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Materials and Methods

Chemicals – 5-hydroxy tryptamine (serotonin), norepinephrine, dopamine, estradiol, dehydrepiandrosterone (DHEA), peroxidase (POD) were procured from Sigma Chemical Co., U.S.A. and all other chemicals were of AR grade and procured from Sisco Research Laboratories Pvt. Ltd., Mumbai, India, Qualigens Fine Chemicals, Mumbai, India, Suvidhanath Laboratories, Vadodara, India and Spectrochem Pvt. Ltd., Mumbai, India. Luteinizing hormone (LH, AFP115368)) and Follicle stimulating hormone (FSH, AFP19896) were kind gifts from Dr. Parlow, NIDDKs National Hormone and Pituitary Program (NHPP), USA.

Animals - Adult virgin female rats of the Charles-Foster strain weighing 180-220 g kept under controlled conditions of light (lights on from 07.00 to 20.00 h) and temperature (24±2°C) and having access to food and water were used. Ovarian cycle was checked daily by vaginal cytology. Animals displaying atleast three 4-day cycles were selected for the experiment.

Assays

17β-hydroxy steroid oxidoreductase and 3α-hydroxy steroid dehydrogenase

(Shivanadappa and Venkatesh, 1997)

17β-hydroxy steroid oxidoreductase (17β-HSOR) plays important role in sex hormone metabolism. The hepatic microsomal 17β-HSOR reduces estrone to 17β-estradiol. The enzyme belongs to the aldo-keto reductase (AKR) superfamily, which involves a push-pull mechanism in which a conserved catalytic tyrosine functions as a general acid-base in catalysis.

$$\text{Estrone} + \text{NADH} + H^{+} \Longleftrightarrow \text{Estradiol} + \text{NAD}$$

3α-hydroxy steroid dehydrogenase, a member of AKR family, which modulates the activities of steroid hormones by reversibly reducing their C3 ketone groups. It is found in low concentrations in the amygdala, brain stem, caudate putamen, cingulate cortex, hippocampus, midbain, and thalamus. In the brain the highest levels are found in the olfactory bulb. Moderate levels are present in the cerebellum, cerebral cortex, hypothalamus and pituitary. Hydroxysteroid dehydrogenases catalyze the interconversion of hydroxyl and carboxyl groups of steroids.

5α dihydro progesterone + NADH + H^+ <=====> 3α hydroxy 5α pregnan 20 one + NAD

Principle

The assay is based on the formation of NADH due to enzymatic oxidation of the steroid alcohol, which is coupled to the reduction of the tetrazolium via diaphorase present in the tissue.

Reagents

1. 50 mM Phthalate buffer (pH 3.0): 2.55 g of potassium hydrogen phthalate dissolved in a mixture of 51 ml N/10 HCl and 2.5 ml Tween 20; pH adjusted to 3.0 and the volume made upto 250 ml with distilled water.
2. 0.1 M Tris-HCl buffer (pH 7.8)
3. 0.25 M of sucrose buffer (pH 7.4)
4. 5 mM NAD
5. Color reagent: 40 mg INT, 10 mg PMS, and 0.5 ml Tween 20 were dissolved in 50 ml distilled water for the standard curve. For the enzyme assay, PMS was omitted from the reagent.
6. The substrate:

17β hydroxysteroid oxidoreductase: 17β estradiol was first dissolved in minimum amount of alcohol and the stock solution (1 mM) prepared in 50 ml Tris-HCl buffer

3α hydroxy steroid dehydrogenase: DHEA was first dissolved in minimum amount of diformamide and the stock solution (1 mM) prepared in 50 ml Tris-HCl buffer

Enzyme

17β hydroxy steroid oxidoreductase (liver was used as the source of enzyme):

Liver microsomal fraction preparation- 10% homogenate was prepared in cold 0.25 M of sucrose buffer (pH 7.4). The homogenate was centrifuged at 1000 g for 15 min at 4^0C. The sediment was discarded and supernatant was centrifuged for 8000 g for 30 min at 4^0C. For further fractionation the above supernatant was centrifuged at 15000 g for 45 min and the pellet thus obtained was dissolved in minimum amount of 0.25 M of sucrose buffer (crude microsomal preparation).

3α hydroxy steroid dehydrogenase: 10% homogenate of hypothalamus/pituitary in 0.25 M of sucrose buffer was centrifuged at 3000 rpm for 20 min and the supernatant was used as enzyme source.

Reagents	**blank**	**Test**
Tris-HCl	1.1 ml	1.0 ml
NAD	1.0	1.0 ml
Substrate	1.0	1.0 ml
Enzyme	----	0.1
INT	0.5	0.5
	Incubate at 37^0C for 1 hr	
Phthalate buffer	2.0	2.0

Centrifuged at 3000 rpm for 20 min; supernatant was read at 490 nm against enzyme blank.

Standard curve: 1 mM solution of NADH was prepared in distilled water. Aliquots of graded concentrations of NADH (0 to 150 nmol) were reacted with the color reagent (0.5 ml) and after color formed, 1.0 ml of phthalate buffer was added to each tube and absorbance read at 490 nm. A standard curve was prepared by plotting NADH concentration vs absorbance.

Unit: nmols of NADH formed/min/mg protein

UDP-Glucoronyl transferase (UDPGT) (Gorski and Kasper, 1977)

The microsomal fraction from the liver possesses higher glucoronyl transferase activity than that from kidney and intestine. Estrone and estradiol are glucoronidated to a longer extent than is estriol by either liver or intestine. The site of conjugation of glucoronuic acid to the estrogens is approximately at C-3. Glucoronating activity in microsomal preparation from the liver of female rats is more active than that from male rats when E3 and E2 are used as substrates. One factor that governs the activity of estrone glucoronyl transferase is the amount of circulating estrogen. The fluctuations in the concentration of estrogens during the estrous cycle apparently do not influence the activity of E3 Glucoronyl transferase.

Principle

UDP-Glucoronyl transferase catalyses transfer of D-glucoronic acid from UDP-α-glucoronuic acid to an acceptor compound with inversion at the C-1 carbon of the sugar to yield the β-glucuronide. The degree of conjugation was based on the disappearance of p-

nitrophenol absorption in the visible region. The rate of g;lucoronide formation was calculated directly from the absorbance change.

Reagents

1. 0.4 M phosphate buffer (pH 7.4)

2. 40 mM MgCl2

3. 1.6 mM p-nitrophenol (PNP)

4. 20 mM UDP-glucoronic acid

5. 0.2 N TCA

6. 0.5 N NaOH

Enzyme:

Crude hepatic microsomal fraction, prepared as discussed above.

Reagents	**Blank**	**Test**
Buffer	0.26 ml	0.18 ml
UDPGA	-----	0.04
MgCl2	0.04	0.04
PNP	-----	0.04
Enzyme	0.1	0.1
Incubated at 37^0 C for 10 min with shaking		
TCA	0.4	0.4

Centrifuged at 2500 rpm for 20 min

0.5 ml supernatant was added to 1.5 ml 0.5 N NaOH; Read the absorbance at 450 nm

A molecular extinction coefficient for p-nitrophenol of 18,200 was used to calculate the amount of phenol conjugated.

Unit: Units of PNP conjugated/min/mg protein

Cytochrome P450 (Omura and Sato, 1964)

Principle

Reduced form of Cytochrome P450 readily combines with CO to form a complex having an absorption maximum at 450 nm when read against its reduced form not treated with CO.

Reagents

1. 0.1 M potassium phosphate buffer (pH 7 .4)

2. Sodium dithionate (After checking its ability to reduce must be used)

3. Carbon monoxide (CO)- CO was generated freshly by the action of concentrated H_2SO_4 and formic acid and purified by passing through KOH dithionate solution.

Procedure

Microsomal suspension was treated in phosphate buffer (1 mg protein/ml) with a few grains of sodium dithionate and taken it in the reference and sample cuvettes and recorded the baseline from 400-510 nm. Transferred the contents of the sample cuvette into a tube and bubbled gently with CO for 60 sec. Transferred back to the sample cuvette and recorded the difference spectrum from 400-510 nm again. The difference in absorbance between 450 and 490 nm was used the calculation of cytochrome P450 content using the extinction difference. Difference spectrum of dithionate-reduced sample with or without CO was used for determination of cytochrome P450 using the extinction coefficient difference of 91 cm-1mM-1.

Unit: nmols/mg protein

Pituitary membrane preparation (Poirier et al., 1974) - The pituitaries were removed immediately and placed in 0.25 M sucrose/ 10 mM Tris Buffer (pH 7.4). Homogenized gently in tissue grinder. 100 ul 0.25 M sucrose/ 10 mM Tris Buffer was used per pituitary. Centrifuged at 600 g for 5 min at 4^0 C. Removed the supernatant and rehomogenized the pellet as described earlier. Centrifuged and saved the supernatant. The supernatants were combined and centrifuged at 10000 g for 30 min at 4^0 C. Resusupended the pellet in 10 mM Tris (no sucrose). Vortexed and centrifuged at 10000 g for 20 min. Discarded the supernatant and pellet contained the membrane. Resusupend in 10 mM Tris (no sucrose). Aliquot was used for protein estimation.

Membrane fluidity (Shinitzky and Barenholz, 1978)

Fluidity specifically refers to the property of the hydrophobic region of the membrane and has been used to express the increased disorder of the fatty acyl chains. Fatty acid chains develop kinks with every double bond. With increase in the number of kinks the packing density of the membrane decreases and subsequently the fluidity of the membrane. Lipid fluidity was assessed by steady state fluorescence polarization of the lipid soluble probe, 1,6-diphenyl-1,3,5-hexatriene (DPH). Depending on the availability of space in the lipid bilayer, DPH gets differentially lodged and when light passes through it, it gets depolarized. The amount of depolarization gives the fluidity of the membrane.

Reagents

1. 0.6μM 1,6-diphenyl-1,3,5-hexatriene (DPH)

2. 10mM Tris HCl (pH 7.4)

3. 0.25 M - 0.32 M sucrose buffer in 10mM Tris HCl (pH 7.4)

Assay

A suspension of pituitary membrane was incubated for 60 min with 0.6μM 1,6-diphenyl-1, 3,5-hexatriene (DPH) in 0.32 M sucrose buffer containing 10mM Tris HCl, pH 7.4. The excitation and emission wavelengths were 360 and 430 nm, with bandwidth 5 nm and 10 nm, respectively. Polarisation values for parallel (vertical) and perpendicular (horizontal) were taken with a Shimadzu RF-540 spectrofluorimeter. The polarisation value (P) was calculated from the equation

$$P = \frac{I_{VV} - G\, I_{VH}}{I_{VV} + G\, I_{VH}}$$

where I_{VV} and I_{VH} are vertical and horizontal components of emitted light, respectively, when emitted with vertically polarised light and G is the correction factor for the emission monochromator.

Na^+K^+ATPase (Floreani et al., 1981)

Principle

Na^+K^+ATPase is an integral membrane bound enzyme and used as a marker for studying membrane changes. It is a complex of two polypeptides α and β and a number of lipid molecules incorporated into the lipid bilayer of the plasma membrane. The enzyme pumps out Na^+ and K^- into the cell against the concentration gradient with a concomitant hydrolysis of intracellular ATP.

$3Na^+$ (in) + $2K^-$ (out) +ATP + H_2O $\rightarrow$ $3Na^+$ (out) + $2K^+$ (in) + ADP +Pi

ATPase was measured by the release of inorganic phosphorus from ATP. The Pi was assayed according to Fiske and Subbarow (1925). To determine the basal Mg^{2+} ATPase

activity 0.2 mM ouabain was added to the incubation medium; the Na^+K^+ATPase activity was calculated as the difference between total ATPase and Mg^{2+}ATPase activity.

Reagents

1. 50 mM Tris HCl (pH7.4)
2. 100 mM NaCl
3. 20 mM KCl
4. 3 mM $MgCl_2$
5. 3 mM ATP
6. 0.2 mM ouabain
7. protein (20-100 ug pituitary membrane)
8. 10%trichloroacetic acid (TCA)

Reagents	**Test** (-ouabain)	**Test** (+ouabain)	**Blank** (-ouabain)	**Blank** (+ouabain)
Tris HCl	0.1 ml	0.1	0.1	0.1
NaCl	0.1	0.1	0.1	0.1
KCl	0.04	0.04	0.04	0.04
$MgCl_2$	0.12	0.12	0.12	0.12
Protein	0.1	0.1	-----	----
DW	0.42	0.32	0.52	0.42
ATP	0.12	0.12	0.12	0.12
Ouabain	-----	0.1	----	0.1
	Incubated for 20 min, at 37^0C in water bath			
TCA	0.1	0.1	0.1	0.1

The tubes were centrifuged at 3000 rpm for 10 min and the aliquot from the supernatant was taken out for phosphorus estimation.

Inorganic phosphorus (Fiske and Subbarow, 1925)

Principle

Orthophosphate reacts with molybdate form phosphomolybdic acid. This product gets reduced to blue coloured molybdenum by a strong reducing agent like ANSA, which is estimated colorimetrically at 660 nm.

Reagents

1. Ammonium molybdate

   4.0 g Ammonium molybdate dissolved in minimum amount of concentrated H_2SO_4 and then made upto 100 ml with distilled water.

2. 1-amino 2-naphthol 4-sulphonic acid (ANSA)

   20 mg triturate (mixture of 0.2 g ANSA, 1.2g sodium sulfite, 1.2 g sodium bisulfite) in 1.0 ml distilled water.

3. Standard

   Dissolve 3.51 mg KH_2PO4 in minimum amount of DW. Add 100 ul concentrated H_2SO4. Make the volume to 10 ml with DW. Dilute this to obtain appropriate working standard concentration (0.8 mg/dl).

	Test	**Blank**
Supernatant	0.5 ml	-----
DW	3.0 ml	3.5 ml
Ammonium Molybdate	0.4 ml	0.4 ml

Incubate for 10 min

ANSA	0.1 ml	0.1 ml

Absorbance was recorded at 660 nm

Standard graph for inorganic phosphate was prepared with KH_2PO4 (range 2-40 ug).

Slope- 0.02 O.D./ ug Pi

Unit- μ mol Pi liberated/min/mg protein

Schiffs base (Tappel, 1975)

The formation of Schiffs base (R_1-N=CH-CH=CH-NH-R_2) was measured to assess the lipid peroxidation in pituitary membrane samples.

Reagents

1. chloroform

2. chloroform-methanol (2:1 v/v),

Assay

Lipids were extracted from pituitary membrane samples with chloroform-methanol (2:1 v/v), dried and redissolved in chloroform. Fluorescence emission was determined at 420 nm (excitation at 360 nm) on spectrofluorometer.

Inorganic peroxides (Bernt and Bergmeyer, 1965)

This procedure measures the oxidation of o-dianisidine after treatment of samples with peroxidase to liberate molecular oxygen. The optical density (436 nm) of the samples was compared with that of H_2O_2 as standard.

Reagents

1. Buffer-enzyme mixture (0.12 M PO_4 buffer (pH 7.0); 40 ug peroxidase (POD)/ml)

Dissolve 2.07 g Na_2HPO_4; 1.09 g NaH_2PO_4 and 6 mg POD in DW and make upto 150

ml.

2. Chromogen (5 mg o-dianisidine HCl/ml DW)

3. Peroxidase reagent – With vigorous stirring add 0.5 ml solution II to 50 ml solution I. Store the mixture in dark bottle. Prevent the growth of bacteria by addition of a few drops of chloroform.

4. H_2O_2 standard solution (20 ug H_2O_2/ml)

a. Dilute 1.0 ml 35% v/v H_2O_2 solution to 250 ml with DW. Check the H_2O_2 content:- dilute 20 ml of the solution with 30 ml DW and 5 ml 1 N H_2SO_4 and titrate with 0.1 N $KMnO_4$ to a permanent pink solution. 1.0 ml of 0.1 N $KMnO_4$ solution is equivalent to 1.70 mg H_2O_2.

b. According to the results of the titration, dilute appropriate volume (between 10 ml and 20 ml) of the solution to 1000 ml with DW.

5. Perchloric acid 0.6 M - Dilute 5.2 ml 70% Perchloric acid to 100 ml DW.

Stability- Solution 3 should be freshly prepared ach day. If the solution 2 becomes turbid, it can be filtered. Always prepare the H_2O_2 standard solution just before use.

Sample- pituitary membrane

Assay system

Reagent	**Test**	**Blank**	**Standard**
Peroxide reagent	5	5	5
DW	–	0.2	--
Standard (H_2O_2)	--	---	0.2
Sample	0.2	---	---

Mixed well, kept at RT for 5 min; Read the absorbance at 436 nm

Unit - ug/H_2O_2/ml

Amine measurements

Amines were estimated by the flourimetric method of Shellenberger and Gordon. 1971.

Reagents

1. 0.4 M/0.2 M perchloric acid
2. 0.1% (wt/vol) Na_2S_2O5 in perchloric acid
3. 0.025% Na_2EDTA in perchloric acid
4. Alumina
5. 10 M NaOH
6. 0.5 M / 0.1 M borate buffer (pH 10): (For 0.5 M buffer preparation, combine 0.5 M boric acid and 0.25 M sodium hydroxide and bring the total volume to 1L with distilled water.)
7. Saturated NaCl solution
8. Butanol
9. 0.05 M phosphate buffer
10. N-heptane
11. 0.1 M phosphate buffer-EDTA solution
12. 2.5 % alkaline sodium sulfite solution
13. Iodine reagent (2.0 g KI and 0.5 g Iodine in 40 ml DW)
14. 0.1 M ninhydrin solution

Tissue sample preparation-

The hypothalamus was dissected out immediately, weighed and homogenized in chilled 0.4 M perchloric acid solution containing 0.1% (wt/vol) Na_2S_2O5 and 0.025% Na_2EDTA at 4^0C in a motor driven glass homogenizer. The homogenates were centrifuged at 4000 g at 4^0C for 15 min. The supernatant was taken and mixed with 25 mg Alumina. The solution was shaken well, centrifuged at 500 g for 5 min and the supernatant was used for 5-HT estimation. Alumina was further washed four times with 5 ml of H_2O, and extracted with 150 µl 0.2 M $HClO_4$ solution. The supernatant was used for the estimation of norepinephrine and dopamine.

Estimation of 5-HT

The fluorescence was developed by reaction with ninhydrin as utilized by Synder et al., 1965. An aliquot (4 ml) was taken and the pH was adjusted to 10 with 0.8 ml 10 M NaOH. It was transferred to a 30 ml stoppered glass tube containing 0.5 ml 0.5 M borate buffer (pH 10), 1.5 ml saturated NaCl solution (~1.5 g) and 15 ml butanol, and vortexed for 10 min. After removing the aqueous phase, the organic phase was then vortexed for 3 min with 2 ml 0.1 M borate buffer (pH 10), previously saturated with NaCl. The butanol layer (10 ml) was transferred to another tube containing 4 ml phosphate buffer (0.05 ng, pH 7) and 15 ml n-heptane. After votrexing for 2 min, 3.6 ml phosphate layer was carefully aspirated into a 15 ml glass tube containing 0.3 ml 0.1 M freshly prepared ninhydrin solution. The tubes were then heated for exactly 30 min at 75^0 C in a water bath. One hour later, the solution was transferred to a quartz cuvette and fluorescence was estimated at 385/490 nm.

Estimation of Norepinephrine and Dopamine

1 ml aliquot of the perchloric acid eluate was made to pH 6.5±0.2 with 1.5 ml of 0.1 M phosphate buffer-EDTA solution. 0.2 ml of the iodine reagent was added (shaking immediately to mix) kept for exactly 2 min, after which 0.5 ml of the alkaline sodium sulfite solution was added. After 2 min. 0.4 ml of glacial acetic acid was added to bring the pH to 4.4 - 4.8 and kept in hot air oven at 100^0C for 3-4 min. Tubes were placed in ice bath for cooling and the NE fluorescence was read at 380/495 nm. Returned the samples to oven and heated at 100^0C for an additional 40 min to develop DA fluorescence. After cooling the tubes DA fluorescence was read at 325/380 nm. The accuracy and precision of the assay were checked by running, in parallel, different concentrations of external and internal standards (125-1000 ng) and blanks with tissue samples. The fluorescent intensity was proportional to standard concentration over the range 0.008-0.5 ug/ml.

LH and FSH estimation (Radio immuno assay)

Principle

Radio immuno assay (RIA) is based upon the competition between unlabelled hormone in the standard or serum/tissue sample and radiolabelled (I^{125}) hormone for the limited binding sites on a specific antibody. At the end of incubation, the antibody bound and free hormone are separated by the second antibody polyethylene glycol (PEG) and separation method. Hormone concentration in the sample is quantitated by measuring the radioactivity (counts/minute, CPM) associated with bound fraction of sample and standards with help of gamma counter.

Reagents

1. Radiolabelled (I^{125}) LH/FSH (Hormones were labeled with I^{125} at Taramani Institute.

Chennai, India according to the modified procedure of Greenwood et al., 1963.)

2. Primary antibodies

3. Secondary antibodies

4. Polyethylene glycol (PEG)-22%

5. LH/FSH standard – 0.01- 5 ng/ml

Sample- serum or pituitary homogenate (10% in 0.25 M sucrose buffer)

Procedure

Reagents	**Total Counts**	**Nonspecific binding**	**Standard**	**Test**
Assay buffer	---	0.4	0.3	0.3
LH/FSH standard	---	----	0.1	---
Serum/tissue sample	---	----	---	0.1
Primary antibody	---	----	0.1	0.1
I^{125} LH/FSH	0.1	0.1	0.1	0.1
Mix gently and incubate the tubes at 37^0Cfor 3 hr.				
Secondary antibody	---	0.025	0.025	0.025
PEG	----	1.0	1.0	1.0

Vortex and keep all tubes at RT for 20 min., then centrifuged at 2000 rpm for 20 min

Calculation-

T= Total counts of 100ul LH/FSH I^{125} only

B0 = CPM of bound with LH/FSH I^{125} in absence of LH/FSH standard (zero binding)

B= CPM of bound with LH/FSH I^{125} in presence of LH/FSH standard

% B/T and % B/B0 was calculated for LH/FSH standards and serum/tissue samples. A logit-log graph was plotted against %B/B0 and concentration of LH/FSH standards. Serum/tissue LH/FSH concentration was calculated from the graph. The intra-assay coefficients of variation for both LH and FSH were determined.

Unit-ng/ml serum or ug/mg pituitary

Metallothionein fraction preparation (Bayne et. al., 1985)

Reagents

1. 0.9% NaCl

2. Ammonium bicarbonate buffer (pH 7.8)

Tissue was homogenized in 0.9% NaCl (10%). Centrifuged at 11000 rpm for 30 min and the supernatant obtained was heated at 70^0 C for 10 min. Centrifuged at 2500 rpm for 10 min and supernatant obtained was loaded in a Sephadex G-75 column. The eluted fractions from the Sephadex G-75 column (0.6X60 cm), using ammonium bicarbonate buffer (pH 7.8), were collected. They were then monitored at 254 and 280 nm in a UV-Visible spectrophotometer. The absorbance obtained at 254 nm was converted into equivalent amounts of protein by interpolating from a standard graph of bovine serum albumin in ammonium bicarbonate buffer read at the same wavelength. The fractions having high absorbance at 254 nm and a comparatively low absorbance at 280 nm, which correspond to the metallothionein, were used for metal analysis.

Metal analysis

Principle

The absorption of energy by atoms in the ground state forms the basis of atomic absorption spectroscopy. When a solution containing metallic species is introduced in the flame the

vapours of the metallic species is formed. Most of the metal atoms remain in the ground state and absorb light of their own wavelength having specific resonance. The amount of light absorbed will be directly proportional to the concentration of the free atoms in the flame given by the Beer- Lambert Law.

$$\textbf{Absorbance} = \log_{10} \frac{I_0}{I_t} = K.C.L$$

I_0 = intensity of incident radiation emitted by light

I_t = intensity of transmitted radiation (amount not absorbed)

C = concentration of sample

K = constant (can be obtained experimentally)

L = path light

Sample preparation

Both tissue and blood samples were digested in reagent grade nitric acid-perchloric acid (2:1) mixture. The digestion was continued till samples become colorless. Then the acid mixture was evaporated and the precipitate thus obtained was dissolved in a few drops of concentrated HCl. The samples were diluted to one ml. with distilled water and then read in GBC 902 double beam atomic absorption spectrophotometer. Sensitivities of the assay for lead and cadmium were calculated.

	lead (Pb)	**cadmium (Cd)**
Range of standard concentration	2.5 – 20 ug/ml	0.2 – 1.6 ug/ml
slit width	1.0 mm	0.5 mm
wavelength	217 nm	228.8 nm

flame	air acetylene	air acetylene
sensitivity	0.06	0.009
cathode lamp	lead	cadmium

$$\text{Ug/ml} = \frac{\text{Total volume in test tube} \times \text{conc. in ppm (as obtained in AAS)} \times \text{total residual volume}}{\text{Amount of sample taken before dilution} \times \text{volume of blood taken (or weight of tissue)}}$$

Reduced glutathione (GSH) (Beutler E & Gelbart T., 1985)

Glutathione (g-glutamylcysteinylglycine, GSH) is highly concentrated intracellular antioxidant, accounts for 90% intracellular non-protein thiol content. Highest concentration of GSH is present in liver. Glutathione exists in two forms: reduced glutathione(GSH) and the oxidized form glutathione disulfide (GSSG). The GSSG/GSH ratio may be a sensitive indicator of oxidative stress (Parris MK 1997). Glutathione status is homeostatically controlled both inside the cell and outside, being continually self-adjusting with respect to the balance between GSH synthesis (by GSH synthetase), its recycling from GSSG (by GSH reductase), and its utilization (by peroxidases, transferases, transhydrogenases, and transpeptidases).

Principle: 5-5' Dithiobis(2 nitrobenzoic) acid (DTNB) is a disulfide compound which is readily reduced by sulfhydryl compounds forming a highly colored yellow anion, which can be read at 412 nm.

Reagents :

1. Precipitating (ppting) reagent : glacial metaphosphoric acid (1.67g),EDTA (0.20g). NaCl (30g) and total volume was made up to 100 ml with distilled water (D/W)
2. 0.3M Na_2HPO_4.

3. DTNB : 40 mg DTNB dissolved in 100 ml 1% sodium citrate.

4. PBS (0.1M, pH 7.4).

5. Standard GSH solution : 2mM GSH.

(Standard range 10-100μg)

Sample preparation –10% tissue homogenate (liver/pituitary/hypothalamus) in Phosphate buffered saline (PBS) (0.1M, pH 7.4).

Procedure:

Reagents	**Blank**	**Tissue**
Sample	-	1.0ml
D/W	1.0ml	-
Ppting reagent	1.5ml	1.5ml
Keep the tubes for 3000rpm for 15min	five minutes then	centrifuge at
Supernatant	0.5ml	0.5ml
Na_2HPO_4 solution	2.0ml	2.0ml
DTNB	0.25ml	0.25ml

Absorbance was read at 412 nm within a minute after the addition of DTNB.

Calculation - Calculation was done according to the slope calculated from the standard graph.

Unit –GSH mg/g of tissue

Lipid peroxidation levels (LPO) (Ohkawa et al., 1979)

Polyunsaturated fatty acids (PUFA) are vulnerable to oxidative damage. ROI generated during various biochemical reactions initiates a chain reaction by abstracting H

atom from PUFA and forms primary stable peroxyl radical and lipid hydroperoxide. Lipid peroxides genearte secondary stable products lipid aldehydes, malondialdehydes, 4-OH alkenals, alkanals, 2-alkanals and 2-4 alkanals etc. Compared to free radicals these aldehydes are stable and can diffuse within or even escape from the cell and attack targets far from the site of their generation. LPO is a good indicator of oxidative damage to the tissues, especially the membrane lipids.

Principle: Lipid peroxidation leads to the formation of an endoperoxide i.e. malondialdehyde (MDA) which reacts with thiobarbituric acid (TBA) and gives thiobarbituric reactive substance (TBARS). TBARS gives a characteristic pink color which can be measured colorimetrically at 532 nm.

Reagent:

1. TBA reagent : TBA(100mg),EDTA(46mg),20%TCA(10ml), 2.5 N HCl (5 ml) total volume was made up to 20ml with D/W .
2. 0.1M Phosphate buffered saline (PBS) - pH 7.4 :
3. 10mM Tetra methoxy propen (TMP) for standard solution.

Standard range (1-10 nmoles)

Sample Preparation –

Tissue - 10% tissue homogenate was prepared in PBS.

Procedure:

Reagents	Blank	Tissue
Sample	-	1.0ml
D/w	1.0ml	-
TBA reagent	1.0ml	1.0ml

Kept in a boiling water bath for 20 min. After cooling, centrifuged at 3000 rpm/20 min. Absorbance were taken against tissue blank at 540 nm.

Calculation – Calculation was done according to the slope calculated from the standard graph.

Unit – nmoles of MDA formed/ g tissue

Superoxide dismutase (SOD) (Kakkar et al, 1984)

SOD is present in all the aerobic organisms. It provides an essential defense against the potential toxicity of molecular oxygen (Beyer et al 1991, Bowler et al 1992, Scandalias 1993). SOD helps to prevent tissue damage by superoxide radicals($O_2^{\bullet -}$). It is a metalloenzyme which catalyzes dismutation of superoxide radicals to hydrogen peroxide (H_2O_2) and oxygen (O_2).

$$2\,O_2^- + 2H^+ \xrightarrow{\text{SOD}} H_2O_2 + O_2$$

Two isoenzymes i.e. Cu-Zn SOD(cytosol) and Mn-SOD(mitochondria) are present in eukaryotic animals which are independently regulated according to the degree of oxidative stress experienced in the respective subcellular compartments but how it is communicated at molecular level is unknown.

Reagents:

1. 0.89%KCl
2. PBS(0.1M, pH 7.4)
3. Sodium pyrophosphate (pH 8.3) - 0.052 mM
4. PMS - 186 µM
5. NBT - 300 µM
6. NADH - 780 µM

Sample Preparation – 4% tissue homogenate in 0.89% KCl was prepared and centrifuged at 3000 rpm for 15 min. Supernatant was used for SOD estimation.

Principle: Mixtures of NADH and phenazine methosulfate (PMS) generate to superoxide under non – acidic conditions via the univalent oxidation of reduced PMS. NBT serve as a detector molecule for superoxide through reduction in to a stable, blue coloured formazone product, which can be measured at 560nm.

Procedure:

Reagents	**Test**	**Control**
Sodium pyrophosphate buffer	- 1.2 ml	1.2 ml
PMS	- 0.1 ml	0.1 ml
NBT	- 0.3 ml	0.3 ml
Diluted enzyme	- 0.01ml /0.02ml	---
D / W	- 1.2 ml / 1.18 ml	1.2 ml
NADH	- 0.2ml	0.2 ml

All tubes were incubated for 90 seconds at 37°C then reaction was terminated by adding 1.0 ml glacial acetic acid and shaken vigorously. Reduced NBT was extracted in 4 ml of n-Butanol. Tubes were centrifuged and absorbance was read at 560 nm against butanol blank.

Calculation –

$$\text{SOD (U/g)} = \frac{OD^{control} - OD^{test}}{OD^{control}} \times 100 \times \frac{60}{0.01} \times \frac{1}{90 \times \text{tissue wt (g/dl)}}$$

Unit – One unit of SOD is defined as the amount of enzyme required to inhibit NBT reduction by 50% as compared to control.

Catalase (CAT) (Hugo A. 1987)

Catalase (CAT) is a heme protein contains four ferriprotoporphyrin groups per molecules. This enzyme is also found in all aerobic organisms and is important in removal of H_2O_2 generated in peroxisomes (microbodies). Highest CAT activity is found in liver and kidney and lowest in connective tissue. In tissue it is mainly present bound to the membranes of mitochondria and peroxisomes, whereas it exist in soluble state in erythrocyte (Hugo Aebi).

Principle: Catalase is a heme containing enzyme which catalyzes dismutation of hydrogen peroxide into water and oxygen. Decomposition of hydrogen peroxide by catalase is measured spectrophotometrically at 240 nm, since hydrogen peroxide absorbs UV light maximally at this wavelength.

$$H_2O_2 \xrightarrow{CAT} H_2O + O_2$$

Reagents:

Phosphate Buffer : (50 m mol/ L . pH 7.0)

a.36 g KH_2PO_4 in 250 ml D/W.

b.13 g Na_2HPO_4 in 300 ml D/W.

Mix both in the ratio 1:1.5 and adjust pH to 7.0

Hydrogen peroxide (30 m mol/ L)

Absolute alcohol (ethanol)

Triton X-100 (10%)

PBS (0.1M. pH 7.4)

Sample Preparation – sample preparation was done according to the method of Cohen et.al., 1970. 10% tissue homogenate was prepared in PBS (pH 7.4), centrifuged at 1000 rpm to remove cell debris. Supernatant was used for enzyme analysis. 10 µl ethanol was added to 1.0 ml supernatant and these tubes incubated in ice water bath for 30 minutes. Just before the assay 10 µl of Triton X-100 and 9 ml of phosphate buffer were added.

Procedure:

Reagents	**Blank**	**Tissue**
Sample	0.2ml	0.2ml
Phosphate buffer	2.8ml	1.8ml
H_2O_2	-	1.0ml

Decrease in absorbance was monitored at 240 nm for 5 sec

Calculation – $\frac{2.303 \text{ X log E1 X dilution factor}}{\Delta t \qquad E2}$

Unit - mmoles of H_2O_2 decomposed /sec/g tissue

Cholesterol estimation (Leffler et al., 1963)

Principle

In this method cholesterol is extracted from the tissue using isopropanol. The aceticacid-$FeCl_3$ reagent acts on cholesterol converting it into cholestradiene, which then reacts with concentrated H_2SO_4 to form colored complex. This is estimated colorimetrically at 540 nm. Range of the method is 75-350 mg.

Reagents

1. $FeCl_3$ reagent– 500 mg $FeCl_3.6H_2O$ in 500 ml of glacial acetic acid or phosphoric

acid.

2. Standard cholesterol – 200 mg/dl in isopropanol

3.Tissue sample – 10% liver homogenate/ pituitary membrane

Assay system

	Test	**Blank**	**Standard**
Standard Cholesterol	---	-----	0.5 ml
Sample	0.5	----	---
$FeCl_3$-Glacial Acetic acid	1.0	1.0	1.0
Conc. H_2SO_4	2.0	2.0	2.0

Mixed well. kept at room temp for 10 min. and read the absorbance at 540 nm.

Range of the method – 75-350 ug

Slope- 0.016 O..D. / mg cholesterol

Glycogen content (Seifter et al., 1950)

Principle

Glycogen reacts with strong alkali and decomposed into glucose, which on further reaction with Anthrone reagent forms coloured complex.

Reagents

1. 30%KOH
2. 0.2% Anthrone reagent
3. Sample- To one gm of liver tissue, 3 ml 30%KOH is added. Heat it in a boiling water bath for 30 min. Take aliquot for glycogen estimation.

Assay

Reagent	**Test**	**Blank**
Sample	1.0	---
KOH	----	1.0
Anthrone reagent	2.0	2.0

The tubes were covered with glass marble, kept for 10 min. in a boiling water bath. After cooling, absorbance was read at 620 nm.

Standard range of the method- 15-150 ug.

Estimation of DNA (Burten, 1956)

Principle

The method of estimation of nucleic acid content in tissue is based on the extraction of nucleic acid by hot acid and further hydrolysis in alkali. In hot acid structural chain formed of deoxy pentose is converted into highly reactive β hydroxy levulaldehyde, which reacts with Diphenyl amine to give blue colored complex which is estimated colorimetrically.

Reagents

1. Phosphate buffered saline (PBS)
2. Diphenylamine
3. Ethyl alcohol 95%
4. Trichloro acetic acid (TCA) 10%

Sample preparation- 10% liver homogenate was prepared in PBS. From this homogenate 1 ml sample was pipetted out and to this 2.5 ml cold 10% TCA was added. Centrifuged at 4000 rpm for 15 min. The supernatant containing acid solution fraction was discarded and pellet was suspended again in 2.5 ml 10% TCA. Centrifuged at 4000 rpm for 15 min and

the pellet was dissolved in 5 ml 95% ethyl alcohol. Centrifuged at 4000 rpm for 15 min. and repeated the same procedure twice. The pellet was then dissolved in 5 ml 5% TCA. heated at 90^0 C for 30 min. Cooled and centrifuged at 4000 rpm for 20 min. The supernatant was used for nucleic acid estimation.

Assay

	Test	**Blank**
Sample	1 ml	---
PBS	----	1.0 ml
DPA solution	2.0 ml	2.0 ml

Kept in boiling water bath for 20 min. and read the absorbance at 660 nm

Standard - Standard graph for DNA was prepared in the range 10-200 ug/ml.

Estimation of RNA (Schneider, 1957)

Principle

Under acidic conditions the ribose sugar present in the RNA is converted into furfural. which then reacts with Orcinol reagent to form, blue/green colored complex.

Reagents

1. Phosphate buffered saline (PBS)
2. Standard RNA
3. Orcinol reagent
4. Ethyl alcohol 95%
5. Trichloro acetic acid (TCA) 10%

Sample preparation-as described for DNA estimation

	Test	Blank
Sample	1 ml	---
PBS	----	1.0 ml
Orcinol Reagent	1.5 ml	1.5 ml

Kept in boiling water bath for 25 min. and read the absorbance at 660 nm

Standard – Standard graph for RNA was prepared in the range 10-80 ug/ml.

Unit -- ug/g tissue

Alkaline phosphatase (ALP) and acid phosphatase (ACP) (Bowers & McComb, 1975)

These hydrolytic enzymes are present in high concentrations in liver, bone, placenta and intestinal epithelium. Increased ALP levels are frequently encountered as a clinical evidence of liver damage.

Principle- The enzyme reacts with p-Nitrophenyl phosphate (PNPP) and converts it into p-nitrophenol (PNP), which is yellow in color. PNP in alkaline medium gives bright yellow coloured phenolic ions, which can be measured at 405 nm.

Reagents

1. PNPP -0.4% (freshly prepared)

2. 0.05 N NaOH

3. 0.05 M Glycine buffer / 0.05 M Citrate buffer

4. Working buffred substrate- equal volume of glycine/ citrate buffer and stock PNPP mixed and pH was adjusted to 10.4.

5. Standard PNP solution – Stock- 1 mm in 0.05 N NaOH (freshly prepared);

Working standard - 0.04 mM made in 0.05 NaOH

Standard range – 0.04-.16 umols

Sample preparation – serum/plasma or hepatic lysosomal fraction

Reagents	**Control**	**Test**
Working buffered substrate	0.4 ml	0.4 ml
Placed in water bath at 37C for 5 min		
Serum/ tissue	---	0.05 ml
Incubated at 37 C for 30 min		
0.05 N NaOH	4.0 ml	4.0 ml
Serum/ tissue	0.05 ml	----

Absorbance was recorded at 405 nm

Calculation – Calculation was done according to the slope calculated from the standard graph.

Unit- μ mols PNP formed/min/l serum or g tissue

Creatinine (Bonsnes and Taussky, 1945)

Creatinine is the end product of creatine metabolism. It is largely formed in the muscle by irreversible and nonenzymatic removal of water from creatine phosphate. It is a waste product and excreted out from the kidney. Increased serum creatinine level is a clinical evidence of renal disease.

Principle

Creatinine reacts with picric acid under alkaline conditions to form a characteristic yellow-orange complex. The color intensity is measured at 520 nm.

Reagents:

1. Saturated Picric acid (40 mM)

2. 0.75 M NaOH

3.Standard creatinine solution was prepared in the range 10-50 ug.

Stock concentration is 100 mg/dL.

Working concentration is 10 mg/ dL

Sample preparation: 1.5 ml of picric acid was added to 0.5 ml of serum and tubes were centrifuged.

Reagents	**Test**	**Blank**
Supernatant	1.0ml	---
Distill Water	1.0 ml	2 ml
Picric Acid	1.0ml	1ml
0.75 m NaOH	1.0 ml	1 ml

Incubated for 20 min at RT and absorbance was recorded at 520 nm

Calculation: – Calculation was done according to the slope calculated from the standard graph.

Unit - mg / dl.

Glutamate pyruvate transaminase (GPT) (Reitman and Frankel, 1957)

Principle

GPT transfers amino group from Alanine to α-keto glutarate (α-KG) and convert it into pyruvate. The pyruvate thus formed is reacted with 2,4 Di Nitro Phenyl Hydrazine. The resulting hydrazone of pyruvate is highly colored and its absorbance at 540 nm is proportional to GPT activity.

L-Alanine + α-keto glutarate $\xrightarrow{\text{SGPT}}$ Pyruvate + Glutamate

Pyruvate + DNPH $\longrightarrow$ dark brown colour in alkaline medium

Reagents:

1. Buffered substrate (pH 7.4): Dissolved 15 g Di potassium Hydrogen Phosphate. 2 g Di Hydrogen Potassium phosphate, 300 mg of α keto glutarate, 17.8 g Alanine in 800 ml DW and made up the volume to 1 litre. Adjusted the pH to 7.4 with NaOH.
2. 2,4 Di Nitro Phenyl Hydrazine (DNPH). Dissolved 200 mg in 250 ml of 1 N HCl and made up the volume to 1 litre with 1 N HCl.
3. 16 g/l (400nM) NaOH.
4. Sodium pyruvate (44 mg%)
5. Sample – serum sample

(Standard range – 22-110 ug)

Reagents	**Test**	**Blank**
Serum	0.05 ml	0.05 ml
Substrate	0.25 ml	-----
Distill water	-----	0.25 ml
Incubate at 37 °C for 30 min.		
DNPH	0.25 ml	0.25 ml
Keep at RT for 20 min.		
0.4 M NaOH	2.5 ml	2.5 ml

Kept at RT for 5 minutes and absorbance was read at 540 nm.

Calculation: Calculation was done according to the slope calculated from the standard graph.

Unit: μmoles of Pyruvate formed/ min/ litre

Hemoglobin (Drabkin and Austin, 1932)

A decrease in hemoglobin below normal range is an indication of anemia. An increase in hemoglobin concentration occurs in hemoconcentration due to loss of body fluid in severe diarrhea and vomiting. High values are also observed in cogenital heart disease in emphysema and also in polycythemia. Hemoglobin concentration drops during pregnancy due to hemodilution.

Principle

When blood is mixed with Drabkin's reagent containing potassium cyanide and potassium ferricyanide, hemoglobin reacts with ferricyanide to form methaemoglobin, which is converted to stable cyanmethaemoglobin by the cyanide. The intensity of the colour is proportional to hemoglobin concentration and it is compared with a known cyanmethaemoglobin standard at 540 nm.

Reagents

Drabkin's reagent - 400 mg potassium ferricyanide, 280 mg potassium dihydrogen phosphate, 100 mg potassium cyanide and one ml nonidet dissolved in 1L DW.

Cyanmethaemoglobin (Hb standard) standard: 15 g/dl (O.D. of this standard at 540 nm corresponds to 15 g/dl hemoglobin)

	Test	Blank
Drabkin's reagent	5.0	5.0
Blood	0.02	0.02

Mixed well, kept at room temperature for 10 min. and read the absorbance at 540 nm.

Absorbance of the standard (15g/dl) was read by pipetting it directly in a cuvette.

Unit: g/dl

Histology

Liver, hypothalamus and pituitary tissues were removed and fixed in Bouins fixative. Histological examination was carried out by standard histological techniques. Sections of 5μm thickness were cut and stained with hematoxylin: eosin. Histological observations were made under the light microscope.

Statistical analysis

Results were subjected to Student's-test, one way analysis of variance (ANOVA). ANOVA followed by Neumans-Keuls multiple comparison test using prism 3.03 software to test the difference among different treated and untreated groups.