MATERIAL & METHODS
3.1 MATERIAL

3.1.1 CHEMICALS

CDNB, DTNB, digitonin, glucose-6-phosphate, GSH, GSSG NADP\(^-\), NADPH, used were from Sisco Research Laboratories Pvt. Ltd., Bombay India. STZ and t-butyl hydroperoxide were obtained from Sigma Chemical Company, USA. TPTZ, \(\alpha\)-tocopherol and glutathione reductase (Sigma) were kindly provided by Professor Ronald R MacGregor, University of Kansas, Medical Centre, USA. Alloxan used was from BDH Chemical Ltd. Poole, England. All other reagents used were of analytical grade.

3.1.2 KITS

Glucose kit used was of Reckon diagnostics, Baroda, India. Uric acid kit was obtained from Span diagnostics, Udhna, India. Benedict’s reagent was from s.d. fine-chem Pvt. Ltd., BOISAR and radioimmunoassay kit for insulin determination was obtained from Bhabha Atomic Research Centre, Bombay, India.
3.2 EXPERIMENTAL DESIGN

3.2.1

PART I : HUMAN DIABETES

Fasting blood samples were obtained from diabetic and non diabetic human volunteers. The diabetic subjects were categorised into insulin dependent diabetics and non insulin dependent diabetics which were further categorised into diabetics (glucose > 120) and controlled diabetics (glucose ≤ 120) depending on plasma glucose levels.

PART II : EXPERIMENTAL DIABETES

Female albino rats 100-140 g body weight were used for carrying out various experimental studies.

(A) Study of differential effect of diabetogen

Rats were divided into three groups. Diabetes was induced in one group by single intraperitoneal injection of streptozotocin (75 mg/kg b.wt.) in citrate buffer (pH 4.5), another group was made diabetic by single intraperitoneal injection of alloxan (150 mg/kg b.wt.) in normal saline. Third group was kept as control that did not receive any treatment.
**Supplementation studies**

Rats were divided into two groups, diabetic and control group. Diabetic rats were further divided into two groups one with supplementation and another without supplementation. Supplemented group received either vitamin C (60 mg/day in drinking water) or vitamin E (200 mg/kg b.wt. in soya oil, intraperitoneally twice weekly) for three weeks or five weeks and both vitamin C and E in conjunction for five weeks after one week of diabetes induction.

**3.2.2 INDUCTION OF DIABETES**

Animals were fasted overnight before injecting diabetogen. Administration of diabetogenic dose of both alloxan and streptozotocin produces triphasic fluctuations in blood glucose levels. An initial brief hyperglycaemic phase develops, which lasts for 4-5 hours during this period, no diet was given which is followed by a short hypoglycaemic phase (12-16 hrs) during this period animals had a access to pelleted diet and glucose was added in drinking water (10%), finally chronic hyperglycemia develops during which glucose water was withheld and thereafter animals had a free access to commercial pelleted diet and water except the vitamin C supplemented group where volume of water was fixed (depending on weather) to ensure proper intake of vitamin C.
3.2.3 SUPPLEMENT

The dose of vitamin C used is as described by Yue et al. (1969) and Young et al. (1992). The route and dose of vitamin E administered is as described by Dillard et al. (1982a, 1982b), Szczeklik et al. (1985) and Morel and Chisolm (1989).

3.2.4 COLLECTION OF SAMPLES

To carry out studies in blood animals were fasted overnight and bled (orbital sinus) under light ether anaesthesia. To obtain liver, animals were fasted overnight and sacrificed by cervical dislocation after respective durations.

3.2.5 PREPARATION OF SAMPLES

Blood was collected in 0.2 M EDTA (15 mg EDTA/10.0 ml blood). Centrifuged at 3000 g aspirated plasma. The sediment left was washed thrice with normal saline and was used to carry out studies on red blood cells (RBCs).

Liver tissue was homogenised in respective buffers for carrying out various studies. Homogenate was used as such to carry out studies in homogenate. Centrifuged homogenate at 1000 g for 10 min. Recentrifuged, the supernatant at 10,000 g for 15 min. The pellet thus obtained was used to carry out studies on mitochondrial fraction. The supernatant was used to carry out enzyme studies.
3.3 METHODS

3.3.1 INDUCTION AND ESTABLISHMENT OF DIABETES

Glycosuria (rats) and hyperglycemia (humans and rats) were taken as indicator of diabetes.

3.3.1.1 Determination of urine sugars

The method of Benedict was employed. The aldehyde group of sugars is oxidized by Benedict’s reagent and cuprous oxide present in Benedict’s reagent precipitates out in the presence of alkali.

Procedure: Placed 5.0 ml of Benedict’s qualitative reagent in a test tube. Added 0.5 ml of urine and boiled over a flame for 3 min, cooled. Observed the change in colour of reagent. Green indicated 0.5%, Yellow 1-1.5% and red >2%, blue indicated absence of glucose in urine.

3.3.1.2 Determination of plasma glucose

Diagnostic kit was used to determine plasma glucose. The method employed is based on the glucose oxidase/peroxidase principle, as described by Trinder (1969). Glucose oxidase oxidizes, glucose to gluconic acid and hydrogen peroxide is generated. Hydrogen peroxide thus produced is acted upon by peroxidase and oxygen is liberated. The liberated oxygen is transferred to chromogen system consisting of 4-aminoantipyrene and phenolic compound to produce red quinoeimine dye. The intensity of colour produced is directly proportional to the concentration of glucose content and is measured photometrically at 505 nm.
Reagents

2. Glucose phenol.
3. Glucose standard (100 mg%).
4. Working reagent: Prepared by mixing (1) and (2) in equal proportions made fresh every time before use.

Procedure

Took 2.5 ml of working reagent, and added 20 μl of plasma into it, mixed well and kept at 37°C for 15 min. Simultaneously blank and standard were also run, blank contained DDW instead of sample and Std. contained glucose Std. Read absorbance of standard and test at 505 nm against reagent blank. Concentration of glucose in sample was calculated by comparing with the absorbance of Std.

3.3.2 ASSESSMENT OF OXIDATIVE STATUS

For practical reasons, neither the rate of oxidant production nor the steady-state levels of reactive oxygen species are easily measured in biological systems. Thus, oxidative stress must be inferred from measurements of oxidative damage as estimated from kinetics of formation, the steady-state levels, or the extent of accumulation of oxidation products in tissues, plasma or urine.

Polysaturated fatty acids are predominantly susceptible to free radical attack. Malondialdehyde (MDA) is formed by decomposition of lipid...
peroxidation products. The thiobarbituric acid (TBA) reaction measures the amount of free MDA present in sample. The levels of TBA reactive substances was taken as an index of lipid peroxidation and hence oxidative stress.

3.3.2.1 Determination of lipid peroxidation

Method of Beuge and Aust (1978) was followed. MDA forms adduct with TBA which absorbs maximally at 532 nm.

Reagents
1. Tris-HCl buffer (pH 7.1) : 150 mM.
2. Ascorbic acid : 1.5 mM
3. FeSO₄ : 1.0 mM
4. Trichloroacetic acid : 10 g%
5. Thiobarbituric acid : 0.375g%

Procedure

0.1 ml of sample (plasma LH, MF) was incubated in 0.1 ml each of Tris-HCl buffer, FeSO₄, ascorbic acid in a final volume of 1.0 ml (volume made by DDW) at 37°C for 15 min. The reaction was stopped by adding 1.0 ml of 10% TCA. Added 2.0 ml TBA and kept in boiling water bath for 15 min. Centrifuged at 3000 g for 10 min. Read absorbance in supernatant at 532 nm against a blank containing all the reagents except the sample. The concentration of MDA in the sample was calculated using an extinction
coefficient of $1.56 \times 10^5 \text{M}^{-1}\text{cm}^{-1}$ and expressed as TBA reactive substances (TBARS) in nmol TBARS/ml for plasma and nmol TBARS/mg protein for LH and MF.

3.3.3 DETERMINATION OF MOLECULAR ANTIOXIDANTS

3.3.3.1 Estimation of vitamin E

Vitamin E was determined employing the method of Martinek (1964). 2,4,6-tripyridyl-S-triazine (TPTZ) binds ferrous iron produced by vitamin E by reduction of ferric iron. The intensity of the colour produced is taken as an index of vitamin E in sample.

Glass ware: All the glassware was soaked in 6 N HCl and rinsed with double distilled water before use.

Reagents

1. TPTZ: 0.12% in n-propanol.
2. Ferric chloride: 0.12% FeCl$_3$$\cdot$6H$_2$O in absolute ethanol.
3. Standard $\alpha$-tocopherol
   Stock standard: 200 mg% in absolute ethanol.
   Working standard: 1 mg% prepared by diluting stock with absolute ethanol.

Procedure

Pipetted 1.0 ml of absolute ethanol into glass-stoppered test tubes. Added 1.0 ml plasma sample slowly with shaking to obtain a finely divided
protein precipitate. Added 1.0 ml of Xylene. Stoppered the tubes tightly and shook vigorously for 30 sec. Simultaneously blank and standard were also run. Blank contained DDW instead of sample, standard contained 1.0 ml each of working std, DDW and Xylene. Centrifuged at 3000 g for 5 min. Pipetted 0.5 ml of Xylene layer (top) into cuvette (1.0 ml), added 0.5 ml of TPTZ reagent and mixed. Read the sample at 460 nm against blank and added 0.1 ml of ferric chloride solution at definite, timed intervals. Then read at 600 nm against blank.

3.3.3.2 Estimation of ascorbic acid (vitamin C)

Vitamin C was determined by method of Roe and Kuether (1943). Ascorbic acid forms hydrazone with 2,4-dinitrophenyl hydrazine which gives absorption maxima at 520 nm in the presence of H₂SO₄.

Reagents

1. DNPH reagent: 2 g 2,4-dinitrophenyl hydrazine, 250 mg thiourea and 30 mg of CuSO₄·5H₂O in 9N H₂SO₄.
2. H₂SO₄: 65%.
3. Activated charcoal.
4. Standard ascorbic acid.

Stock standard: 100 mg% in DDW containing 0.1 ml glacial acetic acid.

Working standard: 1 mg%, prepared by diluting stock std.
Procedure

For plasma

To 0.4 ml plasma, added 1.6 ml 10% TCA, mixed well, allowed to stand for 5 min, centrifuged at 2000 g for 10 min. Simultaneously blank and standard were also run. Blank contained DDW instead of sample and std contained working std. Took 1.0 ml supernatant, added 0.4 ml of DNPH reagent, stoppered the tubes and incubated at 37°C for 3 hrs. Chilled in ice bath and added 1.6 ml of cold 65% H₂SO₄. Mixed well. Allowed to stand for 30 min at room temperature. Read absorbance at 520 nm.

For tissue

Took 4.6 ml of homogenate or MF, added 0.4 ml 50% TCA and 100 mg activated charcoal and filtered. Took 1.0 ml of filtrate, added 0.4 ml DNPH reagent, incubated at 37°C for 3 hrs. Chilled in ice bath and added 1.6 ml of cold 65% H₂SO₄. Kept at room temperature for 30 min. The absorbance was read at 520 nm. Blank and std. were run simultaneously as described previously in plasma. The concentration in sample was calculated by comparing with the absorbance of Std.

3.3.3.3 Estimation of glutathione

GSH was estimated by the method of Beutlar et al. (1963) in plasma and RBC. The tissue GSH was determined by the method of Sedlak and Lindsay (1968) and expressed as non protein thiols (NPSH). The spectrophotometric procedures employed are based on the method of
Ellman who reported SH reduces DTNB to 1 mole of 2-nitro-5-mercaptobenzoic acid, which gives intense yellow colour with a absorption maxima at 412 nm.

3.3.3.3a Determination of plasma and RBC GSH

Reagents

1. Precipitating solution : 1.67 g glacial metaphosphoric acid, 0.2 g disodium EDTA and 30g NaCl in 100 ml DDW.
2. Dilute precipitating solution : Two volumes of precipitating sol and three volumes of DDW.
3. Phosphate solution : 0.3 M Na$_2$HPO$_4$
4. DTNB reagent : 40 mg% in 1% sodium citrate
5. Standard GSH : 0.1 mM in 0.02 M EDTA

Procedure

Plasma : To 1.0 ml plasma added 1.5 ml precipitating sol.

RBC : Took 0.1 ml RBC and added 0.9 ml DDW and 1.5 ml ppt solution.

Kept for 5 min, centrifuged and filtered. Took 1.0 ml filtrate, for blank took 1.0 ml dilute ppt solution and for std took 0.5 ml of std solution and 0.5 ml of dilute ppt reagent. Added 4.0 ml of phosphate solution in each, followed by 0.5 ml DTNB reagent. Read within 10 min at 412nm.
3.3.3.3b Estimation of NPSH in tissue

Reagents
1. EDTA (pH 4.7) : 0.02 M
2. Tris-EDTA buffer (pH 8.9) : 0.4 M.
3. Ellman’s reagent : 0.01 M DTNB in absolute methanol.
4. TCA : 50%
5. Standard GSH : 0.1 mM in 0.02 M EDTA.

Procedure
To aliquot of 5.0 ml of LH or MF (in 0.02 M EDTA) added 4.0 ml DDW and 1.0 ml 50% TCA. Shook intermittently for 10-15 min and centrifuged at 3000 g for 15 min, filtered. To 1.0 ml of filtrate added 2.0 ml of tris-EDTA buffer and 0.05 ml of DTNB. Read at 412 against blank. Concentration of non protein thiols in sample was calculated by comparing the absorbance of known standard run simultaneously.

3.3.3.4 Estimation of uric acid

A diagnostic kit was used (Caraway, 1963). The method employs the property of uric acid to reduce phosphotungstic acid to "tungsten blue" a blue coloured complex, which is measured colorimetrically at 710 nm.

Reagents
1. Sulphuric acid : 2/3 N
2. Sodium Tungstate : 10%
3. Sodium carbonate : 14%
4. Phosphotungstate reagent

5. Standard uric acid

   Stock std : 100 mg%

   Working std : 1 mg%, made by diluting stock before use.

Procedure

   Took 1.0 ml of plasma, 8.0 ml DDW, 0.5 ml sulphuric acid (2/3 N) and 0.5 ml sodium tungstate solution. Mixed and kept for 10 min. Centrifuged. Took 3.0 ml supernatant, and added 1.0 ml of sodium carbonate and 1.0 ml of phosphotungstate to it. For blank took 3.0 ml DDW and for standard 3.0 ml working std instead of supernatant. Mixed well and kept in dark for 15 min. Read against blank at 710nm. Concentration in sample was calculated by comparing with the absorbance of Std.

3.3.4 ASSAY OF ENZYMATIC ANTIOXIDANTS

3.3.4.1 Assay of catalase (EC 1.11.1.6)

Catalase was assayed by the method of Luck (1971). Catalase decomposes H₂O₂ the absorption of H₂O₂ at 240 nm therefore, decreases with time and from this decrease the enzyme activity can be calculated.

Reagents

1. Phosphate buffer (pH 7.0) : M/15.

2. H₂O₂-phosphate buffer : 12.5 mM H₂O₂ in M/15 phosphate buffer.
Procedure

To 3.0 ml of $H_2O_2$-phosphate buffer, added 10-40 µl of post mitochondrial supernatant.

Recorded absorbance at 240 nm against a blank containing the sample in a $H_2O_2$ free-phosphate buffer. The time ‘$t$’ required for a decrease in the absorbance (E) from 0.45 to 0.40 was recorded.

A unit catalase activity was defined as the amount of enzyme which liberates half the peroxide oxygen from a hydrogen peroxide solution of any concentration in 100 seconds and 25°C. Specific activity was expressed as units per mg of protein.

3.3.4.2 Assay of glutathione peroxidase (EC 1.11.1.9)

The activity of glutathione peroxidase was determined according to the method of Paglia and Valentine (1967).

Glutathione peroxidase catalyses the reaction:

\[
\text{GPx} \\
\text{ROOH} + 2\text{GSH} \rightarrow \text{ROH} + \text{H}_2\text{O} + \text{GSSG}
\]

\[
\text{GR} \\
2\text{GSH} \rightarrow \text{GSSG} + \text{NADPH}
\]

Glutathione reductase, generates GSH from GSSG by using NADPH, while the concentration of GSH in the enzymatic cycle remains essentially constant. The decrease in NADPH is taken as index of GSSG formation from GSH by glutathione peroxidase ($E_{340} = 6.22 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$).
Reagents
1. Potassium phosphate buffer (pH 7.0) : 0.25 M
2. Glutathione reductase (from yeast) : 6 µmol activity/min at 25°C in phosphate buffer.
3. Reduced glutathione : 10 mM
4. NADPH : 2.5 mM in 0.1 % NaHCO₃
5. t-butyl hydroperoxide : 12 mM

Procedure

25 µl of postmitochondrial supernatant was incubated, with 100 µl each of buffer, glutathione reductase, reduced glutathione and NADPH in a final volume of 900 µl made with DDW, at 37°C for 10 min. The decrease in absorbance was recorded for 3 min at 30 second intervals by addition of 100 µl of t-butyl-hydroperoxide. Enzyme activity was calculated using extinction coefficient of NADP.

One unit was defined as consumption of 90% GSH within 1 min. Specific activity was expressed as units per gram of protein.

3.3.4.3 Assay of Glutathione reductase (EC 1.6.4.2)

Glutathione reductase was assayed by method of Horn and Bruns (1958). GR catalyses the reaction.

\[ \text{GSSG} + \text{NADPH} + \text{H}^+ \rightarrow 2\text{GSH} + \text{NADP}^+ \]

The decrease in the absorbance due to oxidation of NADPH to NADP⁺ at 340 nm was monitored (\(E_{340} = 6.22 \times 10^3 \text{ M}^{-1}\text{cm}^{-1}\)).
Reagents
1. Phosphate buffer (pH 6.6) : 0.067 M.
2. NADPH : 0.8 mM in 0.1% NaHCO₃.
3. Oxidized glutathione (pH 6.6) : 7.5 mM.

Procedure
Incubated 2.4 ml of buffer, 0.2 ml NADPH, 0.2 ml GSSG at 25°C for 15 min. Added 100μl of post mitochondrial supernatant and made to 3.0 ml with DDW and mixed. The decrease in absorbance was recorded at 340 nm for 3 min at intervals of 30 seconds.

A unit of enzyme activity was defined as the amount of enzyme which reduces 1 μmol GSSG in 1h at 25°C.

3.3.4.4 Assay of glutathione-S-transferase (EC 2.5.1.18)
The enzyme activity was determined by the method of Habig et al. (1974). GST catalyses the conjugation of glutathione to number of compounds. The conjugate formed between glutathione and 1-chloro-2,4-dinitrobenzene was read at 340 nm (E₃₄₀ = 9.6 mM⁻¹cm⁻¹).

Reagents
1. Sodium phosphate buffer (pH 6.5) : 0.2 M.
2. Reduced glutathione : 20 mM
3. CDNB : 20 mM in 95% ethanol.

Procedure
Took 1.5 ml of buffer, 150 μl GSH, 50 μl post mitochondrial supernatant, 1.15 ml DDW and incubated at 30°C for 5 min. Added 150μl CDNB and followed the absorbance at 340 nm for 3 min at intervals of 30 seconds.
A unit of enzyme activity was calculated as the amount of enzyme required to catalyse the formation of 1 \( \mu \text{mol} \) of conjugate per minute at 30°C. Specific activity was expressed as units per mg of protein.

### 3.3.4.3 Assay of glucose-6-phosphate dehydrogenase (EC 1.1.1.49)

Glucose-6-phosphate dehydrogenase was determined by the method of Kornberg and Horecker (1955). The increase in absorption due to reduction of NADP\(^+\) to NADPH by G6PD at 340 nm was monitored \((E_{340} = 6.22 \times 10^{3} \text{ M}^{-1} \text{ cm}^{-1})\).

**Reagents**
1. Triethanolamine buffer (pH 7.6): 50mM in 5mM EDTA.
2. Digitonin : 20 mg%.
3. NADP\(^+\) : 10 mM.
4. Glucose-6-phosphate : 31 mM.
5. Sodium chloride : 150 mM.

**Procedure**

Took 3.0 ml buffer, 0.1 ml NADP\(^+\) and 0.1 ml sample (postmitochondrial supernatant or RBC hemolysate prepared by suspending 0.1 ml of RBC in 0.5 ml digitonin) in a test tube, incubated for 5 min at 25°C. Added 50 \( \mu \text{l} \) glucose-6-phosphate waited for 2 minutes and read absorbance at 340nm against the blank containing buffer and sample alone.

A unit of enzyme was defined as amount that reduces 1\( \mu \text{mol} \) of NADP\(^+\) per hour at 25°C. Specific activity was expressed as units per mg of protein.
3.3.5 ESTIMATION OF PROTEINS

Protein concentration was determined by the method of Lowry et al. (1951). The aromatic amino acids in protein react with the Folin’s reagent to form a coloured complex.

Reagents
1. Folin-ciocalteu’s reagent : diluted 1:1 with DDW.
2. Lowry’s reagent : prepared by mixing 1% CuSO₄, 2% sodium potassium tartarate and 2% sodium carbonate in 0.1N NaOH, in a ratio of 1:1:98.
3. Standard protein solution : 10 mg% BSA.

Procedure
Added 3.0 ml of lowry reagent to 1.0 ml sample (PMS, LH, MF) and incubated at 37°C for 15 min. Added 0.3 ml Folin’s reagent, vortexed and kept at 37°C for 30 min. Read at 670 nm against blank. Concentration of protein in sample was calculated by comparing the absorbance of known standard run simultaneously.

3.3.6 SODIUM DODECYLSULPHATE POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

To study the plasma protein profile, SDS-PAGE was carried out by the method of Laemmli (1970). Sodium dodecylsulphate is a anionic detergent which binds to proteins. About 1.49 g of SDS binds to each gram of protein, giving the protein a constant negative charge per unit mass. Protein-SDS complexes move towards the anode during electrophoresis. The mobility being inversely proportional to the Log₁₀ of their molecular weight.
Reagents

1. Acrylamide solution: 30% acrylamide and 0.8% N,N-methylene bis acrylamide in DDW.
2. Stacking gel buffer (pH 6.8): 0.5 M Tris-Cl
3. Resolving gel buffer (pH 8.8): 1.5 M Tris-Cl
4. Sodium dodecyl sulphate: 10g%
5. Ammonium persulphate: 10 g%
6. Electrode buffer (pH 8.3): 0.025 M Tris, 0.192M glycine, 0.1 g% w/v SDS.
7. TEMED solution (N,N,N',N'-tetramethylethylene-diamine): used as such.
8. Stock sample buffer: 0.06 M Tris-Cl (pH 6.8), 2% SDS, 10% glycerol and 0.25% Bromophenol blue.
9. Staining solution: 25% coomassie blue in a mixture of methanol, glacial acetic acid and DDW in a ratio of 5:1:5.

Preparation of separating gel (10%)

Sol 1 : 8.33 ml
DDW : 10.0 ml
Sol 3 : 6.25 ml
Sol 4 : 250 μl
Sol 5 : 125 μl
TEMED : 12.5 μL
Preparation of stacking gel (4%)  
Sol 1 : 1.3 ml  
DDW : 6.1 ml  
Sol 2 : 2.5 ml  
Sol 4 : 100 µl  
Sol 5 : 50 µl  
TEMED : 10 µL  

Preparation of sample  
Protein (1-2 mg/ml in sample buffer) : 10-50 µl  
10% SDS : 10 µl  
2-Mercaptoethanol : 3 µl  

Heated the mixture in boiling water bath for 3 min. Cooled, centrifuged to settle any insoluble material.  

Procedure  
Glass plates were washed thoroughly. Placed plastic spacers between three edges of the plates and sealed the glass plates together on three sides with the help of agarose and adhesive tape. Poured resolving gel solution between the two glass plates, layered water saturated n-propanol carefully across the top of the gel, allowed to polymerize, decanted off the butanol solution, rinsed with water then carefully poured the stacking gel buffer and inserted sample-well-comb. After polymerization, removed the comb. Removed the spacer across the bottom of the gel. Placed the plates in electrophoretic chamber filled with electrode buffer. Applied the sample into
the wells. Connected anode to the bottom reservoir, turned on current (150 v). Continued until, the tracking dye reached the bottom of the gel. Stained with coomassie blue overnight and destained with destainer.

3.3.7 ASSAY OF INSULIN

Radioimmunoassay kit was used for estimation of plasma insulin (Berson and Yalow, 1968). The method employed is based upon the competition of unlabeled insulin in the standard or sample and radioiodinated (l-125) insulin for the limited binding sites on a specific antibody. At the end of incubation (binding), the antibody bound and free insulin are separated by the second antibody-polyethylene glycol aided separation method. Insulin concentration of samples is quantitated by measuring the radioactivity associated with the bound fraction of sample and standards.

Reagents
1. $^{125}$I-insulin
2. Insulin antiserum (Guinea pig)
3. Insulin free serum
4. Second antibody (Anti-Guinea pig IgG)
5. Polyethylene glycol (PEG)
6. Assay buffer
7. Insulin controls (A and B)
8. Insulin standard : 7.5, 12.5, 25.0, 50.0, 100.0, 200.0, uU/ml.
## Procedure

### Assay flow chart

<table>
<thead>
<tr>
<th>Tube no.</th>
<th>Description</th>
<th>Assay buffer (ml)</th>
<th>Insulin std (ml)</th>
<th>Serum sample (ml)</th>
<th>Insulin free serum (ml)</th>
<th>Insulin antiserum (ml)</th>
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<tbody>
<tr>
<td>1,2</td>
<td>Background</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3,4</td>
<td>Total</td>
<td>0.4</td>
<td>-</td>
<td>-</td>
<td>0.1</td>
<td>-</td>
</tr>
<tr>
<td>5,6</td>
<td>Blank</td>
<td>0.3</td>
<td>-</td>
<td>-</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>7,8</td>
<td>Zero std</td>
<td>0.2</td>
<td>0.1</td>
<td>-</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>9,10</td>
<td>Std 1</td>
<td>0.2</td>
<td>0.1</td>
<td>-</td>
<td>0.1</td>
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</tr>
<tr>
<td>11,12</td>
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<td>0.1</td>
<td>-</td>
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</tr>
<tr>
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<tr>
<td>15,16</td>
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<td>0.2</td>
<td>0.1</td>
<td>-</td>
<td>0.1</td>
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</tr>
<tr>
<td>17,18</td>
<td>Std 5</td>
<td>0.2</td>
<td>0.1</td>
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<td>19,20</td>
<td>Control</td>
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<td>-</td>
<td>0.1</td>
<td>-</td>
<td>0.1</td>
</tr>
<tr>
<td>21,22</td>
<td>Sample</td>
<td>0.3</td>
<td>-</td>
<td>0.1</td>
<td>-</td>
<td>0.1</td>
</tr>
</tbody>
</table>

- Mixed gently, incubated at 2-4°C overnight.
- Added 0.1 ml of $^{125}$I insulin to each.
- Mixed, incubated at room temperature for 3 hrs.
- Added 0.1 ml of second antibody and 1.0 ml of PEG to each (except tubes 1,2).
- Vortexed, kept at room temperature for 20 min, centrifuged at 1500g for 20 minutes. After centrifugation decanted and counted radioactivity in the precipitate on gamma-counter.
Calculated: \[
\% \frac{B}{B_0}
\]

Corrected average count of standard or sample
\[
= \frac{\text{Corrected average count of tubes } 5, 6}{\text{Plotted standard curve}} \times 100
\]

Plotted standard curve: \[
\% \frac{B}{B_0}
\]
on the logit and uU/ml of insulin on logarithmic scale of logit-log graph. Read the sample values from the std curve as uU insulin per ml directly.

3.3.8 ERYTHROCYTE INSULIN RECEPTOR ASSAY

The radioimmunoassay described by Gambhir et al. (1977) was employed. It is based on competitive binding of labelled and unlabeled insulin on cell membrane insulin receptors.

Reagents

1. Buffer G: 50 mM Tris, 50 mM HEPES, 10 mM MgCl$_2.6$H$_2$O, 10 mM CaCl$_2$, 2 mM EDTA, 50 mM NaCl, 5 mM KCl in 0.1% albumin.

2. Dibutyl phthalate: density 1.04.

3. $^{125}$I-insulin

Procedure

Took 400 \( \mu l \) erythrocyte suspension (1.75 \( \times \) 10$^9$ cells in buffer G) and 40 Pg of $^{125}$I-labeled insulin (in 100 \( \mu l \) buffer). Incubated at 15°C for 2.5 h, aliquoted 200 \( \mu l \) of the incubated suspensions into pre-chilled microfuge tubes containing 200 \( \mu l \) of buffer G and 200 \( \mu l \) of dibutyl phthalate. Centrifuged the tubes in microfuge for 2.5 min. Aspirated buffer and dibutyl phthalate layers with a pasteur pipet, leaving about 100 \( \mu l \) of dibutyl...
phthalate on pellet. The blank consisted of 475 µl of buffer and 40 pg of \(^{125}\)I-insulin in 25 µl buffer. To determine bound radioactivity counted in a gamma counter. The radioactivity bound to the cells was determined as follows:

\[
\frac{\text{Erythrocyte pellet radioactivity}}{\text{bound}} \times 100 = \frac{\text{Total radioactivity (in 200 µl of incubated cell suspension)}}{\%
\]

3.4 STATISTICAL ANALYSIS

Statistical analysis of the data was done using students ‘t’ test. Differences with \(p<0.05\) were taken as significant. Levels of significance are indicated at three levels \(p<0.05; p<0.01; p<0.001\).