MATERIAL AND METHODS
The Indian hedgehog, *Parascalops eremicus*, sometimes called the black-spined hedgehog, is one of the seasonally breeding insectivore mammals inhabiting the arid region of Rajasthan. Hedgehogs were collected from the vicinity of Jaipur (Rajasthan) were brought to Chendigarh and lodged in the animal-house, the rooms of which were provided with enough sand to help them to burrow into. These animals, many a time, got infected during transit from Jaipur, which is approximately 250 miles from Chendigarh, and they developed edema on the extremities. However, they were used for experiments only after curing them by giving a daily wash of mild detol and by adding antibiotics to their drinking-water. The animals were fed on fresh or frozen liver and pieces of meat (lamb). To avoid enteric infection or any food contamination, the leftover mutton pieces were removed each morning and the place was cleaned. Water was made available freely. To ensure that each hedgehog gets its own share of food, all the hedgehogs were taken out of their burrows every evening and fed individually under personal supervision. Some extra amount of mutton was also made available to them during the night.
The animals, each weighing approximately 200-350 grams, were used in the present study. The examination of the semen of the animals with different body-weights was made under a phase microscope in a preliminary study to ensure that all the hedgehogs weighing more than 150 g had attained sexual maturity. Each adult hedgehog was dissected each month of the calendar year to study the seasonal fluctuations. The hedgehogs which weighed between 100 and 140 g were found to be immature, as evidenced by the internal examination of their testes. In these immature hedgehogs, their seminiferous tubules were found to be populated with undifferentiated Sertoli cells, and differentiated spermatogonia, but the late stages of spermatogenesis were completely absent from these tubules. Five of such immature hedgehogs were also studied.

The animals were anaesthetized with ether and chloroform and were then fixed to wooden boards. The testes, the rete-testes, the vas deferentia, the epididymides, the prostate and the seminal vesicles were dissected out in saline. Small slices of tissues were cut and fixed in Bouin's, Zenker's, 10 per cent neutral formalin, cold-builline formalin, formal calcium, weak Bouin's, Bell's, Carnoy, Lewitsky saline and Cordes's fixatives. The formal calcium-fixed tissues were post-chromed (Baker, 1953) and washed along with the tissues already fixed in weak Bouin's, which acted as a control, and subsequently were embedded in gelatin. The frozen sections of these hardened gelatin blocks of tissues were cut at
10 microns and stained for lipids. The gelatin-embedded tissues were hardened by keeping the trimmed tissue blocks in cold formal calcium at least for 24 hours.

The tissues fixed in other fixatives were processed for paraffin-embedding and were subsequently sectioned at 7 microns on a rotary microtome. These sections were stained for the routine histological study, following the techniques cited by Palmgren (1962).

To study the topography of the testes, the rete testes, the ductuli efferentes and the epididymides in the breeding and non breeding seasons, serial sections of these organs were cut and were subsequently stained with hematoxylin and alkaline-hematoxylin.

For the histochemical localization of proteins, polysaccharides and nucleic acids, the sections were stained with the respective methods given separately, using the corresponding fixatives mentioned under those techniques.

**Phase optic study**

Thin monolayer tiles of the cells from the seminiferous epithelium were prepared in saline and subsequently the live germ cells were studied and microphotographed with the phase optics (Olympus PM-5-206632) using 10 × ocular and 100 × objective lenses.
Thirty adult healthy hedgehogs were selected for this study. They were divided into six equal groups. Three groups were kept as experimental groups receiving the hormone treatment, whereas the remaining three groups served as experimental and normal controls. For the identification of the hedgehogs belonging to different groups, the animals were painted with six colours for the six groups. The oil paint was applied superficially on the spines in a small area of the back of each animal. Group I received 3 mg of testosterone in 0.2 ml of olive oil per animal every week. Group II served as a control group for Group I and concurrently received an equal amount of vehicle (0.2 ml of olive oil per animal). Group III was administered a weekly dose of 60 mg of fresh pituitary extract in 0.5 ml of saline per hedgehog. The pituitary extract was prepared according to the technique of Carroll and Gregory (1954). Group IV was kept as an experimental control group for Group III and received simultaneously a similar dose of saline only, as was given to animals of Group III. Animals of Group V were administered a combination therapy of testosterone and pituitary extract, keeping the doses similar to those used in the other two experimental groups. Group VI received no treatment whatsoever and was kept as a normal control for all the above five experimental groups.

The treatment of testosterone and pituitary extract
was given for 4 weeks, beginning in the 2nd week of January 1972. One week after the last injections, all the animals were sacrificed within 3 days and the testes, epididymides, prostate and seminal vesicles were dissected out and fixed in Bouin's and Tenzer's. The material was processed for routine paraffin-embedding and sections were cut at 7-10 microns and subsequently stained with iron-hematoxylin, HE and hematoxylin and Masson's trichrome stain.

Preparation of crude pituitary extract
Terrol and Gregory (1962).
Intact pituitaries were procured from the severed heads of freshly slaughtered goats. Each gland was weighed before freezing. The pooled pituitaries were extracted in saline which was prepared in glass-distilled water under sterile conditions. Merthiolate, an antibacterial agent, was added to the pituitary extract, as per instructions of Terrol and Gregory (1962).

Testosterone
A single dose of freshly prepared and thoroughly mixed suspension of 3 mg of testosterone propionate in 0.2 ml of olive oil was administered to each animal at one time.
Histochemical techniques

I. Lipids

(a) Sudan Black B (SB): A saturated solution of Sudan Black B in 70% ethanol or isopropyl alcohol was prepared. Sections of formaldehyde calcium fixed (with post-chroming) material were first thoroughly washed with distilled water and stained with Sudan black (B) solution after a brief rinse in the solvent alcohol (Baker, 1966) at room temperature. The sections were differentiated in the same solvent medium and were subsequently washed with distilled water and mounted in glycerine jelly.

The paraffin sections of the material fixed in Levitsky saline and Kelly's fluid were also stained with Sudan Black B after deparaffinizing and hydrating these sections. Stained sections were then mounted in glycerine jelly. All lipids stained blue with Sudan Black B.

(b) Bound lipids were localized in sections stained with Sudan Black B in material fixed with calcium formal and subsequently treated at room temperature with hydroquinone and phenol, which unmask the bound lipids (Clayton, 1953).

(c) Berenbaum's technique of unmasking bound lipids (1953): To localize masked lipids, paraffin sections of Senker's and Kelly's fixed material were treated with 2 per cent solution of AIB in acetone with a prior rinse in acetone.

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(d) Acid haematin technique for phospholipids (Baker, 1946): The gelatin sections of formaldehyde-fixed material were post-chromed for one hour in dichromate-calcium at 60°C and subsequently were treated with acid haematin solution for 5 hours at 37°C. The differentiation of these sections was carried out in borax ferricyanide for 13 hours at 37°C.

The control sections of weak Bouin's-fixed material were run simultaneously with the formaldehyde-fixed gelatin sections. These control sections before staining were treated with pyridine initially for 30 minutes at 17-22°C and later on for 24 hours at 60°C and subsequently were washed for 2 hours in running water.

A positive reaction in normal acid haematin test and a negative reaction after pyridine extraction confirms the presence of phospholipids.

(e) Mile blue sulphate test (Cain, 1947): Both 1% and 0.62% solution of Mile blue sulphate were used at 60°C to distinguish between the acidic and the neutral lipids. The former stain blue and the latter stain pink.

(f) Oil red O method for neutral lipids (Millie, 1966 from Pearse, 1961): The stock solution of the stain was prepared by adding 0.5 g of oil red O to 100 ml of 93 per cent isopropanol. This stock solution was diluted (6:4) with distilled water and was allowed to stand for 24 hours. Before
the working solution was filtered through whatman No. 42.

Formol-calcium-fixed and gelatin-embedded sections were first washed with distilled water and after a brief rinse with 60% isopropanol were stained for 10 minutes with oil red O. The sections were then differentiated in 60% isopropanol and washed in running water for 10 minutes and mounted in glycerine jelly. The neutral lipids stained red.

Schulz's method for cholesterol (Pearse, 1960):

Fresh frozen sections were cut at 25 microns and washed in distilled water thoroughly. The sections were then treated with 0.5% ferric ammonium sulphate in 0.2 M acetate buffer at 37°C for seven days. The buffer was adjusted to pH 3 by mixing 2 ml of 0.2 M-sodium acetate with 0.1 ml of 0.2 M-acetic acid. The final pH was about 2. The treated sections were washed in three changes of one hour each in acetate buffer and then rinsed in distilled water and were subsequently transferred to 5% formalin for 10 minutes. The sections were then mounted on glass slides and extra water was blotted off from the edges. A drop of a mixture of equal parts of sulphuric and acetic acid was placed on the cover-slip and such cover-slips were applied to inverted slides. The slides were turned right way up and by applying even pressure to the cover-slip, the sections were flattened. The pale violet or red colour developed at the sites of cholesterol turning rapidly into green. The sections were immediately photographed, as the
colour remains stable only for 30-60 minutes.

II Carbohydrates

(a) Periodic acid and Schiff (Pe)

This method was followed, as recommended by Hotchkiss (1943) and Peerse (1960), but the oxidation was done usually in 0.5% aqueous periodic acid for 3 to 5 minutes, as recommended by Casalhoen (1959). This process was followed by Schiff’s reagent for 20 minutes. Nuclei were counterstained with haematoxylin (Sltman, 1963), or with methyl green. In the latter case to retain the nuclear stain, the dehydration was carried out in tertiary butyl alcohol.

To confirm the PE reactivity, owing to 1,2 glycol groups, the following controls and extraction techniques were employed:

(i) Acetylation with KOH reversal: Before oxidizing the sections with periodic acid, they were treated with acetic anhydride-pyridine solution. This solution blocks 1,2 glycol groups (St-Vinas and Casse, 1956; Lillie, 1954) and restores the Schiff’s staining after treating the sections with KOH solution. This test confirms the presence of 1,2 glycol groups of polysaccharides.

(ii) Maltase digestion: The control for glycogen was carried out with malt-Maltase digestion treatment (Lillie, 1954), followed by the staining. To obtain good contrast, the NBT was followed by counterstaining with Sudan Black B (Sltman, 1963).
(iii) Methanol chloroform control: To avoid the ph staining owing to lipids, the sections were treated with methanol-chloroform in the ratio of 1:2 (Corsh, 1949).

(b) Alcian blue reaction for acid mucopolysaccharides: Alcian blue method (Spicer, 1963) considered to be specific for acid mucopolysaccharides was used on Bouin's - and Zenker-fixed material. To differentiate the acid mucopolysaccharides from other polysaccharides, the sections were counterstained with PTA.

(c) Best's carmine stain for glycogen (from Humeason, 1962): A stock solution of carmine was prepared as follows: 2 g of carmine, 1 g of potassium carbonate and 5 g of potassium chloride were added to 50 ml of distilled water and boiled gently, cooled and filtered. 20 ml of ammonia (specific gravity 0.993) was added to the filtrate and kept at 0-4°C. The working solution was prepared by diluting 15 ml of the stock solution with 12.5 ml of ammonia and 12.5 ml of methyl alcohol.

The tissues were fixed at 4°C in pre-cooled Carnoy and Carnoy's fixing solutions and were subsequently washed at room temperature and embedded in paraffin. The sections after demarcating were coated with colloidin and first stained for nuclei in Shrich's hemalum, followed by staining in the working solution of Best's carmine stain. After quick differentiation, these sections were washed in 30% alcohol, dehydrated, cleared and mounted in DPX. Glycogen appeared as red granules, whereas the nuclei were stained blue.
(a) *eyer's mucicarmine* stain for neutral mucopolysaccharides (from Humason, 1962): The stock solution of the stain was prepared by adding 11 g of carmine (c. 175,79), 1 g of ammonium hydroxide powder and 5.5 g of ammonium chloride (anhysrous) to 100 ml of 50% ethyl alcohol. This solution was gently heated in a water-bath with frequent shaking for 2 to 5 minutes. It was then cooled under tap-water and filtered. The working solution was prepared by adding 9 parts of distilled water to 1 part of the stock solution. The sections were then heated for 15-20 minutes by flooding the slides with the working solution. After a quick wash with distilled water, were dehydrated in graded alcohols, cleared in xylene and mounted in DPX. The mucopolysaccharides stained red.

III Proteins

(a) *Mercuric bromphenol blue* stain (Bonhag, 1955): This method was mainly employed on *senker* and *bouin*-fixed material for the localization of proteins in general. The sections were stained in mercuric bromphenol blue solution, differentiated in 1% acetic acid, and dehydrated in tertiary butyl alcohol and mounted in DPX. Some of the sections were counterstained with PA to differentiate the polysaccharides from proteins which were stained clear from blue.

The Luwitsky saline-fixed sections were counterstained with Sudan Black B and PA also. The proteins
stained clear blue, the polysaccharides red and the lipids blue black.

(b) coupled tetrazolium reaction (Danielli, 1947, from Poerse, 1963): This reaction demonstrated the presence of proteins. The reaction is based on the combination of amino acids with tetrazolized benzidine at pH 9 at 4°C and subsequent coupling of residual free diazo group with a phenol or aromatic amine.

To distinguish between the tyrosine, tryptophan and histidine, this reaction was accompanied by its various controls as given below:

(i) **formic acid**: This acid is employed for the oxidation of tryptophan. After this treatment, the indole ring of tryptophan fails to react with coupled tetrazolium. The presence of tryptophan is therefore only indirectly suggested. However, the positive reaction of CF after performic acid control suggests the presence of other amino acids.

Formic acid was prepared by adding 4 cc of 30% hydrogen peroxide and 0.5 cc of concentrated H₂SO₄ to 40 ml of 30% formic acid.

(ii) **sinitrofluorobenzene (SNFB)**: It is used for blocking tyrosine and histidine. A saturated solution of sinitrofluorobenzene in 90% ethanol, saturated with sodium carbonate, was used. The sections were treated with SNFB for 2-16 hours at room temperature.
(iii) **Peroxylation**: A 10 per cent solution of benzoyl chloride in dry pyridine is used for blocking tyrosine and tryptophan.

(c) **Alkaline fast green** (from Pearse, 1960): This method was employed for the detection of basic proteins (Ulfert and Gerschwind, 1953). Formaldehyde-fixed sections were treated with 5 per cent trichloroacetic acid (TCA) for the removal of nucleic acids. After washing, the slides were stained with 0.1 per cent aqueous fast green adjusted to pH 1.0-1.1 with the minimum amount of sodium hydroxide.

Sites stained bright green were basic proteins, mainly histamines and histones.

**Ferric ferrocyanide method for sulphydryl group** (d)

(Chernoff and Freider, 1963): The staining solution was prepared by adding 3 parts of 1% ferric sulphate \( \text{Fe}^{3+} \) to one part of freshly prepared 0.1 per cent potassium ferrocyanide \( \text{K}_3\text{Fe(CN)}_6 \) and its pH was adjusted to 3.4. Formalin-fixed paraffin sections were stained for 25 minutes, differentiated in 2% alkaline 60% alcohol, washed in distilled water, dehydrated in graded alcohols, cleared in xylene and mounted in DPX. The SH groups were stained clear blue.

(d) **The alkaline tetrachloride reaction for SH and SS groups**: The formalin-fixed paraffin sections were brought down to water through descending grades of alcohol. The sections were then incubated for 60 to 90 minutes at 50-60°C in a...
solution of blue tetratolium salt in glycine buffer at pH 12-12.5. Subsequently, the sections were washed in running cold water and then mounted in glycerine jelly. The reducing groups bound to proteins were stained dark blue, whereas those bound to lipids stained redish.

NUCLEIC ACIDS

(a) Pyronin/methyl green reaction (P/MG): The reaction (Jordan and Baker (1956) and Baker and Williams (1965)) demonstrates the presence of deoxyribonucleic acid and ribonucleic acid at the same time. Paraffin sections of chilled Banke (3 hr) and Clarke's fluid were treated with P/MG solution. The presence of RNA and DNA are confirmed only after treating the sections with 5% trichloroacetic acid at 90°C for 15 minutes (Schneider, 1945, from Pearse, 1963).

(b) Feulgen reaction: This reaction (Feulgen and Hansenbach, 1924; Pearse, 1963) demonstrates the presence of DNA. Sections fixed in Banke and Kelly's were hydrolysed for 5 and 3 minutes respectively in 1 M-HCl at 60°C, which released the aldehyde group from oxymetase sugar of DNA. The tissue sections were then treated with Schiff's ( utt, 1963), which reacted with the exposed aldehyde group to give a purple coloration to the nuclear material. At times, sections were counterstained with light green. The presence of DNA was confirmed after extraction with 5 per cent TCA (trichloroacetic acid) at 90°C for 15 to 25 minutes.
Basic fuchsin: 0.5 per cent aqueous basic fuchsin (Gatenby and Beams, 1959) was used on paraffin-fixed material for the localization of basophilia.

(c) Gellocyanin-chromicum chromic medium for nucleic acids (Dinerson, 1951; From Pearse, 1960): The stain was prepared by dissolving 5 g of chromium in 100 ml of distilled water to which 0.15 g of gellocyanin was added and then mixed thoroughly. The solution was warmed gradually to boil (5 minutes), cooled and filtered. The sections after staining in the solution adjusted at pH 1.64 for 2 days at room temperature were washed, dehydrated and mounted in DPX.

Enzyme Localization Techniques

(a) Alkaline Phosphatase - Frozen sections

calcium cobalt Method (Tomori, 1959) from Pearse, 1963:

Formalin-fixed frozen sections were cut at 10 microns and mounted on glass slides without using albumin. Sections were air-dried and incubated for 30 minutes — 4 hours in the substrate medium containing the following:

- 10 ml of 3% sodium B glycerophosphate
- 10 ml of 2% sodium diethyl barbiturate
- 5 ml of distilled water
- 20 ml of 2% calcium chloride
- 1 ml of 5% magnesium sulphate

The control medium did not contain sodium B glycerophosphate.
The slides were washed after incubation, treated with 2-
cobalt solution, washed again and then treated with dilute
yellow ammonium sulphide. To remove the extra amount of the
precipitate, washing was done for 5 minutes and then the
sections were mounted in glycerine jelly. Black deposits in
the section indicated the site of the enzyme activity.

(b) Acid phosphatase (frozen sections)

Lead acetate method (Cane, 1965; From Pears, 1967)
Cryostat sections were air-dried on slides and then the slides were
dipped for 20 minutes at 4°C into the fixative containing the
following:

- 50 ml of 40% formaldehyde
- 5 g of CaCl₂·6H₂O
- 5 g of gum acacia
- 150 g of sucrose
- 500 ml of distilled water

Sections were washed in running water for 5 minutes to remove
the fixative and incubated in the standard Comori's medium
(31.5 mg of sodium B glycerophosphate in 5 ml of 0.1 acetate
buffer at pH 5, to which 5 ml of 0.0003% lead acetate was
added just before use) at 37°C for 15-30 minutes. After
washing the sections in tap-water, they were developed in
buffered (pH 7.3) ammonium sulphide, washed again and then
mounted in glycerine jelly. Black deposits in the tissue
sections indicated the enzyme activity.

d) Thiamine pyrophosphatase (frozen sections)

(Franko and Pasen's method, from Pearse, 1963): The medium was prepared by dissolving 60 mg of Pb (NO$_3$)$_2$ in 45 ml of 0.035 M acetate buffer (pH 5.5), to which was added 5 ml of 5% thiamine pyrophosphate and it was kept for 24 hours at 37°C, then filtered and stored in the cold. Cryostat sections were incubated at 42°C for 50 minutes, subsequently rinsed in 1 per cent acetic acid, blotted, and were dried at 60°C for 5 minutes. The sections were then coated with a thin film of collodion and treated for 2 minutes with dilute yellow ammonium sulphide, rinsed in water and mounted in glycerine jelly.

Brownish black deposits indicated the sites of thiamine pyrophosphatase activity.

In all these enzyme-localization techniques, the controls were run simultaneously in the incubation media lacking the corresponding substrates. The brown or black deposits in the substrate-incubated sections indicated the sites for the enzyme activity. Negative results in the sections incubated in the control media are necessary to confirm the presence of the enzyme activity.

f) Nucleine, acid and thiamine pyro-phosphatase localization in paraffin sections (Bikai & Nave, 1971); Unfixed or
briefly fixed and washed tissue slices were directly incubated in the substrate and the control media. The procedures for incubation for the localization of alkaline, acid and thiamine pyro-phosphatases were similar to those followed for frozen sections and the respective substrates were prepared after Coomer (1960) and Meek and Bradbury (1963). After incubation, the tissues were briefly washed and post-fixed in 10% neutral formalin for 15 minutes, washed again, dehydrated in the ascending grades of alcohol and embedded in paraffin wax. Sections were cut at 7-10 microns and mounted on albuminized slides. After dewaxing, the sections were recounted in Canada balsam. The black and brown deposits indicated the sites of the enzyme activity. The blank control sections confirmed the presence of the enzyme.
Techniques of tissue preparations for electron microscopy

The material used for the ultrastructural study consists of testes, different ducts, epididymides, prostates and seminal vesicles of the sexually active and also of the regressed hedgehogs. These tissues were dissected out in the shortest possible time and were subsequently minced in drops of pre-cooled buffered glutaraldehyde (Luft, 1961; Millonig, 1961b; Karnovsky, 1965; Sowes, 1971) on wax-coated cardboard strips. The same tissues were also simultaneously fixed in Bouin's for the routine histological preparations.

Very small pieces were selected from the minced tissues and were transferred to pre-cooled vials containing buffered glutaraldehyde (used as a primary fixative). The same buffer which was used for the preparation of glutaraldehyde (either cacodylate or phosphate buffers) was also used for washing the tissues and also for the preparation of buffered osmium tetroxide. The post-fixation procedure was followed after fixing the tissues in buffered glutaraldehyde for three hours (Jostrend, 1961; Held, 1971).

Post-fixation procedure (From Electron microscopy techniques manual, 1971).

The tissues after fixing in buffered glutaraldehyde were washed for 4-6 hours in cold buffer with at least 4 one-hourly changes. After the last wash was given, the tissues were kept in a cold buffer overnight.
After draining off the cold buffer, the tissues were post-fixed in cold buffered osmium tetroxide for 1-2 hours. The tissues were subsequently washed thoroughly with the same cold buffer (1 to 6 changes) till all the traces of osmium tetroxide were removed from the tissues.

The tissues were then dehydrated through a graded series of cold ethyl alcohol for 15 minutes each in 30, 50, 70, 90 and 95 per cent sequentially. The dehydration was continued in absolute alcohol for another two hours at room temperature (with at least 3 changes).

The tissues were cleared for 15-30 minutes in a mixture of absolute alcohol and propylene oxide (1:1 ratio) and then in pure propylene oxide.

The tissues were embedded in epon (Luft, 1961) or eukitite (Kernovsky, 1965).

The embedding medium was stirred thoroughly without producing air bubbles with a magnetic stirrer to avoid unsatisfactory embedding or air bubbles being trapped in the tissues.

The embedding was done in four steps of two hours each on a magnetic shaker.

1. Embedding medium + propylene oxide = 1:1
2. Embedding medium + propylene oxide = 2:2
3. Embedding medium + propylene oxide = 3:1
4. The pure embedding medium.
The tissues were subsequently transferred to silastic embedding plates or plastic capsules containing the embedding medium and were kept at room temperature overnight. The embedding was continued for 24 hours at 44°C and for another 41 hours at 60°C. After the embedding was completed the tissues became hard but not brittle and were ready for sectioning. The tissues which remained soft were discarded.

The tissue blocks were properly trimmed under a dissecting microscope after mounting them on the microtome-holders. The instructions for cutting good ultrathin sections were followed from the methods described by Reese (1964), Pawcett (1967), Burm (1970), Dawes (1971) and Cleveert (1974). Before cutting grey or silver-sections, a few thick sections were cut to examine the tissue and select the area for ultrathin sectioning. The remaining tissue was trimmed off and then silver or grey sections were cut on a separate freshly prepared glass knife to avoid any contamination. The thick sections were stained with toluidine blue and Methylene blue.

**The staining of thick section**

**Toluidine blue** : Stock solution -

- 1% toluidine blue (aqueous)
- 2.5% sodium bicarbonate (NaHCO₃) (aqueous)

Fresh stain was prepared each time by mixing of stock solutions (toluidine blue 1 ml + sodium bicarbonate 20 ml). The working stain was filtered before use.
A drop of freshly prepared and filtered stain was placed over the sections on the slide and the sections were allowed to stain for 10 to 20 minutes on a slide-warmer at 47°C, using a microscope to check the degree of staining. The stain was washed off with distilled water. A slight overstaining was obtained with ethanol, when necessary. The section was dried and mounted with a coverslip, using oil or a permanent mounting medium, such as DPX (C.J. Dawes, 1971).

Preparation of ultrathin sections

The scanned area of the tissue block was properly trimmed, so that only the tissue (not the plastic) was visible on the cutting face of the block. Ultrathin sections were cut and floated on a weak acetone solution in distilled water. Only grey or silver sections were taken on the copper grids. The copper grids, used in the study, were of “ethene” type with one surface polished and the other “matte” and were 3mm in diameter with 200 mesh. After the sections were flattened on acetone water in the boat, they were taken up on the copper grids and the pick up of the sections was achieved by lowering the copper grids on to the sections with matte side facing them. The sections stick to the matte surface when it comes closer to the sections and then the grid was taken away from the tough end put on a filter-paper in such a way that the sections remained on the upper surface. The filter-paper absorbs water and the sections become dry and adhere firmly to the copper mesh. These grids were kept covered
with a petri dish till they were stained and were ready to be studied with the electron microscope.

The staining with uranyl acetate was also done while processing the tissue in the following way. Three per cent solutions of uranyl acetate in higher grades of alcohol were prepared (90%, 95% and absolute alcohol) and these grades were used at the time of dehydration and thus the tissue-processing and staining were performed simultaneously.

However, most of the times, the double staining of uranyl acetate and lead citrate of the sections counted on grids had been done.

**Uranyl acetate staining of sections on grids (Stepanek & Ward, 1964).**

The stain was prepared by adding 5 g of uranyl acetate (UO₂(CH₃COO)₂), 2420 to 25 ml of 10% acetone-free methanol with constant agitation. The staining solution was stored at 4°C and was filtered before use.

Staining was carried out in petri-dishes lined with dental wax. Howell's method (1963) for obtaining contamination-free sections after staining with both uranyl acetate and lead stains was followed. Millipore filter of pore size 0.22 μm was used in this method. This filter was fitted into a syringe which was filled with the staining solution to be used on the sections on the grids. With the above syringe, drops of staining solutions were put on the wax-lined petri dishes. The grids were then
floated on these drops with the nette side bearing the thin sections dipping into the fluid. The petri dishes were kept covered while staining to avoid contamination, jeweller's forceps were used to hold the grids during staining & washing. The grids were dipped into drops of uranyl acetate for 10-15 minutes as described above and were subsequently washed by dipping into baths of 100, 70 and 50 per cent methanol solutions sequentially. The above procedure was followed with two washes of distilled water. These washed grids were dried on filter papers keeping the polished sides of the grids down, so that the sections on nette side did not touch the filter papers.

Such dried sections on the grids were kept in tightly covered labelled capsules till they were to be studied under the microscope.

Reynold's lead citrate method for sections on grids (1963):

In a 50 ml volumetric flask, 30 ml of distilled water was taken and 1.33 g of lead nitrate ($\text{Pb(NO}_3\text{)}_2$) and 1.76 g of lead citrate ($\text{Pb}_3$($\text{C}_6\text{H}_5\text{O}_7$)$_2$$\cdot$2$\text{H}_2\text{O}$) from fresh analar salts, were added to water in the above flask. The mixture was shaken and was allowed to stand for 30 minutes, shaking intermittently to ensure complete conversion of lead nitrate into lead citrate. Eight ml of 1N NaOH was diluted to 50 ml with distilled water. The diluted NaOH solution was then added to the mixture in the flask. The lead citrate was dissolved and formed a solution. Any turbidity formed in the process was removed by centrifugation.
The pH of the staining solution was adjusted to 12 ± 0.1 (this solution remains stable for 6 months at room temperature and was stored in glass or polythene bottles). The staining solution was always centrifuged before use.

The sections on grids were then stained in drops of lead citrate in the covered petri dishes for 15 minutes. Double staining was also performed by staining the sections with lead citrate (Reynold, 1963; Venable and Coggeshall, 1965), followed by uranyl acetate for 5 to 30 minutes. Stained sections on copper grids after drying were studied with Philips 201 electron microscope. The photographs of the desired fields were taken on the attached 35 mm camera. The films used were "Fine grain, high resolution film, 5302, Non perforated, 35mm, FRP 426 (Eastman-Kodak)". The prints were prepared, following the developing and printing procedures from The Manual prepared for the department of Harvard Medical School, U.S.A. The final enlarged prints were subsequently studied.