CHEMICAL EVOLUTION OF IRON CONTAINING ENZYMES: MIXED LIGAND COMPLEXES OF IRON AS INTERMEDIARY STEPS

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Abstract. Activities of the iron complexes of evolutionary importance like $K_4[Fe(CN)_6]$, $K_4[Fe(CN)_5(gly)]$, and $K_4[Fe(CN)_5(trigly)]$ have been tested towards some redox reactions of biological significance, namely, decomposition of hydrogen peroxide, dehydrogenation of NADH and ascorbic acid both coupled with reduction of methylene blue. It has been observed that the catalytic activities of iron (II) complexes towards the redox reactions studied at pH 9.18 followed the order, $K_4[Fe(CN)_6] < K_4[Fe(CN)_5(gly)] < K_4[Fe(CN)_5(trigly)]$. Decomposition of H_2O_2 catalysed by cyanocomplexes of iron (II) has been discussed through the formation of an innersphere complex in which loosly bound ligands like, glycine and triglycine are replaced by hydroperoxide ion. A tentative mechanism for the catalysed decomposition of H_2O_2 has been discussed.

Based upon the experimental observations a hypothesis on the evolution of iron containing enzymes has been envisaged as: iron(II) ion → iron(II) cyanide complexes → mixed ligand iron(II) cyanide and amino acid complexes → iron(II) complexes of macromolecules → proenzyme or early enzyme containing iron(II).

1. Introduction

In the recent years numerous studies have been reported on metal complexes possessing enzyme like activities. Complexes of copper [1–3] and iron [4, 5] have been found to perform activities like catalase or peroxidase. However, no experimental efforts seem to have been made for a systematic study on the evolutionary steps of iron containing enzymes.

Egami [6] in his hypothesis on the origin and early evolution of transition metal enzymes has proposed that biometals like, iron, molybdenum, and zinc, the most abundant transition metals in sea water presumably complexed with the accumulated compounds in the primeval sea forming complicated intermediates which subsequently transformed to proenzymes or early enzymes of broad specificity and consequently of low activity. Egami (*loc. cit.*) was of the view that iron containing compounds acted as catalysts in various oxidation-reduction and hydrolytic reactions in the course of chemical evolution.

Beck [7], in view of some organic compounds forming metal complexes during chemical evolution, categorised several reactive compounds as primary and secondary ligands. He considered compounds like H_2O, NH_3, CN^- etc. as primary ligands and amino acids, carboxylic acids, peptides, heterocyclic compounds, and macro-cyclic compounds as secondary ligands. The distinction between the primary and secondary ligands was arbitrary. The primary ligands indicate the species which

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could be present in a fairly early stage of chemical evolution while the secondary ligands are the biologically important molecules having functional groups suitable for complex formation. Beck further proposed that iron which is certainly the most probable candidate for playing crucial role in chemical evolution might have existed in the primeval sea in the form of mixed ligand complexes of cyanide and other ligands of prebiotic importance. Under the prebiotic reducing conditions, iron could have mainly existed in the lower valence state and therefore, low oxidation state iron complexes such as hexacyanoferrate (II) and its related compounds seemed to have played vital role in chemical evolution.

In this paper, results of catalytic activities of iron (II) complexes towards different redox reactions namely, decomposition of hydrogen peroxide, dehydrogenation of NADH and ascorbic acid coupled with reduction of methylene blue are presented. This is in turn an effort to study the evolutionary steps leading to the formation of proenzymes containing iron. The redox reactions pertaining to dehydrogenation processes studied here are supposed to have occurred in the reducing primitive atmosphere. However, selection of hydrogen peroxide for its catalysed decomposition by low oxidation state iron complexes has been undertaken to see the analogy to one reported by iron (III) complexes by Calvin [8].

2. Materials and Method

2.1. *Materials*

Glycine (B.D.H.), triglycine (Sigma), NADH (Sisco), ascorbic acid (Sarabhai Chemicals), methylene blue ((B.D.H.) were used as supplied and were of Anala R grade. All other chemicals used were of reagent grade.

2.2. *Preparation of $K_4[Fe(CN)_5(gly)]$ and $K_4[Fe(CN)_5(trigly)]$*

Glycine pentacyanoferrate(II) was prepared by the method of Toma *et al.* [9] from sodium ammine pentacyanoferrate(II). Ammine pentacyanoferrate(II) was prepared from sodium nitroprusside by the conventional procedure [10]. Similar procedure was followed for the preparation of triglycine-pentacyanoferrate(II) complex also. The procedure essentially involved replacement of ammine by glycine or triglycine molecule in ammine-pentacyanoferrate(II) complex. Sodium ammine pentacyanoferrate(II) (3m mole) was dissolved in 25 ml of distilled water in the presence of five fold excess of ligand (glycine or triglycine) with constant stirring. After 15 min potassium iodide (10 g) was added and stirred for another 15 min. The complex was precipitated with ethanol and the precipitation was repeated once more. The molecular formulae of the complexes have been proposed on the basis of spectral and elemental analysis as $K_4[Fe(CN)_5(gly)]\cdot 4H_2O$ and $K_4[Fe(CN)_5(trigly)]\cdot H_2O$.

For the complex glycine pentacyanoferrate(II), $K_4[Fe(CN)_5(NH_2CH_2CO_2)]\cdot 4H_2O$: Calcd., Fe = 11.47, C = 17.2, H = 2.4%; Found, Few = 11.67, C = 17.0, H = 2.3% where as for the complex triglycine pentacyanoferrate(II) $K_4[Fe(CN)_5(NH_2CH_2CO\cdot NHCH_2CONH\cdot CH_2CO_2)]\cdot H_2O$: Calcd.,

Fe = 10.25, C = 24.0, H = 2.38%; Found, Fe = 11.01, C = 23.75, H = 2.45%.

Infrared spectra of the complexes, i.e., glycinepentacyanoferrate(II) and triglycinepentacyanoferrate(II) were recorded in KBr disc on Perkin–Elmer Spectrophotometer. Cyanide stretching frequency in glycine and triglycine complexes were observed at 2020s and 2040s cm^{-1} respectively. Fe–CN stretching vibration gave absorption band at 570_m cm^{-1} and $400–410_w$ cm^{-1} for both the complexes. The symmetric vibrational peak of carboxylate group for glycine complexes was observed at 1620_m cm^{-1} while reported value is 1590 cm^{-1} whereas for triglycine complex this band was observed at 1610_m cm^{-1}. For both the complexes symmetric vibrational mode of carboxylate group was observed at $1400–1410_m$ cm^{-1}. Electronic spectra showed absorption band at 230 nm and 420 nm for glycine-pentacyanoferrate(II) complex while triglycine-pentacyanoferrate(II) absorption band was observed at 225 nm and 405 nm. The visible absorption band of the complexes have been assigned to d–d transition from $^1A_1$ ground state to $^1E_{(1)}$ excited state. Similar values are reported for many other pentacyanoferrate(II) [11] and cobalates [12]. The visible absorption spectra of triglycine complex is very similar to glycine, ammine, and methyl ammine complexes of pentacyanoferrate(II) indicating coordination through ammine group.

2.3. *Rate Measurements*

(i) *Decomposition of H_2O_2:* Test for catalytic activity of iron(II) complexes for the decomposition of hydrogen peroxide was studied by monitoring the concentration of H_2O_2 at different time intervals. The reaction was initiated by adding known amount of catalyst (1 ml of 0.005M) into hydrogen peroxide solution (25 ml of 0.01M–0.1M) previously thermostated at 40 °C. Aliquots (2 ml) of reaction mixture were withdrawn each time and titrated against sodium thiosulphate iodometrically in acid medium. The data for $[H_2O_2]$ at corresponding time did not fit to any simple rate equation, therefore, initial rate of decomposition of H_2O_2 was determined from the slope of $[H_2O_2]$ vs time plot by scale and mirror method.

(ii) *Dehydrogenation of NADH and Ascorbic acid:* Kinetic measurements on iron(II) complexes catalysed dehydrogenation of NADH (1.17×10^{-3} M) or ascorbic acid (0.2×10^{-3} M) with methylene blue (1.3×10^{-5} M) as hydrogen acceptor were studied by monitoring the decrease in concentration of NADH or methylene blue in terms of absorbance at 340 nm (λ_{max} for NADH) or at 670 nm (λ_{max} for methylene blue) in a 3 ml quartz cell on Unicam SP 500 UV-visible spectrophotometer. All the reaction solutions were flushed with nitrogen for about 10 min before the reaction.

2.4. *Stability of Complexes*

The complexes were found to be stable in buffer used. It was checked by keeping each complex separately into respective buffers at 40 °C for about one hour and then free glycine or triglycine were checked by TLC. Plates were run in butanol:acetic acid:water system (3:3:1) and ninhydrin was used as indicator.

3. Results and Discussion

3.1. *Catalytic Activity of Iron(II) Complexes Towards Decomposition of Hydrogen Peroxide*

Metal ions as such or their complexes have been shown to possess enzyme like activity. Copper complexes with amino acids, amide, amines, diamines, di- and tripeptides, biuret, heterocyclic compounds, some proteins etc. [13–15], copper complexes with 2,2-bipyridyl(1), copper complexes with imidazole(2), have been found to show very high catalytic activity towards decomposition of hydrogen peroxide (catalase like activity). However, relevance of these models towards chemical evolutionary steps of catalase or peroxidase seems to be little as these enzymes do not possess copper in their prosthetic group. Iron(III) complexes with EDTA have been used by Walling *et al.* [4] as model to show catalase like activity. They found $[Fe(III)–EDTA]^{-3}O_2$ as the active species responsible for the decomposition of H_2O_2 by Fe(III)–EDTA complexes.

Catalytic activity of the complexes $K_4[Fe(CN)_6]$, $K_4[Fe(CN)_5(gly)]$, and $K_4[Fe(CN)_5(trigly)]$ towards decomposition of hydrogen peroxide have been studied at three different pH's, 9.18, 7.1, and 4.18 using, borax (0.05M), phosphate (KH_2PO_4, 0.01M + Na_2HPO_4, 0.01M), and phthalate (0.05M) buffers respectively. Table I shows the initial rates for the decomposition of hydrogen peroxide by different complexes studied. It was found that pH 9.18 was most effective for the decomposition of hydrogen peroxide catalysed by iron(II) complexes studied herein. A typical graph showing catalysed decomposition of hydrogen peroxide at pH 9.18 is given in Figure 1.

Addition of only glycine or triglycine was not quite effective in disproportionation of H_2O_2 in comparison with iron(II) complexes studied.

TABLE I

Effect of pH on initial rate of hydrogen peroxide decomposition catalysed by iron(II) complexes

		Initial reaction rate (moles l^{-1} min^{-1})		
Catalyst	pH	4.01	7.01	9.18
Blank		6.48×10^{-5}	3.57×10^{-4}	1.92×10^{-4}
Fe^{2+}		1.05×10^{-4}	4.0×10^{-4}	*
$K_4[Fe(CN)_6]$		1.10×10^{-4}	4.8×10^{-4}	6.3×10^{-4}
$K_4[Fe(CN)_5(gly)]$		1.17×10^{-4}	9.0×10^{-4}	1.3×10^{-3}
$K_4[Fe(CN)_5(trigly)]$		1.25×10^{-4}	1.1×10^{-3}	1.77×10^{-3}

Reaction conditions: $[H_2O_2] = 5.0 \times 10^{-2}$ M; [Catalyst] $= 2.0 \times 10^{-4}$ M; Temp. = 40 °C ± 0.1 °C.

* At this pH hydrolysis of Fe^{2+} took place and hence precipitation occurred.

From Table I, it is evident that the catalytic activity of the complexes towards decomposition of hydrogen peroxide in the pH range 4.01 to 9.18 was found to increase in the order:

$$K_4[Fe(CN)_6] < K_4[Fe(CN)_5(gly)] < K_4[Fe(CN)_5(trigly)].$$

The value of turn over numbers for the decomposition of H_2O_2 by $K_4[Fe(CN)_6]$, $[Fe(CN)_5(gly)]$, and $[Fe(CN)_5(trigly)]$ have been determined as 7.50 and 500 min^{-1} respectively. Subsequent increase in turn over numbers also lent support to the above order for the catalytic activity of the complexes.

On the basis of the experimental observations and keeping in view the fact that out of six cyanides in the inner sphere of iron complex, one cyanide could be reversibly replaced by a suitable ligand as mentioned by Ohno [16] who, however, has studied the exchange of cyanide by a water molecule in hexacyanoferrate(II) in aqueous solution under UV light, a tentative mechanism for decomposition of hydrogen peroxide catalysed by cyano complexes of iron(II) may be written as follows:

$$[Fe(CN)_5L]^{-4} + H_2O_2 \rightleftharpoons [Fe(CN)_5(OOH)]^{-4} + H^+/L^- \quad (1)$$

$$[Fe(CN)_5(OOH)]^{-4} + H_2O_2 \rightleftharpoons \text{Intermediates} \quad (2)$$

$$\text{Intermediates} \rightleftharpoons [Fe(CN)_5(OH)]^{-4} + O_2 + H_2O \quad (3)$$

The proposed mechanism is somewhat similar to the mechanism given by Sigel *et al.* [1] for the decomposition of hydrogen peroxide catalysed by Cu^{+2} and Cu^{+2} complexes of 2,2 bipyridyl. For the catalysed decomposition of hydrogen peroxide by $[Fe(III)(EDTA)]^-$, Oakes *et al.* [17] recently considered various possible pathways in which hepta-coordinated complex such as

$$[\text{EDTA Fe} < \begin{matrix} HO_2 \\ (OH)_2 \end{matrix}]^{-4}$$

has been proposed as one of the intermediates. However, in our studies where decomposition of hydrogen peroxide has been carried out by $[Fe(CN)_6]^{-4}$ and its derivatives, hepta-coordinated intermediates of the type $[Fe(CN)_5(OOH)(H_2O_2)]^{-4}$ are not feasible due to strong ligand field stabilisation of $\bar{C}N$ groups. For the intermediates indicated in steps 2 to 3 of the proposed mechanism, various possibilities may be considered, complexes of iron(III) or peroxo-bridged type could appear. These intermediates could subsequently react and modify with transfer of two electrons in one step, or one electron each in two steps and so on. Futher work is in progress on the elucidation of the mechanism proposed.

Gradual increase in catalytic activity of glycine and triglycine pentacyanoferrate(II) complexes probably depends on the ease by which sixth ligand of the complexes (glycine or triglycine) which are weaker in comparison to $\bar{C}N$ is replaced by H_2O_2 in alkaline medium. Our experimental results suggest (Table I) that gradual increase in pH increases the initial decomposition rate of H_2O_2 catalysed by iron(II) complexes. This fact is probably governed by step 1 of the mechanism which

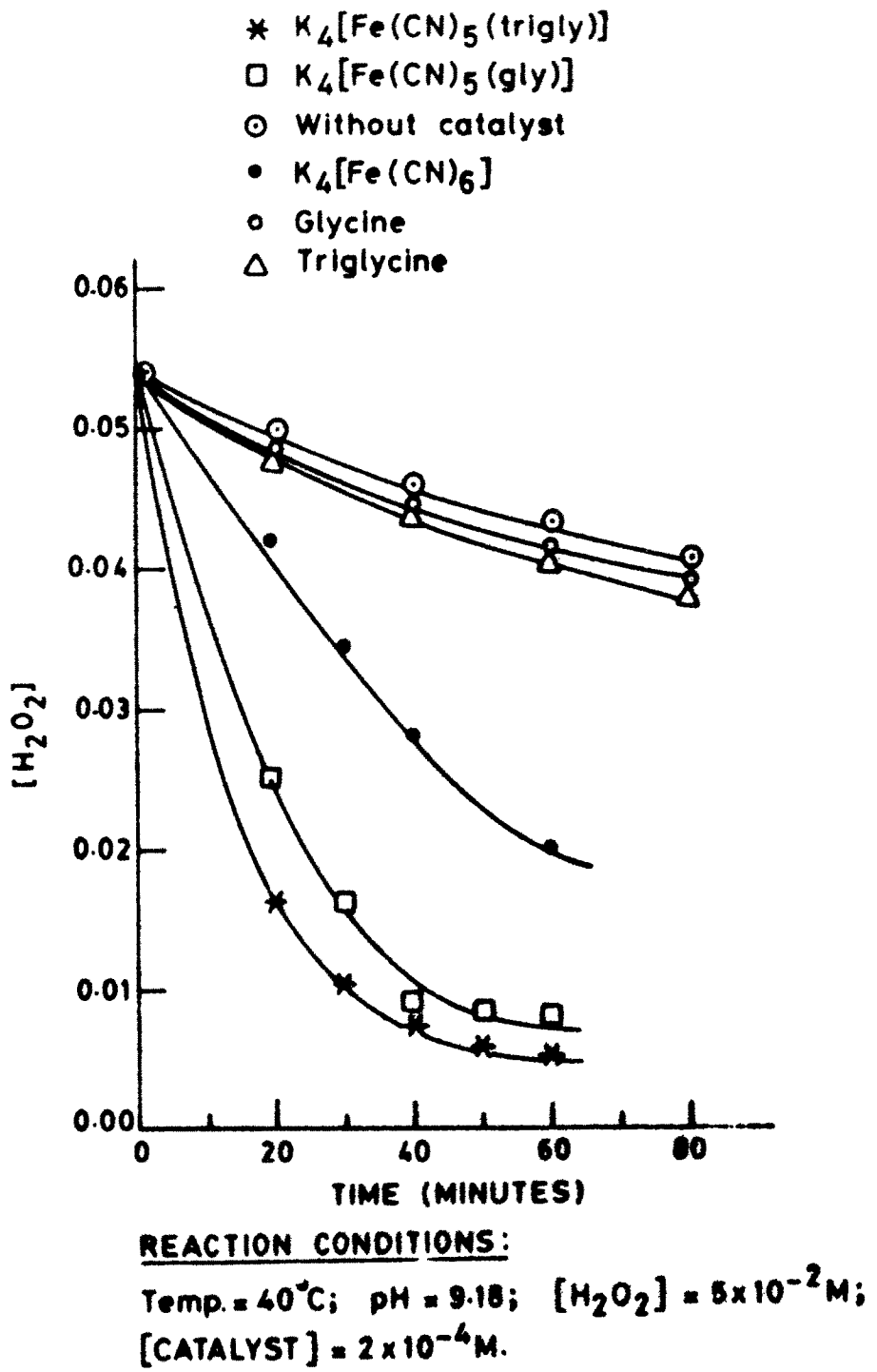
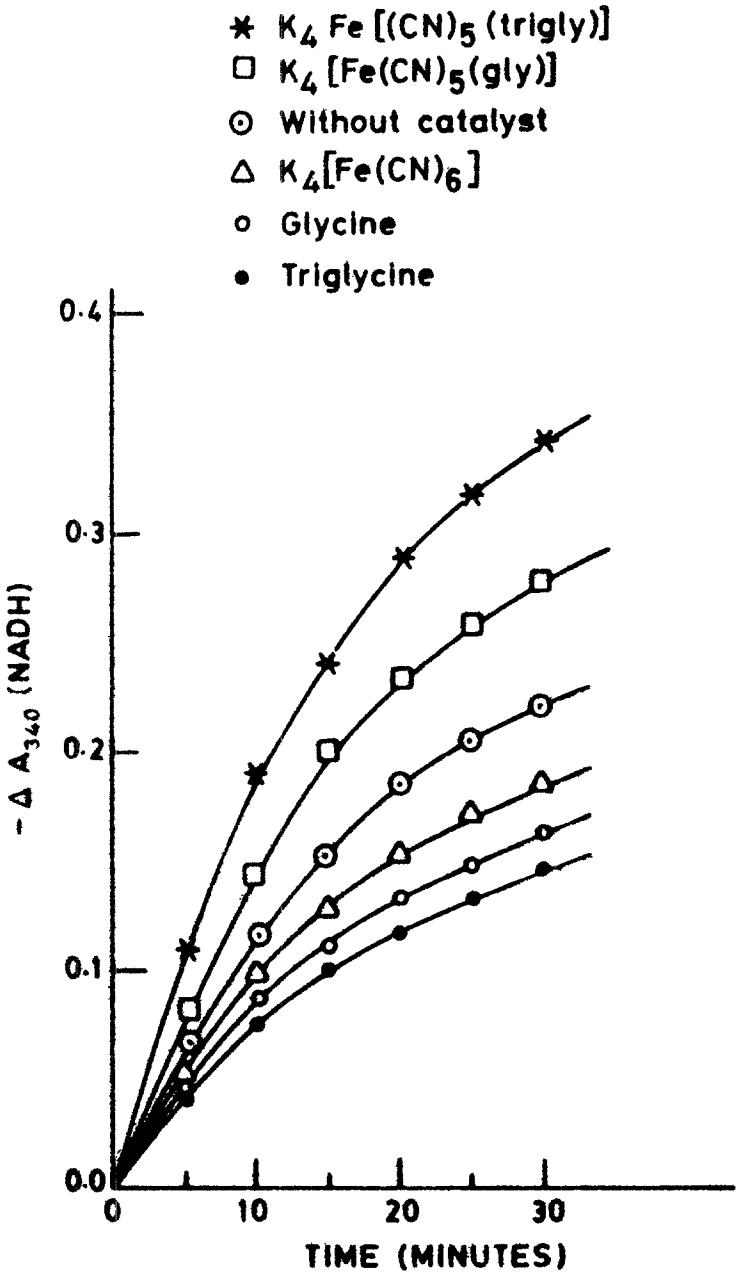


Fig. 1. Catalysed decomposition of hydrogen peroxide.



REACTION CONDITIONS:

Temp. = 40°C; pH = 9·18; [NADH] = $1·17 \times 10^{-3}$M;

[Methylene blue] = $1·3 \times 10^{-5}$M; [Catalyst] = $2·0 \times 10^{-4}$M.

Fig. 2. Catalysed dehydrogenation of NADH.

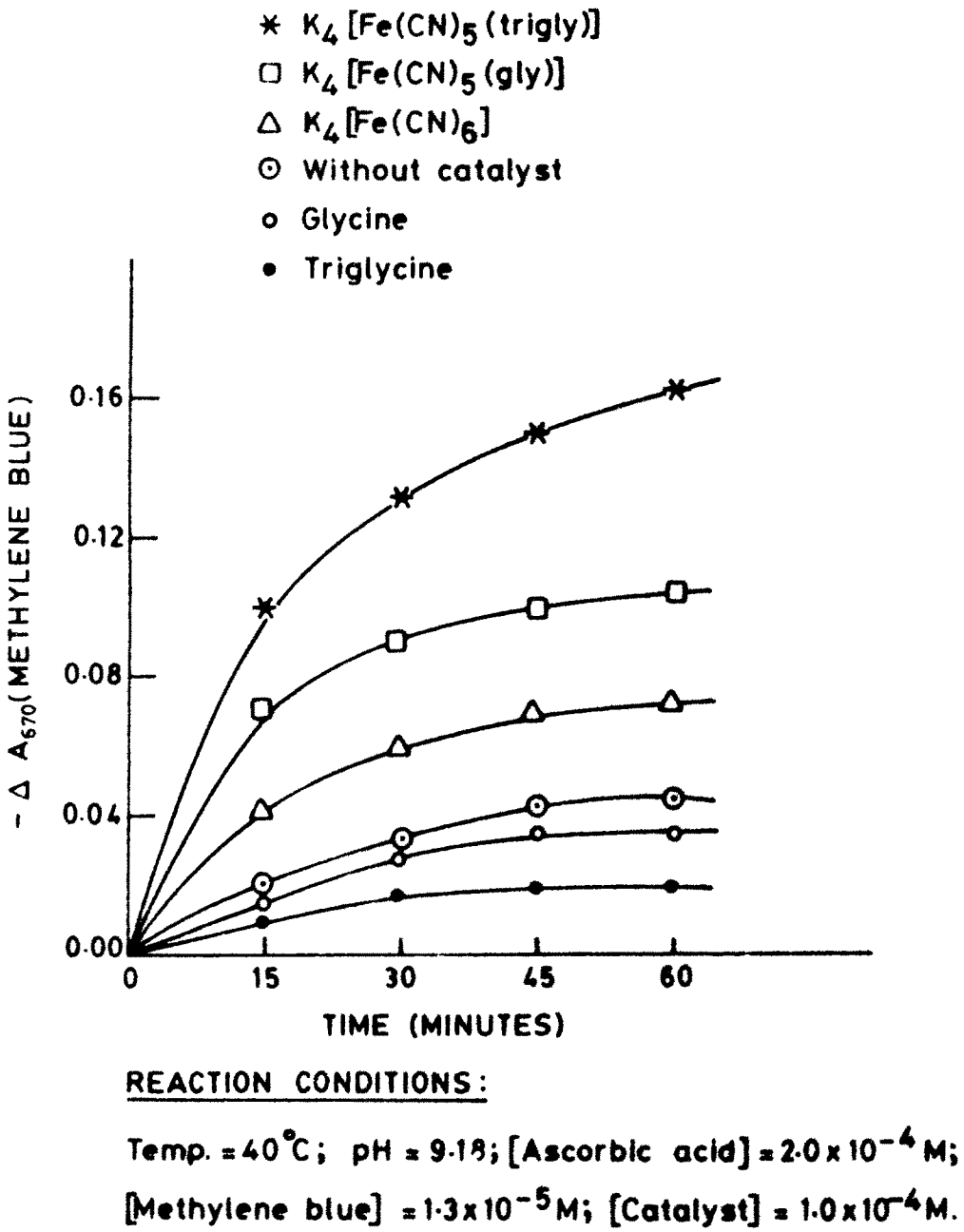


Fig. 3. Catalysed dehydrogenation of ascorbic acid.

directs the reaction in backward side by increasing hydrogen ion concentration in the medium. Since the catalytic activities of the complexes in acidic pH's were comparatively less effective than in alkaline pH, hence experiments were carried out at pH 9.18. Recently imidazole pentacyanoferrate(II) complex which has all the inner valancy saturated been shown to be oxidised by H_2O_2 to imidazole pentacyanoferrate(III) complex but no probable mechanism is discussed by Shephered [17]. It may be recalled that the proposed model of a catalyst responsible for decomposition of hydrogen peroxide resembles very much with that of catalase [18] where iron(III) is strongly attached to four porphyrin nitrogen sites and fifth is imidazole whereas sixth ligand is either CN or H_2O and it is assumed that peroxidase operates by exchange of group at sixth position.

3.2. *Catalytic Activity of Iron(II) Complexes Towards Dehydrogenation of NADH and Ascorbic Acid Coupled with Reduction of Methylene Blue*

In living systems various dehydrogenation reactions are catalysed by different enzymes, and every reaction has its own metabolic importance. The dehydrogenation of NADH has been carried out in living system by the specific iron-flavo enzyme namely NADH dehydrogenase. Physiological electron acceptor for NADH is ubiquinone-1, or ubiquinone-10 while other electron acceptors used in vitro are ferricyanide, methylene blue, phenanzine methosulfate etc. Okihana [19] used resazurin as hydrogen acceptor for dehydrogenation of NADH catalysed by high molecular weight soluble polymers produced by heating eighteen protein amino acids with milk casein composition in a modified sea medium. We have studied the dehydrogenation of NADH and ascorbic acid coupled with reduction of methylene blue. Activity has been tested at three different pH's namely 9.18, 7.1, and 4.1. It was observed from plots in Figure 2 and 3 that catalytic activity of the complexes studied for dehydrogenation of both NADH and ascorbic acid with methylene blue at pH 9.18 followed the order given below; however, reactions studied at acidic or neutral pH did not show a regular pattern.

$$K_4[Fe(CN)_6] < K_4[Fe(CN)_5(gly)] < K_4[Fe(CN)_5(trigly)].$$

Rates of dehydrogenation of both NADH and ascorbic acid were least appreciable when catalysed by glycine and triglycine alone. Efforts to elucidate a possible mechanism for the dehydrogenation of NADH and ascorbic acid catalysed by $Fe(CN)_6^{-4}$ and its derivatives are in progress.

In order to ensure that the catalytic activity observed in decomposition of H_2O_2 and dehydrogenation of NADH and ascorbic acid is only because of $Fe(CN)_6^{-4}$ and its derivatives and not due to traces of uncomplexed metal ions, effect of 10% added concentration of EDTA with respect to catalyst was studied. Addition of EDTA, however, did not change the activity.

It has been pointed out by Ycas [20] that the primitive enzymes were less specific and less reactive. Further primitive enzymes must have catalysed a class of reaction. Our experimental observations also suggest that mixed ligand complex of iron(II) are

able to catalyse a class of redox reactions, i.e., decomposition of H_2O_2 and dehydrogenation of NADH and ascorbic acid coupled with reduction of methylene blue. Further, the catalytic activity of the complexes increases when primary ligands like CN^- are partially substituted by amino acid and peptide molecules respectively. It is obvious from the data presented here that increase in catalytic activity of complexes is quite appreciable and may indicate pathways leading to the evolution of enzymes containing iron.

On the basis of the above experimental observations a tentative pathway for the evolution of iron containing enzymes is proposed as follows:

> Iron(II)ion → Iron(II)cyanide complexes → mixed ligand iron(II)cyanide and amino acid complexes → iron(II) complexes of macromolecules → proenzymes or early enzymes containing iron.

The proposed pathway for the evolution of iron containing enzymes is not contradictory to one proposed by Calvin [8]. The only difference lies in the fact that we have considered much more early stages of evolution where iron was present in its lower oxidation state.

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