The College of Veterinary Science & Animal Husbandry, Anand is a reputed educational and research Institute in India. Established on 16th August, 1964 by the Govt. of Gujarat, the college became a part of Gujarat Agricultural University in the year 1972 and at present is the constituent college of Anand Agricultural University since 2004. It has played a key role in development and growth of livestock sector in Gujarat state. The college shoulders major responsibilities for teaching the subjects of Veterinary and Animal Science; conduct high quality research to enhance the economical productivity and to transfer the technology to the farmers. It has attained status of a prime institution as a centre of repute for academic excellence and quality research in frontier sciences in livestock sector in Western India. The Institute has been appreciated by the Veterinary Council of India.

Academic Programme.....

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<td>B.V.Sc. &amp; A.H.</td>
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<td>M.V.Sc.</td>
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Every year, about 82 students (67 regular + 15 payment/ NRI/ VCI seats and others as per Govt. rules) get admission for B.V.Sc & A.H. degree course. Till date, 2134 students have earned their Bachelor degree. The college has an excellent and highly qualified faculty. The faculty positions include Professors, Associate Professors, Assistant Professors and Teaching Assistants. In addition, the Scientists working at research stations contribute significantly to teaching activity also. The majority of the faculty hold Ph.D. degree in their respective subjects.

The college offers post graduate studies leading to M.V.Sc and Ph.D. degrees in the following major subjects of Veterinary Science and Animal Husbandry. Anatomy, Veterinary Physiology, Veterinary Biochemistry, Veterinary Pharmacology, Veterinary Microbiology, Veterinary Pathology, Veterinary Parasitology, Veterinary Medicine, Veterinary Surgery, Animal Production, Gynaecology & Obstetrics, Veterinary Public Health, Livestock Production, Animal Genetics & Breeding, Animal Nutrition and Reproductive Biology. Till date, 764 students have earned M.V.Sc./M.Sc. degree and 140 students Doctorate degree.

The college campus is spread over 65 acres of land with 17 well established departments, 5 Research Stations at various locations, Molecular Genetics Lab, Veterinary Clinical Complex Livestock farm complex, College Library, indoor and outdoor sports facilities and 4 hostels for the students of UG boys, one for PG boys and two for girls. Information Technology Centre of the University was also located on the campus for few years. The college was the first in the country to establish Instructional farm maintaining important livestock animals for undergraduate teaching and training.
Departments.....

- Veterinary Anatomy
- Veterinary Physiology & Biochemistry
- Veterinary Pharmacology & Toxicology
- Livestock Production and Management
- Animal Genetics and Breeding

- Animal Nutrition
- Veterinary and Animal Husbandry Extension Education
- Veterinary Public Health and Epidemiology
- Veterinary Parasitology
- Veterinary Pathology
- Veterinary Microbiology

- Veterinary Medicine
- Veterinary Surgery and Radiology
- Veterinary Gynaecology and Obstetrics
- Livestock Farm Complex
- Livestock Products Technology
- Veterinary Clinical Complex
Research Stations.....

- Animal Nutrition Research Station
- Livestock Research Station
- Reproductive Biology Research Unit

- Poultry Complex
- Kapila Gau Sanshodhan Kendra, Minawada & Pashupalan Sanshodhan Kendra, Rama Na Muvada

The College runs 50 various Research Schemes (funded by ICAR, DBT, DST, GSBTM State Government and other agency etc.) on targeting various aspects of Livestock health and production.

Molecular Genetics Laboratory at the College is a state of the art laboratory of national repute, with all ultra modern facilities to carry out advance research in Molecular Genetics, Animal Biotechnology and Endo-crinoology. The research programmes focus on general and specific issues related to Veterinary Science and Animal husbandry of the State and the Country keeping in mind the challenges of 21st century.
Extension Activities

The College extends its technical services for the farmers through Veterinary Hospital (60 cases treated/working day), Ambulatory Clinical Services, Poultry Training Centre, Clinical Complex, Disease Diagnosis facilities (20-25 cases/day), T.V. and Radio Talk, as well as through Popular articles in extension journals and newspapers. More than 5000 farmers visited different research stations and Livestock Farm complex every year. The faculty has so far brought out 187 recommendations for Farmer's community and 167 recommendations for Scientific community. The faculty and the PG students also actively participate in Krushi Mahotsav organized every year by the State Government. The NSS wing in the college work for a week in a selected village and explain modern dairy husbandry practices to the farmers and carry out vaccination of animals.

Co-curricular and extra-curricular activities

The Students Representative Council undertakes various activities for cultural programme, debate competition, blood donation camps, sports, essay writing etc. Other forums viz. Vibrant Vets Club, Rotaract club, Red Ribbon Club, Soft Skill Development and Nature club provide ideal platform for development of students' personality and interest. Students are
encouraged to participate at District, State and National level competitions. The Alumni Association also conducts various activities related to the profession's interest and development.

**Encouragement to the students**

Fourteen Gold Medals and Three Cash prizes are offered to the undergraduate and postgraduate students who excel in various areas during their educational programme. Fellowships for the meritorious students are also offered. Placement Cell helps the students in getting suitable job/placement after their degree programme. The facilities at the Computer laboratory are provided to all the students to avail the benefits of the IT tools for their all round career development.

**Other regular features**

- Clinical, Infertility Camps and Ambulatory health services
- Diagnostic services
- Refresher/Short-term courses for field veterinary officers
MAIN ACHIEVEMENTS

- Important human resource created for Livestock/Veterinary Profession by educating 2134 B.V.Sc. & A.H. graduates and 904 post graduates (including 140 Ph.D.)
- First to establish “Instructional Farm” for teaching purpose in the Country
- 100% placement for the students in last five years
- Significant research output given; 167 recommendations for scientific community and 187 recommendations for farmer's community
- First in the World to undertake Whole genome Sequencing of Jafrabadi buffalo; 14, 98, 523 contigs of buffalo genome sequences and deposited to NCBI
- 33,881 genes/DNA segments from animals/birds/microbes sequenced and deposited to NCBI
- Molecular genetics characterization of Livestock breeds of Gujarat
- Development of surgical techniques for cataract in dog, interlocking nail, fracture repair and evaluation of drug eluting stents in rabbits and dogs
- Establishment of modern indoor patient facility for animal hospital
- Standardization of Embryo transfer protocol and production of ET cows and kids
- Mineral mapping of all agro-climatic zones of Gujarat for better nutritional inputs to animals
- Development of Commercial egg type poultry strain
- Trained faculty... ensuring academic environment... happy students... continuous pursuit for academic excellence
- “Best Veterinary College of the year” Award by Times Research Pvt. Ltd. under India Education Excellence Awards 2013

Kindly visit us at
www.aau.in

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Address:
Dean, College of Veterinary Science & Animal Husbandry,
Anand Agricultural University
Anand 388 001, Gujarat, India

(Published in March, 2017)
Laboratory Manual
Veterinary Physiology
(Unit 1 & 2)

Prepared and compiled by
Dr. M. M. Pathan
Dr. S. P. Madhira
Dr. A. M. Pande

Department of Veterinary Physiology & Biochemistry
College of Veterinary Science & Animal Husbandry
Anand Agricultural University
Anand-388 001
CERTIFICATE

This is to certify that Mr/Miss ____________________________
Reg. No. ________________ of First year, B.V.Sc. & A.H. has satisfactorily carried out the required number of practical as shown in this Practical Manual of Veterinary Physiology-I. (4+1) during the year 20__-20__.

Course Teacher

Head of the Department

Date: Date:

External Examiner

Date:
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**Demonstration Practical**

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PRACTICAL NO. 01

AIM: INSTRUMENTS / EQUIPMENT AND GLASS WARE USED IN THE PHYSIOLOGY LABORATORY

(a) Handle carefully and try to remember the names of the following instruments / equipments and glassware used in physiological work.

(b) Draw the clean and neat diagrams of these instruments / equipments and glassware articles using lead pencil only. Neatly label each figure.

(A) GLASSWARE:

Sr. No. Name of the article/glassware
1. Albuminometer
2. Beads (solid glass)
3. Beaker
4. Blood collection tube
5. Burette (Macro and micro)
6. Centrifuge tube (Plane and graduated)
7. Conical flask
8. Cover slip
9. Dropper (plastic)
10. Drop bottle
11. Folin-Wu tubes
12. Funnel (glass and plastic)
13. Glass rod
14. Hemoglobinometer diluting tube (round and square)
15. Hemoglobinometer sucking pipette
16. Measuring cylinder (glass and plastic)
17. Microcapillary
18. Neubauers chamber (Hemocytometer)
19. Petri dish
20. Pipette (Graduated and volumetric)
21. RBC pipette
22. Reagent bottle (glass and plastic, amber color and colorless)
23. Slides
24. Spirit lamp
25. Test tube
26. Ureometer
27. Urine flask
28. Urinometer
29. Volumetric flasks
30. Wash bottle (plastic)
31. Watch glass
32. WBC pipette
33. Westergren pipette
34. Wintrobe tube
(B) INSTRUMENTS / EQUIPMENTS:

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Name of the article/instruments/equipments</th>
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<tbody>
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<td>Centrifuge machine</td>
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<td>Compound binocular Microscope (electrical)</td>
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PRACTICAL NO. 02

AIM: COLLECTION OF WHOLE BLOOD, DEFIBRINATED BLOOD, PLASMA AND SERUM

Depending upon the use, blood is treated with anticoagulant to obtain ‘whole blood’ or plasma and is not treated with anti-coagulant when serum is to be separated. Plasma can be separated by centrifugation of the blood mixed with an anticoagulant. Defibrinated blood is obtained after removal of fibrin clot from the blood and is a mixture not blood cells and serum.

MATERIALS REQUIRED:
1) Experimental subject (Man, animal or a bird)
2) Sterilized blood collection vials and bottles
3) Rubber corks or ordinary corks coated with paraffin wax
4) Clean, dry and sterilized hypodermic syringe and needle (Hypotonic moisture will rupture RBCs)
5) A pair of fine scissors
6) Cotton, wool
7) Anticoagulant of choice (Not required for serum collection)
8) Rectified spirit

COLLECTION OF BLOOD:
Except for analytical methods permitting use of only drop or two of blood drawn from finger tip (man) ear lobe (animals) samples of blood are usually obtained by venipuncture.

1) Collection of blood in drops:
Generally single determination of hemoglobin, total differential count can be carried out from the 1-2 drops of fresh blood obtained from the tip of the ear lobe, finger or toe (human). Following general steps are followed:
1) Clip the hairs from the tip of the ear of the animal.
2) Gently massage the ear to increase the blood circulation that part.
3) Apply spirit or alcohol to clean the area from dust and grease and also to sterilize the area from dust and grease and also to sterilize the area.
4) Select a sterilized sharp needle or lancet. Prick the area sufficiently deep (usually 2 mm) to obtain blood drop easily.
5) Wipe off the first drop of blood with clean and sterilized cotton. The subsequent drops may be used for blood study.
6) Tip of the ear is the most common site preferred collection only small drops of blood in all Species of animal. In birds the tip of the comb is usually used for this purpose.

2) Collection of blood in larger volumes:
Larger volume of blood is invariably obtained by venipuncture. Superficial, most prominent and easily approachable vein is selected for this purpose appropriate needles with suitable gauze and length are used.
Following table gives general idea about the site, name of vein and needle-sizes used for collecting blood from different animal species.
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<th>Length of needle in inch</th>
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<td>20-22</td>
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<td>6</td>
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<td>Rabbit</td>
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<td>3</td>
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</table>

Sufficient care should be taken to see that animal do not get excited while collecting the blood sample. In individual subjects of excitable temperament timely administration of tranquilizer (Promazine hydrochloride) before bleeding is of great help.

**In Large animals:**

Taking all aforementioned precautions, clean the blood collection site and apply spirit or alcohol over it. Raise the vein by blocking its blood flow with the help of thumb or the fist or rope or tourniquet. Selected needle should penetrate the vein from the side and nearly art an angle of 50° degree with the surface of the skin the level or opening of the needle being kept towards the direction of blood flow. Once the needle has penetrated the vein, blood in the form of a jet will be come out (without the use of syringe) into the container. After the blood in required quantity is obtained, release the pressure of thumb, rope of tourniquet. At the time of withdrawal of needle a swab of sterile cotton wool soaked in spirit or alcohol is held over the site and the needle is withdrawn form beneath. Digital pressure on the gauze pad for a few minutes will effectively prevent bleeding form the skin puncture. Styptics should be avoided. Collodion may be applied when larger needles are used.

**In Small animals:**

Method for collection of blood remains same as described for large animals with addition of a few points. With all precautions prepare the site, sterilize it, select the proper needle, and puncture the vein with the hypodermic needle, which is attached to a dry sterile syringe of suitable capacity. As soon as the blood is seen to enter syringe, retract the plunger, slowly, until the desired amount of blood has entered the syringe. With all care withdraw the needle from the vein beneath the cotton pad moistened with spirit or 70% alcohol. After withdraw, detach it form the syringe and into a suitable container eject the blood slowly from the syringe. Apply pressure on the gauze pad for a few minutes to achieve hemostasis.

**COLLECTION OF PLASMA:**
On an average 40-45% volume of plasma is obtained from total volume of blood, depending upon the plasma required calculated quantity of blood is collected in the container having anticoagulant of choice. Blood is gently mixed and is then taken out in a test tube or a centrifuge tube. It is centrifuged for 30 minutes at 3000 rpm. After centrifugation, the supernatant fluid known as plasma is collected in the vial for use. Plasma can be preserved by adding Merthiolate (few drops). The vial can be stored in refrigerator or deep freeze.

Horse blood separates quickly cow, sheep, goat, blood seldom settle dog, cat, pig, blood may settle rapidly or slowly when whole is kept out in the laboratory for separation of plasma.

**COLLECTION OF BLOOD SERUM:**

When serum is desired, place the freshly drawn blood directly into a wider container without anticoagulant. Keep the container in slanting position at room temperature. So, that blood will get coagulated in the form of a gel. After overnight standing, the liquid part is collected from the container. It is centrifuged and clear serum is collected in vial and preserved in the similar manner to that of plasma.

**CLOT RETRACTION:**

It is expressed in percentage. It is the ratio of blood volume to its fluid (serum) volume obtained after the clot is produced, or it is expressed in terms of the volume of the blood. For example if 5 ml of blood clots it will give out 3 ml of serum then clot retraction value is 3/5 and in percentage it is 60%. Clot retraction is reduced in jaundice, thrombocytopenia, and in hemophilia.

**DEFIBRINATED BLOOD:**

Take a conical flask having a glass rod or a few glass beads. Freshly drawn blood is stirred either with a glass rod or with glass beads for 5-10 min. after removal of fibrin clot the remainder fluid is defibrinated blood. Serum can be separated from defibrinated blood after centrifugation.
PRACTICAL NO. 03

AIM: ESTIMATION OF BLOOD HEMOGLOBIN

1. Sahli’s acid hematin method:

PRINCIPLE:
Hemoglobin when treated with dilute hydrochloric acid, acid hematin (a colored hemoglobin compound) is produced. The developed color is diluted gradually to match with the standard colored glass rods. The result is expresses as gram per 100ml of blood.

REQUIREMENTS:
Sahli’s hemoglobinometer containing standard colored rods fixed in comparator box, Graduated hemoglobin diluting tube, Blood sucking pipette with 20cmm mark, 0.1N HCl, Pricking needles, Spirit, Distilled water, Dropper, Stirrer and Cotton.

PROCEDURE:
1. Clean and dry all the glass material of the hemoglobinometer.
2. Fill the graduated hemoglobin diluting graduated tube with 0.1N HCl up to the mark 2g or 10%.
3. If blood sample is not provided, prick the site (ear, ear lobe etc) well with aseptic care. Discard the first oozing out drop. Use next blood drop for filling 20cmm (0.02ml) pipette.
4. Wipe off the blood from out side of the blood sucking pipette (20cmm).
5. Suck 20 cmm or 0.02ml of blood by a clean and dry blood sucking pipette. Adjust the level of the blood to 20cmm (0.02ml) mark given on the blood sucking pipette.
6. Deposit the blood from blood sucking pipette to hemoglobin diluting graduated tube filled with 0.1 N HCl up to 2g or 10% mark.
7. Rinse the pipette with 0.1N HCl filled in hemoglobin diluting graduated tube.
8. Blood and acid content of the hemoglobin diluting graduated tube are thoroughly mixed by rotating the tube between the palms. After about a minute, mixture will be dark brown and clear due to formation of acid hematin.
9. The tube is allowed to stand for 10 to 15 minutes or more for the maximal development of the color (acid hematin). The disadvantage of the acid hematin method is that the color does not develop to a maximum immediately.
10. The acid hematin is then diluted either with distilled water or 0.1N HCl added drop by drop and stirred well every time till the color of the mixture matches with the standard colored rods placed on either side of the diluting tube. While matching the color every time –
    a. Hold the instrument between your eye and light.
    b. Stirrer should not be removed from the tube but held above the level of mixture.
    c. The graduated portion of the diluting tube should not face you but the ungraduated portion only should face to you.
    d. The mixture should appear brown, clear and without blood clots.
11. Express the hemoglobin value in terms of gram percentage per 100 cc of blood. Considering the normal value as 100% the gram % of hemoglobin can be expressed as hemoglobin percentage by calculation.
### Table: Hemoglobin content of the blood of different species

<table>
<thead>
<tr>
<th>Species</th>
<th>Hb (gm per 100ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mare</td>
<td>10 ± 1.50</td>
</tr>
<tr>
<td>Stallion</td>
<td>14.69 ± 1.56</td>
</tr>
<tr>
<td>Cow</td>
<td>12.03</td>
</tr>
<tr>
<td>Sheep</td>
<td>12.40</td>
</tr>
<tr>
<td>Goat</td>
<td>10.90</td>
</tr>
<tr>
<td>Man</td>
<td>13.0 – 15.0</td>
</tr>
<tr>
<td>Pig</td>
<td>11.95</td>
</tr>
<tr>
<td>Dog</td>
<td>1301</td>
</tr>
<tr>
<td>Cat</td>
<td>10.49</td>
</tr>
<tr>
<td>Turkey</td>
<td>10.50</td>
</tr>
<tr>
<td>Cock</td>
<td>13.50</td>
</tr>
<tr>
<td>Hen</td>
<td>9.80</td>
</tr>
</tbody>
</table>
PRACTICAL NO. 04

AIM: DETERMINATION OF PACKED CELL VOLUME (PCV) OR VOLUME PERCENTAGE OF ERYTHROCYTES OR HAEMATOCRIT VALUE

PRINCIPLE:

Blood is composed of cellular elements and plasma. Cellular elements are heavier than plasma. Amongst the cellular elements R.B.Cs are in millions, thrombocytes are in lakhs and W.B.Cs are in thousands per cubic millimeter of blood. When the whole blood filled in narrow, long tube is centrifuged, the heavier cells settle down and pack themselves because of the centrifugal force applied. This packed volume of cell measured is the haematocrit value of the blood. Haematocrit value of the blood is the most accurate and simplest of all the method for determining the presence for absence of anemia or polycythemia and in measuring their degree and type. In conjugation with an erythrocytes count, changes in the size of erythrocytes may be detected. Wintrobe method also furnishes information of volume on alterations in the quantities of leucocytes as well as the colour and capacity of the blood plasma. From the RBC count, haemoglobin estimation and PCV the mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC) can also be calculated.

REQUIREMENTS:

Centrifuge machine (3000rpm), Pasteur pipette, Wintrobe tubes, Blood sample added anticoagulant.

Wintrobe tube is a flat-bottomed narrow glass tube 11 cm. in length of about 2.5 to 3.0 mm inside diameter. It is calibrated by about 10 cm scale with millimeter divisions.

PROCEDURE:

1. Mix the blood gently but thoroughly with care to avoid haemolysis.
2. Suck approximately 1.5 ml of blood in the Pasteur pipette avoiding any air bubble.
3. Now insert the tip of the Pasteur pipette to the bottom of the Wintrobe tube.
4. By gentle pressure fill the tube with slowly, withdrawing the tip of the pipette slowly away from the bottom but keeping inside the blood column up to mark 0 to left 10 on right side. Adjust the blood column up exactly this mark.
5. If ESR is required, its reading must be obtained first.
6. Centrifuge the Wintrobe tube at 3,000rpm for thirty minutes.
7. Remove the tube from the centrifuge machine and note the readings of distinctly separated three zones namely plasma the upper zone, buffy coat the middle white zone, erythrocyte zone the lower red zone.
8. The volume percentage of each compartment is determined directly by observing the height of each compartment in millimeter.
9. The colour and capacity of plasma should be observed and recorded.

Study of three zones:

1. Plasma zone:
   It is an uppermost zone, pale yellow coloured and clear. Depending upon the condition of an animal it may be yellow, red and milky in colour. It can be turbid also. Straw yellow colour to the plasma is imparted in imparted by bilirrubin and is
measured in terms of Icterus Index. The red colour of plasma indicates haemolysis, due to handling mistake or due to some haemolytic disease in the body. Turbid and milky plasma is indicative of lipemia, which is a physiological feature of a laying hen.

2. **Buffy coat:**
   It is a whitish thin layer of the platelets (above) and the leucocytes (below). It is a usually 0.5 to 1 mm thick occurring immediately above erythrocytes column. Increase in zone indicative of leucocytosis and decrease is indicative of leucopenia. A delicate blank line is often observed at the point of contact of RBC and buffy coat, as a result of action of leucocytes upon the haemoglobin is formed. W.B.C number is determined from a column of a buffy coat as under:
   - Each 0.1mm of first 1 mm = 1000 no. of WBCs
   - Each 0.1mm behind 1 mm = 2000 no. of WBCs

3. **Erythrocyte zone:**
   It is red in colour, it represent the collection of red cell due to force of centrifugation. The height of this zone upon the total number and volume of red cells. Under anemic condition this zone is reduced in height, whereas in condition like polycythemia the zone shows increase in the height. These enable to find out roughly the total number of red cells as well as haemoglobin percentage.

   (a) Gram of haemoglobin per 100 ml of blood 
   \[ \text{Gram} = \frac{\text{PCV in millimeter}}{3} \]
   
   (b) Millions of RBCs/cmm of blood.
   \[ \text{Millions} = \frac{\text{PCV in millimeter}}{6} \]

Even under standard condition the packed cell volume contain some ‘trapped’ plasma. In order to obtain the ‘True cell volume’ the packed cell volume is multiplied by a figure between 0.97 to 0.99 depending force of machine used.

**Table: Packed cell volume of domestic animals.**

<table>
<thead>
<tr>
<th>Animal</th>
<th>PCV%</th>
<th>Animal</th>
<th>PCV%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Horse</td>
<td>35-38</td>
<td>Goat</td>
<td>27-32</td>
</tr>
<tr>
<td>Cow</td>
<td>32-35</td>
<td>Buffalo</td>
<td>44.3</td>
</tr>
<tr>
<td>Chicken</td>
<td>30-33</td>
<td>Pig</td>
<td>42.0</td>
</tr>
<tr>
<td>Dog</td>
<td>50.0</td>
<td>Camel</td>
<td>20-33</td>
</tr>
<tr>
<td>Cat</td>
<td>41.3</td>
<td>Fowl</td>
<td>22-30</td>
</tr>
<tr>
<td>Sheep</td>
<td>38.0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
PRACTICAL NO. 05

AIM: DETERMINATION OF ERYTHROCYTE SEDIMENTATION RATE (ESR) OR THE SUSPENTION STABILITY OF BLOOD

PRINCIPLE:
When the sample of the blood added with anticoagulant is allowed to stand in a tube the cells tend to settle down at the bottom leaving the clear supernatant plasma. In side the body such thing does not occur as the blood always circulates and hence the cells and plasma get mixed up every moment.

The rate at which the erythrocytes settle or descend down the vertical column of the blood per unit of time is called erythrocyte sedimentation rate (ESR). The true nature of like sp gr. of the cells, plasma, temperature viscosity etc. affects ESR. Besides these factors stated above certain other factors also govern the rate of sedimentation. Plasma proteins bestow property of stickiness pr cohesiveness to the red cells. As a result of random thermodynamic movements they tend to strike each other and (to stick to each other end) get piled over one another to form a rouleaux each rouleaux consists of 3 to 12 cells. Increased rouleaux formation is believed to occur largely due to changes in the proteins of the plasma (increase in fibrinogen, certain globulin fraction and albumin). These changes affect the surface dehydration or water balance on the surface of the erythrocytes. When rouleaux is formed the density of its mass is increased and it sinks down quickly. An increase in the MCV, a decrease in MCH, spherocytosis all retard the rouleaux formation. Increased in ESR indicates the presence but not the nature of a disease in the body. Increased in the ESR of the two consecutive tests is indicative of increased activity of diseased process. A decrease in ESR is the sign of arrest of the process.

METHODS:

A. WESTERGREN METHOD:

REQUIREMENTS:
The Westergren pipette and stand, Blood sample added with anticoagulant.

Westergren pipette:-
The Westergren pipette is a straight having uniform bore of 3 mm (internal bore 2.5 mm) and a length of about 30 cm (300mm). It is calibrated in millimeters from 0 to 200. It is open at both the ends. It holds about 1 ml of blood.

Westergren stand:-
It is available with a rubber cushion at the base on which the lower end of the Westergren pipette is made to rest. At the upper end is either a spring clip or a screw cap, which fits upon the pipette. It exerts sufficient pressure on the pipette to prevent any leakage of blood at the bottom. The stand allows the pipettes to remain exactly in a vertical position.

PROCEDURE:
1. Inspect the sample of blood provided to confirm the absence of any clot in it. Mix the sample gently.
2. Select dry and clean Westergren pipette and suck the sample slowly exactly up to 0 mark of the pipette.
3. Clean the outer surface of the pipette.
4. Keep the lower end of the pipette pressed on the rubber cushion of the stand and upon the tip of the pipette by removing your index finger with care to avoid any escape of the blood.
5. Fix vertically the tip of the pipette under the pressure of a spring clip. Leave the pipette undisturbed in the stand. Check the level of the blood to 0 mark. If the level is below the 0 mark, refill the pipette. (If the amount of the blood sample is small the pipette may be filled up to any maximum level below 0, the reading noted and ESR measured from that level).
6. Read in millimeter the upper level of the red vertical column of the corpuscles (above which there is clear plasma) every 15 minutes up to one to hour. Sedimentation rate is generally expressed as mm/hour. The test should be made at room temperature between 22-27 ºC.

N.B.: In case of extreme anemia or when the ESR is very high the upper level of the red column is not clearly seen. In such cases the highest point of maximum density of the red column is read.

B. WINTROBE METHOD:

The Wintrobe tube and stand is described in previous practical of PCV.

PROCEDURE:
1. Take clean and dry Wintrobe tube and pasture pipette.
2. Mix the anticoagulant added blood sample prior to filling the pasture.
3. Fill the Wintrobe tube with the help of pasture pipette.
4. Place the tube on the stand in an exact vertical position.
5. Note the time.
6. Take the reading at every fifteen minute up the end of first hour.
7. Record the room temperature also.

This method has the additional advantage that after noting down the ESR the same tube is used for determining the PCV. A chart showing PCV against abscissa and the ESR against the ordinate may be drawn.

Table:

Erythrocyte sedimentation rates of several domestic animals (Dukes Physiology of Domestic Animals, Edi. 8th 1970, page 51).

<table>
<thead>
<tr>
<th>Species</th>
<th>mm</th>
<th>Time</th>
<th>Species</th>
<th>mm</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cattle</td>
<td>2.4</td>
<td>7 hrs.</td>
<td>Chicken</td>
<td>3-10</td>
<td>3 hrs.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1-3</td>
<td>1 hrs.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>10-18</td>
<td>6 hrs.</td>
</tr>
<tr>
<td>Horse</td>
<td>15-38,2-12</td>
<td>20 min.</td>
<td>10 min.</td>
<td>Cat</td>
<td>0.5-15</td>
</tr>
<tr>
<td>Dog</td>
<td>6-10,1-5</td>
<td>1 hr.</td>
<td>½ hr.</td>
<td>Pig</td>
<td>1-14</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0-6</td>
<td>½ hr.</td>
</tr>
</tbody>
</table>
PRACTICAL NO. 6

AIM: COUNTING OF RED BLOOD CELL IN BLOOD

REQUIREMENTS:
Haemocytometer, Microscope, Blood sample added anticoagulant, RBC diluting fluid (normal saline).

PROCEDURE:
1. Adjust the clean dry Neubauer chamber upon the microscope stage and with all care observe the ruled lines under low power using concave surface of the mirror.
2. Mix the blood sample supplied in vial and draw blood with the help of red mouth piece into clean, dry RBC pipette (red bead) exactly up to 0.5 mark if the blood sucked is more then 0.5 mark, adjust to the mark by stroking the tip of the pipette with the tip of the finger. Do not use filter paper, cloth or cotton as they tend to withdraw the plasma (fluid) portion of the blood having a higher concentration of cells. Air bubble should not be present in the blood column of RBC pipette.
3. Wipe off any blood, which adheres to the out side of the pipette.
4. Dip the tip of the pipette in the diluting fluid and suck accurately up to the 101 mark. Repeat with the fresh pipette and fresh sample, if the fluid goes beyond 101 mark or if air bubble enters by accident.
5. Hold the pipette horizontally and roll it between the palms to ensure through mixing.
6. Discard the fluid from the stem of the pipette (three to four drops).
7. Hold the pipette at about 45 degree angle and place a drop from the pipette on the central plat-form near the edge of the cover slip so that the drop, because of the capillary action, spreads up between the central plat-form and cover slip. Repeat this step, in case air bubbles are present or the fluid escapes into the grooves around the plat-form, with cleaned Neubauers chamber.
8. After charging the chamber correctly, allow the cells to settle down for about 2 minutes.
9. Focus properly the central square under low power.
10. Change over to high power focus and adjust the light till the cells and rulings are seen clearly. Use plane mirror, keep the condenser at higher level. Partly close the diaphragm.
11. Move the chamber and bring the upper left corner block of 16 small (tertiary) squares under the visual field.
12. Select any two perpendicular lines of the above square for counting the cells lying on those lines. For example, the cells lying across the upper horizontal and the left vertical line should be counted in the square under consideration, those lying across the lower horizontal and the right vertical border line should not be counted in the square under consideration but in the adjacent square. Note down your reading.
13. Similarly count the cells in four corner square (two upper and two lower) and one central square.
14. Total up the number in each of the five squares. The difference between the counts of any two squares should not be more than 10%. If so, it shows that the cells have not been distributed uniformly over the counting chamber and a fresh preparation has to be made.
15. In any case the counting should not be undertaken if the preparation has dried.
16. Total up the count of cells of all 5 squares (80 small squares).
CALCULATION:

One primary square has 1 mm length, 1 mm width. The primary square is divided into 5 x 5 = 25 secondary squares. Therefore, surfaces area of one secondary square = length x width = 1/5 x 1/5 = 1/25 sq. mm. Therefore, cubic volume of one secondary = surface area x height = 1/25 sq. mm. x 0.1 mm. = 1/250 cmm.

Therefore, cubic volume of five secondary squares = 5 x 1/250 = 1/50

If x is the sum of the total number of RBC counted in five secondary squares then these no. of cells (x) are present in 1/50 cmm of the diluted blood.

Therefore no. of cells in 1 cmm of diluted blood will be x.50 but, blood was diluted 1 in 200 times. The number of cells in 1 cmm of the undiluted blood would therefore be equal to x.10000.

Counting of Poultry Blood Cells: Poultry RBCs are oval in shape and nucleated.

PROCEDURE:
1. Follow all the instructions carefully as described earlier.
2. Suck the blood in RBC pipette up to 1.0 mark.
3. Suck routine diluting fluid up to 101 mark.
4. Mix the content gently and avoid any loss of fluid from the pipette.
5. Charging of the chamber and counting of cells remain same as described earlier.

CALCULATION:

a. Cubic volume of five secondary squares = 5 x 1/250 = 1/50
b. Dilution is 1:100 and hence dilution factor is 100.
c. The number of cell in 1 cmm of the calculation blood would therefore be equal to X.50 (100) = X.5000.

Note: If blood is taken up to 0.5 mark and diluted to 101 mark then factor will be 10,000 (X.50. 200).

Put a decimal before last six digits and the figure obtained gives the value for number of cells in millions per cmm of blood, or in other simple way put decimal before last to figure of the total “X” value obtained after totaling the value of five secondary squares.

Table: Ranges for erythrocytes numbers in blood of domestic animals and man. (Dukes Physiology of Domestics Animal, 8th edi., 1970, page-33)

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Cattle</td>
<td>6-8</td>
<td>Goat</td>
<td>13-14</td>
<td>Cat</td>
<td>6-8</td>
</tr>
<tr>
<td>Horse</td>
<td>9-12</td>
<td>Dog</td>
<td>6-8</td>
<td>Chicken</td>
<td>2.5-3.2</td>
</tr>
<tr>
<td>Sheep</td>
<td>10-13</td>
<td>Pig</td>
<td>6-8</td>
<td>Pigeon</td>
<td>3.5-4.5</td>
</tr>
<tr>
<td>Rabbit</td>
<td>5.5-6.5</td>
<td>Man</td>
<td>5-6</td>
<td>Woman</td>
<td>4-5</td>
</tr>
</tbody>
</table>

Laboratory Manual of Veterinary Physiology _1
PRACTICAL NO. 7

AIM: COUNTING OF WHITE BLOOD CELL IN BLOOD

REQUIREMENTS:

Haemocytometer, Microscope, Blood, Anticoagulant, WBC diluting fluid (Thomas fluid).

PROCEDURE:

1. Adjust the clean dry Neubauers chamber upon the microscope stage and observe the ruled lines under low power using concave mirror. Take due care of the microscope.

2. Mix the blood sample supplied in vial and sucks the blood with the help of white mouth piece through the tip of the pipette into dry clean WBC pipette (white bead) exactly up to 0.5 mark. If the blood sucked is more than 0.5 mark, adjust to the mark by stroking the tip of the pipette with the tip of the finger. Never use filter paper, cloth or cotton as they tend to withdraw the fluid (plasma) portion of the blood leaving a higher concentration of cell. Air bubble should not be present in the blood column of WBC pipette.

3. Wipe off any blood, which adheres to the outside of the pipette.

4. Dip the tip of the pipette in the diluting fluid and suck accurately up to 11 mark.

5. Hold the pipette horizontally and roll it between the palms to ensure through Mixing.

6. Discard the fluid from the stem of the pipette (3 or 4 drops).

7. Hold the pipette about 45 angle and place a drop from the pipette on the central platform near the edge of the cover slip so that the drop, because of the capillary action, spreads up between the central platform and cover slip. Repeat this step if air bubbles are present or if the fluid escapes into the grooves around the platform with cleaned neubauers chambers.

8. After charging the chamber correctly, allow the cells to settle down about 2 minutes.

9. Focus properly any one WBC square under low power.

10. Count the WBC observing rules for counting the cells as detailed in the steps 11 to 15 of the previous experiment for RBC count.

11. Total up the count of cells of all 4 primary squares (64 squares).

CALCULATION:

One primary square has 1 mm length and 1 mm width.
Therefore, surface area of one primary square = Length X Width

= 1 x 1 = 1 sq. mm.

Therefore, cubic volume of four primary squares = Surface area x Height

= Sample area x Height = 1 x 0.1 cmm.

= 1 x 0.1

= 0.1 cmm.

Therefore, cubic volume of four primary squares = 4 x 1/10 = 2/5 cmm
If X is the sum of total number of WBCs counted in four primary squares then these number of X cells are presented in 2/5 cmm. Therefore, number of WBC in 1 cm of undiluted blood will be X.5/2. But blood was diluted 20 times. The number of WBC in 1 cmm of undiluted blood would therefore be equal to X . 5/2 . 20 or X.50

Table :
The leucocytes per cmm of blood of domestic animal (Dukes Physiology of Domestic Animal, 8th edi. 1970 page 46)

<table>
<thead>
<tr>
<th>Animal</th>
<th>Total leucocytes count (range/cmm)</th>
<th>Animal</th>
<th>Leucocytes count (range/cmm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Horse</td>
<td>8000-11000</td>
<td>Chicken</td>
<td>20000-30000</td>
</tr>
<tr>
<td>Cow</td>
<td>7000-10000</td>
<td>Sheep</td>
<td>7000-10000</td>
</tr>
<tr>
<td>Goat</td>
<td>8000-12000</td>
<td>Cat</td>
<td>10000-15000</td>
</tr>
<tr>
<td>Pig</td>
<td>10000-22000</td>
<td>Dog</td>
<td>9000-13000</td>
</tr>
</tbody>
</table>
AIM: PREPARATION OF BLOOD SMEAR

A fresh sample of blood is examined for various purposes under microscope in the form of (a) a drop preparation and (b) in the form of a film preparation. For the very purpose the slide, cover slip, must be thin, completely transparent, scrupulously clean - free from grease and dust.

(A) The drop preparation or wet film preparation

REQUIREMENT:

Puncturing needle, Spirit, Sterile cotton swabs, Cover glass, Slides, Microscope, Vaseline or paraffin.

PROCEDURE:

1) Puncture the site for a small drop of blood with all aseptic precautions.
2) Hold the cover slip by the edge between two fingers and touch it to the drop of blood.
3) Invert the glass slide on it. Do not press. The blood will spread evenly due to the weight of the glass slide itself. Lift the glass slide along with the cover slip.
4) Apply only a little Vaseline or paraffin all round the edge of the cover slip to seal the capillary space. It prevents evaporation.
5) Mount under microscope. Examine first under low power then change over to high power and finally to oil immersion objective.

The drop preparation is extremely useful in studying the colour, size and shape of red cells; number and type of white cell and presence or absence of any parasites.

(B) Film preparation

1) Preparation of a film on a cover glass:

Requirements as usual including two cover slips.

PROCEDURE:

A drop of blood is placed on a cover slip with usual precaution. Invert another one in a crosswise fashion so that their corners are arranged to form a star. After a moment the drop of blood will spread. Separate two cover slips by sliding then horizontally over each other. Dry them.

2) Preparation of a film on a glass slide:

REQUIREMENTS:

As usual with two glass slides. Any one – glass slide can be used as a spreader provided its edge is straight and smooth.
PROCEDURE:

a) Puncture the site and take out the blood drop. Clean the drop from the finger and next oozing out drop should be used for film preparation.

b) A clean dry slide is lifted by holding its edges along its length and touched to the drop end in the center at least 1 cm away from the narrow border.

c) The slide is placed on a level and smooth surface of a table in such a way that the drop is on your right side.

d) Hold the spreader slide along its long edges and is brought towards the blood drop at and angle 30 to 40° (on the right side) with the glass side. The edge of the spreader slide must be smooth.

e) The smooth edge of the spreader slide is made to touch the left end to the drop, immediately blood droop will spread along with of spreader slide.

f) Push the spreader slide ahead towards the left end of the slide with quick but uniform motion and with a light but even pressure. The blood follows the spreader to form a film.

g) Allow it to stand vertically for air-drying. Label it. The preparation of a blood smear would appear to be simple but a better smear is judged from the following criteria.

1. A well-made blood smear has a thin and smooth appearance, jerking movement at the time of spreading produces a wavy film and rough edge of the spreader slide produce streaks or lines in the blood film.

2. Presence of grease or oil produces holes in the blood film.

3. The smear should have long straight edges.

4. When examined under microscope, the cells must have distributed thinly and evenly.

Care of the blood film:

Fix the blood smear with alcohol to preserve the cellular structure. Stain the smear immediately if possible. The film while air drying, should be protected from flies.
AIM: STAINING TECHNIQUE FOR THE BLOOD SMEAR

INTRODUCTION:
Blood smear is stained to study the morphology of blood cells, differential leucocyte count, Arneth cook count and examination of blood parasites. Generally the stain used for staining the blood film is composed of acidic as well as basic dyes. The stain prepared in water is called aqueous stain and those in alcohol are called alcoholic stain. Both, aqueous and alcoholic stains are useful for the staining of the blood smear. The acidic dye used is eosin and the alkaline dye used is heamatoxylin, or gentian violet or methylene blue.
The commonly used stains are:
1. Leishman stain (alcoholic stain)
2. Wright stain (alcoholic stain)
3. Geimsa stain (aqueous stain)
4. JSB stain (aqueous stain)

Leishman’s method:
0.15 g of ready from the stain is dissolved in 100 ml of acetone free, methyl alcohol. The alcoholic solution is kept in sun to warm for three to four weeks (ripening time). The stain improves on keeping and warming.
This stain is prepared from a mixture of methylene blue and eosin. It is a purplish red stain formed by combination of oxidation products of methylene blue and eosin.

PROCEDURE:
1. Select good air dried smears for staining.
2. Place the blood film on a staining rack in such a way that blood film remains upper most.
3. Leishman stain is alcoholic and hence blood Smear is fixed by pouring sufficient stain drop by drop to cover all the film. Count the drop of the stain put over the film.
4. This undiluted stain is allowed to act for ½ minute. During this time blood smear gets fixed with alcohol, present in the stain itself. Omission of this step will wash out the smear during washing.
5. Place double number of drop of distilled water and dilute the stain. A scum appears on the surface.
6. Mix by blowing air through an ordinary pipette and allow it to stain for 10 minutes. Stain should not be allowed to dry. The timing to be allowed for staining depends upon the quality of the stock of the Leishman stain.
7. Wash the excess of the stain with a stream of distilled water or tap water.
8. keep the slide dipped in running tap water or in buffered water at pH 6.8 until the film develops a pink appearance (10 minutes).
9. Pour off the excess water. Wipe the backside of the slide with a clean and dry filter paper. Keep it is a vertical position to drain and dry.
10. Examine the smear under low as well as high powers of the microscope. Examine whether the nuclei of leucocytes have been stained properly, put a drop of cedar wood oil and examine the film under oil immersion lens. Use plane mirror. A well stained nuclei containing nuclei acidic takes basic stain and basic part (cytoplasm) takes acidic stain.
**Wright’s method:**

It is made from a 0.167 g mixture of eosin and methyl violet dissolved in 100 ml methyl alcohol (acetone free). This is an alcoholic stain. The staining procedure is similar to Leishman’s method except that the dilution is made with equal number of drop of distilled water.

**Giemsa’s stain:**

This stain is aqueous stain, therefore, requires separate fixation of blood smear with alcohol prior to the actual staining. After fixation and when the slide is dry, it is kept in coupling jar for staining. Jar is filled with diluted stain (1: 10) and the blood smear is kept in it for one hour. Wash the slide with stream of a distilled water blot dry for examination.

Alcoholic Giemsa stain can be prepared and the procedure that of Leishman stain be followed.

**JSB stain:**

It is aqueous stain. Solution A of methylene blue and solution B of eosin is prepared separately in water.
1. Fix the blood smear with an alcohol.
2. Dip the smear in solution B (eosin) for the 15-20 seconds.
3. Dip the slide in solution A (methylene blue) for 0.30 second.
4. Rinse the slide with water. Wipe the backside.
5. Keep it is a vertical position to drain and dry.
6. Examine the smear.

**Solution A:-**

a) Dissolved 1.3g of medicinal methylene blue and 5 g of anhydrous Na₂HPO₄ in 50 ml distilled water.
b) Bring to a boil and then evaporate on a water bath almost to dryness.
c) Add 6.25 of anhydrous KH₂PO₄ and 500 ml of distilled water. Stir until the stain is completely dissolved.
d) Set aside for 24 hours and filter before use. Solution keeps well.

**Solution B:-**

a) Take 500 ml of distilled water.
b) Dissolved the following phosphate salts in the above 500 ml distilled water.

\[
\text{Na}_2\text{HPO}_4 - 5.00 \text{ g} \\
\text{KH}_2\text{PO}_4 - 6025 \text{ g}
\]
c) Add 1.0 g of eosin water soluble, in the above buffer and dissolve.
AIM: METHODS FOR EXAMINING THE BLOOD FILM FOR DIFFERENTIAL LEUCOCYTES COUNT

A well-stained blood film is necessary for the purpose of differential leucocytes count or Arneth count. While preparing the blood smear, leucocytes tend to lie in different parts of the film. Smaller cells like (lymphocytes) tend to occupy the central periphery. Rest of the leucocytes (eosinophils, monocytes) gets evenly distributed.

METHODS:

The methods employed are
(a) Straight line method,
(b) Cross section method and
(c) Battlement method

(a) Straight line method:

Focus the left corner of the slide and with the help of the screw of the mechanical stage now shift the slide towards the left along the same horizontal axis to bring the adjacent field in view. Note the different leucocytes when slide is moving from right to left. Thus go on counting the cells towards the right along the horizontal edge till you reach the right upper corner of the film. Now shift the slide up (the movement seen in the microscope will be in the opposite direction). Bring the lower adjacent field in view. Then go on counting towards the right till you reach the end. Then shift lower down and go on counting towards the left again and so on.

In this method the results may be reversible if the counting done on line passing through the periphery of the smear is compared with the counting done on a line passing through the central part on the smear. Therefore, this method is not a good method.

(b) Cross section method:

W.B.C. on lines forming a letter W across the whole film is counted in this method. This method is also not better.

(c) Battlement method:

Here every time horizontal field and vertical field is counted alternatively. This is a better method for counting the different leucocytes.
AIM: GENERAL PRINCIPAL FOR COUNTING CELLULAR ELEMENTS OF BLOOD

Introduction:
A cubic millimeter of normal blood contains between 4.5 to 5.5 million red cells and 5 to 6 thousand white blood cells. It is therefore impossible to count them individually without diluting the blood. Therefore, blood is diluted 1:200 times for RBC and 1:20 times for WBC. The number of cells counted in the specified volume is multiplied by constant (10000 for RBC and 50 for WBC). Therefore, every source of error in the entire procedure is multiplied by a corresponding constant value in reporting the count. Possible error in counting method can occur at following steps:
1. Inaccurate calibration of pipettes and counting chamber.
2. Defects in cover slips.
3. Chipped pipette.
4. Failure to discard diluting fluid or blood or both.
5. Inadequate shaking of the pipette.
6. Failure to discard diluting fluid from the capillary before charging the chamber.
7. Flooding of fluid cover slip.
8. Drying of fluid in counting chamber.
9. Errors in counting.
10. Errors in calculations.

Counting of cells is done by either manual method or with the help of electronic counting device. The former method requires large investment of time for a relatively inaccurate result whereas the latter procedure is faster and more accurate.

Principle:
It consists of accurate dilution of a measured quantity of blood with a fluid which is isotonic with blood cells and which will prevent its coagulation and rouleaux formation. The diluted blood is placed in a counting chamber and number of cells in a circumscribed volume is enumerated.

Counting methods (1) Electronic Method (2) Hemocytometer Method.

1. Electronic Apparatus:
“Flow-through system”, this instrument is working on the principle that cells are poor electrical conductor as compared with saline solution. A dilute suspension of red corpuscles in 0.85% NaCl solution (to which 0.2ml of 2.5% solution of albumin has been added to make the suspension stable) is drawn through minute aperture conducting an electric current between platinum electrodes. Each cell passing through the aperture displays an equal volume of solution of electrolytes and thereby modulates the electric current. The resulting pulses are amplified and automatically counted and area displayed as numerical value on the screen. All of the cells contained in 0.5ml; of suspension are counted. The dilution of 1:50000 are found to be optimal.

2. Hemocytometer Method:
Hemocytometer set consists of a dilution pipette (RBC and WBC), Neubauers counting chamber, and Special Cover Glass.
Neubauers Counting Chamber: The counting chamber is heavy glass slide in the center of which are two ruled platforms. These are separated from each other by one moat and from elevated bars on each side by transverse moat. These lateral bars are so ground that a cover slip resting on them lies exactly 0.1mm above the ruled platforms. On each platform is engraved a ruled area divided into 16 smaller squares whereas central square being divided into 400 smaller squares arranged into 25 groups, each of 16 squares separated by triple boundary lines. Each of the largest squares is 1mm on the slide, or sq. mm in area.

RBC diluting pipette: It has red bead in the bulb to assist in mixing and diluting the blood. Mouth piece is of red color, the pipette has marks of 0.5, 1.0 and 101. The first two marks are below the bulb and the last above the bulb.

WBC diluting pipette: It has white bead in the bulb to assist in mixing and diluting the blood. Mouth piece is also of white color. On the pipette marks are of 0.5, 1.0 and 11. The first two marks are below the bulb and the last mark above the bulb.

Special cover slip: It is made up of thick glass. Its weight is such that it will help in spreading a drop of diluted blood to the thickness of 0.1mm only. It will not float over the blood drop due to its own weight. Its length and breadth is also adjusted to that of two lateral bars of the chamber. It’s available in thickness of 0.3, 0.4 and 0.5 mm and in two sizes 16X22 mm and 22X22 mm.

Diluting Fluids:

A. For counting poultry blood cells: For erythrocytes counts, diluting fluid described below can be used. For WBC count different stains are used which stains WBC and RBC differently and makes counting easy. With ordinary WBC diluting fluid, erythrocytes may be hemolysed but the nuclei appear prominently and interfere in distinguishing the leucocytes from the same.

Natt, M. P. and Herrick, C. A. suggested diluting fluid

\[
\begin{align*}
\text{NaCl} & \quad 3.88 \text{ g} \\
\text{Na}_2\text{SO}_4 & \quad 2.50 \text{ g} \\
\text{Na}_2\text{HPO}_4\cdot12\text{H}_2\text{O} & \quad 2.91 \text{ g} \\
\text{KH}_2\text{PO}_4 & \quad 0.25 \text{ g} \\
\text{Formalin (37%)} & \quad 7.50 \text{ cc} \\
\text{Methyl violet 2B} & \quad 0.10 \text{ g} \\
\end{align*}
\]

Made to one liter with distilled water

Following diluting fluid can be used for RBC and WBC counting.

A. Stock solutions:
1. 2% Sodium citrate
2. 0.1% Gentian violet
3. 0.1% Brilliant cresyl blue in ringer’s solution
4. Neutral formalin

Composition of Ringer’s solution:

\[
\begin{align*}
\text{Sodium chloride} & \quad 0.7 \text{ g} \\
\text{Sodium bicarbonate} & \quad 0.07 \text{ g} \\
\text{Potassium chloride} & \quad 0.026 \text{ g} \\
\end{align*}
\]
 Calcium chloride 0.003 g  
 Distilled water 100 ml

**B. Working solution:**
Mix the above solution in the proportion of 1ml of 1, 2ml of 2 and 3 drops of 4 from above stock solution. Made to one liter with distilled water. Mix and filter. The mixture is prepared daily before making the counts.

With the working solution, erythrocytes take a very light blue color. Granulocytes are seen prominently. Heterophils, monocytes and lymphocytes can be identified easily. Thrombocytes can be easily being counted.

**B. For mammalian blood cells:**

**I. RBC diluting fluids:**
A. Haymes fluid:
1. Na$_2$SO$_4$ 2.50 g (as suspending agent lowers surface tension and prevents rouleaux formation)
2. NaCl 0.50 g (prevents hemolysis)
3. HgCl$_2$ 0.25 g (antiseptic and fixative)
4. Distilled water 100 ml

B. Govers fluid:
1. Na$_2$SO$_4$ 6.25 g
2. Glacial acetic acid 16.65 g
3. Distilled water 100 ml

C. Physiological saline:
0.85 % NaCl in distilled water.

**Note:** Haymes fluid because of HgCl$_2$ causes agglutination of bovine erythrocytes. Of the above diluting fluids, physiological saline is the best.

**II. WBC diluting fluid:**
A. Thomas fluid:
1. Glacial acetic acid 2ml (shining to WBC and lyse the RBC)
2. 1% Gentian violet or methyl blue 1 ml (give color to nucleus)
3. Distilled water 100 ml

**Note:** This is hypotonic solution for RBC. The hemoglobin being lost the ghost of the red cells becomes invisible under low power.

**Filling the pipette:**
The blood may be drawn from a freely flowing skin puncture wound. If the drop of blood is too small or the tip of the pipette is not kept immersed air bubbles will enter with the blood. The column of blood be drawn slightly above the desired mark, it may be lowered by blowing out or touching the tip of the pipette to a tip of finger.

**Charging the counting chamber:**
Counting chamber and cover slip should be absolutely clean and free from grease. Cover slip should rest evenly on the supporting platforms. After the pipette is shaken, the first few drops are discarded then the pipette tip is touched to the side of the counting chamber and the fluid allowed to run in flow of fluid into the chamber is controlled by pressure on the rubber tubing. If the cover slip is floated off or fluid escapes into the trenches on the sides of counting platform, clean the chamber and repeat the procedure.
Counting:

Let the cells settle for one or two minutes so that the cells are in same plane. Focus the square to be counted. Adjust the mirror, diaphragm so that the lines and the cells are clearly seen. Start counting from the upper left hand square, count the cells in each of the 16 small squares. In order to avoid counting the same cells twice and in order to correctly count those cells that touch the other boundary lines and ignore those cells that touch the right and lower boundary lines. Cells touching bottom and left center boundary lines are counted. Cells touching bottom and right center boundary lines are not counted.
PRACTICAL NO. 12

AIM: DIFFERENTIAL LEUCOCYTES COUNT (DLC) FROM BLOOD FILM

Enumeration of the number of each type of the white cells is called ‘the differential count’. It is carried out under an oil immersion lens on a stained blood film.

Requirements:
A well stained blood smear, Cedar wood oil, Microscope with an oil immersion lens and mechanical stage, Paper and Pencil.

Procedure:
1. Mount a well stained film on a mechanical stage of a microscope.
2. Focus microscope using low power and a concave mirror.
3. Bring one corner of the film in view.
4. Adjust under high power and then oil immersion. Use plane mirror here. Put a drop of cedar wood oil prior to the adjustment of the field under oil immersion. Raise the condenser. Keep diaphragm opened fully.
5. Use battlement method and identify the leucocytes coming in the field.
6. Enter these cells in a checker board having 100 squares. After identifying each cell, a first letter of the cell is entered in the square ie. N for Neutrophil, B for Basophil, E for Eosinophil, M for Monocyte and L for Lymphocyte.
7. Sum up each type of cells, when 100 leucocytes have been counted. The value gives percentage value of different types of leucocytes.
8. Confirm that the total number of cells counted and the sum total of all types are hundred.

Errors are frequently caused when rare and atypical cells occur in the field. In such cases, such cell is ignored. It is desirable to have a cool and methodical approach. Whenever in doubt decide the following points one after another.

Look to the cytoplasm of leucocytes whether it is granular or agranular.

A. If cytoplasm is agranular – Then the cell is either Lymphocyte or Monocytes.
   1. If the size of this cell is approximately to the nearest erythrocyte, then it is small lymphocytes.
   2. If the size is larger – sufficient to accommodate two or more RBC, then it may be larger lymphocytes or monocytes. They can be differentiated by –
      a. The amount of cytoplasm, which is less in a lymphocyte and more in monocytes.
      b. The cytoplasm of lymphocyte may show the violet stippling granules; while that of monocytes is powdery and may show only one or two red shaped red Auer’s bodies.
      c. The lymphocyte nucleus shows deeply stained chromatin material; while monocytes nucleus may show nucleoli.
      d. The nucleus of monocytes is wrinkled; that of lymphocytes is smooth and solid.

B. If the white cell cytoplasm is granular - Then cells are Neutrophil, Basophil, or Eosinophil.
   1. If acidic granules (red), usually bi-lobed – Eosinophils
   2. If basophilic granules (blue) – Basophils
3. If neutral granules, lobes variable (faintly lilac stained cytoplasm) – Neutrophils

Table:

Percentage of each leucocyte in domestic animals (Dukes’ Physiology of Domestic Animals, 8th Edi, 1970, p46)

<table>
<thead>
<tr>
<th>Animal</th>
<th>Neutrophils</th>
<th>Eosinophils</th>
<th>Basophils</th>
<th>Lymphocytes</th>
<th>Monocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cattle</td>
<td>25-30</td>
<td>2-5</td>
<td>1</td>
<td>60-65</td>
<td>5</td>
</tr>
<tr>
<td>Horse</td>
<td>50-60</td>
<td>2-5</td>
<td>1</td>
<td>30-40</td>
<td>5-6</td>
</tr>
<tr>
<td>Sheep</td>
<td>25-30</td>
<td>2-5</td>
<td>1</td>
<td>60-65</td>
<td>5</td>
</tr>
<tr>
<td>Goat</td>
<td>35-40</td>
<td>2-5</td>
<td>1</td>
<td>50-55</td>
<td>5</td>
</tr>
<tr>
<td>Dog</td>
<td>65-70</td>
<td>2-5</td>
<td>1</td>
<td>20-25</td>
<td>5</td>
</tr>
<tr>
<td>Cat</td>
<td>55-60</td>
<td>2-5</td>
<td>1</td>
<td>30-35</td>
<td>5</td>
</tr>
<tr>
<td>Chicken</td>
<td>25-30</td>
<td>3-8</td>
<td>1-4</td>
<td>55-60</td>
<td>10</td>
</tr>
<tr>
<td>Pig (1 day old)</td>
<td>70</td>
<td>2-5</td>
<td>1</td>
<td>20</td>
<td>5-6</td>
</tr>
<tr>
<td>Pig (6wk older)</td>
<td>30-35</td>
<td>2-5</td>
<td>1</td>
<td>55-60</td>
<td>5-6</td>
</tr>
</tbody>
</table>
PRACTICAL NO. 13

AIM: DETERMINATION OF BLEEDING TIME

The time elapsed between the moment of escape of blood outside vessel and time of cessation of its flow is called the bleeding time.

REQUIREMENTS:

Piece of filter paper, Needle, Cotton, Stopwatch.

PROCEDURE:

1. Choose a suitable site for puncturing the capillaries (ear-lobe).
2. With all aseptic precautions, inflict a puncture would so that the blood flows drop by drop, without any squeezing.
3. Note the time as the first drop appears.
4. With a piece of filter paper gently blot the blood. Do not touch the skin. Do not press the wound.
5. Repeat with a fresh piece of filter paper every ten seconds till it shows the Blot of blood on it.
6. Note the time bleeding stops spontaneously.
7. The time interval between the steps 3 to step 6 is the bleeding time.

The normal bleeding time is 1 to 3 minutes. It is normally always shorter than the coagulation time due largely to the ability of the tissue juices to promote coagulation.
PRACTICAL NO. 14

AIM: DETERMINATION OF CLOTTING TIME AND CLOT RETRACTION TIME

Clotting Time:

PRINCIPLE:
The time elapsed between the moment of escape of blood outside the vessel and the moment of development of the fibrin thread is defined as the ‘coagulation time’. Coagulation time is longer than bleeding time normally. Prolonged coagulation time may be diminished (a) thromboplastin (b) prothrombin (c) fibrinogen and (d) the presence of an anticoagulant in the blood.

REQUIREMENTS:
Thin capillaries, Stopwatch.

PROCEDURE:
1. Collect the venous blood with usual antiseptic precautions. (in case of blood collected from capillaries traumatization of tissue juice occur they shorten the coagulation time. Therefore, method employed with venous blood is more accurate then that employing capillary blood). Note the time.
2. Fill three clean and smooth capillaries with blood.
3. Place the tubes on a table.
4. At the end of 3 minute, break off about 1 cm. length of tubing every 30 seconds.
5. Note the time when fibrin thread are found between two broken ends of the capillaries separated at a distance of 5 mm or more.
6. Calculate the time taken between the appearance of blood and the filling of capillaries and the appearance of fibrin thread. This is coagulation time.

Table:
Coagulation time at 25°C in animals by Amendt with Bürkers method.
(The Physiology of Domestic Animals by H.H. Dukes, 7th edi.)

<table>
<thead>
<tr>
<th>Animal</th>
<th>Coagulation time</th>
<th>Animal</th>
<th>Coagulation time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cow</td>
<td>4.5</td>
<td>Goat</td>
<td>2.5</td>
</tr>
<tr>
<td>Rabbit</td>
<td>4.0</td>
<td>Ox</td>
<td>6.5</td>
</tr>
<tr>
<td>Dog</td>
<td>2.5</td>
<td>Horse</td>
<td>11.5</td>
</tr>
<tr>
<td>Pig</td>
<td>3.5</td>
<td>Man</td>
<td>5.0</td>
</tr>
<tr>
<td>sheep</td>
<td>2.5</td>
<td>Cat</td>
<td>1-3</td>
</tr>
</tbody>
</table>

Clot Retraction Time:

PRINCIPLE:
Retractibility of a blood clot closely parallels the number of the thrombocytes. When these are numerically reduced, it is delayed, partial or poor. Retractibility is independent of the coagulation time. The blood may coagulate in normal time but retractibility may be very poor. It is prolonged in thrombocytopenia purpura but normal in hemophilia.
**PROCEDURE:**

1. Collect venous blood and place 3 to 5 ml of blood in a chemically clean test tube.
2. Stopper with cotton and place in an incubator at 37 ºC.
3. Observed the tube at 1 hr, 8 hr and 24 hrs.
4. Normally, retraction of clot and expression of serum are appreciable after 1 hour and marked after 18 hours. Occasionally, however, the clot of even normal blood fails to separate from the walls of the tube. If the clot is loosened with a platinum wire, however, retraction occurs promptly.
PRACTICAL NO. 15  

DATE:

AIM: DETERMINATION OF BLOOD GROUPS

Many blood groups systems have been elucidated. The important are ‘ABO system’ ‘MN and P system’ and ‘Rh system’. The study of genetics, (origin and development of man) study of association and proneness to diseases study of association with animal health and production there by helping in the selection of animal at early age for future production. General principle & technique of blood grouping is described here.

REQUIREMENT:

Slides, 0.9% saline, Type anti-sera A, B, and anti-Rh factor, Glass marking pencil.

METHOD:

(A) Slide Technique – ABC System.

1. Make a large slide with wax pencil into three equal compartments.
2. Place one drop of anti-A grouping serum in the left compartment, one drop anti-B serum in the central compartment and one drop of anti A+B serum in the right compartment.
3. Add to each serum a small quantity of whole blood equal to one half of the volume of serum.
4. Mix well with the aid of separate applicator.
5. Observe for agglutination for two minutes, not large. No heat should be applied to the slide.
6. In doubtful cases repeat the test with one drop washed erythrocytes.
7. Interpretation for the test is given below.

Interpretation of the slide method test.

<table>
<thead>
<tr>
<th>Anti-A Serum</th>
<th>Anti-B Serum</th>
<th>Anti-A+B Serum</th>
<th>Blood Group Identified</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>-</td>
<td>+</td>
<td>A</td>
</tr>
<tr>
<td>-</td>
<td>+</td>
<td>+</td>
<td>B</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>+</td>
<td>AB</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>-</td>
<td>O</td>
</tr>
</tbody>
</table>

Note: Sign + means agglutination

(B) Rh Testing: Slide method

The red blood cells, to be tested should not be more that three day old.

1. Warm the slide and put a drop of Anti-D on it.
2. Add one drop of oxalated blood mix and read the reaction. In doubtful cases, repeat the test with one drop washed cells.
3. Clear agglutination should appear within one minute.
4. Negative result must be confirmed by the Albumin.
**Albumin Replacement Technique:**

1. Prepare a 2 percent calls suspension of the red blood cells to be tested in isotonic saline.
2. Add one volume of the anti-Rh (Anti-D) serum, to one of the test cell suspension, in precipitation tube.
3. Incubate at 37 degree for one hour.
4. Centrifuge and remove the supernatant saline serum mixture leaving the button of cells, at the bottom undisturbed.
5. Add one volume of 20% bovine albumin on the surface of the bottom of cells. Do not shake.
6. Incubate at 37˚C for ten minutes.
7. Centrifuge and read the results by gently shaking the tube. Negative results must be conformed under the microscope.
8. Keep the following controls each time you do the test controls
   (i) O Rho (D) positive cells.
   (ii) O Rho (D) negative cells.
AIM: DETERMINATION OF OSMOTIC FRAGILITY RANGE FOR THE GIVEN SAMPLE OF BLOOD

When erythrocyte are placed in any solution the effect of osmosis is observed over the cell if the osmotic pressure of the solution is either low (hypotonic) compared to the osmotic pressure of the red cells the term used for solution depending upon its relative osmotic pressure of solution with that of erythrocytes are (a) isotonic (b) hypotonic (c) hypertonic.

When erythrocyte s are placed in hypotonic solution the water absorbed by the cell and the biconcave flat cell tends to become spherical. As a result total mass of the cells increases while surface area remains same which leads to change in the shape of the cell from biconcave to biconvex. Generally biconcave cell accommodates larger quantities of water as compared to biconvex. Therefore, biconcave cells can stand very low hypotonic solution. The volume of cells- biconcave or biconvex will go on increasing to a certain limit, after which the cell meshwork is stretched and ultimately the cell membrane ruptures which is accompanied with the escape of hemoglobin – that is they get hemolysed. Thus the rupture (fragility) of erythrocyte due to lower osmotic pressure (osmosis) of the solution is called osmotic fragility. The osmotic fragility is expressed in terms of concentration of saline solution in which they hemolysed. The results are expressed in the range between highest and lowest strength of saline solution. The highest concentration of the range is one when the few cells start hemolysing. The lowest concentration of the range is the concentration of first step containing saline in which all the cells are hemolysed (100% hemolysed).

If the erythrocyte are more fragile (due to shape, degree of sphereocytosis or any other reason), they show hemolysis even in higher concentration of the saline.

Normal osmotic range for human erythrocyte is 0.40 – 0.33 i.e. normal blood shows slight hemolysis at 0.40 (g% NaCl). The fragility is greater under venous blood condition, as the diameter of venous blood erythrocyte is larger compared to that of arterial blood erythrocytes.

REQUIREMENTS:
Test tubes, Test tube stand, Dropper, Glass pencil, 0.85% NaCl solution, 1% NaCl solution, Distil water and Blood sample (oxalated).

PROCEDURE:
1. Label all the clean and dry test tube as I, H, 1, 2…..up to 9 and W.
2. Pipette accurately 1% NaCl, 0.85% NaCl, solutions and distilled water in the following proportions.
3. Shake the tube well to mix the content.
4. Add a blood drop of uniform size each tube with the help of a dropper.
5. Mix the blood with the saline by complete but gentle inversion. Shaking may induce haemolysis and yield false results.
6. Keep them on the stand undisturbed.
7. Examine preferably after two hours or centrifuge the tube after an hour.
8. Examine the tubes without disturbing them.
9. The test tube labeled I (isotonic) and H (hypertonic) will not show any change in colour of solution because of absence haemolysis in this tube.
10. Tube labeled W will show complete (100%) hemolysis.
11. Other tube from 1 to 9 will show different intensity of red color depending upon the degree of hemolysis.
12. Tube I has clear colorless supernatant because of absence of hemolysis. Red blood cell can maintain their normal shape and size in this solution. This can be studied under microscope. Such solution, which does not alter the morphology of erythrocyte, is called isotonic solution. In other words such solution preserves normal shape and size of the cell.

13. Match the supernatant of tube I with other tube in series. Find the tube, which contain faint red colored supernatant. The faint red coloration of the supernatant is indicative of staining of hemolysis. Here all erythrocytes have not hemolysed but very few have hemolysed. The solution is called hypotonic solution. This is the first higher reading of osmotic fragility in hypotonic range. Suppose it is tube No.4 containing 0.46% NaCl solution. Any solution in the series having strength higher than 0.46% NaCl will not show hemolysis.

14. As the strength of sodium chloride in the solution decrease the solution become more and more hypotonic in the subsequent tubes. As a result more and more erythrocyte get hemolysed resulting in to increase in the degree of hemolysis reaching maximum to 100%. This can be spotted with the help of W tube.

15. In a series of test tube find the first tube of higher concentration which contains red colored supernatant having its intensity similar to that of red colored supernatant of W tube. Comparing of red colored supernatant of the other tubes can identify this. This is the second reading of the osmotic fragility in hypotonic range showing the concentration in which all erythrocyte have got hemolysed complete hemolysis. For example, if it is tube No. 7 containing 0.34% NaCl solution. Any solution the series containing less than 0.34% NaCl will show complete hemolysis.

Suppose the result obtained in this experiments are 0.46% and 0.34%. This the osmotic fragility ranges. Both above solution of NaCl are hypotonic for erythrocyte but in 0.46% the degree of hemolysis is less which increases subsequently in the other tube and is complete in the solution of 0.34% NaCl and below it.

16. Tube H (hypertonic) has cleared colorless supernatant as that of I tube indicating absence of hemolysis. But then erythrocyte shrink in H tube but they do not shrink in I tube. Shrinkage results into crenation of cells, which can be demonstrated under the microscope. Such solutions are hypertonic solutions.

<table>
<thead>
<tr>
<th>Test tube No.</th>
<th>1% NaCl (ml)</th>
<th>D. W. (ml)</th>
<th>0.85% NaCl (ml)</th>
<th>Total Volume (ml)</th>
<th>Total Strength (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>-</td>
<td>-</td>
<td>5.0</td>
<td>5.0</td>
<td>0.85</td>
</tr>
<tr>
<td>H</td>
<td>5.0</td>
<td>-</td>
<td>-</td>
<td>5.0</td>
<td>1.0</td>
</tr>
<tr>
<td>1</td>
<td>2.9</td>
<td>2.1</td>
<td>-</td>
<td>5.0</td>
<td>0.58</td>
</tr>
<tr>
<td>2</td>
<td>2.7</td>
<td>2.3</td>
<td>-</td>
<td>5.0</td>
<td>0.54</td>
</tr>
<tr>
<td>3</td>
<td>2.5</td>
<td>2.5</td>
<td>-</td>
<td>5.0</td>
<td>0.50</td>
</tr>
<tr>
<td>4</td>
<td>2.3</td>
<td>2.7</td>
<td>-</td>
<td>5.0</td>
<td>0.46</td>
</tr>
<tr>
<td>5</td>
<td>2.1</td>
<td>2.9</td>
<td>-</td>
<td>5.0</td>
<td>0.42</td>
</tr>
<tr>
<td>6</td>
<td>1.9</td>
<td>3.1</td>
<td>-</td>
<td>5.0</td>
<td>0.38</td>
</tr>
<tr>
<td>7</td>
<td>1.7</td>
<td>3.3</td>
<td>-</td>
<td>5.0</td>
<td>0.34</td>
</tr>
<tr>
<td>8</td>
<td>1.5</td>
<td>3.5</td>
<td>-</td>
<td>5.0</td>
<td>0.30</td>
</tr>
<tr>
<td>9</td>
<td>1.3</td>
<td>3.7</td>
<td>-</td>
<td>5.0</td>
<td>0.26</td>
</tr>
<tr>
<td>W</td>
<td>-</td>
<td>5.0</td>
<td>-</td>
<td>5.0</td>
<td>0.00</td>
</tr>
</tbody>
</table>
**INFERIENCE:**
   
   All solution that is prepared for intravenous injection should be isotonic in order to preserve the morphology of erythrocytes.

   In certain pathological condition this destruction is greatly increased, usually resulting in hemolytic anemia. Under these circumstances it is of clinical value to determine whether excessive haemolysis is due chiefly to increased fragility of the erythrocytes, as in congenital hemolytic jaundice, or to a toxic haemolytic agent in the blood causing the excessive haemolysis or erythrocyte of comparatively normal fragility.

**Table:**

**Osmotic fragility range of erythrocytes of different species of animals.** (Dukes Physiology of Domestic Animal. Edi. 8th, 1970, page 36.)

<table>
<thead>
<tr>
<th>Species of animal</th>
<th>Strength of NaCl solution (g per 100 ml)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>For initial heamolysis</td>
<td>For complete heamolysis</td>
<td></td>
</tr>
<tr>
<td>Cattle</td>
<td>0.59</td>
<td>0.42</td>
<td></td>
</tr>
<tr>
<td>Dog</td>
<td>0.45</td>
<td>0.36</td>
<td></td>
</tr>
<tr>
<td>Goat</td>
<td>0.62</td>
<td>0.48</td>
<td></td>
</tr>
<tr>
<td>Horse</td>
<td>0.59</td>
<td>0.39</td>
<td></td>
</tr>
<tr>
<td>Pig</td>
<td>0.74</td>
<td>0.45</td>
<td></td>
</tr>
<tr>
<td>Sheep</td>
<td>0.60</td>
<td>0.45</td>
<td></td>
</tr>
<tr>
<td>Cat</td>
<td>0.58</td>
<td>0.47</td>
<td></td>
</tr>
<tr>
<td>Chicken</td>
<td>0.40</td>
<td>0.28</td>
<td></td>
</tr>
<tr>
<td>Rabbit</td>
<td>0.57</td>
<td>0.45</td>
<td></td>
</tr>
</tbody>
</table>

**Table:**

**Osmotic pressure of blood (average in equivalent concentration of NaCl, grams per 100 ml)** (Dukes, H.H., 7th edition. Page 27)

<table>
<thead>
<tr>
<th>Species</th>
<th>Osmotic Pressure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sheep</td>
<td>0.978</td>
</tr>
<tr>
<td>Goat</td>
<td>0.955</td>
</tr>
<tr>
<td>horse</td>
<td>0.927</td>
</tr>
<tr>
<td>cow</td>
<td>0.936</td>
</tr>
<tr>
<td>Dog</td>
<td>0.933</td>
</tr>
<tr>
<td>Man</td>
<td>0.945</td>
</tr>
</tbody>
</table>
PRACTICAL NO. 17

AIM: DETERMINATION OF VISCOSITY OF BLOOD / PLASMA / SERUM

PRINCIPLE:
Viscosity is the frictional force present in all fluids which are flowing. Fluids are called as Newtonian and non-Newtonian fluid. Newtonian fluids show a definite and constant relation between the resistance offered and the force applied. But such relation is absent in all body fluids. Therefore, all body fluids are non-Newtonian in nature. In such fluids the applied force is utilized in changing the shape and the orientation of the molecule of the liquid. Therefore, in absence of any coefficient of viscosity one can measure only the apparent viscosity of fluids. The shape of the molecule decides viscosity more than the size and the weight. E.g. rod shape of fibrinogen gives a more viscous solution as compared to the one of albumin of same concentration.

Apparent viscosity is determined under a given temperature and pressure by nothing the time of flow of a given volume of blood/plasma/serum through a specially prepared Ostwalds viscometer and comparing the time taken by the same volume of distilled water to pass through the same tube under conditions prevailing for blood. The unit of viscosity is poise.

REQUIREMENTS:
Retort stand, Ostwalds viscometer, Stop watch, Pipette, Blood sample added anticoagulant, Distilled water.

PROCEDURE:
1. Fix the Ostwald viscometer to a retort stand in a perfectly vertical position.
2. Take 20cc of distilled water and suck through rubber tubing up to the mark ‘X’ above the small bulb.
3. Start the stopwatch immediately when the distilled water crosses the mark ‘X’ above the bulb.
4. Stop the watch when the meniscus reaches the mark below the small bulb. Note the time (in seconds) taken by water to pass from X to Y mark.
5. Dry the viscometer and repeat the experiment with 20ml of well mixed blood instead of distilled water.
6. Note the time taken (in seconds) for blood.

N.B. While performing the experiment remember the following points:
a. Volume of blood and water should be kept the same.
b. Room temperature should be kept same both for blood and distilled water timings.
c. Viscometer should not be changed.

CALCULATION:
Viscosity ratio = Time ratio X Density ratio
Therefore,

\[
\text{Viscosity of blood} = \frac{\text{Blood time (sec)}}{\text{Water time (sec)}} \times \frac{\text{Density of blood}}{\text{Density of water}}
\]
Taking density of water as unity and that of blood as 1.06

\[
\text{Blood time (sec)} \times 1.06 \\
\text{Water time (sec)} \times 1.06
\]

Table: Viscosity of blood of different species of animals:

<table>
<thead>
<tr>
<th>Animal</th>
<th>Viscosity (poise)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cow</td>
<td>6.09</td>
</tr>
<tr>
<td>Sheep</td>
<td>4.10</td>
</tr>
<tr>
<td>Goat</td>
<td>4.30</td>
</tr>
<tr>
<td>Dog</td>
<td>4.00</td>
</tr>
<tr>
<td>Pig</td>
<td>4.20</td>
</tr>
<tr>
<td>Cat</td>
<td>4.60</td>
</tr>
<tr>
<td>Fowl</td>
<td>5.90</td>
</tr>
</tbody>
</table>
PRACTICAL NO. 18

AIM: MEASUREMENT OF BLOOD PRESSURE AND CENTRAL VENOUS/ARTERIAL PRESSURE (SPHYGMOMANOMETRY)

BLOOD PRESSURE:
Blood pressure is the lateral pressure exerted by blood on the wall of the blood vessels it is measured (a) Experimentally (Direct method) and (B) Clinically (Indirect method).

(A) **Experimental or Direct method:**
For measuring the blood pressure by direct method mercury manometer is used, carotid or femoral artery of an anaesthetized animal is cut open and “T” shaped arterial Cannula is attached to it the Cannula joins with mercury manometer by means of a rubber tube The manometer with the rubber tubing in the arterial side is filled with 3.8% sodium citrate solution. The inner limb of the manometer carries a float with a recording tip, which directly writes on the smoked paper of the kymograph. The sodium citrate acts as an anticoagulant for the blood entering in the rubber tubing usually mean blood pressure is obtained. Animal used in the experiment is generally sacrificed. In the experimental or direct method, detailed method detailed methodology is given as under.

MEASUREMENT OF CENTRAL VENOUS PESSURE (CVP) AND ARTERIAL PESSURE:
Materials and requirements:
1. Animal (Dog or calf)
2. Anaesthetic agent (Nembutal sodium)
3. Cannula (Arterial and venous)
4. Research kymograph
5. Plasma expander
6. 3.8% sodium citrate solution

1. Preparation of animals
The hairs over neck and medium aspect of the thigh region should be shaved. The skin is thoroughly washed with soap and water and applies disinfectants.

2. Anaesthetizing the animals
If needed, phenobarbitone sodium at a rate of 75 mg/kg body weight is given intravenously (I/V) as a preanesthetic Thiopentane sodium 2.5% solution is injected I/V with the animal enter into the stage of general/surgical anesthesia. In case of animals regaining consciousness the same is injected in smaller amount

3. Placement of Central Venous Catheter
(a) Keep anaesthetized animal in supine position on the table.
(b) A skin incision of 1 inch is given on medial aspect of right thigh 1 to 2 inches away from the pubic bone.
(c) Examine femoral vein and artery running parallel to each other in this region
(d) Dissecting the underlying connective tissue and muscles expose 1 to 2 inches portion of femoral blood vessels
(e) The medial end of femoral vein is ligated and a V shaped nick incision is given in the femoral vein.
(f) In order to facilitate the introduction of the venous polyethylene catheter into the femoral vein proceed as follows-
(i) One end of the full curved needle is first introduced into the vein which is slightly lifted in order to make space for the entry of venous catheter.

(ii) Through a three-way stopcock cannula which connects the water manometer on the other end, the catheter is introduced into the femoral vein below the needle.

(iii) Once the tip of the catheter enters into the vein to a distance of 1 cm or so the needle is withdrawn and the venous catheter is advanced slowly towards the heart with the tip positioned in the main stream of the vein. The catheter is pushed further until it reaches the inferior vena cava, in case of any difficulty, withdraw it back, reintroduce in the passage. The fluctuations start appearing in the column of fluid (plasma expander or saline) filled in the water manometer. These fluctuations indicate the correct placement of venous catheter.

(g) The zero reference point of the manometer is fixed with the help of a special pointer devised for the purpose.

(h) The zero reference point of the saline manometer scale is placed at the level of right atrium. Which is one half ways between the sternum & spinal cord?

(i) Before recording the CVP the manometer is filled in with the plasma expander or saline up to 200mm. thereafter connect the manometer with the venous catheter by turning the stop cock.

(j) The column of fluid in the manometer gradually drops to the pressure in the venous reservoir, which is taken as initial CVP.

4. **Fixation of the arterial cannula:**
   
   (a) Give the skin incision of 1-inch length just lateral to the trachea on left side of the neck
   
   (b) Bluntly dissect the underlying connective tissue and muscles to expose the common carotid artery.
   
   (c) Lift up the artery and remove the fascia attached to it
   
   (d) Ligate the cephalic end of the carotid artery.
   
   (e) Empty about an inch of the carotid artery by pushing the blood towards heart and apply an artery forceps on the cardiac end of the artery to check the flow of blood.
   
   (f) Hold the carotid with index finger and give a small V shaped nick incision on it with the help of sharp, pointed scissors.
   
   (g) Insert and fix the arterial cannula and into the carotid artery by tying it with the help of silk thread.
   
   (h) Fill the whole recording system (arterial cannula, polyethylene tube empty portion of U tube of mercury manometer with 3.8% sodium citrate solution.
   
   (i) Remove the artery forceps from the carotid artery to connect it with mercury manometer.
   
   (j) With the help of writing device fitted in the mercury manometer record the arterial pressure on the smoked paper fitted in the research kymograph. The result of arterial pressure expressed as mm Hg.

**(B) Clinical or Indirect method:**

In this method for measurement of B.P. Sphygmomanometer is used. The principle is that blood flow through large sized artery is obstructed by means of air compression exerted through an air tight rubber bag (cuff) wrapped around forearm. The bag is slowly inflated with a rubber bulb. When the air transmitted through the artery is equal to or little more than the blood pressure, the blood flow stops. When the pressure is slowly released from the rubber bag the blood enters through the obstacle into the blood vessels. This can be studied by three methods:
B.1 Feeling the pulse  
Palpatory method

B.2 Observing the oscillation of mercury level  
Oscillatory method

B.3 Hearing with a stethoscope the hissing sound produced in the segment of the artery distal to the obstruction
Auscultatory method

B.1 Palpatory method:

The sphygmomanometer cuff is tied round the forearm and the pulse is felt at a suitable site (generally in the region of wrist). The cuff is then inflated after closing the screw wall of the rubber bulb rapidly to compress the artery with the pressure usually little more than the expected systolic pressure. As the compressed pressure is raised beyond the systolic pressure the pulse disappears. Screw valve is loosened to let out the air in order to reduce the air pressure. At this time try to feel the pulse and watch the mercury level. The mercury level at which the pulse first reappear is taken as the systolic blood pressure reading. The palpatory method gives only the systolic pressure measurement. Though the result obtained by this method is very accurate but form a sound control over the methods.

N.B. Diastolic pressure cannot be determined by the palpatory method for B.P. measurement.

B.2 Oscillatory method:

In this method the screw valve of the rubber bulb is closed and the air is pumped to a level about that of the expected systolic pressure. Then the air pressure on the cuff is slowly reduced by opening the screw valve carefully, while all the time watching the mercury level in the manometer. When the pressure becomes equal to the systolic level the mercury level starts oscillating. The mercury level at the commencement of the oscillation is taken as the systolic blood pressure reading. The reduction of air pressure within the cuff is further continued. There will be an increase in the amplitude of oscillation of mercury level. This oscillation reaches a climax (zenith) and then the amplitude gets reduced finally to disappear. The pressure at which the mercury level shows maximum amplitude of the oscillation is taken as the diastolic pressure.

B.3 Auscultatory method:

A clinical stethoscope along with mercurial sphygmomanometer is needed for recording B.P. by this method. The air pressure in the cuffs is raised as usual beyond the expected systolic level. The chest piece of the stethoscope is placed on the area of the blood vessel (Brachial artery on the medial side). No sound is heard, as the artery remains collapsed due to higher air pressure created in the cuff. Decrease the pressure slowly and carefully by operating the screw valve of the bulb. As the blood pressure becomes equal or just less than the systolic blood pressure the blood is able to slip towards the artery through the obliterated segment of the vessel during each systole. Each sound synchronizes with the systole of the heart. These sounds are named as ‘korotkoff’ sounds after the Russian scientist (Nicolai Sergeevich Korotkoff in 1905). As the air pressure in the cuff is decreasing progressively the characters of sound changes. They are recognized under four phases:

First Phase: The sound appears suddenly and is usually clearly heard as trapping or click sound. The phase continues till pressure is reduced by about 10 to 14 mm Hg. It is taken as the criterion of the SYSTOLIC B.P.
Second Phase: It begins at the end of first sound and at the beginning of second phase. It is of murmural quality and continues during the fall of next 15 to 20 mm Hg pressure.

Third Phase: At the end of second phase, sound becomes clear and louder, indicating the onset of third phase. It continues during the fall of next 5 to 7 mm Hg pressure.

Fourth Phase: At the beginning of this phase sound becomes muffled suddenly. It continues for a fall of about 5 to 6 mm Hg pressure. The reading of mercury level at appearance of muffle sound is taken as the DIASTOLIC B.P.

Fifth Phase: At the end of the fourth phase the sound may slowly and suddenly disappear.

It is impossible to give any satisfactory explanation in terms of physical signs of sound (vibrations) for these sounds and their relation to the blood pressure. The empirical observations have been found reasonably accurate from the direct experimental measurement of blood pressure in animals and men.

Calculations:

\[
\text{Mean pressure} : \quad \frac{\text{Systolic pressure} + \text{Diastolic pressure}}{2}
\]

Or

\[
\text{Mean pressure} : \quad \frac{1}{3} \times \text{Pulse pressure} + \text{Diastolic pressure}
\]

(Dukes’ Physiology of Domestic Animal, 12th Ed.)

Pulse pressure : \quad \text{Systolic pressure} - \text{Diastolic pressure}
PRACTICAL NO. 19

AIM : IN-VITRO SALIVARY DIGESTION AND ACID HYDROLIZATION OF THE RAW AND COOKED STARCH

Principle:
The ptyalin is a salivary alpha-amylase, which is present in the majority of animals, including human subjects except sheep, goat and dog. Ptyalin splits the starch into maltose at body temperature. The acid does the same by utilizing heat as energy source. The hydrolization of the starch can be tested by the change in the color after addition of the iodine and the end products with the Fehling’s or Benedict’s reagent.

Material and Requirements:
1. Boiled starch solution 1%
2. Saliva / raw pealed potato
3. Gram’s iodine solution (0.2gm iodine grind with 0.4gm potassium iodide in 60ml of water)
4. Benedict’s reagent (CuSO₄.5H₂O 17.3 gm, Na Citrate 173.0 gm and Na carbonate 100.0 gm. Dissolve the citrate and carbonate in 700ml of water and filter. Dissolve the copper sulphate in 100 ml of water separately and add slowly to the first solution with stirring constantly. Dilute to one liter with distilled water.)
5. Water bath at 37 ºC
6. Test Tubes
7. Dilute hydrochloric acid

Procedure:
1. Take 1 ml of boiled starch solution in a tube and to it add equal volume of your own saliva.
2. Incubate this tube for 15 min. at 37 ºC temperature in the water bath.
3. In another tube, take 1 ml of starch solution and to it add 1 ml of dilute HCl solution. Keep this tube in boiling water bath for 30 minutes
4. After elapse of requisite time take out both the tubes and divided the contents into two portions.
5. One portion of both the tubes is tested for the presence of unhdyrolized starch with Gram’s iodine solution (1ml). If you get blue color, it shows that some starch is still left unhdyrolyzed and complete hydrolization is indicated by the absence of blue color.
6. Another portion of both the tubes is subjected to the Benedict’s test by taking 5 ml of the boiled reagent and adding eight drops of the portion to be tasted. After boiling and subsequently cooling down under running tap water the results are read as follows:
   a. No color - Indicates no reduction called negative test.
   b. Green color – Indicates the hydrolization of starch upto the level of dextrin.
   c. Pink/Red color – Indicates hydrolization upto maltose and
   d. Brick red color – Indicates complete hydrolization of the starch into maltose and glucose.
7. The similar experiment can be repeated using cut portion of raw potato. The raw start of the potato does not give blue color with iodine.
   (It is because the raw potato contains starch where alpha and beta amylo are not separated. In boiled starch both get separated and alpha amylo reacts with starch giving blue color.)
AIM: IN-VITRO PROTEIN DIGESTION BY TRYSIN

Principle:
The trypsin is an endopeptidase produced from pancreas which hydrolyzes native protein in the small intestine at pH 7.0 to 9.0.

Material and Requirements:
1. Native protein such as casein (milk protein) and fibrin threads which can be obtained after allowing the blood to get coagulated.
2. Trypsin solution (1%)
3. Sodium carbonate (1%)
4. Ninhydrin solution (1%)
5. Water bath at 37 ºC
6. Test Tubes

Procedure:
1. Mix in a tube 1 ml of native protein solution (Fibrin threads), 2 ml trypsin solution and 2 ml of 1% sodium carbonate solution. Keep the pH between 7.0 and 9.0. Alkaline pH can also be maintained by sodium hydroxide solution.
2. Incubate the above mixture at 37 ºC for 30 min to one hour.
3. Observe the dissolution of fibrin and its conversion into proteoses, peptones, polypeptides, short chain polypeptides and dipeptides.
4. The end products of tryptic digestion can also be tested by adding 1% Ninhydrin solution to obtain pink color.

Observation:
PRACTICAL NO. 21

AIM: IN-VITRO PROTEIN DIGESTION BY PEPSIN

Principle:
The pepsin is an endopeptidase which converts native proteins into proteoses, peptones and polypeptides at the pH of 1.5 to 3.2.

Material and Requirements:
1. Milk (calcium casein) or Egg albumin
2. Diluted hydrochloric acid (0.4 ml HCl in 100 ml distilled water)
3. Pepsin solution (1%)
4. Water bath at 37 ºC
5. Test tubes

Procedure:
1. To a milk sample add 2 ml of pepsin solution and 2 ml of HCl acid (0.4%).
2. Incubate the mixture of milk, pepsin and HCL acid at 37 ºC for 30 min to one hour.
3. Observe the formation of curd (calcium paracasein) and proteoses are liberated.
4. Calcium paracasein is further acted upon by the pepsin and finally proteoses, peptones and polypeptides are formed.

Observation:
PRACTICAL NO. 22

AIM : TO OBSERVED THE REDUCTION IN THE SURFACE TENSION AND EMULSIFICATION OF FAT BY THE BILE SALTS

Principle:
Bile salts cause the emulsification of fat by reducing the surface tension and thereby allowing the action of pancreatic lipase on the fat for its proper digestion and absorption.

Material and Requirements:
1. Sweet oil
2. Bile salts solution (Sodium taurocholate or glycocholate)
3. Sulphur powder
4. Test tubes
5. Distilled water

Procedure:
1. Take 5 ml of distilled water in a test tube and add fine sulphur powder to it. The sulphur powder does not sink down.
2. In a separate tube take distilled water and to it add equal amount of bile salt solution and then add sulphur powder in it. The sulphur powder now sinks down due to reduction in the surface tension of the fluid.
3. In a separate test tube take some distilled water and to it add a few drops of the sweet oil and mix thoroughly. The big fat globules are seen in the test tube.
4. Add few ml of bile salt solution and again mix it thoroughly. The big fat globules are now converted into fine fat droplets, which are known as emulsification of fat due to reduction in the surface tension of water.

Observation:
PRACTICAL NO. 23

AIM : EXAMINATION OF RUMEN FLUID

Examination of rumen fluid is routinely carried out in buitaric practice as they enable biochemical disorders of fare stomach digestion to be confirmed or excluded. Rumen fluid should be collected by using rumen fluid extraction pump or direct paracentesis from rumen. Rumen fluid can be kept in room temperature for 9 hrs and in refrigerator for 24 hrs.

**Physical examination**: Take 100 ml of rumen fluid in a beaker and conduct the following testes in a well lighted area.

1. **Color**:

<table>
<thead>
<tr>
<th>Normal color</th>
<th>In grazing</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yellowish brown</td>
<td>In straw feeding</td>
</tr>
<tr>
<td>Gray/brownish green</td>
<td>In concentration and straw feeding</td>
</tr>
<tr>
<td>Abnormal color</td>
<td>Rumen acidosis, simple inactivity of flora and fauna</td>
</tr>
<tr>
<td>Slightly milky</td>
<td>Chronic rumen acidosis</td>
</tr>
<tr>
<td>Milky green</td>
<td>Acute rumen acidosis</td>
</tr>
<tr>
<td>Greenish dark</td>
<td>Vagus indigestion, decomposition of food</td>
</tr>
</tbody>
</table>

2. **Odour**:

<table>
<thead>
<tr>
<th>Normal</th>
<th>Aromatic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abnormal</td>
<td>Inactive rumen juice</td>
</tr>
<tr>
<td>Acrid/acidosis</td>
<td>Lacto acidosis/pyloric</td>
</tr>
<tr>
<td>Foul/putrid</td>
<td>Protein overfeeding</td>
</tr>
<tr>
<td>Slightly ammonical</td>
<td>Rumen alkalosis</td>
</tr>
<tr>
<td>Musty/fecal</td>
<td>Vagus indigestion</td>
</tr>
</tbody>
</table>

3. **Consistency**:

<table>
<thead>
<tr>
<th>Normal</th>
<th>Slightly viscous</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abnormal</td>
<td>Extremely viscous</td>
</tr>
<tr>
<td>Watery</td>
<td>Inactive rumen fluid</td>
</tr>
<tr>
<td>Foaming</td>
<td>Abomasal dilation of frothy bloat</td>
</tr>
<tr>
<td>Mixture of watery &amp; foam</td>
<td>Rumen decomposition</td>
</tr>
<tr>
<td>Slimy pulp</td>
<td>Overfeeding</td>
</tr>
<tr>
<td>Semi liquid</td>
<td>Vagus indigestion</td>
</tr>
</tbody>
</table>

4. **Sedimentation Activity Time (SAT)**:

100ml freshly collected rumen contents are observed as it settles in a glass cylinder.

<table>
<thead>
<tr>
<th>Normal</th>
<th>4 to 8 minutes, Fine food particles and infusaria begin to settle at once. Large and more fibrous particles carried upward forming broad upper layer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abnormal</td>
<td>Rapid sedimentation and absent or retarded flotation \ Inappetance, starvation and feed without nutritive value</td>
</tr>
<tr>
<td></td>
<td>Rapid flotation is abundant foam and solid components remain in suspension for long time \ Decomposition rumen</td>
</tr>
<tr>
<td></td>
<td>Absence of solid particles and gas bubbles, hence no sedimentation and flotation \ Vagus indigestion</td>
</tr>
</tbody>
</table>
Biochemical examination:

1. pH:
   It should be measured immediately, using indication paper in field condition.

<table>
<thead>
<tr>
<th>Normal</th>
<th>5.5 – 7.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abnormal</td>
<td>Up to 8.5</td>
</tr>
<tr>
<td></td>
<td>After starvation for 24 hrs, urea poisoning, rumen decomposition</td>
</tr>
<tr>
<td></td>
<td>4.3 – 7.0</td>
</tr>
<tr>
<td></td>
<td>Hydrochloric acidosis</td>
</tr>
<tr>
<td></td>
<td>4.0 – 5.5</td>
</tr>
<tr>
<td></td>
<td>Rumen acidosis</td>
</tr>
</tbody>
</table>

2. Cellulose Digestion Test (CDT):
   Take 10ml of strained rumen fluid and add 0.3 ml of 16% cellulose or single strand of unmercerized cotton thread in the rumen fluid. The lower end of the cotton thread is tied with a glass bead or other weight which must immerse in rumen fluid. Then tightly close the test tube and incubate at body temperature either in incubator or near a light bulb.

Significances: normally digestion of cellulose takes place within 48-54 hrs. So in fully active rumen fluid the weight in the lower end of cotton thread will fall to the bottom of the tube within that time due to digestion of cotton thread. If the thread has not broken within the normal time, it should be interpreted as cellulose digestion time is delayed due to inactive rumen fluid.

3. Glucose Fermentation Test:
   It is performed in a fermentation sacchrometer. Take 10ml of rumen fluid and add 0.5ml 16% glucose solution in the sacchrometer and kept at 39ºC. The result is read after 30-60 minutes.
   Normally rumen fluid fermented the glucose and result is formation of gas.
   - ml gas / hr – Rumen fluid containing active microflora.
   - No gas – Rumen fluid containing microflora and acute rumen acidosis.
   - Decreased gas – Rumen decomposition, rumen alkalosis, acute rumen acidosis, Hydrochloric acidosis.
   - Normal / increased gas – Latent rumen acidosis.
   - Increased gas – Foamy bloat.

4. Redox potential or Methylene Blue Reduction Time (MBRT) test:
   This is measured by using a redox dyes methylene blue. Take 20ml of freshly collected rumen fluid and add 1ml of 0.03% methylene blue solution and mix. Measure the time required for decoloration of the sample using a plain rumen fluid as a basis for comparison.
   - Normal flora - Within 3 minutes
   - Inactive flora due to ration, poor in structure, inappetance – More than 15 min.
   - Rumen acidosis - < 5 min if pH > 5.2
   - > 5min if pH < 5.2
   - Hydrochloric acidosis - > 5 min.
PRACTICAL NO. 24

AIM: COUNTING OF RUMEN BACTERIA AND PROTOZOA

Principle:
The rumen bacteria and protozoa are not present at the time of birth. These organisms develop with age and type of food given to the animals and research the normal adult number at 9 to 13 weeks of age. The number of rumen bacteria varies from 15-80 billion per ml of rumen liquor. They are beneficial to host due to their role in the synthesis (vitamin B complex, microbial cell protein, fatty acids) and break down (lipids, protein, carbohydrates) reactions observed in rumen. Rumen liquor is collected from fistulated animals or stomach tube or ruminal fluid extraction pump or from a slaughter house animal. Normal rumen fluid pH is 5.5-7.0.

Ciliates and flagellates are found as ruminal protozoa. Ciliates are more in number. Most of protozoa of rumen are of family Ophryscolecidae. Protozoa are useful to host due to their beneficial effects like a) improvement of digestibility – protein, organic matter, b) increase in VFA production.

Material and Requirements:
1. Strained rumen liquor,
2. Hemocytometer slide with Neubauer counting chamber,
3. Microscope,
4. Gentian Violet crystals,
5. Formalin / formaldehyde solution.

Procedure:
I. Counting of the protozoa:
There are various methods like colorimetric, spectrophotometric available for counting of the rumen bacteria and protozoa where optical density of the strained rumen fluid is determined at 600nm wavelength and optical density of 0.2 is equal to one billion cells per ml of rumen fluid. However, through less accurate the direct counting of the microorganisms by using Neubauer counting chamber is more convenient.

1. Collect the rumen liquor.
2. Strain (filter) through muslin cloth, choose cloth (2-3 layers) and get a clear strained rumen liquor (SRL). SRL may be diluted five times.
3. Add a few drops of formaldehyde solution in the rumen liquor to kill the microorganisms and a few crystals of gentian violet to give identifiable strain to the organisms.
4. Charge the already cleaned hemocytometer Neubauer counting chamber covered with cover slip.
5. After keeping for a few minutes undisturbed, focus the slide under high power to count the number of microorganisms in five medium squares like red cell counting. Counting of bacteria is easier than protozoa due to bigger size of the later.
6. Count the bacteria and protozoa separately.

Calculation:
The volume of five medium squares used for microbial count is 1/50 cu.mm.
To express the number of micro-organism per ml of rumen liquor, the number of bacteria or protozoa are to be multiplied by 50000 (50 X 1000) X dilution factor, if any. SRL may be diluted five times.

II. To evaluate the motility of protozoa in SRL:
(a) Clean a glass slide and place a drop of SRL and cover it with a cover slip.
(b) Adjust under low power and observe the motility of protozoa.
(c) The SRL sample is evaluated as follows:
<table>
<thead>
<tr>
<th>Motility</th>
<th>Grade</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) Vigorous</td>
<td>++++ (80-100%)</td>
<td>Excellent</td>
</tr>
<tr>
<td>2) Abundant</td>
<td>+++ (60-80%)</td>
<td>Very good</td>
</tr>
<tr>
<td>3) Moderate</td>
<td>++ (40-60%)</td>
<td>Good</td>
</tr>
<tr>
<td>4) Few</td>
<td>+ (20-40%)</td>
<td>Fair</td>
</tr>
<tr>
<td>5) Negligible</td>
<td>&lt; 20%</td>
<td>Poor</td>
</tr>
</tbody>
</table>

### III. Counting of the bacteria:

An air dried smear of rumen fluid is stained Gram’s method and observed under the microscope. In the normal pH of rumen fluid, Gram negative bacteria is dominating. Rumen acidosis, proliferation of gram positive cocci and rode at the expense of gram negative bacteria.

#### List of some common rumen microbes:

<table>
<thead>
<tr>
<th>Name</th>
<th>Functions</th>
<th>Name</th>
<th>Functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteroides succinogens</td>
<td>Attacks cellulose</td>
<td>Cillites:</td>
<td></td>
</tr>
<tr>
<td>Ruminococcus flavifaciens</td>
<td>Fiber digestion</td>
<td>Isotricha intestinalis</td>
<td>Ingest sugars, produce Acetate, Butyrate, Lactate and small amount of Propionate</td>
</tr>
<tr>
<td>R. albus</td>
<td>Fiber digestion</td>
<td>Epidinium eucaudatum</td>
<td></td>
</tr>
<tr>
<td>Bacteroides amylophilus</td>
<td>Starch digestion</td>
<td>Diplodenum dentatum</td>
<td></td>
</tr>
<tr>
<td>Succinimonas amylolytica</td>
<td>Starch digestion</td>
<td>Dasytricha caudatum</td>
<td></td>
</tr>
<tr>
<td>Anaerovibrio lipolytica</td>
<td>Lipolytic</td>
<td>Ophryoscolex purkynei</td>
<td></td>
</tr>
<tr>
<td>Peptostreptococcus elsdenii</td>
<td>Lactate fermenter</td>
<td>Flagillates:</td>
<td></td>
</tr>
<tr>
<td>Celllobacterium cellulosolvens</td>
<td>Cellulose digestion</td>
<td>Monocercomonas ruminatum</td>
<td>-</td>
</tr>
<tr>
<td>Butyrivibrio fibrisolvens</td>
<td>Starch digestion</td>
<td>Cellimastix frontalis</td>
<td>-</td>
</tr>
<tr>
<td>B. alactacidigens</td>
<td>Starch digestion</td>
<td>Chilomastix spp.</td>
<td>-</td>
</tr>
<tr>
<td>Methano-bacterium ruminatum</td>
<td>Methane production</td>
<td>Pentatrichomonas hominis</td>
<td>-</td>
</tr>
<tr>
<td>Streptococcus bovis</td>
<td>Starch digestion</td>
<td>Monocercomonas bovis</td>
<td>-</td>
</tr>
<tr>
<td>Eubacterium ruminatum</td>
<td>Sugar, Xylan</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
PRACTICAL NO. 25

AIM: ESTIMATION OF AMMONIA IN RUMEN LIQUOR

Principle:
The proteins present in the diet of ruminants are fermented by the rumen microbes and converted into volatile fatty acids and ammonia. Ammonia in rumen liquor, therefore is an indicator of degree of proteolysis of feeds in rumen. Its concentration is ranging from 2-100 mg/100 ml of rumen liquor. Ammonia present in the rumen is distilled and titrated against the acid for its qualification.

Method – I Microkjeldahl method

Materials and Requirements:
1. Microkjeldahl apparatus
2. Pipettes and conical flasks
3. Burette
4. 10N sulphuric acid solution
5. 40% sodium hydroxide solution
6. 2% Boric acid solution
7. Tabhiro’s indicator
8. N/10 sulphuric acid solution

Procedure:
1. Prepare SRL as described in the previous experiment.
2. Take 9.5 ml of SRL add 0.5 ml of 10N sulphuric acid solution in a tube.
3. Mix thoroughly and centrifuge it at 2500 rpm.
4. Wash the apparatus with distilled water and finally collect the distillate in 10 ml of 2% boric acid solution with a few drops of Tabhiro’s indicator.
5. Take 2 ml of supernatant in the distillation apparatus.
6. To it add an excess (3 to 4 ml) of 10% sodium hydroxide in the microkjeldahl apparatus.
7. Keep the heater “On” and as steam starts coming out close the pinch cocks.
8. Collect the distilled in a conical flask containing 10 ml of 2% boric acid solution and add a few drops of Tabhiro’s indicator.
9. Titrate the distilled against N/10 sulphuric acid to end point.

Calculation:
The amount of rumen liquor used for the determination of ammonia nitrogen content is 1.9 ml.
Therefore, $\frac{9.5}{10} \times 2 = \frac{19}{10} = 1.9$ ml
If, for example, the amount of N/10 sulphuric acid used is 25 ml to reach the end point then –
$$N_1V_1 = N_2V_2,$$
$$\frac{1}{10} \times 25 = N_2 \times 1.9$$
$$N_2 = \frac{25 \times 1/10}{1/1.9}$$
$$N_2 = 1/1.31$$

$1/1.31$ N NH$_3$ per liter of rumen liquor
To convert the value into gm/liter the value base to be multiplied by the equivalent weight of NH$_3$ is
$$= \frac{1}{1.31} \times 17 = 12.9 \text{ g/lit}$$
To convert into meq/lit the value has to be multiplied by $1000 = 12.9 \times 1000 = 12900$ meq/lit of rumen liquor.

Method – II Conway microdiffusion method
Conway microdiffusion cell is made up of an inner well and outer well. When SRL is incubated with saturated potassium carbonate in the outer chamber, ammonia is released from SRL. Boric acid solution present in central well absorbs liberated ammonia and is titrated with N/70 sulphuric acid.

**Materials and Requirements:**
1. Conway microdiffusion dish with lid
2. Pipettes, glass rod
3. Saturated potassium carbonate solution
4. Sulphuric acid N/70
5. Boric acid solution

**Procedure:**
1. Take clean, dry Conway microdiffusion cell (with lid). Prepare duplicate set for each sample.
2. Pipette 1 ml of boric acid solution in the central well.
3. Pipette 1 ml of saturated potassium carbonate and 1 ml SRL in the outer well.
4. Immediately place the lid on the dish tightly.
5. Mix the contents in the outer well, through gentle rotary mixing. Take care that the inner well contents do not mix with outer wall contents.
6. Incubate for two hours at 37°C.
7. Open the lid. Fill micropipette with N/70 H$_2$SO$_4$. Titrate boric acid solution in central well with N/70 H$_2$SO$_4$. Titration is complete when a faint pink color appears. Mix the contents every time with glass rod.
8. Calculate the average amount of N/70 H$_2$SO$_4$ used in titration.

**Calculation:**
1. 1 ml N/70 H$_2$SO$_4$ titrates 0.2 mg NH$_3$, therefore xml N/70 H$_2$SO$_4$ titrates 0.2 mg X xml burette reading.
2. The amount of ammonia in 100ml SRLs
   \[= 0.2mg \times \text{xml} \times 100/0.5 = x.40 \text{mg}\]
PRACTICAL NO. 26

DATE:
AIM: ESTIMATION OF TOTAL VOLATILE FATTY ACID CONTENT IN THE RUMEN LIQUOR

Principle:
The end products of microbial fermentation of the roughage are the volatile fatty acid and ammonia. The TVFA so produced can be distilled and then titrated against the N/50 NaOH to qualify the VFA present in the strained rumen liquor (SRL).

Material and Requirements:
1. Markham distillation set
2. SRL
3. Burettes
4. Phenolphthalein indicator
5. N/50 sodium hydroxide solution
6. Pipettes and conical flasks
7. Saturated magnesium sulphate solution in N sulphuric acid solution
8. 1N sulphuric acid solution

Procedure:
1. Collect the rumen fluid.
2. Filter it through four muslin cloth. The liquid so obtained is known as SRL.
3. Centrifuge clean SRL at 3000 rpm for 10 min to get clear SRL.
4. Take an aliquot of 10 ml of clean SRL.
5. Deproteinise it by adding 10 ml of saturated magnesium sulphate solution.
6. Mix it thoroughly and filter through Whatman’s NO.1 filter paper or centrifuge at 3000 rpm for 10 min.
7. Take an aliquot of 0.5 ml of filtrate in Markham steam distillation and put the stopper. Seal it by adding distilled water. Keep the heater “on” for boiling. Close the pinch cock as soon as steam starts coming out.
8. Clean the apparatus thoroughly and collect 50 ml of distillate to run a blank.
9. Collect 50 ml of distillate in a conical flask twice in one sample.
10. Titrate the distillate against N/50 sodium hydroxide adding 2 drops of phenolphthalein indicator to the end point.

Calculation:
The amount of rumen liquor used for the determination of VFA is 2.5 ml.
Therefore, \( \frac{10}{20} \times 5 = 2.5 \text{ ml} \)
If, for example, the amount of N/50 NaOH used is 25ml to reach the end point then –
\[
\frac{N_1}{V_1} = \frac{N_2}{V_2} \]
\[
\frac{1}{50} \times 25 = N_2 \times 2.5 \\
N_2 = \frac{25 \times 1}{50} \times 2.5 \\
N_2 = \frac{1}{2} = 0.2
\]
Therefore, 0.2 N total VFA per liter of rumen liquor, for meq/lit = 0.2 N \times 1000
PRAC TICAL NO. 27

AIM: RECORDING OF THE RUMEN MOTILITY

Principle:
The orderly and synchronized movements of the reticulum and rumen is essential for mixing of the fresh ingesta with that already present in the stomach and propelling the food downwards and simultaneous uplifting of the gases in the dorsal sac and towards cardia for eructation (belching). The movement of reticulo-rumen is brought about by the primary (mixing) cycle and secondary (eructation) cycle of rumen contraction. Primary cycle consists of biphasic reticular contraction followed by monophasic contraction of the dorsal ruminal sac and the followed by the monophasic contraction of the ventral ruminal sac, which re-occur after the interval of one minute. The secondary cycle, which occur at the interval of 2 minutes are confined to the rumen and consist of contractions of the dorsal sac. Primary movements of the rumen may or may not be followed by the secondary movements. Undulation of paralumber fossa caused by these contractions can be identified by auscultation, palpation, observations of the left paralumber fossa and the left lateral abdominal region.

Material and Requirements:
1. Animal
2. Service crate procedure
3. Stop watch

Procedure:
The ruminal motility can be identified and counted either by visual inspection or by palpation by proceeding through the following methods.

A. Visual inspection method:
1. Secure the animal in a service crate in comfortable position.
2. Observe the movements of the left paralumber fossa and left lateral abdominal region.
3. During contraction of the rumen there is alternate rising and sinking of left paralumber fossa in conjunction with the abdominal surface ripples.
4. The number of ripples moving up per minute gives the number of rumen contractions.

B. Palpatory method:
1. After securing the animals in a travis, put your fist in the left flank in the paralumber fossa.
2. Press the fist in the paralumber fossa while fist passes touching the last rib.
3. When the fist is pushed outwards, it is recorded as ruminal movement.
4. Count the movements for three minutes.
5. Practice it in different animals during various conditions, such as resting, feeding and rumination etc.
6. Normally three movements/ 2 minutes is observed. Incase of impaction, trauma and anorexia ruminal motility is suspended.
PRACTICAL NO. 28
AIM: AUSCULTATION OF THE RUMINAL AND RETICULAR SOUNDS

Principle:
Auscultation of the abdomen is an essential part of the clinical examination of ruminants. The ruminal and reticular sounds are predominant abdominal sounds, which indicate the nature of the intra-ruminal contents and the frequency and amplitude of the gastrointestinal movements. The intensity, duration and frequency of the sounds get increased after feeding and excitement which should be noted.

Material and Requirements:
Animal, Service Crate, Stethoscope

Procedure:
1. Secure the animal comfortably in a secure crate.
2. Auscultate in the left paralumbar fossa for ruminal sounds.
3. Ruminal sound consists of a lift of the flank with a fluid gargling/churning sound followed by a second more pronounced lift accompanied by a booming gassy sound.
4. Auscultation over the lower left sibs gives the reticulate sounds.
5. Reticular sounds are fainter fluid sounds of reticular contraction just prior to the contractions of the dorsal and ventral ruminal sacs.
6. Auscultate these sounds repeatably and differentiate the ruminal sounds from reticular sounds.
DEMONSTRATION NO. 01

AIM: RECORDING AN ECG IN VARIOUS ANIMALS

REQUIREMENTS:
ECG machine, Jelly or salt solution (MgSO4), Electrodes, Graph paper.

PROCEDURE:
1. Read the instruction supplied along with ECG machine.
2. Get acquainted the operation of the machine with different control switches.
3. Insert the necessary transformer and stabilizers to obtain steady current at the required voltage.
4. Earth the instrument.
5. The subject should be under a complete physical and mental rest.
6. Obtain good electrical contacts of electrodes with the body surface by applying jelly or cotton soaked saturated salt solution. All electrodes are fixed simultaneously.
7. Select the electrodes - for obtaining the required recording with the help of switches.
8. Always calibrate the machine before obtaining the lead records.
9. Obtain a record of ECG at different leads.
10. Study the record given to you and note down the observations in the following table.

Name:  
Age:  
Sex:  
Date:  

<table>
<thead>
<tr>
<th>Observation to be made</th>
<th>Lead 1</th>
<th>Lead 2</th>
<th>Lead 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) P wave</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(a) Positive or Negative</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(b) Round or Pointed</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(c) Height (mm)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(d) Duration (msec)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The ECG is recorded using (a) Bipolar leads and (b) Unipolar leads. The specific arrangement of each pair of electrodes connected between two point of the body is called LEAD. Non-polarized electrodes are used to obviate the impedence of the skin. They are obtained by using a non corrosive metal wrapped in cotton or lint soaked in strong saline (sat. MgSO4) or by the use of a special jelly serving the same purpose.

Bipolar leads:
Two similar electrodes are placed on the body surface and potential difference between these two electrodes is recorded. Following are three standard bipolar leads right arm, left arm, left leg (but not right leg)

<table>
<thead>
<tr>
<th>Leads</th>
<th>Connections</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lead I record</td>
<td>Right arm (-ve) and Left arm (+ve)</td>
</tr>
<tr>
<td>Lead II record</td>
<td>Right arm (-ve) and Left leg (+ve)</td>
</tr>
<tr>
<td>Lead III record</td>
<td>Left arm (-ve) and Left leg (+ve)</td>
</tr>
</tbody>
</table>
If two acromion processes of right and left arms and the pubic region are connected by a line it forms a equilateral triangle with the heart in the center. It is called as Einthoven Triangle.

Unipolar leads:
In this method one of the two electrodes is placed at a constant potential. It is called “Indifferent electrodes”. The other electrodes is called an “active electrodes” which record the changes of potential of the body surface area here it is placed. It records the potential changes of the area as compared to the constant potential of the indifferent electrodes. The indifferent electrodes is to be placed on the body where the electric potential changes do not reach the surface and the electrical state is constant. It is not possible to obtain such a place on the body surface. To overcome this difficulty Wilson connected the three bipolar lead electrodes together through a non-inductive resistance of 5000 ohm. This is described as the “Wilson Central Terminus” or “indifferent electrode”. This arrangement is based on the principle that the algebraic sum of the potential difference occurring under the bipolar leads should be zero at any moment.

\[ I + (-II) + III = 0 \]

Above Gold Berger’s arrangement consists of disconnecting that extremity from the central terminus, on which the exploring electrodes is placed. This procedure gives a record, which is increased in amplitude. Therefore a prefix ‘a’ standing for the word ‘augmented’ is placed before unipolar limb leads (aVR, aVL and aVF). Generally augmented limb leads are used to day. Augmented limb lead equals to one half of the unaugmented lead.

For unipolar leads there are nine different locations as given below:-
(a) Chest leads or precordial leads V1 to V6 = 6 leads
(b) Limb leads -a VR (right arm ) = 1 leads
- a VL (left arm) = 1 leads
- a VF (left foot) = 1 leads

Total = 9 leads

(A) Chest leads:
A record is obtained by using the unipolar method when the active electrode is placed on the pericardium. It is designated as VC. The letter V means a ‘Victor’. For chest leads following recording from V1 to V6 are obtained.
V1 is recorded when active electrode is placed in the 4th intercostals space to the right of the sternum.
V2 is recorded in the same space but to the left of the sternum.
V3 is obtained midway between V2 and V4.
V4 is obtained in the fifth intercostals space on the midclavicular line.
V5 is obtained at same level as V4 but on the anterior axillary line.
V6 is obtained at the same level but on the mid -axillary line.

(B) Limb leads:
These are three augmented limb lead aVR (right arm), aVL (left arm), and aVF (left foot) recording. They are obtained by disconnecting the right forearm, left forearm and left foot each time from the central terminus and placing the exploring electrode on each terminus and placing the exploring electrode on each of them in turn respectively. In both the
methods of recording discussed above various waves of ECG are same. The letters P, Q, R, S, T and U name them. The recorded waves during each cardiac cycle comprises of two depolarization waves and one repolarization waves. Auricular repolarization wave is not obtained as a separate wave in ECG. The record runs at isoelectric levels between P, QRS complex, T and U waves. In ECG theoretically a process of activation is followed by a downward deflection of equal magnitude and duration. The ECG does not show paired opposite waves of depolarization and repolarization. The reasons are that
(1) The positive and negative processes is the sum total of all the positive and negative processes occurring at the time in the volume conductor.
(2) The second reason is the cardiac muscle undergoes sustained contraction and the recovery process is disturbed by this mechanical factor.

<table>
<thead>
<tr>
<th>Observation to be made</th>
<th>Lead</th>
</tr>
</thead>
<tbody>
<tr>
<td>(2) PR interval</td>
<td>1.</td>
</tr>
<tr>
<td>Duration (from beginning of P to beginning of Q or R)</td>
<td>2.</td>
</tr>
<tr>
<td></td>
<td>3.</td>
</tr>
<tr>
<td>(3) QRS complex</td>
<td></td>
</tr>
<tr>
<td>(i) Q wave – whether prominent or insignificant</td>
<td></td>
</tr>
<tr>
<td>(ii) R wave – height (mm)</td>
<td></td>
</tr>
<tr>
<td>(iii) S wave – height (mm)</td>
<td></td>
</tr>
<tr>
<td>(iv) QRS interval (mSec)</td>
<td></td>
</tr>
<tr>
<td>(4) ST segment</td>
<td></td>
</tr>
<tr>
<td>(i) Duration (mSec)</td>
<td></td>
</tr>
<tr>
<td>(ii) Above or below the isoelectric line and how much</td>
<td></td>
</tr>
<tr>
<td>(5) T wave</td>
<td></td>
</tr>
<tr>
<td>(i) Positive or Negative</td>
<td></td>
</tr>
<tr>
<td>(ii) Height (mm)</td>
<td></td>
</tr>
<tr>
<td>(iii) Duration (mSec)</td>
<td></td>
</tr>
<tr>
<td>(6) U wave</td>
<td></td>
</tr>
<tr>
<td>(i) Present or Absent</td>
<td></td>
</tr>
<tr>
<td>(ii) Positive or Negative</td>
<td></td>
</tr>
<tr>
<td>(7) Rate of the heart</td>
<td></td>
</tr>
<tr>
<td>(8) Axis deviation</td>
<td></td>
</tr>
<tr>
<td>(i) Height of R wave in lead I</td>
<td></td>
</tr>
<tr>
<td>(ii) Height of S wave in lead III and vice versa</td>
<td></td>
</tr>
</tbody>
</table>

P Wave:
The first wave whether positive or negative is called the ‘P wave’. It produces atrial depolarization. It is recorded when cardiac impulse originated at SA node spreads through the auricular muscle fibers to reach the A.V. node. The beginning of this deflection indicates that the impulse has started pervading the auricles and reaches the A.V. node at the top of the P
wave. The end of the deflection indicates that the auricles have come back to a resting state. It is always positive in lead II and I; it is usually tallest in lead II. In lead II and lead I it, lasts for 0.08 to 0.1 sec and has a maximum height of 0.5cm i.e.0.5mv. It may be diphasic in lead III and is always inverted in aVR.

QRS complex:
At the end of the P wave the record runs along an isoelectric for a very short interval (0.08 sec). It then shows QRS complex (0.08 sec to 0.12 sec and height between 1.8 to 2cms i.e. 1.5mV to 2mV). It is produce by ventricular depolarization. The first positive wave following the P wave is called the ‘R wave’. The negative wave, which precedes R, is labeled as Q and the negative wave which follows the R wave is labeled as ‘S’. The whole QRS complex need not be present in every record.

Q wave:
It indicates the commencement of the invasion of ventricle. It is small in lead I but may be wider and deep in lead III. It is affected by the position of the diaphragm. It is always more deep during experiment than during inspiration.

R wave:
It is due to the activation processes suddenly pervading both the ventricles. It is tallest in lead II and smallest in lead III .in aVR it is again in significant.

S wave:
Whether R is small and insignificant the S wave is always prominent comparatively. It has same significant as that of R wave, which is guide of the functional activity of the ventricles. It is prominent in lead III and in aVR. The mean electrodes axis can be determined from the values of QRS complex above and below isoelectric line in the lead I & III. High value above the base line in lead I and below the base line in lead III indicates left axis deviation. In right axis deviation it is vice-versa.

PR interval:
It is the time interval between the top of the P wave (from the beginning of the P wave) and the beginning of the R wave (beginning of the Q wave when prominent). It indicates the true conduction time of the bundle of his. In health it varies between 0.13 to 0.16 sec. and should never exceed 0.2 sec.

T wave:
The wave following QRS complex whether positive or negative is labeled as ‘T’. It occurs at the end of ventricular systole and is associated with the repolarization phenomena. It is always in the same direction as that of the major deflection of the QRS complex i.e. always positive in lead III and I and always negative in a VR. It lasts for the 0.24 sec. interval. Its voltage is more than that of the P wave (about 1 mv).

ST segment:
At the end of the S wave the record runs on the isoelectric level for 0.08 sec. and then shows the T wave. ST segment and T wave is produced due to ventricular repolarization.

U wave:
It is the wave following the ‘T’ wave after another isoelectric interval for 0.08 sec. the wave is inconstant. It is recorded due to repolarization of papillary muscle. It lasts for 0.08
sec. it has always the same polarity as that of the T wave. Its voltage is about 0.02 mv. It indicates a state of increased excitability of heart tissue.

Normal ECG intervals:

<table>
<thead>
<tr>
<th>ECG</th>
<th>Duration (sec.)</th>
<th>Events</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Average</td>
<td>Range</td>
</tr>
<tr>
<td>PR interval</td>
<td>0.18</td>
<td>0.12 - 0.20</td>
</tr>
<tr>
<td>QRS complex</td>
<td>0.08 - 0.12</td>
<td>-</td>
</tr>
<tr>
<td>QT interval</td>
<td>0.40 - 0.43</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ST interval (QT-QRS)</td>
<td>0.32</td>
<td>-</td>
</tr>
</tbody>
</table>

No wave of auricular repolarization is recorded as its manifestations are normally submerged in the QRS complex.
DEMONSTRATION NO. 02

AIM: MEASUREMENT OF CARDIAC OUTPUT

PRINCIPLE:
Certain amount of blood is pumped out by each ventricle of each beat into the circulation is called cardiac output, which can be measured by the Fick’s principle. As per the Fick’s principle the unit of time is equal to the arterial level of the substance minus the venous level (A.V. difference) times the blood flow.

Procedures used for measuring cardiac output:
In animals the cardiac output can be measured with the help of
1. Cardiometers
2. Heart-Lung preparation
3. Dye method (or thermo dilution method)
4. Fick’s principle using O₂ or CO₂
5. Physical method (Ballistocardiography)

The following two methods are most commonly used for measuring cardiac output

A. **Fick’s Method:**

Materials and requirement:
1. Experimental animal
2. Douglas bag
3. Gas analyzer

Procedure:
1. The total O₂ consumption and CO₂ output per minute is determined with Douglas bag.
2. Calculate O₂ content of arterial blood from Hb% assuming 95% saturation.
3. Collect the alveolar air, measure its CO₂ tension, which should be identical with that of arterial blood.
4. Now, collect a sample of mixed venous blood from the right atrium by introducing a fine rubber catheter into the cubital vein and pushing it gradually into the right atrium and measure its O₂ content (%).
5. Alveolar air is again collected after holding the breath for 5 seconds. The CO₂ tension of this air should be same as that of venous blood.

Calculation:

\[
\text{Cardiac output (ml/min)} = \frac{\text{O}_2 \text{ consumption (ml/min)}}{\text{Difference of O}_2 \text{ content in arterial and venous blood}}
\]

B. **Indicator Dilution Method:**

Materials and requirement:
1. Animal
2. Dye
3. Colorimeter
4. Graph paper
Procedure:
1. A known quantity (5mg) of Evans blue (non-diffusible dye, known as T-1824) or indocyanine green or radioactive isotope should be injected slowly into the cephalic vein.
2. Collect serial blood samples at different intervals from the artery and estimate concentration of dye with the help of colorimeter.
3. Plot concentration of the dye in each sample on a semi logarithmic paper. The main concentration of the dye is calculated from the curve. The volume flow in liters per second is determined with help of following formula.

Calculation:

\[
F = \frac{D}{C \times T}
\]

Where \( F \) = Volume flow in liters per second
\( D \) = The quantity of dye (Evans blue) injected.
\( C \) = The mean concentration of dye
\( T \) = The duration in seconds of the first passage of dye through the artery.

Note: In case of dogs, horses, cows, men and other species of animals the cardiac output is linearly related to the body weight by the equation:

\[
\text{Cardiac output} = 0.1017 \times \text{b.w. (kg)} \times 0.99
\]
DEMONSTRATION NO. 03

AIM: RECORDING OF \( \text{PaO}_2 \), \( \text{PvO}_2 \), \( \text{PaCO}_2 \) AND \( \text{PvCO}_2 \)

OBJECT:
Manometric determination of blood gases by Van Slyke blood gas analyzer.

PRINCIPLE:
The gases are chemically released from the blood. The amount of gas getting dissolved in blood is dependent upon the partial pressure of gases in liquid. The pressure of a gas at a fixed volume is measured manometrically.

MATERIALS AND REQUIREMENTS:
1. Van Slyke instrument
2. Blood samples
3. Solution A (Neutral Saponin Ferricyanide) – Dissolved 6.0 gm of potassium ferricyanide, \( \text{K}_3\text{Fe(CN)}_6 \) and 3.0gm of saponin in one liter of distilled water.
4. Solution B (0.1N lactic acid plus 99ml distilled water)
5. 1.0N sodium hydroxide (4.0 gm NaOH per 100ml distilled water) – used to absorb carbon dioxide.
6. Mix 2.0 gm of sodium hydrosulphite (Na\(_2\)S\(_2\)O\(_4\)) and 2.0 gm sodium anthraquinone beta sulphonate. Dissolved the above mixture in 10ml of 1.0 N potassium hydroxide (5.6gm KOH in 100ml of distilled water).
7. Caprylic alcohol
8. two 10ml syringes with short rubber tubes and attached clamps
9. Mineral oil

PROCEDURE:
1. Collect the blood in heparinised tube from artery and vein as per requirement.
2. Place the mercury bulb in such a position so that cup is full of mercury.
3. Place the mercury in a position to lower the mercury down almost up to the bottom of the cup.
4. Place 10 ml of 1.0N NaOH into the cup and let it flow down into the chamber.
5. Put a few drops of mercury in the cup and let a little of it be trapped in the bore of the stop cock (mercury seal). Remove excess of the NaOH from the cup.
6. Place the mercury bulb in the lower position so that mercury level in the chamber goes down up to 50 ml mark.
7. Close the lower stopcock and shake for 3 min. The gases escape after shaking.
8. The solution of NaOH is now air free, which is collected under oil.
9. Take some oil in a syringe and with the help of rubber tube place the oil into the cup over mercury seal. Keep some oil in the syringe.
10. Place the mercury bulb in the upper position to bring up the NaOH into the cup. Suck up the NaOH solution into the syringe having oil at both ends.
11. Similarly, make the solution hydrosulphide solution free and collect it under oil in the syringe.
12. Wash the chamber with lactic acid to remove alkali.
13. The mixture of A and B solution used for hemolysing red cells is made air free by taking 7.5 ml of the mixture of solution A and B (5 ml each) in the cup. Place 4 drops of caprylic alcohol on top.
14. Place the mercury bulb a position to bring down this solution in the chamber and thereafter seal the stop cock as describe earlier.
15. Shake the solution for 3 minutes.
16. Place the mercury bulb in a position to bring up the fluid in the up leaving about 1.5 ml of the fluid in the chamber.
17. Take exactly 1 ml of heparinized blood in a syringe having small rubber tubing on the tip. Place the tip directly on the bore of the stopcock in the bottom of the cup.
18. Place the mercury bulb at the level of W tube and open the lower stop cock.
19. Now, open the upper stop cock so as to suck the blood in the chamber.
20. Wash through 1 ml of the mixture of solution A and B already kept on the cup. Remove this fluid from the cup. Make the mercury seal described earlier.
21. Place the mercury bulb to lower the solution up to 50 ml mark at the bottom of the chamber.
22. Shake it for 34 minutes.
23. Place the mercury bulb in such a position to raise the solution up to 2ml mark at the apex of the chamber.
24. Read the column of mercury in the manometer as PO. This pressure is created by all the gases liberated from the blood.
25. Note the temperature in thermometer at this point.
26. Place the mercury bulb in a position to lower down by 5 ml in the chamber and pour 2ml of 0.1N NaOH into the cup under oil seal.
27. Draw exactly 1 ml of NaOH into the chamber slowly drop by drop in 30 sec time.
28. Place the mercury bulb in a position to bring up the solution exactly up to 2 ml mark in the apex of the chamber.
29. Read the column of mercury in the manometer as P1. This pressure represents the pressure of oxygen and nitrogen in the blood.
30. Since, the CO2 has been absorbed by NaOH, the pressure exerted by CO2 can be found out by PO-P1.
31. Remove the excess of NaOH from cup and put 2 ml of hydrosulphide solution to enter into the chamber very slowly.
32. Allow 1 ml of hydrosulphide solution to enter into the chamber very slowly.
33. Keep it as such for 2 minutes to absorb the total oxygen present (absorption time).
34. Place the mercury bulb in a position to bring up the solution exactly at 2 ml mark in the apex of the chamber.
35. Read the mercury column in the manometer as P2.
36. The oxygen pressure can be worked out by P2-P1.
37. Repeat the same for arterial and venous blood separately.
38. Drain the solution out of apparatus and wash it with strong alkaline hydrosulphide solution.
39. Finally rinse and fill the chamber with water until further use.

**Precautions:**
1. Never open then upper stopcock with mercury bulb at lower position with the lower stopcock opened; else the mercury and solution will be drawn into the manometer.
2. Before undertaking the experiment, learn handling of the instrument.
DEMONSTRATION NO. 4

AIM: DEMONSTRATION OF USE OF SPIROMETER

This practical involves following aspects
(a) To obtain basal metabolic rate (BMR) and resting energy metabolic rate.
(b) To obtain respiratory quotient
(c) To obtain different volumes of air in a respiratory process

Requirements:
Spirometer, Oxygen cylinder, Water, Patient, Soda lime, Easy chair, Thermometer, Barometer, Nose clips

Speed of the drum: One inch in one minute

Introduction:
‘Spirare’ means to breathe. The word spirometry means a measurement of various (Dynamic) volumes of air breathed in and out. The same instrument can be useful for all objects listed above. Volumes of air can be measured from the volumes of air expired and inspired. Ratio of volume of CO$_2$ produced and oxygen consumed will give RQ value. Measurement of metabolic rate of (a) resting (b) post absorptive (c) mentally alert human subjects is carried out in (d) a thermally neutral environment. The measurement of human metabolic rate under above basal conditions is known as BMR. It is usually expressed as calorie of heat production per square mm body surface area per hour. But in case of animals, above basal state is seldom achieved with assurance and therefore instead of BMR measurements of resting energy metabolism are more common. Resting metabolism refers to heat production when the animal is at rest, usually in recumbent position. It is measured before the morning feeding, under usual farm or laboratory conditions. Here, many species are not in post absorptive state under these conditions and they are not in a thermally neutral environment. Usually for measurement of energy metabolism two methods existing are:
(1) Direct calorimetry – simple in theory, difficult in practice
(2) Indirect calorimetry – simple in theory and practice

Here respiratory gases exchange is estimated either by close circuit devices or open circuit devices. Main difference in open and close circuits is that former method allows the animal to breathe atmospheric air whereas in the later method the animal has to re-breathe the same air.

Procedure:
I. Obtaining Basal Metabolic Rate (BMR) & Resting Energy Metabolic rate:

1) Note height, weight, age and sex of the patient.
2) Set the instrument in quite surroundings.
3) Fill one and half liter of water in the water cabinet after closing water outlet.
4) Fill the soda lime bottle, weight and connect with the expiration pipe end opening in the oxygen chamber.
5) Close the top of the soda lime bottle with the lid bearing valve, which allows the entry of expired air only into oxygen cabinet.
6) Place bell like inverted vessel in water cabinet.
7) Fill oxygen from O2 cylinder into cabinet through air inlet. Water compartment will not allow the escape of sir filled in oxygen cabinet under bell like vessel.
8) Make patient in comfortable position (either in easy chair or bed).
9) Fix mouthpiece to the inspiration and expiration tubings. Adjust writing stylet properly.
10) Note room temperature, barometric pressure at the start of experiment.
11) Apply mouthpiece to the patient and close the nose with clips. Now ask the patient to respire. Put the drum in rotation (one inch per minute).
12) Inspiration will take air from oxygen cabinet and hence volume of the air is reduced and is recorded on the rotating drum. At expiration the breathed out air passes through soda lime bottle (CO₂ is absorbed) and lifts the bell when it passes through valve into oxygen cabinet. The effect is recorded over the drum.
13) The use of oxygen by the animal body decreases the volume of the respiratory gas mixture filled in oxygen cabinet.
14) Remove mouthpiece and nose clips after six minutes of experimental period. Stop the drum.
15) Find out oxygen consumption for six minute from the graph. Find out CO₂ formed from the weight difference in soda lime bottle.
16) Correct the value of oxygen consumed from standard chart at a room temperature and barometer pressure to a STP (Standard Temperature and Pressure). Find surface area from body weight and height, with the help of standard chart.
17) The corrected value of oxygen when multiplied by a constant 4.825 (thermal equivalent of oxygen) gives total amount of calories produced per whole body total surface area.
18) Convert value for sq. mm. body surface and for one hour of time.
19) Compare this value with standard chart showing relation of age and sex with metabolic rate.
20) The observed difference in table value and calculated value is expressed in percentage.
21) BMR depending upon the result is expressed either positive or negative value in comparison to normal table value.

II. Obtaining & Respiratory Quotient (RQ):

It is calculated from the CO₂ produced and O₂ consumed by the animal. The RQ of any carbohydrate is 1.0, fat (tripalmitin) is 0.7 and protein 0.8. in ruminants, anaerobic fermentation in the rumen produces large amount of CO₂ and methane. This CO₂ cannot be distinguished from CO₂ which arises from metabolism in the tissues; under these circumstances some correction is necessary before any significance can be attached to the RQ.

IV. Obtaining different respiratory volumes:

(A) Tidal volume:
1) Note the initial level of the Spirometer. It should be zero.
2) Instrument the subject to breathe in through the nose and to breathe out through the mouthpiece every time, for a period of half a minute.
3) Note the number of breathe given out.
4) Read the Spirometer reading.

(B) Inspiratory capacity:
1) Bring down the Spirometer bell to any arbitrary level after it is filled with the fresh atmospheric air.
2) Record this initial level accurately.
3) Instruct the subject to breathe in only once from the Spirometer maximally and forcefully (but only once) at end of a normal expiration. Note the reading.
4) Repeat for three times. Find out the difference with the initial reading.

(C) Vital capacity:
1) Measure the maximum amount of air collected by breathing out forcefully after taking in a maximal deep breath from air.
2) Take three reading at the interval of one minute in between.
DEMONSTRATION NO. 5

DATE:

AIM: STUDY OF DIFFERENT ELECTRICAL CIRCUITS USED IN EXPERIMENTAL PHYSIOLOGY LABORATORY

Apparatus:
1. Stimulating or electrical apparatus: These include electricity source (low voltage unit), induction coils, electrodes, short circuiting key, mercury key and vibrating reed.
2. Recording or mechanical apparatus: In this group – recording drum, stand, heart lever, moist chamber, muscle lever and tuning fork are included.

Circuit-1: Simple circuit without drum in circuit.

Arrange the following as directed and draw a diagram of the complete circuit.

Primary circuit: Mains, mercury key and primary coil to form the primary circuit. Primary circuit will be completed when the mercury keys is closed.
Secondary circuit: Secondary coil short circulating key and electrodes from the secondary circuit.

This arrangement of primary and secondary circuits is called simple circuit. It is employed when make and break stimuli of varying strength are applied to living nerve-muscle preparation or recording its response.

Circuit-2: Drum in circuit.

Arrangement of the primary circuit: The drum is included in the circuit to form a primary circuit, other things remaining the same as before. There is no change in the arrangement of secondary circuit. Primary circuit is complete when mercury key is closed and contact arms of the drum touch contact spring. This circuit is used in recording simple muscle curve (SMC) and recording the effect of temperature, load, two successive stimuli on SMC and the study of essential features of fatigue.

Circuit-3: Circuit inclusive of tetanus set or vibrating reed.

In this circuit the drum is excluded from primary circuit. The drum is kept rotating but separated from electrical connections. The vibrating reed is included in the primary circuit in place of drum. The secondary circuit remains as before. This circuit is used in experiments demonstrating the genesis of tetanus.

Circuit-4: Circuit inclusive of hammer.

Drum is excluded from primary circuit. The drum is kept rotating but separate from any electric circuits. Neef's hammer is included in the primary circuit to obtain 30 minimal or 60 sub maximal stimuli per second for giving tetanising shock to the muscle. This circuit can also be used to give tetanising shocks to vagus nerve or to the crescent.
AIM: PITHING AND REMOVAL OF FROG NERVE-MUSCLE PREPARATION

The nerve muscle preparation obtained from the living frog is used in experimental work to study the general physiological properties of both muscle and nerve. Pithing is preferred to anaesthesia because the latter is likely to affect the various systems thus vitiating experimental results.

The reasons for using frog in physiological experiment are:
1. It is cold-blooded animal.
2. It is comparatively cheap and easily available.
3. The dissected out nerve-muscle preparation retains its vitality at the room temperature for a few hours.
4. Its activity and recording obtained are basically similar to those of the nerve-muscle preparation obtained from warm blood animals which are (a) costlier (b) difficult to procure and (c) require special temperature regulation mechanism in the laboratory for working.

A. Pithing the frog:
Pithing is a process of destroying the central nervous system of the frog. The individual organs continue to maintain their vitality for some time. The activity can only be elicited on direct stimulation with electrical, chemical or physical stimuli to nerves or tissues.

Requirements: Experimental frog and a pithing needle.

Procedure:
1. Wash the frog under tap water and hold it in the left hand.
2. Hold the frog with its dorsal surface towards you and with the help of left hand index finger press its nose down as far as possible.
3. Feel the depression with index finger of right hand in the mid line at the level of a line passing through the posterior borders of tympanic membranes.
4. Pierce the center of the depression with pithing needle through the skin and ligaments to the spinal canal then pass it upward into the skull through foramen magnum.
5. Move the needle from side to side destroying and separated the brain from spinal cord.
6. Withdraw the needle and then pass it downwards into the spinal cord. Destroy the spinal cord. Observe the violet contractions of the limbs while cord is being destroyed. This is due to the mechanical stimulation of nerve cells in the spinal cord. Complete relaxation of hind limbs and absence of reflex movement of the limbs upon pinching the skin of limbs with a pointed needle indicate successful pithing.
7. The bleeding from pithing hole after withdrawal of the needle is checked by plugging it with a small piece of cotton moistened with frog saline.

B. Dissection of nerve-muscle preparation:

Requirements: A pair of scissors, rat tooth forceps, rectangular tray, enameled iron bowl, cotton, frog saline, pin for fixing tissues, bone cutter, strong and thin thread (wax thread).

Procedure:
1. After pithing the frog perfectly, place it in a dissecting tray with its abdomen facing upwards.
2. Lift up the abdominal skin with a forceps and incise it up to the jaw. (Note: If cutaneous stimulation due to cutting causes reflex movements it indicates that the pithing is not perfect.)

3. Incise the skin on both forelimbs and hind limbs. Reflect the skin flaps on their sides.

4. Cut the muscles of abdomen taking sufficient care that the central vein is not injured. Reflect the muscle flaps on their sides.

5. Move all abdominal viscera and locate the thick whitish nerve trunks (sciatic nerve) emerging from the vertebral column and running their course towards the hind limbs on either side.

6. Cut transversely the vertebral column with a bone cutter a little above the place of emerging of sciatic nerves from the vertebral column. Discard the entire anterior portion of the body of the frog.

7. Pull out the skin carefully from the entire hind portion of the body of the frog.

8. Cut open the pelvic girdle with a bone cutter.

9. Hold the tip of the urostyle (prolongation of the vertebral column) with a forceps and lift it up. Cut off its muscular attachments at the lower end.

10. Make two parallel incisions with scissors on either side of the urostyle extending it upwards up to the origin of the sciatic plexus. (Sciatic plexus usually consists of 7th, 8th and 9th spinal nerves).

11. Lift urostyle with forceps and cut attachments with the last 9th vertebra with a bone cutter.

12. Cut the segment of the vertebral column very carefully in its medium line with a small bone cutter or a pair of scissors. Take care that the instrument in use for this step, does not slip on one side and damage the nerves of that side.

13. Hold the vertebral column with the fingers and clean the sciatic nerve downwards removing the extra attachments up to the thigh.

14. The nerve goes deep below the pyriformis and ilio-cooccygeous muscles at the junction of the thigh and then it emerges out on outer side of the thigh between the muscle masses consisting of triceps femoris, biceps or ilio-fibularis and semi-from outside to inside.

15. Pull apart the muscle along the shining line with the help of two thumbs and locate the thick trunk of sciatic nerve.

16. Trace the nerve up to ilio-pubic line and down to the knee joint. Cut the small branches going to the muscles while clearing the main nerve trunk.

17. Identify tendo-achillis just above its attachment to the bone and separate it from the tissues below. Pass a ligature with the help of an aneurysm needle between the bone and the tendon and tie it at its lowest level around the tendon.

18. Cut the tendon below this ligature to separate it from its bony attachments.

19. Hold the end of the ligature and tear off the muscle from bone so that it is separated up to knee joint.

20. Cut the tibio-fibula close to knee joint with a bone cutter.

21. Cut all the thigh muscle just above knee joint. Cut the femur bone with a bone cutter and finally complete the separation with a pair of scissors. Take care to avoid any injury to the nerve during this operation.

22. Separate the joint along with gastrocnemius muscle from remaining attachments.

C. General instructions to be followed during dissection of nerve muscle preparations:
1. Do not allow the nerve to come in contact with blood, body fluids, skin and other freshly cut tissues.
2. Keep the muscle and nerves moistened with 0.65% NaCl solution (frog saline) all the time and nerve allow them to dry.
3. The nerve should never be pinched during dissection.
DEMONSTRATION NO. 7

AIM: EFFECT OF PASSING A SINGLE SHOCK INTO A NERVE MUSCLE OR TO CALCULATE THE TIME TAKEN FOR DIFFERENT PHASES OF THE SIMPLE MUSCLE CURVE (SMC)

Apparatus required:
Mercury key, Induction coil, connecting wires, electrodes, short circuiting key, recording drum, stand, moist chamber with muscle lever, tuning fork and cotton.

Electric circuit:
Circuit No. 2 with the drum in circuit as above practical.

Speed of drum:
Obtain fast speed with following combinations
i. Pulley No.1 of the shaft and pulley no.4 of the drum
ii. Gear position: fast

Strength of induced current: Minimal current.

Procedure:
1. Check the primary and secondary circuits for their correctness. Fix the nerve-muscle preparation (after load position) in the moist chamber as in the previous experiment.
2. Rotate the cylinder in such a way that contact arms touch the contact spring of the drum.
3. By making and breaking the primary circuit with the help of the mercury key, adjust the distance between the coils in such a way that only break shock is effective in producing a muscle contraction. This means a minimal stimulus is adjusted.
   Note: When the cylinder is made to rotate, the contact arms touch and leave the contact spring quickly and thus make and break circuits are established instantaneously. When the drum is revolving at a fast speed the make and break stimuli follow each other so rapidly that second stimuli falls in the refractory period of the first (make) stimulus and as a result of (break) stimulus only one response is obtained.
4. Keep the writing point away from the smoked surface of the drum and adjust the height of contraction to 6-8cm. For this purpose adjust the strength of current.
5. Adjust the position of the smoked drum in such a way that the muscle curve will not be recorded at the paper joint.
6. Record a base line by rotating on base line the drum with hand.
7. Record a simple muscle curve by closing the mercury key. Open the mercury key after simple curve is recorded.
8. Mark the point with stylet when contact arm touches the contact spring. This point A is the point where a stimulus is given to muscle for recording SMC.
9. Mark point B when the curve is lifted from the base line.
10. Take line from highest point of SMC to the base line. Highest point is marked as C and on base line as C'. CC' present the maximum height of SMC. Record the height with a thread and foot rule.
11. Mark point D when the curve is coming down and touches the base line.
12. Mark E for any mechanical curve recorded due to jumping of writing stylet.
13. Remove the writing stylet away from the drum.
14. Take tuning fork and strike it again any hard surface to make it to vibrate.
15. Vibrations recorded below the base line are having frequency of 100 vibration/sec. These are called as time tracing as each small curve is equal to 0.01 sec.

16. Calculate number of curves between various points on SMC. Each curve represents 0.01 seconds.

17. Calculate time between:
   - AB: Latent period
   - BC: Contraction period
   - CD: Relaxation period
   - AD: Total time taken by SMC
DEMONSTRATION NO. 8  
AIM: TEMPERATURE EFFECT ON CONTRACTION OF FROG'S NERVE MUSCLE PREPARATION

Apparatus required:
Mercury key, Induction coil, connecting wires, electrodes, short circuiting key, recording drum, stand, moist chamber with muscle lever, thermometer, tuning fork and cotton.

Electric circuit:
Circuit No. 2 with the drum in circuit as above practical.

Speed of drum:
Obtain fast speed with following combinations
i. Pulley No.1 of the shaft and pulley no.4 of the drum ii. Gear position: fast

Strength of induced current: Minimal current.

Procedure:
1. Check the primary and secondary circuits for their correctness. Fix a freshly prepared nerve-muscle in the moist chamber as before. Make the lever after loaded. Fill the muscle chamber with frog saline solution kept at room temperature.
2. Adjust the height of contraction to approximately 6-8cm and record a SMC. Measure the temperature of the saline with thermometer and the record this temperature on the graph. Mark the point of stimulus.
3. Drain off the saline from the muscle chamber and replace it by saline having tempt about 20°C. Wait for a few minutes and then record the SMC on the same base line and at the same point of stimulus. Measure the exact tempt of saline and write it on the graph.
4. Similarly record the muscle response at 10°C, 25°C, 35°C and 40°C taking all care not to pour saline having tempt more than 40°C in the moist chamber.
5. Take the tome tracing calculate latent period, contraction period, relaxation period and height of contraction for each curve. Record height of each curve in cms. Calculate the results and enter them in a tubular form as given during the practical class.
6. Change the point of stimulus by shifting the contact arms. Drain off the saline. Touch the pointer, gently on the smoked surface. Now fill up the muscle chamber with saline as 45°C. Observe a vertical line being recorded as the lever goes up due to shortening of the muscle (heat rigor).
7. Study the physical characteristic of muscle after rigor and compare with fresh muscle. Record your observations.
AIM: TO STUDY THE ESSENTIAL FEATURES OF FATIGUE OR TO RECORD CONTRACTURE IN A FROG'S NERVE MUSCLE PREPARATION OR TO PROVE THAT THE FIRST SEAT OF FATIGUE IS NOT MUSCLE

Apparatus required:
- Mercury key, Induction coil, connecting wires, electrodes, short circuiting key, recording drum, stand, moist chamber with muscle lever, tuning fork and cotton.

Electric circuit:
- Circuit No. 2 with the drum in circuit as above practical.

Speed of drum:
- Obtain fast speed with following combinations
  i. Pulley No.1 of the shaft and pulley no.4 of the drum
  ii. Gear position: fast

Strength of induced current: Minimal current.

Procedure:
1. Connect the primary and secondary circuits and check them for the correctness.
2. Dissects out a fresh nerve-muscle preparation and fix it properly in a moist chamber.
3. Apply 10 grams weight as an initial load and arrange muscle lever in free loaded position.
4. Control the height of the muscle contraction by arranging the secondary coil at a proper distance from the primary coil.
5. Adjust the writing point on the smoked surface of the drum and draw a base line. Then record a SMC with the stimulus, which has been already adjusted.
6. Move the writing point away from the drum surface and allow the muscle to contract 10 times. Do not record these contractions on the drum surface.
7. Adjust the writing point on the drum surface at the same previous point of stimulus and record a curve and number it as two after the first.
8. Again move the writing point away from the writing surface and allow the muscle to undergo to contraction and then record the curved three by keeping the point of stimulus constant.
9. Repeat the above procedure till a feeble contraction is recorded on the drum surface. Mark the curve, every time, by a higher number.
10. Record the point of stimulus, latent period, contraction period, height of contraction and relaxation period of each of the curves obtained during the experiment. Record the time tracing on the drum for this purpose.
11. Select one more point of stimulus on the same base line. Adjust the writing point on the smoked surface of the drum. Apply the stimulus directly to the muscle and record its response. Observe the SMC obtained on the smoked surface. This proves that the muscles are not the first seat of fatigue. Fatigue is a progressive loss of irritability due to repeated activity.
Laboratory Manual
Veterinary Physiology
(Unit 3 & 4)

Prepared and compiled by
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CERTIFICATE

This is to certify that Mr/Miss ______________________________ Reg. No. ________________ of First year, B.V.Sc. & A.H. has satisfactorily carried out the required number of practical as shown in this Practical Manual of Veterinary Physiology-I, (4+1) during the year 2020 - 2021.

Course Teacher

Head of the Department

Date: Date:

External Examiner

Date:
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PRACTICAL NO. 1

AIM: GROSS EXAMINATION AND MOTILITY EVALUATION OF SEMEN

Semen evaluation is necessary for assessing the quality and potential fertility of semen sample. Evaluation of semen consists of following main criteria.

1. **Gross or Macroscopic examination:** Volume, colour, consistency, specific gravity, gross activity, electro conductivity and density.

2. **Microscopic examination:** Mass activity, individual motility, total sperm counts, live and dead sperm counts, morphological abnormalities of spermatozoa.

3. **Microbial examination:** Total bacterial count (examination), and isolation and identification of pathogenic microorganisms in semen.

4. **Biochemical and Metabolic tests:** pH, initial fructose content, fructolytic index, Methylene blue reduction test (MBRT), Resazurin reduction test (RRT), oxygen uptake, pyruvate utilization, total protein, macro-micro minerals activity test, Catalase test.

5. **Physical tests:** Resistance to cold and hot shocks, resistance to 1% sodium chloride solution, impedance change frequency.

**Gross/ macroscopic examination of semen:**

**Volume**

Ejaculate volume is of prime importance in artificial insemination. Ith higher volume a larger population of females can be inseminated. Volume is measured immediately after collection directly form graduated semen collection tube it is affected by following factors

- Age of animal
- Species and breed
- Individuality
- Restrain or false mount
- Type of thrust
- Type of ejaculate
- Frequency of semen collection
- Pathology of genital tract

**Colour**

Colour of semen indicates normal and abnormal activity of spermatogenic tract and its passage in the male. In case of bull, ram, buck and cock semen is concentrated and the color is milky or creamy white and opaque. In stallion, boar and dog, the spermatozoa are much less concentrated and the color is nearly white to gray and translucent abnormal colour of semen denotes certain pathological condition of genitalia. Yellowish colour indicates presence of pus or urine in the semen, reddish or pinkish colour that of blood or degenerative tissues in genital tract

**Consistency**

Normal bull semen may be thick creamy or milky in consistency depending upon the concentration of spermatozoa a rough estimate of sperm concentration can be made form
Density of semen ejaculate gives a rough estimate count and is viewed macroscopically by holding the tube containing semen against normal bright light. This is later confirmed by actual counting of sperm numbers by haemocytometric method. Density is recorded as under.

**Hydrogen ion concentration**

pH of semen indicates the rate of sperm metabolism. Seminal PH upon nature and degree of anaerobic metabolism of spermatozoa forming free acids, availability of carbohydrate substrate and buffering capacity of medium.

The pH is measured by
- Use of indicator paper
- pH meter
- Capillator with bromothymol blue or bromocrysol purple as indicators

**Microscopic examination of spermatozoa**

I. **Mass motility of spermatozoa**

The mass activity can be defined as en-mass motility of the spermatozoa in the semen. It is observed under low power microscope (10 X) in a fresh sample, immediately after its collection. It is commonly used as a measure of the fertilizing ability of spermatozoa.

**Procedure:**

1. Take a drop of freshly collected neat semen on a clean dry microscopic glass slide (without cover-slip).
2. Put the slide on the thermoregulatory stage of microscope (temp.37°C).
3. Observe a swarming mass of waves and eddies under the low power microscope.
4. Grade the degree of movement of all viable sperms in the semen from 0 to +4 as under:
   - 0 = spermatozoa are immotile (flat semen).
   - +1 = sperms movement slightly vigorous but no major waves (eddies).
   - +2 = Wave formation with slight whirl, which moves slowly across the field.
   - +3 = Rapid and vigorous waves with whirl and eddies, which change with great rapidly.
   - +4 = Extremely rapid movement and churning of whirl and eddies.

Initially flat semen samples generally gain progressive motility upon dilution with a suitable buffer to the extent of 80% (+3). The semen sample with optimum mass activity +2.5 and above can be permitted for use in AI program.

II. **Individual activity**

Sperm motility of the freshly ejaculated or stored semen can be used to detect gross difference in the sperm quality.

**Procedure:**

Semen is diluted in Ringer’s solution, normal saline or any other buffer solution (1:100) and kept in water-bath at 35-37°C.
1. A drop of diluted semen is taken on a clean dry micro-slide and examined at 37°C with cover-slip under low as well as high power microscope.

2. Observe the direction and speed of individual sperm in motion and express it as percent of the progressively/forward motile spermatozoa.

3. Observe, if any, abnormal type of sperm movement like circulating, oscillating, reverse or back movements. This indicates weak or damaged sperms in the given sample.

Note:

The middle piece of the sperm is believed to be the motor part as with loss of the head the middle piece and tail continue to move forward in a straight line. When tail is injured motility may cease or the sperm moves in circle of backward. A motility score grading is done from 0 to 5.

---

It is not possible to be a true scientist, without being honest in daily life

---- S.N. Ekbote

**Schematic diagram of spermatozoa cell**
PRACTICAL NO. 2

AIM: DETERMINATION OF SPERM CONCENTRATION IN A GIVEN SEMEN SAMPLE

The number of spermatozoa in a given ejaculate can be determined by one of the following methods.

1. Direct sperm count of semen by haemocytometer.
2. Use of photo-electric colorimeter.
3. Macroscopically by consistency or density of semen.
4. Opacity method by comparing with standard opacity solution.
5. Comparison of the packed cell volume of the semen ejaculates after centrifugation against a direct count by haemocytometer.

Sperm Count by Haemocytometer Method

This is a time consuming but most accurate method for determining the sperm concentration, in comparison to any of the above methods. The diluting fluid used is as under:

A) Dilution fluids for sperm count in R.B.C’s methods

1. 3% Chlorazene solution. (Salisbury et al.)
2. Citrate buffer (Hukeri,1960) - It contains
   Eosin yellow   -100mg
   Sodium citrate -2.9g
   Distilled water -100ml
   Commercial formalin -2 drops

The diluting fluid will kill all the spermatozoa and hence facilitate immediate estimation of sperm concentration in neat as well as extended semen.

Procedure

1. Adjust the clean, dry, absolutely grease-free Neubauer’s chamber upon microscope stage. Observe the ruled lines under a low power using concave surface of mirror.
2. Mix the given semen sample gently and draw the semen into a clean, dry, RBC pipette (red bead) exactly upto the 0.5 mark.
3. If the semen sucked is more than 0.5 mark adjust to the mark by striking the tip of the pipette with the help of index finger.
4. Wipe off semen adhered outside the tip of the pipette by cotton.
5. Suck the diluting fluid exactly upto the 101 mark.
6. Hold the pipette horizontally and roll it between the palms.
7. Discharge initial few drops of diluting fluid from the pipette before charging the Neubauer's chamber.
8. Charge the chamber by putting a drop between cover-slip and Neubauer's chamber. Allow fluid to spread under the cover-slip.
9. After charging chamber correctly, allow the sperm to settle down (wait for about 2 min).

10. Focus RBCs counting squares of the counting chamber.

11. Change over to and count all the sperm in each of the 16 squares of five secondary squares (four corner and one central square).

12. Sum up all cells and multiply with $10^7$.

**Calculation**

1) Surface area of five secondary squares is $= (1/5 \times 1/5) \times 5$

2) Cubic area of five secondary squares is $= 0.1(1/25) \times 5 = 1/50$ cmm

3) Dilution is 200 times.

4) Number of sperms counted in five secondary $(16 \times 5 = 80$ tertiary) squares is $N$ in $1/50$ cmm

5) Hence in 1 cmm area, it will be $N \times 50 \times 200$

6) In order to convert 1 cmm to 1 ml, values are multiplied by 0.001 ml i.e. $Nx50x200 \times 1000 = NX \times 10^7$

**B) Sperm Count by WBCs Method:**

1. Draw the neat semen carefully upto 0.1 mark.

2. Mix the semen with 9.9 ml of physiological saline solution to make the dilution 1:100 and mix.

3. Take 1.0 ml of above diluted semen; add 9.0 ml 0.05% Eosin solution.

4. Transfer this semen to ice-chamber to kill sperms by cold.

5. Change the Neubauer's chamber and count the sperm in four primary (64 secondary) squares of the corners.

6. Take out the average for one primary (16 secondary) square.

7. Multiply this overall average count by $10^7$ get actual sperm cont per ml of semen.

---

*Basic research is when I’m doing what I don’t know I am doing*

------- Werner Van Braun
PRACTICAL NO. 3  

DATE:  

AIM: DETERMINATION OF LIVE AND DEAD SPERM PERCENTAGE IN A GIVEN SEMEN SAMPLE

The presence of high percentage of live, progressively, motile, vigorous spermatozoa in the ejaculate usually indicate good quality semen. The percent of live sperm can be determined by means of differential staining of semen smears.

Percent of live sperm in semen depends mainly upon the frequency of collection, age, health of bull and season.

The dead and live staining is based upon the difference between dead and live cells in absorbing certain dyes. The differential stain most commonly used now-a-days is Eosin-Nigrosin strain. The sperms that are dead at the time of staining will take up the eosin stain and appear pink. Procedure:

1. Keep the stain and the semen sample at the same temperature in a range of 35° to 38°C.
2. Place a small drop of semen (either diluted or neat) on a clean glass-slide.
3. Put 2-3 drops of stain near the semen on the same slide.
4. Spread the Eosin-Nigrosin stain towards the semen with the help of platinum loop or by a corner of another slide.
5. Then immediately prepare a few medium thick smears of stained semen.
6. Allow them to dry in air.
7. View the sperms under oil emulsion lens (100X) and count like DLC.
8. Nearly 200 sperms are generally counted and the percentage of live sperms is then computed by formula as -

\[
\text{Per cent live sperms} = \frac{\text{No. of live sperms present}}{\text{Total sperms counted}} \times 100
\]

Composition of Eosin-Nigrosin stain

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eosin-Y</td>
<td>1.67 gm</td>
</tr>
</tbody>
</table>
| Nigrosin water soluble         | 10.00 gm | pH 6.8 to 7.0
| Sodium citrate dihydrate       | 2.9 gm   |
| Distilled water                | 100.00 ml|

Dissolve the ingredients in distilled water by heating the flask in water-bath under a condenser, allow it to cool and then filter. This stain is stable at room temperature for more than one year. It is widely accepted, simple and effective vital stain used for counting the percentage of live/dead sperms.

Other stains occasionally used are:

1. Eosin-Opal blue stain (Lasley et al., 1942): It consists of 2 parts.
   (a) 2% Eosin in 1/8 M Phosphate buffer
        (80.4 CC of 1/8 M Na₂HPO₄ and 19.6 CC of 1/8 M KH₂PO₄)
(b) One part Opal blue in one part 1/8 M Phosphate buffer (80.4 CC)
Mix Soln. A and B: 1:1, heat and filter

2. Eosin-Fast Green stain (Mayer et al., 1957)
   Fast green FCF grade : 2.0 gm
   Eosin (or Erythrocin) : 0.8 gm
   1/8 M Phosphate buffer : 100 ml
   \[ \text{pH 7.3} \]

3. Eosin-Aniline blue stain (Shaffer and Almquist, 1948)
   Eosin-Y : 1.0 gm
   Aniline blue : 4.0 gm
   1/8MPO\textsubscript{4} buffer : 100 ml
   \[ \text{pH 7.2} \]

4. Rose-Bengal stain
   Rose bengal : 3.0 gm
   Commercial formalin : 1.0 ml
   Distilled water : 100 ml
   \[ \text{pH 7.2} \]

This stain is toxic to the sperm and hence may give higher abnormalities of secondary type in semen smear.

Biologists must always keep in mind that what they see was not designed but evolved

----- Francis Crick
AIM: DEMONSTRATION OF ESTROUS CYCLE IN DIFFERENT SPECIES

The cyclic change represented by the female at a regular interval of time is known as estrous cycle. In primates, such cycle is known as menstrual cycle. The estrous cycle has got different phases namely the proestrus, estrus, metestrus and diestrus. The duration or length of estrous cycle in farm animals is on an average 18-22 days. There are two main phases according to predominant ovarian structure or circulating hormone, viz.,

1. Follicular or Estrogenic phase, i.e. proestrus + estrus phases.
2. Luteal or Progesteronic phase, i.e. metestrus + diestrus phases.

The estrous cycle involves following physiological & cytological changes.

1. The development of the follicle.
2. Ovulation of the follicle and release of ova.
3. Formation and regression of corpus luteum.

Proestrus:

Proestrus is the stage in the beginning of estrous cycle, where the necessary changes are taking place for exhibiting the estrus. The vestibule and posterior vagina show congestion, edema and on anterior part of vagina large wide mucus cells and edematous stroma are seen. Cervix shows mucus secreting cells; in uterus cells become tallest and in uterine tube cilia shows activity, edematous swelling, granules in epithelial cells and cytoplasmic projections from the cells. Observe proestrus symptoms in different species.

Estrus:

The second stage is called as estrus phase/period, wherein the animal shows all the cytological and physiological changes related to exhibition of symptoms of estrus and it also shows receptivity for the male. Estrus stage is characterized by further congestion, edema of vaginal part, cells becomes columnar with abundant leucocytes. Cervix cells are loaded with mucus, and cervix becomes edematous and hyperaemic. Cervical mucus is secreted from cervix as a transparent watery-white sticky substance. Uterus also gets congested due to increased blood circulation. There is also edematous swelling. Uterine tubes also show active cilia and the fimbriated end shows amoebic activity for collecting the ova if released during estrus stage. Observe estrus symptoms in different species.

Metestrus:

It is 3rd phase. Here animal's receptivity for male and signs of estrus are declined, congestion decrease and animal looses interest in male. Observe the animals in metestrus.

Diestrus:

It is the 4th stage, in which corpus luteum develops at the site of ovulation. All estrus signs are abolished, and animal if becomes pregnant, this phase extends upto its gestation period. Otherwise it terminates within 15-16 days and again a stage of proestrus comes.

Metestrus and diestrus collectively forms a post-estrus phase. In the cow, this stage/phase is characterized by cornification of the vaginal cells, leucocytes are also seen and congestion is decreasing. Different species of animals are having different length and duration of estrous cycle and its phases.

The animal will show estrous cycle at the time of puberty. Certain time is allowed to lapse after puberty for animal to breed. This time, which is a proper time for breeding animal,
is known as sexual maturity. At puberty it is not advisable to breed/conceive the animal, since the reproductive organs are not fully developed, but at maturity genital development is complete and maturity is more appropriate for making animal pregnant. In dog, cat, rat to identify stage of estrus, vaginal smear is prepared whereas in other domestic animals vaginal smear is not much valid.

**Table 1: Duration of Various Phases of Estrous Cycle in Domestic Animals**

<table>
<thead>
<tr>
<th>Species</th>
<th>Total Length of cycle(days)</th>
<th>Duration of Phases of estrous cycle</th>
<th>Ovulation time</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Proestrus (days)</td>
<td>Estrus (days)</td>
</tr>
<tr>
<td>Cow</td>
<td>21</td>
<td>3-4</td>
<td>1</td>
</tr>
<tr>
<td>Buffalo</td>
<td>21</td>
<td>3-4</td>
<td>1</td>
</tr>
<tr>
<td>Sheep &amp; Goat</td>
<td>18-20</td>
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<td>1-2</td>
</tr>
<tr>
<td>Horse</td>
<td>21-28</td>
<td>2</td>
<td>5-6</td>
</tr>
<tr>
<td>Camel*</td>
<td>21-23</td>
<td>2-3</td>
<td>3-4</td>
</tr>
<tr>
<td>Pig</td>
<td>21</td>
<td>2</td>
<td>2-3</td>
</tr>
<tr>
<td>Bitch</td>
<td>2 months</td>
<td>7-9</td>
<td>7-9</td>
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</tbody>
</table>

* Induced ovulator; all others - spontaneous ovulators.

*As knowledge increases wonder deepens*

--- *Charles Morgan*
PRACTICAL NO. 5

DATE:

AIM: STUDY OF RADIO-IMMUNO-ASSAY (RIA) TECHNIQUE

Antibodies, antigen and many small organic molecules such as drugs are most often measured by sensitive procedures that take advantage of radioactive labels on the antibodies or antigen (RIA) or of enzymes linked to antibodies (ELISA). RIA is one of the most sensitive techniques for deleting antigen or antibody.

The technique was first developed by two endocrinologists, S. A. Berson and Rosalyn Yalow, in 1960 to determine levels of insulin-anti-insulin complexes. This technique is useful for measuring hormones, serum proteins, drugs and vitamins at concentrations of 0.001 micrograms per milliliter or less. Yalow got Nobel Prize in 1977, after Berson's death.

Determination or detection of any minute quantity of substance with the help of radio-isotope under the principle of immunology is called radio immuno assay (RIA). This assay is also called as saturation analysis where reagent (antibody) is very less in quantity compared to analyte (antigen), so the reagent gets saturated. While in biochemical analysis the reagent will be always in excess.

The RIAs are often based on competition for antibodies (Ab) between a radioactive "indicator" legend (Ag* or L) and its unlabeled counterpart (Ag) in the test sample; the higher the level of Ag, the less Ag* or L is bound. In Radio Immuno Assay, the amount of labelled antigen binding with antibody will be inversely proportional to the concentration of unlabelled antigen present in the serum sample. The concentration of Ag is readily determined by comparison with a calibration curve prepared with purified Ag at known concentration.

\[
[\text{Ag}] = \frac{\text{Total Ag (Ag + Ag*)}}{\text{Ag*}}
\]

The labeled antigen (Ag*) is mixed with antibody at a concentration that saturates the antigen-binding sites of the antibody molecule and then increasing amounts of the test sample containing labeled antigen of unknown concentration are added. The antibodies can bind equally to unlabeled and labeled antigen, and hence two kinds of antigen complete for available binding sites on the antibody. With increasing concentration of unlabeled antigen more labeled antigen will be displaced from the binding sites.

The antigen is generally labeled with gamma-emitting isotopes such as I^{251}, but beta-emitting isotopes such as tritium (3H) are also routinely used as labels. The amount of labeled antigen bound is precipitated to separate it from free antigen (not bound to Ab), the radioactivity in the precipitate or supernatant is measured from a prepared standard curve.

Solid-Phase RIAs

This method makes separate of Ag-Ab complex easier from the unbound antigen. The antibody can be immobilized on

(1) Sepharose beads, the amount of radiolabeled antigen bound to the beads can be measured after the beads have been centrifuged and washed.

(2) Polystyrene on polyvinyl-chloride wells and the amount of free-labeled antigen in the supernatant is determined in a radioactive counter.

(3) The antibody is immobilized on the walls of microtiter wells and the amount of bound antigen determined (Hepatitis virus).

The basic principle behind the Radio Immuno Assay is, the readily detectable radiation from the tracer isotopes coupled with the highly specific reactions of an antigen.
with its antibody are made use to attain a high degree of detection with high sensitivity and specificity in the estimation.

\[
\text{Ag} + \text{Ab} \rightleftharpoons [\text{AgAb}] + [\text{Ag}\!*\text{Ab}] \text{ Ag} + \text{Ag}*
\]

<table>
<thead>
<tr>
<th>S.N.</th>
<th>Ag</th>
<th>Ab</th>
<th>Ag*</th>
<th>Total [Ag]</th>
<th>Bond [Ag]</th>
<th>Free [Ag]</th>
<th>B/T [Ag]</th>
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<td>12</td>
<td>84</td>
<td>0.13</td>
<td>0.14</td>
</tr>
</tbody>
</table>

Requirements of RIA:

I) Standard - Hormone
II) Tracer - Labelled hormone
III) Binder - Antibody
IV) Separator - Separating chemical

Standard:

Based on the standard curve the quantification of analyte will be done. Standard hormone will also have part in the immunology reaction; hence it should have following characteristics.

a) Chemical purity
b) Physical and immunological homogenicity
c) Estimate of biological activity
d) Known value of mature and concentration of contaminants

Tracer

It has got its own importance in RIA. It is dependent on the choice of label and on the assay system. Ability of the system to measure picogram quantity can be exploited only if the antigen can be labelled to high specific activity of isotope.

Radio Isotopes Used for RIA

- \(^{14}\text{C}\) 5730 year Half life Beta 0.156 Mev.
- \(^{3}\text{C}\) 12.33 year Half life Beta 0.0186 Mev.
- \(^{125}\text{I}\) 60 days Half life gamma 0.0355 Mev.
- \(^{131}\text{I}\) 8 days Half life Beta -0.606 Mev.
  gamma 0.30 Mev.
Binder

The binder in RIA is always antibody (IgM and IgG). Antibodies (Ab) are produced against required antigen either in rat or rabbits. There are two methods of antibody production (i) polyclonal (ii) monoclonal. Monoclonal antibodies are more specific than polyclonal antibody.

Separator

After equilibration in the reaction, there is need for separating bound fraction from free fraction, otherwise it will produce error due to free-labelled antigen. There are three major methods for separating bound form from free form.

1) Adsorption of free antigen to a suitable adsorbent
   e.g. Dextran coated charcoal
        Albumin coated charcoal

2) Precipitation of antigen-antibody bound fraction
   e.g. Poly ethylene glycol
        Second antibody method

3) Solid phase technique
   e.g. Antibody coated plastic tubes
        Antibody coated plastic glass beads

After separating bound fractions, i.e. antigen-antibody bound, these bound fractions are measured in the gamma counter for radiation. A graph is plotted for known standards (standardization) and than value of unknown samples can be obtained from the graph.

You cannot teach a man anything you can only help him find it within himself

------ Galileo
Objective:

Estimation of serum progesterone by enzyme linked immunosorbent assay (ELISA) technique.

This technique is similar in principle to RIA, but depends on an enzyme rather than a radioactive label. An enzyme conjugated with an antibody reacts with a colorless substrate to generate a colored reaction product. Such a substrate is called a chromogenic substrate. A number of enzymes employed for ELISA are alkaline phosphatase, horseradish peroxide, and beta-galactosidase. The assays' accuracy is equal to RIA. ELISA method is simple and cheap, yet sensitive technique as compared to, costly, cumbersome and health hazardous radio-immuno-assay (RIA) technique. ELISA technique allows qualitative detection as well as quantitative measurements of either antigen on antibodies. In case of quantitative measurements a standard curve based on known concentrations of antigen on antibodies is prepared from which the unknown concentration of a sample can be determined. There are various types of ELISA techniques, viz.,

1) Indirect ELISA
2) Sandwich ELISA
3) Competitive ELISA
4) Chemiluminescence
5) ELISPOT Assay

(1) **Indirect ELISA**

Micro liter well contains antigen-coaling serum or sample containing primary antibody (Ab₁) is added to micro liter well. Allow antibodies to react with antigen attached to the well. Wash away free Ab₁. The presence of antibody bound to antigen is detected by adding an enzyme-conjugated secondary anti-isotype antibody (Ab₂), which binds to the primary antibody. Free Ab₂ is washed away and substrate for the enzyme is added. The amount of colored reaction product that forms is measured by specialized spectrophotometer plate readers, which can measure the absorbance of all the wells of 96-well plate in less than a few seconds.

(2) **Sandwich ELISA**

In this technique, the antibody (not the antigen) is immobilized on micro titer well. A sample containing antigen is added and allowed to react with the immobilized antibody. Wash the wells, add a second enzyme-linked antibody specific for a different epitope on the antigen is labeled and allowed to react with the bound antigen. Remove by washing free second antibody, if present any. Add substrate and the colored reaction product formed is measured.

(3) **Competitive ELISA**

In this technique antibody is first incubated in solution with a sample containing antigen. The antigen-antibody mixture is then added to an antigen coated micro titer well. The more antigen present in the sample, the less free antibody will be available to bind to the antigen-coated well. Addition of an enzyme-conjugated secondary antibody (Ab₂) specific for the isotype of the primary antibody can be used to determine the amount of primary antibody bound to the well as in indirect ELISA.
(4) Chemiluminescence

Measurement of light product by chemiluminescence during certain chemical reactions provides a convenient and highly sensitive alternative to absorbance measurements in ELISA assays. A luxogenic (light generating) substrate takes the place of the chromogenic substrate in conventional ELISA reaction. Oxidation of the luminol compound by \( \text{H}_2\text{O}_2 \) and the enzyme horseradish peroxidase produces light. Detection limit can be increased at least ten-fold by switching from a chromogenic to a luxogenic substrate, and with the addition of enhancing agents, more than 200-fold (\( 5 \times 10^{-8} \) moles of antigen detectable).

(5) ELISPOT Assay

It allows determination of number of cells in a population that are producing antibodies specific for a given antigen or an antigen for which one has a specific antibody. The plates are coated with the antigen recognized by the antibody of interest or with the antibody specific for the antigen whose production is being assayed. A suspension of the cells under investigation is then added to the coated plates and incubated. The cells settle on to the surface of the plate, and secreted molecules reactive with the capture molecules are bound by the capture molecules in the vicinity of the secreting cells, producing a ring of antigen-antibody complexes around each cell that is producing the molecule of interest. The plate is then washed and an enzyme-linked antibody specific for the secreted antigen or specific for the species of the secreted antibody is added and allowed to bind. Afterwards, suitable chromogenic or chemiluminescence-producing substrate reveals the position of each antibody or antigen-producing cell as a point of color or light.
AIM: EFFECT OF CLIMATE ON ANIMALS AND ANIMAL PRODUCTION

Introduction: All species of animals do not suit all the geographical regions with different climate. Hence camel and sheep are suited for arid zones, but not for Tibet/cold regions. Similarly, yak is not suited for Rajasthan (arid zone), but it can survive in Himalayas.

The major components of climate affect animal’s performance through neuroendocrine system. This acts on the body to bring about homeostasis or equilibrium through body heat balance, thermoregulation, balance of water and electrolytes, circulatory balance and cardiovascular activity.

Indirect effect of climate on animal is through changes in vegetation (nutrition) including all form of food, water, shelter, shade etc. Animal husbandry practices try to minimize the effect of climate on animal’s performance by providing uniform condition (such as feed, water, shade, shelter etc) throughout the year. This is a costly process and yet a private animal owner cannot do it because it is costly and resources (green feeds) are not available throughout the year eg in summer.

Now a days prediction of animal diseases is done by forecasting the weather. This helps in taking the precautionary measures in advance and protects the animal. This is called weather based forecasting of diseases.

Effects heat stress on animals:
1. Animal’s performance decreases.
2. Availability of feed and water is limited.
3. Voluntary feed and water intake decreases.
4. Milk production of European breeds decreases when the temp is above 21°C. In the Jersey and Brown Swiss breeds it declines at 24-27°C but in Zebu it declines only above 32-34°C.
5. Animal present under sun show shade seeking behaviour, when ambient temp is greater than body temperature.
6. Production of concentrated urine.
7. Animals standing dispersed.

Effects cold stress on animals:
1. Conservation of core temperature of body.
2. Occupy warmer spots.
3. Piloerection
4. Peripheral vasoconstriction occurs
5. Thermogenesis by shivering.
6. Generally cold is considered good because animal can eat more and is comfortable, but zero and sub zero temp leads to stress in animals.

Effects of solar radiation:
1. Direct exposure to sun rays results in decreased heat load on animal and body temp increases.
2. Decrease in feed intake.
Exercise:
To study the effect of physiological responses in animals exposed to heat, one animal is kept in the shed and other animal is kept outside the shed in open.
1. Record rectal temp of both the animals by inserting clinical thermometer in the rectum. The tips of the thermometer should touch the mucus membrane of rectum. Keep it in rectum for one to two minutes.
2. Record respiration rate of both the animals by keeping palm in front of the nostrils of the animal. Recording the number of times the air is breathed out in one minute or else counts the movements of paralumber fossa in one minute.
3. Record pulse rate of animals by palpating ventral coccygeal artery for one minute.

Recorded data:

Place of work:
Species of animal:
Breed of animal:
Control animal no:
Experimental animal no:
Date of work:
Time:

<table>
<thead>
<tr>
<th>Parameters</th>
<th>In shed</th>
<th>Outside</th>
</tr>
</thead>
<tbody>
<tr>
<td>Environmental temp</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rectal temp</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Respiration rate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pulse rate</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Interpretation:
AIM: HEAT TOLERANCE - CONCEPT, MEASUREMENT AND REGULATION

Concept of Heat tolerance:
Heat tolerance is the animal ability to escape adverse consequence of the direct operation of hot condition ie it is a measure of its ability to withstand heat when all other factors are constant. Indian tropical breeds of cattle are heat tolerant in comparison to temperate breeds of cattle. In a breed, animals may be uniform anatomically but when they are subjected to hot conditions, wide variations are found in the extent and nature of responses. This is due to the physiological status of the animal, feeding pattern and health etc.

Reactions on exposure to heat:
1. Respiration rate: For the heat regulation, the evaporative heat loss takes place from the respiratory passages. The rate of heat loss is increased by increase in respiratory rate. It is volume of respired air per minute which is important for heat loss than number of respiration per minute.
2. Sweating: The heat loss through sweating is a major channel of heat loss. Indian breeds of cattle and camel sweat profusely in hot environment.
3. Rectal temperature: It is a reliable index of deep body temperature especially under warm conditions. It is one of the most significant reactions to heat stress.
5. Feed consumption: In hot environment there is decline in feed intake.
6. Productive traits: In milch animals, which are susceptible to heat stress, there is decline in milk yield. In beef cattle there is no decline in weight gain in heat tolerant animals. In draft animals there is decline in work capacity in hot climate.
7. Reproductive efficiency: when temperate cattle are brought up in tropical climate, the male and female fertility is reduced due to tropical hot climate. Same in buffaloes reproductive efficiency decline in summer.

Heat tolerance tests (Heat Tolerance Coefficient, HTC):
The following are the heat tolerance tests, which evaluate an animal in a numerical value. Thus comparison can be made between the animals of the same breed or between animals of different breeds.

1. Iberia Heat Tolerance Test:
This test meant for cattle. This test is carried out in open when the temperature is between 85 to 95°F and there is bright sunshine. The animals are exposed from 10am to 3pm and rectal temperature and respiration rate is recorded before and after the exposure. This is carried out for three days with nearly the same atmospheric conditions. Atmospheric temperature, radiation, humidity and air movement are also to be recorded.

\[ HTC = 100 - 1 \cdot (RT - 101) \]

RT – Average rectal temperature of six reading. 101°F is the average temperature of the cattle. When the two animals have the same heat tolerance coefficient (HTC) then the animal, which has got higher respiration rate, is less heat tolerant.
2. Born Test:
   In this test the animals are kept in the open in natural conditions. It is carried out just like Iberia Heat Tolerance test. Animals are not disturbed in this test.

3. Benezera’s Coefficient of Adaptability (BCA):
   It is based on the body temperature and respiratory rate responses after exposure for 7 hours on three consecutive days.

   \[ BCA = \frac{\text{Body temp °C}}{38.33} + \frac{\text{Respiration rate}}{23} \]

   A value of 2 shows maximum adaptability and value over 2 a state of lower adaptability.

4. Dairy Search Index (DI):
   It takes into consideration rectal temperature, respiration rate and pulse rate with appropriate weightage. The animals are exposed for seven hours on sunny cloudless day. The index is obtain by,

   \[ DI = 0.5 \frac{X_1}{X} + 0.3 \frac{Y_1}{Y} + 0.2 \frac{Z_1}{Z} \]

   Where, \( X_1, Y_1 \) and \( Z_1 \) are rectal temperature, pulse rate and respiration rate after exposure and \( X, Y \) and \( Z \) the same parameter before exposure respectively.

Observation table:

<table>
<thead>
<tr>
<th>Physiological response</th>
<th>Animals</th>
<th>Normal values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Experimental</td>
<td>Control</td>
</tr>
<tr>
<td>1. Rectal Temp (°C)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. Respiration rate/min</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3. Pulse rate/min</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
AIM: THI AS A TOOL FOR ASSESSMENT OF HEAT STRESS

The Temperature Humidity Index (THI) is also known as discomfort index because when the temperature and humidity both are high then it causes discomfort to the animals like crossbreds and buffaloes. Due to high humidity the evaporation does not take place from the body.

We can classify the **temperature** into three categories –
1. Hot temperature (hot by more than 30°C),
2. Warm temperature (between 20 to 29°C),
3. Cold (below 10°C)

Likewise **humidity** can also be classified as per the dew point –
1. Wet (above 20 dew point),
2. Humid (between 16 to 19 dew point),
3. Dry (less than 15 dew point)

Based on this condition Thom et al. (1959) derived the formulas for calculating the THI. Modified formulas for calculating THI as below

\[
\text{THI} = 0.72 (\text{Cdb} + \text{Cwb}) + 40.6 \\
\text{THI} = (0.55 \text{Cdb} + 0.2 \text{Cdp}) 1.8 + 32 + 17.5 \\
\text{THI} = \text{Cdb} + (0.36 \text{Cdp}) + 41.2 \\
\text{THI} = 0.8 \text{Cdb} + \text{RH} (\text{Cdb} -14.4) + 46.4
\]

Where, Cdb is temperature of dry bulb, Cwb is temperature of wet bulb, Cdp is temp. dew point; \{Cdp = Cdb – [(100 - RH)/5]\} and RH is relative humidity.

<table>
<thead>
<tr>
<th>THI value</th>
<th>Level of heat stress</th>
<th>Effect on animal (in cattle)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 72</td>
<td>Comfortable</td>
<td>Normal</td>
</tr>
<tr>
<td>72-79</td>
<td>Mild</td>
<td>Little discomfort to the animals</td>
</tr>
<tr>
<td>80-89</td>
<td>Medium</td>
<td>depress the feed intake and decrease milk yield of the animals</td>
</tr>
<tr>
<td>90-98</td>
<td>Severe</td>
<td>Major level of discomfort</td>
</tr>
<tr>
<td>&gt; 98</td>
<td>Danger</td>
<td>Animal may die</td>
</tr>
</tbody>
</table>

Berry et al. (1964) used the value of THI and calculate milk production decline in cow (MDEC, kg/cow per day)

\[
\text{MDEC} = 1.075 - 1.736 \text{M} + 0.0247\text{M} \times \text{THI}
\]

Where M is normal level of milk production of cow (kg/cow per day)
PRACTICAL NO.: 10  DATE:

AIM: UTILITY OF STUDYING ETHOLOGY

Ecology: The study of animals and plants in relation to each other and to their surroundings. It is the study of relationship of organism to their environment.

Ethology: It is the branch of science which deals with study of animal behavior.

Ethogram: Rating or quantitation of animal behavior on a given scale is called ethogram. It is a detailed description of behavioral feature of a particular species.

Scope:
In any live stock farming system animal welfare is important with regards to health and production. Behaviour of animal is an immediate expression of deprivation, discomfort and disease. People working with farm animals have to understand the language of animal behaviour. The knowledge of applied farm ethology is required.
1. To breed and raise livestock.
2. To increase productivity.
3. To remove stress and fulfill behavioral need of animals.
4. To assess heritability of behavior.
5. To make optimal use of advances in farm management.

The veterinarian’s concern is –
1. To diagnose the disease/abnormality.
2. To examine and treat animals.
3. To treat behavior abnormality.
4. To reduce stress related diseases.
5. Designing animal housing which is comfortable.

Animal are sentient creatures. They show unmistakable signs of exhaustion, fright, frustration, pain, anger and other emotions which can be observed in their voice, expression, reaction etc ie their behaviour.

Some definition related to animal behaviour:

Instinctive or Innate behaviour: Animal behaviour present since birth. It is automatic and reflex in nature. It is a natural tendency to behave.

Learned behaviour: Learned behaviour in response to environmental stimuli.

Conditioned behaviour: Conditioning is a process by which an animal forms a association between a previously neutral stimulus or behavioural response and previously significant stimulus.

Imprinting: It is a rapid and relatively stable learning, which takes place in early life of an animal (critical period).

Critical period or Sensitive period or Optimum period: In an animal soon after birth there is a limited time period during development when the organization of a system is early modified eg attachment of a newborn to its mother ie mother-young bonding.
Following major behavioural activities are studied in cattle and buffaloes:

1. Eating or Ingestive behaviour
2. Rumination behaviour
3. Resting / Idling behaviour
4. Drinking behaviour
5. Sleeping behaviour
6. Walking/Roaming/Loitering behaviour
7. Posture behaviour
8. Eliminative behaviour

Domestication of animals is a contact between animal and man, whereby animal gives up its freedom in exchange of its welfare and protection. Animal scientists have come to realize that ethology can help in animal welfare leading to better production. The environmental factors such as social companionship, regular feeding, rest, sleep etc are very important in the lives of animal. In the intensive system of livestock rearing also, animal should have following five freedoms.

1. To get up
2. To lie down
3. To turn around
4. To stretch its limbs and
5. To groom normally

Animal welfare activities emphasize on these points and people (consumers) refused to eat meat from calves, which were not provided with above freedoms.
AIM: PREPARATION OF AN ETHOGRAM

An ethogram is a detailed description of the behavioural feature of a particular species usually concerning single individuals or interaction between pair of individuals.

<table>
<thead>
<tr>
<th>Behaviours</th>
<th>Characteristic pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ingestive</td>
<td>Grazing, browsing, ruminating, licking salt, drinking</td>
</tr>
<tr>
<td></td>
<td>Suckling – nudging udder with nose, suckling, wiggling tail</td>
</tr>
<tr>
<td>Shelter-seeking</td>
<td>Moving under trees into burns</td>
</tr>
<tr>
<td></td>
<td>Huddling together to keep off flies</td>
</tr>
<tr>
<td></td>
<td>Crowding together in extreme cold weather</td>
</tr>
<tr>
<td></td>
<td>Pawing ground and laying down</td>
</tr>
<tr>
<td>Investigatory</td>
<td>Raising head, directing eyes, ears and nose towards disturbance</td>
</tr>
<tr>
<td></td>
<td>Nosing an object or another sheep</td>
</tr>
<tr>
<td>Allelomimetic (groups)</td>
<td>Walking, tunning, grazing and bedding down together</td>
</tr>
<tr>
<td></td>
<td>Following one another</td>
</tr>
<tr>
<td></td>
<td>Bouncing stiff-legged past an obstacle together (sheep)</td>
</tr>
<tr>
<td>Agonistic (conflict, fighting, escape)</td>
<td>Pawing, butting, showing with shoulders</td>
</tr>
<tr>
<td></td>
<td>Running together and butting (rearing and butting in goats)</td>
</tr>
<tr>
<td></td>
<td>Bunching and running, lying immobile</td>
</tr>
<tr>
<td></td>
<td>Freezing (in young kids only)</td>
</tr>
<tr>
<td></td>
<td>Snort and stamp foot (sneeze in goats)</td>
</tr>
<tr>
<td>Eliminative</td>
<td>Urination posture: squatting of females, arching back, bending legs (male lambs)</td>
</tr>
<tr>
<td></td>
<td>Defecation: wiggling tails</td>
</tr>
<tr>
<td>Care giving (epimelectic)</td>
<td>Licking and nibbing placental membranes and young</td>
</tr>
<tr>
<td></td>
<td>Arching back to permit nursing, nosing lamb at base of tail</td>
</tr>
<tr>
<td></td>
<td>Circling newborn lamb</td>
</tr>
<tr>
<td></td>
<td>Baaaing when separated from lamb</td>
</tr>
<tr>
<td>Care soliciting (et-epimelectic)</td>
<td>Baaaing (distress vocalization by lamb when separated, hungry, hurt or caught)</td>
</tr>
<tr>
<td></td>
<td>Baaaing of adult when separated from flock</td>
</tr>
<tr>
<td>Sexual (male)</td>
<td>Courtship: following female, pawing female, hoarse baa or grumble, nosing genital region of female</td>
</tr>
<tr>
<td></td>
<td>Flehman reaction: sniffing female urine, extending neck with upcurled lip, running tongue in and out, rubbing against side of female, biting wool of female, herding or pushing female away from other males</td>
</tr>
<tr>
<td></td>
<td>Copulation: wiggling tail (rare), mounting, thrusting movements of hindquarters</td>
</tr>
<tr>
<td>Sexual (female)</td>
<td>Courtship: rubbing neck and body against male, mounting male (rare)</td>
</tr>
<tr>
<td></td>
<td>Copulation: standing still to receive male</td>
</tr>
<tr>
<td>Play</td>
<td>Sexual: mounting (by either sex), agonist-playful butting</td>
</tr>
<tr>
<td></td>
<td>Allelomimetic: running together, gamboling (bouncing stiff-legged and turning in all together)</td>
</tr>
<tr>
<td></td>
<td>Game playing – jumping off and on rock or log together</td>
</tr>
</tbody>
</table>
PRACTICAL NO.: 12

AIM: TO STUDY DRINKING BEHAVIOR IN FARM ANIMALS

It depends upon the need of the water by the body. Water is regulated by the body water compartments and total 20% of the blood that is circulating depends upon the thirst mechanism. Main purpose of drinking water is to maintain osmotic pressure of the extra cellular fluid. The thirst mechanism is mainly regulated by osmoreceptors located in the hypothalamus.

**Camel:** It is an animal of desert having fat storage in hump and can metabolise fat to set metabolic water. Also they have anatomical structure like pouch are located in their rumen/stomach, which may stored water. They have capacity to drink water in large quantity in a short time and can live for a longer time under dehydrated condition.

**Canines:** They protrude their tongue, roll it into a spoon form and withdraw tongue with water into the mouth. The frequency increases during thirst.

**Birds:** The drinking behaviour of birds is different. They put their beak inside the water and trap water into the beak and lift head upward so that water can enter into oesophagus. They do not have soft palate hence do not create a negative pressure but by gravitational force drink water.

**Cattle:** This animal drinks water about 45-60 liter per day. They have rumen capacity about 150-200 liter. During grazing and rumination processes they secret around 120 liter of saliva which are to be added to stomach capacity, they don’t have any proper or defined storage of water. Requirements of water in different physiological state like pregnancy, lactation, they need water as per requirement.

**Exercise:**
1. On farm study the drinking behaviour of cows and buffaloes.
2. In a tabular form enlist water requirement of various species.
3. Why camel and sheep require less water?
4. How do birds save water?
5. Why does milch cow require more water than dry?
6. Which hormone affects water conservation and how?
AIM: TO STUDY THE INGESTION BEHAVIOR IN FARM ANIMALS

**Grazing**: Eating grass from the ground.

**Nibbling**: Eating here and there in small quantities.

**Browsing**: Grazing from top of the tress or leaves.

**Grazing behaviour**:
- **Horse**: They graze grass between their incisors and tear it off.
- **Cattle**: They use their mobile rasp like tongue (prehensile organ) and encircle the grass and engulf. Cattle cannot graze closer than ½’ above the soil.
- **Goat**: These browse the grass. They do not have bitter receptors and hence they enjoy wide variety of plants.
- **Pigs**: They cannot graze efficiently due to presence of snout.

**Factors affecting grazing**:
1. The total grazing time increases when the quantity and quality of pasture is poor.
2. Daily grazing time.
3. The distance of grazing.
4. Total time spent is more in sheep than in cattle.
5. Farm animals selectively reject the pasture contaminated by their own feces.

**Ingestive behaviour in cattle – summary**:

<table>
<thead>
<tr>
<th>Behaviour</th>
<th>Parameters</th>
<th>Values (over 24hrs period)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grazing</td>
<td>Grazing time (hrs)</td>
<td>4-9</td>
</tr>
<tr>
<td></td>
<td>Total no of bites</td>
<td>24000</td>
</tr>
<tr>
<td></td>
<td>Rate of grazing (bites/min)</td>
<td>50-80</td>
</tr>
<tr>
<td></td>
<td>Amount grazed</td>
<td>10% of animal weight</td>
</tr>
<tr>
<td></td>
<td>Grazing distance (km)</td>
<td>3-4</td>
</tr>
<tr>
<td>Rumination</td>
<td>Rumination time (hrs)</td>
<td>4-9</td>
</tr>
<tr>
<td></td>
<td>No of rumination periods</td>
<td>15-20</td>
</tr>
<tr>
<td></td>
<td>No of boluses</td>
<td>360</td>
</tr>
<tr>
<td></td>
<td>No of bites/bolus</td>
<td>48</td>
</tr>
<tr>
<td>Drinking</td>
<td>No of drinks/day</td>
<td>1-4</td>
</tr>
<tr>
<td>Activity</td>
<td>Time spent lying down (hrs)</td>
<td>9-12</td>
</tr>
<tr>
<td></td>
<td>Time spent loafing (hrs)</td>
<td>8-9</td>
</tr>
</tbody>
</table>

**Observation / Exercise**:
1. Observe the orientation of animals when they graze.
2. Count the no. of bites/min.
3. Count rumination/min.
4. Draw a diagram showing grazing cows/buffaloes.
PRACTICAL NO.: 14

AIM: RECORDING GROWTH MEASUREMENT IN GROWING CALVES

Growth: Growth is the increase in body size/body mass of animal or increase in the protein content of body or increase in the DNA content or increase in cell number (hyperplasia) or cell size (hypertrophy).

Why to measure the body growth?
1. To know whether animal is growing as per the standard growth charts available (within normal range) for the given age group of a species.
2. To know whether the feed being supplied to animal is sufficient to bring about normal growth or not.
3. To know whether the feed is to be increased/supplemented if growth is not normal.

Variations in growth rate in different breeds and species:
Different animal species grow at a different pace and attain the adult body weight in different spans of time eg a cow-calf weights from 22-35kg at birth depending upon breeds and attains adult body size of 300-450 kg depending upon breed type. The growth curve of animal is not linear if plotted against age. But it is sigmoid ‘s’ shaped ie first linear, then plateau (stable) and again linear.

Methods/Criteria of growth measurements:
Body weight/size (kg): On a farm the growing animals are generally weighed every week to keep a check whether they are gaining weight or not. Adults are weighed every month. The animals are neither fed nor watered before weighing. Generally animals are weighed on weighbridge.

Heart girth (cm): The heart girth is measured immediately behind the fore limbs. The girth of the body at heart indicates increase or decrease in size. Formula are available for different species whereby one can calculate the weight by measuring heart girth alone, if weighing bridge is not there.

Height at withers (cm): It is also another measure to know the growth of long ones ie height of animal.

Crown-rump length, CRL (cm): Measurement of length of calf/ or any other animal from crown (forehead) to the base of the tail (a straight line).

Curved crown-rump length, CCRL (cm): Measurement of length of calf/ fetus/ or any other animal from crown (forehead) to the base of the tail (curved).

Observation table:

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Date</th>
<th>Date of birth</th>
<th>Age (wks)</th>
<th>Heart girth (cm)</th>
<th>Height (cm)</th>
<th>Body weight (kg)</th>
<th>Calculated weight (kg)</th>
<th>Rectal temp.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Total body length (cm): ---------------- from mouth to tail.

Calf No.: Birth weight:

Observation on same calf for ---------------- weeks.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>1st week</th>
<th>2nd week</th>
<th>3rd week</th>
<th>4th week</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (kg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Height at withers (cm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heart girth (cm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CRL (cm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCRL (cm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total length (cm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tail length (cm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Calculation:

Absolute growth (kg/day) = \[
\frac{(\text{Wt. at } ---- \text{ week of age}) - (\text{Wt. at } ---- \text{ week of age})}{\text{No. of days}}
\]

Relative growth (kg/day) = \[
\frac{(\text{Weight at } ---- \text{ week of age}) - (\text{Weight at } ---- \text{ week of age})}{\text{No. of days}}
\]

Results and Interpretation:

1. The growth rate of the given calves of -------------- breeds are as per standard norms.
2. The growth rate of the calf no. --------- of given breed -------------- is --------------.

Exercise:

1. Weight the calf and measure the heart girth and height of 10 calves of different age groups and plot a curve of weight and heart girth. Calculate the weight by formula.
2. Weigh the calf and measure the heart girth and height of same calf for 5 weeks. Record your observation and plot a curve. Calculate the weight by formula.
STANDARD GROWTH CHART FOR CATTLE CALVES
PRACTICAL NO. 15

DATE:

AIM: PHYSICAL AND PHYSIOLOGICAL EXAMINATION OF URINE

Examination of urine samples involved three aspects:

1. **Physical**: The simplest of all procedures conducted on urine is the physical examination, which provides considerable amount of information.

2. **Chemical**: Chemical examination of urine provides information on chemical substance present (physiological and pathological substances)

3. **Microscopic aspects**: Microscopic examination of urine is helpful to detect the presence of microscopic particles (cells, crystals) which have escaped from being detected either in physical or chemical examination.

**PHYSICAL EXAMINATION OF URINE:**

Recode the following observation on the given urine sample

1. Volume
2. Color
3. Odour
4. Transparency or Turbidity
5. Specific gravity
6. Reaction to litmus
7. Total solids
8. pH
9. Osmolality
10. Freezing point

(1) **VOLUME:**

The volume of urine excreted is taken for the total volume excreted within 24 hrs. The volume of urine excreted varies with the substance to be excreted, temperature and exercise. Volume of urine voided by domestic animals and man is given below:

<table>
<thead>
<tr>
<th>Animal</th>
<th>Urine volume (ml/kg.b.wt./day)</th>
<th>Animal</th>
<th>Urine volume (ml/kg.b.wt./day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cattle</td>
<td>17-45</td>
<td>Cat</td>
<td>10-20</td>
</tr>
<tr>
<td>Horse</td>
<td>3-18</td>
<td>Dog</td>
<td>20-100</td>
</tr>
<tr>
<td>Sheep</td>
<td>10-40</td>
<td>Swine</td>
<td>5-30</td>
</tr>
<tr>
<td>Goat</td>
<td>10-40</td>
<td>Man</td>
<td>8.6-28.6</td>
</tr>
</tbody>
</table>

When total volume of urine excreted is more than the normal condition it is called polyuria, e.g. diabetes mellitus and diabetes insipidus. Many diuretic drugs and low ADH levels in blood increase ‘urine’ volume. When urine excretion is decreased the condition is referred as oligouria. Urine volume is decreased by fluid loss (vomiting, diarrhea, hemorrhage, fever etc), formation of oedema by impaired circulation (heart disease) and in acute nephritis. The condition in which there is total suppression of urine formation is called anuria. Anuria occurs in collapse in some cases of acute nephritis and in complete obstruction to the urinary outflow.

(2) **COLOR:**

Physiological urine possesses straw yellow color depending on the concentration of urochrome. Urine sometimes has abnormal color also under different conditions:

<table>
<thead>
<tr>
<th>Abnormal color</th>
<th>Inference</th>
</tr>
</thead>
</table>
| Dark yellow    | If urine is concentrated, amount of urochromes volume is increased and urine appears darker then normal. During febrile condition and jaundice the
(3) ODOUR:
The normal odour of urine is derived from the volatile organic acids present. An odour of ammonia may appear if urine is being converted to ammonia by bacterial action the urine possess sweetish odour or fruity odours when ketone bodies are present in the urine.

(4) TRANSPARENCY OR TURBIDITY:
Transparency is recorded as clear, flocculant or cloudiness of urine. Urine excreted from most of species is usually clear. In horse urine is normally quite thick and cloudy due to the presence of calcium crystals and mucus. Normal urine from other species of animals although clear on being voided, may become cloudy as if cools and precipitation of crystals occurs. Urine may become turbid due to presence of leucocytes, erythrocytes, epithelial cells, bacteria, mucus, fat and crystals.

(5) SPECIFIC GRAVITY:
The specific gravity of urine varies with the relative proportion of dissolved matter and water. In general greater the volume, lower is the sp. gr. Specific gravity is an indication of the degree of tubular resorption or concentration by the kidney. Higher sp. gr. occurs in diabetes mellitus and albuminuria. Normal specific gravity of urine in domestic animals is as under:

<table>
<thead>
<tr>
<th>Animal</th>
<th>Range of sp. gr.</th>
<th>Animal</th>
<th>Range of sp. gr.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cattle</td>
<td>1.025-1.045</td>
<td>Cat</td>
<td>1.020-1.045</td>
</tr>
<tr>
<td>Horse</td>
<td>1.020-1.050</td>
<td>Dog</td>
<td>1.015-1.045</td>
</tr>
<tr>
<td>Sheep</td>
<td>1.015-1.045</td>
<td>Swine</td>
<td>1.010-1.030</td>
</tr>
<tr>
<td>Goat</td>
<td>1.015-1.045</td>
<td>Man</td>
<td>1.002-1.040</td>
</tr>
</tbody>
</table>
(6) TOTAL SOLIDS:
A total solid in urine is derived by multiplying last two figures of sp. gr. by a constant 2.5 (Long’s coefficient). This gives gm solids per liter of urine.

(7) OSMOLALITY:
It is the number of osmoles of a solute per kg of solvent (water) and it is directly related to the depression of the freezing point of an aqueous solution. A change in osmolality of urine may not be reflected in the specific gravity.

(8) FREEZING POINT:
It is a measure of the salt concentration of the urine and is not materially influenced by large molecular substances such as proteins. Higher the water content, the freezing point will be closer to zero. The molecular concentration of urine is much higher than that of blood plasma. This means that kidney performs work in the formation of urine. It also depends on fullness of bladder, inflammation of the urinary tract, disturbances in innervations of bladder increased irritability of the bladder and weakening of the sphincter.

<table>
<thead>
<tr>
<th>Species</th>
<th>Freezing point</th>
</tr>
</thead>
<tbody>
<tr>
<td>Horse</td>
<td>-177 to -200 ºC</td>
</tr>
<tr>
<td>Goat</td>
<td>-157.3 to -363.8 ºC</td>
</tr>
<tr>
<td>Cat</td>
<td>-5 ºC</td>
</tr>
</tbody>
</table>

(9) pH:
Physiological pH of urine varies from about 4.8 to 7.5 with an average about 6.6. The night urine of human has low pH because of the respiratory acidosis that develops during sleep. In the morning and after meals the pH rises (alkalinetides). The pH of a pooled specimen is therefore often in the range of pH 6.0 but on standing urine becomes alkaline due to liberation of ammonia by the urea splitting bacteria. The pH, therefore, must be measured in fresh urine. During starvation urine is acidic due to tissue catabolism. Depending upon the food habits, pH of urine varies. It is acidic in carnivores, alkaline in herbivores and either acidic or alkaline in omnivores. Nursing calves and foals urine is acidic.

Carnivores have highly acidic urine pH (6.7) due to formation of sulphuric acid and phosphoric acid. Herbivores urine pH is alkaline due to formation of alkaline carbonates (7.4-8.4). Porcine urine pH is either acidic or alkaline.

Pathologically acidity may result from starvation, fever, as a consequence of acidosis and following prolonged muscular activity. Administration of acid salts (NaCl, Ammonium Chloride) may acidify the urine under pathological conditions. Alkaline urine occurs as a result of retention of urine in the bladder due to cystitis or obstructive lesions. It is also observed during metabolic alkalosis, ingestion of salts (sodium, potassium salts of acetate, bicarbonate, citrate and urate).

Normal pH of urine in domestic animals is as under:

<table>
<thead>
<tr>
<th>Species</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Horse</td>
<td>7.0-8.0</td>
</tr>
<tr>
<td>Cow, Sheep, Goat</td>
<td>7.4-8.4</td>
</tr>
<tr>
<td>Dog, Cat</td>
<td>5.0-7.0</td>
</tr>
<tr>
<td>Pig</td>
<td>5.0-8.0</td>
</tr>
<tr>
<td>Human</td>
<td>4.8-7.5</td>
</tr>
</tbody>
</table>
EXAMINATION OF NORMAL URINE FOR PHYSIOLOGICAL CONSTITUTES:

Mix the urine sample well. Allow it to attain room temperature. Fill the urinometer cylinder with urine approximately within one inch from the top. Place carefully the urinometer float in urine filled cylinder and spin it free by gripping the tip of urinometer float between thumb and other fingers so that the float seeks its proper level. Urinometer float should not touch the walls of urinometer cylinder. Read the bottom meniscus at the highest pint and record. While measuring the specific gravity avoids the collection of any bubbles on the top of the urine otherwise they interfere with the accurate reading of the meniscus.

If urine is not in sufficient quantity, it may be diluted with distilled water. The amount of water added to dilute the urine is recorded and the dilution factor is calculated. In such case the last two figures of the specific gravity are multiplied by the dilution factor to get correct specific gravity. If the double diluted urine shows sp. gr. as 1.015 then the correct sp. gr. of the urine is 1.030.

TEST FOR PHYSIOLOGICAL CONSTITUTES OF URINE:

<table>
<thead>
<tr>
<th>TEST</th>
<th>OBSERVATION</th>
<th>INFERENECE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. CHLORIDE TEST</td>
<td>A white cloudy precipitate of silver chloride is formed.</td>
<td>Chloride ions are present in major amount.</td>
</tr>
<tr>
<td>Take 3 ml of dilute nitric acid in a clean dry test tube. Add 2ml of silver nitrate solution. Mix the contents. Finally add 3 ml of urine.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. SULPHATE TEST</td>
<td>White precipitate of barium sulphate is formed.</td>
<td>Greater portion of this sulphate is derived from the sulphur of sulphur containing amino acids on their oxidation in the body.</td>
</tr>
<tr>
<td>Take 1 ml of dilute HCl in a clean and dry test tube. Add 2 ml of barium chloride solution to it. Mix the contents. Add 3 ml of urine.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3. PHOSPHATE TEST</td>
<td>Yellowish color or precipitate is observed.</td>
<td>Phosphorous is present mostly in inorganic form in very small quantity.</td>
</tr>
<tr>
<td>Take 3 ml of conc. Nitric acid in a clean and dry test tube and add 3 ml of ammonium molybdate reagent and mix the contents. Add 3 ml of urine and heat the mixture.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4. CREATININE TEST</td>
<td>A mahogany red color of creatine picrate appears.</td>
<td>Creatinine is an anhydride form of creatine, which is found in muscle. Creatinine is formed during muscle activity it forms a</td>
</tr>
</tbody>
</table>
| **5. HIPPURIC ACID TEST**  
Take 5 ml of urine in clean and dry test tube. Make the urine neutral to litmus test by adding dilute acid solution (HCl) or dilute alkaline solution (NaOH) drop by drop. Add 3 drops of neutral ferric chloride and mix the contents. | **metabolic waste product.**  
Chocolate color or a precipitate of ferric salt of hippuric acid is formed.  
Hippuric acid is a metabolic excretory product of benzoic acid, which is conjugated with glycine and excreted in the urine as hippuric acid. |
| **6. URIC ACID & URATES TEST**  
Take 3 ml of urine in a clean and dry test tube, few drops of folin uric acid reagent and sodium carbonate mixture. | A deep blue color is produced.  
It is found in urine of men and apes. In the urine of other animals oxidative product of uric acid is excreted. |
| **7. INDICAN TEST (OBERMAYER TEST)**  
Take 5 ml of Obermayer reagent in a clean and dry test tube then take 5 ml of urine. Mix the contents. Let stand for 5 minutes. Add to the mixture 3 ml of chloroform. Pour the contents from one tube to the other continuously for ten minutes. | The chloroform layer is blue color is formed.  
The indican is the metabolic product of tryptophan. This is oxidized to indigo blue, which is soluble in chloroform. |
| **8. UREA TEST**  
Take 5 ml of urine in a clean and dry test tube. Add 4 drops of phenolphthalein indicator and small pinch of urea (jack bean meal). Mix the contents. Keep for 15 minutes if urine is acidic neutralize with one drop of 5% NaOH. | Pink color is observed.  
Development of pink color due to hydrolysis of urea and production of ammonia. |
| **9. AMMONIA TEST**  
Take clean and dry test tube. Add to it 10 ml of urine, few drops of phenolphthalein and dilute NaOH solution till faint pink color is obtained. Dip a glass rod in the solution of phenolphthalein. Hold this glass rod in the path of vapors coming out when the mixture of the test tube boiled. Take care that glass rod should not touch the sides of the test tubes. | Phenolphthalein dipped end of the glass rod turns red indicate presence of ammonia. |
AIM: PATHOLOGICAL AND MICROSCOPIC EXAMINATION OF URINE

CHEMICAL TESTS FOR PATHOLOGICAL CONSTITUTES:

Many of the substances considered as pathological constitutes of urine are present in very small amounts in all normal urine. Their pathological significance is dependent on the amount present then their qualitative presence or absence in the urine. The usual qualitative tests give negative tests if substances are presents in very small amounts. It is always advisable to carry out the tests on the well mixed proportion of 24 hrs, collection of urine, which is properly preserved.

TESTS FOR PROTEINS:

<table>
<thead>
<tr>
<th>TEST</th>
<th>OBSERVATION</th>
<th>INFEERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) HELLERS TEST</td>
<td>White ring appears at the junction of urine and acid.</td>
<td>Albumin is present.</td>
</tr>
<tr>
<td>1. Take 2ml of pure white conc. HNO3 in a clean and dry test tube.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. Add carefully 3ml of urine from side of the test tube to layer on acid. Keep for 3 minutes.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(2) MODIFIED HEAT COAGULATION TEST</td>
<td>Cloudiness appears</td>
<td>Albuminuria, albumin is present</td>
</tr>
<tr>
<td>Take 5ml of urine in a test tube, and 1ml of saturated NaCl solution. Add 5 drops of glacial acetic acid. Heat contents to boil.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(3) SULPHOSALICYLIC TEST</td>
<td>Turbidity or precipitates appears</td>
<td>Protein is present.</td>
</tr>
<tr>
<td>Take 1ml of urine and 3ml of sulphosalicylic acid (3%) solution.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: If urine is cloudy, it should be centrifuged or filtered before using for test. A transitional proteinuria may occur as physiological or functional reaction. Pathological proteinuria is observed in nephritis, nephrosis, pyelitis, urethritis, urolithiasis. Albumin occurs in certain poisoning cases (phenol, arsenic, lead mercury, terpentine, sulfonamide acid, ether) and fever. The proteins of low mol. wt. and small size pass through glomerulus pores (hemoglobin, albumin and globulin). About 80000-100000 pus cells/ml and 24000 RBC in per ml urine give a positive test for protein.

TESTS FOR SUGAR:

<table>
<thead>
<tr>
<th>TEST</th>
<th>OBSERVATION</th>
<th>INFEERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>(4) BENEDICTS TEST</td>
<td>Red to blue precipitation formed</td>
<td>Glucose present (Glucosuria)</td>
</tr>
<tr>
<td>Place 5ml of Benedict’s qualitative reagent in a test tube. Add 8-10 drops of urine. Mix well and boil over flame for 1 to 2 minutes.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(5) FEHLINGS TEST</td>
<td>Red to greenish precipitates will be formed</td>
<td>Glucosuria, glucose is present in urine.</td>
</tr>
<tr>
<td>Place each 1ml of Fehling’s reagent I and II in a clean and dry test tube then add 2ml of urine sample. Heat over the flame.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Precipitation interference
Precipitate | Reaction | Glucose amount approx.  
--- | --- | ---
Red | ++++ | 3%  
Reddish yellow | +++ | 2%  
Greenish yellow | ++ | 1%  
Yellowish green | + | 0.7%  
Green | 0 | 0.3-0.5%  
Bluish green | Trace | 0.2-0.25%  
Greenish blue | Trace | 0.1%  
Blue | 0 | None

Note: Substance containing free keto or aldehyde groups reduces the soluble cupric salt to insoluble cuprous oxide. If glucose load in the blood exceeds the renal threshold, glucose may appear in the urine. Physiological glucosuria may occur following heavy meal of carbohydrates. It is transient condition. Pathological glucosuria occur in diseases like diabetes mellitus, pancreatitis, hyperthyroidism, hyperactivity of adrenal cortex, chronic liver disease, in hyperpituitarism, increased intracranial pressure, enterotoxaemia in sheep, due to impairment of the tubular reabsorption or lowering of renal threshold for glucose. Glucosuria may occur from increased epinephrine secretion (fear, excitement and restraint).

False positive test for glucose may be caused by antibiotic (streptomycin, chlortetracycline, tetracycline, penicilline and chloramphenicol), other reducing sugars, ascorbic acid, morphine, chloral hydrate, formaldehyde, glucuronic acid. Glucoranates, uric acid and salicylates.

**TESTS FOR KETONE BODIES:**

<table>
<thead>
<tr>
<th>TEST</th>
<th>OBSERVATION</th>
<th>INFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>(5) ROTHERA’S TEST</td>
<td>A reddish purple or potassium permanganate color develops</td>
<td>Ketone bodies present (ketonuria)</td>
</tr>
</tbody>
</table>

Note: Ketone bodies in small amount (acetoacetic acid, acetone and beta-hydroxybutyrate acid) results from lipid breakdown and accumulation of acetyl CoA results under condition of depletion of liver glycogen (starvation, diabetes, vomiting for long time, severe diarrhea, high fat and low carbohydrate diet) as a result of low availability of oxaloacetate needed to enter in TCA cycle.

In small domestic animals (dog, cat) the principle causes of ketosis is diabetes mellitus. In ruminants, ketosis is frequent due to production of VFAs as a passage of lactose (glucose + galactose) through milk. Ketosis is observed in pregnant ewes carrying twin lambs, due to insufficient supply of diet containing carbohydrate.

**TESTS FOR BILE SALTS:**

<table>
<thead>
<tr>
<th>TEST</th>
<th>OBSERVATION</th>
<th>INFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>(6) HAY’S TEST</td>
<td>The sulphur granules sink to bottoms of the test tube</td>
<td>Bile salts present</td>
</tr>
</tbody>
</table>
### (7) GMELIN TEST
Take 2ml of urine (unfiltered) in a test tube and run 1-2 ml yellow nitric acid. Take care that two fluids do not mix.

| Various color rings are found at the line of contact as the oxidation proceeds. Starting from urine to the acid, the colors are green, blue, violet, red and finally yellow. | Bile pigment present |

### (8) IODINE TEST
Take 2ml of 0.5% alcoholic solution of iodine. Urine acidified with acetic acid is run down the side of the test tube.

| Bile gives emerald green color which diffused into the urine. | The color is due to compound of iodine with bile and is a oxidation product. |

### TESTS FOR BILIRUBIN:

<table>
<thead>
<tr>
<th>TEST</th>
<th>OBSERVATION</th>
<th>INFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>(9) HUNTER’S TEST</strong></td>
<td>Presence of red color indicates bilirubin</td>
<td>Bile pigments present</td>
</tr>
<tr>
<td>Take 2ml of 10% BaCl₂ and add 5 ml urine in a centrifuge tube. Centrifuge the precipitate. Remove supernatant. Add little water in it for wash the precipitate. Again centrifuge the contents. Discard the wash water and add 0.5ml of alcohol and 0.5ml of phosphate buffer with mixing after each addition.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Note:** The chief bile pigment in serum of domestic animals is bilirubin. The kidney does not excrete unconjugated bilirubin but is able to excrete conjugated bilirubin. Bilirubin conjugates are not found normally in urine of cat, pig, sheep and horse. About 25% of normal cattle have traces of bilirubin.

### TESTS FOR BLOOD:

<table>
<thead>
<tr>
<th>TEST</th>
<th>OBSERVATION</th>
<th>INFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>(10) BENZIDINE TEST</strong></td>
<td>Greenish blue color develops</td>
<td>Blood present in urine (hematuria or hemoglobinuria)</td>
</tr>
<tr>
<td>Take 3ml of glacial acetic acid, add pinch of benzidine dihydrochloride and 2ml of urine and 1 ml of freshly prepared 3% H₂O₂ (1:10).</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Note:** Hemoglobinuria occurs as result of an excessive hemolysis of erythrocytes following malaria, leptospirosis, piroplasmosis or babesiosis, photosensitization, chemical hemolytic agents (copper, mercury), consumption of certain plants, post-parturient hemoglobinuria, clostridium hemolyticum, severe burns, incompatible blood transfusion, hemolytic diseases of new born, ingestion of large amount of clod water by cattle (iopathic hemoglobinuria), snake venom poisoning and spider bites.

### MICROSCOPIC EXAMINATION:

Mix the urine sample thoroughly. Approximately 15ml of urine is centrifuged at low speed (1000 to 1800 RPM) for approximately 3 to 5 minutes. Pour off the supernatant fluid
by inverting tube without wiping lid of tube. Mix sediment with the small amount of urine leftover in the tube. Pour a drop on a slide and cover with a cover slip. Under subdued light examine with low power lens the entire cover glass area. Examine under high power to identify structures. There may be organized or unorganized elements. Organized elements in the sediment of animal urine seldom have any clinical significance.

Crystals are not present when urine is voided but form after it cools, either urine gets supersaturated at the cooler temperature or because changes in the reaction alter the solubility of the substances.

**Crystals in acid urine:**

Amorphous urates and uric acids are present most commonly. Less commonly the crystals of calcium oxalate and hippuric acid are present. Calcium oxalate crystals are soluble in HCl and not in acetic acid. Urine oxalates are soluble in NaOH and not in acid.

**Crystals in alkaline urine:**

Triple and amorphous phosphates, calcium carbonate (especially in the horse) and on rare occasions ammonium urate crystals are present. Triple phosphate crystals are ammonium magnesium phosphate dissolves in dilute acetic acid without effervescence. Calcium carbonate crystals dissolve in dilute acetic acid with effervescence.

If calculi are present determination of the crystal types present in the sediment becomes important in order that therapy can be instituted to prevent reoccurrence.

<table>
<thead>
<tr>
<th>Leucocytes</th>
<th>Under normal condition, a few leucocytes are present (1 in male, 5 in female). More than 10 leucocytes in high power field (HPF) is indication of inflammation. Leucocytes are often called ‘pus cells’</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erythrocytes</td>
<td>It is indicative of hemorrhage in genitourinary tract if blood is found during the onset of urination; it is due to urethral lesion. If present in last portion of voided urine, bleeding is probably in the bladder</td>
</tr>
<tr>
<td>Epithelial cells</td>
<td>Squamous cells are derived from urethra, bladder and vagina in particular. Cells from renal tubules are smaller. They are slightly larger than leucocytes</td>
</tr>
<tr>
<td>Salts</td>
<td>They are cylindrical structures with parallel sides and blunt rounded ends. They appear only in acid urine. They are therefore uncommon in herbivores urine</td>
</tr>
<tr>
<td>Microorganism</td>
<td>Bacteria, yeast, fungi and protozoa may occasionally be seen in the urine</td>
</tr>
<tr>
<td>Parasites</td>
<td>Parasitic ova include bladder worm of the cat, dog and fox, swine kidney worm, the giant worm of the goose milk</td>
</tr>
<tr>
<td>Spermatozoa</td>
<td>The presence of the spermatozoa indicates contamination of the specimen with semen</td>
</tr>
</tbody>
</table>

Over and above these organized elements urine also contains many times unorganized elements in the sediments eg. Fat droplets, crystals and pigments.
AIM: ESTIMATION OF TITRABLE ACIDITY IN URINE (FOLINS METHOD)

PRINCIPLE:
The urine is titrated against standard sodium hydroxide solution using phenolphthalein as an indicator. Potassium oxalate is added to precipitate the calcium which otherwise will interfere with the result (as it goes precipitated as calcium oxalate).

EQUIPMENT:
1. Burette with stand
2. Conical flask (250ml capacity)
3. Glass funnel and beaker
4. Measuring cylinder (25ml capacity)

REAGENTS:
1. Sodium hydroxide (1/10N) solution
2. Phenolphthalein indicator
3. Potassium oxalate powder
4. Sample of urine

PROCEDURE:
1. Take clean and dry burette and fix vertically on retort stand rinse with 0.1N NaOH solution and fill it with 0.1N NaOH solution.
2. Adjust the level of solution filled (0.1N NaOH) in burette, remove air bubbles if present in the burette.
3. Place 25ml urine sample may be a single urination sample or from a pooled volume of urine collected for 24 hrs in a clean conical flask and drop in it 1 or 2 glass beads.
4. Add approximately 5-10gm of potassium oxalate powder to the conical flask. Mix the contents with rotation.
5. Add 1 or 2 drops of phenolphthalein as an indicator in the flask containing urine for titration. Mix the contents.
6. Pipette drop by drop 0.1N NaOH from burette to flask till faint unmistakable pink color is present on shaking (at least for 2 minutes).
7. Continue to pipette out 0.1n NaOH from burette to flask till faint unmistakable pink color is present on shaking (at least for 2 minutes).
8. Note burette reading the time. It is final burette reading.
9. Repeat the experiment taking 25ml urine again. Record initial and final burette.

<table>
<thead>
<tr>
<th>Burette reading</th>
<th>Reading 1</th>
<th>Reading 2</th>
<th>Reading 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Final</td>
<td>ml</td>
<td>ml</td>
<td>ml</td>
</tr>
<tr>
<td>Initial</td>
<td>ml</td>
<td>ml</td>
<td>ml</td>
</tr>
<tr>
<td>Difference</td>
<td>ml</td>
<td>ml</td>
<td>ml</td>
</tr>
</tbody>
</table>

Average of three burette reading (ml) =

CALCULATION:
\[
\text{ml of NaOH} \quad \text{ml of urine sample}
\]

Percentage acidity = \[ \frac{\text{ml of NaOH}}{\text{ml of urine sample}} \times 100 \]
INFERENCE:
In natural urine the acid titrated consist almost entirely of the acid phosphate pool H$_2$PO$_4$ the reaction being as follows;

$$\text{H}_2\text{PO}_4 + \text{OH} \rightarrow \text{HPO}_4 + \text{H}_2\text{O}$$

Small amount of acidity may be contributed by acid organic salts (urates, oxalates) and keto acids if present. Titrable acidity is lower in vegetable diet (base forming diet) compared to diet containing much meat, milk, rice while wheat product (acid forming foods). It is increased during fasting and decreased shortly after a meal due to alkali netide. Bacterial decomposition of urine in urinary bladder may be decrease the acidity. Administration of mineral acids, acid phosphates or ammonium chloride may increase the acidity.
PRACTICAL NO. 18

AIM: ESTIMATION OF AMMONIA IN URINE (FORMAL TITRATION METHOD)

PRINCIPLE:
Natural ammonia salt, amino acids react with excess of neutral formalin solution and liberates in acidic substance hexamethylene tetramine. The amount of later formed is directly proportional to the amount of ammonia salt present in the natural urine. These can be calculated by amount of 0.1N NaOH required to completely neutralize the hexamethylene tetramine formed in the urine by the action of neutral formalin.

EQUIPMENTS:
1. Burette
2. Burette stand
3. Funnel
4. Beaker and conical flask (25ml capacity)
5. Measuring cylinder (25ml capacity)

REAGENTS:
1. Sodium hydroxide 0.1N solution
2. Neutral formalin solution
3. Phenolphthalein indicator
4. Potassium oxalate powder
5. Urine sample

PROCEDURE:
1. Carry out all the steps systematically as in case of estimation of titrable acidity in urine. The end point signifies that urine in solution is neutral.
2. To this neutral mixture, add 100ml of neutral formalin solution to the urine titrated in step 1.
3. Mix the contents by rotation and observe the disappearance of pink color.
4. After 2 to 3 min, titrate the mixture against 0.1N NaOH solution from the burette. Repeat the steps till faint pink color persists.
5. Record the quantity of 0.1N NaOH required to neutralize the hexamethylene tetramine completely.
6. Repeat the whole procedure twice and record the observations.
Note: If the given urine is alkaline then neutralize it with 0.1N HCl solution.

<table>
<thead>
<tr>
<th>Burette reading</th>
<th>Reading 1</th>
<th>Reading 2</th>
<th>Reading 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Final</td>
<td>ml</td>
<td>ml</td>
<td>ml</td>
</tr>
<tr>
<td>Initial</td>
<td>ml</td>
<td>ml</td>
<td>ml</td>
</tr>
<tr>
<td>Difference</td>
<td>ml</td>
<td>ml</td>
<td>ml</td>
</tr>
</tbody>
</table>

Average of three burette reading (ml) =

CALCULATION:
10ml of 0.1N NaOH used in titration neutralized the hexamethylene tetramine and is equal to 17mg of ammonia. Suppose x ml of 0.1N NaOH is used then

Mg% of Ammonia and amino acid nitrogen = x ml X 17 X 100
PRACTICAL NO. 19

AIM: ESTIMATION OF CREATININE IN URINE

PRINCIPLE:
A measured amount of urine is treated with picric acid and sodium hydroxide. The creatinine combines with the picric acid to form an orange colored picramic acid (Jaffe reaction). A known amount of creatinine is also similarly treated and the colors are compared in suitable colorimeter.

EQUIPMENTS:
1. Colorimeter
2. Graduated pipettes
3. Test tubes
4. Wash bottle containing distilled water

REAGENTS:
1. Standard creatinine solution containing 0.1mg creatinine (1mg/0.1ml) – Dissolve 100mg creatinine in 10ml of distilled water, 10mg/ml concentration
2. 0.1N Hydrochloric acid
3. Picric acid solution (1%)
4. Sodium hydroxide solution (10%)

PROCEDURE:
1. Take three test tubes. Mark them with T (for sample), S (for standard) and B (for blank).
2. Pipette the following solution as shown in the table
3. Mix the contents shaking gently.
4. Keep for 15 minutes for reaction.
5. Dilute the solution by adding distilled water up to 10ml mark.
6. Compared the unknown against the standard in a colorimeter at 520nm wavelength by set instrument ‘ZERO’ with blank solution.

<table>
<thead>
<tr>
<th>Reagents (ml)</th>
<th>T</th>
<th>S</th>
<th>B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water</td>
<td>-</td>
<td>-</td>
<td>0.1</td>
</tr>
<tr>
<td>Urine sample</td>
<td>0.1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Standard creatinine</td>
<td>-</td>
<td>0.1</td>
<td>-</td>
</tr>
<tr>
<td>Picric acid (1%)</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>NaOH solution (10%)</td>
<td>0.15</td>
<td>0.15</td>
<td>0.15</td>
</tr>
<tr>
<td>Total volume</td>
<td>2.25</td>
<td>2.25</td>
<td>2.25</td>
</tr>
</tbody>
</table>

CALCULATION:
\[
\text{mg of creatinine per 100ml of urine} = \frac{\text{O.D. of Unknown}}{\text{100 O.D. of standard}} \times 1 \text{ mg} \times \frac{1}{0.1}
\]
**INTERPRETATION:**

The adult human urine contains 1 to 1.8g creatinine per day. In heavy meat diet the content increase. It is entirely a waste product. Exercise increases creatinine content as result of release from muscle stores/ it increases in muscle wasting disease and fever.

**Normal values of urine creatinine in animals are:**

- Dog: 30 – 80 mg/kg/day
- Cattle: 15 – 20 mg/kg/day
- Sheep: 10 mg/kg/day
- Pig: 20 – 90 mg/kg/day
PRACTICAL NO. 20

AIM: ESTIMATION OF INORGANIC PHOSPHORUS IN URINE (FISKE AND SUBBROW METHOD)

PRINCIPLE:
Phosphate reacts with molybdic acid to form phosphomolybdic acid. On treatment with 1, 2, 4 amino naphthosulphonic acid the phosphomolybdic acid is selectively reduced to produce a deep blue color (molybdenum blue), which is probably a mixture of lower oxides of molybdenum. This color is read in suitable photometer against a standard phosphate solution prepared in the same way.

EQUIPMENTS:
1. Colorimeter
2. Pipette graduated
3. Volumetric flask (100ml capacity)
4. Wash bottle

REAGENTS:
1. Distilled water
2. Molybdate reagent
3. 1,2,4 Aminonaphtho sulphonic acid (ANSA)
4. Standard phosphate solution containing 0.4mg phosphorus in 5ml
5. Urine sample

PROCEDURE:
1. Clean three test tubes. Mark as S (for standard), T (for test) and B (for blank).
2. Pipette simultaneously following solution in all tubes.
3. Make to 10ml mark with distilled water and mix the contents carefully.
4. Read after five minutes in colorimeter at 660 to 720 nm wavelength.

<table>
<thead>
<tr>
<th>Reagents (ml)</th>
<th>T</th>
<th>S</th>
<th>B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard phosphorus</td>
<td>-</td>
<td>0.5</td>
<td>-</td>
</tr>
<tr>
<td>Urine sample</td>
<td>0.2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Molybdate reagent</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>ANSA solution</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
</tr>
<tr>
<td>Distilled water</td>
<td>8.4</td>
<td>8.1</td>
<td>8.6</td>
</tr>
<tr>
<td>Total volume</td>
<td>10.0</td>
<td>10.0</td>
<td>10.0</td>
</tr>
</tbody>
</table>

CALCULATION:
mg of phosphorus per 100ml of urine = \( \frac{\text{O.D. of Unknown}}{\text{O.D. of standard} \times 0.04 \times \text{mg of phosphorus per 5ml}} \)

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TEMPERATURE HUMIDITY INDEX CHART

Temperature Humidity Index (THI) is determined by equation from the relative humidity and the air temperature.

The principle of THI is that as the relative humidity at any given temperature increases, then the comfort factor decreases.

It becomes progressively more difficult for the body to cool itself. Results show that milk production begins to be affected above a THI of 78 which will occur at 27°C and 80% relative humidity, or 31°C at 40% relative humidity.

Say temperature = 33.3°C
Relative humidity = 85%
Reading is 89
This figure is on the edge of moderate heat stress, going into severe.

Source: Dr Frank Wiersama (1990) Dept. of Ag Eng, The University of Arizona, Tucson, Arizona
HEAT LOSSES FROM THE BODY

- Conduction
- Natural Convection
- Radiation
- Forced Convection
- Evaporation
- Exhalation of warm and moist gases
- Fuel

Warm and moist air