

## **IFCC Professional Scientific Exchange Programme**

---

**Elizabeth A Frank Biochemical  
Diagnostic Laboratory, Mysore -  
570 021 Karnataka, India Fax:  
+91-821-521948 Email:**

---

## **Paroxonase and its Role in Cardiovascular Disease**

---

**Dr. Frank, Clinical Chemist,  
visited as a short time scholar  
the Dept. of Pathology and Lab.  
Medicine at the Louisville School  
of Medicine, Kentucky, USA.  
Her investigations and training  
was supervised by Associate  
Professor James J Miller, Ph.D.,  
and Stanley S. Levinson, Ph.D.**

---

### **Introduction**

Arteriosclerosis is a major cause of morbidity and mortality not only among North Americans but also among Asians. While the initiation and progression of the disease is well understood, little is known about the cardio protective mechanisms. The oxidation of LDL is central to current theories concerning the initiation and progression of atherosclerosis (1) Equally, the potential protective role of high-density lipoprotein (HDL) has been emphasized. The inverse correlation that exists between plasma HDL- cholesterol concentrations and the risk of coronary artery disease (CAD) has led to the identification of " reverse cholesterol transport " pathway.( 2 ). In this pathway the lipoprotein particles return excess cholesterol to the liver and allow for its excretion mainly as bile acids. Although it is clear that the reverse cholesterol

transport and the HDL particle are inversely related to CAD, metabolism of HDL is poorly understood. HDL was shown to inhibit LDL oxidation in vitro by several laboratories (2). Recent evidence suggests that several enzymes residing on the HDL could contribute to this activity. It has been shown that HDL associated Paraoxonase (PON1) retards the oxidation of LDL by preventing the generation of lipid peroxides (3).

PON1 is a calcium dependent esterase whose mechanism of action is incompletely elucidated. It was originally found to be responsible for the hydrolysis of Paraoxon, a catabolic product of the insecticide parathion. PON is also able to hydrolyze other substrates such as phenyl acetate. However the physiological substrate for PON is still unknown. PON is widely distributed among tissues such as Liver, kidney, intestine and also serum, were it is associated with HDL.

This project was designed to study the role of paraoxonase in the cardio protective mechanism of HDL. Our objective was to investigate whether the HDL associated paraoxonase was responsible in retarding oxidation of LDL. This study will eventually be able to answer many questions like for example whether possible interactions between Apolipoprotein and Paraoxonase is responsible in protecting LDL from oxidation. Hence we first tried to purify paraoxonase from serum and then use it in studies involving oxidation of LDL in vitro.

We also studied the levels of serum paraoxonase in relation to lipid profiles in a randomly selected patient population. Although from this study we are unable to draw definite conclusions about the role in lipid oxidation, we have made a number of interesting observations which when pursued will eventually prove conclusively the in vivo role of paraoxonase in HDL

### **Materials and Methods.**

All chemicals used were from Sigma - Aldrich USA .

Patients used in this studies was randomly selected from among the patients from Louisville medical school. All patients selected were between the age group on 21 to 80 years. Among the 30 patients studied 17 were female and the rest were males.

### **Paraoxonase assay:**

Activity of paraoxonase was assayed by following the formation of p nitro phenyl by its absorbance at 405nm (4, 5). The assay buffer contains 0.132 M Tris base, 1.32 m M CaCl<sub>2</sub> and 2.63 M NaCl. Addition of 200 ml of 1.2 m M paraoxon and 10 ml of serum initiated the assay. Absorbance was continuously monitored at 405 nm. A molar extinction coefficient of  $18.05 \times 10^3$  was used for calculation using paraoxon as substrate (Biggs 1954) , 3.6 mM of phenyl acetate was also used as substrate. A molar extinction co-efficient of 1310 was used when Phenyl acetate was used as a substrate. 1 unit of paraoxonase is defined as a nmol of 4 nitrophenol formed per minute.

### **Purification of serum Paraoxonase:**

150 ml (or 25 ml) serum is diluted with 150 ml (or 25 ml) of 25 m M tris Hcl buffer pH8.0 containing 2 M Nacl, 0.5 mM CaCl<sub>2</sub> and 2.5  $\mu$ M EDTA. This diluted serum was fractionated on a Cibacron Blue 3 GA - Agarose column (6). The column was washed with 4 M Nacl. The column was then eluted with 0.2% Sodium deoxycholate in water.

The fractions containing paraoxonase activity were further fractionated by detergent DEAE - trisacryl M chromatography. The Trisacryl M columns were equilibrated with equilibration buffer 15 mM Tris HCl , pH 7.5, 1 m M CaCl<sub>2</sub> and 0.1% nonidet P-40 . The desalted, concentrated sample was adjusted to equilibration buffer conditions by the addition of 25% NP-40. The concentrated sample was loaded on to the column, which was then washed to baseline OD with equilibration buffer. The adsorbed protein was eluted with a linear gradient from 0 to 0.125 M Nacl in column equilibration buffer.

Cholesterol Triglycerides, HDL-Cholesterol, APO A, and APO B were assayed on the Roche Cobas Mira using Sigma diagnostic kits.

### **Preparation of LDL :**

LDL was prepared from pooled human serum using heparin-agarose affinity columns as follows: . Heparin (1 ml) was mixed well by inversion with Agarose and allowed to settle overnight. The column was allowed to drain . The column was washed with the elution buffer ( 0.7 % NaCl ) The wash was discarded . Apo B content of pooled human serum was measured using immunonephelometry . Serum containing 1.25-2 g/L of Apo B (200ul) was loaded on to the Heparin - agarose column followed by 50 ul of the alpha elution buffer (0.7% NaCl) . Alpha proteins were eluted first with 2 ml of the alpha elution buffer. This allowed complete elution of albumin and the alpha protein (HDL) . The column was then eluted with the beta buffer (2.9% NaCl) to give the LDL fraction. The heparin agarose column was reused three times.

### **Lipoprotein Oxidation**

CuSO<sub>4</sub> (20 ul of 250 uM) was added to LDL fraction(300ul) and mixed vigorously resulting in oxidized lipoproteins. particle . Assessment of lipoprotein oxidation was monitored by formation of conjugated dienes at 234 nm using Genysis 5 system.

### **Peroxidation Assay**

Peroxidation of lipids was monitored using Xylengthol orange. Peroxides oxidize Fe<sup>2+</sup> to Fe<sup>3+</sup> in acidic solution. Fe<sup>3+</sup> in the presence of xyelnol orange forms a Fe<sup>3+</sup> - Xylengthol orange complex which absorbs at 500 nm. In this assay the reagent contained 900 ml of pure methanol, 100 ml of 250 m M H<sub>2</sub>SO<sub>4</sub> 880 mg of BHT ( to inhibit further oxidation within the assay itself, 76 mg of Xylengthol orange and 98 mg of ammonium iron (II) sulfate hexahydrate . LDL samples (0.1 ml , oxidized with cu<sup>2+</sup>) were mixed with 0.9 ml of reagent and incubated for 30 minute at room temperature and the colour developed is measured at 560 nm.

## **Results**

### **Purification of Serum Paraoxonase**

The scheme of purification of human serum paraoxonase of a typical run is shown in Table 1

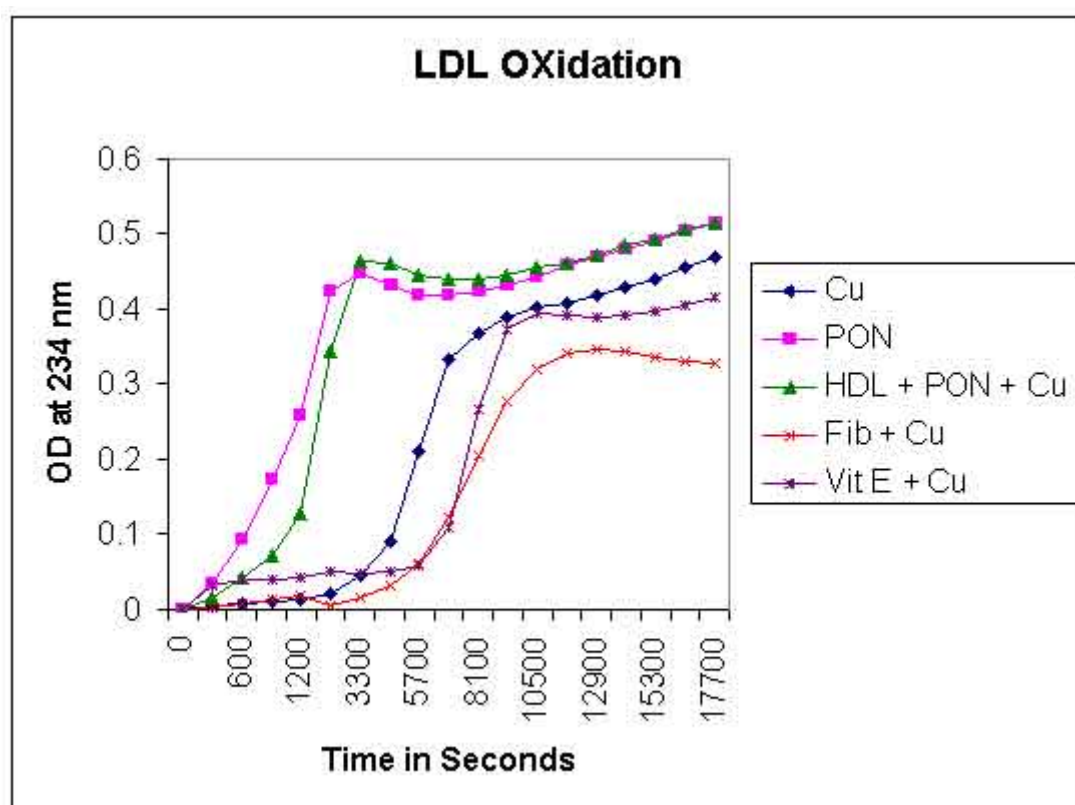


Figure 1

The Lipid components of the fractions are shown in Table 2

The Fractions eluted from the DEAE column with arylesterase activity were used as paraoxonase and was used to study its effect on LDL-Oxidation.

## Oxidation of LDL

The oxidation of LDL by copper in the absence and presence of added Paraoxonase and Cibacran blue fraction of a typical experiment as shown in Fig. 1

Cu- Copper , PON-Paraoxonase , Vit E-Vitamin E

Formation of conjugated dienes reflects oxidation of PUFA composing the core of lipoprotein .The process of oxidation spans three phases.1) An initiation or lag phase . 2) A propagation phase during which time lipid peroxides are formed and double bonds produced. 3) Decomposition phase, which is recognized when the oxidation reaches a plateau during which aldehydes and ketones are produced from fatty acid degradation.

The Oxidation of LDL by copper ions was followed by conjugated diene formation and showed a typical hyperbolic curve with an initial Lag phase followed by an propagation phase and finally a plateau. In the presence of added antioxidants like Vit E and fibrinogen (Fib) the oxidation of LDL was abolished. However paraoxonase (PON) did not abolish the oxidation of LDL.