MATERIALS AND METHODS

Experimental Model

Fifteen adult male (seven month old, 300-350 g) and thirty adult virgin female (six month old, 250-300 g) albino rats (Spragu-Dawley strain) obtained from Animal House, Jawaharlal Nehru University, were used in the present investigation. Every male rat was kept with two females (in plastic cages with wire cover and rice husk padding) for mating for 10 days. At the end of the period males were removed and kept individually in separate cages. Every pregnant female was kept in a separate cage until delivery. During mating and pregnancy, rats were fed stock pellet diet (supplied by Gold Mohur laboratory, Lepton India Ltd., Bangalore) and water ad libitum. At birth, the weight of each pup, as well as, the mother was recorded. A daily record of both was maintained until day 30 (Table 1, Fig. 1). The animals were divided, randomly into two groups. The control group (C) consisted of one mother and eight pups fed on laboratory formulated diet (Table 2) ad libitum. The experimental group (E) comprised one mother with sixteen pups and given 50% of the same diet consumed by control. At 21 days mothers of both groups were removed. The pups were continued to be fed on the same pattern (Table 3, Fig. 2). Both groups
were given water ad libitum. During the experiment, rats were maintained in an air conditioned room (25°C) with artificial light-dark cycle for 12 hours daily.

Experiment was carried out at 10, 15, 20, 25 and 30 day old rats at 5 day interval from day 10 to day 30.

Rats were killed by decapitation. Both hind limbs of every animal were skinned and the muscles, gastrocnemius, soleus and EDL from each limb were dissected (Hebel and Stromberg, 1976), the abdominal cavity was quickly opened and the whole diaphragm was dissected. All muscles were chilled in an ice-bath and immediately weighed on Mittlor-AE 160 balance. The muscles were kept individually in 0.25M KCl solution and stored at -70°C, till processed (maximum 15 days).

BIOCHEMISTRY

Reagents and Buffers:

I. DNA Extraction

1. 0.1 mM EDTA: 0.372 gm of EDTA (sodium salt) was dissolved in 1 litre of distilled H2O.

2. 20% sodium dodecyl sulfate (SDS): 20 gm of SDS was dissolved slowly in 100 ml of distilled water. No refrigeration is needed.
3. Chloroform/isoamyl alcohol (24:1 v/v): To prepare one litre of the mixture, 1920 ml of chloroform was mixed with 80 ml of isoamyl alcohol.

4. Citrate saline (CS) buffer (pH 7.0): 2.94 gm of Na citrate and 9 gm of NaCl were dissolved in a 1 litre of distilled H$_2$O. pH was adjusted to 7.0 by 4N-HCl.

5. 0.15M-Na Citrate buffer (pH 7.0): 44.1 gm of Na-citrate was dissolved in 800 of distilled water. The pH was adjusted to 7.0 with 4N-HCl (20 to 30 drops). The solution was diluted to 1 litre with distilled water.

6. 0.1M-Na Phosphate buffer (pH 7.0) + 0.3MNaCl: 13.7 gm of NaH$_2$PO$_4$·H$_2$O was dissolved in 800 ml of distilled H$_2$O. The pH was adjusted to 7.0 with 6N-NaOH. Solution was diluted into 1 litre with distilled H$_2$O. Then 17.5 gm of NaCl was added to the solution and dissolved thoroughly.

II. DNA Estimation

1. 0.15 mg/ml DNA standard solution:
15 mg of DNA (Sigma) was dissolved in 100 ml of 0.1 M phosphate buffer (pH 7.4) until the solution is clear and all DNA is dissolved.
2. Diphenylamine reagent:
0.75 gm of diphenylamine (Sigma) was dissolved in
50 ml of glacial acetic acid and 0.75 ml of concen-
trated H₂SO₄ was added. Just prior to use 0.25 ml
of cold 1.6% acetaldehyde was added (diphenylamine
reagent must be prepared fresh shortly before use
and it should be handled carefully.)

3. 1.6% acetaldehyde:
1.6 ml of acetaldehyde was diluted to 100 ml with
distilled H₂O. Solution was made fresh weekly and
kept refrigerated.

4. Cuvette washing solution (50% ethanol, 5% acetic
acid): 425 ml of distilled H₂O, 525 ml of 95% ethanol
and 50 ml conc. acetic acid were mixed and kept in a
wash bottle.

III. RNA Extraction:

1. Extraction buffer (50 mM - Tris-HCl, pH 7.5, 150 mM-
NaCl, 5 mM-EDTA, 5% SDS):
5.055 gm Tris-HCl (Sigma), 8.766 gm NaCl, 1.861 gm
EDTA and 50 gm SDS were dissolved in one liter of
distilled water. The pH was adjusted to 7.5 with
4N-HCl. Buffer is stored at room temperature.

2. Proteinase K:
A concentration of 50 mg proteinase K/1 litre extraction
buffer was used.

3. Phenol/chloroform/isoamyl alcohol (50:50:1, by volume): Phenol (Sigma) was melted in water-bath at 60°C. 500 ml of melted phenol was mixed with 500 ml of chloroform and 5 ml of isoamyl alcohol.

4. Re-extraction buffer (50 mM Tris-HCl, pH 7.5, 5 mM-EDTA): 6.055 gm of Tris-HCl (Sigma) and 1.861 gm of EDTA were dissolved in one litre of distilled water. pH was adjusted to 7.5 by 4N-HCl.

5. 4M-NaCl solution:
23.376 gm of NaCl was dissolved in 100 ml of distilled H₂O.

6. Buffer (50 mM-Tris-HCl, pH 7.5, 0.2% SDS):
6.055 gm of Tris-HCl (Sigma) and 2 gm of SDS were dissolved in one litre of distilled water. pH was adjusted to 7.5 with 4N-HCl.

IV. RNA Estimation:

1. 5% Trichloroacetic acid (TCA) solution:
5 gm of TCA was dissolved in 100 ml of distilled water.

2. 0.05 mg/ml RNA standard solution:
5 mg of RNA (Sigma) was dissolved in 100 ml of 0.1M phosphate buffer (pH 7.0).
3. **85/15 \( H_2SO_4/H_2O \) (v/v):**

For preparation of one litre of the mixture, 150 ml of distilled water was added slowly (dropwise with shaking) to 850 ml of conc. \( H_2SO_4 \). Utmost caution should be taken during preparation.

4. **Modified orcinol reagent:**

0.35 ml of 6% Orcinol solution in water was added to 5 ml of concentrated HCl in a dark bottle. The preparation is prepared fresh immediately before use.

V. **Protein Extraction:**

1. Tris-Mg acetate buffer, pH 8.0 (0.02 M-Tris HCl pH 8.0, 0.02 M-sucrose, 0.1 M KCl, 0.05 M NaCl, 0.006 M Mg acetate, 0.001 M EDTA):

   2.4 gm of Tris-HCl (Sigma), 6.84 gm sucrose, 7.44 gm KCl, 2.92 gm NaCl, 1.28 gm Mg acetate and 0.372 gm EDTA were dissolved in 1 litre of distilled \( H_2O \).

   The pH was adjusted to 8.0 with 4N-HCl.

2. **20% Trichloroacetic acid (TCA) solution:**

   100 gm of TCA was dissolved in 500 ml of distilled \( H_2O \).

3. **Ethanol/Ether (1:1 v/v):**

   100 ml of absolute ethyl alcohol was mixed with an equal volume of ether. The mixture should be prepared fresh weekly and stored in the fridge.
4. **1N-NaOH solution**: 
40 gm of NaOH was dissolved in one litre of distilled H$_2$O.

VI. **Protein Estimation**: 

1. **2% Na$_2$CO$_3$ in 0.1N-NaOH**: 
20 gm of Na$_2$CO$_3$ (anhydrous) and 4 gm of NaOH were dissolved in one litre of distilled H$_2$O.

2. **2.7% K-Na tartrate**: 
2.7 gm of K-Na tartrate was dissolved in 100 ml of distilled water and kept refrigerated.

3. **1% CuSO$_4$**: 
1 gm of CuSO$_4$·5H$_2$O was dissolved in 100 ml of distilled water.

4. **1N-Folin-Ciocatiteu (Phenol reagent)**: 
2N-Folin Ciocatiteu (commercial) was diluted (1:1) with distilled water.

**EXPERIMENTAL PROCEDURE**

According to Alexander et al. (1984), caution must be exercised to prevent denaturation during the isolation of DNA. For this reason all glassware that will come in contact with the DNA should be rinsed with 1 mM EDTA solution and the preparation should be kept cold (by using an ice bucket). The use of a chelating agent such as EDTA,
serves to remove Mg$^{++}$ which is required for the action of DNase. In the absence of the required ion and in the cold, the degradative enzyme becomes nonfunctional. It is also important to handle the DNA gently to avoid mechanical shearing.

**DNA extraction**

DNA extraction was carried out by the method of Alexander et al. (1984) with slight modifications. One gram of muscle was chopped into small pieces by fine scissors. To this 3.5 ml of citrate saline buffer (pH 7.0) was added and the muscle was homogenized by adding sufficient amount of liquid nitrogen. The muscle was crushed in porcelain mortar until a complete homogenate was obtained. The homogenate was poured into 35 ml plastic centrifuge tubes and centrifuged (in Sorvall RC-5 Superspeed Centrifuge at 6500 rpm) for 15 minutes at 4°C. The top lipid layer was removed with a plastic spatula and the supernatant was discarded. The pellet was resuspended in a beaker with 6 ml of 0.15M sodium citrate buffer (pH 7.0) and placed on a magnetic stirrer (using teflon coated magnetic bar) and 0.6 ml of 20% sodium dodecyl sulfate (SDS) solution was added slowly (dropwise) with a Pasteur pipette (addition
should take one minute). After addition of SDS, suspension was allowed to stir for additional 5 minutes, the suspension would become increasingly viscous. The suspension was placed in 55°C water-bath and allowed to heat for 15 minutes and occasionally stirred with glass rod. 0.6 gm of NaCl was added and the solution kept at 55°C, with stirring continued until the salt was dissolved. The solution was removed from the water-bath and cooled to room temperature in an ice-bath. 7 ml of chloroform/isoamyl alcohol (24:1; v/v) was added and the solution was shaken vigorously for 10 minutes. Both layers of solution were poured into centrifuge tubes and centrifuged at 10,000 rpm for 15 minutes at 4°C. Top layer was saved and transferred into a beaker. The white interphase and bottom layers were discarded. Two volumes of 95% ethanol were added and the solution was stirred with glass rod. The gelatinous precipitate was wound over the glass rod. Another 2 ml ethanol was added to collect any additional precipitate that might wind into the rod. The solvent was gently pressed out by turning the rod against the side of the beaker. The wound DNA was rinsed with 95% ethanol and then with acetone (AR Grade) until the washings were no longer
turbid. DNA was removed from the rod, dried in a small beaker in a discicator overnight and the dry weight was recorded.

**DNA Estimation**

DNA was estimated by diphenylamine reaction (Alexander et al., 1984) with slight modifications. A standard curve was constructed by preparing a series of concentrations ranging from 0 to 0.5 mg of DNA (Calf thymus DNA, Sigma Co., U.S.A.) by using 0.15 mg/ml standard DNA solution. 1 mg of dried unknown DNA was dissolved in 1 ml of 0.1 M Na phosphate buffer + 0.3 M NaCl (pH 7.0) and placed on a stirrer with a topohon coated magnetic bar overnight in a refrigerator. Aliquot of 100, 200, and 300 ul of DNA solution from every muscle of both control and experimental was brought to a final volume of 2 ml with distilled H₂O. 2 ml of diphenylamine reagent was added to every sample and the solution was allowed to stand for 17 hours at 30°C (Burton, 1956). The optical density was read at 600 nm (using Schimadzu UV-260 Spectrophotometer) and DNA content in every sample was recorded.
RNA Extraction:

RNA extraction was carried out by the method of Theologis et al. (1985) with slight modification. One gm of muscle was chopped into small pieces by fine scissors. To this 2.5 ml of 50mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 5% SDS was added and the muscle was frozen by adding sufficient amount of liquid nitrogen. The muscle was crushed in porcelain mortar until a complete homogenate was obtained. Proteinase K (25 mg/500 ml extraction buffer) was added and the homogenate was stirred for 30 minutes at room temperature. An equal volume of phenol/chloroform/isoamyl alcohol (50:50:1, by volume) was added, and after stirring for 20 minutes, the phases were separated by centrifugation (10,000 rpm for 10 minutes at 4°C) and the aqueous phase was removed. The organic phase was re-extracted with 1.5 ml of 50 mM Tris-HCl (pH 7.5), 5 mM EDTA for 30 minutes at room temperature and the combined aqueous phases were re-extracted with an equal volume of phenol/chloroform/isoamyl alcohol (50:50:1, by volume).
by vigorous stirring. The final aqueous phase was adjusted to 0.4 M NaCl (by using 4 M NaCl stock solution) and the nucleic acids were precipitated with 2.5 volume ethanol at -20°C overnight. RNA was collected by centrifugation (10,000 rpm for 10 minutes at 4°C) and dissolved in 1 ml of 50 mM Tris-HCl (pH 7.5), 0.2% SDS.

**Estimation of RNA Content:**

It was carried out according to the modified orcinol test by Almog and Shirey (1978) with some modification. A standard curve was constructed by preparing series of concentrations ranging from 0-0.1 mg of RNA (Sigma Co., USA) by using 50 μg/ml standard RNA solution (Alexander et al., 1984). One ml of the RNA sample (to be estimated) was incubated with 4 ml of 85/15 (v/v) H₂SO₄/H₂O for 24 hours at 40°C. Then 0.1 ml of the modified orcinol reagent was added. The mixture was incubated with shaking at 100°C for 30 minutes. It was cooled at room temperature. The RNA content of the sample was measured at 500 nm (using Schimadzu UV-260 spectrophotometer).

**Protein Extraction:**

Protein extraction was carried out according to the method of Fagan et al. (1987) with some modification.
Muscle was chopped in an ice-bath. It was homogenized in 29 volumes of magnesium acetate Tris buffer (0.02 M Tris HCl, pH 8.2; 0.02 M sucrose, 0.1 M KCl, 0.05 M NaCl, 0.006 M magnesium acetate, 0.001 M EDTA) in iced Potter-Elvehjem homogenizer at medium speed for 2 minutes. Similar volume of 20% Trichloroacetic acid (TCA) was added to the homogenate and the sample was kept at 4°C for 24 hours. Samples were centrifuged at 14,000 rpm for 5 minutes at 4°C. Supernatant was discarded and the pellet washed twice with ethanol/ether (1:1, v/v). 29 volumes of 1N-NaOH was added to the sample and kept to dissolve at 4°C for 24 hours. 1ml of sample was diluted 30 times by 1N-NaOH and incubated at 37°C for 30 minutes. Aliquots of 100, 200 and 300 μl of the diluted sample of each muscle, both control and experimental, was used for protein analysis.

**Protein Estimation**

The estimation of protein content was carried out according to the method of Lowry et al. (1951). A standard curve was constructed by preparing series of tubes containing protein concentrations ranging from 0-200 μg by using a standard solution containing 400 μg/ml bovine serum albumin (BSA) (Sigma Co., USA). The final volume in each tube (protein and water) did not exceed 0.5 ml.
The volume of every protein sample (to be estimated) was also brought to a final volume of 0.5 ml of distilled water. For every sample, a mixture of 4.9 ml of 2% \( \text{Na}_2\text{CO}_3 \), 0.05 ml of 2.7% sodium potassium tartrate and 0.05 ml of 1% \( \text{CuSO}_4 \) was prepared (the mixture should be prepared fresh and \( \text{CuSO}_4 \) must be added last). 5 ml of the mixture was added to every sample at a regular interval of 30 seconds and mixed thoroughly on a Vortex mixer. Samples were incubated for exactly 10 minutes at room temperature. At the end of incubation, 0.5 ml of phenol reagent was added to every sample at an interval of 30 seconds and mixed rapidly on a vortex mixer until the reaction was over. After addition of reagent to the last tube, samples were incubated at room temperature for 30 minutes. The protein content was measured at 700 nm (using Schimadzu UV-260 Spectrophotometer).

**Calculations and Statistical Analysis:**

The nucleic acid or protein content/muscle pair was calculated by dividing the content of total muscle pairs by the number of pups in each experiment (i.e., 8 in control and 16 in experimental). Also the nucleic acid or protein concentration (in mg/gm fresh muscle tissue) was calculated by dividing the nucleic acid or protein content of one muscle pair (in mg) by the muscle pair
weight (in gm). Rate of increase in body weight and muscle weight as well as nucleic acid and protein content was calculated as the difference between the values of every two successive ages. Also, the percentage loss in body weight, muscle weight and nucleic acid and protein content in experimental compared to control was calculated by dividing the difference between control and experimental by the control values, then multiplying the product by 100.

Every experiment has been done three times and all the results expressed as mean ± S.D. Significance of testing was performed by the student's 't' test (Kilpatrick, 1973).

\[
't' = \frac{|X_1 - X_2|}{\sqrt{\frac{(S_1)^2}{n_1} + \frac{(S_2)^2}{n_2}}}
\]

where, \(X_1\) is the mean value of control group.
\(X_2\) is the mean value of experimental group.
\(S_1\) is the standard deviation of control group.
\(S_2\) is the standard deviation of experimental group.
\(n_1\) represents no. of samples in control.
\(n_2\) represents no. of samples in experimental.

The level of significance between the two groups has been calculated at the degree of freedom \(n^*\)

\[
where, \ n^* = (n_1 + n_2) - 1
\]
Materials:

Dissection Tools: Sterilized dissecting tools were used for dissection of femur bones from the rats.

Glassware: Sterilized glassware was used.

Slides: Slides were kept overnight in chromic acid, following day washed with soap and water and stored frozen in distilled water. They were brought to 4°C before use.

Distilled Water: Double or triple distilled water was used throughout the work.

Chemicals:

Colchicine solution: A stock solution of 0.5% colchicine was prepared and stored at 4°C. For intraperitonial injection 300 ug/100 gm body weight of colchicine was administered.

Hypotonic Solution: 0.075M potassium chloride was prepared in distilled water.
Carnoy's fixative: Absolute methanol and glacial acetic acid were mixed in the ratio of 3:1. Fresh fixative was prepared each time and used cold (4°C).

Trypsin (Difco): A stock solution of 0.2% trypsin in phosphate buffer saline (PBS) was prepared. For G-banding it was diluted to 0.008% with distilled water.

Buffers:

i) 0.05M - phosphate buffer (pH 6.8).

ii) Phosphate buffer saline (pH 8.6).

Giemsa: A) Stock solution: 1 gm of Giemsa stain powder was dissolved in 66 ml of glycerol and kept at 55-60°C for 90 minutes. To this 66 ml of absolute methyl alcohol was added and kept overnight at 37°C. The stain was stored at room temperature.

B) Working solution: 5% Giemsa in 0.05M phosphate buffer (pH 6.8) was used for banding. 20% Giemsa in phosphate buffer (pH 6.8) was used for routine staining and
Chromosome Preparation:

It was carried out by according to the method described by Preston et al. (1987) with slight modifications. Two hours prior to the sacrifice of rats animals were numbered randomly and colchicine (1 mg/ml) was administered intraperitoneally at dose of 300 ug/100 gm of body weight. The rats were sacrificed after 2 hours by cervical dislocation. The femurs were quickly removed and muscle was cleaned away from the bone. Each femur was cut from both ends and the bone marrow was flushed from the femur into prenumbered centrifuge tubes, which corresponded to the animal number, with a hypodermic syringe fitted with a 22-g needle and dispersed with the same needle using 0.075M-KCl hypotonic solution (prewarmed to 37°C). Tubes were incubated for 20 minutes in 37°C. The tubes were centrifuged at 900 rpm for 5 minutes. Supernatant was removed by gentle aspiration and small volume of supernatant was left above the pellet. The pellet was suspended in Carnoy’s fixative (3:1 absolute methanol: glacial acetic acid), which is prepared fresh immediately before use and precooled to 4°C, with a fine tipped Pasteur pipette. The cells were kept for fixation, for 3 hours at 4°C. They were centrifuged at 900 rpm for 5 minutes and the supernatant was discarded. The pellet was washed thrice with the fixative (at 900 rpm for 5
minutes each). Finally, the pellet was suspended in suitable amount of fresh fixative and drops of suspension were dropped on to clean, wet, chilled slide. Slides were quickly blotted on the back and sides and blown once, then gently flame dried for better chromosome spreading (Upadhyaya and Verma, 1975). Minimum of 5 slides per animal were prepared and the slides were immediately coded with a random number which has been correlated with the animal number.

Giemsa Staining:

For chromosome scanning, slides were stained with 20% Giemsa in phosphate buffer (pH 6.8) for 30 minutes (Upadhyaya and Verma, 1975). Quality of staining was checked on test slide before all slides were stained. Properly stained slides were dried thoroughly, cleared in xylene and mounted with D.P.X. After drying up, slides were analysed microscopically for chromosome number and the mitotic index.

G. Banding:

For G-banding the slides were prepared in the conventional way as described earlier. Trypsin procedure (Seabright, 1972) as modified by Upadhyaya and Verma (1975) was used.
Trypsin Procedure:

Slides one to seven days old, were treated with 15% H₂O₂ for 10 minutes, rinsed in normal saline for 10 seconds and treated with 0.008% trypsin (Difco) in phosphate buffered saline for 10-15 seconds at controlled temperature of 0°C. The slides were again rinsed in normal saline for 10 seconds and stained with 5% Giemsa in phosphate buffer (pH 6.8) for 5 minutes. After drying, slides were cleared in xylene and mounted with D.P.X.

Methods of Scoring Metaphase Plates:

The total number of cells in interphase and the number of cells that were in metaphase were calculated after counting at least 1000 cells from randomly chosen fields per slide using a binocular microscope (Orthopan) at magnification (x400). The mitotic index (MI) was calculated from the formula,

\[
MI = \frac{\text{Total no. of metaphase plates}}{\text{Total no. of Cells}} \times 100
\]

HISTOLOGY

Materials:

a. Fine dissecting tools, cork and zero size pins.
b. Microscope slides and staining jars.
c. Ethyl alcohol, xylene, paraffine wax and D.P.X.

Solutions:

a. Buffered neutral formalin solution: 100ml of
37-40% formaline solution was diluted to 1 litre with distilled water, then 4g of sodium phosphate monobasic and 6.5g of sodium phosphate dibasic were dissolved in the formalin solution.

b. Mayer's egg albumin: 50ml of egg white was mixed well with similar volume of glycerin. The mixture was filtered through coarse filter paper. Crystal of thymol was added to the mixture for preservation.

c. Acid alcohol: 10 ml of concentrated H₂SO₄ was added to 1 litre of 70% alcohol.

d. Ammonia water: 2.3 ml of 28% ammonium hydroxide solution was added to 1 litre of tap water.

Stains:

a. Harris' hematoxylin stain: 5 gm of hematoxylin was dissolved in 50ml of absolute ethyl alcohol. 100 gm of ammonium or potassium alum was dissolved, by heat, in 1 litre of distilled water. Both solutions were mixed and brought to a boil as rapidly as possible. The heat should be limited to less than 1 minute and the mixture was stirred often. The solution was removed from heat and 2.5 gm of red mercuric oxide was added slowly to it. The solution was reheated to a simmer until it became dark purple; then removed from heat and the vessel containing the stain was plunged into a basin of cold water until it got cold. 2.4 ml of glacial acetic acid per 100 ml of stain solution was added to increase the precision of the nuclear stain. The stain was filtered before use.
b. Eosin Stain:

1% stock alcoholic eosin: 1 gm of eosin (water soluble) was dissolved in 20 ml of distilled water, then 80 ml of 95% alcohol was added to the stain.

Working eosin solution: 1 part of eosin stock solution was mixed with 3 parts of 80% alcohol. Just before use, 0.5 ml of glacial acetic acid was added to each 100 ml of the stain and the stain was stirred properly.

**Dissection and Fixation of Muscle:**

The nature of cytological analysis necessiated the use of standardized fixation and histological techniques (Rowe, 1968). Each rat was killed in chloroform vapour. The muscles; gastrocnemius, soleus and EDL from each hind limb were removed, after the onset of rigor mortis, in a standardized fashion. The abdominal cavity was quickly opened and the whole diaphragm was dissected out. Each muscle was pinned to cork board and fixed individually in formalin (100 ml/1 l) buffered with 0.1M phosphate buffer, pH 7.4 (Luna, 1968) for at least 10 days before being used for examination of muscle fiber size (Layman et al., 1981). The muscles were then washed for a minimum of 12 hours in running water before processing.
Processing of tissue (dehydration, clearing, impregnating and embedding):

The method described for biopsies by Luna (1968) was adopted with slight modification. This method was recommended for processing minute pieces of tissues to prevent the tissue from becoming hard and brittle, due to prolonged periods in the various agents. The muscle was dehydrated in 80% ethyl alcohol (for 15 minutes), 95% alcohol twice (15 minutes each), then cleared in xylene twice (15 minutes each) and impregnated in paraffin wax (BDH) twice, (15 minutes each).

Sectioning

Transverse and longitudinal sections 5μ thick from each muscle, were cut by using a microtome. Sections were floated in 55°C water-bath for stretching.

Attaching section to slides (section adhesive):

A small drop of Mayer's egg albumin was smeared over the surface of the slide with the finger and the excess was rubbed off with the heal of hand, or it can be applied with a clean foam-rubber sponge. According to Luna (1968) a sponge is usually preferred so that the epithelial cells from the finger will not adhere to the slide and produce artifacts when slides are stained.
Staining Procedure:

The method described by Luna (1968) was adopted with slight modification. Sections were deparaffinized in xylene for 5 minutes; then hydrated to water through a series of ethanol concentrations (100%, 95%, 80%, 70%, 50% and 30% respectively), for 5 minutes each. Slides were hydrated in water for 5 minutes, stained with Haris' hematoxylin for 1-2 minutes, rinsed in tap water and differentiated in acid alcohol (three to ten quick dips). Sections were checked up for the differentiation under the microscope, the nuclei should be distinct and the background very light or colourless. Slides were washed in tap water very briefly then dipped in ammonia water (for 3-5 minutes) until sections were bright blue. Sections were stained with eosin for 15 seconds to 2 minutes depending on the age of eosin. For even staining results, slides were dipped several times before allowing them to set in the eosin for the desired time (Luna, 1968). Slides were dehydrated in 95% alcohol twice (for 2 minutes each) to remove the excess eosin stain, then dehydrated in absolute alcohol twice (3 minutes each). Sections were cleared in xylene twice (2 minutes each), mounted in D.P.X covered with glass coverslips and kept at 37°C oven for drying. These slides were used later for examination of muscle fiber size.