MATERIAL AND METHODS
CHAPTER-III

MATERIAL AND METHODS

The summary of the experimental technique and the methods of analysis adopted during the course of present investigation have been given below:

Location of experiment and its climate

The experimental site is located in the main campus of the Narendra Deva University of Agriculture & Technology Narendra Nagar (Kumarganj), Faizabad, which is 42 km from Faizabad on Raibareilly Faizabad Road. This site falls on a latitude of 24.47° and 26.56° North and longitude at 81.12° and 83.98° East on an elevation of 113 meter above sea level in the Gangetic alluvium of eastern Uttar Pradesh. The area lies in sub tropical zone which is characterized by hot and dry summer and sufficiently cold winter.

Selection of birds

For the purpose of present investigation 100 laying birds of about 5 months of age of White plymouth rock breeds were selected from the stock available at Instructional Livestock Farm of Narendra Deva University of Agriculture & Technology, Narendra Nagar (Kumarganj), Faizabad U.P. While selecting the birds every care was taken to have birds of nearly identical (as for as possible) in body weight, age and stage of sexual maturity.
Plan of works

100 white plymouth rock birds about 5 months of age was selected for these studies. The birds selected were randomly divided in to four groups of 25 birds each. The birds are kept in Cage house. Feed and water trays were washed daily. Feed and fresh drinking water was offered daily in all group of birds. The birds were fed stresroak in the drinking water in various groups as given below:

Group I : Control (Fed drinking water without stresroak)
Group II : 8.0 ml/100 birds/day in drinking water
Group III : 10.0 ml/100 birds/day in drinking water.
Group IV : 12.0 ml/100 birds/day in drinking water.

Stresroak was given 1 to 10 days of every month.

1. Nutrient utilization

(a) Feed consumption

A measured quantity of feed was provided to a group every day up to 20\textsuperscript{th} week of age daily. Residues left was weighed and deducted from the total feed given during the day to obtain the feed consumption for a day for the whole group and the average consumption per bird was calculated.

Feed consumption/bird/day = \frac{\text{Feed offered-Residue of feed}}{\text{Number of birds}}

FEED CONVERSION EFFICIENCY

\text{Feed efficiency for egg production,} = \frac{\text{Total feed consumption during the test period in (g)}}{\text{Egg production during the test period}}
CHEMICAL ANALYSIS OF FEED AND DROPPING

The analytical techniques followed for estimation of dry matter and proximate principles in the present study were those recommended by A.O.A.C. (1980) and are as follows:

**Estimation of dry matter**

100 g of each sample was taken in a previously weighed petridish and placed in the hot air oven at 100 °C for at least 6 hours. Then the dishes were cooled at room temperature and weighed. This operation was repeated till a constant weight was attained. Now the dry matter content per 100 g of sample was calculated as follows:

- Weight of tray (g) = x g
- Weight of tray + sample (g) = y g
- Weight of sample before drying (g) = y - x g
- Weight of tray + dried sample (g) = z g
- Weight of dried sample (g) = z - x g
- Percentage of dry matter = \( \frac{z-x}{y-x} \times 100 \)

DM % or per cent (%) DM = \( \frac{\text{Weight of dried sample}}{\text{Weight of fresh sample}} \times 100 \)

**Estimation of crude protein**

In the proximate analysis system crude protein is generally estimated by Micro-Kjeldahl method. Crude protein includes true protein nitrogen and non-protein nitrogen multiplied by 6.25.
Reagents

1. Digestion mixture (K$_2$SO$_4$ and Cu SO$_4$ in the ratio of 9:1)
2. Nitrogen free concentrated H$_2$SO$_4$
3. 40% NaOH solution
4. N/100 NaOH solution
5. N/100 H$_2$SO$_4$ solution
6. Methyl red indicator

Procedure

(i) Digestion

(a) Took 0.5-2.0 g of representative sample and transferred into Kjeldahl Flask.
(b) Added 20-30 ml con. H$_2$SO$_4$
(c) Added 2-5 g digestion mixture
(d) Placed the flask on digestion bench and heated till the solution became clear blue.
(e) Removed the flask and cool it.

(ii) Distillation

(a) Added 5-10 ml distilled water to the Kjeldahl Flask and transferred the solution to 100 ml volumetric flask with repeated washing till the volume is reached.
(b) Took 10 ml aliquate and transferred it to micro-kjeldahl assembly.
(c) Took 10 ml of N/100 H$_2$SO$_4$ in a conical flask with the help of pipette.
(d) Added 2-3 drops of Methyl red indicator.
(e) Set this conical flask under the condenser.

(f) Added just sufficient volume (10-20 ml) of 40% NaOH solution to the distillation assembly to make contents alkaline. Put stopper immediately.

(g) Allowed the distillation for 10-15 minutes.

(iii) Titration

(a) Removed the conical flask after washing the tip of the condenser with distilled water in to the flask.

(b) Back titrate the flask contents with standard alkali (N/100 NaOH) till the end point was reached (red to pink).

(c) Recorded the volume of alkali used in filtration to calculate the volume of standard H$_2$SO$_4$ (N/100) used for ammonia absorption.

Calculation

\[
1 \text{ ml N/100 H}_2\text{SO}_4 = 0.00014 \text{ g Nitrogen}
\]

\[
\% \text{ CP} = \frac{V \times 0.00014 \times D \times 100}{W \times A}
\]

Where,

\(V\) = Volume of N/100 H$_2$SO$_4$ take volume of N/100 NaOH used for titration

\(D\) = Dilution factor (volume made in volumetric flask)

\(W\) = Weight (g) of sample

\(A\) = Aliquate taken
Estimation of Ether extract

Ether extract includes all the portion of a feed which are soluble in ether. The ether extract content of the sample was estimated by extracting it with a fat solvent like petroleum ether, benzene, chloroform diethyl ether etc. Ether was continuously volatilised at 55-60 °C, condensed and allowed to pass through the sample in a soxhlet apparatus.

Procedure

1. Weighed a small quantity of the sample (usually less than 5 g) into a pre-weighed extraction thimble (completely dried), having porosity permitting rapid passage of ether.

2. Removed water from the sample by placing it over night at 105 °C in a drying oven. Cooled in desicator and weighed.

3. Placed the thimble in the soxhlet apparatus in a straight direction so that the condensed ether may drop on it.

4. Checked the flask under the soxhlet’s apparatus to see if they are ¾ full of petroleum ether. Make sure that water is running through all the condensers. Extraction period may vary from four hours at a condensation rate of 5-6 drops per second to 16 hours at a 2 to 3 drops per second.

5. Took out the thimble and kept it at room temperature for evaporation of ether and then kept for over night in the oven at 100-150 °C.
6. Removed the thimble from hot air oven, cooled it in a desicator and weighed.

Calculation

Weight of sample = (Weight of thimble + sample) - Weight of thimble.

Weight of fat = (Weight of thimble + sample) - (Wt. of thimble + sample after extraction)

\[
\text{Ether extract (\%)} = \frac{\text{Weight of fat}}{\text{Wt. of sample}} \times 100
\]

Total carbohydrate

The values of total carbohydrate were obtained by subtracting the sum of crude protein, ether extract and total ash.

ORGANIC MATTER

Organic matter was obtained by deducting total ash (on dry matter basis) from the total dry matter.

Nitrogen balance

It was calculated by subtracting the sum of nitrogen of dropping and nitrogen of egg from nitrogen intake.

\[
\text{N intake} - (\text{N dropping} + \text{N egg}) = \text{Nitrogen balance}
\]

Calcium balance

It was calculated by subtracting the sum of calcium of dropping and calcium of egg from calcium intake.

\[
\text{Ca intake} - (\text{Ca dropping} + \text{Ca egg}) = \text{Ca balance}.
\]
Phosphorus balance

It was calculated by subtracting the sum of phosphorus of dropping and phosphorus of egg from phosphorus intake. \[ P_{\text{intake}} - (P_{\text{dropping}} + P_{\text{egg}}) = P_{\text{balance}} \]

Egg Production

(a) Age at laying of first egg of each bird was recorded.

(b) Egg production: Egg production was recorded as the number of eggs laid by a female up to 100 days of laying. The records of birds which died during the period of measurement were not included for analysis of data.

(c) When a bird laid her first egg, she was weighed for recording her mature body-weight. The first egg and last three eggs were also weighed to find out an average egg weight of the birds.

(d) Egg quality

Egg quality traits were measured after 60 days of maturity, one egg from each bird was taken for egg quality studies.

The eggs were broken by means of a sharp knife on a perfectly labeled thick glass plate and following egg quality traits were evaluated.

External egg quality

1. Egg Weight

The egg of each bird were weighed on each day by "egg weighing balance" up to 0.1 g accuracy.
2. Egg length

Egg length was measured by using Vernier callipers.

3. Egg girth

Girth of the egg was measured by using vernier callipers.

4. Shell thickness

Thickness of the shell was measured at three places by using screw gauge.

5. Shape index

The length and width of the eggs were measured by Vernier callipers up to the fraction of 1/10\textsuperscript{th} of the mm. The length of the egg was measured at two poles of the egg and the width at the maximum circumference of the egg. The shape index was calculated by formula described by “Shuliz” (1953).

\[
\text{Shape index} = \frac{\text{Greatest width}}{\text{Greatest length}} \times 100
\]

6. Shell weight

The shell weight was recorded on the egg weighing balance after collecting all fractions of the egg shell on the balance.

7. Vitelline membrane thickness

Vitelline membrane thickness of the membrane was measured at one place by using screw gauge.
Internal egg quality

1. Yolk weight

The yolk was transferred to a whatman filter paper without breaking the yolk membrane. After making it free from the surrounding albumen and chalazae, the weight was recorded and the weight of filter paper was deducted.

2. Yolk index

The height of the yolk was taken at its highest point with a micrometer. Diameter of yolk was measured at two different places using the vernier callipers. The average of the two readings was used as its width for calculating yolk index. Yolk index was calculated using the formula:

\[
\text{Yolk index} = \frac{\text{Height of yolk}}{\text{Average width of yolk}}
\]

3. Albumen weight

Albumen weight was obtained by reducing yolk and shell weight from total egg weight.

4. Albumen index

The height of the albumen was measured at about middle of the thick albumen and about one cm away from yolk by using triploid micrometer (AMES). Length of the thick albumen at the broadest point was taken by using the vernier callipers up to 0.01 cm accuracy and the average was used as width of the albumen. Albumen index
was calculated by using the formula of Heiman and Carver (1936) as follows:

\[
\text{Albumen index} = \frac{\text{Albumen height}}{\text{Av. width of albumen}}
\]

5. Haugh unit score

Since the average height of albumen and the weight of eggs has already been recorded the haugh unit was calculated according to the formula of haugh unit score.

\[
\text{Haugh unit score} = 100 \log (H + 7.57 - 1.7W.37)
\]

Where,

\[
H = \text{Height of albumen in mm} \\
W = \text{Weight of the egg in gram}
\]

Collection of Blood Sample

The blood sample was collected under aseptic condition from the wing veins in the morning hours between 8.00 to 10.00 A.M. before providing feed and water. Ethylene diamethyl tetra acetic acid disodium salt (E.D.T.A.) or heparin were used as anticogulant. Haematological, biochemical constituents and Enzyme activity measurements.

(a) Haematological parameters

1. Haemoglobin

Haemoglobin was estimated in sample of blood with the help of photoelectric haemoglobin binometer (Erma Japan Model 202).
This instrument works on Cyanmethaemoglobin principle described by Drapkin (1944).

Reagent solution

1. 0.2 g potassium ferricyanide
2. 0.05 g potassium cyanide
3. 1.0 g sodium bicarbonate

Procedure

With sohli’s pipette 0.2 ml blood was added into a test tube containing 5 ml of reagent solution. The test tube was left for 20 minutes at room temperature after shaking. The zero point of instrument was adjusted with the help of reagent blank and the values of haemoglobin were directly read on the scale expressed in g.

Haematocrit value

Determination of haematocrit value

The haematocrit value (P.C.V.) of the blood sample were determined by “Wintrobe haematocrit method”.

Principle

By centrifugation, the blood was separated into three distinct compartments, namely:

(i) the erythrocyte mass at the bottom or P.C.V.;

(ii) a white to gray layer of leukocytes and thrombocytes occurring immediately above the red cell mass and called the buffy coat;
(iii) the blood plasma.

Procedure

(i) The wintrobe haematocrit tube was filled by means of a pipette having a long narrow delivery tip. The tip was inserted to the bottom of the haematocrit tube and blood was forced out by pressure on the rubber bulb. The pipette was slowly withdrawn. When the column of the blood reaches the level of the 'O' mark on the left side of scale, the proper amount of blood had been deposited.

(ii) By the above procedure the other haematocrit tubes were also filled.

(iii) Balanced the tube on a physical balance

(iv) Centrifuged the haematocrit tube in centrifuge machine at 3000 rpm for 30 minutes.

(v) After 30 minutes switched off the centrifuge machine and took out the tubes, reading of the right side scale was taken.

Packed cell volume (P.C.V.)

P.C.V. was measured in wintrobes haematocrit tube as described by Schalm (1965). The haematocrit tubes were filled up to zero mark with the help of pasture pipette. The haematocrit tubes were centrifuged for 45 minutes at 4000 rpm and the P.C.V. values were recorded and expressed in percentage.
Total leucocyte count (T.L.C.)

The method described by Olson (1937) was followed for counting of leucocytes. The leucocyte count was made by counting the number of cells in each of the 4 large corner squares having Neubauer ruling and expressed in thousands per Cu. Mm.

Calculation

\[
\text{Cell count} \times 20 \ (1:20 \text{ dilution}) \times 10 \ (0.1 \text{ mm} \ \text{depth}) \quad \frac{\text{Leucocyte/Microliter}}{4 \ (\text{number sq. mm counted})}
\]

Differential leucocyte count (D.L.C.)

A dry unfixed blood smear on the slide was stained for 2 minutes with undiluted weight's stain.

After two minutes the stain was diluted with the volumes of distilled water. Mixed well and allowed to stain for 10 minutes. The slide was washed and dried at room temperature. The cell were counted under oil emersion lens of the microscope (100 x). The value was presented in percentage.

Red blood corpuscles (R.B.C.)

A measured quantity of blood was diluted with a fluid which was isotonic with blood and prevents its coagulation. The diluted blood is spread on a counting chamber and the number of cells in a circumscribed volume was counted under the microscope.
Reagent

A solution of 1% formaline (40% formaldehyde) in 31.3 g/litre trisodium citrate.

Calculation

Red blood cells/mm² of blood = Number of cells in 5 squares \times 10,000

White blood corpuscles (W.B.C.)

The blood was diluted (20 times) with a diluent which destroys the red blood cells. The diluted blood was placed in a neubear counting chamber for counting.

Reagent

Diluting fluid-mix 2.0 ml glacial acetic acid and one drop of gention violet in 100 ml of distilled water.

Calculation

WBC/mm² of blood = Total number of cells in four large corner squares \times 50

Total serum protein

Protein content was analysed by conventional Kjeldahl method (A.O.A.C., 1960). 1.0 ml serum was digested in the presence of digestion mixture (CuSO₄ 20% Na₂SO₄ 80%) and H₂SO₄. After complete digestion, the digested material was transferred into distillation flask. An aliquate was distilled with 40 per cent NaOH and ammonia liberated was collected in about 10 ml of 2 per cent boric
acid solution containing Tashiro’s indicator. Ammonia trapped with boric acid was titrated against N/7 H$_2$SO$_4$. The amount of nitrogen was multiplied by a factor 6.25 to get the protein value in the serum.

Cholestrol

Method described by Zack (1957) was adopted for estimation of serum cholestrol.

Reagent

(i) Glacial acetic acid
(ii) Conc. H$_2$SO$_4$
(iii) Stockferric chloride

840 mg of ferric chloride was dissolved in few ml of glacial acetic acid and the volume was made up to 100 ml with glacial acetic acid.

(iv) Ferric chloride precipitating reagent

Stock ferric chloride solution was diluted to 1:10 with glacial acetic acid.

(v) Ferric chloride blank

1.7 ml stock ferric chloride solution was diluted to 20 ml with glacial acetic acid.

(vi) Chloride standard

100 mg pure cholestrol was dissolved in few ml of glacial acetic acid and the volume was made up 100 ml with glacial acetic acid.
(vii) Working standard of cholesterol

2 ml of stock cholesterol standard pure 1.7 ml of stock ferric chloride solution was diluted to 20 ml with glacial acetic acid

Procedure

0.1 ml serum was added to every 6 ml of ferric chloride precipitating reagent and the test tube was shaken gently and filtrate was obtained by using No. 40 filter paper. 2 ml of working standard of cholesterol and 4 ml of ferric chloride blank solution was taken in test tube (this serves as final standard) and 3 ml of each filtrate chloride final standard blank solution (Serves as blank) were taken in test tube and 2 ml of conc. H$_2$SO$_4$ was added to each by the side of test tube. After the formation of a ring at the junction, the test tube was shaken gently to have uniform distribution of heat. After cooling, the readings were taken in photometer using blue filter.

Calculation

\[
\text{Cholesterol mg/100 ml of blood} = \frac{\text{Reading of unknown} \times \text{Conc. of standard}}{100} \times \frac{\text{Reading of standard}}{0.05}
\]

Glucose

The method of folin and WU (1920) was adopted for this estimation.

Procedure

One ml of blood was taken in a test tube and 7 ml of distilled water, 1 ml 10 per cent sodium tungstate and 1.0 ml of 2/3 N H$_2$SO$_4$ were added and mixed properly. The mixture was kept for 10 minutes and filtered through what man No.1 filter paper.
From the above filtrate 2 ml was taken in the fillen WU sugar tube. Similarly two other tubes were used, one for standard (2 ml of sugar solution containing 0.2 ml glucose) and other for blank (2 ml distilled water). To each tube, 2 ml of alkaline copper solution (40 gm CuSO₄ and water added up to 1 litre) was added. The tube were kept in a rapid boiling water bath for 8 minutes and then were cooled in running water with out shaking.

To each, 2 ml of phosphomolybic acid reagent was added and the contents were diluted to 25 ml mark with water and mix thoroughly. The densities of the solution were determined at 420 milimicron with photoelectric calorimeter (Baush and Lamb).

Calculation

\[
\text{Densities of unknown x mg glucose in standard} = \frac{\text{Densities of unknown}}{\text{Densities of standard}} \times 100
\]

Serum calcium

Samples of blood were analysed, using the titrimetric method. Calcium was precipitated directly from the serum as oxalate and the latter was titrated with potassium permangnate.

In a centrifuge tube, 1 ml of clear serum, 3 ml of distilled water and 1 ml of 4 per cent ammonium oxalate solutions were taken and mixed thoroughly. The sample was left to stand overnight, mixed again and centrifuged for 5 minutes at 1500 rpm. The supernant liquid was poured off carefully and left to drain in a rack for 5 minutes on a pad of filter paper. Now side of the tube was washed with dilute ammonia (2%), centrifuged and drained off. The process was repeated thrice. The precipitate was dissolved in 2 ml normal
sulphuric acid and kept in a boiling water bath for 1 minute and calcium oxalate was titrated with 0.01 N potassium permanganate solution to a definite pink colour.

Calculation

1.0 ml of 0.01 N KMnO₄ is equivalent to 0.2 mg of calcium. \((X-b) \times 0.2 \times \frac{100}{S} \times \text{mg calcium per 100 ml serum}\)

Where,

\(X = \text{Number of ml of permanganate solution required in the titration.}\)

\(b = \text{Number of ml of permanganate solution required to titrate 2 ml of sulphuric acid solution.}\)

\(S = \text{Quantity of serum taken for analysis.}\)

Phosphorus in blood serum

*(Fiske and Subba Row, 1925)*

Principle

When the protein free filtrate (Obtained by precipitating blood protein with trichloroacetic acid) is treated with acid molybdate solution, the phosphate present in the filtrate from phosphomolybdic acid. The phosphomolybdic acid is reduced by the addition of 1,2,4-aminonaphtol sulfonic acid reagent to form a blue colour whose intensity is proportional to the amount of phosphorus present.
Reagents

1. 10 per cent trichloroacetic acid-dissolved 10 g of reagent grade trichloroacetic acid in water and dilute it to 100 ml.

2. Molybdate solution-same as used in magnesium estimation.

3. Aminanaphthol sulfonic acid reagent-same as used in magnesium estimation.

4. Stock standard phosphate solution-dissolved 0.351 g (Weight exactly) of pure dry monopotassium phosphate (KH₂PO₄) in distilled water and transferred to a litre volumetric flask. Added 10 ml of 10 N H₂SO₄ and made the volume up to the mark with distilled water. Mixed well and keep it in a well stoppered bottle (Solution is stable for long period). This solution contains 0.4 mg of phosphorus in 5 ml.

5. Standard phosphate solution-took 10 ml of stock standard phosphate solution and made the volume up to 100 ml in a volumetric flask. This solution contains 0.04 mg of phosphorus in 5 ml.

Procedure

1. Took 8 ml of 10 per cent trichloroacetic acid in a small flask and added slowly with stirring 2 ml of whole blood, plasma or serum, put stopper and shake well.

2. Filtered through an ashless filter paper.

3. Took 5 ml of the filter in a 10 ml graduated centrifuge tube. Added 1 ml of the molybdate solution and mixed properly.
4. Prepare a standard taking 5 ml of dilute standard phosphate solution (reagents) and 1 ml of molybdate in a second centrifuge tube marked at 10 ml.

5. Prepared a blank taking 5 ml of distilled water and 1 ml of molybdate solution in a third centrifuge tube marked at 10 ml.

6. Added 0.4 ml of amino naphthol sulfonic acid reagent to each tube. Made the volume to 10 ml by adding distilled water (3.6 ml) to each tube. Mixed properly and allow it to stand for five minutes.

7. Recorded the optical density in the photoelectric colorimeter, with the blank set at zero, at 660-720 nm.

Calculation

\[ \text{Serum phosphorus (mg/100ml)} = \frac{\text{Density of unknown} \times 0.04}{\text{Density of known}} \times 100 \]

ENZYME ACTIVITY

(i) Serum Glutamic-Pyruvic transaminase (SGPT) SGPT is measured by colorimetric method.

Solutions

(i) Phosphate buffer (pH 7.4)

11.30 g of dry anhydrous disodium hydrogen phosphate and 2.7 g of dry anhydrous potassium dihydrogen phosphate per litre in water. Check with a pH meter or indicator paper, store at 4°.

(ii) GPT substrate (200 mM-alanine; 2 mM-a-Ketoglutarate)

Dissolved 9.0 g of alanine in 90 ml of water with addition of about 2.5 ml of N-sodium hydroxide to adjust the pH to 7.4 Acid
0.146 g of a-Ketoglutoric acid, dissolved it by adding a little more NaOH and adjust the pH to 7.4. Make to 500 ml portions and store frozen at -15 °C.

(iii) Stock pyruvate standard (20 mM)

Added 220 mg of sodium pyruvate per 100 ml in phosphate buffer. Store at -15 °C in 1ml aliquats.

(iv) Working pyruvate standard (4mM)

Dilute stock standard 1 in 5 with phosphate buffer and store at -15 °C. Prepared freshly each week.

(v) 2, 4 dinitrophenyl hydrazine (1mM)

Dissolve 19.8 mg of dinitrophenyl hydrozine in 10 ml of conc. Hcl and make to 100 ml with water. Keep in a brown bottle at room temperature.

(vi) 0.4 N-Sodium hydroxide

16 g of NaOH per litre in water.

Method for SGPT

Test : Warm 0.5 ml of substrate in a water bath at 37 °C for 3 min added 0.1 ml of serum, mixed gently and incubate for 30 minutes exactly. Removed the tubes from the bath and immediately added 0.5 ml of dinitrophenyl hydrazine (DNPH) solution and mixed well.

Control

Mixed 0.5 ml of substrate with 0.5 ml of DNPH solution and added 0.1 of serum.
Standard

Mixed 0.1 ml of working pyruvate standard with 0.4 ml of substrate. 0.1 ml of water and 0.5 ml of DNPH solution.

Blank

Mixed 0.5 ml of substrate, 0.1 ml of water and 0.5 ml of DNPH in a test tube. Allow the DNPH to react in all tubes for 20 minutes at room temperature; then add 5 ml of 0.4 N NaOH, mix well and leave for a further 10 minutes.

The pyruvate formed in 30 minutes by 0.1 ml serum is-

\[
\frac{\text{T-C}}{\text{S-B}} \times 0.4 \text{ u mole}
\]

Thus, the pyruvate formed per minute per litre of serum is:

\[
\frac{\text{T-C}}{\text{S-B}} \times \frac{1}{30} \times \frac{1000}{0.1} = \frac{\text{T-C}}{\text{S-B}} \times 133 \text{ u mole}
\]

The calculated pyruvate is converted into international units per litre.

Serum Glutamic Oxalic Transaminase (SGOT).

Solution

(i) Phosphate buffer (pH 7.4)
(ii) GOT substrate (200mM-DL-aspartic acid; 2mM-a Ketoglutarate)

Dissolved 13.3 g of DL-aspartic acid in the minimum amount of N-NaOH which will dissolve it and produce a solution with a pH of 7.4. About 90 ml is required. Added 0.146 g of -Ketoglutaric acid and dissolved it by adding a little more sodium hydroxide. Adjust the pH to 7.4 and make to 500 ml with phosphate buffer. Divided into 5-10 ml portions and stored frozen at -15°C.

(iii) Stock pyruvate standard (20mM)
(iv) Working pyruvate standard (4mM)
(v) 2,4 dinitrophenyl hydrazine (1mM)
(vi) 0.4 N-Sodium hydroxide

Method for SGOT

Test

Warm 0.5 ml of substrate in a water bath at 37°C for 3 minutes. Added 0.1 ml of serum, mixed gently and incubate for 60 minutes exactly. Removed the tubes from the bath and immediately added 0.5 ml of dinitrophenyl hydrazine (NDPH) solution and mixed well.

Control

Mixed 0.5 ml of substrate with 0.5 ml of DNPH solution and added 0.1 ml of serum.

Standard

Mixed 0.1 ml of working pyruvate standard with 0.4 ml of substrate, 0.1 ml of water and 0.5 ml of DNPH solution.
Blank

Mixed 0.5 ml of substrate, 0.1 ml of water and 0.5 ml of DNPH in a test tube. Allow the DNPH to react in all tubes for 20 minutes at room temperature; the added 5 ml of 0.4 N-NaOH, mixed well and left for a further 10 minutes.

Compared the colours at 510 nm or with on II ford 624 green filter. The pyruvate formed by the serum is responsible for the difference between test and control (T-C).

The pyruvate in 0.1 ml of the working standard (0.4 u mole) produced the difference between standard and Blank (S-B). So the pyruvate formed in 60 minutes by 0.1 ml of serum is:

\[
\frac{T-C}{S-B} \times 0.4 \text{ u mole}
\]

Thus, the pyruvate formed per minutes per litre of serum is:

\[
\frac{T-C}{S-B} \times 0.4 \times \frac{1000}{60} \times \frac{1}{0.1} = \frac{T-C}{S-B} \times 67 \text{ u mole}
\]

The calculated pyruvate is converted into international units per litre.

Alkaline phosphatase

Alkaline phosphatase was determined by measuring the phenol with 4 amino antipyrine (4-amino-phenazone).

To each of the tube labeled 'test and control' 1.0 ml of 0.1 M carbonate and bicarbonate buffer, 1.0 ml of 0.01 M disodium phenyl phosphate solution (substrate) were added and kept in water bath at
37°C for 5 minutes. 0.05 ml of plasma was added to the tubes labeled test then incubated. After 15 minutes of incubation 0.8 ml of 0.5 N NaOH was added to all the tube; 0.05 ml of plasma was added to the control and mixed. 1.2 ml of 0.5 M sodium bicarbonate, 1.0 ml of 0.6 per cent 4-amino antipyrin solution and 1.0 ml of 2.4 per cent potassium ferricyanide were added to all the tubes in the order given and shake well after each addition. The blank was prepared in the same way as test sample, except the buffer was added instead of plasma.

Optical densities were recorded at 510nm and alkaline phosphatase activity was calculated in terms of K.A. units/100 ml plasma by the formula:

\[
\text{Alkaline phosphatase} = \frac{\text{Reading (test-control)}}{\text{Reading of (standard-blank)}} \times \frac{100}{0.03} \times \frac{0.05}{0.05}
\]

One king Armstrong units of phosphatase is the amount of enzyme which will set free 1 mg of phenol in the given times under the condition of test.

Acid phosphatase

Solution

(i) Buffer (pH 4.9) Dissolved 42 g of crystalline citric acid in water. Added 376 ml of N- NaOH and make upto 1 litre with water. Check the pH. Preserved with a few drops of chloroform and kept at 4°C.
(ii) M tartrate: Dissolved 15 g of L (+) tartaric acid in about 70 ml water. Added 18.5 ml of 10 N-NaOH, adjust the pH to 4.9 and make to 100 ml with water. Stored in a dropping bottle at 4°C.

(iii) Stock phenol standard (1 mg/ml).

(iv) Working phenol standard (1 mg/100ml)

(v) 0.5 N- Sodium hydroxide

(vi) 0.5 N- Sodium bicarbonate

(vii) 4-Amino antipyrine

(viii) Potassium ferricyanide

Method

For total acid phosphate, mixed 1 ml citrate buffer and 1 ml of substrate in a test tube and place in a water bath at 37°C. After 3 minutes added 2 ml of serum, mixed gently and incubate for 1 hours exactly. Stop the reaction by the addition of 1 ml of 0.5 N-NaOH.

For the determination of the prostatic fraction, prepared a second tube in exactly the same manner except for adding 1 drop of M-tartrate solution before pipetting the serum.

Control

To 1 ml buffer, added 1 ml substrate 1 ml of 0.5 N-NaOH, mixed well and then add 0.2 ml of serum.

Standard

Mixed together 1.2 ml of buffer, 1 ml of phenol standard (1 mg/100ml) and 1 ml of 0.5 N-NaOH.
Mixed together 1.2 ml of buffer, 1 ml of water and 1 ml of 0.5 N- NaOH.

To all tubes, added 1 ml of 0.5 N-NaHCO₃ followed by 1 ml of amino antipyrine solution and 1 ml of potassium ferricyanide solution, mixed well after each addition.

Compared to reddish brown colours produced immediately at 510 nm or with an 11 ford 624 green light filter, avoiding exposure to strong sunlight.

Calculation

The amount of phenol present in the standard tube in 10 ug.

\[
\frac{T-C}{S-B} \times 10 \text{ ug.}
\]

Hence 100 ml of serum would liberate

\[
\frac{T-C}{S-B} \times 5 \text{ mg of phenol}
\]

Since, 1 king-Arm strong unit is the production of 1 mg of phenol in 60 min. under the conditions of the test,

\[
\text{Acid phosphatase} = \frac{T-C}{S-B} \times 5 \text{ (K.A. Units per 100 ml)}
\]

Hatchability percentage

Five females and one male were bred in individual sire pens and fertile eggs were collected after a period of 10 days to have
optimum fertility. On 7\textsuperscript{th} day of incubation the presence of an embryo was recorded by candling as fertile egg and the percentage of hatchability were calculated as follows:

\[
\text{Hatchability (\%)} = \frac{\text{Number of chicks hatched}}{\text{Number of eggs set}} \times 100
\]

Vital body organs measurement

At the end of the experiment 15 birds from each group have been slaughtered and vital body organs measurements have been made and pathological lesions were recorded, if any.

Statistical analysis

Calculation of means, standard error and analysis of variance had been followed as described by Snedecor and Cochran (1968).