3.1 Location of experiment

The study was carried at Mohanpur village under P S Hannghata, Dist - Nadia, State - West Bengal, India situated in Latitude 22° 56' N, Longitude 88° 32' E and Altitude 975 m above MSL, having tropical climate.

3.2 Animals

36 healthy Black Bengal nannies (adult female) of breedable age between 7-10 months and body weight 8-11 kg (with known date of birth and birth weight) were selected from farmers’ stock. Ear was tagged for proper identification of the animal.

3.3 Climate

The climate of the location is hot and humid. The mean annual precipitation (rainfall) was 1837.55 mm during the period of investigation. During summer season, maximum temperature rises up to an extent of 40°C. The relative humidity ranges from 60 to 97 percent throughout the year but almost lies at 87 percent and above during maximum days. The whole period of the year was divided into three seasons, “summer” consisting of March, April, May and June, “Monsoon” for July, August, September and October and “winter” for November, December, January and February. Summer, monsoon and winter seasons were marked as S1, S2 and S3 respectively in the present experiment.

3.4 Husbandry Practices

The female goats were reared in farm shed and allowed for free grazing daily from morning to noon and afternoon to evening till sunset. Only supplementary feed (tree leaves, chaffed paddy straw with rice polish, rice gruel and water) was provided to the animals during monsoon. Six adult and healthy bucks were maintained in the farm for breeding purposes with similar husbandry practice. The animals were fed mainly on grazing and allowing tree leaves during the rainy days. Little amount of concentrate (within the range of 50gm) comprising of Maize (crushed)- 70%, Mustard oil cake- 15%, Mung churm- 14% and Mineral mixtures, Vitamins along with salts- 1% were offered at the last stage of pregnancy and continued till few days after parturition. All the does were
Black Bengal Buck (male goat)

Black Bengal Doe (female goat)
kept on the floor made of soil or mud. The goat houses were sheded with paddy straw or corrugate sheet. A separate well-ventilated dry house for the animals was maintained. The male breeding stocks were confined in a separate place to record the assured information regarding breeding. The animals were dewormed twice a year before rainy season (during May) and after rainy season (during November) and the following rearing procedures were adopted to raise the good quality kids:

i) Cleaning the nostrils and mouth just after the birth of the kid and painting the naval cord with 2% iodine solution

ii) Allowing the kid for licking by the mother

iii) Feeding the colostrums to the kids within 15 minutes of birth and four to five times within 12 hours of birth

To assess the pattern of reproductive performances of 36 nannies (adult female), the following studies were considered:

3.5 Recording of body weight

The body weight was taken in an Avery Balance for Black Bengal or Spring Balance in three months interval prior to feeding of concentrate and collection of blood. The body weight at 0 day was done within 24 hours after birth and taken up to 9 months of age.

3.6 Reproductive Performances

The following reproductive traits of does were studied as described by Ahmed (1992).

3.6.1 Study of puberty and first kidding

For this aspect, the age at puberty, body weight at puberty, age at first kidding and body weight at first kidding of nannies were recorded.

a) Age at puberty (days): calculated from the date of birth to the date of sexual maturity (showing signs of first estrus)

b) Body weight at puberty (kg): Body weight was recorded on the date of the sexual maturity by “Salter Spring” balance

c) Age at first kidding: It was calculated from the date of birth of the animal (dam or mother) to the date of kidding (by the animal)
Goats are going out for grazing

A female goat giving birth the offspring (kid)

Goats are going out for grazing
d) Body weight at first kidding: It was recorded just after first kidding except the does that gave birth late night. In those cases, the body weights of the does were recorded in the next morning within 6 a.m.

3.6.2 Study of estrus

A total of 115 oestrus cycles were studied. Clinical manifestations and examinations of animals were noted carefully for estrus detection. The behavioural oestrus symptoms were noted by observing (i) continuous bleating, (ii) vulval oedema, (iii) discharge of cervical mucus through vulval opening, (iv) wagging (moving) of tail (v) frequent urination (vi) Parading by apronised buck to detect oestrus does were done. The glass speculum was also used for inspection of the vagina or uterus. The estrus characteristics were recorded as mentioned below:

a) Length of estrus cycle (days): It was determined as the period between the days of preceding estrus to the end of the next estrus.

b) Duration of estrus (hours): It was determined by noting the phases between the starting time of onset of estrus symptoms to the ending time of the heat symptoms.

c) Season of estrus: It was recorded by counting the incidences of estrus in different seasons i.e. summer, monsoon and winter.

d) Breeding: The oestrous does were allowed by the bucks maintained by the farmer.

e) Pregnancy diagnosis: It was done by abdominal palpation. In this method, the animal is kept in natural standing position and the abdominal wall is pressed on both the sides to feel the fetal mass after ninety days of pregnancy.

3.6.3 Study of gestation period and post partum estrus

To study the gestation period, recording of (i) date of service, (ii) parturition date and (iii) date of next estrus after parturition and (iv) post partum open period were done. Those were measured as below:

a) Gestation period (days): It is the interval between the date of successful service to the date of kidding.

b) Post partum estrus (days): It is the period which accounted from the date of kidding to the date of next estrus.
Female goat taking rest with kids

Kids are suckling milk from mother
3.6.4 Study of kidding interval and kidding season

Numbers of consecutive kiddings were recorded to study the kidding interval and kidding season.

a) Kidding interval (days). - It is the interval between two consecutive kiddings.

b) Kidding seasons. It was recorded by counting the incidences of kidding in different seasons i.e., summer, monsoon, and winter.

3.6.5 Study of birth pattern, sex ratio, conception rate, kidding rate and litter size:

a) Birth pattern: It is classified by different birth patterns viz. single, twin, triplet, quadruplet, pentaplet according to number of kids born per kidding.

b) Sex ratio: The ratio between the male and female kids born in a year.

\[
\text{c) Conception rate (\%) } = \frac{\text{Number of does conceived}}{\text{Number of does bred}} \times 100
\]

\[
\text{d) Kidding rate (\%) } = \frac{\text{Number of kids born in the year}}{\text{Number of kidding occurred in the year}} \times 100
\]

\[
\text{e) Litter size: } = \frac{\text{Number of kids born in a year}}{\text{Number of does kidded in that year}} \times 100
\]

3.7 REPRODUCTIVE FAILURE.

Incidences and percentage of reproductive failure for abnormal kidding due to abortion and still birth were recorded. To assess the extent of reproductive failure, the following studies were considered:

3.7.1 Haematological study:

Estimation of (i) Haemoglobin percentage (Hb %),

(ii) Packed cell volume (PCV),

(iii) Total erythrocyte count (TEC),

(iv) Total leukocyte count (TLC) and

(v) Differential leukocyte count (DLC)
A female goat with single kid

A female goat with twin kids
2) Estimation of –(i) Blood glucose, (ii) Total serum protein and (iii) Serum cholesterol

3) Estimation of serum macro minerals- (i) Calcium (Ca), (ii) Phosphorus (P) and (iii) Magnesium (Mg)

4) Estimation of serum micro minerals- (i) Copper (Cu), (ii) Iron (Fe) (iii) Zinc (Zn) and (iv) Manganese (Mn)

5) Estimation of Alkaline phosphatase , SGOT and SGPT

3.8 COLLECTION OF BLOOD

Blood samples were collected randomly both from 12 pregnant and 6 non-pregnant animals for haematological and biochemical observations

3.8.1 Collection and processing of blood sample

Blood samples were collected aseptically from jugular vein of each animal using sterilized glass syringe and kept in three different sets of sterile glass tubes containing heparin as anticoagulant. The stored blood sample was used for estimation of haemoglobin, serum electrolytes. First set was estimated for haemoglobin and second set was for plasma collection for mineral estimation, total serum protein, serum cholesterol.

3.8.2 Collection of plasma

The blood sample was centrifuged at 3000 r p m for 10 minutes for separation of plasma. The clear supernatant fluids thus obtained by centrifugation were pipetted out and collected in separate sterilized vials, labeled property and stored at -20°C for further biochemical estimation

3.8.3 Estimation of percentage of haemoglobin

It was estimated by alkali haematin method of Wu (1922). 05 ml of blood was measured in a micropipette and was blewed from the pipette into 10 ml of 0.1N hydrochloric acid. It was allowed to stand at room temperature for at least one hour to complete colour development. After stipulated period it was treated with 1/10 th volume of 10 % sodium hydroxide solution and was read in spectrophotometer at 520 nm against the standard alkali haematin solution with water as a blank

49
A female goat with triplet (three) kids

A female goat with quadruplet (four) kids
Calculation

Reading of standard

\[ \text{grams haemoglobin/100 ml of blood} = \text{X} \times 0.75 \times \frac{100}{0.5} \times \frac{10}{100} \]

Reading of unknown

3.8.4 Haematocrit value (HV) or Packed cell volume (PCV)

The ratio of RBC to plasma is expressed as the haematocrit value. This was estimated by an instrument called haematocrit. It consists of a specially prepared graduated capillary tube (wintrobe's) of uniform bore in which a specimen of blood treated with an anticoagulant was taken. It was centrifuged at 3000X for 30 minutes until plasma and corpuscles were completely separated and sedimented corpuscles showed no further shrinkage in the volume. From the graduations on the tube, the proportion of plasma and corpuscles can be known.

Calculation

\[ \text{Height of red cell column} \]
\[ \text{Packed cell volume} = \frac{\text{Height of red cell column}}{\text{Total height of blood}} \times 100 \]

3.8.5 Total Erythrocyte count (TEC)

The method involves an accurate dilution of a measured quantity of blood with a diluting fluid, e.g., isotonic, to prevent coagulation. The diluted blood was placed in a counting chamber and the number of cells in a circumscribed volume was enumerated under the microscope.

20 μl of blood was taken in a micropipette and mixed up with 398 μl of RBC diluting fluid in a proportion 1:200 in a glass tube. The diluted blood was mixed in a mechanical mixer to allow the air bubble to mix the suspension.

A clean dry Neubauer counting chamber was fixed with the fluid and was covered with the cover glass. The chamber was left undisturbed for about 2 minutes.

The counting was done in 5 groups of 16 small squares of counting chamber in a Neubauer glass slide including the cells touching the bottom and left hand margin of the small squares. Care was taken to avoid introduction of any air bubble.
Calculation

\[
\text{No of R B C counted (X) X dilution}
\]
\[
\text{No of R B C / mm}^3 \text{ of blood} = \frac{\text{No of R B C counted (X) X dilution}}{\text{Area counted & depth of the fluid}}
\]

Preparation of the diluting fluid

10 ml of 40 % formalin solution was made up to 1 litre with 32 gm/litre of di-sodium citrate solution

3.8.6 Total Leucocyte Count (TLC)

The method involves an accurate dilution of a measured quantity of blood with a diluting fluid i.e. isotonic the blood and prevents its coagulation. The diluted blood was placed in a counting chamber and the number of cells in a circumscribed volume was enumerated under the microscope.

20 \mu l of blood was taken in a micropipette and mixed up with 398 \mu l of W B C diluting fluid in a proportion 1:200 in a glass tube. The diluted blood was mixed in a mechanical mixer to allow the air bubble to mix the suspension.

A clean dry Neubauer counting chamber was fixed with the fluid and was covered with the cover glass. The chamber was left undisturbed for about 2 minutes.

The cells were counted in four large squares at any one area of the counting chamber including the cells touching the margin of the square.

Calculation

\[
\text{No of W B C counted (X) X dilution}
\]
\[
\text{No of W B C / mm}^3 \text{ of blood} = \frac{\text{No of W B C counted (X) X dilution}}{\text{Area counted & depth of the fluid}}
\]

Preparation of diluting fluid

2% acetic acid solution was made which was coloured pale violet with gentian violet.

3.8.7 Differential leukocyte count (DLC)

Differential leukocyte count was made from a prepared thin blood film stained with Giemsa as per following procedure.
The dried thin smear slide was fixed was methanol for 15 – 20 minutes. After fixation the slide was allowed to dry in air. Then the fixed slide was stained with Giemsa for one to one and half hour. After staining the slide was washed with tap water and allowed for air drying. Now the slide was observed under microscope.

The percentage of leukocyte was calculated by counting total number of leukocytes from the slide.

**Preparation of Giemsa stain**

This was done by mixing of 15-20 ml of concentrated Giemsa with 15-20 ml of ph 7 buffer solution and 60-70 ml of distilled water. The whole solution was then well mixed and filtered. After that it was used for staining.

**3.9 Blood Biochemistry**

The blood samples were collected aseptically from jugular vein. A part of blood sample was kept in a vial with anti-coagulant and utilized for estimation of sugar following the method of Nelson and Somogy as described by Nath (1990). The remaining part of blood was allowed to clot in order to collect the serum. The serum was separated and utilized for the biochemical estimation of Serum Protein (Biuret method) and Cholesterol following the method of Tarbutton and Gunter (1974) as described by Nath (1990).

**3.9.1 Determination of Blood sugar**

Blood sugar was determined from the protein free filtrate through a reaction with an alkaline copper-tartrate reagent and by production of blue color with arsenomolybdate. The intensity of color was measured through a colorimeter (Systronics, India).

In a neutral glass test tube 0.1 ml of blood was taken and 3.5 ml of glass distilled water was added. It was mixed thoroughly and 0.2 ml of 5% Zinc sulphate was added. Again it was mixed thoroughly and kept for 3 minutes. After scheduled period 0.2 ml of 0.1 (N) NaOH was added. It was shaken well and kept for 5 minutes. Further it was centrifuged to get a clear supernatant. This supernatant acted as stock.
Now three test tubes were taken and marked as blank, unknown and standard. The following components were added as per table in three different test tubes:

<table>
<thead>
<tr>
<th></th>
<th>Blank</th>
<th>Unknown</th>
<th>Standard</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water,</td>
<td>2.0</td>
<td>1.0</td>
<td>1.5 (ml)</td>
</tr>
<tr>
<td>Supernatant</td>
<td>---</td>
<td>1.0</td>
<td>--- (ml)</td>
</tr>
<tr>
<td>Working standard</td>
<td>---</td>
<td>---</td>
<td>0.5 (ml)</td>
</tr>
<tr>
<td>Alkaline copper-tartrate reagent</td>
<td>2.0</td>
<td>2.0</td>
<td>2.0 (ml)</td>
</tr>
</tbody>
</table>

All the contents were mixed and the tubes were placed in boiling water bath for 9 minutes. It was cooled quickly and 1 ml of arsenomolybdate reagent was added to each tube. It was mixed, allowed effervescence to subside and then diluted to 12.5 ml with water. It was mixed again and kept for 20 minutes for stabilization of color. The intensity of color thus developed was read for unknown and standard at 680 nm (red filter) against Blank in a colorimeter (Miltan & Ray, Spectronic 20 USA).

**Calculation**

\[
\text{Mg sugar per 100 ml of blood} = \frac{U \times O \times D \times 100}{S \times O \times D} \times 100
\]

**Alkaline copper-tartrate reagent** - It is a combination of solution-A which contains 12.5 gms of anhydrous sodium carbonate, 10 gms of sodium bicarbonate, 12.5 gms of sodium-potassium tartarate and 100 gm of anhydrous sodium sulfate solution and solution-B which contains 75 gm of CuSO₄·5H₂O and 0.25 ml of concentrate sulfuric acid solution mixed at the proportion of 25:1 immediately before work.

**Arsenomolybdate reagent** - It is prepared with 25 gms of ammonium molybdate, 3 gms of disodium hydrogen arsenate and 21 ml of sulfuric acid.

### 3.9.2 Determination of Serum Protein

Total serum protein was estimated by using Biuret method, as described by Nath (1990).
Estimation of protein

To 2.8 ml of glass distilled water 0.2 ml of serum was mixed in the case of test. 3.0 ml of glass distilled water was taken in another test tube as blank. 5.0 ml of biuret reagent was then added to each tube and was mixed thoroughly. The intensity of colour was read in a Systronic colorimeter (India) at 540 nm (green filter) after 30 minutes at room temperature. The quantity of protein was estimated through a standard curve prepared in the same way using known protein—Bovine serum albumin.

3.9.3 Determination of Serum Cholesterol

Serum Cholesterol was estimated following the method of Tarbutton and Gunter (1974) as described by Nath (1990).

Autozyme new cholesterol is a reagent set for determination of total cholesterol based on enzymatic method using cholesterol esterase, cholesterol oxidase, and peroxidase.

**Principle**

\[
\begin{align*}
\text{Cholesterol Esterase} & \\
\text{Cholesterol Esters} & \rightarrow \text{Cholesterol + Fatty Acids} \\
\text{Cholesterol Oxidase} & \\
\text{Cholesterol + O}_2 & \rightarrow \text{H}_2\text{O}_2 + \text{cholest-4-en-3-one} \\
\text{Peroxidase} & \\
2\text{H}_2\text{O}_2 + 4 \text{Aminoantipyrine + phenol} & \rightarrow \text{Red quinoneimine Dye +H}_2\text{O}
\end{align*}
\]

**Working solution**

Reconstitute enzyme & diluents mixed by gentle swirling and stored at 2°–8°C.

**Procedure**

Serum was collected from concerned animal and stored at 2°–8°C. When in use the serum and working solution was brought to room temperature.

Now the three test tubes were marked as unknown, standard, and blank. The different test tubes were filled with components as per table.
The tubes were incubated for 10 minutes at room temperature (25° – 30°C). After incubation, the absorbance of assay mixture was measured against blank at 510 nm. The final color was stable for two hours.

**Calculation:**

\[
\text{Total cholesterol in mg\%} = \frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times 200 \text{ (standard concentration)}
\]

### 3.9.4 Determination of inorganic phosphorus:

Plasma phosphorus was estimated following the method of Fiske and Subbarow (1925). When protein free filtrate (obtained by precipitating blood proteins with trichloroacetic acid) is treated with acid molybdate solution, the phosphate reagent in the filtrate forms phosphomolybdic acid. The phosphomolybdic acid is reduced by 1,2,4-amino napthol sulphonic acid to develop a blue colour which is measured by using a spectrophotometer at a wavelength of 700 nm. The value of phosphorus is measured as mg/dl.

### 3.9.5 Determination of Serum Ca, Mg, Cu, Fe, Zn and Mn

Serum minerals were determined as per method of Sandel (1950) and modified by Arneza et al. (1977) utilizing Atomic Absorption Spectrophotometer with the help of specific separate lamp for different minerals viz. Ca, Mg, Cu, Fe, Zn and Mn.

**Composition of triacid mixture**

- Conc. Nitric acid – 9 parts
- Conc. Sulfuric acid – 2 parts
- Conc. Perchloric acid – 1 part
1ml of plasma was digested with 20ml tracid mixture on hot plate at 180-200C temperature till the dense white colour fumes of tracids mixture disappeared. Then it was transferred to a 50ml volumetric flask by several washing through whatman filter paper no 42. Washing of each sample was done by triple distilled water and made up to the final volume of 50ml. Each sample was transferred to a separate sterilized plastic vials. Thereafter the clean solution was placed in Atomic Absorption Spectrophotometer (Perkin Elmer A Analyst 100) with standard solution of different concentration of elements in order to estimate the final concentration of minerals. The concentration of major element was expressed in mg/dl and concentration of trace element were expressed as µg/ml.

3.9.6 Methodology of SGOT and SGPT Estimation

Principles

Animal tissues including serum contains enzyme called transaminase which catalyzes the reactions such as the following:

(a) \( \alpha \)-keto-glutarate + asparate = Oxaloacetate + glutamate

(b) \( \alpha \)-keto-glutarate + alanine = pyruvate + glutamate

The enzyme involved in the first reaction is conventionally abbreviated ‘GOT’ and the transaminase in the second is abbreviated as ‘GPT’. The SGOT and SGPT were estimated colorimetrically as stated below.

Procedure

- 0.50 ml of the desired substrate was pipetted in a test tube and placed in the BOD incubator at 37°C until temperature equilibrium was reached.
- 0.10 ml of serum added, mixed and incubated for exactly 60 minutes for GOT and 30 minutes for GPT.
- 0.50 ml of 2, 4, dinitrophenylhydrazine reagent was added, thereby stopping the reaction. It was kept in room temperature for 20 minutes.
- 5.0 ml of 0.4 (N) sodium hydroxide was added and mixed thoroughly by inversion.
- After 5 minutes, the samples were put in a photoelectric colorimeter (Erma, model-A, E-II) and for each sample the absorbance at 530 μm was determined against a distilled water blank.

- The reading was referred to table 3(a) to determine the GOT and GPT activity of the serum as "Karmen Units"/ml.

**Calculation**

The units of transaminase activity of the serum was determined from the appropriate calibration curve as established below.

If a value greater than 182 GOT Karmen units/ml of Serum, or 125 GPT Karmen units/ml of serum is obtained, the test is repeated using serum diluted appropriately with water and adjusted the calculations accordingly.

**Reagents**

- **Phosphate Buffer**: 0.1 (M), pH – 7.5
  
  420 ml of 0.1 (M) di-sodium phosphate was mixed with 80 ml of 0.1 (M) potassium di-hydrogen phosphate.

- **Aspartate glutarate substrate**: (pH – 7.5 for SGOT)
  
  0.92 gm of α-keto-glutaric acid and 2.66 gm of L-aspartic acid were placed in a small beaker, 1(N) sodium hydroxide was added until the solution is complete.

  This substrate was then adjusted to pH 7.5 with sodium hydroxide transferred quantitatively with buffer to a 100 ml volumetric flask and diluted to the mark with buffer solution stored in a refrigerator, where it was stable for 1 year.

- **α-keto-glutarate-L-alanine substrate**: (pH – 7.5, for SGPT)
  
  0.092 gm of α-keto glutaric acid and 1.78 gm L-alanine were placed in a small beaker, 1(N) sodium hydroxide was added until the solution was complete.

  Adjust to pH 7.5 with sodium hydroxide to a 100 ml volumetric flask and diluted to the mark with buffer solution stored in a refrigerator, where it was stable for 1 year.

- **2, 4, dinitrophenylhydrazine**: (1 mM/litre)
  
  0.0198 gm of 2, 4, dinitrophenylhydrazine was dissolved in 100 ml 1(N) hydrochloric acid.
• **Sodium hydroxide:** 0.4 (N)

16 gm of sodium hydroxide was dissolved in water, cooled and diluted to 1 litre, and stored in a polythene bottle.

**Standardization**

A standard pyruvate solution 2 mM/litre is prepared by dissolving 0.02220 gm of sodium pyruvate in 100 ml of phosphate buffer. A calibration curve as was prepared as follows.

**Table containing data for preparing standard curves**

<table>
<thead>
<tr>
<th>Tube No.</th>
<th>Standard pyruvate solution (ml)</th>
<th>Aspartate glnamate substrate (ml)</th>
<th>Water (ml)</th>
<th>GOT units/ml</th>
<th>GPT units/ml</th>
<th>A or % T obtained</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0 0</td>
<td>1 0</td>
<td>0 2</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>0 1</td>
<td>0 9</td>
<td>0 2</td>
<td>20</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0 2</td>
<td>0 8</td>
<td>0 2</td>
<td>55</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>0 3</td>
<td>0 7</td>
<td>0 2</td>
<td>95</td>
<td>83</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0 4</td>
<td>0 6</td>
<td>0 2</td>
<td>148</td>
<td>125</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>0 5</td>
<td>0 5</td>
<td>0 2</td>
<td>216</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

• Into 6 test tubes pipetted the solution indicated in columns No 2, 3 and 4 of the above chart and placed in the BOD incubator at 37°C for exactly 60 minutes for GOT and 30 minutes for GPT.

• 10 ml of 2, 4, dinitrophenylhydrazine was added, mixed and allowed to stand for 20 minutes at room temperature.

• 10.0 ml 0.4 (N) sodium hydroxide was added and mixed thoroughly by inversion.
After a minimum of 5 minutes, all samples were read against a distilled water blank of 530 μ A or % T was recorded in the last column of the above chart and prepared calibration table or charts as desired. The unit obtained is equivalent to 1 "Karmen Unit", which was defined thusly: 1 karmen unit of GOT will cause decrease in A at 530 μ of 0.001/minute/ml of serum under the conditions of Karmen at 37°C and per cm light path. One unit of GPT was the same but with alanine substrate.

3.9.7 Methodology of Acid and Alkaline estimation

There were different processes to estimate serum phosphatase activity. It was virtually impossible to compare accurately one result with another if they had been obtained by different methods. If the serum phosphatases activity of two samples, which had identical concentrations of serum phosphatases were measured, the results would be identical only if the same test method was used in each case, together with the same incubation temperature, substrate and buffer. If any of these factors vary, the estimated activity would not be identical, despite the fact that they were expressed in the same unit. For this reason when the phosphatases activity in a sample were estimated by different laboratories the results were often completely different.

Various methods had been proposed for the determination of phosphatase activity of serum. For alkaline phosphatase, the modified method of Bodansky (1933) outlined by Oser (1971) had perhaps the widest application.

In this method, the phosphate liberated on incubation of serum with buffered glycerophosphate at pH 8.6 was used as an index of phosphatase activity, one Bodansky unit corresponded to the liberation of 1 mg of inorganic phosphate per 100 ml serum during one hour period of incubation under these conditions.

In the present experiment, the incubation procedure of the Bodansky method was used for a alkaline phosphatase determination, with modification to permit the use of the method of Fiske and Subba Row (1925) for the determination of phosphate liberated; for acid phosphatase, the conditions prescribed by Shinowara, Jones and Reinhart (1942) were followed, likewise modified to permit the use of the Fiske and Subba Row (1925) phosphate method.
Reagents required for alkaline phosphatase determination

- Alkaline phosphate substrate (pH 8.6)
  a) Sodium β glycerophosphate
  b) Sodium diethyl barbiturate
  c) Petroleum ether

Into a 100 ml volumetric flask, 3 ml of petroleum ether, 80 ml of distilled water, 0.5 gm of sodium β glycerophosphate, 0.424 gm sodium diethyl barbiturate and water to volume were introduced successively and kept in the refrigerator. The pH of the final solution was checked always before the actual chemical procedure and if it was not 8.6, adjusted to that value by the addition of dilute acid or alkali as necessary.

- 30% Trichloro acetic acid

30 gm of reagent grade trichloro acetic acid was dissolved in distilled water and diluted to 100 ml. This solution was stable indefinitely. To prepare a 5 percent solution, 1 volume was diluted with 5 volumes of distilled water.

- Amino napthol sulfonic acid reagent
  a) 15% Sodium bisulfite
  b) 1, 2, 4-Amino napthol sulfonic acid
  c) 20% Sodium sulfite

15% Sodium bisulfite: To 30 gm of reagent grade sodium bisulfite, taken in a beaker, 200 ml of distilled water was added and stirred to dissolve and if turbid allowed to stand well stoppered for several days and filtered and then kept well stoppered.

20% Sodium sulfite: 20 gm of reagent acid was added. After this, 5 ml of 20% sodium sulfite was added, stoppered and shaken until the powder was dissolved. If solution was not complete, more sodium sulfite was added, 1 ml at a time, with shaking, avoiding an excess. The solution was then transferred to a brown glass bottle and stored in the refrigerator. This solution was usable for about four weeks.
• Standard phosphate solution
  a) Monopotassium phosphate
  b) Concentrated sulfuric acid

10N Sulfuric acid  90 ml of concentrated sulfuric acid was carefully added to 260 ml of distilled water. To check, 1 ml of this solution was diluted to 10 ml in a volumetric flask, mixed and titrated a 10 ml portion against standard 1 N sodium hydroxide. From the titration results, the original solution if necessary was adjusted to make it exactly 10N.

Standard phosphate solution  · Exactly 0.351 gm of pure dry mono potassium phosphate was dissolved in distilled water and transferred quantitatively to a one litre volumetric flask. 10 ml of 10 N sulfuric acid was added, diluted to the mark with distilled water and mixed. This solution contains 0.4 mg of phosphorus in 5 ml. It was stable indefinitely. 6.25 ml of the stock phosphate standard, containing 0.5 mg of phosphorus was transferred to 100 ml volumetric flask, 16.7 ml of 30 per cent trichloro acetic acid was added and diluted to 100 ml with distilled water and mixed. This solution was stable indefinitely. 8 ml of this diluted standard contains 0.04 mg of phosphorus in 5% trichloro acetic acid.

• Ammonium molybdate solution

25 gm of reagent grade ammonium molybdate was dissolved in about 200 ml of distilled water. In a one litre volumetric flask 300 ml of 10 N sulfuric acid was placed and to this molybdate solution was added and diluted with washing to 1 litre with distilled water and mixed. This was stable indefinitely.

• Reagents required for acid phosphatase determination

All the reagents are same as in the alkaline phosphatase determination except the acid phosphate substrate.

Acid phosphate substrate: This was identical with the alkaline phosphate substrate already described except that sufficient acetic acid was incorporated to bring the pH to 5.0. Into a 100 ml volumetric flask, 3 ml of petroleum ether, about 80 ml of distilled water, 0.5 gm of sodium β glycerophosphate, 0.424 gm of sodium diethyl barbiturate and 5 ml of 1N acetic acid was introduced successively and distilled water was added to bring the
aqueous meniscus to the 100 ml mark. Kept in the refrigerator. The pH of the final solution should be checked and if it was not 5.0, adjusted to that value by the addition of dilute acid or alkali as necessary.

**Procedure for alkaline phosphate determination**

**Incubated sample**

- 9 ml of “alkaline phosphate” substrate was measured into a glass stoppered 12 ml centrifuged tube and was placed in BOD incubator at 37°C until the fluid reached the incubator temperature.

- Then the substrate was removed from the incubator and 1 ml of freshly separated serum kept at room temperature was added to it and thoroughly mixed and again placed at incubator and the reaction was allowed to proceed for an hour at 37°C.

- Just after one hour of incubation the tube was removed and cooled in ice water for several minutes. Then 2 ml of 30% trichloro acetic acid was added to it and mixed thoroughly and let stand a few minutes and centrifuged at 3000-5000 RPM for 10-15 minutes.

**Control sample**

At or near the end of the incubation period, a control sample was prepared as follows:

- 9 ml of substrate was measured into a 12 ml centrifuge tube.

- 2 ml of 30% trichloroacetic acid was added to it before the addition of enzyme preparation (serum) and thoroughly mixed.

- 1 ml of freshly separated serum was added to it, stoppered and shaken well and centrifuged as before.
8.0 ml of the supernatant from each of the experimental and control tube was taken carefully without disturbing the precipitate in centrifuge tube graduated at 12 ml.

In a third similar container, 8 ml of standard phosphate solution containing 0.04 mg of phosphorus, was taken.

In a fourth or blank tube 8 ml of 5 per cent trichloro acetic acid was taken.

When all the tubes were ready, to each 1 ml of ammonium molybdate solution and 0.4 ml of aminonaphthol sulfonic acid was added successively, diluted immediately to 10 ml with distilled water, mixed and allowed to stand for 5 minutes for colour development.

**Procedure for acid phosphatase determination**

All the procedures are same like alkaline phosphatase determination but only acid phosphate substrate was used instead of alkaline phosphate substrate.

**Calculation**

Reading of the unknown, control and the standard were taken in a photo-electric colorimeter (Erma-model A E-II). Before each study colorimeter was set to zero density, with the blank by using 660 red-filter (Erma). Methods followed as per the determination of inorganic phosphate (Fiske and Subba Row, 1925).

The calculation:

\[
\frac{\text{Density of unknown}}{\text{Density of standard}} \times 0.04 \times \frac{3}{2} \times 100 = \text{mg of inorganic phosphate per 100 ml serum per hour (Control or incubated)}
\]

The phosphatase activity is the difference between the inorganic phosphate content of the incubated and control samples expressed in mg of phosphorus per 100 ml per hour and also expressed in Bondasky unit per 100 ml of serum per hour.

3.8.3 Estimation of enzyme activity on growth

To study the effect of different stages of growth on enzyme activity (SGPT, SGOT, Acid and Alkaline phosphatase) in blood serum, 10 each of healthy kid of similar...
age group (one month old) irrespective of sex were taken from three groups and blood samples were collected from one month to 12 month of age

3.9.8 Estimation of enzyme activity on oestrus cycle

To study the effect of oestrus cycle and different phases of oestrous cycle, 10 healthy female goats with known normal oestrus cycle were taken and blood samples were collected in the 0, 6, 14 and 18th day of oestrus cycle. Here these particular days were taken because day '0' indicates day of 1st sign of heat, 6th day reflected start of CL function and decrease of oestrogen level, 14th day showed maximum activity of CL and highest progesterone function and in 18th day regression of CL started in the normal oestrous cycle in doe (McDonald, 1989). Oestrus was detected four (4) times a day i.e., at 6 am, 11 am, 4 pm and 7 pm. Oestrus detection was done by observing the sign of oestrous as described by Jainuddin and Hafez (1994) and using a proven buck. The behaviour of goat in the presence of proven buck was considered to be the sole criteria for the sign of oestrous.

3.9.9 Estimation of enzyme activity on pregnancy

To study the effect of pregnancy on enzyme activity, 10 healthy pregnant doe of similar age group in advance pregnancy (4 months of pregnancy) were taken for the study. Pregnancy diagnosis was done by abdominal ballotment procedure, non return of oestrous cycle and increase in body weight.

3.9.10 Estimation of enzyme activity on non pregnant doe

To study the effect of non pregnant phase on enzyme activity, 6 healthy non pregnant goats of similar age group were taken for the study.

3.10 Statistical Analysis

One way analysis of variance technique was used to study the effect of season on body weight sex wise, season wise, birth type etc. and reproductive performances (like age at puberty, body weight at puberty, age of first kidding, body weight of first kidding, length of oestrous cycle, duration of oestrous, season of oestrus, gestation period, post partum oestrous interval, kidding interval and kidding pattern). Duncan’s Multiple Range Test (DMRT) was used when F statistic was found significant either at
1% or 5% level of significance. For hematological (Hb, PCV, TEC, TLC and DLC), biochemical (glucose, protein and cholesterol) and blood minerals (Ca, Mg, P, Cu, Fe, Zn, and Mn) parameters, independent sample t-test was used to find out the significant difference between pregnant and non-pregnant goats in different seasons.

Effect of different month and season on distribution of oestrus and kidding pattern of Black Bengal goats was estimated by using Chi Square ($\chi^2$) Test. All these analysis was done by using SPSS package (Standard package for Social Science, Duncan, 1955).