MATERIALS & METHODS

4.0 Materials & Methods :

4.1.1 Experimental animals :

Seven to eight weeks old inbred male swiss mice, average weight of each being 20 ± (SD2) gm, were housed in a temperature controlled (25.5 ± 0.5°C) room with 50-60% humidity and were exposed to 14 hr. cycle of light and darkness. All the animals had access to mouse feed (pellets) supplied by Lipton India Ltd., Calcutta and Water ad libidium.

4.1.2 Mode of treatment :

Mice were injected intraperitoneally 65 mg/kg body weight of streptozotocin (Sigma, USA) dissolved in 0.5 M citrate buffer (pH 4.5) [148]. Hyperglycaemia was observed within 24-48 hrs.

4.1.3 Design of Experiments :

Animals were divided into two groups of fifteen. One group was kept as control and another was made diabetic. Blood samples were drawn from orbital veins and heart. Experiments were performed after 3 weeks of injection of Streptozotocin. Animals were sacrificed after proper anaesthesia with ether. Mice with blood sugar over 250 mgm% were taken.

4.2 Carbohydrate Metabolism :

4.2.1 Estimation of blood sugar :

Blood sugar was estimated by glucose oxydase method [149].

Blood glucose was measured by One Touch instrument of Life Scan inc. Johnson & Johnson Company, Milpitas, California, 95035, USA.

Procedure :

1. Insert test strip and close door.
2. Press on /off button.
3. Check code.
4. Obtain blood sample.
5. Open door, apply blood and close door.
6. Reading.

4.2.2 Estimation of liver glucose-6-phosphatase :

Test sample - 5% weight/volume of liver homogenate in sucrose solution [150].

Reagents :

(i) Sucrose solution (0.25 M) - 8.56 gm in 100 ml of water.

(ii) Glucose-6-phosphate solution (0.1 M) - 0.0336 gm. in 1 ml. water.

(iii) Maleic acid buffer - 116 mgm. Maleic acid is dissolved in water, pH is adjusted to 6.5 with 1(N) NaOH and made upto 10 ml.

(iv) 10% TCA.

(v) Ammonium Molybdate solution - 0.75 gm of ammonium molybdate is dissolved in 20 ml. of water, 10 ml of 10 (N) H_2SO_4 is added, and volume made upto 40 ml with water.

(vi) Metol solution - 1 gm in 100 ml of 3% sodium bisulphite.

(vii) Standard phosphate solution - 0.022 gm of H_2PO_4 is dissolved in 100 ml of water. Few drops of chloroform is added.

(viii) Ascorbic acid.

Procedure :

Centrifuge tubes are taken and reagents are added according to following protocol (All in ml.)

Tubes	Sucrose EDTA Sol.	Gl-6-PO_4	Buffer		Sample	Standard PO_4 Sol.	
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
	0.2	0.1	0.1	Mix and bring to 37°C in water bath	0.1	0.1	Mix and incubate at 37°C for 10 mins.

Ascorbic Acid TCA Sol.	Sample		Supernatant fluid	Ammonium Molybdate sol.	D.W.	O.D. 680nm
(9)	(10)	(11)	(12)	(13)	(14)	(15)
2	0.1	1. Keep in ice for 5 mins. 2. Centrifuge for 3 mins. at 3000 rpm. 3. Pipette clear supernatant fluid to another centrifuge tube.	1	0.5	1	

$$\text{Volume Activity} = \frac{\text{E sample - E control}}{\text{E standard}} \times \frac{1500}{t}$$

[U/L] at 37°C
t = 10 mins

4.2.3 Estimation of liver Glucose-6-phosphate dehydrogenase :

Reagents :

(i) Physiological saline with EDTA :

0.9 gm of NaCl was dissolved in distilled water and made to 100 ml. 0.025 gm EDTA - Na_2H_2, $2H_2O$ was dissolved in physiological saline and made to 100 ml.

(ii) Triethanol amine buffer (50 mM; pH 7.5)

0.93 gm of triethanolamine hydrochloride and 0.2 gm EDTA- Na_2H_2, $2H_2O$ were dissolved in 50 ml distilled water, pH was adjusted at 7.5 with 0.1 (N) NaOH and diluted to 100 ml with distilled water.

(iii) Glucose-6-phosphate solution (40 mM)

13 mg or 0.013 gm Glucose-6-phosphate, sodium salt (Gl-6-PO_4, Na_2) was dissolved in 1 ml distilled water.

(iv) $NADP^+$ solution - (30 mM β-NADP)

0.0025 gm NADP, Na_2H was dissolved in 0.1 ml 1% $NaHCO_3$ solution.

Procedure :

(i) Preparation of Enzyme

0.35 gm of liver tissue and EDTA physiological saline solution were taken in an ice cold homogeniser and homogenised for 2 min in an ice bath (The EDTA physiological buffer was added according to 0.04 ml/mg of wet liver tissue (i.e., 2.5% w/v). Then the content was centrifuged for 20 min. (30 min.) at 0-15°C (0°C) and 15,000 rpm (7,600 rpm) smallest unit of cold centrifuge (lower gradient) = 400 rpm and clear supernatant fluid was decanted. The time between the biopsy and the start of centifugation should not be more than 5 min [151].

(ii) Assay Procedure (in ml)

Tubes (1)	Triethanol Amine Buffer (2)	Sample (3)	NADP Sol. (4)	(5)	Glucose-6-phosphate Sol. (6)
	2.4	0.5	0.05	Mix and incubate for 5 min. at 25°C	0.05

After setting 100% T at 365 nm with the blank test solution was placed in cuvette. After waiting for an extinction increase of about 0.020 readings were taken at every 2 min interval for 10 mins.

4.2.4 Estimation of Liver Pyruvic acid :

Lu, 1939; Friedman and Flangen, 1943; Nath Mukherjee, 1957.

This method is based on the formation of hydrazone of Pyruvic acid with 2'4 dinitro-phenyl hydrazone. This gives a reddish colour with strong alkali which is estimated by colorimeter. Other associated substances like acetone, aceto acetic acid etc. giving the same colour in lesser degree are eliminated by extraction in an organic solvent like toluene and re-extraction in sodium carbonate solution [152].

Preparation of Reagents :

1. Trichloro acetic acid- (TCA), 10% (w/v) : prepare by dissolving 10 gm in water and making to 100 ml. Keep in refrigerator.

2. 2'4 - dinitrophenyl (DNPH), 100 mg/100 ml : Dissolve 100 mg of finely powdered substance in 2 (N) HCl and make to 100 ml with the same solvent, filter if necessary and keep in refrigerator.

3. Toluene, Pure grade, benzene or xylene also may be used.

4. Sodium carbonate solution, 10% : Dissolve 10 gm of Na_2CO_3 in water and make to 100 ml. Store in a polythene bottle.

5. Sodium hydroxide, 1.5 (N) : Prepare from more concentrated solution of known strength.

6. Standard solution of Pyruvic acid, 100 mgm/100 ml of 0.1 (N) H_2SO_4. Dissolve 100 mgm of freshly distilled pyruvic acid (or 125 mgm of Sodium pyruvate or 107 mgm of lithium pyruvate) in 100 ml of 0.1 (N) H_2SO_4.

Method :

1. Take 8 ml of TCA in a 15 ml graduated centrifugal tube.
2. Deliver 2 ml of liver homogenate into TCA.
3. Mix immediately and thoroughly and centrifuge. Keep in refrigerator till use.
4. Bring the supernatant and TCA to room temperature. Transfer 3 ml of supernatant to tube marked T.
5. Add 3 ml of TCA to another tube marked C.
6. Add 1 ml of DNPH to each. Mix and keep for 10 min at 37^0C.
7. Add 3 ml of toluene to each tube and mix the liquids by bubbling and current of air for 5 min. using a cappilary pipette.
8. Remove the lower aquaous layer as completely as possible.
9. Add 6 ml of Sodium Carbonate and mix thoroughly by passing air.
10. When 2 layers separate out clearly, separate the lower carbonate layer with a pasteaur pipette into 2 similarly marked pipette.
11. Add 5 ml of 1.5 (N) NaOH and mix.
12. Wait for 10 min at 37^0C.
13. Take reading of T against C at 520 nm.

Interpretation :

Normal range of whole blood pyruvic acid by the above method is 0.3 - 0.9 mgm/100 ml. There is no sex difference. Newborns have higher values. Values also increase after meals and exercise. Plasma has slightly higher concentration than whole blood.

In thiamine deficiency the value may be increased to 2.3 mgm/100 ml. Thiamine is an essential factor for the oxidation of pyruvic acid and an increase in the level of pyruvic acid indicates thiamine deficiency. It is also elevated in diabetes mellitus, congestive heart failure, diarrhoea, in some liver disease and some infections.

4.2.5 Estimation of liver Glycogen :

Biochemistry Laboratory Techniques, Sterling Chaykin John Wiley and Sons, Inc. 1966, Page 88.

The characterisation of Glycogen [153, 154, 155] **:**

The anthrous method (a general colorimetric method for the determination of carbohydrates). Carbohydrates are dehydrated by conc. H_2SO_4 to form furfural and a variety of other degradation products. Furfural will condense with anthrone (9, 10-dehydro-9, oxanthracene) to form a blue coloured complex. The blue colored complex is subject to quantitation through colorimetry. Since various carbohydrates have different dispositions with regard to the formation of furfural, the colour yield varies from one carbohydrate to another. This method is particularly useful in the estimation of di and polysacharide, since hydrolysis of these compounds prior to assay is unnecessary. For example the colour yield per mole of glucose is the same for glucose, maltose and glycogen.

Experimental procedure :

To 1.0 ml of a solution containing carbohydrate (0-100 μg) add 4.0 ml of anthrone reagent (0.2% w/v anthrone dissolved in conc. H_2SO_4). Quickly and thoroughly mix the two solutions, taking care not to spatter the concentrated acid, cover each tube with a marble to prevent loses of water by evaporation. Heat the mixture in a boiling water bath for 10 mins allow the reaction tubes to cool and read their adsorbance at 620 mμ against a reagent blank.

1. Verify that Beer's law hold over the concentration range suggested above (various number of the class should choose different standard sugars, such as glucose, maltose, glycogen, a methyl α-glucoside)

2. Determine the purity of the glycogen isolated from mouse liver.

$$\text{Glycogen concentration} = \frac{\text{glucose std. conc.}}{1.11} \times \frac{E_{620} \text{ of glycogen reac. mixture}}{E_{620} \text{ of glucose reac. mixture}}$$

4.2.6 Estimation of Lactic Acid :

4.2.6.1 Blood lactic acid estimation :

Serum lactic acid was estimated by the method of Barker and Summerson (1941)[156].

In this method protein free filtrate is treated with copper sulphate and solid calcium hydroxide to remove glucose. A portion of the filtrate from this process is treated with sulphuric acid to convert the lactic acid into acetaldehyde, the amount of which is measured by means of the purple colour given by p-hydroxy diphenyl in the presence of copper.

Calculation :

Since 5 ml of standard (Standard solution of zinc or lithium lactate. Dissolve 0.213 gm of pure dry lithium lactate in about 100ml of water, add 1ml of conc. H_2SO_4 and make up to a litre with water. Dilute 1 in 20 to obtain a standard containing 10 μg/ml) contains 50 μg (0.05 mgm), $^1/_{10}$th of which is used for developing the colour and since the colour developed in the determination corresponds to 0.02 ml of blood.

$$\text{Mgm. lactic acid per 100 ml blood} = \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times 0.005 \times \frac{1007}{0.02} \text{ i.u. x } 25$$

4.2.6.2 Tissue lactic acid estimation :

Reagents :

(i) 20% $CuSO_4$, $5H_2O$

(ii) 4% $CuSO_4$, $5H_2O$

(iii) Solid $Ca(OH)_2$ powder

(iv) Conc. H_2SO_4

(v) 1.5% p-hydroxy diphenyl in 0.5% NaOH

1.5gm of p-hydroxy diphenly was dissolved in 100 ml of 0.5% NaOH.

The sample : Liver/muscle homogenate 10% w/v in distilled water.

Procedure :

1. Removal of protein :

(i) To 2 ml of tissue homogenate, 1ml of 10% TCA was added and shaken well.

(ii) The tube was centrifuged at 1000 g for 15 mins. and the supernatant was removed.

2. Treatment with copper and calcium :

(i) To 1 ml of supernatant 1ml of 20% $CuSO_4$, 5 H_2O and 8 ml distilled H_2O were added to make total volume 10 ml.

(ii) Approximately 1 g powdered $Ca(OH)_2$ was added and then the tube was shaken vigorously by a cyclomixer.

(iii) The mixture was allowed to stand at room temperature for 30 mins with occasional shaking.

(iv) Then it was centrifuged at 1000 g for 15 mins. and supernatant was removed.

3. Formation of Acetaldehyde :

(i) To 1ml of the supernatant fluid (taken in a test tube) 0.05ml of 4% $CuSO_4$, $5H_2O$ was added, tube was kept in an ice and water bath for 2 min.

(ii) Then 6.0 ml of conc. H_2SO_4 was added slowly with occasional shaking.

(iii) Then the test tube was placed in a boiling water bath for 5 mins.

(iv) Then it was cooled below 20^0C

4. Development of colour :

(i) To the test tube 0.1 ml of p-hydroxydiphenyl was added and shaken well.

(ii) The tube was placed in a water bath (25-30^0C) for 30 minutes and meanwhile it was shaken at least once to redisperse the precipitated reagent.

(iii) Excess reagent was dissolved by heating the tube in a boiling water bath for 90 sec. and cooled in cold water.

5. Measurement of colour :

The resultant solution was read at 560 nm against conc. H_2SO_4 as blank

4.2.7 Estimation of liver Succinic dehydrogenase :

Reduction of indophenol - The enzymatic oxidation of succinate by indophenol has been followed spectrophotometrically at 600 mμ. A cuvette of 1 ml capacity was filled with 0.98 ml containing 10 μmoles of phosphate (pH - 7.4), 0.5 mgm of H. Serum albumin, 2 μmoles of KCN, 10 μmoles of succinate and 20 γ [γ = 0.001 mgm] of 2, 6-dichloroindophenol. Then (0.02 mgm) 5-15 γ of enzyme in 0.02ml were added and reading were taken at 30 sec. intervals against blank containing all components except succinate [157].

Specific activity to be defined as micromoles of indophenol reduced/mgm of protein/min. at 38^0C. The O.D. change at 600 mμ divided by 19.1 is the value for micro moles of indophenol reduced.

Reagents :

Sodium Succinate (0.1M) = 0.27 gm/10 ml of distilled water

Dichlorophenol indophenol = 0.6 mgm/1 ml of distilled water

KCN - 0.0195 gm/5ml

BSA - 10 mgm in 2ml

Protocol :

	Blank (ml)	Test (ml)
Buffer	0.1	0.1
Ser Albumin	0.5	0.5
KCN	0.1	0.1
Succinate	-	0.1
Indophenol	-	0.1
Distilled water	2.2	2.0
Enzyme	0.1	0.1

4.2.8 Estimation of Serum Lactate Dehydrogenase :

The spectrophotometric method measures the increase or decrease in extinction at 340 mμ due to the change in the amount of $NADH_2$. The reduced forms of nicotinamide-adenine dinucleotide and nicotinamide-adenine dinucleotide phosphate have an absorption peak at the wave length whereas the oxidized forms have very little absorption. Wroblewski and La Due (1955) using pyruvate and $NADH_2$ measured the decrease in extinction [158].

$$\underset{\text{Pyruvate}}{CH_3COCOO^-} + NADH_2 \rightleftharpoons \underset{\text{Lactate}}{CH_3CHOH.COO^-} + NAD$$

Reagents :

(i) Phosphate buffer, pH 7.4, 0.1 M : Dissolve 18.97 gms. of anhydrous dipotassium hydrogen phosphate & 2.69 gms. of anhydrous potassium dihydrogen phosphate in water to 1 lit.

(ii) Reduced nicotinamide-adenine dinucleotide, 2.5 mg per ml phosphate buffer (freshly prepared).

(iii) Sodium pyruvate 2.5 mgm/ml. (keep in refrigerator and have to discard if any contamination occur) dissolved in H_2O.

Technique :

2.7 ml of phosphate buffer into a spectrophotometer cell (1 cm.) add 0.1 ml of serum, 0.1 ml of $NADH_2$. Allow to stand for 20 minutes to reduce any keto acids already present in the serum. Add 0.1 ml of pyruvate.

Read the extinction for 5 min at intervals of 15 to 30 seconds at room temperature (24-27°C)

[0.1 ml Serum + 0.1 ml of $NADH_2$ → 20 mins. + 0.1 ml of pyruvate]

Calculation :

The unit of activity is defined as that which produces a decrease of 0.001 in extinction/ minute at 340 mμ and is reported per ml. of serum.

$$\text{Hence : Units/ml} = \frac{\text{Change in extinction/min}}{0.001} \times \frac{1}{\text{Volume of serum used}}$$

$$= \text{Change in extinction/min} \times 10{,}000$$

To convert to International unit.

4.3 Lipid metabolism :

4.3.1 Estimation of Blood Cholesterol :

Blood cholesterol was estimated by Sackett method [159]. This is a modification of Bloor's method in which blood is added to an ethanol-ether mixture, which precipitates proteins and extracts the cholesterol. The supernatant fluid obtained in centrifuging is evaporated, the cholesterol taken up in chloroform and determined colorimetrically by the Libermann-Buschard reaction.

4.3.2 Estimation of serum LDL, VLDL, and HDL :

Serum lipoproteins were estimated by selective precipitation method of Miller et. al.(1982). Different fractions of lipoprotein were precipitated by sodium dodecyl sulphate and centrifuged. Clear supernatant was analysed for total cholesterol by using Ferric chloride [160].

4.3.3 Estimation of serum and liver triglyceride :

Serum and liver (10% w/v) was dissolved in isopropanol and shaken with active alumina to remove phospholipids, triglycerides remaining in solution. This is then hydrolysed by alkali and liberated glycerol is oxidised to formaldehyde by periodate. The formaldehyde is condensed with acetyl acetone in presence of ammonia to form yellow colored. 3, 5-diacetyl, 1,6 dihydrobetidine which is measured in a colorimeter [161].

Preparation of reagents :

1. Isopropanol, analytical grade, aldehyde free.

2. Aluminium oxide, active : This should be thoroughly washed with isopropanol to remove fine granules and then dried.

3. Saponification mixture : Dissolve 0.5 g of KOH in 3.75 ml of water and 1.25 ml of isopropanol and mix. Prepare fresh.

4. Periodate solution : Dissolve 0.95 gm of ammonium acetate in 8 ml of water. Add 0.75ml of glacial acetic acid and 8 mgm of sodium metaperiodate. Dilute 12.5 ml with water and mix. Prepare fresh.

5. Acetyl acetone solution : Mix 0.4 ml of acetyl acetone in 100 ml of isopropanol and shake to dissolve. Prepare fresh.

6. Standard solution of triolein, 100 mgm per 100 ml. Dissolve 0.1 g of triolein in 100 ml of isopropanol.

Method :

Arrange 5 centrifuge tubes fitted with glass stoppers and label them B (for blank), S (for standard) and others.

		B	S	Others
Aluminium oxide	gm	0.8	0.8	0.8
Isopropanol	ml	4.8	4.8	4.8
Serum/Liver	ml	-	-	0.2
Water	ml	0.2	-	-
Standard Triolein solution	ml	-	0.2	-

Put the stoppers shake vigorously for 15 to 20 mins. Mechanical shaking is preferable, without removing stoppers, at 2000 r.p.m. for 10 min. to get a clear supernatant in each. Next arrange another 5 tubes marked as before and add as under.

		B	S	Others
Respective supernatant	ml	2	2	2
Saponification mixture	ml	0.5	0.5	0.5
		Mix and wait for 10 mins and then add		
Periodate solution	ml	1.5	1.5	1.5
Acetyl acetone solution	ml	1.5	1.5	1.5

Mix and incubate in the dark at 65-70^{0}C for 15 min. Cool to room temperature and take readings for unknown and standard respectively against blank at 415 nm (violet filter).

Calculation :

$$\text{mgm of triglyceride per 100 ml of serum/liver} = \frac{v}{S} \times 100 \ (v = \text{unknown})$$

Results may also be obtained from a calibration curve prepared with different concentrations, say from 30 to 150 mgm of triolein per 100 ml in isopropanol.

4.3.4 Estimation of liver HMG CoA reductase :

HMG CoA reductase was estimated by homogenising the liver in potassium phosphate buffer pH 7.2 containing 20 mM dithiothritol. Reductase activity was determined on the whole homogenate to optimize the recovery of the enzymatic activity as recommended by Field. (1982) [162].

Total activity was calculated by the protein recovered. Protein was estimated by the method of Lowry. All statistical analysis were done by Student's T test.

4.4 Detoxicating microsomal enzymes :

4.4.1 Estimation of glutathione in liver and blood :

Glutathione level was determined by the method of Grunert and Philips (1951) [163] in liver tissue homogenate and blood.

Principle :

The observation by Grunert and Philips that the colored complex in the analysis for glutathione was stable for only 15 secs. prompted an investigation for the purpose of determining the factors which would prevent the rapid fading of color. The instability of the complex has been noted by Biorich. when the use of nitroprusside for the analysis of glutathione was first

reported. The qualitative inhibition of fading by the use of cyanide suggested the posibility that the reagent would be of value in the quantitative determination of glutathione. With this in mind the nitroprusside method of analysis for glutathione was reinvestigated in order to obtain the maximum degree of stability for the coloured complex and to determine the optimum concentration of reagent and condition.

Reagents :

Metaphosphoric acid solution - 3% Metaphosphoric acid in saturated solution of sodium chloride - 2%

Sodium chloride granules

Sodium nitroprusside solution - 0.067 M 20 mgm/ml (stored in brown bottle from sunlight)

Sodium carbonate - Sodium cyanide solution - 1.5 M sodium carbonate and 0.067 M sodium cyanide. This solution was stable.

Procedure :

Blood (0.5 ml) obtained by heart or venous puncture was haemolysed in 1ml of water containing a pinch of saponin in a centrifuge tube. Upon completion of haemolysis 2.5 ml of 3% metaphosphoric acid was added followed by sufficient sodium chloride to saturate the solution (1.5 gm). After shaking well the sample was centrifuged, filtered and 2 ml aliquot removed from the filtrate for analysis when liver and other tissues were analysed.

100 - 200 mgm of freshly excised CO_2 frozen tissue was homogenised with Polter-Elrehjen homogeniser in 3 ml of 3% metaphosphoric acid and 1 ml of water. The solution was then saturated with sodium chloride and treated as the blood sample. All manipulations were carried out at 3^0C.

To an Evelyn tube containing 6 ml of saturated sodium chloride was added the 2 ml aliquot. After equilibration at 20^0C for 5-10 min 1 ml of sodium nitroprusside solution was added followed immediately by 1 ml of the sodium cyanide solution. The intensity of the resulting colour was measured in the Evelyn colorimeter with a 520 mμ filter within 1 min. 2 ml of 2% metaphosphoric acid saturated with sodium chloride was used for reagent blank.

4.4.2 Estimation of Glutathione reductase in liver :

Glutathione reductase activity was measured by a modification of the method of Carlburg and Manervik [164].

The reaction mixture (1 ml) consisted of 0.1 M potassium phosphate (pH 7.6), 0.1 nM NADPH, 0.5 nM EDTA, 1 nM oxidised glutathione and a suitable amount of cytosol preparation (6 mgm protein per ml). The reaction mixture was incubated at 30°C for 5 min. before initiating the reaction by the addition of cytosol. The enzyme activity was determined by measuring the disappearance of NADPH at 340 nm.

4.4.3 Estimation of Glutathion-S-Transferase in liver [165] :

Glutathione-S-transferases (E.C. 2.5.1.18) are thought to play a physiological role in initiating the detoxication of potential alkylating agents including pharmacologically active compounds. These enzymes catalyse the reaction of such compounds with the -SH group of glutathione, there by neutralizing their electrophilic sites and rendering the products more water soluble. Glutathione conjugates are thought to be metabolised further by cleavage of the glutamate and glycine residues followed by acetylation of the resultant free amino groups of the cystenyl residue to provide the final product a mercapturic acid. The mercapturic acids i.e., S-alkylated derivatives of N-acetyl cystein are then excreted.

Spectrophotometric assay methods :

Enzyme activity with aromatic substrates was usually determined by monitoring changes in absorbance in a Cary dual beam spectrophotometer. A complete assay mixture without enzyme was used as control. Assays were conducted in a thermostated cell compartment at 25°C in 0.1 M potassium phosphate at a pH at which the non enzymatic reaction was minimal.

The concentration of GSH was 5 nM, except in systems with trans-4-phenyl-3-butene-one and ethacrynic acid (0.025 GSH) and with 1-Chloro-2-4 dinitrobenzene (1 nM GSH). The concentration of the specific substrates was limited by either high absorbance or low solubility. All assays were linear function of protein concentration and of time for atleast 3 min when the amount of enzyme used resulted in an absorbance change of less than 0.05/min substrates of limited water solubility were prepared in ethanol. The final ethanol concentration in the assay solution was always less than 4%.

Activity units : A unit of activity is defined as the amount of enzyme catalysing the formation of 1 μmole of product per min.under the condition of the specific assay. Specific activity is defined as the units of enzyme activity per mgm of protein as measured by the method of Lowrey et al.

Conditions for spectrophotometric enzyme assays in 0.1 M potassium phosphate and 5 mM GSH at 25°C.

Substrate	Conc. in in mM	pH	λ max (nm)	$mM^{-1} cm^{-1}(\lambda\varepsilon)$
1, 2 Dichloro 4-nitro benzene	1.0	7.5	345	8.5
1-Chloro, 2, 4-dinitro benzene	1.0	6.5	340	9.6
4-nitro pyriamic-N-amide	0.2	7.0	295	7.0
p-nitro benzyl chloride	1.0	6.5	310	1.9

Detailed method using 3 : 4 dichloronitrobenzene as substrate :

The substrate chosen for the estimation of enzyme activity was 3 : 4 dichloronitro benzene, becuase of the change in the ultraviolet absorption spectrum which occurs when this compound is converted in S-(2-Chloro-4-nitrophenyl) glutathione.

A cell containing all the constituents of the reaction mixture except 3 : 4 dichloro nitro benzene was used as the blank so that the rate of increase of E at 344 mμ is a measure of the rate of formation of S-(2-Chloro-4-nitophenyl) glutathione.

This increase was measured on a Unicom S.P.500 spectrophotometer with a Unicom SP 570 constant temperature cell housing. The reaction mixtures contained GSH (5 mM), various concentrations of 3 : 4 dichloronitro benzene (added 0.1 ml of ethanol and enzyme in a total volume of 3 ml of 0.1M pyrophosphate buffer, pH 2.0. The enzyme was diluted so that the rate of increase in E was less than 0.2/min. The reactions were carried out at 37°C the constituents being brought to this temperature before the determination and the reaction was started by the addition of the enzyme solution to the cell.

After stirring, readings at 344 mμ were taken at 30 secs. interval for 5.5 min. begining 30 secs. after mixing. Under these conditions the initial reaction rates were constant for at least 1.0 min and 1 unit of enzyme is defined as that amount which will form 1 μmole of S (2-Chloro-4-nitrophenyl) glutathione/min. Specific activity is expressed as units/mgm of protein.

Protein was estimated by Lowry et al method.

Method followed in this experiment :

Reagent :

(i) 0.1 M potassium phosphate buffer (pH 7.5)

(ii) GSH (5 mM)

(iii) 2-5 dichloronitro benzene 1 mM (dissolved in phenol) in a total volume of 3 ml.

GSH 5 mM :

$$\frac{5}{1000} \times 307.33 \text{ g in } 1000 \text{ ml}$$

1000 ml - 5.1 gm

3 ml - 0.004609 gm

Now dissolve (0.004609 x 10) g GSH i.e., 0.04609 g in 10 ml water.

Then 1 ml will contain 0.004609 g and this will satisfy the conc. reqd. in 3 ml.

2, 5-Dichloronitro benzene 1 mM or 0.001 M

MW - 192 g

1000 ml - 0.192

$$3 \text{ ml} - \frac{0.192 \times 3}{1000} = 0.000576 \text{ gm}$$

0.12 ml - 0.000576 gm

$\therefore$ 2 ml - 0.0096 gm.

Weigh 0.0096 g and dissolve in 2 ml ethanol 0.1 M potassium phosphate buffer calculated in the same manner.

(Liver was homogenised in 0.1 M potassium phosphate buffer).

10% homogenate

Protocol :

Tubes	Buffer	GSH	2, 5-Dichloronitro benzene	Enzyme
Blank	4 ml	2 ml	0.24 ml	-
Test	2 ml	1 ml	0.12 ml	0.1 ml

Reading at 344 nm.

Calculation :

$$\frac{\text{Mean extinction coefficient}}{\text{mgm of protein}} = \text{Unit activity of GSH-s-trans/mgm of protein/min.}$$

4.4.4 Estimation of hepatic UDP- glucoronyl transferase activity :

Mice liver microsomes were prepared by homogenising the liver tissue in 4 vols. of ice cold 0.1 M KCl. The homogenate was filtered through nylon gauze and rotated at 8000 g for 30 mins. The supernatant was then spun at 1,05,000 g for 1 hour in an ultracentrifuge. The microsomal pellet was resuspended in a vol. of 0.1 M KCl equivalent to the original net weight of liver with p-nitrophenol as the substrate, glucoronyl transferase activity was measured spectrophotometrically at 400 mμ [166]. Statistical evaluation was carried out using Student's T test.

4.5 Peroxidation :

4.5.1 Estimation of lipid peroxidation in liver, brain and kidney :

Lipid peroxidation in liver, brain and kidney was measured by the thioberbeturic acid assay [167]. In this method malondialdehyde (MDA) formed from the breakdown of polyunsaturated fatty acids reacts with thiobarbituric acid to give a red species absorbing at 535 nm. Tissues were homogenised in KCl (potassium chloride, pH 7.4) solution in a Potter-Elvehjem Teflon glass homogeniser for 1 minute to make a 10% w/v homogenate. The malondialdyhyde concentration of the sample can be calculated by using an extinction coefficient of $1.56 \times 10^5\ M^{-1}\ Cm^{-1}$ according to Wills, 1969.

Reagent :

(1) TCA-TBA-HCl Reagent-15 g TCA and 0.375 g TBA (Thio barbituric acid) were dissolved in 100 ml 0.25 (N) HCl. This solution was mildly heated to assist in the dissolution of the thiobarbituric acid.

(2) 0.15 M KCl

Test sample -Tissue homogenate in 0.15 M KCl.

Liver-10%, Kidney-10%, Brain-10%.

Tube	Tissue homogenate	Distilled water	TCA-TBA-HCl
B	-	1.0 ml	2.0 ml
Sample	1.0 ml	-	2.0 ml

(i) All tubes were vortexed for few seconds.

(ii) Then all were heated for 15 mins in a boiling water bath.

(iii) All were cooled in room temperature/refrigerator.

(iv) All were centrifuged at 1000 g for 10 min.

(v) Supernatant liquid in each tube was removed by pasteur pipette in cuvette and read at 535 nm against blank.

Results :

Molar extinction coefficeient = 1.56×10^5

1 (M) 1000 ml → 72 g MDA (Malondialdehyde)

1 ml → 72/1000 MDA (Malondialdehyde)

3 ml → $\frac{3 \times 72}{1000} = 0.216$ g MDA

1.56×10^5 O.D. ≡ 0.216 g MDA

1 O.D. ≡ $\frac{0.216}{1.56 \times 10^5}$ g MDA

X O.D. ≡ $X \times 0.13846 \times 10^{-5}$ g MDA

10% Tissue homogenate.

∴ 1 ml tissue homogenate or 0.1g tissue gives = $X \times 0.13846 \times 10^{-5}$ g MDA

1 g tissue gives $= X \times 0.13846 \times 10^{-4}$ g MDA

$$\therefore \text{ n moles of MDA} = \frac{X \times 0.13846 \times 10^{-4}}{72} \times 10^{-9} = X \times 192.305$$

$\therefore$ n moles of MDA per gm. of tissue = O.D. x 192.305

4.5.2 Estimation of Catalase in liver :

Catalase activity was determined by the method of Cohen et al 1970 [168].

Ethanol increases the observable catalase level by decomposing complex II which is an inactive complex of catalase with H_2O_2.

Preparation of enzyme sample :

1. An isotonic solution containing NaCl and 0.01 M sodium phosphate buffered at pH 7.4. Prepare a stock concentration solution containing
   (a) 0.405 gm NaCl
   (b) 0.061425 gm. Na_2HPO_4
   (c) 0.010935 gm. NaH_2PO_4
   (d) Make upto 4.5 ml with distilled water
   (e) Dilute 9 : 100 to obtain required buffer.
2. Individual livers were removed and homogenised in the cold (10 strokes) in 10 volumes of isotonic buffer in a glass homogeniser tube equipped with a Teflon pestle.
3. The homogenate was centrifuged for 5-10 mins. at 700 g to remove nuclei and cell debris.
4. To an aliquot of the supernatant fluid ethanol was added to a final concentration of 0.17 M (0.01 ml Ethanol/ml).
5. The samples were then incubated for 30 mins. in ice water bath. This procedure decomposes Complex II.
6. After 30 mins., 10% Triton x-100 was added to a final concentration of 1.0%.
7. Cold isotonic buffer was then added to produce a 100 fold dilution of the original homogenate. The solubilisation of catalase by Triton is rapid and dilution can be made within minutes.

Reagents for the next step :

1. 0.01 M phosphate buffer, pH 7.0. Prepare stock concentrated solution by mixing 9 parts 1 M KH_2PO_4 with 10 parts 1 M K_2HPO_4. Dilute 1 : 100 to obtain required buffer.

2. 6 mM H_2O_2 (0.06 ml 30% H_2O_2/100 ml, 0.01 M phosphate buffer pH 7.0).

3. 6 (N) H_2SO_4

4. 0.01 (N) $KMnO_4$ (316 mgm/ltr.)

Procedure :

Reactions are carried out in an ice water bath (0-2°C). The enzyme catalysed decomposition of H_2O_2 is measured.

1. Place duplicate 0.5 ml aliquots of cold catalase samples (A) into cold 20-40 ml test tubes.

2. Include duplicate blanks consisting of 0.5 ml distilled water.

3. Initiate the enzymic reactions sequentially at fixed intervals by adding 5.0 ml of cold 6 mM H_2O_2 and mix thoroughly (vortex).

4. After exactly 3 mins. stop the reactions sequentilly at the same fixed intervals by rapidly adding 1.0 ml 6 (N) H_2SO_4 and mixing (vortex).

Spectrophotometry - The H_2O_2 is measured by reacting it with a standard excess of $KMnO_4$ and then measuring the residual $KMnO_4$ spectrophotometrically.

(i) Prepare a spectrophotometric standard by adding 7 ml of 0.01 (N) $KMnO_4$ to a mixture of 5.5 ml buffer and 1.0 ml 6 (N) H_2SO_4.

Read the absorbance at 480 nm. (For convenience in performing later calculations we adjust the digital read out of the Gylford model 300 spectrophotometer to read 1.0 absorbance unit for the standard. Distilled water then reacts in the range of 0.3 absorbance unit). A distilled water reading is checked at intervals to ensure spectrophotometer stability.

(ii) The remaining enzyme reaction samples (A) and the blanks (B) are taken one at a time. To each add rapidly 7 ml of $KMnO_4$ reagent. Mix thoroughly (vortex). Read at 480 mμ within 30-60 secs. On longer standing turbidity developes. To avoid precipitation of MnO_2 in the cell flush with distilled water between samples.

Tube	Enzyme	Dist. H_2O ml	H_2O_2 buffer (ml)	Buffer (ml)		H_2SO_4 (ml)	$KMnO_4$ (ml)
Blank	-	0.5	5.0	-	Vortex	1.0	7.0
Stamd.	-	-	-	5.5	and after	1.0	7.0
Test	0.5	-	5.0	-	3 mins. add	1.0	7.0

Calculation and expression of results :

Under the conditions described the decomposition of H_2O_2 by catalase follows first order kinetics as given by the equation.

$K = \text{Log}(S_0/S_3) \times 2.3/t$

K = First order reaction rate constant

t = time interval over which reaction is measured (viz. 3 mins).

S_0 = Substrate concentration at zero time.

S_3 = Substrate concentration at 3 mins.

S_0 = Subtract the absorbance of the reaction system blanks from the spectrophotometric standard (St.)

S_3 = Subtract the absorbance of the reaction samples (A) from (St). Results can now be expressed in terms of the first order reaction rate constant (K).

4.5.3 Estimation of Cyt-P-450 mono oxygenase in liver :

The enzyme assay was performed using the method of Omura and Sato [169]. Cytochrome P 450 mono oxygenase is the carbon monoxide binding pigment of liver microsomes.

Experimental procedure :

Microsomal preparation - Liver of male mice was thoroughly perfused in situ with 0.9% NaCl solution. The liver was homogenised with 4 volumes of isotonic (1.15%) KCl solution.

(1) The homogenate was centrifuged at 12,000 g for 25 min in a refrigerated centrifuge and the precipitate was discarded.

(2) The microsome was sedimented by centrifugation at 78,000 g for 90 min in a Hitachi Model 40 P preparative ultra centrifuge.

(3) The firmly packed pellet of microsomes was resuspended in isotonic KCl solution and again centrifuged as above.

The washed microsomes were finally suspended in isotonic KCl, usually at a concentration of 10 mgm of protein per ml. The resultant microsomal suspensions were stored at 4^0C and used within 2-3 days.

The microsomal preparations thus obtained were found to be practically free of absorbed haemoglobin.

Measurements of difference spectra :

Difference spectra of microsomal preparations were measured in a Cary Model 14 spectrophotometer with cuvettes of 1 cm. optical path. Microsomal preparations usually containing 2 mgm. of protein per ml. of 0.1 M phosphate buffer (pH 7.0), were placed in both the sample and reference cells. After recording the base line, the content of sample cell was treated with various reagents and the spectral difference thereby induced was measured. When CO was used, it was carefully bubbled through the sample for about 20 seconds, this was sufficient to saturate the sample with the gas. Reduction of samples with dithionite was effected with a few milligrams of solid $Na_2S_2O_4$.

All spectrophotometric measurements were made at room temperature (20-25^{0}C).

Photo dissociation of CO compound of P-450 :

Microsomes suspended in 0.1 M phosphate buffer, pH 7.0, were placed in a 1 cm. square cell and reduced by a few milligrams of solid sodium dithionate. A suitable amount of water saturated with CO was added to the suspension to convert reduced P-450 to the CO compound.

The final concentration of CO was calculated from the solubility of CO in water at 20^{0}C.

The sample cell was then placed in the sample compartment of a Cary Model 14 spectrophotometer and illuminated from the side with a 100 watt tungsten lamp through a set of condenser lens and a red glass filter. The filter transmitted only red light of wave lengths longer than 520 mμ. An interference filter with a sharp transmittance max at about 450 mμ was inserted between the sample cell and the measuring photo tube to absorb the red light and to pass the light from the monochromater (450 mμ). The reference cell containing the same suspension was placed in the reference cell compartment without illumination. The photo dissociation was

measured by following the absorbance change at 450 mμ caused by illumination of the sample with the red light. Control experiments using reduced microsomes were carried out without CO addition to eliminate the effect of any red light leaking through the blue interference filter or blue light leaking through the red glass filter.

Reagents :

(1) $Na_2S_2O_4$ - Sodium dithionite.

(2) CO was prepared from formic acid and concentrated sulphuric acid by the conventional method and purified by bubbling through a KOH solution.

Calculation :

The concentrations of various Cyt-P-450 fractions were determined by the method of Onura and Sato from the reduced, CO complexed difference spectrum using an extinction coefficient of 91 mM^{-1}. The specific content of Cyt-P-450 in the various fractions was expressed as nmoles per mgm of protein.

4.6 Miscellaneous :

4.6.1 Estimation of Blood Fibrinogen :

Fibrinogen was determined by the method of Lempert [170].

Reagents :

(1) Calcium Chloride - 2.5% w/v (0.5 ml) [Calcium Chloride in dehydrated form must be used, otherwise significant error will occur].

(2) Sodium hydroxide - N/4 (10 ml).

(3) Sulphuric Acid - N (amount required for neutralization)

(4) Folin ciocaltin reagent (undiluted) - 1 ml.

(5) Sodium carbonate - 20% w/v - 6 ml.

(6) Standard tyrosine - 0.5 ml of tyrosine solution containing 20 mgm of tyrosine per 100 ml of 0.1 (N) HCl.

Apparatus and instruments :

(1) Centrifuge tube
(2) Pipette
(3) Small beaker
(4) Glass rod
(5) Incubator (37°C)
(6) Filter paper & funnel
(7) Digestion tube
(8) Boiling water bath
(9) Colorimeter

Method :

Blood is drawn from heart and taken in a centrifuge tube containing sodium citrate (as anticoagulant). It is better if 2 ml blood is taken. It is then centrifuged for 15 minutes. Plasma is separated and 0.5 ml of it is taken in a small beaker. It is diluted with 14 ml of distilled water and then mixed with 0.5 ml of 2.5 % Calcium chloride solution. A fine glass rod is placed in the liquid and its is allowed to stand in an incubator at 37°C for overnight in order to get a clot.

The rod is rotated to collect the clot into it. Then the rod is pressed against the side of a beaker to squeeze out any solution and to compress the clot. Care should be taken to pick up the rod as any small pieces of clot may have become detached. The clot is dried by pressing carefully against a piece of filter paper. The clot is transferred to the tube in which the digestion is carried out. The clot is washed with several proportions of distilled water, dissolved in 5 ml of $^{N}/_{4}$ sodium hydroxide (in a boiling water bath) nutralized with normal H_2SO_4 and then 0.5 ml of Folin ciocaltin reagent is added followed by 3 ml of 20% soidum carbonate. Volume is made upto 25 ml of distilled water and the solution is finally transferred into a small beaker or conical flask and it is placed in an incubator at 37°C for an hour.

As standard 0.5 ml of a tyrosine solution is used and treated in the same way as the test, Standard and unknown (test) solution are read at 680 nm wave length with water as blank.

Calculation :

Since 1 mgm of tyrosine is equivalent to 16.4 mgm of fibrinogen.

mgm of fibrinogen/100 ml of plasma

$$= \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times \frac{100}{0.5} \times 0.1 \times 16.4$$

$$= \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times 328$$

4.6.2 Estimation of serum Mono amine oxidase :

$$RCH_2NH_3 + O_2 + H_2O \rightarrow RCHO + NH_4 + H_2O_2$$

Assay Method :

Benzaldehyde production from the oxidative deamination of Benzylamine allows a convenient spectrophotometric assay of enzyme activity [171].

Reagents :

Phosphate buffer (Na_2HPO_4 - KH_2PO_4) 0.2 M, pH 7.2 Benzylamine in 0.2 M phosphate buffer. 0.1 M final pH 7.2 may be prepared from either redistilled benzylamine or recrystallized benzylamine hydrochloride.

Procedure :

Serum or purified enzyme preparations and 0.1 ml of buffered 0.1 M benzylamine and a sufficient volume of 0.2 M phosphate buffer pH 7.2 to provide a final volume of 3.0 ml are added and a silica cell (1 cm light path) increases in optimal density at 250 nm are measured at 25°C in a recording.

Spectrophotometer, with thermostatically controlled cuvette chambers against a reaction mixture that contains no benzylamine.

Definition of Enzyme units and specific activity, one unit of activity is defined as the amount of enzyme catalysing a change in absorbance of 0.001/minute at 250 nm corresponds to the production of 0.25 nM of benzyldehyde/min. at 25°C. Specific activity expressed as units of enzyme/milligram of protein.

Protocol :

	Phosphate buffer + Benzylamine	**Phosphate buffer**	**Enzyme**
Blank	0.5 ml	14.5 ml	-
Test	0.1 ml	2.8 ml	0.1 ml.

4.6.3 Estimation of Acetyl Choline Esterase in brain :

Assay was made by the method of Biggs et al 1958 [172]. Cholinesterases hydrolyses esters of choline to give choline and an acid. Acetylcholine esterase would produce choline and acetic acid from acetyl choline bromide.

$$CH_3COO\ (CH_2)N^+\ (CH_3)_3 + H_2O \rightarrow CH_3COOH + CH_2OH.CH_2N^+\ (CH_3)_3.$$

The change of color of the indicator, bromothymol blue caused by the liberated acetic acid is read in the photoelectric colorimeter.

Preparation of reagent :

(1) Buffer : Dissolve 3.09 g of Na-barbitone, 0.34 g of Potassium dihydrogen phosphate and 43.84 g of NaCl in water and make to 250 ml.

(2) Buffer indicator solution : Dissolve 100 mgm of bromothymol blue in 2 ml of 2 (N) NaOH and wash into a litre volumetric flask with 150 ml of buffer. Dilute to about 950 ml with water. Adjust pH to 8 by adding 0.5 (N) HCl (about 16 ml necessary) and then dilute to a litre with water.

(3) Acetylcholine bromide solution : Prepare a 15% solution in water.

(4) Acetic acid : Prepare accurate by 0.15 (N) solution. Dilute 1 ml of this with 9 ml of water before use.

Method :

Measure in a tube 2 ml of buffer indicator solution and 2.2 ml of water; mix and then add 0.1 ml of tissue homogenate and 0.2 ml of acetylcholine bromide solution. Mix throughly and immediately read in the photoelectric colorimeter against red filter (620 nm). Incubate exactly for 30 min at 37°C and read again.

The difference of the 2 readings (u) corresponds to the amount of acid liberated.

The units of cholinesterase activity is defined to be the micromoles of acetic acid liberated from acetyl choline by 1 ml of tissue homogenate in 30 min at 37°C.

Preparation of standard curve :

To get a calibration curve, prepare a series of dilutions of acetic acid by measuring 0, 1, 2, 3, 4 .....14 ml of 0.015 (N) acid and making up the volume of each to 15 ml with water. 1 ml of each solution is mixed separately with 2 ml of buffer indicator. 1.4 ml of water and 0.1 ml

of tissue homogenates. These tubes will then represent 0, 10, 20, 30 .....140 units of activity. Read the tubes using the 1st tube with no acetic acid, as blank in the colorimeter against red filter (620 nm) and plot the readings against units of activity. Results are obtained from this curve.

Interpretation :

Normal range - 90 to 150 units per or 3.0 - 5.0 I.units/ml.

Low values are found in Uraemia, Tuberculosis and malnutrition and cachexia.

Reduced value in liver disease has limited significance. Low values are also found in poisoning by organo phosphorus compound.

4.6.4 Estimation of Blood Urea :

Estimation of Blood Urea by Diacetyl Monoxime Method [173] :

When urea is heated with substances such as diacetyl - (CH_3-C-C-CH_3), containing two adjacent carbonyl groups, coloured complexes are formed. Techniques employing this reaction have been devised for determining urea. Readings are taken at 480 nm wavelength against blank.

4.6.5 Estimation of Protein :

Protein was estimated by the method of Lowery et al [174].

4.6.6 Estimation of Selenium in Pancreas, Liver, Blood :

Selenium content was measured according to the method given by Mabuchi and Nakahara [175]. Sodium selenite was purchased from Loba Chemie Indoaustranal Co., Bombay-5, India. Selenium feeding was started 1 week before streptozotocin injection.

4.6.7 Vanadium :

Ammonium monovanadate was purchased from Sigma. Dose of vanadate solution used was 0.05 μg/0.1 ml. Vanadium feeding was started 1 week before streptozotocin injection.

4.7 Chromosome preparation and G banding technique from mice [176] :

(1) 40 mg of choline powder dissolved in 100 ml of distilled water followed by pasting in morter and stored at 4°C temperature (0.04% Colchicine).

(2) 1% sodium citrate solution - 1 gm sodium citrate in 100 ml single distilled water - hypotonic solution.

(3) Fixative - Glacial acetic acid one part and 90% ethanol or methanol (Acetoethanol = 1 : 3)

(4) Giemsa stain - stock solution - 1 gm of giemsa powder is pasted with 66 ml glycerol and kept at 60°C. Another 66 ml methanol is to be added later. Glycerol incubation at 60°C for 2 hours.

Filter the solution and store at 4°C for 15 days.

Pretreatment :

0.4% Colchicine is to be injected intraperitoneally in the adenoma at a rate of 1 ml/100 gm body weight after cleaning the site with ethyl alcohol. Leave for 45 mins. to 1 hour and 15 minutes.

Tissue extraction :

Colchicine treated animal is to be anaesthetized by cervical dislocation or by chloroform treatment. Femur of either side is to be dissected out and the bone marrow tissues is to be dissected out and the bone marrow tissues is to be collected in glass centrifuge by flushing the tissue with 1% sodium citrate at 37°C (by glass syringe with needle). The suspension is left for 10 mins. and centrifuged at 2000 rpm for 10 mins. Supernatant is to be discarded. Pellet is collected and mixed with acetomethanol. (3-4 ml at 1 : 3 ratio) and centrifuge again for 10 mins. at 2000 rpm. Discard supernatant. Mix aceto alcohol with the pellet. Leave for some time.

Preparation of slides :

Clean grease free glass slides are to be kept in chilled 50% ethanol for 4-5 hours. The suspension was placed in drops one after another uniformly along chilled slides held in horizontal position (1 foot height) to spread them out. The slides were flame dried.

Staining :

Staining is to be done in diluted Giemsa (one part Giemsa and 10-20 parts of distilled water) at pH 6.8 for about $1^1/_2$ hour. Slides were rinsed in distilled water and dried in air and leave for at least two days prior to observation.

4.8 Histopathology of Pancreas :

Procedure :

(i) Fix the small tissue (Pancreas) from mice in Bouin's fixative for 24 hours.

(ii) Dehydrate through 70% alcohol overnight.

(iii) Dehydrate through 90% alcohol (4 changes at 30 min interval).

(iv) Dehydrate through 100% alcohol (4 changes at 30 min interval).

(v) Immerse in Cedar wood oil for 7 days.

Microtomy :

(i) Remove oil with xylol for 10 minutes.

(ii) Infiltrate in paraffin (melted) - xylol mixture (1 : 1) in paraffin bath (incubator) at 58°C for 1 hour.

(iii) Infiltrate in paraffin in paraffin bath at 58°C for $1^1/_2$ hour.

(iv) Embed in paraffin, trim the block and make 5 μm thick paraffin section by microtome.

Staining :

(i) Dissolve paraffin in xylol for 10 minutes.

(ii) Hydrate through 100% alcohol (1 min), 90% alcohol (1 min), 70% alcohol (1 min) and distilled water (5 min).

(iii) Stain in haematoxylin (Delafield) (aqueous) for 5 minutes.

(iv) Wash in tap water for 5 minutes.

(v) Dehydrate through 70% alcohol (10 min), 90% alcohol (5 min).

(vi) Stain in eosin for 1 min.

(vii) Wash in 90% alcohol for 1 min.

(viii) Dehydrate in 100% alcohol for 10 minutes.

(ix) Immerse in xylol for 5 minutes.

(x) Mount in DPX, Examine.

Photomicrography : Film : ILFOR ASA : 100