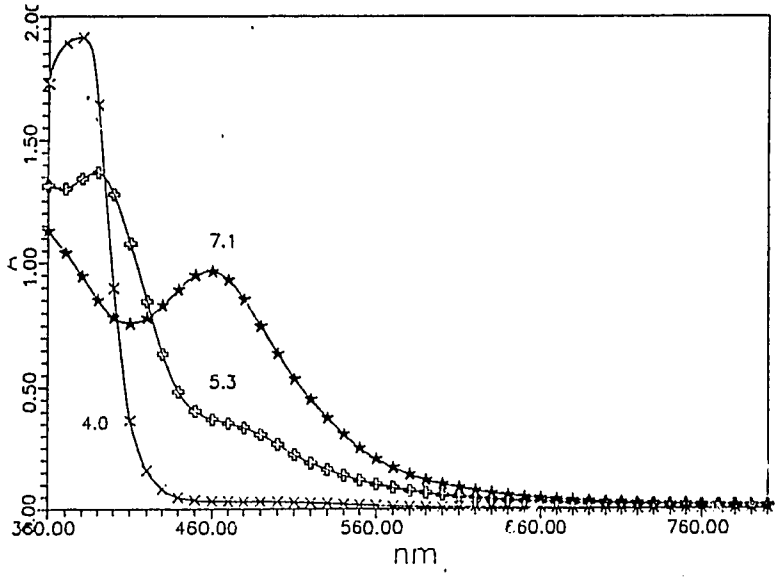


Figure 8a. Visible Spectra of 1:1-Fe(III)-chelate of 1-[pyridoxylidene]-2-[2'-pyridyl]hydrazine (**10**) at pH 4.0, 5.3, and 7.1

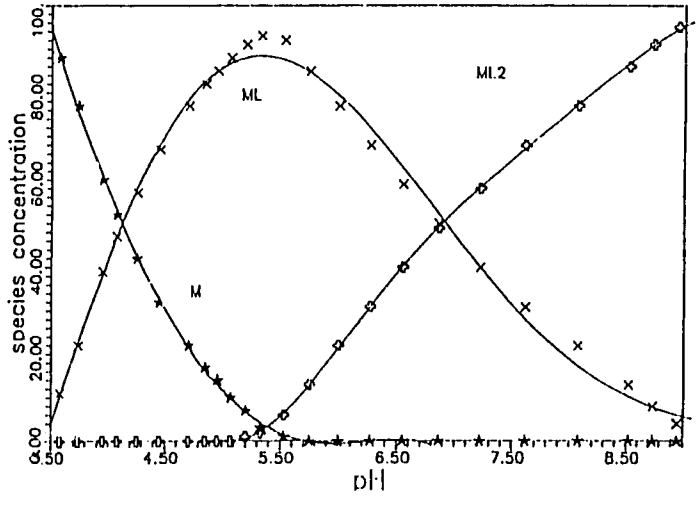


Figure 9a. Species distribution plot over pH 3.5 - 8.5 for 1:1-Fe(III)-chelate of 1-[pyridoxylidene]-2-[2'-pyridyl]hydrazine (**10**) at 37°C in 0.15 M KNO_3

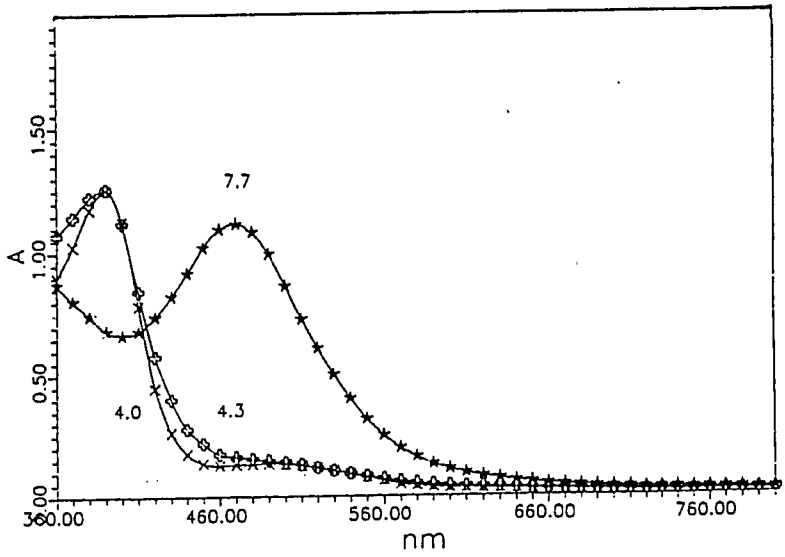


Figure 8b. Visible Spectra of 1:1-Fe(III)-chelate of 1-[N-methylpyridoxylidenium]-2-[2'-pyridyl]hydrazine iodide (**25**) at pH 4.0, 4.3, and 7.7.

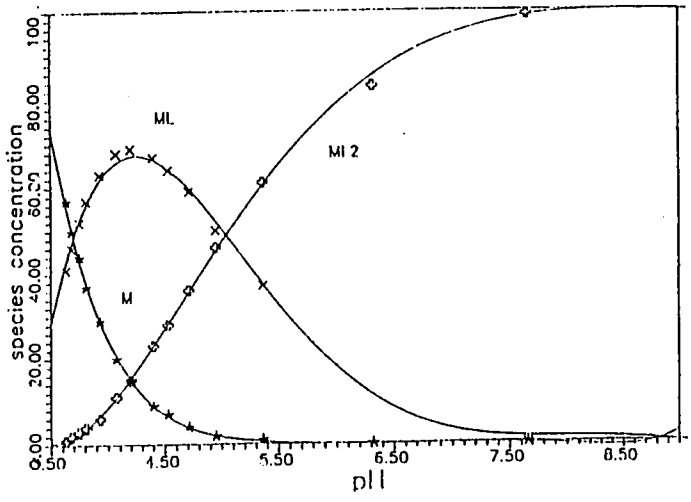


Figure 9b. Species distribution plot over pH 3.5 - 8.5 for 1:1-, and 1:2-Fe(III)-chelate of 1-[N-methylpyridoxylidenium]-2-[2'-pyridyl]hydrazine iodide (**25**) at 37°C in 0.15 M KNO_3

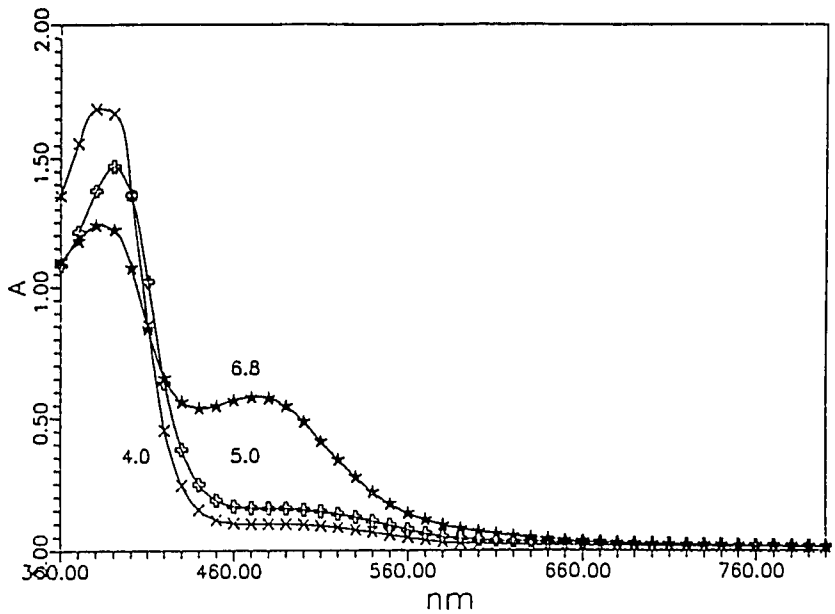


Figure 8c. Visible Spectra of 1:1-Fe(III)-chelate of 1-[N-Ethoxycarbonylmethylpyridoxyl idenium]-2-[2'-pyridyl]hydrazine bromide (29) at pH 4.0, 5.0, and 6.8.

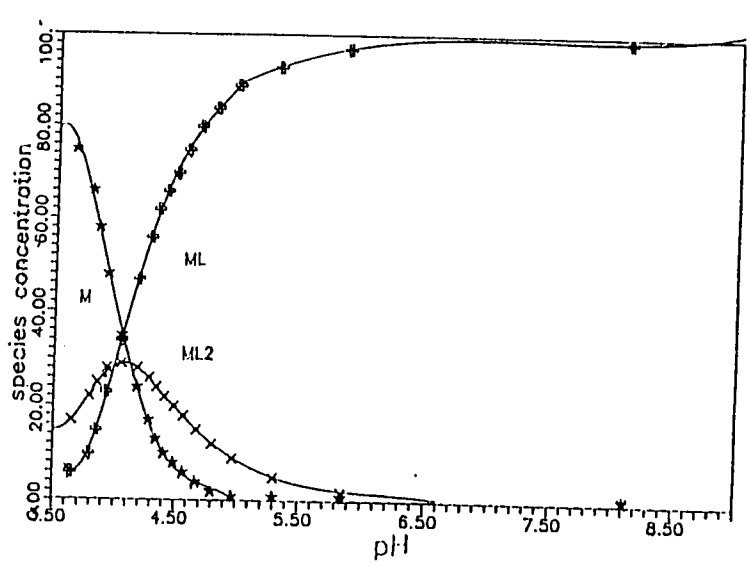


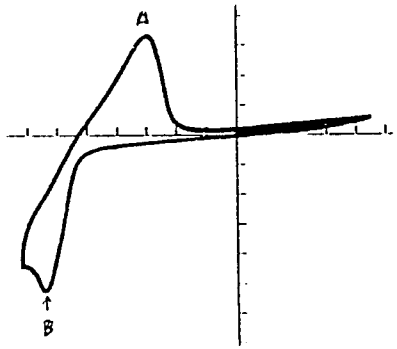
Figure 9c. Species distribution plot over pH 3.5 - 8.5 for 1:1-Fe(II)-chelate of 1-[N-ethoxy-carbonylmethylpyrdoxylidenium]-2-[2'-pyridyl]hydrazine bromide (29) at 37°C in 0.15 M KNO_3

Redox Potentials of Antimalarials

Determined by Cyclovoltametry

CYCLIC VOLTAMETRY OF AN ANTIMALARIAL CHELATOR AND OF ITS Fe(II)-CHELATE

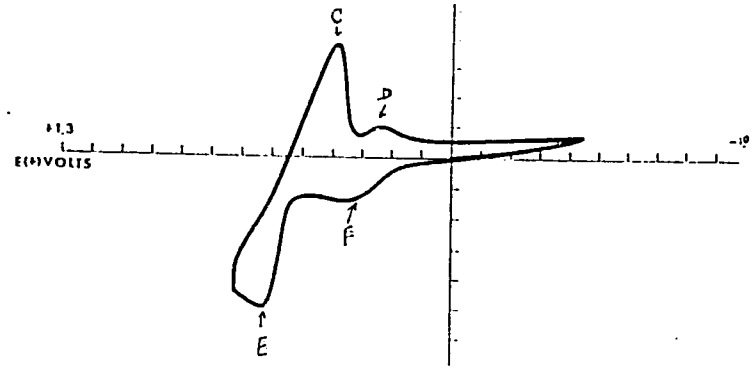
in water containing 0.5 M KCl at ambient temp.



concentration : 5 μM; *pH* = 4.41

peak **A, Red** Potential : + 0.30 volt; 85 μA

peak **B, Oxid** Potential: + 0.63 volt; 132 μA



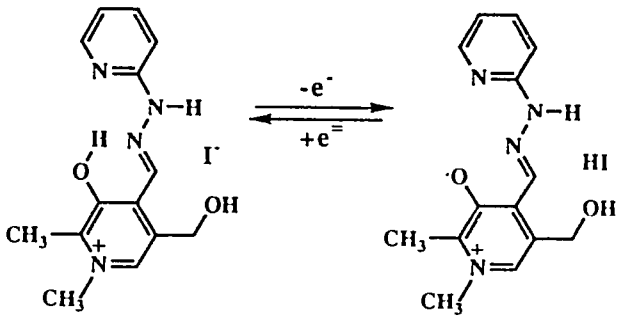
Conc.: ligand = 5μM; Fe^{2+}= 2μM; *pH* = 2.07

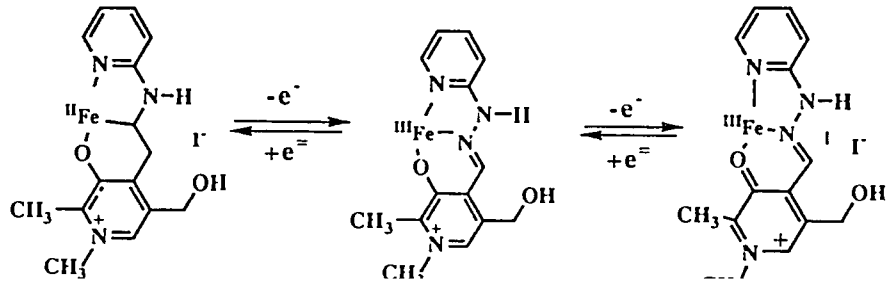
peak **C, Red** Potential : + 0.38 volt; 95 μA

peak **D, Red** Potential: + 0.23 volt; 25 μA

peak **E, Oxid** Potential : + 0.62 volt; 125 μA

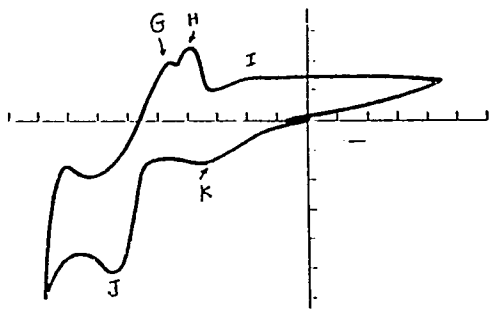
peak **F, Oxid** Potential : + 0.36 volt; 37 μA

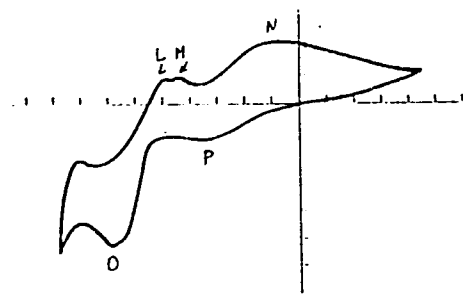


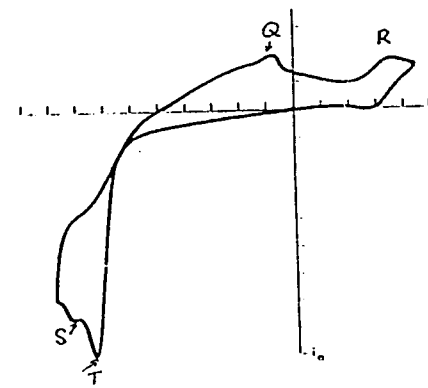


CYCLIC VOLTAMETRY OF AN ANTIMALARIAL Fe(II)-CHELATE OF 1-[N-METHYL-PYRIDOXYLIDENE]-2-[2'-PYRIDYL]HYDRAZINE IODIDE

Solvent: Water; in KCl 0.5μM; Temp.: Ambient







Conc.: ligand = 5μM; Fe^{2+}= 2μM; *pH* = **5.87**

peak **G, Red** Potential : + 0.46 volt; 47 μA
peak **H, Red** Potential: + 0.38 volt; 60 μA
peak **I,** Red Potential : + 0.20 volt; 37 μA
peak **J, Oxid** Potential : + 0.63 volt; 130 μA
peak **K, Oxid** Potential :+ 0.35 volt; 42 μA

Conc.: ligand = 5μM; Fe^{2+}= 2μM; *pH* = **7.44**

peak **L, Red** Potential : + 0.48 volt; 20 μA
peak **M, Red** Potential: + 0.44 volt; 20 μA
peak **N, Red** Potential: + 0.10 volt; 40 μA
peak **O, Oxid** Potential : + 0.68 volt; 132 μA
peak **P, Oxid** Potential : + 0.32 volt; 37 μA

Conc.: ligand = 5μM; Fe^{2+}= 2μM; *pH* = **10.83**

peak **Q, Red** Potential : + 0.06 volt; 50 μA
peak **R, Red** Potential: - 0.34 volt; 45 μA
peak **S, Oxid** Potential : + 0.81 volt; 190 μA
peak **T, Oxid** Potential : + 0.72 volt; 225 μA

Project Activities

Over the entire project period the activities included: i) participation in national and international symposia dedicated to subjects related to the project, and ii) publication of research accounts in international scientific journals. They list as following:

List of meetings:

Plenary Lecture: "Mechanism-Based Approaches Towards Chemotherapy of Thalessemia and Malaria", delivered at Xth International Symposia on Medicinal Chemistry, held in Budapest, Hungary, August 1988.

Symposium Lecture: "C-Centered Radicals As Antimalarials", XIth International Symposium on Medicinal Chemistry, held in Jerusalem, Israel, 2-7 September 1990.

Symposium Lecture: "From Pyridoxal To Antimalarials Active Against Drug-Resistant Plasmodium falciparum", held in Jomien, Thailand, 11-14 December 1990.

Poster: "Malarial Iron-Based Antimalarials and Free Radicals", 10th International Conference on Iron and Iron Proteins, held in Oxford, England, 27-31 July 1991. Abst. of Papers pp. P116.

Poster: "Antimalarials as Inhibitors of Ribonucleotide Reductase", The 3rd NIH sponsored Symoposium on "The Development of Iron Chelators for Clinical Use", held in Gainsville, Florida, USA, May 20-22, 1992.Abst. of Papers, pp.46-47.

Symposium Lecture: "A New Chemotherapeutic Model System Against Falciparum Malaria (FCR-5)", XIIth International Symposium on Medicinal Chemistry, held in Basel, Switzerland, 13-17 September 1992. Abst. of Papers, OC-06.5, pp.79.

Plenary Lecture: "Mechanism-Based Antimalarials: The Iron Connection"; and Poster: "Avidity for Fe^{2+} and Fe^{3+} Ions by Tridentatte Chelators Based on Pyridoxal-Betaines", Second International Symposium on Applied Bioinorganic Chemistry, held in Guangzhou, China, December 8-12, 1992. Abst. of Papers B-76, and B-77, pp. 151 and 152.

Publications:

1. S. Sarel, S. Avramovici-Grisaru, C. Hershko, E.N. Iheanacho, D.T. Spira, "Trends in Medicinal Chemistry 88' ", Edited by H. van der Goot, G. Domany, L. Pallos, and H. Timmermann, **1989,** Elsevier Science Publishers B.V., Amsterdam

2. E.N. Iheanacho, A. Samuni, S. Avramovici-Grisaru, S. Sarel, and D.T. Spira, *Trans,Roy.Soc.Trop.Med.Hyg.*, **1990,** 84, 213-216

3. E.N. Iheanacho, S. Sarel, A. Samuni, S. Avramovici-Grisaru,, and D.T. Spira, *Free Rad Res.Communs.*, **1991,** 15, 1-10.

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Impact, Relevance and Technology Transfer

In retrospect, the rationale that initiated this project proved to have a sound basis in reality. Indeed, it opened new venues in the combat against the drug-resistant strains (FCR-3) of the malaria parasites which are responsible for the rapid spread of falciparum malaria in South East Asia and South America.

The greatest impact of results produced in this project is likely to be on the minds of the researchers dedicated to malaria chemotherapy, since the new discoveries are based on hitherto unknown mechanism of action. The new antimalarial agents were shown to be parasiticidal, killing the parasites within minutes or hours, depending on structure and concentration.

The results fully warrant a new project aimed at enhancing the scale and deepening the depth of testings in model animals.

The collaborators from Thailand learned many lessons during the joint endeavor. They have acquired new skills in modern experimental chemistry (ESR, cyclovoltametry etc.), molecular biology, and coputational chemistry. In estimation of parasitemia they have learned to use the more accurate ^{3}H-hypoxanthine incorporation method which is highly reliable. The training acquired via involvement in the process of data production, analysis of data, and discussion of results immensely improved the capabilities of the collaborating country scientists.

Project Productivity

The project accomplished almost all of the proposed goals, except the *in vivo* testings.

Future Work

The new conceptual findings in the project opened interesting venues to antimalarial drug design implicating intracellular generation of harmfull free radicals by iron-dependent inducers. This prompts the use of new models for drug design to provide more effective antimalarials operating by hitherto unknown mechanisms.Towards this end more work should be done on five relevant topics conducive to therapeutically safe agents active against drug-resistant falciparum malaria. The ordering of topics below is tentative, requiring more thought for preferences in design a working research plan.

(i) in vivo testings of chelating agents which exhibited remarkable *in vitro* antimalarial activity.

(ii) Structure-activity-relationship study to uncover factors determining the antimalarial activity of pyridoxal-based chelators.

(iii) Synthesis and assessment of antimalarial activity of a new series of metal-chelates where the metal equals: Fe(II), Fe(III), Cu(II), Co(III), and Pd(II), and the respective ligands are: pyridoxal 1-phthalazyl hydrazone, and/or di-pyridoxal 1,4-phthalazyl di-hydrazone and other related analogues.

(iv) Inhibition tests of chelating agents against haem polymerase [27] which blocks the detoxification of ferri-protoporphyrin IX, and poisons the parasite food vacuole.

(v) To uncover facors affecting transmembrane transport of chelating agents and their metal-chelates through blood red-cells.

(vi) The importance of affinity of chelating agents to Fe(II) and/or Fe(III) to effect antimalarial activity.

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MECHANISM-BASED APPROACHES TOWARDS CHEMOTHERAPIES OF THALESSEMIA AND MALARIA*

Shalom Sarel, Schelly Grisaru, Chaim Hershko, Gabriela Link, Dan Spira and Eugene Iheanacho

Hebrew University of Jerusalem, Jerusalem, Israel

SUMMARY

The synthesis, coordination chemistry and biological activities of a new class of iron chelators based on pyridoxal are described herein. These comprise two types of aryl hydrazones of pyridoxal represented by the general formulae (I) and (II). Both function in vitro and in vivo as tridentate chelators. They are essentially non-toxic and compare with desferrioxamine (desferal, DF) with regard to affinity and specificity for iron. Unlike desferrioxamine, they are orally effective and considerably less expensive. They represent excellent candidates for remedy of iron overloads in thalassemia and blood transfusional patients.

Depending on structure, some of the new chelators can kill cultures of chloroquine-resistant strains of P. falciparum at 2.10^{-6} concentration. Their mode of action is believed to involve an intraerythrocytic iron-catalyzed Fenton-type reaction which places increased "oxidative stress" both on the real cells and on the more sensitive parasitic invaders.

INTRODUCTION

The thalassemias are among the commonest inherited blood disorders in the world, of which beta-thalassemia major (known also as Cooley's anaemia) alone is responsible for 100,000 child deaths per year.[1,2,3]. Most significantly, it is also among the five major haemoglobin abnormalities that confer resistance to Plasmodium falciparum,[4] the main causative agent of human malaria which affects one quarter of the world's population and is responsible for 1,000,000 deaths per year in Africa alone.[5] The five genetically controlled traits are:

1. Sickle-cell anaemia, the normal haemoglobin A ($\alpha_2\beta_2$) in the red cell being replaced by a chemical variant HbS.
2. Thalassemia, in its α-form, the red cells contain the totally ineffective haemoglobin variant HbH (β_4).
3. Hereditary persistance of foetal haemoglobin (HPFH), part of the thalassemia syndrome in which the red cells contain an unstable haemoglobin variant HbF ($\alpha_2\gamma_2$).
4. Haemoglobin E disease, part of the thalassemia syndrome, containing HbE variant, an unstable haemoglobin.
5. Favism, common among Mediterranean and Middle Eastern populations, in which the red cells are deficient of the enzyme glucose-6-phosphate dehydrogenase (G-6-PD), controlling the redox transitions: GSSG + 2NADPH ⇌ 2GSH + 2NADP

* Dedicated to the memory of Professor Edgar Lederer (5 June 1908 - 19 October 1988)

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The growth of P. falciparum in culture has been shown to be inhibited on exposure to the above haemoglobin variants.[6] The normal person has no obvious defence mechanism against invasion by the malaria parasite. Evolutionwise, the genetic alteration of normal haemoglobin appears to be the only survival means for the body to combat the malaria parasites.[3,7]

Immunity to malaria could also be promoted by induction state of iron-deficiency. The case in point is the resistance to malaria acquired by anaemic women during pregnancy which fades away upon administration of iron.

This communication deals with chemotherapeutic strategies aimed at (i) designing orally effective and inexpensive chelators capable of removal of toxic accumulation of iron in thalassemic and iron overloaded patients, and (ii) the molecular engineering of orally effective, safe, and inexpensive antimalarials, mechanistically implicating iron.

THALASSEMIA

Thalassemia is a syndrom[2] of inherited abnormalities of haemoglobin which arises from defects in the genes controlling the manufacture of either alpha- or beta-haemoglobin chains. It occurs not only in people of Mediterranean extraction but also in the Middle East, the Indian subcontinent, South-East Asia and Africa and among immigrant populations from these areas. If the gene for production of β chains is abnormal, the blood disorder is called β-thalassemia, and when the α chains are affected, it is known as α-thalassemia. Reduced production of the affected globin chain leads to an excess of the other which tends to precipitate particularly in the nucleated red cells in the bone marrow resulting in distorting the vault of the skull and facial bones.

The anaemia in β-thalassemia major becomes apparent soon after birth. The child fails to thrive, is prone to infection, developing bony distortions and giving mongoloid appearance. These children die young.

IRON OVERLOAD

The picture can be dramatically modified by regular blood transfusions to suppress the abnormal bone marrow activity and to maintain normal levels of haemoglobin. About 200mg of iron is estimated to accumulate as a result of each unit of blood transfused since it cannot be excreted from the body when the transfused cells are ultimately destroyed. Most patients die in their teens from liver or heart failure. As the body has no means of significantly increasing iron excretion, about 1 or 2 individuals in a population of 10,000 require treatment with chelating agents for safe removal of toxic amounts of iron from the body. The iron overload can successfully be treated by applying the naturally occurring chelator desferrioxamine (DF) which safely removes surpluses of iron from the body. Although DF is satisfactory with regard to

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affinity and specificity for iron and it is essentially non-toxic, it is unsatisfactory in other respects, such as its poor absorption from the gastrointestinal tract and it is expensive. It has a very short half-life and has no effect on plasma ferrokinetics. This prompted studies which brought about the uncovering of some novel candidates, representing a new class of chelators based on pyridoxal of the general structures (I) and (II).

CHEMICAL SYNTHESIS

The syntheses of (I) and (II) were straightforward, as delineated in Chart 1. The pyridoxal is condensed with the corresponding aryl or acyl-hydrazine to form (I), and when required the latter was exposed to the action of alkyl halide or an α-bromoester to yield (II).[8,9]

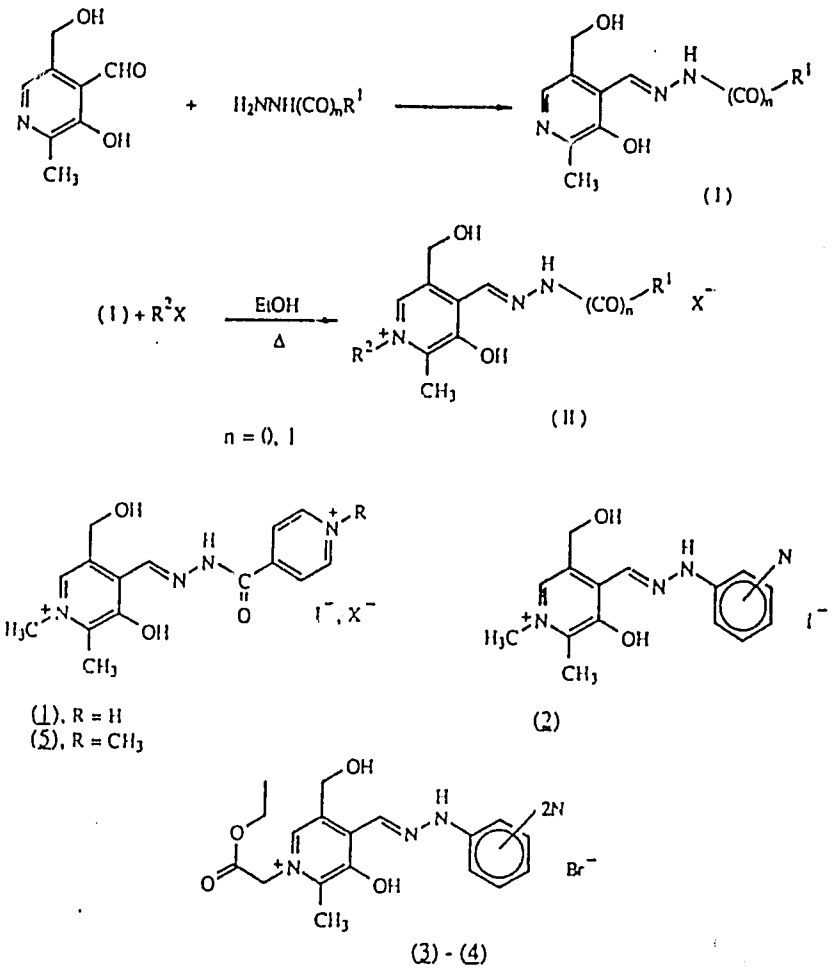

CHART 1

BIOLOGICAL TESTINGS

The biological testings involved hypertransfused rats which were labelled by intraveneous injection of ^{59}Fe-ferritin. The ability of the new chelators to mobilize radio-iron from hepatic parenchymal stores followed from a single subcutaneous injection of 40mg of the compound into the labelled rat. The radio-iron was excreted totally, mainly in the stool. The oral effectiveness was assessed from administration of 40mg of the chelator by the gastric tube followed by estimation of total radio-iron excretion.[8,10,11] The results of some selected compounds, (1) to (5), are given in Table 1.

Table 1

Radio-iron Distribution in %						
Code Number	Blood	Liver	Spleen	Urine	Faeces	Total Excretion
Control	27.4±1.6	12.1±0.6	0.47±0.03	0.1±0	3.1±0	3.2±0
Desferral	8.7±1.6	2.7±0.3	0.14±0.03	71.9±3.6	13.5±0.7	85.4±4.3
1	33.3±2.1	12.8±0.2	0.56±0.02	3.7±0.2	22.7±1.1	26.4±1.3
2	13.3±2.3	6.8±0.4	0.51±0.02	39.0±3.1	27.0±2.2	66.0±5.3
3	6.4±0.4	3.3±0.4	0.16±0.04	29.5±0.6	57.2±1.1	86.7±1.7
4	4.2±0.2	2.9±0.2	0.06±0.03	28.9±1.0	67.3±2.4	96.2±3.4
Comp.5*	25.1±2.2	8.2±0.8	0.36±0.03	30.6±1.12	8.6±0.3	39.2±1.6

*pyridioxol isoniccotinoyl hydrazone dimethiodide

From Table 1 it can be seen that when the tested compounds were given orally to the animals enhanced excretion of radio-iron took place, considerably above controls. The observed in vivo activities of two compounds were comparable to and even exceeded that of desferrioxamine (which were given by injection). These results represent a very significant progress in providing new chelators of potential clinical usefulness in the management of patients with transfusional iron overload.

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COORDINATION CHEMISTRY

Study of the coordination chemistry of these compounds was undertaken with a view to uncovering factors underlying the biological activities. Chemical and spectroscopic evidences indicate that the new chelators function as tridentate ligands, forming two sets of metal complexes with metal:ligand ratios of 1:1 and 1:2,[12] depending on the pH. Thus, 1:1-complexes are stable at pH values below 4, whereas the 1:2 complexes are highly stable at pH values above 5.5 (Chart 2). The apparent binding constants with metals were measured (see Table 2), following spectroscopic methods.[13] From Table 2 it can be seen that the affinity for iron is quite high at neutral pH. Looking at Mössbauer spectroscopic data[14] presented in Table 3 reveals that iron(II) in the complexes tends to lose one electron to form high-spin Fe(III)-complexes.

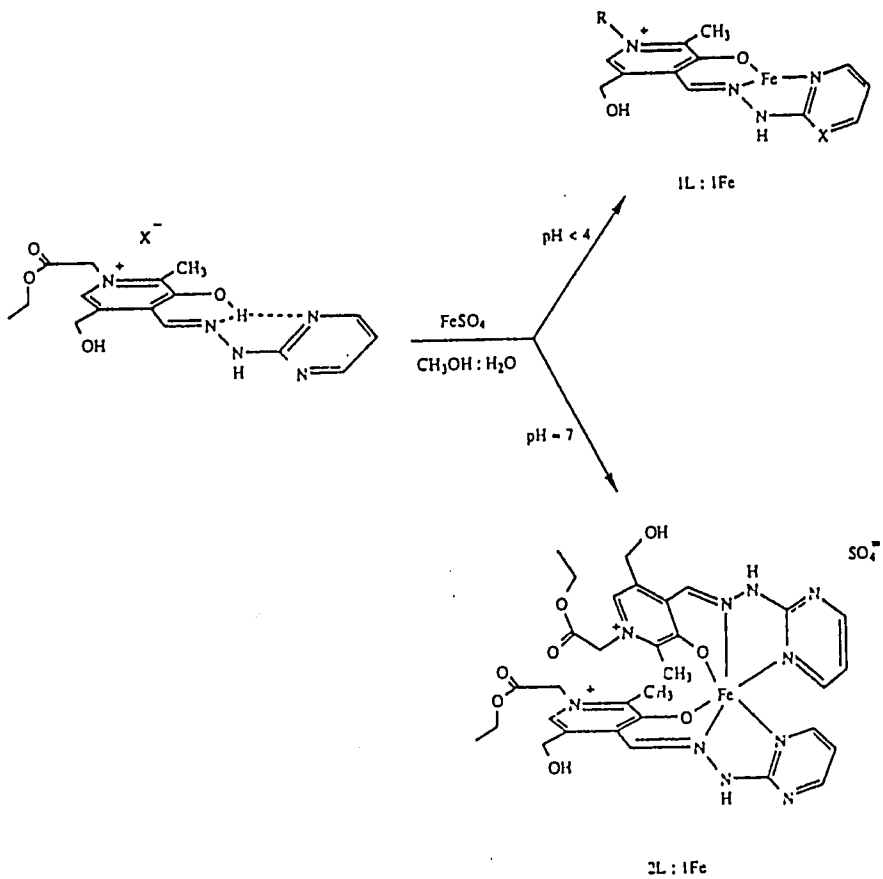


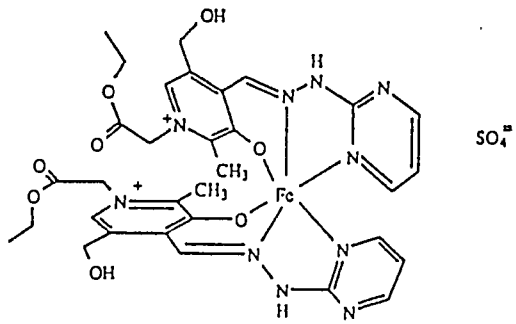
CHART 2

Table 2. CHELATION OF (I) AND (II) WITH FERROUS AND CUPRIC IONS IN H_2O at 10^{-4}M CONCENTRATION AND pH 6.50 ± 0.15[13]

	COPPER CHELATION				IRON CHELATION			
LIGAND	RATIO Cu:L	λ mμ	ε x 10^3	K apparent	RATIO Fe:L	λ mμ	ε x 10^3	K apparent
DESFERRIOXAMINE	-	-	-	-	1:1	402	18	4.5 x 10^3
PIH	1:1	405	14	2.3 x 10^4	1:2	453	16	3.8 x 10^8
PPH (I)	1:2	443	12.8	9 x 10^{10}	1:2	420	2.15	3 x 10^9
PPH*	-	-	-	-	1:2			1 x 10^8
PPHE (II)	1:1	460	70	2 x 10^4	1:2	439	42	1 x 10^{10}

(*) With ferric ions at comparable conditions

Table 3. MOSSBAUER RESULTS



Form	%	I.S.	Q.S
	Fe^{2+} : 30	0.93(1)	2.38(1)
MeOH insoluble			
	Fe^{3+} : 70	0.370(6)	0.810(6)
	Fe^{2+} : 50	0.94(1)	2.26(2)
EtOH soluble			
	Fe^{3+} : 50	0.39(2)	0.84(2)

MALARIA

Malaria is caused by protozoan parasites belonging to the genus Plasmodium, of which the four species: P. falciparum (often fatal), P. vivax, P. malariae and P. ovale are essentially exclusive to human beings. They have a complex life cycle in both the insect and the vertebrate host. Our practical attention was directed at the three-stage development sequence: ring forms, trophozoite and schizont.

Malaria has undergone a resurgence over the past decade,[15,16] and, despite its high incidence, no revolutionary new drugs have been introduced over the past three decades. The widespread appearance of chloroquine-resistant strains of P. falciparum[17] prompts the development of new antimalarial drugs, operating most desirably by entirely new mechanisms, to replace those to which P. falciparum has become resistant. In conformity with this line the most logical approach would be of making use of parasite metabolism for targeting the

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drug action at vulnerable sites of the organism. Towards this end two different approaches have been adopted. One, making use of the susceptibility of the malaria parasites to free oxygen radical-reduced oxidative stress.[6,18] The second, to affect the iron pool of the parasite controlling the biosynthesis both of metallo-protein oxidases[19] and of the iron-dependent ribonucleotide diphosphate reductase (RdR)[20] by applying chelators inhibitory to these enzymes. This might exert selective inhibition on the growth of P. falciparum. Cultures of the chloroquine-resistant strain of P. falciparum (FCR-3) were suspended in solutions containing chelators and then cultured for 3 days. Growth of parasites was determined by either slide counts or by incorporation of ^{3}H-hypoxanthine. The parasite growth was monitored by microscopic slide counts. The effects of one representative of the new chelators on the growth of P. falciparum choroquine-resistant strains are demonstrated in Figures 1 and 2. The effect of added Cu^{2+} ions is shown in Figure 3. Table 4 summarizes the data on inhibitory effects of some of the new chelators on the growth of P. falciparum and on the enzyme activity of RdR with a view to their capability of removal of iron from the body.

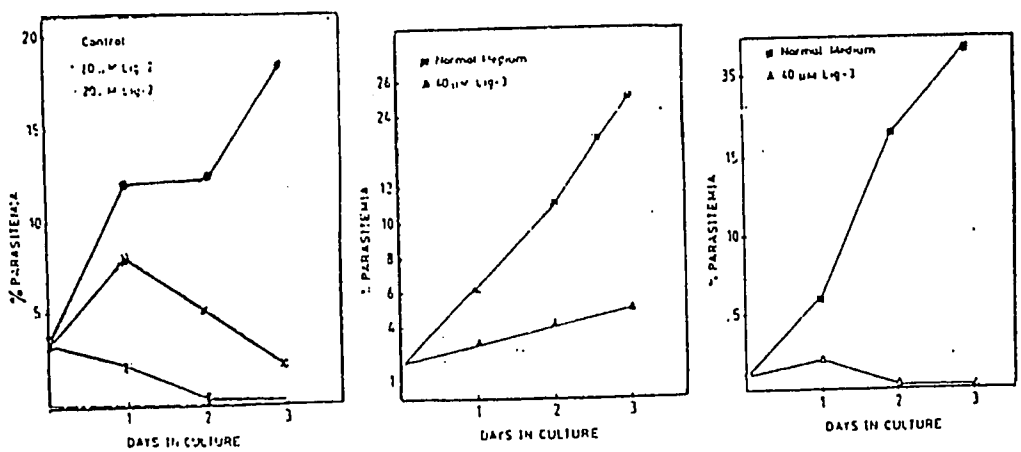

Fig. 1. Growth curves of P. falciparum in human red cells as a function of Lig-2 concentration. A synchronous of 3% schizonts was used as the starting parasitemia.

Fig. 2. Growth curves of parasites pre-treated with 40 μM Lig-3 and normal medium respectively for 2 hours before culturing.

Fig. 3. Pre-complexing chelator with copper salt: the chelator was pre-complexed with copper. This was then used in medium for growing the parasite as in previous experiments. 1% mu culture was used for initial parasitemia.

TABLE 4. CYTOXICITIES AND INHIBITORY EFFECTS OF SOME CHELATORS ON THE GROWTH OF *P. falciparum* AND ON ACTIVITY OF RIBONUCLEOSIDE DIPHOSPHATE REDUCTASE (RdR)

CHELATOR Code No.	% EXCRETION OF RADIO-IRON IN RATS	% CYTOXICITY ON P388 CELLS OF 10µg/ml[21]	% INHIBITION OF *P. falciparum* at $2 \cdot 10^{-5}$M	% INHIBITION OF RdR[21] 10^{-3} M HYDROXYUREA = 81%	
				CONC. IN MOLES	INHIBITION IN %
SG-6	35.4	-	16.6 at $2 \cdot 19^{-5}$M	$1 \cdot 10^{-6}$	none
SG-9	86.7	-	90.9	$1 \cdot 10^{-5}$	none
SG-11	34.2	100	93.4	$8 \cdot 10^{-5}$	72
SG-14	-	100	16 at $2 \cdot 10^{-5}$M	$2 \cdot 10^{-5}$	77
SG-15	96	13 (non-toxic)	90.3 at $2 \cdot 10^{-5}$M	$1 \cdot 10^{-5}$	79
SG-18	-		88.6 at $2 \cdot 10^{-5}$M	$1 \cdot 10^{-5}$	none
SG-32	-	none	100	(10^{-5} (10^{-4}	38 78

ANTIMALARIAL MODE OF ACTION

The data presented in Table 4 provides no clue for understanding the impressive antimalarial activity displayed by the chelators. The clue, however, emerged when the effect of antioxidants[23] on antimalarial activity was searched. Towards this end desferrioxamine was selected since it effectively blocks the iron-catalysed Fenton-type reaction,[18] generating highly toxic oxygen species such as H_2O_2, superoxide ($O_2^{\cdot-}$), and hydroxyl ($\cdot OH$) radicals. As suspected, the presence of desferrioxamine entirely blocked the antimalarial activity of the pyridoxal-based chelators. This suggested that the latter functions most likely as electron-transfer oxidants, mediating transfer of an electron from iron(II) to an appropriate "acceptor". When the latter is molecular oxygen, a uni-valent reduction process ensues, generating the toxic oxygen species (see Chart 3).

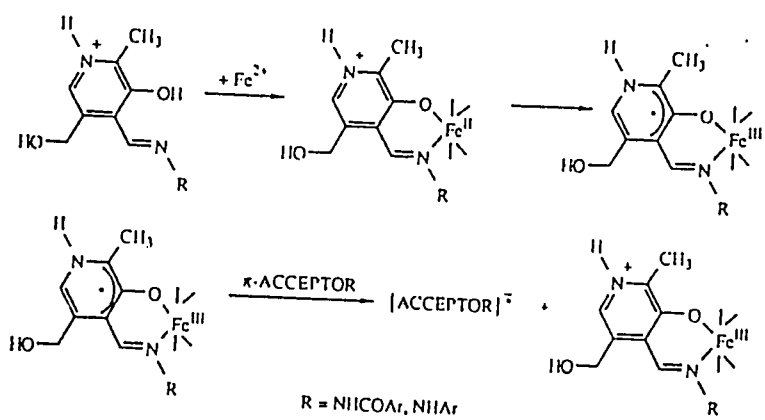

CHART 3. Suggested mechanism for pyridoxal induced electron transfer process.

The superoxide radical is efficiently and specifically scavanged by superoxide dismutase (SOD), catalyzing the disproportionation of $O_2^{\cdot-}$ into H_2O_2

$$O_2^{\cdot-} + O_2^{\cdot-} + 2H^+ \longrightarrow H_2O_2 + O_2$$

in the presence of ferrous ions the Haber-Weiss reaction takes place:

$$H_2O_2 + O_2^{\cdot-} \xrightarrow{Fe^{2+}} O_2 + OH^- + \cdot OH$$

The toxic oxygen species are formed intraerythrocytically in large quantities when the red blood cells are abnormal as in the case of sickle cell anaemia and thalassemia.[23] This implies enhanced resistance to malaria parasites by haemolytic anaemia patients, as is the case.

Corroboration of this mechanistic view was forthcoming from Electron Spin Resonance studies of cultures of P. falciparum exposed to the simultaneous action of both the chelator and iron ions. The appearance of strong ESR signals was attributed to carbon-centered free radicals as portrayed by Figures 4 and 5.

Fig. 4. Electron spin resonance spectra of ligand Fe(II) complex in the presence of P. falciparum, and α-phenyl-N-tert-butylnitrone (PNB) as a spin trap. A_N = 15.75 G, A_H = 4.25 G (carbon-centered spin adduct).

CONCLUSION

Among the metals playing vital roles in health and diseased organisms,[24] iron is the most important one. This communication amply underlines the importance of implicating iron in designing successful chemotherapeutical strategies against parasites capable of developing resistance to drugs. In Rudyard Kipling's words: "Cold Iron":

"Gold is for the mistress - silver for the maid - copper for the craftsman, cunning at his trade. Good! said the Baron, sitting in his hall, but Iron - Cold Iron - is the master of them all".

80

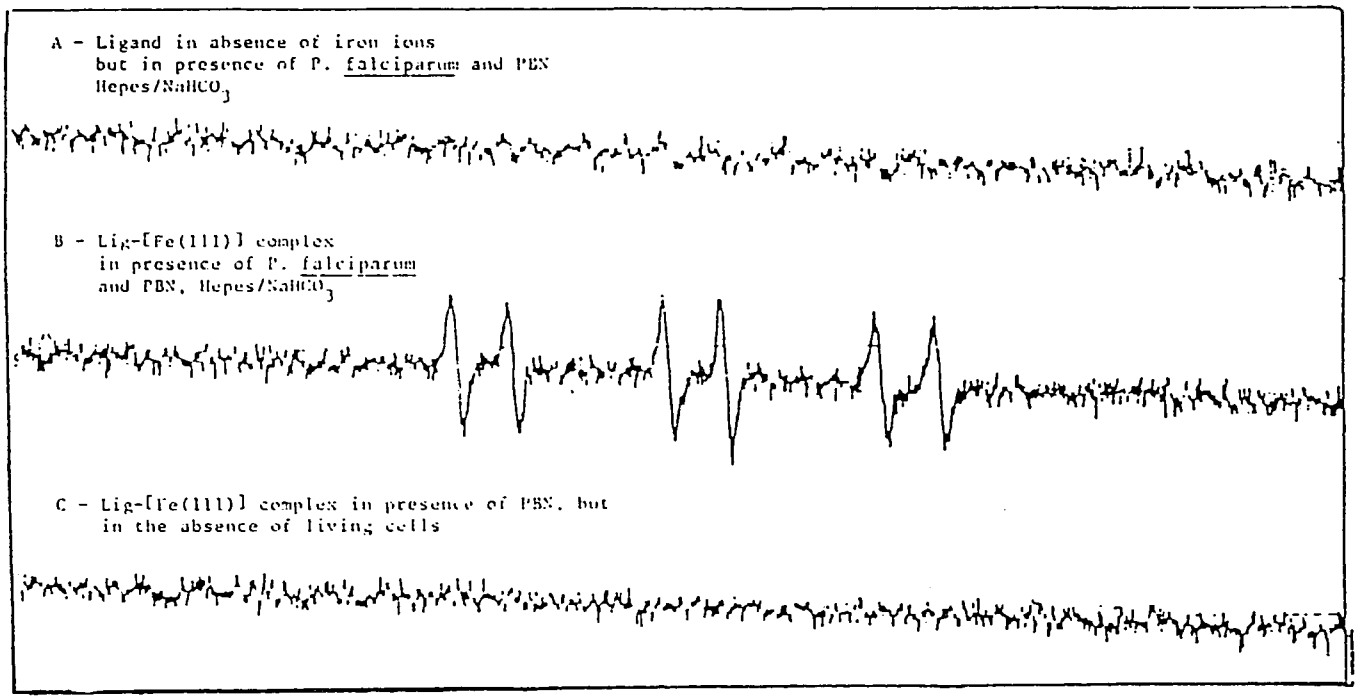

Fig. 5. ESR spectra of Ligand-Fe(III) complexes in the presence (A) and (B) and in the absence of P. falciparum (C), PBN as a spin trap.

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Inhibition of *Plasmodium falciparum* growth by a synthetic iron chelator

Eugene N. Iheanacho[1], Amram Samuni[2], Schelly Avramovici-Grisaru[3], Shalom Sarel[3] and Dan T. Spira[1] [1]*The Kuvin Centre for the Study of Infectious and Tropical Diseases;* [2]*Department of Molecular Biology; and* [3]*Department of Pharmaceutical Chemistry, Hebrew University–Hadassah Medical School, Jerusalem, Israel*

Abstract

The susceptibility of the chloroquine-resistant malaria parasite *Plasmodium falciparum* (FCR-3) to a pyridoxal-based iron chelator was tested. 10 μM of the chelator 1[N-ethoxycarbonylmethyl-pyridoxylidenium]-2-[2'-pyridyl] hydrazine bromide (code name L2-9) effectively inhibited growth *in vitro* of the parasites. Presaturation of the chelator with either ferric or ferrous iron partially blocked the inhibitory effect. Two hours' exposure of parasites to 20 μM L2-9 was sufficient to inhibit their growth irreversibly. Desferrioxamine blocked the inhibitory effect of L2-9. It is suggested that the chelator may be acting by generating free radicals in complexing intracellular iron.

Introduction

Drug-resistant malaria has now become a major health problem. World-wide malaria eradication appears to be less likely, and millions of lives are in jeopardy as a result of this threat. The fast growing prevalence of drug-resistant malaria therefore calls for rational biochemical approaches for the development of new antimalarial chemotherapy. The exquisite sensitivity of malaria parasites to oxidant stress and radical-inducing drugs makes this approach a logical candidate for antimalarial activity.

With the possible exception of lactobacilli, iron is an essential growth element to virtually all living organisms (Neilands, 1981), and its importance and influence on infections is well documented (Weinberg, 1984). An invading pathogen must compete with its host for iron. If the pathogen cannot obtain sufficient iron, its proliferation may be retarded and the infection attenuated (Payne & Finkelstein, 1978). Host defences may involve withholding iron: serum iron levels fall with inflammation, and phagocytic cells, which accumulate at sites of inflammation, bring with them lactoferrin, a protein which sequesters iron (Roeser, 1980). The drop in serum iron levels of mammalian hosts during bacterial infections (Kluger & Rothenburg, 1979) can be regarded as a mechanism of non-specific resistance. A similar decrease in serum iron has been observed in malarial infections in man (Usanga, 1983). Many microorganisms show enhanced virulence *in vivo* or faster growth *in vitro* when the concentration of available iron is increased (McFarlane *et al.*, 1970; Murray *et al.*, 1980; Loo & Lalonde, 1984). Iron chelators have, therefore, been shown to affect the growth and development of parasites both *in vitro* and *in vivo* (Raventos-Suarez *et al.*, 1982; Fritsch *et al.*, 1985, 1987).

Corespondence to: Dan T. Spira, The Kuvin Centre for the Study of Infectious and Tropical Diseases, Hadassah Medical School, P.O.B. 1172, Jerusalem 91010, Israel.

Desferrioxamine (DFO), an iron-specific chelating agent, inhibits growth of the malarial parasite, *Plasmodium falciparum*, in cultures (Raventos-Suarez *et al.*, 1982; Fritsch *et al.*, 1985; Peto & Thompson, 1986). However, the poor oral absorption and short half-life of DFO in the plasma limit its usefulness as an antimalarial agent. There is therefore a need continually to explore the antimalarial properties of other iron chelators for possible clinical usefulness. In the present work, the effect of a new pyridoxal-based iron chelator on the growth and development of a chloroquine-resistant strain of *P. falciparum* was studied. The results showed that parasite growth could be effectively inhibited by this iron chelator, which has no effect on normal red blood cells; it seemed to be acting not by merely depriving the parasite of iron but primarily by initiating an adverse biochemical reaction which led to parasite death.

Materials and Methods

P. falciparum, chloroquine resistant strain FCR-3, was obtained from the stock of a continuous line maintained in our laboratory. Normal red blood cells, group A^+ or O^+, were obtained from healthy donors who had no prior contact with malaria. Serum and plasma from healthy donors were obtained from the Hadassah Hospital blood bank. The pyridoxal-based iron chelator 1-[N-ethoxycarbonylmethyl-pyridoxylidenium]-2-[2'-pyridyl] hydrazine bromide, code-named L2-9, was synthesized by us. Iron chelates were prepared by mixing L2-9 anaerobically with either $FeSO_4$ or $Fe_2(SO_4)_3$ at 1:1 molar ratios. Radiolabelled [^{3}H]hypoxanthine (specific activity 10 Ci/mM) was purchased from NEN Corporation (Boston, Massachusetts, USA).

Parasites were grown in 9 cm Petri dishes using the candle jar method (Jensen & Trager, 1977). They were cultured in group A^+ or O^+ red blood cells in RPMI 1640 medium containing 25 mM HEPES buffer, 2 g/litre $NaHCO_3$, 100 μg/ml gentamycin (Sigma) and 10% human serum or plasma type A. Parasites were synchronized by a combination of sorbitol treatment (Lambros & Vanderberg, 1979) and gelatine sedimentation (Jensen, 1978). Parasitaemia was determined by both microscopical counts of the number of parasites per 1000 cells and by [^{3}H]hypoxanthine incorporation (Golenser *et al.*, 1981). The cells were harvested with a MASH II® automatic cell harvester on glass fibre filters and their radioactivity determined in a Beckman LS 7500 liquid scintillation counter.

All experiments were begun with highly synchronized parasite cultures of either schizonts or ring stages. Since the inoculum varied from experiment to experiment, parasite growth depended on the inocu-

lum and [^{3}H]hypoxanthine incorporation and growth curves were comparable only within a given experimental set and not between different experiments. All experiments were repeated at least thrice. All microscopic and radioactive counts presented here are averages of at least triplicate determinations.

Results

In the first series of experiments parasites were cultured for 3 d in the presence of varying concentrations of L2-9. The results (Fig. 1) show that 20 μM L2-9 completely inhibited parasite growth in less than 24 h, while 10 μM L2-9 achieved nearly total inhibition on day 2. To study the effect of iron, the experiment was repeated with 20 μM preformed iron chelates of L2-9-Fe(II) and L2-9-Fe(III). As seen in the Table, Fe(III) and, to a lesser extent, Fe(II) blocked the inhibitory effect of L2-9. To investigate the possibility that L2-9 affects the red blood cells rather than the parasites, normal erythrocytes were pre-incubated with 40 μM L2-9 for 24 h and subsequently washed with RPMI 1640 before being infected with the parasites. Chelator-treated red cells sustained parasite growth as effectively as untreated normal cells, indicating that L2-9 does not affect parasite-free erythrocytes.

To measure the minimal exposure time required for drug-induced killing of parasites, synchronized schizonts were suspended in 20 μM L2-9 and incubated at 37°C for 1 h or 2 h. Thereafter, the cells were washed twice and cultured in the normal way. The results showed that L2-9 had no effect on the parasite in the first hour but an inhibition of more than 75% occurred within the second hour of incubation (Fig. 2). To test which growth stage of the parasite is susceptible to the drug, 2 h pulse treatment was given at various times throughout the growth cycle. Cultures were synchronized and adjusted with fresh erythrocytes to 0·1% parasitaemia and 4% haematocrit. At times corresponding to specific growth stages of the parasite, the cells were exposed to 20 μM L2-9 for 2 h, washed thrice and re-cultivated. The results (Fig. 3) showed that, while there was marked growth inhibition at the period corresponding to the late trophozoites and schizonts, there was no inhibition at the ring stage, suggesting that the inhibitory effect of L2-9 is stage-dependent. A differential count of the various parasite stages after 2 h pulsing showed no deviation from the normal growth pattern. This means that parasites surviving the 2 h treatment continued their normal life cycle, implying that L2-9 does not modify the parasite's growth.

Because desferrioxamine (DFO) is known to sequester iron ions, its effect on the inhibitory activity of L2-9 was tested. The parasite-infected erythrocytes were exposed to DFO and L2-9 (40 μM each) for 2 h. DFO alone had a minimal inhibitory effect on the parasites, but in combination with L2-9, DFO fully blocked the inhibitory effect of L2-9. A 15 min pre-incubation of parasites with DFO was necessary for the effect of DFO on L2-9 to be manifested (Fig. 4).

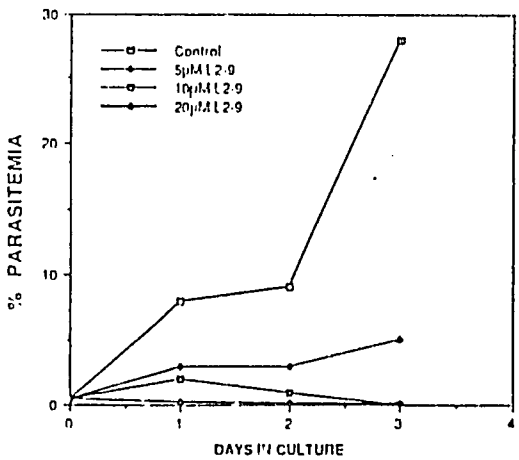

Fig. 1. Inhibition of parasite growth by various concentrations of L2-9. Parasites (*P. falciparum*) were synchronized by gelatin sedimentation and grown in RPMI growth medium containing various concentrations (5, 10, & 20 μM) of L2-9. These concentrations were maintained throughout the 3 days of culture by incorporating the appropriate amount of L2-9 in the fresh medium added each day. Parasitaemias shown are the means of triplicate determinations.

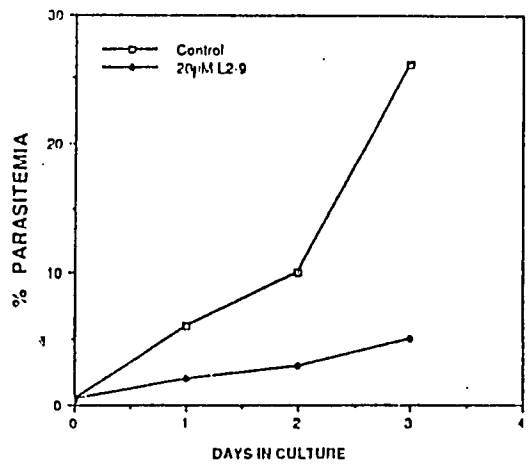

Fig. 2. Effect of parasite pre-exposure to L2-9. Gelatin synchronized parasites were suspended in RPMI washing solution containing 20 μM L2-9 for 2 h. Thereafter, the parasites were washed thrice in RPMI washing solution and cultured. The medium was replaced each day with fresh RPMI growth medium.

Table. Effect of pre-complexing iron with L2-9 on its antiplasmodial activity*

Culture media	[^{3}H]hypoxanthine (cpm)	Percentage incorporation
Normal medium	8298	100
10 μM L2-9	1331	16
20 μM L2-9	302	3
20 μM L2-9+Fe(II)	6682	80
20 μM L2-9+Fe(III)	7679	92

*Gelatin-synchronized parasites were grown in RPMI growth medium containing either only L2-9 or its complexes with iron. L2-9 was chemically pre-complexed with either Fe(II) or Fe(III) and these were used in the growth medium. [^{3}H]hypoxanthine was added 24 h after the start of the experiment, and the cells were harvested 24 h later. The results shown are the means of triplicate counts.

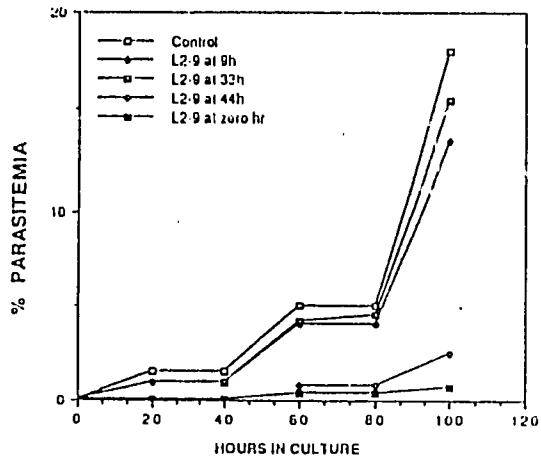

Fig. 3. Effect of L2-9 on parasites at various growth stages. Parasites were synchronized by gelatin sedimentation and suspended in RPMI washing solution containing 20 μM L2-9 for 2 h at various time intervals. The times corresponded to the various growth stages of the parasite.

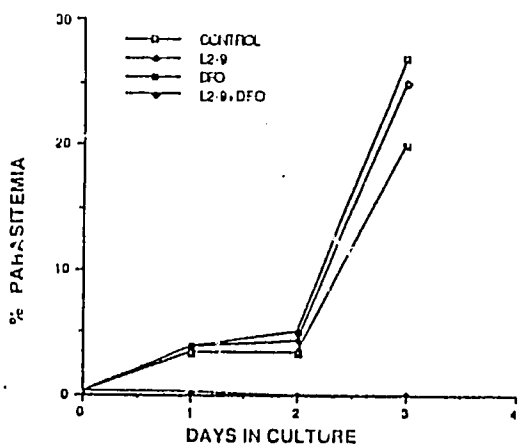

Fig. 4. Effect of desferrioxamine on L2-9 antiplasmodial activity. Parasites were synchronized by gelatin sedimentation and suspended in RPMI washing solution containing 40 μM of desferrioxamine (DFO), L2-9, or a mixture of both, for 2 h. After the 2 h incubation, the cells were washed and cultured in RPMI growth medium.

Discussion

This study has shown that L2-9, a pyridoxal-based iron chelator, at a concentration of 20 μM inhibited *P. falciparum* growth *in vitro*. If the drug exerts its antiparasitic effect by sequestering the iron essential for parasite growth, pre-saturation of the chelator with exogenous iron should diminish or abolish its antiparasitic action, as shown for DFO (RAVENTOS-SUAREZ *et al.*, 1982; FRITSCH *et al.*, 1985; PETO & THOMPSON, 1986; HERSHKO & PETO, 1988). The present results indeed showed that presaturating the chelator with iron greatly reduced its antiparasitic action. This finding apparently supports the assumption that L2-9 acts by depriving the parasite of necessary iron. This conclusion, however, does not account for the finding that Fe(III), despite its lower solubility, was more effective than Fe(II) in abolishing the inhibitory action of L2-9. Moreover, this conclusion does not agree with the effect of DFO on the chelator. If L2-9 inhibits the growth of parasites by simply withholding iron, its combined effect with DFO would be expected to be either additive or synergistic. On the contrary, a combination of DFO and L2-9 was antagonistic. This antagonistic effect depended on the sequence of addition of DFO and L2-9 to the culture, and was manifested only when DFO, which has a higher affinity for iron, was added at least 15 min before the addition of L2-9. Early removal of chelatable iron from the erythrocyte-parasite complex appeared to protect the parasite, suggesting that iron ions were instrumental in the antiparasitic activity of L2-9. Parasite killing could involve free radical intermediates generated by L2-9 upon its binding with iron. Considerable controversy exists as to whether natural sequestered forms of iron or possibly other transition metal ions are effective in catalyzing the Fenton and Haber-Weiss reaction (AMBRUSO & JOHNSTON, 1981; GUTTERIDGE *et al.*, 1982; HALLIWELL, 1982; ITO *et al.*, 1983; WINTERBOURN, 1983; BALDWIN *et al.*, 1984; VERCELLOTTI *et al.*, 1985; FRITSCH *et al.*, 1987). However, iron activity strongly depends upon the nature of its coordinating ligand. Different ligands alter the redox potential, the activity and binding mode of a complexed transition metal (RICHARD, 1984). Since iron ions readily undergo redox reactions which could lead to free radical formation (GUTTERIDGE *et al.*, 1979; BUTCHER *et al.*, 1983), it is tempting to speculate that a similar mechanism is operative also in the antiparasitic action of L2-9. L2-9 is a redox-active compound. In cyclic voltametry in water, it exhibits a redox transition at approximately +1·0 V versus calomel. Indeed L2-9 induces carbon-centred radicals as proven by electron spin resonance spectroscopy. The radical is initiated by the internal redox reaction of the L2-9 iron complex during an Fe(II)—Fe(III) transition (unpublished observations).

It is, therefore, very likely that L2-9 exerts its antiparasitic action in a dual manner, withholding iron from the parasite and generating free radical intermediates, both mechanisms leading to the death of the parasites.

Acknowledgement

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GROWTH INHIBITION OF *PLASMODIUM FALCIPARUM* INVOLVING CARBON CENTERED IRON-CHELATE RADICAL ($L^{\cdot}$, X^{-})-Fe(III) BASED ON PYRIDOXAL-BETAINE. A NOVEL TYPE OF ANTIMALARIALS ACTIVE AGAINST CHLOROQUINE-RESISTANT PARASITES

EUGENE N. IHEANACHO,† SHALOM SAREL,‡ AMRAM SAMUNI,§ SCHELLY AVRAMOVICI-GRISARU‡ and DAN T. SPIRA†

†*The Kuvin Centre for the Study of Infectious and Tropical Diseases,*
§*Department of Molecular biology, Hebrew University – Hadassah Medical School,*
‡*Department of Pharmaceutical Chemistry, Hebrew University School of Pharmacy, Jerusalem, Israel*

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Malaria parasites have been shown to be more susceptible to oxidative stress than their host erythrocytes. In the present work, a chloroquine resistant malaria parasite, *Plasmodium falciparum* (FCR-3) was found to be susceptible in vitro to a pyridoxal based iron chelator – (1-[*N*-ethoxycarbonylmethyl-pyridoxlidenium]-2-[2'-pyridyl] hydrazine bromide – (code named L2-9). 2h exposure to 20 μM L2-9 was sufficient to irreversibly inhibit parasite growth. Desferrioxamine blocked the drug effect, indicating the requirement for iron. Oxygen however, was not essential. Spectrophotometric analysis showed that under anoxic conditions, L2-9-Fe(II) chelate undergoes an intramolecular redox reaction which presumably involves a one electron transfer and is expected to result in the formation of free radical. Spin trapping coupled to electron spin resonance (ESR) studies of L2-9-iron chelate showed that L2-9-Fe(II) produced free radicals both in the presence and absence of cells, while L2-9-Fe(III) produced free radicals only in the presence of actively metabolising cells.

KEY WORDS: red blood cells, *Plasmodium falciparum*, iron chelates, free radicals, electron spin resonance.

ABBREVIATIONS: RBC, red blood cells; DFO, desferrioxamine; ESR, electron spin resonance.

INTRODUCTION

The wide spread of multidrug resistant strains of the malarial parasite – *P. falciparum* that are now resistant to the quinine-related antimalarials calls for fundamental change in the design of novel chemotherapeutical agents. Clinical observations have shown that iron metabolism and malarial infection are closely interrelated,[1,2] and several studies have been designed to explore the effect of iron chelators on the growth of *P. falciparum*.[3-6] The binding of iron to redox-active sequestering agents could give

Correspondence address: Professor Shalom Sarel, Department of Pharmaceutical Chemistry, Hebrew University School of Pharmacy, P.O. Box 12065, Jerusalem 91120, Israel.
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rise to opposite biological effects, namely, either to enhancement of an oxidative (free radical) damage to the cell,[7] or inversely to an inhibition of such an effect. Mechanistically, chelators can operate by several distinctly different modes. One mode may involve mediation in transferring of the metal–ion to a cell receptor, invoking a damaging process to the latter. Another mode may invoke modification of the electrochemical gradient of the metal–ion. Alternatively, the chelator may act as a catalyst for converting essential cell metabolites into harmful free radicals by promoting a single-electron-transfer (SET) process.

In vivo and *in vitro* observations suggest that oxidative stress plays a dominant role in the defense against parasitic infections. Malarial parasites have been shown to be more susceptible to oxidative stress than their host erythrocytes.[8-11] Injection of malaria infected mice with alloxan which is known to increase in vivo the production of highly reactive oxygen species such as $O_2^{\cdot-}$, H_2O_2, and $^{\cdot}OH$ markedly reduced the parasitemia.[12,13] There is also evidence that activated macrophages contribute through reactive oxygen intermediates (ROI) and other secretory products to rodent immunity against malaria.[14] The concept of free radical induced damage and the greater susceptibility of parasitized erythrocytes to oxidant damage provides a number of possibilities for malaria control.

In the present study, the chloroquine-resistant strain of *P. falciparum* is shown to be susceptible to 20 μM of an iron chelator (L2-9 = L^+, X^-) based on pyridoxal-betaine. Most significantly, the latter was observed to inhibit the growth of parasites after 2 hours of incubation.[3] This inhibition could be reversed by equimolar concentration of desferrioxamine (DFO). This result could be rationalized by viewing the chelator as functioning not only as a metal sequestrant capable of depriving the parasite of essential iron, but also as an inducer of free radical intermediates which lead to the death of the parasite. To substantiate this rational, we undertook an electron spin resonance (ESR) study (using spin traps such as PBN and DMPO) of L2-9. Most significantly, we observed that whereas the Fe(II)-chelate [(L^+, X^-)-Fe(II)] induces the production of carbon-centered free radicals both in the presence and in the absence of cells (parasites), the corresponding Fe(III)-chelate [(L^+, X^-)-Fe(III)] could do so only in the presence of living cells.

MATERIALS AND METHODS

Chemicals: The ligand, 1-[*N*-ethoxycarbonylmethyl-pyridoxylidenium]-2-[2′-pyridyl] hydrazine bromide (code named L2-9) was synthesized by exposing pyridoxal pyridyl hydrazone to the action of ethyl bromoacetate. Desferrioxamine B methanesulphonate (DFO) was obtained from CIBA. Horsham, Sussex-UK. α-phenyl-*N*-*tert*-butylnitrone (PBN) and 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) were purchased from Aldrich. DMPO was purified before use by distillation or by activated charcoal. The DMPO concentration in aqueous solutions was determined spectroscopically. $Fe_2(SO_4)_3$ and $FeSO_4$ were obtained from Fisher Scientific Co. (Silver Spring, MD); RPMI 1640 from GIBCO (Grand Island, NY USA); HEPES from Sigma Chem. Co. (USA). All other chemicals were of analytical grade.

Parasites: *P. falciparum* (FCR-3) was obtained from a stock of a continuous line maintained in human A^+ erythrocytes in RPMI 1640 medium containing 10% plasma.[15] Parasites were synchronized by either the sorbitol treatment,[16] gelatine sedimentation[17]

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or a combination of both. Before use, parasites were washed twice with RPMI 1640 without plasma and once with HEPES/$NaHCO_3$ buffer (pH 7.2), and resuspended in the HEPES/$NaHCO_3$ buffer to a concentration of 10^9 cells/ml.

Influence of DFO on the inhibitory potential of L2-9. Parasites were synchronized by gelatin sedimentation and adjusted to 0.4% parasitemia and 4% haematocrit. This was dispensed equally into 15 ml centrifuge tubes. To these were added either 4 ml of RPMI 1640 normal growth medium, L2-9, DFO or 2 ml each of DFO and L2-9. For the L2-9/DFO mixture, parasites were first suspended in 2 ml of 80 μM DFO for 15 min, and then 2 ml of 80 μM L2-9 was added to give a final L2-9/DFO concentration of 40 μM. All four tubes were incubated at 37°C for 2 h. After the 2 h incubation, the cells were washed thrice with RPMI washing solution and cultured in normal growth medium. Parasitemia was determined daily by microscopical counts of number of parasites per 1000 cells.

ESR measurements: Spectra were recorded with a Varian E4 X-band spectrometer (9.5 GHz) with a field modulation frequency of 100 kHz, using non-saturating microwave power and modulation amplitude of 1 G. Measurements were made using a gas permeable teflon capillary which was inserted inside quartz tubing and placed in spectrometer.

Spectroscopic measurements: Measurements were made with a dual beam Kontron model Uvikon 860 spectrophotometer at room temperature in quartz curvettes with optical path length of 1 cm. In order to prevent hydrolysis, iron was initially dissolved in sulphuric acid at pH 2.0 and complexed to the chelator anaerobically at the acid pH, [L2-9]/[iron] = 1 : 1. The concentration was adjusted to 0.5 mM L2-9 with tripple distilled water. Just before use, the pH of the complex was adjusted to 7.2 using HEPES buffer to give a final L2-9-Fe concentration of 50–100 μM and 40 mM buffer concentration.

RESULTS

The exposure of the parasites to 20 μM L2-9 for 2 h resulted in inhibition of the *P. falciparum* growth. This inhibitory effect could be reversed by adding an equimolar concentration of DFO. This reversal action was observed only when the parasites were suspended first in DFO medium for 15 min followed by addition of the chelator (L2-9). If the parasites were added to a mixture containing DFO and the pyridoxal-based chelator, no effect of DFO was observed (Figure 1).

Spectrophotometric studies showed that under anoxic conditions and at pH 5.0–5.6, the reaction between the ligand and Fe(II) gives rise to a green coloured complex characterised by an absorption band $\varepsilon^{507\,nm}_{L2\text{-}9\text{-}Fe(II)} = 5.25\,mM^{-1}cm^{-1}$. Within 30–60 min and even in the absence of oxygen, the green L2-9-Fe(II) chelate gradually changes to a red L2-9-Fe(III) chelate having an extinction coefficient $\varepsilon^{474\,nm}_{L2\text{-}9\text{-}Fe(III)} = 19.05\,mM^{-1}cm^{-1}$ (Figure 2). The L2-9-Fe(III) generated from L2-9-Fe(II) under anoxic conditions was spectrometrically similar, though not equal to that formed by the direct chelating of L2-9 to Fe(III) ion, having $\varepsilon^{44\,nm}_{L2\text{-}9\text{-}Fe(III)} = 60.8\,mM^{-1}cm^{-1}$. The anoxic conversion of L2-9-Fe(II) to L2-9-Fe(III) indicates some intramolecular redox process which presumably involves a one electron transfer and is therefore expected to yield some

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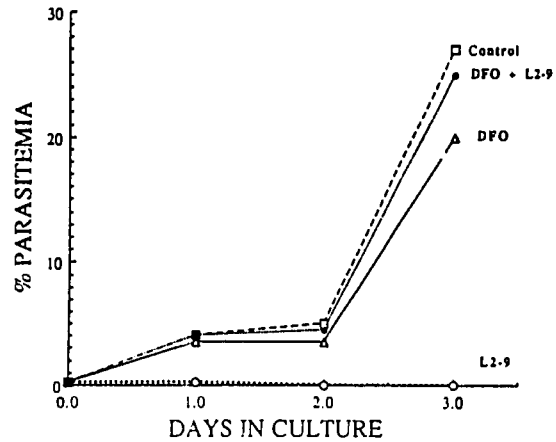

FIGURE 1 Effect of Desferrioxamine (DFO) on the antimalarial activity of L2-9. Gelatin synchronized parasites *P. falciparum* culture was dispensed in aliquots of 5 ml of either normal medium (control), L2-9, DFO or L2-9 + DFO. All concentrations were 40 μM at final hematocrit of 4% and a parasitemia of 0.2%. They were incubated at 37°C for 2 h. In the case of L2-9 + DFO, DFO was first added and left to stand for 15 min before addition of L2-9. After incubating for 2 h, parasites were washed thrice with RPMI 1640 washing solution and cultured in RPMI complete medium. Parasitemia was determined daily by microscope counts of number of parasites per 5×10^3 erythrocytes. Each value represents an average of 3 counts.

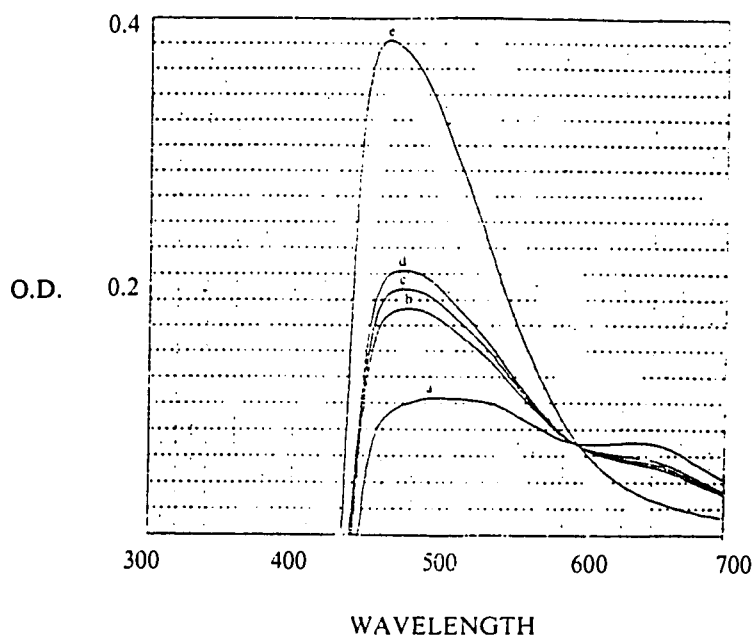

FIGURE 2 Absorption spectra of the anaerobic oxidation of Fe(II) to Fe(III) by L2-9. 1 mM $Fe(NH_4)_2(SO_4)_2$ in 10 mM sulphuric acid at pH 2.0, was complexed to L2-9 anaerobically in a ratio of [L2-9]/[iron] = 1 : 1. The concentration was adjusted to give 50 μM (L2-9)-Fe(II) in 40 mM HEPES buffer pH 7.4. Change in spectra with time after complexing were (a) 20 min; (b) 1 h; (c) 2 h; (d) 3 h; and (e) 20 h. The assay was performed anaerobically at room temperature using a dual beam Kontron model Uvikon 860 spectrophotometer.

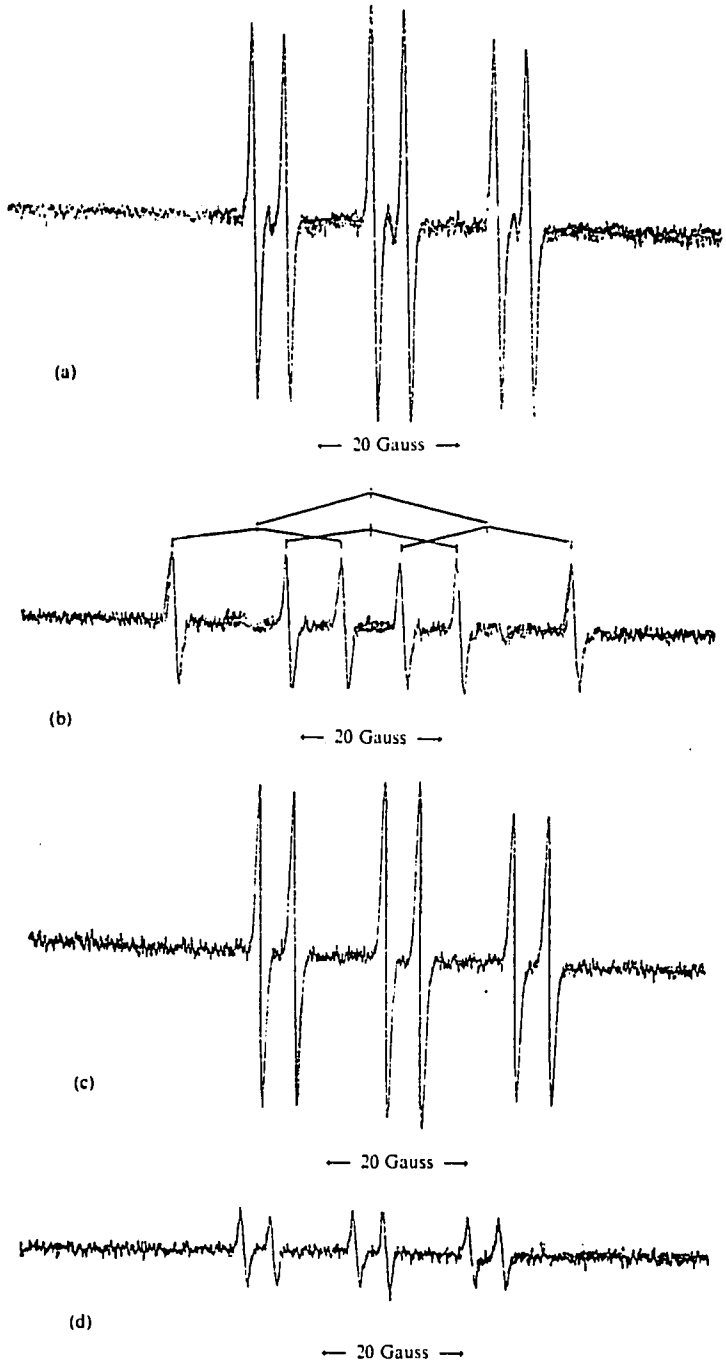

FIGURE 3 Electron spin resonance (ESR) spectra of L2-9-Iron complexes. L2-9-Fe(II) was prepared anoxically and immediately scanned for ESR signal in the presence and absence of cells, (a) 15 mM PBN; (b) 135 mM DMPO; (c) in the presence of 15 mM PBN and 10^9 *P. falciparum* schizonts/ml; (d) (L2-9)-Fe(III), 15 mM PBN and 10^9 *P. falciparum* schizonts/ml.

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reduced intermediate of the ligand L2-9. A tentative explanation for this observation is that Fe(III)-($L^{\cdot}$, X^{-}) tends to enter into a demonstration reaction, as follows: 2Fe(III)-($L^{\cdot}$, X^{-}) → Fe(II)-(L^{+}, X^{-}) + Fe(III)-(L^{+}, X^{-}). To investigate this possibility, the reaction was studied using electron spin resonance (ESR) spectroscopy. When the ligand-iron II complex was anaerobically prepared and scanned for the presence of free radicals, no ESR signal was detected. Because the lack of any observable ESR signal could be due to instability of the active intermediates, the spin trapping technique was utilized. L2-9-Fe(II) was anaerobically incubated in the presence of spin traps such as PBN and DMPO. In the presence of either PBN or DMPO, six lines ESR signals were accumulated and persisted. Both ESR spectra were characteristic of a carbon centered spin adduct having a_N = 15.75 G and a_H = 4.25 G of PBN-R$^{\cdot}$ (Figure 3a) and a_N = 15.75 G and a_H = 23.5 G of DMPO-R$^{\cdot}$ (Figure 3b) respectively. The same ESR signals were observed when the experiments were repeated under aerobic conditions but no spin adducts of $^{\cdot}OH$ or $O_2^{\cdot-}$ were detected.

L2-9-Fe(III), unlike L2-9-Fe(II), did not elicit any detectable spin adduct. It was therefore anticipated that in the presence of metabolising cells, which are capable of reducing Fe(III) to Fe(II), the generation of free radicals by both chelates would be potentiated. When the experiments were repeated using *Plasmodium falciparum* (10^9 schizonts/ml), the chelates of both Fe(II) and Fe(III) gave rise to ESR signals identical to those observed in cell-free systems (Fig. 3c and 3d). However, the spin adduct signal generated by L2-9-Fe(II) was bigger than that formed by L2-9-Fe(III).

DISCUSSION

In this study we have shown that the chelator L2-9 (L^{+}, X^{-} in chart) induces the production of free radicals in the presence of Fe(II). In the presence of metabolising cells or reducing agents, free radicals were produced by both Fe(II) and Fe(III). It thus appears that L2-9 inhibits the growth of parasites not just by the simple chelating process of iron withholding, but by the generation of free radicals. The death of parasites after two hours of incubation with L2-9 is attributable to some active intermediates induced by the chelator in the presence of Fe(II) rather than a simple nutritional deprivation of iron.

The production of oxygen-derived free radicals via the iron catalyzed Haber–Weiss reaction is well documented. However, considerable controversy exists as to whether the natural sequestered forms of iron or possibly other transition metal ions are effective in catalyzing this reaction.[18-23] The activity of iron is dependent upon the ligand to which the metal is complexed, since various chelators alter the redox potential and the reactivity of the bound metal ion to varying extents.[24,25] Iron chelators such as EDTA and DTPA inhibit or increase lipid peroxidation stimulated by chelatable iron depending on the ratio of chelator to iron, whereas DFO inhibits at all concentrations tested.[26-28] Clark et al.[29] have reported the in vivo and in vitro killing of *Plasmodium vinckei vinckei* and *Plasmodium falciparum* by *t*-butyl hydroperoxide (*t*-BHP) which generates oxygen derived free radicals. It was also shown that alloxan, a generator of reactive oxygen intermediates, cleared mice of lethal infection with *Plasmodium vinckei*.[13] In both experiments, pre-treatment with DFO for 15 min reportedly abolished the action of *t*-butyl hydroperoxide and alloxan. The present results suggest that free radical intermediates are instrumental in L2-9 toxicity

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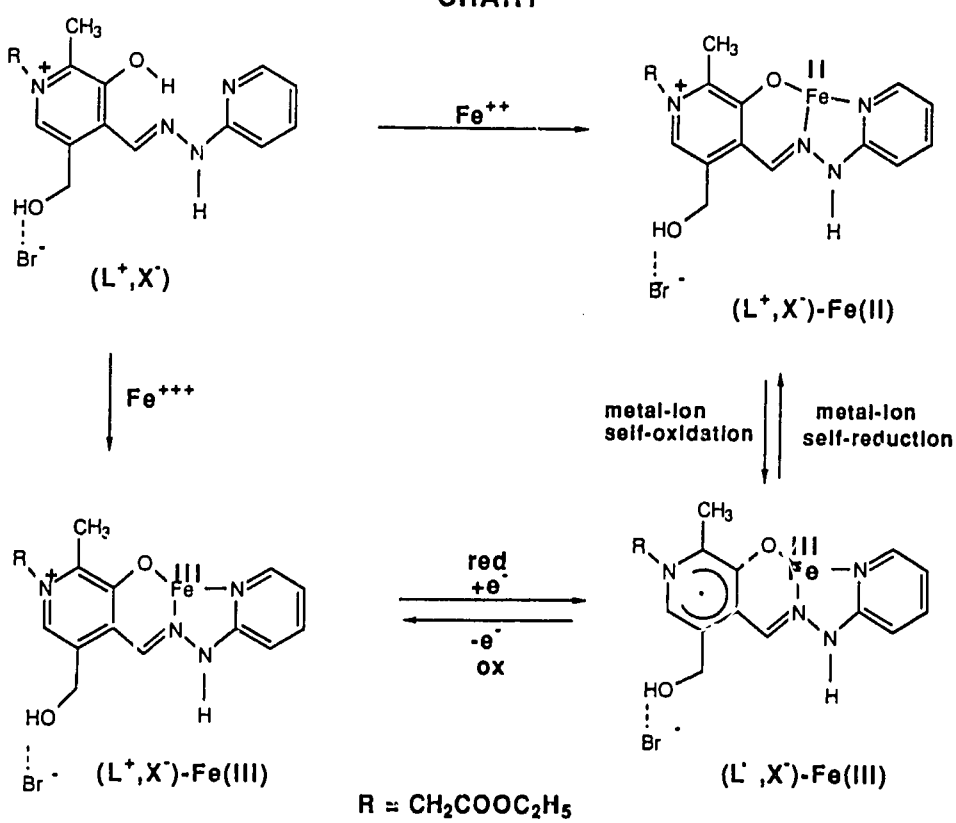
towards the parasites. The failure, however, to detect DMPO-OH spin adduct in the present study and the anoxic toxicity of L2-9 do not support the hypothesis that oxygen-derived radicals such as ·OH or superoxide, play any role in the drug's effect.

Oxidant generating drugs have been previously shown to have anti-malarial properties,[30] but the precise mechanism by which the human malarial parasite is killed by a variety of radical generating systems still remain unclear. The data produced here permits to suggest that the death of the parasite is likely caused by a sort of damaging process arising from the break down of an essential component of the cell, such as the DNA macromolecule. Evidences indicating damage to DNA by L2-9-Fe(II) is forthcoming.[31] This is substantiated by the appearance of ESR signals only when living cells are present in a mixture of the chelate L2-9-Fe(III) with spin traps such as PBN or DMPO, indicating that living cells are involved in a type of single electron-transfer [SET] as expressed by Eq. 3. The tendency of the DNA macromolecule to function as an electron-transfer oxidant (an electron acceptor) in redox reactions, is well documented.[32,33] It is plausible therefore to suggest that the iron chelate assumes most likely the role of an electron-transfer-reductant (an electron donor) in the latter process.[34,35] Accordingly we attribute reductive properties to the carbon-centered free radical of structure (L, X^-)-Fe(III) (see chart). The latter most likely arises from a reversible intramolecular SET shift (Eq. (2)), involving an initially formed (L^+, X^-)-Fe(II) intermediate (Eq. (1)). The $(DNA)^{\cdot-}$ anion radical (eq. (3)) is known to be highly labile, tending to fragment and thus leading to the death of

CHART



the cell.

$$[H_2L^+, X^-] + Fe^{2+} \longrightarrow 2H^+ + [L^+, X^-]\text{-Fe(II)} \quad (1)$$

$$[L^+X^-]\text{-Fe(II)} \xrightarrow[\text{electron transfer}]{\text{intramolecular}} [L^{\cdot}, X^-]\text{-Fe(III)} \quad (2)$$

$$[L^{\cdot}, X]\text{-Fe(III)} + \text{DNA (parasite)} \ \underline{\text{SET}} \ [L^+, X^-]\text{-Fe(III)} + [\text{DNA}]^{\cdot-} \text{ parasite} \quad (3)$$

$$\text{Parasite-}[\text{DNA}]^{\cdot-} \longrightarrow \text{Decomposition products} \quad (4)$$

Because of the low concentration of the ligand (20 μM) for effective antimalarial activity, its mode of action could not be explained in terms of mass-action alone. It could however be understood in terms of steady-state theory, by which the truly active species, namely, the carbon-centered free radical ($L^{\cdot}$, X^-)-Fe(III) is regenerated in the presence of living cells as soon as consumed. The free radical regeneration could be envisioned to result from a cascade of redox events involving ubiquitous cell reductants by (red-(I)-H_2), (red-(II)-H_2), etc., with matching electrochemical gradient as expressed by Eqs. (5) and (6).

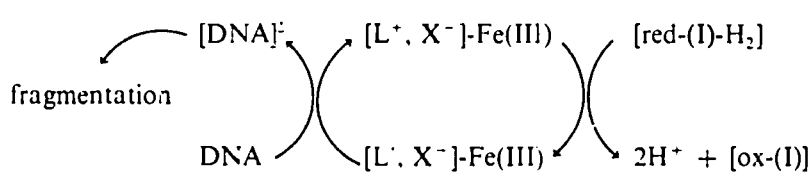
$$2[L^+, X^-]\text{-Fe(III)} + [\text{red-(I)-}H_2] \longrightarrow 2H^+ + [L^{\cdot}, X^-]\text{-Fe(III)} \quad (5)$$

$$[\text{ox-(I)}] + [\text{red-(II)-}H_2] \longrightarrow [\text{red-(I)-}H_2] + [\text{ox-(II)}] \quad (6)$$

Essentially it suggests that the antimalarial free radical species ($L^{\cdot}$, X^-]-Fe(III) plays a pivotal role in catalyzing the generation of electrons from ubiquitous cell reductants, and in mediating the transfer of the electrons to the parasite-DNA, by a multi-stage ET process, as outlined below:

$$[\text{red-(I)-H2}] + 2\text{DNA-parasite} \xrightarrow{\text{catalyst}} [\text{ox-(I)}] + 2H^+ + 2[\text{DNA-}]^{\cdot-} \text{ parasite.} \quad (7)$$

The novel type of lipophilic-hydrophilic chelator based on pyridoxal-betaine (L2-9) described here is a representative of a family of new antimalarials operating by a hitherto unknown mechanism involving the generation and transfer of electrons from cell reductants to the parasite DNA (Eqs. (3–7)). The death of the parasite is believed to follow from the breakdown of the resulting DNA anion radical ($[DNA]^{\cdot-}$) (eqn. 4).[36,37]



The greater susceptibility of parasitized erythrocytes to oxidant damage[14,38] and the relative abundance of iron in parasitized cells provide a number of possibilities for the design of new anti-malarial drugs based on intracellular iron dependent radical inducers.

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Fe (II)-CHELATES BASED ON REDOX-ACTIVE PYRIDOXAL-BETAINES AS C-CENTERED RADICALS CAUSING SINGLE- AND DOUBLE-STRAND SCISSIONS TO DNA

EUGENE N. IHEANACHO†, SHALOM SAREL‡, AMRAM SAMUNI*, SHELLY AVRAMOVICI-GRISARU‡, and DAN T. SPIRA†

*†The Kuvin Centre for the Study of Infectious and Tropical Diseases; *Dept. of Molecular Biology; Hebrew University – Hadassah Medical School; ‡Dept. of Pharmaceutical Chemistry; Hebrew University School of Pharmacy, Jerusalem – Israel*

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The ability of 1-[N-Ethoxycarbonylmethylpridoxylidenium]-2-[2'-pyridyl]hydrazine bromide code name - [L2-9 = L^+X^-]-FE(II) chelate {L2-9-Fe(II)} to induce breaks both in the 43kb linear double-strand λ phage DNA, and in the 4363 base pair supercoiled pBR322 plasmid DNA is herein described. Neither the free ligand nor FE(II) alone demonstrated any effect on the DNA. The cleaving ability is shown to occur instantaneously under strictly anaerobic conditions, either in the presence or absence of the enzyme catalase. It is also shown to be dose dependent. Thus, at λ DNA:L2-9-Fe(II) molar ratio of 3.7:1.0, the linear DNA is randomly cleaved into fragments ranging from 23.1kb to 4.3kb, whereas at approximately 1:1 molar ratio, the range extends down to 2.5kb fragments. By contrast, at 1:2.7 [plasmid DNA]: chelate-Fe(II) molar ratio, a single-strand nick was observed, and a double strand break was noted at a 1:50 ratio ([plasmid DNA] : chelate-Fe(II)). A multi-stage redox cycling involving a carbon-centered (L^+X^-)-Fe(III) radical capable of transferring an electron to the DNA to form high unstable [DNA]$^-$ anion-radical is invoked to explain the degradation of the chain macromolecule. Possible modes for regeneration of the chelate-Fe(III) radical both at the cell-free and at the cell levels are proposed.

KEY WORDS: Iron chelates, free radicals, DNA breakage.

INTRODUCTION

The increase in drug resistant strains of the malarial parasite – *P. falciparum* underscores an urgent need for the development of new chemotherapeutic strategies against malaria. Though immunopreventive and immunocurative strategies are still being experimented upon, the prospect for the development of an effective vaccine looks bleak.[1] Chemotherapy therefore remains the major weapon for reducing malaria morbidity and mortality. Elucidation of the mechanism of resistance and the mode of action of chemotherapeutic agents are important in the development of new chemotherapeutic strategies.

Iron chelation may form the basis of a new class of antimalarials. Laboratory and field observations lend support to this possibility.[2-5] The binding of iron to redox

Correspondence Address: Prof. Shalom Sarel, Department of Pharmaceutical Chemistry, Hebrew University School of Pharmacy, P.O. Box 12065, Jerusalem 91120, Israel.

active sequestering agents may give rise to opposite biological effects, namely, either to the enhancement of an oxidative (free radical) damage,[6] or inversely, to an inhibition of such effect. Mechanistically, chelators can operate by several distinctly different modes. One mode may involve mediation in transferring of the transition metal-ion to a cell receptor, invoking a damaging process to the latter. Another mode may involve modification of the electrochemical potential of the metal-ion and as a consequence to affect essential redox processes in the cell. Alternatively, the chelator may act as a catalyst for converting essential cell metabolites into harmful free radicals by promoting single electron transfer (SET) processes.

Free radical attack on DNA has been shown to have a serious effect on living organisms by causing strand breaks in the DNA.[7,8] The tendency of the DNA macromolecule to function as an electron-transfer oxidant is well documented.[9,10] Cochrane *et al.*[11] have shown in studies with isolated DNA that hydrogen peroxide (H_2O_2) generates hydroxyl radicals (·OH), following Fenton reaction at the vicinity of the DNA and that the amount of the ·OH formed correlates with the percentage of DNA strand breaks.

By use of a representative of a new class of iron chelators based on pyridoxal-betaine – (1-[N-ethoxycarbonylmethyl-pyridoxylidenium]-2-[2'-pyridyl]hydrazine bromide) – (code named L2-9), we have developed a model for a new chemotherapeutic strategy against the chloroquine resistant malaria parasite – *Plasmodium falciparum* (FCR_{3IC}). Previously, we have shown that the antimalarial action of the new chelator involves a carbon-centered free radical chelate (L2-9FE(II)→[L2-9]·-Fe(III)).[12] In the present study, we show that the free radicals generated by the chelator and Fe^{2+} affects the DNA. Nick translation and electrophoresis of L2-9-FE(II) complex treated DNA showed that the latter suffers both single and double strand fissions only by the chelator-iron complex and not by either the chelator, or the iron (Fe^{2+}) alone.

MATERIALS AND METHODS

Chemicals

The iron chelator – L2-9[13] was synthesized by exposing pyridoxal pyridyl hydrazone[14] to the action of ethyl bromoacetate. Ferric and ferrous sulphates were obtained from Fisher Scientific Co. (Silver Spring, MD, USA); RPMI 1640 from GIBCO (Grand Island, NY, USA); HEPES from Sigma Chemical Co (St Louis, MO, USA); Nick translation System Kit from NEN research Products (Boston, MA, USA); Calf thymus (CT), Lambda (λ) DNA, Plasmid (pBR322), catalase and mineral oil from Sigma Chemical Co. All other chemicals were of analytical grades.

Chelator-iron (II) complex [L2-9-FE(II)] was prepared immediately before use by mixing 1mM L2-9 with 1mM $FeSO_4$ (v/v) to give a 500μM complex. This was diluted to the required working concentrations (0.5–40μM) with RPMI 1640 medium without plasma (washing solution).

Nick translation. The method used as per the instructions in the manufacturer's manual for Nick translation system [^{32}P]. Briefly, the following components were added as follows: [α-^{32}P]dCTP – 5μl; Nick translation buffer – 5μl; cold deoxynucleoside triphosphate mixture (-dCTP) 4μl; either L2-9-Fe(II) treated or untreated CT DNA – 2μl (the final concentration in the reaction mixture was 0.5μg);

translation grade water – 7μl; DNA polymerase I (Pol I, 0.6 units/μl – 2μl. These gave a total reaction mixture of 25μl. This was incubated at 10°C for 2h. The reaction was terminated by the addition of 200μl Nick translation stop buffer. The mixture was then passed through a sephadex G-50 column using a 1ml syringe. The effluent was collected and 10μl sample placed on a Whatman GFC filter paper and radioactivity counted.

Gel electrophoresis (Neutral gel). 2μl of λ DNA (0.5μg/μl) was incubated with 8μl of various concentrations (0.5μM–40μM) of freshly prepared L2-9-Fe(II) complex for 2h. After the 2 h incubation period, 0.5μg of the treated DNA was run in an agarose gel (0.8%) using 50mM Tris-Acetate-EDTA (TAE) pH 7.5 as the running buffer. The plasmid (pBR322) was treated likewise with the L2-9-FE(II) complex and run in 1% agarose gel in TAE buffer pH 7.5. The gel was run at 5V cm^{-1} for 3h.

To measure the time course of DNA cleavage by the chelate, 1μl of plasmid pBR322 (0.5μg/λ) was incubated with 5μl of 40μM freshly prepared L2-9-Fe(II) complex for various lengths of time (0–60 minutes). The reaction was stopped by the addition of 2μl loading buffer containing 1mM EDTA. To mimic the effect of an anoxic condition, a thin layer of mineral oil (heavy white oil) was placed over one of the reaction mixtures and incubated for 60 minutes. To test if hydroxyl radicals (•OH) was involved in L2-9 induced radical damage, catalase (60μg) was added to one of the reaction mixtures and incubated for 60 minutes. The mixtures were run in 0.8% agarose gel in TAE buffer pH 7.5 at 1.5V cm^{-1} for 14 hours (overnight) at room temperature.

RESULTS

Using the nick translation method of Kelly *et al.* (1970),[17] the ability of L2-9-Fe(II) complex to induce nicks in DNA was tested. The result (Table) showed that L2-9-Fe(II) complex induced nicks in DNA. CT DNA treated with L2-9-FE(II) complex incorporated [^{32}P]dCTP 3 times as much as the untreated one. CT DNA treated with DNase I incorporated [^{32}P]dCTP only twice as much as the L2-9-Fe(II) teated one. L2-9-Fe(II) could therefore be said to have introduced nicks or breaks in the DNA half as effectively as the DNase I.

TABLE I
Incorporation of [^{32}P]dCTP into L2-9-Fe(II) treated CT DNA

CT DNA treated with:	Counts
RPMI medium (without chelate)	136492 ± 15121
L2-9-FE(II) in RPMI medium	322504 ± 31754
DNase I	619230 ± 60332

Counts are expressed as mean ± SD† of triplicate determinations. CT DNA was suspended in either 40μM L2-9-Fe(II) in RPMI medium or RPMI medium alone for 3h and thereafter used for the nick translation reaction.

†Standard Deviation.

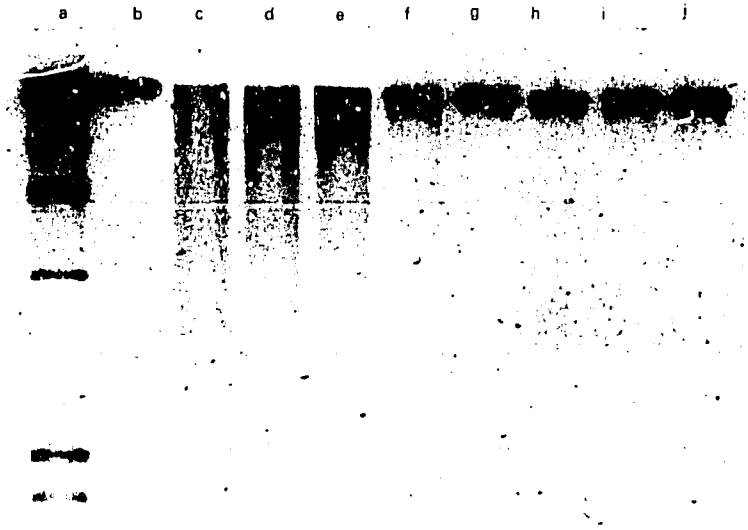


FIGURE 1 Gel electrophoretic pattern of λ phage DNA treated with L2-9-Fe(II) complex. λ phage DNA was suspended for 2h in RPMI medium (without plasma) containing various concentrations of L2-9-Fe(II). The treated DNA was then separated on a 0.8% agarose gel, stained with ethidium bromide and visualized under UV illumination. Lane a, DNA digested with HindIII, lane b, DNA in medium without L2-9-Fe(II), lanes c-h, DNA in medium containing 40, 20, 10, 5, 1, and 0.5μM L2-9-Fe(II) complex respectively. Lanes i, and J, DNA in medium containing 40μM L2-9, and Fe(II) alone, respectively. The final amount of the DNA applied to each well was 0.5μg.

The ability of the chelator-iron complex to induce nicks in DNA was also tested by gel electrophoretic analysis of both λ phage and pBR322 plasmid DNA. Figure 1 shows the gel electrophoretic pattern of λ phage DNA treated with the chelator-Fe(II) complex. The cutting ability of the complex is dose dependent. 40μM of the complex formed fragments ranging from 2.5kb to 23.1kb. The 20 and 10μM complexes formed less number of fragments, while the 5 and 1μM complexes formed only fragments of 9.4 and 23.1kb (using λ DNA cut with restriction endonuclease HindIII as the marker). Introducing nicks in a supercoiled plasmid would result in either its relaxation (for single strand nicks) or its linearization (for double strand nicks). When pBR322 plasmid was incubated with the complex for 2h, both single and double strand breakers were induced by 40, 20, and 10μM complexes; while the 5, 1, and 0.5μM complexes induced only single strand breaks (Figure 2). Neither L2-9 nor Fe(II) alone had any effect on the DNA.

The time course of DNA cleavage by the chelate was followed by incubating 0.5μg pBR322 with 40μM complex for times 0–60 minutes. The result (Figure 3) showed that the DNA cleavage was rapid, occurring in less than 5 minutes. Oxygen was not necessary for the cleavage to occur, and catalase had no effect on it, indicating the non-participitation of hydroxyl radicals in the L2-9 induced radical damage.

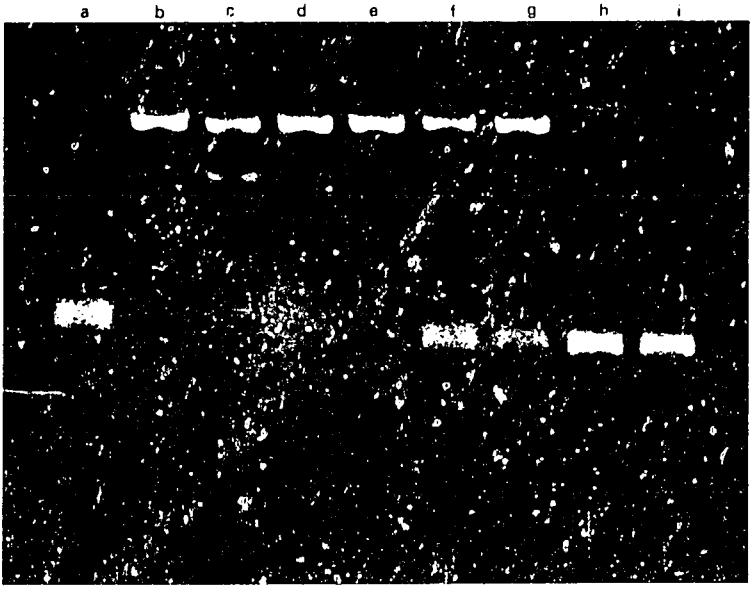


FIGURE 2 Gel electrophoretic pattern of plasmid pBR322 treated with L2-9-Fe(II) complex. Plasmid pBR322 was suspended for 2h in RPMI medium (without plasma) containing various concentrations of L2-9-Fe(II). It was separated on a 0.8% agarose gel, stained with ethidium bromide and visualized under UV illumination. Lane a, plasmid in medium without iron chelate, lanes b-g, plasmid in medium containing 40, 20, 10, 5, 1 and 0.5μM L2-9-Fe(II) complex respectively. Lanes h, and i, plasmid in medium containing 40μM of L2-9 and Fe(II) alone respectively. The amount of DNA in each well was 0.5μg.

DISCUSSION

From Figures 1 and 2, it is evident that L2-9-Fe(II) [= (L^+,X^-)-Fe(II)] is able to induce breaks both in the high molecular λ phage DNA (26.57×10^6 mol. wt., from *E.coli* strain C600), a 43000 base pair linear double strand DNA, and in a lower molecular pBR322 plasmid DNA (2.7×10^6 mol. wt., from *E.coli* strain RPI), a 4363 base pair supercoiled DNA. Neither the free ligand (L2-9) nor Fe(II) alone exhibited any effect on the DNA. The cleaving ability of (L^+,X^-)-Fe(II) is shown to be both instantaneous and dose-dependent. At molar ratio 3.7 : 1.0 of λ DNA : (L^+,X^-)-Fe(II), the macromolecule is randomly cut into fragments ranging from 23100 to 4360 base pairs, and at aproximately 1:1 – molar ratio, the range extends down to 2500 base pair fragments.

In contrast to the observed chain breaking yield (DNA/Fe-chelate × 100) of 370% in λ DNA, the yield of macromolecular scission in the supercoiled DNA plasmid is considerably lower (37–4%). Thus, at [pBR322 plasmid DNA] : $[(L^+,X^-)$-Fe(II)] molar ratio of 1 : 2.7, the supercoiled DNA undergoes a single strand nick leading to its relaxation, whereas at 1 : 50 molar ratio, a double-strand scission takes place giving rise to its linearization. From Figure 3 it can be seen that these DNA chain cuts occur

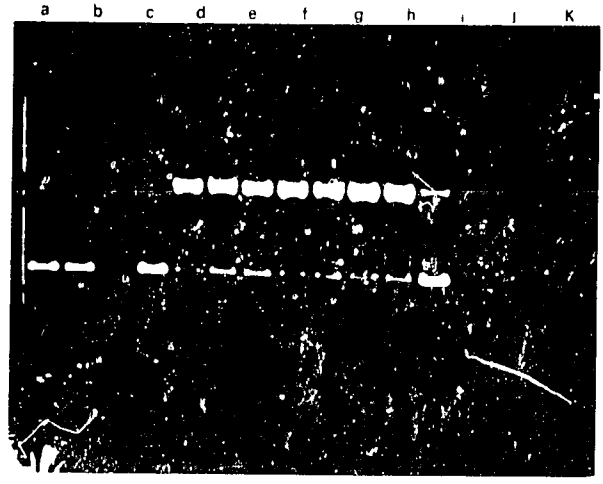
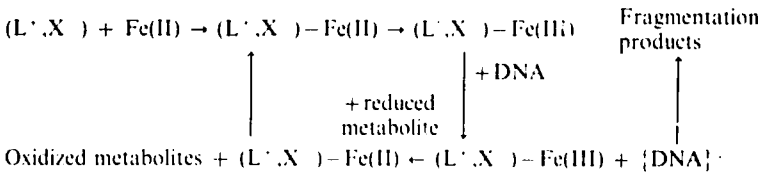


FIGURE 3 Gel electrophoretic pattern of plasmid pBR322 treated with 40μM L2-9-Fe(II) complex fo times ranging from 0-60 minutes. 0.5μg of plasmid pBR322 was incubated with 5μl of 40μM fresh prepared L2-9-Fe(II) complex for various lengths of time (0-60 minutes). The reaction was stopped by th addition of 2μl loading buffer containing 1mM EDTA. It was separated on a 0.8% agarose gel, stained wit ethidium bromide and visualized under UV illumination. Lane a, untreated plasmid; lane b, untreate plasmid under anerobic condition; lane c plasmid treated with 100μM L2-9 alone; lane d, treated wit 40μM chelate for 60 minutes; lane e, treated with 40μM chelate + 60μg catalase for 60 minutes; lane treated with 40μM chelate under anaerobic condition for 60 minutes; lanes g-k, treated with 40μM chelate and incubated for 30, 20, 10, 5, and 0 minutes respectively. The amount of DNA in each well was 0.5μg The gel was run at 1.5V cm⁻¹ for 14 hours (overnight) at room temperature.

instantaneously under anaerobic conditions, and that they are unaffected by th presence of the enzyme catalase, implying the uninvolvement of reactive oxyge species (ROS) in the chain breakings.

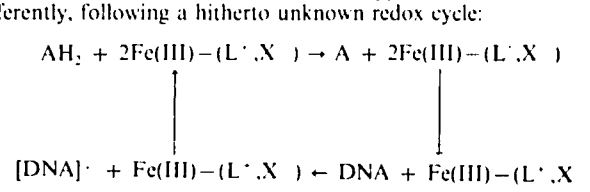
ESR study[12] has shown that (L·,X)-Fe(II) exist as a transient carbon-centered free radical presumably of L·,X)-Fe(III) structure, and its X-ray crystal analysis[14,15] indicated that the ligand (L·,X) utilizes all available hydrogen acceptors and donors for intra- and inter-molecular hydrogen contacts. On the basis of data now available, it is permissible to invoke a multi-stage process for the macromolecular chain degradation initiated by (i) binding (L·,X)-Fe(II) to DNA chain, followed by (ii) single-electron-transfers (SET): (L·,X)-Fe(II) → (L·,X)-Fe(III) + DNA → (L·,X)-Fe(III) + [DNA]⁻, and (iii) degradation of the anion — radical [DNA]⁻ into [base pair fragments]⁻, then finally by (iv) redox-cycling: [base pair fragment]· + (L·,X)-Fe(III) → DNA-fragments + (L·,X)-Fe(III), to start all over again. The discrepancy in the chain breaking yields underlined above could be rationalized in terms of redox gradients, being higher for the linear [DNA]⁻ than for the circular/relaxed [DNA]⁻ species.



The mechanistic scheme portrayed above could reasonably be invoked to explain the *in vitro* cytocidal effect of L2-9 on the drug resistant malarial parasite – *Plasmodium falciparum*. It implies that the ligand sequesters first the endogenous cellular Fe^{2+} to form the respective Fe(II)-complex, since pre-treatment of the parasite culture with a stronger iron chelator – desferrioxamine – obliterates the antimalarial properties of L2-9.[5]

The (L˙,X)-Fe(II) complex is prone to an internal electron transfer to form the respective radical species capable of delivering an electron to the DNA after its binding to the macromolecule to yield an unstable [DNA]˙ which decomposes as soon as formed (see chart). It is of interest to compare the mode of action of (L˙,X)-Fe(III) with that of the iron chelate radical-cation Fe(II)-Dox˙⁺,[21] originating from sequestration of the anti-tumour antibiotic doxorubicin (DOX) (adriamycin) with Fe^{3+}, following the sequence: Fe(III)-Dox → Fe(II)-Dox˙⁺. Whereas the former does not require molecular oxygen to damage DNA, the latter by contrast, does require O_2 to produce ROS via the reaction: Fe(II)-Dox˙⁺ + O_2 → Fe(III)-Dox + $O_2^{\cdot-}$ (ROS). The ligand in (L˙,X)-Fe(II) functions apparently as an internal electron transfer oxidant, whereas DOX in Fe(III)-Dox functions as an internal electron transfer reductant.[21] The antimalarial action of dialuric/alloxan,[22] divicine,[23] and butyl hydroperoxide,[24] seems also to involve ROS[25,26] via redox cycling.[27] In the latter, electrons appear to emerge from endogenous cellular reductants, AH_2, which in combination with a redox metal (Cu/Fe) complex and oxygen can generate ROS *via* $3AH_2 + 2O_2 \rightarrow 3A + 2H_2O + 2{\cdot}OH$ (net reaction).

Antimalarial chelators based on redox-active pyridoxal-betaine seems to operate quite differently, following a hitherto unknown redox cycle:



Conceptually, the ability of L2-9 to interact with and cut DNAs (λ phage and plasmid) was reasonably extended to explain its antimalarial action *in vitro*. This offers an interesting venue to antimalarial drug design implicating intracellular generation of harmful free radicals by iron-dependent inducers. The rapid spread of multi-drug resistant strains of the malarial parasites prompts the use of new models for drug design to provide more effective antimalarials operating by hitherto unknown mechanisms. It is highly likely that L2-9, or its analogues could demonstrate other antiparasitic and/or antitumor activity of potential therapeutic utility.

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