Glycation of HDL by glyoxal and glucose carried out at different concentrations of glyoxal and glucose for up to 7 days are shown in Fig.3.1 (A through D) and Fig.3. 2 (A and B). Fig.3.1A compares the changes in PON activity over a period of 7 days in the presence of 10mM glyoxal and glucose.

![Graph showing PON activity changes over 7 days](image)

**Fig.3.1A Changes in PON activity of HDL subjected to Glycation using 10mM glyoxal and Glucose**

HDL was glycated in the presence of 10mM glyoxal or glucose for up to 7 days. PON activity was measured using phenylacetate as substrate. The results are expressed as percent of PON activity taking the PON activity of control HDL on day 1 as 100%. Results are mean of 3 determinations.

From this figure glyoxal appears to be protecting the PON activity from inactivation.

Fig.3.1B compares the changes in PON activity over a period of 7 days in the presence of 50mM glyoxal and glucose. At 50mM concentration glucose did not inactivate PON whereas glyoxal inactivated PON activities.
Chapter III

Results

Fig. 3.1B Changes in PON activity of HDL subjected to Glycation using 50mM glyoxal and Glucose

HDL was glycated in the presence of 50mM glyoxal or glucose as described in legends to Fig. 3.1A.

Fig. 3.1C compares the changes in PON activity over a period of 7 days in the presence of 100mM glyoxal and glucose. At 100mM conc, glyoxal almost completely inactivated PON activity whereas glucose did not inactivate PON.

Fig. 3.1C Changes in PON activity of HDL subjected to Glycation using 100mM glyoxal and Glucose
HDL was glycated in the presence of 100mM glyoxal or glucose as described in legends to Fig. 3.1A.

Fig.3.1D compares the changes in PON activity over a period of 7 days in the presence of 500mM glyoxal and glucose. At 500mM concentration glucose protected PON activity for 7 days.

![Graph showing changes in PON activity](image)

**Fig.3.1D Changes in PON activity of HDL subjected to Glycation using 500mM glyoxal and Glucose**

HDL was glycated in the presence of 500mM glyoxal or glucose as described in legends to Fig.3.1A

PON activity in the presence of increasing concentration of glyoxal and glucose are shown in Fig.3.2A and 3.2B. In Fig.3. 2A, the activity of PON in the presences of glyoxal and glucose on day 1 are compared. Glyoxal inhibited PON1 activity in a dose dependent manner, whereas glucose did not bring about dose dependent inactivation. The changes in PON activity on day 7 are shown in Fig.3.2B
Fig. 3. 2A PON activity of HDL glycated with Glyoxal or Glucose for one day.

Paraoxonase activity of HDL determined using Phenyl acetate as substrate in the presence of increasing concentration of glyoxal and glucose on day 1. Data from fig3.1A, B, C, D

Fig. 3. 2B PON activity of HDL glycated with Glyoxal or Glucose for seven days

Paraoxonase activity of HDL in the presence of increasing concentration of glyoxal and glucose on day 7. Data from fig3.1A, B, C, D
On day 7, the PON activity was less than 1/5th of the control activity at 50mM glyoxal. However in the presence of glucose there appears to be a dose dependent increase in the PON activity.

The effect of glyoxal and glucose on PON activity at day 60 is shown in Fig. 3.3. PON activity was decreased by both glyoxal and glucose even at 10mM concentration.

**Fig 3.3** PON activity of HDL glycated with Glyoxal or Glucose for sixty days

**PON activity of HDL in the presence of increasing concentration of glyoxal and glucose on day 60**

The effect of glyoxal and glucose incubated for 14 days enzyme activity towards PON against different substrates is shown in Fig. 3.4A (A through D) at two concentrations namely 100mM and 1M.

At 100mM a concentration, paraoxonase hydrolyzing activity of HDL was not affected by glucose or glyoxal whereas at 1M concentration, the glyoxal completely inhibited PON activity whereas glucose was protective of PON activity. (Fig. 3.4A).
Fig 3.4A Paraoxonase activity of HDL glycated with Glyoxal or Glucose

HDL incubated with glyoxal or glucose for 14 days at 100mM and 1M concentration. PON activity was determined using paraoxon as substrate as described in the methods.

Fig 3.4B Thiolactonase activity of HDL glycated with Glyoxal or Glucose

Thiolactonase activity of HDL incubated with glyoxal or glucose for 14 days at 100mM and 1M concentration. Activity of Thiolactonase was determined using homocystein thiolactone as substrate as described in methods.
The thiolactonase hydrolyzing activity at 100mM concentration of glucose and glyoxal unaffected but at 1M concentration, the thiolactonase activity increased enormously when HDL was modified by glyoxal but not glucose.(Fig 4B). The epoxide hydrolase activity deceased with increasing glyoxal concentration as well as glucose concentration (Fig.3.4C).

![Epoxidase activity of HDL glycated with Glyoxal and Glucose](image.png)

**Fig.3.4C Epoxidase activity of HDL glycated with Glyoxal and Glucose**

Epoxidase activity of HDL incubated with glyoxal or glucose for 14 days at 100mM and 1M concentrations. Epoxide hydrolase activity of HDL determined as described in the methods.

The Tryptophan fluorescence changes brought about by glucose and glyoxal are shown in Fig.3.5. At 1M concentration glucose increased the fluorescence compared with glyoxal significantly (P>0.05).

Fluorescence of AGE products formed by glyoxal was significantly higher than that by glucose at 1M conc (Fig.3.6)
Fig. 3.5. Tryptophane fluorescence of HDL glycated with Glyoxal or Glucose

Changes in trp fluorescence of HDL proteins incubated with 100mM and 1M glyoxal or glucose. Results are mean ±SD (n=3)

Fig.3.6 AGE formation in HDL glycated with Glyoxal or Glucose

AGE fluorescence of HDL proteins incubated with 100mM and 1M glyoxal or glucose. Results are mean ±SD (n=3)
The percentage of amino groups modified by TNBS in the presence of and absence of glyoxal and glucose are shown in Fig. 3.7. At 100mM concentration of glucose or glyoxal, about 8% of the amino groups were modified by glucose or glyoxal and the remaining 92% were available for modification by TNBS. At 1M concentration only about 45-55% of the amino groups were available for modification.

![Graph showing amino group modification in HDL glycated with Glyoxal or Glucose.]

**Fig. 3.7 Amino group modification in HDL glycated with Glyoxal or Glucose.**

Percent aminogroup modified by glyoxal or glucose in HDL. HDL was incubated with 100mM and 1M concentration of glyoxal or glucose for 14 days. The fluorescence of AGE determined as described in the methods.

The thiol groups in the HDL after treatment with glyoxal and glucose are shown in Fig. 3.8. At 100mM concentration of glyoxal more thiol groups have reacted with glyoxal than at 1M concentration whereas, only at 1M concentration glucose was able to react with thiol groups. Consequently less thiol groups are available to react with DTNB.
Fig. 3. 8 Thiol Groups in HDL glycated with Glyoxal and Glucose

Thiol groups in HDL after treatment with glyoxal or glucose. HDL was incubated with 100mM and 1M concentration of glyoxal or glucose for 14 days. Thiol groups were determined in HDL as described in the methods.

The Carbonyl content of HDL subjected to glycation is shown in Table 3.1. Glucose did not increase the carbonyl content of HDL but Glyoxal increased it about 73 fold.

Table 1. Carbonyl content of Control and Glycated HDL

<table>
<thead>
<tr>
<th></th>
<th>Carbonyl content (µmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.7 ± 0.15</td>
</tr>
<tr>
<td>Glycated</td>
<td></td>
</tr>
<tr>
<td>Glyoxal</td>
<td>272.3 ± 170.1</td>
</tr>
<tr>
<td>Glucose</td>
<td>3.68 ± 0.34</td>
</tr>
</tbody>
</table>

HDL was glycated for one week with 200mM glyoxal or glucose.
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Results

The elution of PON activity from Cibachron blue sepharose column is depicted in Fig. 3.9

![Graph showing recovery of PON and protein of HDL from Cibachron Blue Sepharose column.](image)

Fig. 3.9 Recovery of PON and protein of HDL from Cibachron Blue Sepharose column

Elution of esterase and paraoxonase activities from cibacron blue sepharose column as described in methods. Esterase activity and paraoxonase activity in the loaded, and eluted were determined as described in methods. Results are mean ±SD (n=3).

The total protein bound to the column in case of control and glycated HDL was the same. The PON activity of PA substrate was bound more in the control sample than the glycated sample. Whereas the PON activity measured by paraoxon substrate was the same for control and glycated samples. The recovery of protein was quantitative but the recovery of PON (PA) was 62 ± 11.5% in the control and 66 ± 6.5 in the glycated HDL. The recovery of PON activity was about 10-fold higher than the activity loaded (11.6 ± 3 fold for control and 10.8 ± 1.8 fold for glycated HDL).
Electrophoretic pattern of HDL proteins before and after glycation are shown in Fig.3.10 (A and B. Fig.3.10A gives the SDS-PAGE pattern and Fig.3.10B gives the native PAGE pattern. In native PAGE some differences could be observed in the mobility of the bands.

Fig.3. 10 Polyacrylamide gel electrophoresis of HDL and glycated HDL.

HDL was glycated with 100 mM for 7 days. The HDL was subjected to SDS-PAGE (Fig 10A) or native PAGE (Fig 10B) as describd in methods. Lane 1. Control  Lane 2. Glycated

The mass spectral pattern of glycated HDL with reference to control HDL are shown in Fig.3. 11A and B. Significance observations from the mass spectrograph are the loss of 15KD fragment after glycation.
Fig 3.11A Mass spectrum of HDL.

HDL was subjected to MALDI-TOF as described in methods.

Fig 3.11B Mass spectrum of glycated HDL.

HDL was subjected to glycation using 100 mM glyoxal for 7 days. The glycated HDL was dialysed and subjected to MALDI-TOF as described in methods.
There was a significant decrease (P<0.05) in HDL-Cholesterol after glycation, and is shown in Fig.3.12.

![Graph showing HDL-C mg/ml]

**Fig.3. 12 Effect of glycation of HDL on HDL-C.**

HDL was glycated using 100mM glyoxal or glucose. The HDL-C was determined as described in the methods.

**Circular Dichroism**

CD spectra of HDL and Glycated HDL are shown in Fig.3.13. The Difference spectra were calculated by subtracting the [0] values of glycated HDL from that of control HDL and plotted against the wavelength (Fig3. 14).

The CD spectrum of HDL showed a typical spectrum for protein rich in α helix. On glycation with glyoxal or glucose the overall shape of the spectrum did not change. However the difference CD spectra showed an increase in β structure in HDL. There was no difference when the HDL was modified with glyoxal or glucose.
Fig.3.13 CD spectra of HDL and glycated HDL.

HDL was glycated with glyoxal (100mM) or glucose (100mM) for 2 days. The HDL was dialysed and analysed by CD. CD spectra for HDL and Glycated HDL were recorded in a Jasco J-810 recording spectrometer.

Fig.3.14 Difference CD spectra

The \( \theta \) values of glycated HDL were subjected from that of control HDL and the difference was plotted against wavelength.
Non enzymatic glycation of umbilical vein

Fig.3.15 shows the histology of the umbilical cord treated with glyoxal. There are no apparent changes in the stained section of the umbilical vein (Fig.3.15 B). However, the vein exposed to glyoxal had fluorescent compound which was not found in the control vein.(Fig.3.15D)

Fig.3.15. Fluorescence detection of AGE in glycated umbilical Vein

Human umbilical vein was subjected to glycation using 100 mM glyoxal for 2 days. The umbilical cord was fixed in Bovin’s fixative and stained. A.control B.glycated (Hematoxylin-Eosinstain) C.control D.glycated (Fluorescence).

Inhibition of Glycation by antioxidants

The inhibition of glycation of HDL in the presence of Vitamin C is shown in Fig.3.16A. The inhibition of glycation of HDL in the presence of Vitamin E is shown in Fig.3.16B.
Fig. 3.16A. Protection of PON from inactivation by Vitamin C.

HDL was glycated with glyoxal or glucose in the absence or presence of 57μM vitamin C. results are mean±SD (n=3).

Fig. 3.16B protection of PON from inactivation by Vitamin E

HDL was glycated with glyoxal or glucose in the absence or presence of 23μM and 93 μM vitamin E
Fig. 3. 16C. Protection of PON from inactivation by quercetin.

HDL was glycated with glyoxal or glucose in the absence or presence of 330 µM and 660 µM Quercetin.

Vit C and Quercetin were able to protect glyoxal induced inactivation of PON but not glucose induced inactivation whereas Vit E was able to protect PON by both glucose and glyoxal induced inactivation.

**Studies with antibodies to glycated ApoA1**

The electrophoretic pattern for the isolation of ApoA1 by SDS-PAGE is shown in Fig. 3.17. The ApoA1 was subjected to nonenzymatic glycation with glyoxal and used to raise antibodies in the rabbit. ApoA1 was antigenic to rabbits as well as hens.

The presence of antibody in the rabbit serum was confirmed by taking serum before inducing the production of antibody and compared with that obtained after induction of antibody synthesis. (Fig. 3.18).
Fig 17 SDS PAGE of HDL and ApoA1.

HDL was subjected to SDS-PAGE. The band corresponding to ApoA1 was cut, electroeluted and concentrated. Lane 1. Molecular weight markers  Lane 2,3 HDL Lane 4. Purified ApoA1 Lane 5 Purified ApoA1 silver stain

Fig.3.18 Antibody titre

Antibodies against glycated ApoA1 was raised in rabbit. The pre induction blood and post induction blood was drawn from ear vein and tested for antigen-antibody reactions by ELISA as described in methods.
The post bleed serum showed a dose dependent increase in the optical density by ELISA whereas for the same antigen, there was no dose dependent increase in the optical density in the pre bleed serum sample.

In order to check for cross reactivity, the post bleed serum was tested against glycated ApoA1 and BSA. BSA did not cross reacted with the antibody. Whereas glycated ApoA1 showed a concentration dependent increase in optical density (Fig 3.19)

![Graph showing the cross reactivity of antibody to glycated ApoA1 against BSA.](image)

**Fig.3. 19 Cross reactivity of Antibody to glycated ApoA1 against BSA.**

The antibody to glycatd ApoA1 was raised in rabbit as described in methods. The Antibody was diluted and tested against glycated ApoA1 and BSA as antigens.

**Purification of Antibody**

The elution profile of the antibody from rabbit serum on protein A sepharose column is shown in Fig.3.20. The protein was eluted in one asymmetric peak. It was pooled into two fractions and the fraction 1 consists of tube No 3 -12 and the fraction 2 consists of tube number 13-18.
Fig.3.20 Purifition of antibody on Protein A Sepharose.

Antibody to glycated ApoA₁ was purified on protA sepharose column as described in methods.

The antibody was tested against two different samples of glycated ApoA₁. Fig.3.21 shows the optical density at one concentration of antibody namely 2000-times dilution. The two different samples of glycated apoA₁ gave two different optical density values. However, the sample having lower OD of the two ApoA₁ samples gave an optical density significantly higher than that of the control (P=0.018)

Fig.3.21. Specificity of antibody to different glycated samples of ApoA₁

ApoA₁ prepared at different times and glycated with glyoxal were tested for their antigeni-Antibody reaction against pre-bleed and post bleed serum.
Chapter III

The reaction was also tested at two dilutions of antibody. A 10000 dilution factor gave a lower optical density than at 2000 dilution factors. However in both the cases glycated sample 1 gave optical density values significantly higher than the respective control value (Fig.3.22) but was less than that of sample.

Fig.3.22. Specificity of Antibody to different glycated samples of ApoA1

ApoA1 was prepared at different times and glycated. The glycated ApoA1 samples were tested at two dilutions of antibody.

“Checker board” analysis of antigen-antibody reaction is shown in Fig.3.23.

Fig.3. 23 Checker-Board analysis for antigen-antibody reaction.

The Antibody to glycated ApoA1 was diluted 1:0, 1:5, 1:10 and 1:50, and tested against glycated ApoA1 at concentrations 0.5ng to 100ng as described in methods.
Fig. 3.24 Antige-Antibody reaction

Glycatd ApoA1 was taken at concentrations of 0.5ng, 5ng, 50ng, 100ng and tested against antibody diluted 1:0, 1:2000 and 1:10,000 as described in legends to Fig 23.

With increasing antigen concentration there was an increase in optical density with increasing antibody dilutions, there was an increase in optical density except for 10,000X dilution. The maximum optical density was at 2000X diluted antibody and is shown in Fig.3. 24.

In order to estimate the glycated HDL in diabetics, 30 diabetics and 10 control subjects were taken for the study. The subject characteristics of the diabetics and the controls are given in Table.3.2.
Table 3.2 Subject characteristics of diabetics and controls

<table>
<thead>
<tr>
<th></th>
<th>Diabetics</th>
<th>Control</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>30</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Age (Years)</td>
<td>52 ± 11.9</td>
<td>25.3 ± 5.7</td>
<td>0.000</td>
</tr>
<tr>
<td>Total Cholesterol (mg/dl)</td>
<td>178.8 ± 22.7</td>
<td>162.9 ± 27.3</td>
<td>0.040</td>
</tr>
<tr>
<td>Total Triglycerides (mg/dl)</td>
<td>220.8 ± 130.4</td>
<td>130.5 ± 57.0</td>
<td>0.021</td>
</tr>
<tr>
<td>HDL-C (mg/dl)</td>
<td>42.7 ± 4.9</td>
<td>46.8 ± 7.7</td>
<td>0.027</td>
</tr>
<tr>
<td>LDL-C (mg/dl)</td>
<td>110.0 ± 23.1</td>
<td>99.8 ± 24.0</td>
<td>0.121</td>
</tr>
<tr>
<td>HbA1c (percent)</td>
<td>7.56 ± 2.46</td>
<td>5.33 ± 0.41</td>
<td>0.003</td>
</tr>
<tr>
<td>PON (PA) Units/Lt</td>
<td>138.8 ± 48.6</td>
<td>92.0 ± 30.1</td>
<td>0.000</td>
</tr>
<tr>
<td>PON (Units/Lt)</td>
<td>235.5 ± 123.0</td>
<td>577.3 ± 262.6</td>
<td>0.000</td>
</tr>
<tr>
<td>Glycated HDL (ng/ml)</td>
<td>2.34 ± 1.00</td>
<td>2.86 ± 0.86</td>
<td>0.075</td>
</tr>
</tbody>
</table>

Graphical representation of glycated HDL in control and diabetic serum is shown in Fig. 3.25.

Fig. 3.25 Quantitative detection of glycated ApoA1 in control and diabetic subjects.

Control and Glycated HDL was tested for the presence of glycated ApoA1 using antibody as described in methods.
The geometric mean of control and diabetic samples was not significantly different. However, the diabetic samples had some very high values.

Correlation between HDL-C and Glycated HDL of diabetics is shown in Fig.3. 26.

Fig.3. 26 Correlation between HDL-C and Glycated HDL

Glycated HDL and HDL-C showed a complex relationship. At low HDL concentration the Glycated HDL did not correlate with HDL-C. At high HDL-C the glycated HDL showed a negative correlation.

Correlation of Glycated HDL and HbA1c is shown in Fig.3.27. Glycated HDL correlated positively with HbA1c.

Fig.3. 27 Correlation between HbA1c and Glycated HDL
When subjects with high HbA1c and those with low HbA1c were compared, except for their HbA1c there were no significant differences. (Table 2)

**Table.3.3 Comparison between diabetics with high HbA1c and normal HbA1c**

<table>
<thead>
<tr>
<th></th>
<th>High HbA1c</th>
<th>Low HbA1c</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>10</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>HbA1c (percent)</td>
<td>10.50 + 1.81</td>
<td>5.51 + 0.37</td>
<td>0.000</td>
</tr>
<tr>
<td>PON (PA) Units /lt X1000</td>
<td>134.0 + 56.6</td>
<td>145.8 + 44.0</td>
<td>0.289</td>
</tr>
<tr>
<td>PON Units/lt</td>
<td>259.9 + 150.9</td>
<td>191.7 + 73.4</td>
<td>0.084</td>
</tr>
<tr>
<td>Glycated HDL ng/ml</td>
<td>2.50 + 0.86</td>
<td>2.00 + 1.10</td>
<td>0.125</td>
</tr>
</tbody>
</table>

Patients with HbA1c >8 were used as High HbA1c group and those with HbA1c <6 were used as low HbA1c group

Even though the blood glucose was under control in the low HbA1c group, the serum PON activity was not different from those of high HbA1c group. Even the glycated HDL was not significantly different.

**Cross reactivity of other antibodies**

![Image of cross reactivity experiment](image)

**Fig.3.28 Cross reactivity of IgY**

Cross reactivity of IgY was tested against different antigens. A-serum, B-Control HDL, C-Glycated HDL, O-IgY
In testing for cross reactivity of antibody raised against oxidized HDL, precipitin bands were seen for both glycated as well as non glycated HDL and for control serum (Fig.3.28). Since the antibody is polyclonal antibody raised in the chick all mammalian proteins would be highly antigenic. Hence even if normal HDL remained in the oxidized HDL as trace contaminant, it should show up as two distinct proteins and antibodies would be formed for both.

Antibodies to oxidized HDL were raised in the rabbit. This antibody was used to determine the cross reactivity against control and glycated proteins like HLD, LDL, BSA and Apo A1(Fig.3.29).

![Graph showing cross reactivity against antibody to oxidized HDL](image)

**Fig.3.29 Cross reactivity against antibody to oxidized HDL**

Oxidized HDL was used to raise antibodies in rabbit. The antibody was diluted 1:8000 and tested against different antigens.

The antibody to oxidized HDL did not cross react with glycated HDL or glycated ApoA1. Antibody to glycated ApoA1 was also raised in the hen egg(IgY) and tested against control and glycated proteins. (Fig.3.30).
Fig. 3.30 Cross reactivity of glycated HDL by antibody raised against oxidized ApoA1 in hen

Oxidised HDL was used to raise antibodies in hen (IgY). Antibody was diluted 1:8000 and tested against different antigens.

The IgY reacted with glycated ApoA1 as well as glycated BSA.

Fig. 3.31 Precipitation of glycated HDL by antibody.

HDL of control subjects was treated with antibody to precipitate glycated HDL if any. The PON activity in the precipitate was determined as described in methods. Results are mean ±SD (n=3)
Chapter III

HDL was glycated with glyoxal and glucose and then treated with the antibody. The precipitated HDL was separated and tested for PON activity as well as for its pro-inflammatory activity on DCFH fluorescence. Fig.3. 31 shows the PON activity in the precipitate.

HDL of control subjects was treated with antibody to precipitate glycated HDL if any. The PON activity in the precipitate was determined as described in methods. Glyoxal gave significantly higher activity in the precipitate compared with the control (P<0.05) whereas there was no difference in the activity of HDL of control and glucose catalyzed glycation.

The DCFH fluorescence of control and glycated precipitate of HDL is shown in Fig.3.32

![Precipitate](image)

**Fig.3. 32 DCFH fluorescence**

The conditions of the experiment are as described in legends to Fig.3. 28. The effect of precipitated HDL was tested on DCFH fluorescence as described in methods.

There was no significant differences in the DCFH fluorescence (P>0.05).

The PON activity in the supernatant of HDL after precipitation with antibody is shown in Fig.3.33. The supernatant PON activity in both glyoxal and glucose treated HDL decreased, suggesting that the total activity was significantly reduced by precipitation.
Fig.3.33 PON activity in the supernatant after precipitation with antibody to glycated HDL

The conditions of the experiment are as described in legends to Fig.3.29. The supernatants were used to assay PON activity.

DCFH fluorescence of the supernatant of HDL after removing the precipitate is shown in Fig.3.34.

Fig.3.34 DCFH Fluorescence

The conditions of the experiment are as described in legends to Fig.3.29. The supernatant was used in the DCFH fluorescence study.
When glycated serum was treated with the antibody and the precipitate was removed, the PON activity of the HDL remaining in the supernatant increased. (Fig.3.35). However the DCFH fluorescence did not decrease (Fig.3.36).

**Fig.3.35.** PON activity in precipitate and supernatant of serum treated with antiglycated ApoA1 antibody

Serum was treated with antiglycated ApoA1 antibody. The precipitate and supernatant were separated. PON activity in the precipitate and supernatant was determined.

**Fig.3.36.** DCFH Fluorescence of serum

DCFH fluorescence was determined in the precipitate and supernatant obtained as described in the legends to Fig.3. 35.
Studies on diabetes subjects

Study of HbA1c distribution

In order to study the distribution of HbA1c and its relation to duration of diabetes, a sample of diabetics consist of 305 males and 181 females of age 21 to 86 years was surveyed.

The age distribution is shown in Fig.3.37.

![Age distribution of diabetics](image)

**Fig.3. 37 Age distribution of diabetic subjects.**

**Diabetic males (305) and females (181) in the study were classified based on the age.**

The median age for males was 40-50 years where as females it was 50-60 years. More females than males were in the higher age groups. The mean age of males was 49.7 +/- 12.2 and females was 52.0 +/- 11.3
Fig. 3.38 Variation of HbA1c levels in diabetes with increased duration of diabetes.

The subjects described in legends to Fig 3.37 were classified according to the duration of diabetes in years. Results are mean ±SD.

The duration of diabetes and HbA1c is shown in Fig.3.38. With the increased duration of diabetes HbA1c increased reaching a maximum of 9.64%. With increased duration greater than 14 years the HbA1c actually decreased.

The distribution of patients according to their HbA1c levels is shown in Fig.3.39.

Fig. 3.39 Frequency distribution of diabetic males and females according to their HbA1c levels.

The subjects described in legends to Fig3.37 were classified according to their HbA1c level.
Both males and females showed a bimodal distribution with reference to the HbA1c levels. However more females had HbA1c greater than 9%.

The distribution of hemoglobin (Hb) among males and females is shown in Fig.3.40. About 60% of the females had Hb levels between 11% and 12% while about 53% of the males had between 13% and 14%.

![Graph showing distribution of hemoglobin (Hb) among males and females](Fig.3.40)

**Fig.3.40. Distribution of hemoglobin (Hb) among the study subjects.**

The percent distribution of the subjects according to their blood levels of hemoglobin.

Pearsons correlations coefficient between Hb and HbA1c is shown in table3.

**Table 3.4: Pearson correlation coefficient for Hb Vs HbA1c**

<table>
<thead>
<tr>
<th>Subjects</th>
<th>r</th>
</tr>
</thead>
<tbody>
<tr>
<td>Males</td>
<td>0.022</td>
</tr>
<tr>
<td>Females</td>
<td>0.033</td>
</tr>
</tbody>
</table>

The Hb levels and HbA1c levels did not correlate.
Table 3.5 Pearson’s correlation coefficient for Age Vs HbA1c

<table>
<thead>
<tr>
<th>Subjects</th>
<th>r</th>
</tr>
</thead>
<tbody>
<tr>
<td>Males</td>
<td>0.043</td>
</tr>
<tr>
<td>Females</td>
<td>-0.013</td>
</tr>
</tbody>
</table>

The correlation coefficient between age of the individual and HbA1c is shown in table 3.5. Age of the individual did not correlate with the HbA1c. The duration of diabetes Vs HbA1c is shown in table 3.6.

Table 3.6 Pearson’s correlation coefficient for duration of diabetes vs HbA1c

<table>
<thead>
<tr>
<th>Subjects</th>
<th>r</th>
<th>(Critical r)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Males</td>
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<td>0.195</td>
</tr>
<tr>
<td>Females</td>
<td>0.338</td>
<td>0.296</td>
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</table>

The duration of diabetes correlated with HbA1c.

Correlation between HbA1c and PON

The subject characteristics of the study group are shown in table 3.7.

Table 3.7 Subject characteristics

<table>
<thead>
<tr>
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<tbody>
<tr>
<td>Gender</td>
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</tr>
<tr>
<td>Males</td>
<td>59.7 %</td>
</tr>
<tr>
<td>Females</td>
<td>40.3 %</td>
</tr>
<tr>
<td>Age</td>
<td>50.7 ± 13.5 (17-79)</td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>187.5 ± 34.9 mg/dl</td>
</tr>
<tr>
<td>Total Triglycerides</td>
<td>175.0 ± 90.4 mg/dl</td>
</tr>
<tr>
<td>HDL-C</td>
<td>45.3 ± 7.3 mg/dl</td>
</tr>
<tr>
<td>HbA1c</td>
<td>7.21 ± 1.97 %</td>
</tr>
<tr>
<td>PON</td>
<td></td>
</tr>
<tr>
<td>Phenylacetate</td>
<td>218.9 ± 88.9 μmole/min/ml</td>
</tr>
<tr>
<td>Paraoxonase</td>
<td>123.2 ± 61.0 nmol/min/ml</td>
</tr>
</tbody>
</table>
The correlation coefficient of HbA1c Vs PON is shown in table.3.8.

**Table.3.8 Correlation of HbA1c Vs PON**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>r</th>
<th>Critical r</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenylacetate</td>
<td>0.000</td>
<td>0.164</td>
</tr>
<tr>
<td>paraoxon</td>
<td>0.086</td>
<td>0.164</td>
</tr>
</tbody>
</table>

HbA1c did not correlate with PON activity

Glycation of erythrocytes by glyoxal and glucose is shown in Fig.3.41

**Fig.3.41 Glycation of washed erythrocytes by glyoxal or glucose**

Erythrocytes collected from normal subjects were washed with saline and treated with glyoxal or glucose for 5 days. HbA1c levels were then determined as described in methods.

Glyoxal and glucose were both able to glycate erythrocytes invitro. The amount of HbA1c increased with concentration of glucose. However at 100mM glyoxal HbA1c reduced. This is probably because of toxicity of glyoxal resulting in erythrocytes lysis and not because of inhibition of glycation.
Effect of variables on the determination of blood glucose values

In order to evaluate the effect of type of the sample namely plasma or serum and the time between drawing of blood and its blood glucose determination is shown in Fig.3.42. Fig.3.42 gives a comparison between Fluoride plasma, EDTA plasma and serum when analyzed within 10min after drawing the blood and up to 8hrs after drawing the blood.

![Effect of time on the quantitative determination of blood glucose.](image)

**Fig.3. 42  Effect of time on the quantitative determination of blood glucose.**

Plasma or serum was used to determine the blood glucose levels immediately, after 4 hrs and after 8hrs. The results are mean ±SD.

Serum values of blood glucose were 1.15 lower than the plasma values when fluoride plasma was used. EDTA plasma gave the least value. On storing, EDTA plasma glucose values decreased with rime to a maximum extent followed by serum values. Fluoride plasma glucose values remained constant to a greater extent than the other two.
Correlation between Plasma glucose values and the serum values of the same individuals is shown in Fig.3.43. There was a linear correlation between serum glucose and plasma glucose values.

**Fig.3.43 Corelation between Plasma glucose values and the corresponding serum values**

Blood from subjects was drawn and divided into two portions one was allowed to clot and to the other fluoride was added. Plasma glucose and serum glucose of each individual as assayed as described in methods.