Material and Methods
CLINICAL :

Control: 70 apparently healthy and normal volunteers (35 males and 35 females, age ranging between 20-50 years) were selected and they served as normal controls. Total 12 ml of blood was collected from each volunteer in the fasting condition between 9 to 10 A.M. lying in supine position. Out of this 6 ml of blood was put in a heparinised tube, the plasma was separated by centrifugation and utilized for the estimation of cortisol (Mattingly, 1962), monoamine oxidase (McEwen and Cohen, 1963 and modified by McEven, 1971) and the erythrocytes were used for the estimation of acetylcholinesterase (Ellman et al., 1961). Serum was separated from the rest of the blood and was used for the determination of the activities of glutamate oxaloacetate transaminase (Reitman and Frankel, 1957), glutamate pyruvate transaminase (Reitman and Frankel, 1957) and lactate dehydrogenase (Wroblewski and La Due, 1955). The details of the assay procedures employed in the present study are described later.

Anxiety neurosis: Fifteen patients suffering from anxiety neurosis were selected for this study from the psychiatric clinic of J.N. Medical College, Aligarh Muslim University, Aligarh. Detailed clinical history of each case was recorded and the diagnosis was confirmed by their symptoms, behaviour and case history. The blood was collected from each case twice, before the commencement of
treatment and after one month of treatment and was subjected for the estimations of cortisol, AChE, MAO, GOT, GPT and LDH.

**Cancer Breast**: This series included 15 patients of cancer of breast in which follow-up study was also done after one month of treatment (surgery, chemotherapy, radiotherapy). The diagnosis was confirmed by surgery and histopathological examination. Blood from each case was collected and subjected for the estimation of cortisol, MAO, AChE, GOT, GPT and LDH.

**Cancer Liver**: In this series 15 cases of cancer liver were included. These cases were admitted in the surgical ward of J.N. Medical College Hospital, Aligarh Muslim University. Detailed clinical history of each case was recorded and diagnosis was confirmed by surgical and histopathological examinations. Blood from each patient was collected at the time of admission and after 15 days of therapy and was subjected for the estimation of MAO, cortisol, AChE, GOT, GPT and LDH.

**EXPERIMENTAL**

The experimental models were studied to establish a correlation between clinical and experimental findings. The effect of stress on the development of DMBA induced mammary cancer and the prophylactic effects of indigenous drugs on initiation and promotion of DMBA induced carcinogenesis were evaluated on the
experimental models in terms of biochemical parameters as discussed below. Standardized methods of inducing mammary cancer (Rogers et al., 1990) and restraint stress (Hasan et al., 1980) were employed. After the termination of the experiment the rats were sacrificed, and the blood and tissue samples were collected according to the following procedures:

**Collection of Plasma, Serum and Erythrocytes**

After the termination of the experiment, the rats were anaesthetized by injecting sodium pentobarbitol (50mg/kg body weight, intraperitoneal). Heparin was used as anticoagulant for collection of plasma and RBC. Blood was centrifuged at 1000g for 10 minutes at 4°C, after centrifugation plasma/serum was collected carefully.

Serum was utilized for the estimations of LDH, GOT and GPT, while plasma for cortisol and MAO determinations.

**Preparation of Erythrocytes**:

To obtain erythrocytes, heparinized blood was centrifuged at 1000g for 5 minutes. The buffy coat was removed, the red blood cells were suspended in 10 volumes of cold 0.1M phosphate buffered saline pH 7.4. Cells were washed in this manner 5 times to remove plasma proteins. During each wash a small portion of the top of the cell pellet was removed to ensure maximal removal of leukocytes. The activity of acetylcholinesterase and osmotic fragility were estimated in the erythrocytes.
Collection of Tissues:

Brain, heart, liver, kidney and spleen were dissected out immediately from each rat after the injection of sodium pentobarbital (50mg/kg body weight, i.p.). The tissues were washed with chilled normal saline. The tissue samples were subjected for the estimation of AChE, GST, SOD and glutathione. The homogenization, was carried out as follows:

Preparation of tissue homogenates:

The homogenate of various rat tissues (heart, liver, kidney, brain and spleen) were prepared as follows:

1) For AChE activity: 20 mg tissues/ 1ml 0.1M phosphate buffer pH 8.0.

ii) For GST activity: 10% (w/v) in 0.2M phosphate buffer pH 6.5.

iii) For GSH, and SOD: 10% (w/v) in chilled 0.15 M KCL.

Quantitative Estimation of Proteins:

The procedure described by Lowry et al., (1951) was followed. Suitable aliquot of the protein samples (Washed erythrocytes, plasma and clear supernatant of different tissues) were diluted to 1.0 ml with distilled water. To this a 5.0ml of freshly prepared copper reagent was added (copper reagent was prepared by mixing 0.5% copper sulphate, 1% (w/v) sodium potassium tartarate, 2% (w/v) sodium carbonate in 0.1N NaOH in 1:50 ratio). After incubation for 10 minutes at room temperature, 0.5ml of 1N folin's reagent was added.
The contents were rapidly mixed and colour intensity of the reaction was read after 30 minutes against a reagent blank at 660 nm. The concentration of proteins in different samples were determined using a standard curve with BSA.

1. Restrained stress and Dimethylbenz(a)anthracene induced mammary cancer:

Total 140 female albino rats (Spraque-Dawley strain), weighing around 40g±5g of same age (45-55 days) were selected. Before experimentation, the rats were deprived of food for 12 hours and water was supplied *ad libitum*. Through out the experiment the animals were supplied with food and water *ad libitum*. The animals were divided into following groups:

**Group I** (20 rats): These rats were kept at laboratory conditions and were given food and water *ad libitum* till the termination of the experiment. These animals received 1 ml of sesame oil once instead of DMBA and served as controls.

**Group II** (100 rats): This group of rats were kept at laboratory conditions for 129 days (so that the age of the stress treated and DMBA infused rats remains same.) and then exposed to restraint stress by standardized method (Hasan *et al.*, 1980). The rats were divided into five groups of 20 rats each and they were immobilized in individual cages of their size for 6, 12, 18, 24 and 30 hours at laboratory conditions (Figs. 7 & 8).
Fig. 7: Cage used for immobilization stress treatment.

Fig. 8: Rat immobilized in a body size cage.
Group III (20 rats): These rats were treated with (DMBA) dimethylbenz(a) anthracene (Sigma Chemical Co.) by gavage (30mg/kg body weight dissolved in 1ml sesame oil). The standardized method of tumor induction (Rogers et al., 1990) by a single dose of DMBA administration was followed. The weight, palpability and dietary intake of all these rats were monitored every ten days after 5 weeks of DMBA administration. The rats were sacrificed after 130 days of DMBA administration.

2. The effect of stress on DMBA induced cancer:

The effect of stress (both pre and post) was seen on the DMBA induced mammary carcinogenesis in female rats in terms of above mentioned biochemical parameters. For this study, the animals were divided into following groups:

A. Effect of Pre-stress treatment:

Group-I(A): (20 Rats): These rats were exposed to 24 hours restraint stress prior to the oral administration of DMBA (30mg/kg body weight dissolved in 1ml sesame oil). The rats were monitored for their body weight, intake of diet and palpability every ten days after 5 weeks of DMBA administration. This group served as pre-stress treated rats.

Group-II(A): (20 rats): These rats were infused with DMBA (30 mg/kg body weight dissolved in 1ml sesame oil) along with the group I(A) and served as control group (cancerous without stress) for the
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To evaluate the effect of stress on DMBA induced carcinogenesis, the rats (S-D) were divided into following groups

<table>
<thead>
<tr>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
<th>V</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control rats (20)</td>
<td>Stress treated rats (20)</td>
<td>DMBA infused rats (60)</td>
<td>Pre-stress treated DMBA infused rats (20)</td>
<td>DMBA infused post-stress treated rats (20)</td>
</tr>
<tr>
<td>The rats were kept at laboratory conditions and given single dose off 1ml sesame oil by gavage.</td>
<td>Rats were exposed to 24 hours immobilization after 129th day of commencement of the experiment.</td>
<td>Rats were given DMBA (30 mg/kg body weight dissolved in 1 ml sesame oil) by gavage.</td>
<td>On the 1st day, the rats were exposed to 24 hours immobilization stress and then were given DMBA by gavage (single dose, 30 mg/kg body weight, in 1 ml sesame oil)</td>
<td>On the 1st day, the rats were infused with DMBA (30 mg/kg body weight, single dose, in 1 ml sesame oil) and then they were exposed to 24 hours immobilization.</td>
</tr>
</tbody>
</table>

All these rats were then fed food and water ad libitum throughout the experiment

| Sacrificed at 130th day. | Sacrificed at 130th day. | Divided into three groups of 20 rats each and sacrificed on 100th, 110th (controls for IV and V group) and 130th day. | Sacrificed on 110th day of DMBA infusion. | Sacrificed on 100th day of DMBA infusion. |

No. in paranthesis indicate the number of rats.
pre-stress DMBA induced cancerous rats, group I(A) above. The weight, palpability etc. was monitored for this group too as mentioned earlier. Both these groups were sacrificed after 110 days of DMBA treatment due to the deteriorating conditions of group I(A) rats. Blood and tissue samples were collected and were subjected for the assay of cortisol, MAO, GOT, GPT, LDH, SOD, GST, AChE and reduced glutathione (total, free and protein bound GSH).

B. Effect of Post-stress treatment:

**Group-I(B):(20 Rats):** Each rat of this group received DMBA (30mg/kg body weight dissolved in 1ml of sesame oil) by gavage and then exposed to restraint stress (immobilization) for 24 hours as described earlier. The animals were supplied with food and water ad libitum and their weight, palpability and food intake was checked every ten day after 5 weeks of DMBA treatment. This group served as post-stress treated rats.

**Group-II(B):** (20 Rats): Each rat received DMBA (as mentioned above) by gavage and served as cancerous control group for group I(B) (Post-stress treated). The rats of both the above groups had to be sacrificed after 100 days of commencement of the experiment due to the fast deteriorating condition of group I(B).

**Effect of indigenous drugs, Garlic (Allium sativum) and Sage (Salvia officinalis) on DMBA induced cancer in rats:**

140 female Spraque-Dawley rats weighing around 40 ±5gm and 40 days ±5 days old were included in this series. These rats were
The rats (S-D) were divided into four different groups to evaluate the prophylactic and curative effects of indigenous drugs.

<table>
<thead>
<tr>
<th>I Control rats (20)</th>
<th>II DMBA infused (Cancerous) rats (20)</th>
<th>III Pre-drugs treatment (40)</th>
<th>IV Post-drugs treatment (40)</th>
</tr>
</thead>
<tbody>
<tr>
<td>The rats were kept at laboratory conditions and given 1 ml saline for 20 days, by gavage.</td>
<td>DMBA (30 mg/kg body weight) was given orally, dissolved in 1 ml sesame oil.</td>
<td>Rats were divided into two groups of 20 rats each. One group received garlic (1 ml, 25 gm/100 ml), while another received salvia (25 gm/100 ml) for 20 days prior to DMBA (30 mg/kg body weight, in 1 ml sesame oil) infusion.</td>
<td>40 rats were given DMBA orally in 1 ml sesame oil (30 mg/kg bodyweight). Then were divided into two groups of 20 rats each. One group received garlic (1 ml for 20 days of 25 g/100 ml) while another received salvia (1 ml for 20 days, 25 g/100 ml).</td>
</tr>
</tbody>
</table>

Food and water supplied to all these rats *ad Libitum* and were sacrificed on 130th day of experimentation.
divided into following groups. Fresh garlic was bought from the local market while salvia was procured from Jordan (Middle East). (Fig. 9).

**Group I (20 rats):** In this group, each rat received 1ml of 0.1% saline daily for 20 days. They were kept at laboratory conditions and were given food and water *ad libitum* till the termination of the experiment (served as normal controls).

**Group II:** This group included 60 rats. These rats were further divided into three subgroups of 20 rats each to assess the preventive role of salvia and garlic (indigenous drugs) on the initiation of carcinogenic effect of dimethylbenz(a)anthracene (DMBA).

**Sub-group II(A):** One ml of saline given orally once a day for twenty days prior to DMBA infusion and these served as controls (cancer control) for subgroups II(B) and II(C).

**Sub-group II(B):** One ml of garlic extract (25gms/100ml of 0.1% saline) was administered orally once a day for twenty days prior to cancer induction by DMBA infusion.

**Sub-group II (C):** One ml of salvia extract (25gm/100ml in 0.1% saline) was given orally for 20 days prior to cancer induction. Thereafter, the treatment was stopped 24 hours prior to the DMBA oral infusion (30mg/kg body weight in 1ml sesame oil).
Group III: The remaining 60 rats were included in this group and were again divided into three different subgroups of 20 rats each, to determine the preventive effect of salvia and garlic on the promotion of DMBA induced cancer.

Sub-group III (A): One ml of 0.1% saline was given orally for 20 days after DMBA infusion and these served as cancerous control for subgroups III(B) and III(C).

Sub-group III(B): Garlic (same dose as given to sub-group II(B) animals) was administered orally once a day for 20 days after 24 hours of an oral infusion of DMBA (30mg/kg body weight).

Group III (C): Salvia solution (in the same dose as given to sub-group II(C) animals) was given orally once a day for 20 days after 24 hours of a single dose oral infusion of DMBA.

All the six groups of animals were sacrificed after 130 days of commencement of experiment. Blood and tissue samples were collected from each rat. Blood samples were used for the assay of AChE, MAO, cortisol, SGOT, SGPT, LDH and osmotic fragility. The tissues from brain, heart, liver, kidney and spleen were subjected for the estimation of AChE, GST and SOD. The levels of total, free and protein bound GSH were also estimated in the above tissues. The biochemical procedures are discussed later.

Throughout the study students 't' test was used for statistical analysis.
The estimations of Biochemical Parameters:

Acetylcholinesterase (AChE):

(Ellman et al., 1961)

Principle:

The enzyme catalyses the hydrolysis of substrate (acetylthiocholine iodide) into thiocholine and acetate. The thiocholine thus formed reacts with dithiobisnitrobenzoate to give yellow colour. The absorbance of this yellow colour is measured in the spectrophotometer at 412 nm.

\[
\text{Acetylthiocholine iodide} \xrightarrow{\text{AChE}} \text{Thiocholine} + \text{Acetate}
\]

\[
\text{Thiocholine} + \text{dithiobisnitrobenzoic acid} \rightarrow \text{yellow colour}
\]

(absorbed at 412nm).

Reaction:

\[
(\text{CH}_3)_3NCH_2CH_2SCOCH_3 \xrightarrow{\text{AChE}} (\text{CH}_3)_3N^+CH_2-CH_2-S^- + \text{CH}_3\text{COO}^- + 2\text{H}^+
\]

(acetate)

\[
(\text{CH}_3)_3N^+\text{CH}_2\text{CH}_2\text{S} + \text{R-S-S-R} \rightarrow (\text{CH}_3)_3\text{NCH}_2\text{SSR} + \text{RS}
\]

(DTNB) (yellow colour)

Reagent:

i. 0.1M Phosphate buffer pH 8.0 and 0.1M, phosphate buffer pH 7.0.

ii. Substrate; Acetylthiocholine iodide 0.075M (21.67mg/ml) stable for 10-15 days if kept refrigerated.

iii. Sodium bicarbonate AR.

iv. Reagent: Dithiobisnitrobenzoic acid (DTNB). 0.01M of the 5,5' dithiobis-2-nitrobenzoic acid 39.6 mg were dissolved in 10ml of pH 7.0 phosphate buffer (0.1M) and 15 mg of sodium
bicarbonate were added. The reagent was made in buffer pH 7.0 in which it was more stable than in that of pH 8.0.

v. Working blood solution: 10 μl (0.01ml) of washed erythrocytes were suspended in 12.0 ml of 0.1M phosphate buffer of pH 8.0 (dilution, 1:1200). Since the AChE is bound on the cell membrane of RBC, hemolysis was not necessary.

**Method for RBC:**

Exactly 3.0ml of the suspension of washed RBC (diluted 1:1200) were pipetted into a cuvette, 25 μl of DTNB reagent was added. The cuvette was then placed into the spectrophotometer and its slit was adjusted so that the absorbance (at 412nm) of the suspension in the cuvette was zero. 20μl of the substrate was added to this cuvette. Changes in the absorbance at 412nm were recorded for at least 6 minutes period. Protein was estimated in the rest 3ml suspension of RBC by the method of Lowry *et al.* (1951).

**Calculations:**

\[
\text{Moles substrates hydrolyzed/min/mg protein} = 0.0882 \times \frac{600}{13.600} \times \frac{A}{\text{Protein concentration(mg)}} = (8.82)10^{-5} \times \frac{A}{\text{Protein concentration(mg)}}
\]

where \(8.82\times10^{-5}\) = factor for dilution and extinction coefficient.

**Method for Tissues:**

The tissues were homogenized (20mg/ml) in phosphate buffer (pH 8.0, 0.1M). The muscular tissues were minced considerably before homogenizing. 0.4ml aliquot of this homogenate was added
to a cuvette containing 2.6ml of phosphate buffer (pH 8.0, 0.1M). To this, 100μl of DTNB reagent was added. The absorption was set to zero at 412 nm and 20 μl of the substrate was added. Changes in absorbance were recorded and the change in absorbance per minute was calculated. The rates were calculated as follows:

\[
R = \frac{A}{1.36 \times 10^4} \times \frac{1}{(400/3120)C_o}
\]

\[
= 5.74 \times 10^{-5} \frac{A}{C_o}
\]

where \(R\) = rate in moles substrates hydrolysed per minute per gm. of tissues.

\[A\] = Change in absorbance per min.

\[C_o\] = Original concentration of tissues (mg/ml).

**Plasma Monoamine Oxidase (MAO):**

**Principle:** The measurement of MAO activity depends on the conversion of amine to aldehyde. The substrate benzylamine is converted into benzaldehyde which is extracted in the organic layer (cyclohexane) and is measured spectrophotometrically at 242nm.

\[
\text{MAO} \quad \text{RCH}_2\text{NH}_2 \quad \text{MAO} \quad \text{RCH}_2\text{NH}_2 \\
\text{RCHO + NH}_3
\]

**Reagents:**

i. Phosphate buffer 0.2M, pH 7.2
ii. Benzylamine 8mM in 0.2M phosphate buffer pH 7.2
iii. Perchloric acid 60%
iv. Cyclohexane AR

**Method:** To 1.0ml of plasma, 1.25ml of phosphate buffer and 0.25ml of substrate (benzylamine) were added. This was incubated in
Dubnoff's metabolic shaker for 3 hours. The reaction was stopped by the addition of 0.25ml of 60% PCA. A control tube was also run along with it, in which substrate was added after stopping the reaction. The mixture of both the control and the experimental tubes were properly shaken and 2.5 ml of cyclohexane was added (for aldehyde extraction). It was stirred and allowed to stand for 15 minutes at room temperature. The emulsion was centrifuged and the cyclohexane layer was separated (This step was repeated with 2.5ml of cyclohexane). The absorbance of the cyclohexane extracts were measured at 242nm in silica cells against plain cyclohexane.

**Contents of the Tubes:**

<table>
<thead>
<tr>
<th>Control Tube</th>
<th>Experimental Tube</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td>plasma</td>
</tr>
<tr>
<td>Buffer</td>
<td>Buffer</td>
</tr>
<tr>
<td>PCA</td>
<td>Benzylamine</td>
</tr>
<tr>
<td>Benzylamine</td>
<td>PCA</td>
</tr>
<tr>
<td>1.0ml</td>
<td>1.0ml</td>
</tr>
<tr>
<td>1.25ml</td>
<td>1.25ml</td>
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<tr>
<td>0.25ml</td>
<td>0.25ml</td>
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<tr>
<td>0.25ml</td>
<td>0.25ml</td>
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</tbody>
</table>

**Calculations:**

The difference of 0.01 in optical density in between control and experimental samples using 1ml plasma is equivalent to one provisional unit (P.U.). The results were expressed in terms of P.U. (provisional unit)/ 1ml of plasma.

**Plasma Cortisol:**
(Mattingly, 1962)

**Principle:**

A very high fluorescence is given by cortisol at either a very
high or a very low pH. Thus, cortisol is extracted and the fluorescence is measured after dissolving it in a strongly acidic medium.

**Reagents:**

i. Cortisol standard 1 ug/ml.
ii. Dichloromethane AR
iii. Fluorescence reagent: volumes of H₂SO₄ is mixed in cold with 3 volumes of ethanol.

**Method:**

Two ml each of plasma, water and standard cortisol solution (1 μg/ml) were pipetted in glass stoppered extraction tubes which served as unknown tube, blank and standard tubes respectively. 15ml of dichloromethane was added in each tube, and the tubes were rotated gently from one end to another for 20 minutes. The phases then were allowed to separate and the supernatent aqueous layer was discarded. The organic layer was transferred to a tube containing 5 ml of the "fluorescence reagent" and the contents were shaken for 20 minutes. After 20 minutes the fluorescence of the aqueous layer was measured at excitation = 470 and emission =530nm.

The concentration of cortisol was expressed in terms of μg/100ml.

**Glutathione:**

(Ellman, 1959, modified by Sedlack and Lindsay, 1968).

**Principle:**

The determination of total, free and protein bound sulfhydryl groups were based on the reduction of 5-5', dithiobis-2-nitrobenzoic acid (DTNB) by -SH group of glutathione (GSH) in alkaline medium
to reduced one mole of 2-nitro 5-mercaptobenzoic acid per mole of -SH group. The reaction was measured at 412nm.

**Reagents:**

i. Standard: Glutathione (reduced) $2 \times 10^{-3}$M.

ii. 0.02M EDTA.

iii. 0.15 M KCL.

iv. 0.2M Tris-EDTA buffer, pH 8.2

v. 0.4M Tris-EDTA buffer, pH 8.9.

vi. 0.01M DTNB (dissolved in absolute alcohol).

vii. 10% TCA.

**Procedure:**

**Total reduced glutathione (GSH-total):** Various tissues (heart, brain, liver, kidney and spleen) of the rats were homogenized in chilled 0.15M KCL and the volume of the homogenate was adjusted to 10% (w/v). To 0.1ml of homogenate, 1.5ml of 0.2M Tris-EDTA buffer (pH 8.2) and 0.1ml DTNB were added. The solution was mixed properly and the volume was made upto 10 ml with 8.3 ml absolute alcohol. The solution was centrifuged at 6000g for 5 minutes in cold. The precipitate was discarded and the absorbance of the clear supernatant was read at 412nm. A calibration curve with different concentrations of GSH (200-1600 μmoles) was obtained according to the same procedure as described above. The standard curve was used for the calculation of total -SH group present in the samples and the results were expressed as μmole of -SH group/gm tissues.
Non-Protein bound glutathione (GSH-free) :

1.0 ml of tissues homogenate (10%) prepared in 0.1 M KCl was deproteinized by adding 1.0 ml of 10% TCA. The tubes were kept at 4°C for 20 minutes and then centrifuged at 6000g for 5 minutes. 0.5 ml of the clear supernatant was mixed with 0.5 ml water, 2 ml of 0.4 M Tris-EDTA buffer pH 8.9 and 0.1 ml of 0.01 M DTNB (prepared in absolute methanol) with proper stirring. The absorbance was read at 412 nm within 30 minutes of the addition of DTNB. For the calibration curve, different concentrations of GSH (200-1600 μmoles) were also run by the same procedure as described above.

Protein bound reduced glutathione (GSH-protein bound) :

Protein bound sulfhydryl groups were determined by subtracting free GSH from total GSH, as described by Sedlak and Lindsay (1968).

Protein bound GSH group = Total GSH group−Free GSH group.

Glutathione-S-Transferase (GST) :
(Habig et al., 1974).

Principle:

The enzyme activity is measured by following the increase in absorbance at 340 nm of CDNB-GSH conjugate generated as a result of GST catalysis between glutathione and 1-Chloro-2, 4-dinitrobenzene (CDNB).

CDNB +GSH $\xrightarrow{GST}$ CDNB-GSH Conjugate.
**Reagents:**

i. 1.0mM Glutathione (Reduced).

ii. 0.2M phosphate buffer pH 6.5

iii. 1.0mM CDNB (prepared in acetone).

**Procedure:**

Different tissues (brain, heart, liver, kidney and spleen) of rats were homogenized in chilled 0.2M phosphate buffer pH 6.5 (10% w/v) and centrifuged in cold for 15 minutes at 1500g. To 0.1ml of tissue supernatant, 2.7 ml 1mM glutathione solution (in 0.2M phosphate buffer pH 6.5) and 0.2 ml of 1.0mM CDNB were mixed. The change in absorbance at 340 nm was recorded at room temperature against blank (containing all the reagents except the enzymes). Protein content in enzyme source was also determined by the method of Lowry et al. (1951).

**Calculation:**

The values were calculated on the basis of molar extinction coefficient of CDNB (9.6x10^3 M^-1 Cm^-1) and specific activity of enzyme was expressed in μmoles of GSH-CDNB conjugate formed per minute per mg protein.

\[
\text{GST activity} = \frac{\text{O.D.} \times 625 \text{ (Factor)}}{\text{protein concentration (mg)}} = \text{Units/1mg protein}
\]

O.D. = Change of optical density per minute.

Where 625 is a factor for dilution and extinction coefficient.
Superoxide Dismutase (SOD):
(Markland & Markland, 1974)

Principle:
The SOD activity determination is based on the conversion of O₂ (the products of pyrogallol autoxidation) to H₂O₂, which is measured at 420 nm.

Reaction:

Autoxidation

\[
\text{Pyrogallol} + \text{O}_2 \xrightarrow{\text{SOD}} \text{Oxidation product} + \text{O}_2
\]

\[
2\text{O}_2 + 2\text{H}^+ \xrightarrow{\text{SOD}} \text{O}_2 + \text{H}_2\text{O}_2
\]

Reagents:

i. 0.05M Tris succinate buffer (pH 8.2).
ii. 0.05M Succinic acid solution.
iii. 8mM Pyrogallol solution (freshly prepared).

Procedures:

2.85 ml of 0.05M Tris-succinate buffer pH 8.2 and 0.05 ml of tissues supernatant, mixed and incubated at 25°C for 20 minutes. The reaction was started by adding 0.1 ml pyrogallol solution. Changes in absorbance per minute were immediately recorded for an initial period of 3 minutes at 420 nm. A reference set, consisting of 0.05 ml distilled water instead of the homogenate sample was also run similarly.
Calculation:

\[
\text{SOD Activity} = \frac{(\Delta A / \text{min. ref.} - \Delta A / \text{min. Sample}) \times 30}{\Delta A / \text{min. ref.}/2 \times 0.05 \times 10}
\]

where \(\Delta A / \text{min. ref.}\) = change of absorbance per minute in reference set.

and \(\Delta A / \text{min. sample}\) = Change of absorbance per minute in sample set.

Activity Unit: One unit of enzyme is defined as the amount of enzyme which causes a 50% inhibition of pyrogallol autoxidation under assay conditions.

Transaminases:
(Reitman and Frankel, 1957):

Glutamate oxalacetate (GOT): (EC.2.6.1.1.) and glutamate pyruvate transaminase (GPT): (EC.2.6.1.2.)

Principle:

The transamination involves the transfer of \(\alpha\)-amino group from \(\alpha\)-amino acid to \(\alpha\)-oxoacids, usually 2-oxoglutarate. The major 2-oxoglutarate linked transaminases are aspartate transaminases (GOT) and alanine transaminases (GPT).

The reactions:

\[(A) \text{ For GOT:}\]

\[
\begin{align*}
\text{H-C-NH}_2 + \text{COO}^- & \rightarrow \text{C}=\text{O} \quad \text{SGOT} \\
\text{CH}_2 & \quad \text{CH}_2 \\
\text{COO}^- & \quad \text{COO}^- \\
\text{L-Aspartate} & \Rightarrow \text{\(\alpha\)-Oxoglutarate} \\
\end{align*}
\]

Oxaloacetate L-Glutamate
(B) *For GPT* :

\[
\begin{align*}
\text{L-Alanine} & \quad \text{CH} \quad \text{COO}^- \\
\text{C}=\text{O} \quad & \quad \text{C}=\text{O} \\
\text{H-C-NH}_2 + \quad & \quad +\text{H-C-NH}_2 \\
\text{CH}_3 \quad & \quad \text{CH}_3 \\
\text{CH}_2 \quad & \quad \text{CH}_2 \\
\text{COO}^- \quad & \quad \text{COO}^- \\
\text{SGPT} \quad & \quad \text{SGPT} \\
\end{align*}
\]

The pyruvate formed by GOT transamination, reacts with 2,4-Dinitrophenyl hydrazine (DNPH) to give a brown coloured hydrazone, which is measured colorimetrically at 510 nm.

The oxaloacetate is decarboxylated spontaneously to pyruvate by GPT, which is again measured by hydrazone formation.

**Reagents:**

i. Phosphate buffer 0.1M pH 7.4.

ii. 200 mM L-aspartic acid.

iii. 200 mM L-alanine.

iv. 2 mM α-ketoglutarate.

v. Stock pyruvate standard: (20 mM) in 0.1M phosphate buffer pH 7.4.

vi. Working pyruvate standard: (4 mM) freshly prepared from the above stock.

vii. 1 mM 2,4-dinitrophenyl hydrazine.

viii. 0.4 N sodium hydroxide.

**Methods:**

**Glutamate Oxaloacetate Transaminases : (GOT)**

**Test (1):** 0.5ml of substrate (aspartic acid; α-Ketoglutarate) were warmed in a water bath at 37°C for 3 minutes prior to the addition
of 0.1ml of serum. Mixed gently and incubated again for 60 minutes exactly. The tubes were removed from the water bath and 0.5ml of DNPH solution were added and Mixed well.

Three other test tubes (control, standard and blank) were prepared as follows:

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Tube I (C)</th>
<th>Tube II (S)</th>
<th>Tube III (B)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(aspartic acid; α-Ketoglutarate)</td>
<td>0.5ml</td>
<td>0.4ml</td>
<td>0.5ml</td>
</tr>
<tr>
<td>Working pyruvate</td>
<td>--</td>
<td>0.1ml</td>
<td>--</td>
</tr>
<tr>
<td>Water</td>
<td>--</td>
<td>0.1ml</td>
<td>0.1ml</td>
</tr>
<tr>
<td>DNPH</td>
<td>0.5ml</td>
<td>0.5ml</td>
<td>0.5ml</td>
</tr>
<tr>
<td>Serum</td>
<td>0.1ml</td>
<td>--</td>
<td>--</td>
</tr>
</tbody>
</table>

In all the above tubes, the DNPH was allowed to react for 20 minutes at room temperature, then 5ml of 0.4 N sodium hydroxyde were added, mixed well and the reactions were allowed to proceed for another 10 minutes. The optical density of all the tubes were measured at 510nm.

The pyruvate formed per minute per litre of serum

\[ \frac{\text{T-C}}{\text{S-B}} \times 0.4 \times \frac{1}{60} \times \frac{1000}{0.1} \]

\[ = \frac{T-C}{S-B} \times 67 \text{ μmoles.} \]

The calculated pyruvate was converted into international units per litre.
2. **Glutamate Pyruvate Transaminases (GPT)**

The procedure is same as in GOT. While L-alanine was used as substrate instead of L-aspartate and the incubation time in (T) test was reduced to 30 minutes (instead of that of 1 hour for GOT).

The pyruvate formed per minute per litre of serum

\[
\frac{T-C}{S-B} \times 0.4 \times \frac{1}{30} \times \frac{1000}{0.1}
\]

= \( \frac{T-C}{S-B} \times 133 \) µmoles.

The calculated pyruvate was converted into international units per litre.

**Lactate dehydrogenase (LDH):**
(Wroblewski and La Due, 1955).

**Principle:**

Pyruvate is reduced to lactate at pH 7.4, 37°C in the presence of LDH. The progress of the accompanying oxidation of NADH to \( NAD^+ \) is monitored continuously, by measuring the rate of decrease of absorbance at 340 nm spectrophotometrically.

**The reaction:**

\[
\begin{align*}
\text{CH}_3 & \quad \text{CH}_3 \\
H-C-OH & + NAD^+ \quad \text{LDH} \\
C=O & \quad C=O \\
C-O & \quad O_\cdot \\
\text{L-Lactate} & \quad \text{Pyruvate}
\end{align*}
\]

**Reagents:**

i. 0.1M phosphate buffer pH 7.4.

ii. NADH (2.5mg/ml 0.1M phosphate buffer pH 7.4).

iii. Sodium pyruvate (2.5mg/ml) in 0.1M phosphate buffer pH 7.4.
Procedure:

To 2.4 ml of 0.1M phosphate buffer (pH 7.4), 0.1ml of non-hemolyzed serum and 0.1ml NADH were added. The reaction was allowed to proceed at room temperature for 20 minutes. then 0.1ml of sodium pyruvate was added, and the change in optical density at every 30 second interval was recorded at 340 nm for 5 minutes.

The optical density was measured against blank (consisting of all reagents except sodium pyruvate) giving 100% transmittance.

Calculation:

Decrease in O.D. of test solution in 5 minutes x 1000 = Units LDH activity per ml serum.

Osmotic fragility of erythrocytes:

(Orcutt et al., 1995)

The erythrocytes of blood collected after centrifugation were washed five times with normal saline (0.9%) (The leukocytes and the buffy coat were removed after centrifugation). The washed RBCs were diluted in 0.1M phosphate buffer pH 8.0 (1:1200) and were suspended in different concentrations (0.1% to 0.9%) of saline. This leads to the release of hemoglobin of erythrocytes. The extent of fragility (hemolysis) of RBC membranes in the presence of various concentrations of saline was measured in terms of quantity of hemoglobin released. The release of hemoglobin was monitored colorimetrically at 545 nm (Orcutt et al., 1995).
**Procedure:**

To 0.2 ml of washed and diluted (1:1200) suspension of RBC, 2.3 ml of 0.1M phosphate buffer pH 8.0 containing different concentrations of saline (0.1% to 0.9%) was added (in different test tubes) and incubated at room temperature for 30 minutes, then was monitored at 545 against 0.1M phosphate buffer pH 8.0 (as blank).

The pH 8.0 of phosphate buffer was selected because at this pH, the hemoglobin of rat showed maximum solubility (Luque et al., 1992).