APPENDIX 1

QUANTIFICATION OF AFLATOXIN B1 BY THIN LAYER CHROMATOGRAPHY
(Romer, 1975)

REAGENTS

- 0.2 M NaOH
- 0.41 M Ferric chloride
- 0.03% H$_2$SO$_4$
- Potassium wash solution: Dissolved 1.12 g KOH and 10 g KCl in water and made up to 1 litre.
- Aqueous acetone - acetone : water (85:15)
- Developing solvent – Chloroform : Acetone : Water (88:12:1)

PROCEDURE

To 25.0 g sample in a conical flask, 100 ml of distilled water was added and blended for 2 minutes. To this 150.0 ml acetone was added and again blended for 2 minutes. The contents were filtered through Whatman no.1 filter paper. 75 ml of the filtrate was transferred to a conical flask containing 3 g cupric carbonate. Added 100 ml of ferric gel prepared by adding 85 ml of 0.2 M NaOH to 15 ml of 0.41 M FeCl$_3$ to the flask containing extract and cupric carbonate. The contents were mixed slowly and filtered through Whatman No.1 filter paper. 100 ml of this filtrate was taken in a 250 ml separating funnel and 100 ml of 0.03% H$_2$SO$_4$ and 10 ml of chloroform were added, mixed slowly and the chloroform layer was collected into a 100 ml beaker. Another 10 ml of chloroform was added to the separating funnel and the above step was repeated and both the chloroform extracts were combined. In a second separating funnel,
100 ml of potassium wash solution was taken and the chloroform extract was added and mixed slowly. The chloroform layer was collected through anhydrous sodium sulfate bed drop by drop to remove moisture. The chloroform extract was dried in an oven at 50°C. The dried residue was dissolved in 0.2 ml chloroform and spotted on TLC plate along with the standard. The fluorescence intensities of the sample and the standard spots were compared and the ones matching with each other were identified. The aflatoxin B1 content was calculated in the following way

\[
\text{Aflatoxin B1 content (ppb)} = \frac{S \times C \times D}{T \times W} \times 1000
\]

where, 
- \( S \) = Standard which compares with the sample in fluorescent intensity
- \( C \) = Concentration of standard (1µg/ml)
- \( D \) = Dilution factor in ml
- \( T \) = sample which compares with standard in fluorescent intensity
- \( W \) = Effective weight (g) = \( \frac{25 \times 75 \times 100}{250 \times 175} = 4.286 \)

APPENDIX 2

INGREDIENTS OF THE BASAL DIET FED TO THE RATS
(Raghuramalu et al., 2003)

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Amount (g/100 g)</th>
</tr>
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<tbody>
<tr>
<td>Wheat flour</td>
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</tr>
<tr>
<td>Green gram flour</td>
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<td>Yeast</td>
<td>1 g</td>
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<tr>
<td>Greens</td>
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<tr>
<td>Groundnut oil</td>
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<tr>
<td>Cord liver oil</td>
<td>1 drop</td>
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<tr>
<td>Whole milk</td>
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APPENDIX 3

DETERMINATION OF TOTAL RED BLOOD CELL (RBC) COUNT
(Chaudhari, 2000a)

PRINCIPLE

The method involves an accurate dilution of a measured quantity of blood with a fluid which is isotonic with the blood and which will prevent its coagulation. The diluted blood is placed in a counting chamber and the number of cells in a circumscribed volume is enumerated under a microscope.

PROCEDURE

Added 0.02 ml blood to 3.98 ml of diluting fluid. Charged the Neubauer chamber with well-mixed dilute block. Counted the total number of red cells in the small square in the central ruled area of Neubauer counting chamber using 40X objective of the microscope.

APPENDIX 4

DETERMINATION OF TOTAL WHITE BLOOD CELL (WBC) COUNT
(Chaudhari, 2000b)

PRINCIPLE

The method involves an accurate dilution of a measured quantity of blood with a fluid which destroys the red blood corpuscles. The diluted blood is placed in a counting chamber and the number of cells in a circumscribed volume is enumerated under a microscope.

PROCEDURE

Added 0.02 ml blood to 3.38 ml of diluting fluid, charged the Neubauer counting chamber with well-mixed dilute blood. Counted the total number of white blood cells in the four large corner squares of chamber after 3 - 4 minutes.
APPENDIX 5

DETERMINATION OF HEMOGLOBIN (Hb) IN BLOOD
(Drabkin and Austin, 1932)

PRINCIPLE

When blood is diluted with an alkaline solution of potassium cyanide and potassium ferricyanide, hemoglobin is oxidized to methemoglobin, which then combines with cyanide to form cyanmethemoglobin that is measured colorimetrically at 540 nm.

REAGENTS

Drabkin's reagent- This reagent contains 0.05 g of potassium cyanide, 0.2 g of potassium ferricyanide and 1 g of sodium bicarbonate in 1 litre of distilled water (pH 9.6). Cyanmethemoglobin standard- The solution was obtained commercially and had a concentration of 16 g/dl.

PROCEDURE

The reaction mixture in a volume of 5.02 ml contained, 5.0 ml of Drabkin's reagent and 0.02 ml of blood. The reaction mixture was kept at room temperature for 5 minutes to ensure the completion of the reaction and read at 540 nm against a reagent blank.

The hemoglobin content was expressed as g/dl.

APPENDIX 6

ESTIMATION OF PROTEIN
(Lowry et al., 1951)

PRINCIPLE

The CO-NH group (peptide bond) present in the protein molecule reacts with copper sulphate in alkaline medium to give a blue colour, which was read at 620 nm.
REAGENTS

- Copper sulphate, 0.5%
- NaOH, 0.1 N
- Sodium carbonate 2% in 0.1N NaOH
- Sodium potassium tartarate, 1%
- Alkaline copper reagent: Prepared by mixing 0.5ml copper sulphate, 0.5 ml sodium potassium tartarate and 50ml sodium carbonate.
- Folin phenol reagent: Diluted 1:1 with distilled water.
- Standard protein solution: 100mg% bovine serum albumin in 0.1N NaOH
- Working standard: Diluted stock standard 1 in 10 using 0.1N NaOH.

PROCEDURE

To 0.2 ml serum or liver / kidney tissue extract, 1.8 ml of 0.1N NaOH and 5.0 ml alkaline copper reagent were added and kept for 15 minutes. Then 0.5 ml alkaline copper reagent were added and kept for 15 minutes. To this, 0.5 ml diluted Folin phenol reagent was added, mixed and kept for 30 minutes and read at 675 nm. To the blank 0.2 ml water and to the standard 0.2 ml working standard were added instead of serum or the tissue extracts and treated as above. Read at 520nm.

The amount of protein was expressed as g/dl serum.

APPENDIX 7

ESTIMATION OF BLOOD GLUCOSE
(Raghuramulu et al., 1983)

PRINCIPLE

Glucose reacts with ortho-toluidine in glacial acetic acid to form a green chromogen which is measured at 620 nm.
REAGENTS
- Ortho-toluidine, 30 ml
- Thiourea, 750 mg
- Made up to 500 ml with glacial acetic acid. Kept at room temperature for 24h and then used.
- Standard, 100 mg of glucose in 100 ml of water.

PROCEDURE

To 0.2 ml of blood added 1.8 ml of distilled water. Mixed well; from this took 0.5 ml and then added 5.0 ml of ortho-toluidine. Kept it in a boiling water bath for 10 minutes, cooled and took the readings at 620 nm. For standard, took 0.2 ml of the 100 mg % of glucose and treated as above.

The glucose levels were expressed as mg/dl.

APPENDIX 8

ESTIMATION OF SERUM CHOLESTEROL
(Zlatkis et al., 1953)

PRINCIPLE

Sample is treated with ferric-chloride acetic acid reagent to precipitate the protein. The protein free filtrate containing cholesterol ferric chloride is treated with concentrated H_2SO_4. The reaction involves the 3-OH-ene part of the cholesterol molecule. It is first dehydrated to form cholestra 3 - 5 diene and then oxidized by H_2SO_4 to link two molecules together as bis-cholestra 3 - 5 diene and this material is sulphonated by H_2SO_4. The colour developed is read at 560 nm.

REAGENTS
- Ferric chloride, 0.05% solution of FeCl_3.6H_2O in acetic acid.
- H_2SO_4
Stock cholesterol standard: 100 mg in 100.0 ml acetic acid.

Working standard: The stock standard was diluted 1 to 25 with ferric chloride-acetic acid reagent.

Acetone-ethanol reagent (1:1)

PROCEDURE

0.1 ml of serum was added to 10 ml of the ferric chloride acetic acid reagent in a stoppered centrifuge tube. Mixed and allowed to stand for 10-15 minutes for the proteins to flocculate. Transferred 5.0 ml of the clear supernatant fluid to stoppered centrifuge tube after centrifugation. For standard, 0.1 ml of physiological saline was mixed with 10 ml of the cholesterol standard and 5.0 ml was transferred to a second stoppered centrifuge tube. As blank 5.0 ml of the ferric chloride-acetic acid reagent was taken. 3.0 ml of sulphuric acid was added to all three tubes, stoppered and mixed. Allowed to stand for 20 to 30 minutes. The serum and standards were read against the blank at 560 nm in a spectrophotometer.

The amount of cholesterol was expressed as mg/dl serum.

APPENDIX 9

ESTIMATION OF SERUM URIC ACID

(Caraway, 1963)

PRINCIPLE

Serum is deproteinised by tungstic acid. Uric acid in the protein filtrate when treated with phosphotungstic acid and sodium carbonate gives a blue colour.

REAGENTS

- Sodium tungstate, 10%
- Sulphuric acid, 2/3N
Phosphotungstic acid : 50 gm of sodium tungstate was dissolved in 400 ml water. To this 40 ml 85% phosphoric acid was added, refluxed gently for two hours, cooled and made upto 500ml.

Sodium carbonate, 10%

Stock uric acid standard : 60 mg lithium carbonate was dissolved in 15 - 20 ml of water, heated to about 60°C and poured on to 100mg uric acid, stirred and dissolved. To this mixture, 2.0 ml of formalin and 1.0 ml of 50%v/v acetic acid was added and the volume was made upto 100 ml.

Working standard : 1.0 ml of stock standard was diluted to 100 ml.

PROCEDURE

To 0.5 ml of serum, 2.0 ml of water, 1.0 ml of sodium tungstate and 1.0 ml of 2/3 N sulphuric acid were added, mixed and centrifuged. To 2.5 ml of the supernatant, 1.0 ml of phosphotungstic acid and 1.0 ml of 10% sodium carbonate were added and kept at room temperature for 30 minutes and the colour developed was read at 700 nm. A series of standards were treated in a similar manner along with a blank.

The amount of uric acid was expressed as mg/dl serum.

APPENDIX 10

ESTIMATION OF THE ACTIVITY OF SERUM ASPARTATE TRANSAMINASES (AST)
(Mohum and Cook, 1957)

PRINCIPLE

The determination of AST activity is based on the transamination of aspartic acid to ketoglutaric acid.

REAGENTS

- Phosphate buffer (100 mM) with α-oxoglutaric acid (2 mM)
- Aspartate transaminase (Substrate) : 100 mM L-aspartic acid was added to the phosphate buffer (100 mM)
PROCEDURE

1 ml of substrate was pipetted into two tubes and placed in a water bath at 37°C for few minutes. To one tube, the test, 0.2 ml of serum was added and shaken gently. Exactly 1 hr later with the test tubes still in the bath, 1.0 ml dinitro phenyl hydrazine was added to both, and 0.2 ml serum to the other (control). Allowed to stand for 20 minutes at room temperature. 10 ml 0.4N NaOH was added to all the tubes, mixed well and the colour develop was read at 520 nm after 5 minutes in a colorimeter. For standard, 1.0 ml working standard was taken and made up to 1.2 ml with water and proceeded as above. For blank, 1.2 ml water was taken and proceeded as above.

The enzyme activity was expressed as µmoles of oxaloacetate liberated/min/L (U/L).

APPENDIX 11

ESTIMATION OF THE ACTIVITY OF SERUM ALANINE TRANSAMINASE (ALT)
(Mohum and Cook, 1957)

PRINCIPLE

The determination of ALT activity is based on the transamination of alanine to pyruvic acid.

REAGENTS

- Phosphate buffer (100 mM) with α-oxoglutaric acid (2 mM)
- Alanine transaminase (Substrate) : 200 mM DL-alanine was added to the phosphate buffer
- 2.4 Dinitro phenyl hydrazine (1 mM) in 1N HCl
Sodium hydroxide, 400 mM
Pyruvate standard (2 mM) working standard-diluted 1 in 20.

PROCEDURE

1.0 ml of substrate was pipetted into two tubes and placed in a water bath at 37°C for few minutes to reach its temperature. To one, the test, 0.2 ml of serum was added and shaken gently to mix. Exactly after 30 minutes, with the test tubes still in the bath, 1.0 ml dinitro phenyl hydrazine was added to both, and 0.2 ml serum to the other (control). Allowed to stand for 20 minutes at room temperature. 10 ml 0.4 N NaOH was added to both the tubes, mixed well and was read at 520 nm after 5 minutes in a colorimeter. For standard, 1.0 ml working standard was taken and made up to 1.2 ml with water and proceeded as above. For standard blank, 1.2 ml water was taken and proceeded as above. The enzyme activity was expressed as μmoles of pyruvate liberated/min/L (U/L).

APPENDIX 12

ESTIMATION OF THE ACTIVITY OF SERUM ALKALINE PHOSPHATASE (ALP)
(Varley, 1988a)

REAGENTS

Disodium phenyl phosphate, 0.01 M
Sodium carbonate-sodium bicarbonate buffer, 0.1 M
Buffered substrate for use prepared by mixing equal volumes of the above two solutions (pH 10)
Sodium hydroxide, 0.5 N
Sodium bicarbonate, 0.5 M
Stock standard phenol solution: 100 mg of phenol per 100.0 ml of solution.
Working standard: Stock standard was diluted 1 in 10.
4-amino-antipyrine, 0.6% in water
Potassium ferricyanide, 2.4 g per 100 ml in water.
PROCEDURE

2.0 ml of buffered substrate was measured into each of two test tubes and placed in a water bath at 37°C for few minutes. Then to one (the test) 0.1 ml of serum was added and incubated for exactly 15 minutes. Removed from the bath and added 0.8 ml of 0.5 N sodium hydroxide and 1.2 ml of serum to the second tube (the blank). To both tubes 1.0 ml of amino antipyrine reagent and 1.0 ml of potassium ferricyanide were added. For standard, 1.1 ml of buffer and 1.0 ml of phenol standard containing 1.01 mg of phenol and for the standard blank, 1.1 ml of buffer and 1 ml of water were taken. Then to both sodium hydroxide, bicarbonate, amino antipyrine and ferricyanide were added as above. Read at 520 nm.

The enzyme activity was expressed as U/L. One unit is the amount of enzyme that transforms one micromole of substrate per minute in standard conditions.

APPENDIX 13

ESTIMATION OF THE ACTIVITY OF GAMMA GLUTAMYL TRANSFERASE (GGT)
(Varley, 1988b)

PRINCIPLE

Gamma glutamyl transferase catalyses the transfer of the gamma glutamyl group from gamma glutamyl peptides to suitable acceptors. The p-nitroaniline released from the substrate by GGT was monitored by following the increase in absorbance at 405 nm.

REAGENTS

 buffering: Prepared by mixing tris (120 mM/l), magnesium chloride, (12 mM/l) and glycyl - glycine (90 mM/l) pH 7.8.

substrate: 1.28 g L-γ-glutamyl-4-nitroanilide in 0.15 M/l HCl and made to 100 ml with the acid.
PROCEDURE

100 µl liver extract and 1.0 ml buffer were warmed to 37°C. The reaction was started by adding 0.1 ml substrate. Mixed and monitored the reaction continuously at 405nm in 1cm cuvette so as to obtain the change in absorbance/minute.

The enzyme activity was expressed as U/L. One unit is the amount of enzyme that transforms one micromole of substrate per minute in standard conditions.

APPENDIX 14

ESTIMATION OF THIOBARBITURIC ACID REACTIVE SUBSTANCES [TBARS] IN PLASMA
(Yagi, 1987)

PRINCIPLE

Malondialdehyde (MDA) and other thiobarbituric acid reactive substances (TBARS) react with thiobarbituric acid (TBA) in acidic conditions. The reaction generates a red coloured complex, which can be read at 553 nm.

REAGENTS

- NaCl solution, 0.9%
- H₂SO₄, 12 N
- Phosphotungstic acid, 10%
- TBA in acetic acid, 0.67%
- n-Butanol

PROCEDURE

To 0.05 ml of plasma, 4.0 ml of H₂SO₄ and 0.5 ml of phosphotungstic acid were added and mixed. The mixture was kept at room temperature for five minutes and then centrifuged at 3000 x g for 10 minutes. The supernatant was discarded and to the sediment, 2.0 ml of H₂SO₄ and 0.3 ml of phosphotungstic acid were added and mixed. The mixture was again centrifuged at 3000 x g for
10 minutes. The sediment was resuspended in 4.0 ml of distilled water and 1.0 ml of TBA reagent. The reaction mixture was heated at 95°C for 60 minutes. The tubes were cooled and 5.0 ml of n-butanol was added, mixed well and centrifuged at 3000 x g for 15 minutes. The butanol layer was collected and the absorbance was measured at 553 nm.

Values were expressed as nmol MDA released/mL/15minutes.

APPENDIX 15

ESTIMATION OF THIOBARBITURIC ACID REACTIVE SUBSTANCES [TBARS] IN TISSUES
(Okhawa et al., 1979)

PRINCIPLE

Malondialdehyde and other TBARS are quantified by their reactivity with thiobarbituric acid (TBA) in acidic conditions. The reaction generates a pink coloured chromophore, which can be read in a colorimeter at 535 nm.

REAGENTS

- Sodium dodecyl sulfate, 8.1%
- Acetic acid, 20% adjusted to pH 3.5 with NaOH
- Thiobarbituric acid, 0.8%
- n-Butanol and pyridine mixture (15:1, v/v)
- Stock MDA solution: 1, 1, 3, 3-Tetramethoxypropane (184 µg/mL).

PROCEDURE

4.0 ml of reaction mixture containing 0.4 ml of the tissue homogenate, 1.5 ml of 0.8% TBA, 1.5 ml of acetic acid (20%, pH 3.5) and distilled water was kept for 1 hr in a boiling water bath at 95°C. After 1 hr the reaction mixture was moved from the water bath, cooled and added 1.0 ml of distilled water. To this, 5.0 ml of butanol : pyridine mixture (15:1) was added, mixed thoroughly
and centrifuged at 3000 rpm for 10 minutes. Absorbance of the clear supernatant was measured at 532 nm against Butanol: pyridine mixture. The MDA was calculated with the help of a standard graph made by using different concentration (1-10 nmol) of 1, 1, 3, 3-tetramethoxypropane in 1.0 ml distilled water.

The values were expressed as nmol/mg protein.

APPENDIX 16

ESTIMATION OF LIPID HYDROPEROXIDES IN TISSUES

(Jiang et al., 1992)

PRINCIPLE

This method is based on the rapid peroxide-mediated oxidation of ferrous ion (Fe²⁺) to ferric ion (Fe³⁺) under acidic conditions in presence of xylenol orange. The Fe³⁺ xylenol orange complex was measured spectrophotometrically at 560 nm.

REAGENTS

- Fox reagent: 100 μM xylenol orange, 4 mM butylated hydroxytoluene, 25 mM sulphuric acid and 250 μM ammonium ferrous sulfate were added to 90 ml methanol and 10 ml of H₂SO₄ (250 mM)

PROCEDURE

To 0.1 ml of tissue lipid sample extracted with methanol, 0.9 ml of Fox reagent was added, mixed well and incubated at room temperature for 30 minutes and the absorbance was read in a colorimeter at 560 nm. By using the molar extinction co-efficient of 4.6x10⁴ M⁻¹ cm⁻¹, the amount of hydroperoxides produced was calculated.

The levels of hydroperoxides were expressed as mmoles/mg tissue.
APPENDIX 17

ESTIMATION OF CONJUGATED DIENES IN TISSUES
(Klein, 1970)

PRINCIPLE

Conjugated dienes, formed during the rearrangement of double bonds in polyunsaturated fatty acids absorb light at 233 nm. The oxidation index, the ratio of the absorbance at 233 nm to absorbance at 233 nm was computed which reflect the extent of peroxidation in lipid samples.

REAGENTS

- Chloroform
- Methanol
- Cyclohexane

PROCEDURE

To 1.0 ml of tissue homogenate, 5.0 ml of chloroform–methanol reagent (2:1) was added, mixed thoroughly and centrifuged for 5 minutes. 3.0 ml of lower layer was evaporated to dryness. To this, 1.5 ml of cyclohexane was added and the absorbance was read at 233 nm against a cyclohexane blank. The values were the ratio of $A_{233}$ to $A_{215}$.

APPENDIX 18

ESTIMATION OF SUPEROXIDE DISMUTASE ACTIVITY IN TISSUES
(Kakkar et al., 1984)

PRINCIPLE

The assay is based on the inhibition of NADH-Phenazine methosulfate nitroblue tetrazolium formazan formation. The reaction is initiated by the addition of NADH. After 90 seconds incubation, the reaction was stopped by adding glacial acetic acid. The colour developed at the end of the reaction was extracted into n-Butanol layer and measured at 520 nm.
REAGENTS

- Sodium pyrophosphate buffer, 0.052 M, pH 8.3
- Phenazine methosulfate (PMS), 186 μM
- Nitroblue tetrazolium (NBT), 300 μM
- Reduced nicotinamide adenine dinucleotide (NADH), 780 μM

PROCEDURE

To 0.5 ml of tissue homogenate prepared in sodium pyrophosphate buffer, 1.0 ml of water, 2.5 ml ethanol and 1.5 ml of chloroform were added. All the reagents were cooled before adding to the tissue homogenate. The reaction mixture was shaken for 90 s at 4°C and then centrifuged. In a separate test tube, 1.2 ml sodium pyrophosphate buffer, 0.1 ml PMS, 0.3 ml NBT and appropriately diluted supernatant were taken. The reaction was started by the addition of 0.2 ml NADH. The mixture was incubated at 30°C for 90s and the reaction was stopped by the addition of 1.0 ml glacial acetic acid. The reaction mixture was stirred vigorously, shaken with 4.0 ml butanol and was allowed to stand for 10 minutes. After centrifugation the colour intensity of the chromogen in butanol layer was measured in a colorimeter at 520 nm. A control was performed in a similar manner but without the enzyme source.

The enzyme activity was expressed as U/mg protein. One unit is the enzyme required for 50% inhibition of NBT reduction/minute.

APPENDIX 19

ESTIMATION OF CATALASE ACTIVITY IN TISSUES
(Sinha, 1972)

PRINCIPLE

Dichromate in acetic acid was reduced to chromic acetate, when heated in presence of H₂O₂ with the formation of perchromic acid as and
unstable intermediate. The catalase preparation is allowed to split $H_2O_2$ for various periods of time. The reaction is stopped at different time intervals by the addition of dichromate- acetic acid mixture in hot conditions. The remaining $H_2O_2$ forms $H_2O_2$ chromic acetate which is determined colorimetrically at 590 nm.

**REAGENTS**

- Phosphate buffer, 0.01 M, pH 7.0
- Hydrogen peroxide ($H_2O_2$) 0.2 M
- Potassium dichromate, 5%
- Dichromate-acetic acid reagent: Potassium dichromate and glacial acetic acid were mixed in the ratio of 1:3. From this, 1.0 ml was diluted again with 4.0 ml acetic acid.
- Standard hydrogen peroxide : 0.2 mM

**PROCEDURE**

To 0.1 ml of the tissue homogenate, 0.9 ml of phosphate buffer and 0.4 ml of hydrogen peroxide were added. At 15, 30, 45 and 60 seconds, the reaction was arrested by the addition of 2.0 ml of dichromate acetic acid mixture. The tubes were kept in a boiling waterbath for 10 minutes and then cooled. The colour developed was read at 590 nm. The standards in the concentration range of 20-100$\mu$M were also performed as that of the test samples.

The specific activity of the enzyme was expressed as U/mg protein. One unit is the moles of $H_2O_2$ utilized / minute.
APPENDIX 20

ESTIMATION OF GLUTATHIONE PEROXIDASE ACTIVITY IN TISSUES
(Rotruck et al., 1973)

PRINCIPLE

A known amount of enzyme preparation is allowed to react with \( \text{H}_2\text{O}_2 \) and glutathione (GSH) for a specified time period. The GSH content remaining after the reaction is measured by Ellman’s reaction.

REAGENTS

- Tris-HCl buffer, 0.4 M, pH 7.0
- Sodium azide solution, 10 mM
- TCA, 10%
- EDTA, 0.4 mM
- \( \text{H}_2\text{O}_2 \), 1.0 mM
- Reduced glutathione (GSH), 2.0 mM

PROCEDURE

To 0.2 ml of Tris-HCl buffer, 0.2 ml EDTA, 0.1 ml sodium azide and 0.2 ml tissue homogenate were added and mixed thoroughly. To this mixture, 0.2 ml GSH and 0.1 ml \( \text{H}_2\text{O}_2 \) were added. The mixture was incubated at 37°C for 10 minutes after which the reaction was arrested by the addition of 0.5 ml of TCA. The tubes were centrifuged and the remaining GSH content was quantified in a colorimeter at 340 nm.

The specific activity of the enzyme was expressed as U/mg protein. One unit is the \( \mu \)g of GSH utilized / minute.
APPENDIX 21

ESTIMATION OF GLUTATHIONE-S-TRANSFERASE IN TISSUES
(Beutler, 1986)

PRINCIPLE
The glutathione-S-transferase (GST) activity was measured by the increase in absorbance by the addition of 1-chloro-2,4-dinitrobenzene at 340 nm.

REAGENTS
- Phosphate buffer (0.5 M, pH 6.5)
- 1-chloro-2,4-dinitrobenzene (CDNB) in 95% ethanol- 25 mM
- Reduced glutathione (GSH), 20 mM

PROCEDURE
The reaction mixture contained 200 µl phosphate buffer, 20 µl CDNB and 680 µl water and was incubated at 37°C for 10 minutes. Then 50 µl GSH was added and mixed well. Then 50 µl of tissue extract was added and the change in absorbance per minute was read at 340 nm. To the blank 50 µl of water was added instead of tissue homogenate.

The GST activity was expressed as U/mg protein. One unit is the µmol of CDNB-GSH conjugate formed/minute.

APPENDIX 22

ESTIMATION OF VITAMIN C IN PLASMA AND TISSUES
(Ornaye et al. 1979)

PRINCIPLE
Ascorbic acid is oxidized by copper to form dehydroascorbic acid and diketogulonic acid. These products are treated with 2,4-dinitrophenylhydrazine to form the derivative bis-2,4-dinitrophenylhydrazone, which undergoes rearrangement in presence of sulphuric acid to form a product with an absorption band that is measured at 520 nm.
REAGENTS

- TCA, 10%
- H₂SO₄, 65%
- DNPH-Thiourea-Copper sulfate reagent (DTC): This reagent was prepared by dissolving 0.4 g thiourea, 0.05 g copper sulfate and 3.0 g 2,4-dinitrophenylhydrazine in 100 ml of 9 N H₂SO₄.
- Stock standard: 100 mg L-ascorbic acid was dissolved in 100 ml of 5% TCA.
- Working standard: 1 in 10 dilution with 5% TCA is made to obtain a concentration of 0.1 mg/ml

PROCEDURE

1.5 ml of ice-cold TCA was added to 0.5 ml of plasma or tissue homogenate, mixed well and centrifuged at 1800 x g for 10 minutes. 0.5 ml of the supernatant was taken and 0.1 ml DTC reagent was added, mixed well and the tubes were incubated at 37°C for 3 hours. To the mixture, 0.75 ml of ice-cold 65% H₂SO₄ was added and the tubes were kept at room temperature for 30 minutes. Along with test samples, standards containing 10-50µg ascorbic acid were processed in a similar manner along with a blank containing 0.5 ml TCA. The colour developed was read at 520 nm.

The amount of ascorbic acid (Vitamin C) was expressed as mg/dl plasma and µg/mg protein and µg/g tissue.

APPENDIX 23

ESTIMATION OF VITAMIN E IN PLASMA AND TISSUES

(Desai, 1971)

PRINCIPLE

Ferric ions are reduced to ferrous ions in presence of tocopherols and a pink coloured complex is formed with a more sensitive reagent such as bathophenanthroline. Orthophosphoric acid is added as a chelating agent to
reduce carotene interference by preventing its oxidation and stabilization of color by binding excess ferric ions and thus preventing their photochemical reduction. Absorbance of the stable chromophore is measured spectrophotometrically at 536 nm.

REAGENTS

- Bathphenanthroline reagent: 0.2% solution of 4,7-diphenyl-1,10-phenanthroline in absolute ethanol.
- Ferric chloride reagent: FeCl₃ (0.001M) in purified absolute ethanol.
- Orthophosphoric acid reagent: o-Phosphoric acid (0.001M) in purified absolute ethanol.
- Standard: α-Tocopherol 1-10 µg per ml of purified absolute ethanol.

PROCEDURE

3.0 ml of hexane extract was evaporated to dryness. To the residue, 1.0 ml ethanol, 0.2 ml bromophenanthroline reagent and 0.2 ml FeCl₃ reagent were added and mixed well. They were kept for one minute after which 0.2 ml o-phosphoric acid reagent was added, mixed and read at 536 nm in the colorimeter. The experiment was repeated with plasma.

The amount of α-Tocopherol (Vitamin E) was expressed as mg/dl plasma and µg/mg protein and µg/g tissue.

APPENDIX 24

ESTIMATION OF PLASMA β-carotene
(Bayfield and Cole, 1980)

PRINCIPLE

Vitamin A reacts with trichloroacetic acid and undergoes protonation to form anhydrovitamin and the transient blue colour of the carbonium ion was measured colorimetrically at 620 nm.
REAGENTS

- TCA solution saturated with chloroform
- Light petroleum ether
- Distilled ethanol, 95%
- β-carotene standard: 20 mg of synthetic crystalline carotene was dissolved in 4.0 ml of chloroform and made up to 100 ml with petroleum ether.

PROCEDURE

In a glass stoppered centrifuge tube, 1.5 ml of plasma was taken. 1.5 ml of ethanol was added to the plasma and shaken in a mechanical shaker with 3.0 ml of light petroleum ether for 2 minutes. The contents were allowed to stand for 10 minutes to obtain a clear supernatant solution. 2.5 ml of the supernatant was then dried and the residue was re-dissolved in 2.5 ml of chloroform. 1.0 ml of this solution was mixed with 2.0 ml of TCA reagent and the optical density was read at 620 nm without delay to prevent solvent evaporation and destruction of carotenoids by light. A set of standards were treated similar to that of the test.

The amount of β-carotene was expressed as mg/dl plasma.

APPENDIX 25

ESTIMATION OF REDUCED GLUTATHIONE IN PLASMA AND TISSUES
(Beutler and Kelly, 1963)

REAGENTS

- Phosphate buffer (0.2M, pH 8.0)
- TCA, 5%
- Ellman's reagent
- Standard glutathione solution
PROCEDURE

A known weight of tissue was homogenized in phosphate buffer. From this, 0.5 ml was pipetted out and precipitated with 2.0 ml of 5% of TCA. 1.0 ml of the supernatant was taken after centrifugation and 0.5 ml of Ellman’s reagent and 3.0 ml of phosphate buffer were added. The yellow colour developed was read at 412 nm. A series of standards were treated in a similar manner along with a blank containing 3.5 ml of buffer.

The GSH was expressed as mg/dl plasma, mg/100 g tissue and μmol/g tissue.

APPENDIX 26

INGREDIENTS OF THE BROILER FEED
(STARTER AND FINISHER DIET)

<table>
<thead>
<tr>
<th>Name of the ingredient</th>
<th>Starter diet (g)</th>
<th>Finisher diet (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maize</td>
<td>47</td>
<td>31</td>
</tr>
<tr>
<td>Jowar</td>
<td>0.0</td>
<td>20</td>
</tr>
<tr>
<td>De-oiled rice bran</td>
<td>0.0</td>
<td>4</td>
</tr>
<tr>
<td>Refined sunflower oil</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>Soybean meal</td>
<td>31</td>
<td>20</td>
</tr>
<tr>
<td>De-oiled groundnut cake</td>
<td>5</td>
<td>16</td>
</tr>
<tr>
<td>Di-calcium phosphate</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Shell grit</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Supplements</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vitamin AB₂D₃K mix</td>
<td>0.010</td>
<td>0.010</td>
</tr>
<tr>
<td>Vitamin B complex</td>
<td>0.020</td>
<td>0.020</td>
</tr>
<tr>
<td>Choline chloride</td>
<td>0.000</td>
<td>0.030</td>
</tr>
<tr>
<td>Trace mineral mixture</td>
<td>0.160</td>
<td>0.160</td>
</tr>
<tr>
<td>Salt</td>
<td>0.480</td>
<td>0.480</td>
</tr>
<tr>
<td>Lysine</td>
<td>0.010</td>
<td>0.051</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.253</td>
<td>0.127</td>
</tr>
</tbody>
</table>

Vitamin AB₂D₃K mix-One gram supplement contained 82500 IU of vitamin A, 50 mg of vitamin B2, 12000 IU of vitamin D3 and 10 mg of vitamin K.
Vitamin B complex – It contained 8mg of vitamin B1, 16 mg of vitamin B6, 80mg of vitamin B12, 80mg of vitamin E, 120 mg of niacin, 8 mg of folic acid, 80mg of calcium pantothenate and 86 mg of calcium.

Trace mineral mixture- one gram of the mixture contained 54 mg manganese, 52 mg of zinc, 20 mg of iron, 2 mg of iodine and 1 mg of cobalt.

APPENDIX 27
PRODUCTION OF AFLATOXIN
(Shotwell et al.,1966)

To 25 g broken rice, 7.0 – 10.0 ml of tap water was added, autoclaved and inoculated with the spores of Aspergillus parasiticus. The contents were mixed, plugged with cotton and kept in dark for 7 days. On the 7th day, the flasks were autoclaved and the fermented rice was collected and dried overnight at 80°C. The dried fermented rice was powdered and stored in dark place.

APPENDIX 28
ESTIMATION OF SUPEROXIDE DISMUTASE IN TISSUES
(Marklund and Marklund, 1974)

PRINCIPLE
Superoxide dismutase activity was measured by the increase in absorbance due to inhibition of autooxidation of pyrogallol brought about by the enzyme in tissue homogenate.

REAGENTS

Pyrogallol, 0.2 mM
Tris-cacodylic acid buffer, 50mM (pH 8.2)
Diethylenetriaminetetraacetic acid, 1.0mM
PROCEDURE

The reaction mixture of volume 1.0 ml contained the tissue homogenate, 0.2 mM pyrogallol in 50 mM Tris-cacodylic acid buffer containing 1.0 mM diethylenetriaminepentaacetic acid. The increase in absorbance was recorded at 420 nm.

The enzyme activity was expressed as U/mg protein. One unit of enzyme activity has been defined as the amount of the enzyme which inhibits the autooxidation of pyrogallol by 50 percent.

APPENDIX 29

ESTIMATION OF CATALASE IN TISSUES
(Aebi, 1984)

PRINCIPLE

The UV light absorption of hydrogen peroxide can be easily measured between 230 - 250 nm.

REAGENTS

- Phosphate buffer 50mM, pH 7.0
- 30 mM Hydrogen peroxide in phosphate buffer

PROCEDURE

The reaction mixture of 1.0 ml contained 0.02ml tissue homogenate in 0.88 ml of phosphate buffer and 0.1 ml of 30 mM hydrogen peroxide in phosphate buffer.

The specific activity of catalase was expressed as U/mg protein. One unit is μmoles of hydrogen peroxide consumed/minute.
APPENDIX 30

ESTIMATION OF GLUTATHIONE PEROXIDASE IN TISSUES
(Paglia and Valentine, 1967)

PRINCIPLE

The glutathione peroxidase activity was measured based on nicotinamide adenine dinucleotide phosphate oxidation at 340 nm.

REAGENTS

- Phosphate buffer (50mM, pH7.0)
- EDTA, 1 mM
- Yeast glutathione reductase
- Reduced glutathione, 0.3 mM
- NADPH, 0.2 mM
- Hydrogen peroxide, 1.5 mM

PROCEDURE

The incubation medium contained in a final volume of 1.0 ml, 50 mM sodium phosphate buffer, 1.0 mM EDTA, 0.24u/ml yeast glutathione reductase, 0.3 mM glutathione (GSH), 0.2 mM NADPH, 1.5mM hydrogen peroxide and the tissue homogenate. The reaction was initiated by adding NADPH and its oxidation was monitored at 340 nm by observing the decrease in OD/min for 3 minutes.

The enzyme activity was expressed as U/mg protein. One unit of enzyme activity has been defined as nmoles of NADPH consumed/minute, protein based on an extinction co-efficient of 6.22 mM^{-1}cm^{-1}.
APPENDIX 31

ESTIMATION OF GLUTATHIONE-S-TRANSFERASE IN TISSUES
(Habig et al., 1974)

PRINCIPLE

Glutathione-S-transferase (GST) activity is measured by following the increase in absorbance at 340 nm, using 1-chloro-2,4-dinitro benzene as the substrate.

REAGENTS

- 0.3 M phosphate buffer, pH 6.5
- Reduced glutathione, 30 mM
- 30 mM 1-Chloro-2,4- dinitrobenzene (CDNB) in 95% ethanol

PROCEDURE

The reaction mixture contained 1.0 ml phosphate buffer, 0.1 ml CDNB, 0.1 ml tissue homogenate and 2.09 ml of distilled water. The reaction mixture was incubated at 37°C for 5 minutes. The reaction was started by the addition of 0.1 ml of 30mM glutathione (GSH). The absorbance at 340 nm was followed for 5 minutes. The reaction mixture without the homogenate served as the blank.

The GST activity was expressed as U/mg protein. One unit is μmol of CDNB-GSH conjugate formed /minute.