1) Studies on the effects of an aqueous extract of
Caries papaya seed on male rats for 7 and 15 days.

2) Carica papaya seed extract + ascorbic acid feeding
for 7 and 15 days.

3) Extract treatment for 7 days + 1 and 2½ months
withdrawal.

Animals: Colony-bred healthy adult male albino rats
(Rattus norvegicus) of Holtzman strain were utilized for
various experiments. The animals used were weighing between
150 - 250 g. All the animals used were of proven fertility,
and were housed in a controlled environment at a constant
temperature of 26-28°C and were exposed to 12-14 day light
hours. They were maintained on a standard diet of pellets
(Hindustan Lever Ltd; Bombay, and water ad libitum. The
animals were grouped as follows:
<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatment</th>
<th>No. of animals used</th>
<th>Day of autopsy</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Normal (Control rate)</td>
<td>35</td>
<td>Batches of 5 sacrificed along with each group of treated rats</td>
</tr>
<tr>
<td>II</td>
<td>Seed extract treatment (7 days)</td>
<td>40</td>
<td>8th day</td>
</tr>
<tr>
<td>III</td>
<td>Seed extract treatment (15 days)</td>
<td>35</td>
<td>16th day</td>
</tr>
<tr>
<td>IV</td>
<td>Ext. Treatment + ascorbic acid (AA) feeding for 7 days</td>
<td>35</td>
<td>8th day</td>
</tr>
<tr>
<td>V</td>
<td>Ext. treatment + A. (15 days)</td>
<td>35</td>
<td>16th day</td>
</tr>
<tr>
<td>VI</td>
<td>Ext. 7 days + 1 month withdrawal</td>
<td>35</td>
<td>38th day</td>
</tr>
<tr>
<td>VII</td>
<td>Ext. 7 days + 2-2.5 months withdrawal</td>
<td>45</td>
<td>67th to 83rd day</td>
</tr>
</tbody>
</table>

Preparation of aqueous extract of Carica papaya seeds:

Fully ripened fruits of Carica papaya were chosen for the experimental purpose. The seeds were air-dried for a few days, and ground in a pestle and mortar. An aqueous suspension of 500 mg of the powder was made in 100 ml of double distilled water and sonicated for 30 minutes. After cooling, the extract was filtered through Whatman filter...
paper no. 1 to remove any suspended particles. The filtrate was tested by Dragendorff's reagent (Sarwar and Rakhshit, 1960), for the presence of water soluble alkaloids in the extract. The extract was preserved in the cold for 5-6 days only for the present study.

**Dosage:** Each animal received 1 mg of the extract/0.2 ml/day for 7 and 15 days, so that the dosage was 5 mg/kg body weight. The extract was injected i.m. in the thigh region.

In groups IV and V animals 50 and 100 mg/day/rat of ascorbic acid was fed orally along with the treatment for 7 and 15 days.

At the end of each treatment, the body weight of each animal was recorded separately and they were sacrificed by cervical dislocation. The testis, caput and cauda epididymides, seminal vesicles, and vas deferens of either side from each animal were excised and the surrounding tissue was cleared of fat, blood vessels, and nerves. The vas deferens was studied in two regions separately, proximal vas deferens (VDP, which is nearer to the cauda epididymis) and distal vas deferens (VDD, nearer to the sex accessory complex).

Each tissue was weighed on a torsion balance to the nearest milligram and used for various estimations separately.

**A. The parameters studied were as follows:**

1) **Body weight:** The body weight of control and treated rats were recorded separately.
(2) Absolute organ weights: The absolute weights of testis, caput and cauda epididymides, proximal and distal vas deferens and seminal vesicles and ventral and dorso-lateral prostate of control and treated rats were recorded (in mg).

(3) Spermatosoma count and motility: Testis and cauda epididymides of known weights were teased gently in a known volume of physiological saline to release the spermatosoma from the tubular elements. The tissue components were removed and the clear sperm suspensions were used for evaluating the sperm count and motility.

The spermatosoma density were determined according to the method of Prasad et al. (1972) using W.B.C. counting Neubauer chamber of a haemocytometer and were expressed as million spermatosoma/ml suspension.

The sperm motility was assessed visually and the percentage of motile sperms were calculated per unit area.

(4) Fertility test: Control and treated males were caged individually with normal cycling females (proestrus or estrus) in the ratio of 1:3 to allow normal mating overnight. On the following morning the successful mating was indicated by the presence of spermatozoa in the vaginal smear or by the formation
of the vaginal plug. After 6 days, the uteri of each female were exposed under light ether anaesthesia and the implantation sites were examined. Presence of implantation sites was taken as a criterion of successful insemination and fertility test was considered to be positive. The absence of these sites revealed negative fertility test.

B. Biochemical parameters:

Several androgen sensitive parameters were investigated for a comparative assessment in different animals as follows. For all biochemical estimations, a minimum of 6 replicates were done for each tissue and parameter and data was statistically analysed using Student's "t" test.

(1) Succinate dehydrogenase (SDH) (E.C.1.3.99.1):

Succinate dehydrogenase activity was assayed by the modified tetrazolium reduction method of Beatty et al. (1968). The different tissues viz., caput and cauda epididymis, and the two regions of vas deferens (VDP and VDD) were weighed and homogenized separately in 3 ml of cold distilled water and aliquots were used for the estimations.

An electron acceptor 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-tetrazolium chloride (INT) was used which takes up the electrons released by the enzyme succinate dehydrogenase from the substrate, sodium succinate. Thus, INT is reduced.
to a red formazan which was extracted with ethyl acetate and read colorimetrically at 420 nm.

The reaction mixture contained 1 ml of 0.2 M phosphate buffer (pH 7.6), 1 ml of 0.1 M sodium succinate and 1 ml freshly prepared INT solution. The blank tube contained 1 ml of distilled water instead of INT solution. After 15 minutes (caudal and cauda epididymis) to one hour (VDA and VDB) of incubation at 37°C, 0.1 ml of 30% TCA was added to terminate the reaction. The formazan was extracted into 7 ml of ethyl acetate by vigorous shaking for 30 seconds. The solution was then centrifuged for 5 minutes at 1500 r.p.m. The supernatant was then transferred to other test tubes and the intensity of colour was measured at 420 nm on Spectronic 20 Bausch and Lomb colorimeter. The colour was stable for at least 20 minutes.

The standard curve prepared was linear from 12 µg to 100 µg of INT.

The calculation of the SDH activity was done using the regression formula:

\[ \lambda = 1.856 + 152.21Y \]

where, \( \lambda \) = the concentration of SDH in µg.

\( Y \) = optical density of the unknown sample.

The enzyme activity was determined by substituting the value of \( \lambda \) in the formula.
MConcentration) x Dilution

$$\text{GOD activity} = \frac{\lambda \text{ Concentration}}{\text{Tissue weight}} \times 100 \div \text{Protein value}$$

The enzyme activity was expressed as µg formazan formed/100 µg fresh tissue weight/15 minutes or one hour/µg protein.

(2) Acid phosphatase (ACP) (L:3.1.3.2):

The acid phosphatase activity was estimated by employing the method of Belfield and Goldberg (1971) based on the amount of phenol liberated by hydrolysis of the substrate, disodium phenyl phosphate at a specific pH.

The different tissues, viz., caput and cauda epididymides and proximal, distal regions of vas deferens (UD & VDD) were used for the assay.

A known amount of tissue was homogenized in 3 ml of cold double distilled water. 0.05 ml of tissue homogenate was incubated with 1 ml of citrate buffer substrate mixture (pH of buffer 4.9; 21 gm of citric acid in 1 N NaOH; substrate 10 m mol/litre disodium phenyl phosphate) at 37°C ± 2°C for 1 hour. The enzymic activity was terminated by the addition of 1 ml of carbonate-bicarbonate-4-aminantipyrine-arsenate reagent (4-AAP, 0.6 gm/100 ml; sodium arsenate, 3.75 gm/100 ml) followed by 1 ml potassium ferricyanide (2.45 gm/100 ml) and the optical density (CD) was read at 510 nm on a spectronic 20 Bausch and Lomb
Colorimeter. The blank was run along with the sample containing 0.05 ml of cold distilled water instead of the tissue homogenate and similarly, in the control test tubes, 4-AAP arsenate reagent was added before the incubation of the tubes at 37°C and buffer was added after the incubation period was over. The standard was run containing phenol in place of the homogenate. A linear curve was obtained for phenol concentrations used (10 µg to 50 µg). The concentration of standard used was 0.05 mg/0.1 ml.

The activity of acid phosphatase was derived as King-Armstrong Units/100 ml homogenate (µg phenol liberated/hour) and calculated as follows:

\[
\text{ACP activity} = \frac{\text{O.D. of Aliquot - O.D. of Blank}}{\text{Concentration of standard} \times \text{Aliquot volume} \times 100}
\]

= King-Armstrong Units.

The enzyme activity was expressed as µ moles/litre/mg protein by multiplying King-Armstrong units by the factor 7.9 of Selfield and Goldberg (1971) and then dividing it by the protein value obtained for the respective tissue.

(3) Protein:

The protein levels in the tissues were determined by the method of Cornell et al. (1949). Tissues of known weights
weights were homogenized in 3 ml of double distilled water. In each sample test tube, 1 ml of tissue homogenate was added, whereas in blank, the homogenate was replaced by 1 ml of double distilled water. In all the tubes 4 ml of niuret reagent (1.5 g CuSO₄·5H₂O; 6.0 g sodium potassium tartrate and 10% NaOH; made up to 1 litre) was pipetted, mixed and the tubes were allowed to stand for 30 minutes at room temperature. The optical density of the resultant colour was read in a Colorimeter at 540 nm.

The calculation was done using the regression formula:

\[ X = 0.077 + 18.7Y \]

where, \( X \) = Concentration of protein in mg and 
\( Y \) = Optical density of the unknown sample.

The value was substituted in the formula:

\[
\frac{\text{Concentration x Dilution}}{\text{Fresh tissue weight in mg and expressed as mg protein/100 mg fresh tissue weight}} \times 100
\]

(3a) Assay of SDH, ACP and Protein concentrations in whole sperm suspensions (sperm suspension + luminal fluid) and sperm pellet of caput and cauda epididymides of control and 7 day seed extract treated rats were carried out as follows:

weighed tissues of caput and cauda epididymides were
separately teased in known volumes (4 ml) of 0.9 % saline. The tissues were removed gently by filtering and each assay was carried out in the whole sperm suspension and luminal fluid thus obtained. In order to separate the sperm pellet of caput and cauda epididymides, the whole sperm suspension was centrifuged at 1,000 to 4,000 r.p.m. for 10 minutes. The supernatant was drained off carefully and to the sperm pellet, 4 ml of 0.9 % saline was added and the assays were carried out.

(4) Fructose

The method of Forsman et al. (1973) was used for estimating fructose concentrations in the seminal vesicle. The modified method is specific for fructose as it shows no reaction with glucose or glucose phosphatase. When heated with concentrated hydrochloric acid (HCl), fructose forms oxy-methyl furfural which gives a red colour with resorcinol. A known weight of the tissue was homogenized in 10 ml of 5 % (0.9 N) perchloric acid. To the sample tubes 1 ml of homogenate, 0.5 ml of 0.1 % (0.09 N) resorcinol dissolved in 95 % alcohol and 1.5 ml of 30 % hydrochloric acid were added. In the blank tube, 1 ml of homogenate was replaced by 1 ml of 5 % perchloric acid. The tubes were heated in a water bath at 80°C for one hour and cooled to room temperature. The colour intensity was read on a Spectronic 20 Bausch and
Lamb Colorimeter at 410 nm wavelength. The concentration of fructose was calculated by using the regression formula.

\[ X = 781.09 Y + 10.99 \]

Where, \( X \) = concentration of fructose in \( \mu g \)
\( Y \) = O.D. of unknown sample.

The concentration was expressed as \( \mu g/\text{mg fresh tissue weight} \).

**Calculation:**

\[
\text{Fructose concentration} = \frac{\text{Concentration of unknown sample}}{\text{Dilution}} \times \text{Fresh tissue weight (mg)}
\]

and was expressed as,

\( \mu g \) fructose formed/\( \mu g \) fresh tissue weight.

(5) **Determination of Total ascorbic acid (TAA), Dehydro-ascorbic acid (DHA) and Reduced ascorbic acid (RAA):**

The 2,4-dinitrophenyl hydrazine procedure of Roe and Kuether (1943) was employed to measure the total ascorbic acid. Ascorbic acid is oxidised to dehydroascorbic acid by shaking with 'Norit' in the presence of acetic acid. After coupling with 2,4-dinitrophenyl hydrazine, the solution is treated with sulphuric acid to produce a red colour which is measured colorimetrically. This 2,4-dinitrophenyl hydrazine
method which measures total ascorbic acid could be partitioned into the dehydro and reduced forms by including a simultaneous test in which norit treatment is omitted. The latter procedure measures only dehydroascorbic acid. The reduced ascorbic acid content was obtained by difference.

A known weight of the tissue (Caput, cauda, VDP and VDD) was homogenized in a known volume (10 ml) of 'norit' extract to estimate total ascorbic acid. The norit extract was prepared by using 0.5 gm norit per 25 ml of trichloroacetic acid (TCA). The mixture was shaken well, allowed to stand for 15 minutes and filtered through whatman 42 filter paper. For estimation of dehydro ascorbic acid (DHA), the tissue homogenate was made in 6 % TCA instead of 'norit' extract.

To 4 ml of the above homogenates taken in test tubes, 1 drop of 10 % thio urea (10 gm in 50 % alcohol made up to 100 ml) was added followed by 1 ml of 2,4-dinitrophenyl hydrazine reagent (2 gm in 9 N HCl, made up to 100 ml, filtered and kept in cold condition). In the blank tube, 4 ml of the 6 % TCA was substituted instead of the homogenate. In the standard, 4 ml of ascorbic acid solution in TCA was used (50 mg of ascorbic acid was dissolved in 6 % TCA and was made upto 50 ml. From this 1 ml was taken and was made upto 100 ml with 4 % TCA. The concentration of this solution was 10 µg ascorbic acid/ml of TCA. The test tubes were kept
in a boiling water bath for 15 minutes. After the incubation time was over they were transferred to an ice-bath and 5 μl of 85% H₂SO₄ was added drop wise to each test tube. They were allowed to stand for 30 minutes. The readings were taken at 540 nm in a Spectronic 20 Bausch and Lomb Colorimeter.

The concentration of ascorbic acid was calculated by the formula:

\[
\text{D.O. of unknown} \times \frac{\text{D.O. of Std.}}{\text{Dilution}} \times \frac{\text{Aliquot volume}}{\text{weight of tissue in mg}}
\]

The concentration was expressed as mg ascorbic acid/g fresh tissue weight.

The calculation as well as units for DHA were same as for TAA. The concentrations of RAA was obtained by subtracting the value of DHA from that of TAA.

(6) Glutathione:

A modification of the nitroprusside method of Grünert and Phillips (1951) was adopted for the determination of glutathione. The most important modification introduced by the above authors was the use of cyanide to obtain maximum stability of the coloured complex.

The epididymides and vas deferens of known weights were homogenized in 3 ml of 3% metaphosphoric acid and 1 ml
of distilled water. This 4 ml mixture was saturated with sodium chloride and centrifuged. From the mixture, 2 ml aliquots were used for the analysis.

To test tubes containing 6 ml of saturated sodium chloride, the above 2 ml aliquot was added. After equilibrium at 20°C for 5-10 minutes, 1 ml of sodium nitroprusside solution (0.067 M, 20 mg/ml) was added. This solution was stable when stored in a brown coloured bottle away from direct sunlight. Then immediately 1 ml of the sodium-carbonate - sodium cyanide mixture was added (1.5 M sodium carbonate and 0.067 M sodium cyanide). The intensity of the resulting colour was measured in a Spectronic 20 Bausch and Lomb colorimeter at a wavelength of 520 nm within a minute. Two ml of 2% metaphosphoric acid saturated with sodium chloride was used for the reagent blank.

The level of glutathione was calculated using the formula:

\[ X = 272.01 Y - 2.32 \]

where, \( X \) = Concentration of glutathione in \( \mu g \)
\( Y \) = O.D. of the unknown sample.

Glutathione levels were calculated by substituting the value of \( X \) in the formula.

\[
\frac{\text{Concentration of the sample}}{\text{weight of the tissue in mg}} \times \frac{\text{Dilution}}{\text{Aliquot volume}} \times 100
\]

and was expressed as \( \mu g/100 \text{ mg fresh tissue weight} \).
(7) Cholesterol:

The estimation of cholesterol was carried out by following the method of Pearson et al. (1953). To the test tubes containing 5 ml of colouring reagent mixture (4 gm of P-toluene sulphonic acid in 100 ml mixture of glacial acetic acid : acetic anhydride; 40 : 60), 0.2 ml of tissue homogenate prepared in 1 ml of glacial acetic acid was added. In the blank tube, the tissue homogenate was substituted by 0.2 ml of glacial acetic acid. The standard tube had 0.1 ml of cholesterol solution (100 μg/0.1 ml) and 0.1 ml of glacial acetic acid together with 5 ml of colouring reagent. The contents were mixed and 1 ml of concentrated sulphuric acid was added to each tube and the colour was allowed to develop. The O.D. was read at 620 nm within 7 to 12 minutes after adding H₂SO₄. The cholesterol content was calculated as follows:

\[
\frac{(O.D. \text{ of sample} - O.D. \text{ of blank})}{(O.D. \text{ of standard} - O.D. \text{ of blank})} \times \frac{\text{conc. of standard (100 μg)}}{1000 \times \text{weight of the tissue in mg}} \\
\times 5 \times 100
\]

where, Concentration of standard = 100 μg

Dilution factor = 5

Concentration of cholesterol was expressed as μg

cholesterol/100 μg fresh tissue weight.
The biochemical estimation of ascorbic acid was carried out according to the method of Chinoy, J.J. et al. (1976). A known amount of the tissue was taken and the homogenate was prepared in 16 ml of ice-cold carbon-dioxide saturated, deionized glass distilled water. The following estimations were carried out in caput and cauda epididymides and VDP and VDD separately by dividing the homogenate into four equal parts.

(a) Free ascorbic acid (AA):

One part of the homogenate (4 ml) was mixed with an equal volume of buffered metaphosphoric acid (2 parts of 3% H3PO4 : 1 part of 10.55 gm of citric acid in 1 N NaOH) at pH 3.6. The free ascorbic acid concentration was estimated colorimetrically at 520 nm using the dye 2,6-dichlorophenol indophenol.

(b) Bound ascorbic acid or Ascorbigen (ARG):

The second part of the homogenate (4 ml) was mixed with 15% w/v H3PO4 solution in the ratio of 2:1 and the resulting mixture was hydrolysed in a water bath at 75°C for 15 minutes to release the bound ascorbic acid. After cooling the solutions, citric acid, NaOH buffer, pH 3.6 (31.35 gm of citric acid in 3 N NaOH) was added. The ascorbic acid in the system was determined with the above mentioned
dye. The total ascorbic acid minus free ascorbic acid gave the value of ascorbigen in the homogenate.

(c) Ascorbic acid utilization (AAU):

To the remaining two parts of the homogenate (8 ml) a known amount of freshly prepared ascorbic acid solution (0.1 mg/ml) made in CO₂ saturated glass distilled water in the ratio of 1:1 was added. The mixture was incubated at 30 ± 2°C for 1 hour. Immediately after incubation, the mixture was divided into two sub parts and in one, the unutilized ascorbic acid was estimated by the method described under AA.

(d) Ascorbic acid - macromolecule (AA-MM) complexing:

After incubation of the homogenate with externally added AA, the remaining second sub part of the mixture was hydrolysed using the same procedure as mentioned earlier under 'b'. Deducting from this quantity the total amount of ascorbic acid unutilized, gave the value of ascorbic acid released from the bound state.

The level of each of the above mentioned parameters were calculated using the regression formula:

\[ x = 0.1075 - 0.1099 y \]

where, \( x \) = Concentration of ascorbic acid in mg and \( y \) = Optical density of the unknown sample.
The free ascorbic acid (AA), ascorbigen (ASG), ascorbic acid utilization (AAU) and ascorbic acid macromolecule (AA-M) complexing in the tissue were expressed as mg/gm fresh tissue weight.

(9) Ascorbic acid free radical (AA-FF) forming special peroxidase (E.C. 1.11.1.7):

The ascorbic acid free radical (AA-FF) forming special peroxidase which is an isoenzyme of peroxidase, was assayed by the method of Chinoy, H.J. (1973), which is based on the induced reduction reaction of Gurevich (1963).

The free radical of ascorbic acid, monodehydroascorbic acid (MDHA) produced as a result of special peroxidase activity is a much more powerful reducing agent than AA. The special peroxidase catalyses the formation of the free radical of ascorbic acid in the presence of hydrogen peroxide and ascorbic acid which is monovalently oxidised. MDHA reduces O-dinitrobenzene (OHB) to a yellow compound O-nitrophenyl-hydroxylamine which reacts quantitatively with ammonia to give a violet compound.

A known amount of tissue was homogenized in 10 ml of ice-cold distilled water. To each 2 ml aliquot of the homogenate, the following solutions were added in sequence: 1 ml of 1 M sodium nitrate was added inorder to inhibit the catalase activity, followed by 1 ml of saturated aqueous
solution of p-dinitrobenzene, 1 ml of freshly prepared ascorbic acid (5 mg/ml), and 0.2 ml of 20 volume hydrogen peroxide. In the blank, hydrogen peroxide was substituted by 0.2 ml of distilled water. The solutions were incubated at 37°C for 20 minutes. The optical density of the resulting yellow colour was recorded at 450 μm in a Spectronic 20 Bausch and Lomb colorimeter. The enzyme activity was calculated by the formula:

\[
\frac{O.D. \text{ of the sample} \times \text{Dilution}(1,0)}{\text{Fresh tissue weight in mg \times 2}} \times 1000
\]

Special peroxidase activity/20 minutes/gm fresh tissue weight.

C(1) Histological study:

Histological study was carried out by using the standard technique of Haematoxylin and Eosin staining. The tissues of normal and experimental animals (testis, epididymides, seminal vesicles and vas proximal and distal regions (VDP and VDD) were fixed immediately in alcoholic Bouin's fluid after autopsy of the animals. After 16 hours of fixation, the tissues were transferred to 70 % alcohol, and a pinch of lithium carbonate was added to remove the yellowness. Tissues were washed thoroughly in 70 % alcohol and dehydrated through ascending grades of alcohol, embedded in paraffin and sections of 5-7 μ in thickness were cut. These sections were stained in haematoxylin and eosin and
The histocytometric measurements were done on the stained slices using an 'ocular' eye-piece and a micrometer scale.

C(ii) Histochemical localization of dehydrogenases:

The histochemical localization of 3β and 17β hydroxysteroid dehydrogenases (HSDs) was carried out by the method of Tao and Lofts (1977) using Nitro-BT as an electron acceptor.

Fresh frozen sections of testis mounted on slides were used after semi-drying them. Testosterone and pregnenolone (General Biochemicals, U.S.A.) were used for detection of 3β and 17β steroid dehydrogenases. The media consisted of 4.7 ml of phosphate buffer (0.2 M, pH 7.5), 0.8 ml of [%] (6 mg/ml) in distilled water, 2 ml of Nitro BT (1 mg/ml in dimethyl formamide). The sections were incubated at 37°C for 3 hours. Controls were run concurrently using the same medium as above, but without steroids. After incubation, the sections were rinsed in distilled water, fixed in 10% formalin and washed in distilled water and mounted in glycerine jelly.

D Scanning Electron Microscopy:

The methods of Chinoy and Sanjeevan (1980a) and Chinoy and Chinoy (1981) were used for scanning electron
microscopic observations of rat spermatozoa under normal and physiologically altered conditions.

Spermatozoa from cauda epididymis and vas deferens of normal and different treated rats were used for scanning electron microscopy. The cauda epididymis and vas deferens from each side of the animal was teased gently in 1 ml of physiological saline, in order to flush out the spermatozoa.

The suspension devoid of tissue components were centrifuged at 1,000 r.p.m. for 15 minutes and the supernatants were discarded. The sperm pellets were fixed in 3% glutaraldehyde in 0.1 M Sorensen's phosphate buffer (pH 7.2) at 4°C for 1-5 hours. Post-fixation washings were done two to three times with phosphate buffer followed by distilled water. The samples were dehydrated in ethanol series and were subsequently air-dried. The samples were coated with gold (100 - 150 Å) in an A&1 vacuum coating unit and scanned in a Cambridge Stereoscan Microscope (84 - 10) at an accelerating voltage of 5-10 KV.

E. Recordings of isolated vas deferens:

The recordings of the contractile pattern of the isolated vas deferens in toto from adult rats, under normal as well as altered physiological conditions was carried out using a thermostatic double organ bath of 25 ml capacity, maintained at 34 ± 3°C for 30 minutes, according to the method of Chinoy and Chinoy (1979; 1983 b). Freshly prepared Kreb's -
Finger - Bicarbonate (KRB) solution contained glucose as the substrate: (NaCl, 6.92 g; KCl, 0.35 g; MgCl₂, 0.30 g; CaCl₂, 0.30 g; KH₂PO₄, 0.16 g; NaHCO₃, 2.10 g; Glucose, 2.10 g) (dissolved in 1 litre of glass distilled water).

The bath contained Kreb's solution bubbled with 95% O₂ and 5% CO₂. The animal was decapitated, the freshly excised vas deferens tissue was immersed in a petri-dish. The two ends of the vas were tied with silk threads so as to prevent the contents from flowing out of its lumen. One end of the segment (Proximal vas) was anchored to the oxygen tube and that of the distal vas end was tied to the frontal writing lever, which was adjusted at 500 mg tension for rat vas deferens. The tissue was allowed to stabilize for about 30 minutes in the glass tissue chamber filled with 25 ml KRB-glucose solution. This medium was bubbled with gas-mixture mentioned above and was maintained at a constant temperature and pH of 37°C and 7.4 respectively. Due to the orientation of the preparations in the experimental set up, their contractile activity reflected preferentially that of their longitudinal muscle fibres. After 30 minutes of stabilization, the spontaneous contractility if any, was recorded first for 2-5 minutes and thereafter, the response to 1-10, 10-100 and 100-500 μg doses of adrenaline tartarate (1:1,000 w/v adrenaline; Seth Pharmaceuticals Pvt Ltd., Calcutta) was recorded for one minute each. A rest period of 1 minute was given after each fresh wash of KRB-solution in between each stimulation.
The mean amplitude (in mm) of the individual response to each dose of adrenaline in normal and experimental conditions were calculated, for the left and right vas deferens, and a graph was plotted with doses on the X-axis and mean amplitude obtained for different doses on the Y-axis.

E(1) **Testosterone estimation by Radioimmunoassay (RIA) method:**

Serum testosterone levels were determined according to the method given in the Method Manual of WHO Special Programme of Research, Development and Research Training in Human Reproduction (Suli et al. 1984). The assay is a conventional RIA method that utilizes dextran-charcoal for separation of free from antibody bound hormone according to the method of Castro et al. (1974).

**Collection and storage of samples:**

Serum was used for the assay of testosterone. Blood of control and treated rats were collected by cardiac puncture under light ether anaesthesia. The samples were left at room temperature for 2 hours and stored overnight at 4°C. The serum was separated by centrifugation at 1000-2000 r.p.m. for 20 to 30 minutes and was preserved at -18°C to -20°C until the sample analysis was done. Before aliquots were taken for analysis, the samples were completely thawed and carefully mixed.
Testosterone antiserum: The antiserum raised in sheep against testosterone-3 carboxyoxime derivative coupled with bovine serum albumin (BSA) and supplied by WHO was used.

Testosterone standard: Provided by WHO as a solution at a concentration of 25.6 n mol/L in assay buffer, was used. Since the standard should not be stored frozen it was stored at 4°C until the assay was done.

Testosterone tracer (Labelled testosterone): *1, 2, 6, 7* 3H-testosterone of specific activity of 9.25 M Bq (250 uCi) was provided by WHO in sealed tubes.

Charcoal reagent: Provided in sealed bags and stored at room temperature was used for the assay.

Gelatin reagent: Provided in bags and stored at room temperature until required.

Dextran-IEaagent: Provided in bottles, and stored at room temperature until required.

Other reagents needed for the assay were:

1) Glass distilled water or deionized water.
2) Buffer S.
3) Scintillation cocktail.
4) Diethyl ether.
Preparation of Steroid Hormone Assay Buffer (E):

1) 2.35 g Sodium dihydrogen phosphate (anhydrous; \( \text{NaH}_2\text{PO}_4 \)) (M.W 120).
2) 11.6 g Disodium hydrogen phosphate (Anhydrous; \( \text{Na}_2\text{HPO}_4 \)) (M.W 142).
3) 8.8 g sodium chloride (NaCl).
4) 0.1 g Thiomersal (Mercaptoal).
5) 1.0 g Gelatin (Provided by Matched Reagent Programme).

* If the hydrated forms are used, then the amounts taken must be increased in proportion to the degree of hydration.

Dissolved the salts in distilled water to 1 litre. The gelatin was dissolved in a small volume of warm water, before being added to other reagents. The pH of the buffer should be between 7.2 and 7.4.

The buffer thus prepared was stored at 4°C and was used as a diluent for all reagents in the steroid assays.

Charcoal suspension:

Dissolved 0.0625 g of dextran in 100 ml assay buffer (5) in a stoppered container, and then 0.625 g of charcoal was added to it and shook vigorously for 30-60 sec. This charcoal suspension was stored at 40°C.

Diethyl ether was used for the extraction of the steroid hormone. It was purified using alumina column.
Assay Procedure:

Reagents were assed to the tubes as follows:

Total Count Tubes: (2 tubes) 100 µl of the working dilution of $^3$H-testosterone and 800 µl of buffer were added to the total count tubes. These tubes did not have charcoal reagent. At the time of counting, the contents of these two tubes were added directly to counting vials. This gave the total radioactivity added to the assay tubes.

Ether blanks: (2 tubes) 500 µl of sample from a vial containing reconstituted ether residues only; 100 µl of tracer; 100 µl of antiserum.

Non-specific binding tubes: (2 tubes) 100 µl of the working solution of $^3$H-testosterone and 600 µl of buffer were added to these tubes. The tubes were separated in the normal way at the end of the assay.

Standards and Unknowns (Samples): 500 µl of standard or serum sample was added; to this 100 µl of tracer and 100 µl of antiserum were also added.

Charcoal separation:

1. To each tube was added 200 µl of the charcoal reagent.
2. Vortex mixed the tubes and they were allowed to stand for 30-35 minutes at 4°C.
3. The tubes were centrifuged at a minimum of 500 g for 10 minutes.
4. The supernatant was decanted off carefully into scintillation vials. There should be minimum delay between centrifugation and decanting of supernatant.

To ensure that drift does not occur at the separation stage the following precautions were taken when adding charcoal.

(i) Kept all the tubes at 4°C.

(ii) The charcoal suspension was kept stirred during addition to the tubes.

(iii) Charcoal was added rapidly to the tubes, so that the time the incubation medium is in contact with the charcoal is not too different across the assay.

Counting of radioactivity: The vials were vortexed and allowed to equilibrate for at least 4 hours at room temperature before counting. The counting was done in a Packard-Tricarb Liquid Scintillation Spectrometer Model-3255 with automatic recording device.

Calculations: The levels of testosterone were calculated using standard curve plotted (% bound fraction against concentrations of standard on a semilog paper) or in an IBM computer in normal as well as in different experimental animals. The sensitivity of the assay was 5 pg per tube.
R(2) Radioimmunoassay of r FSH and r LH hormones:

The assay is a conventional RIA that utilizes liquid phase second antibody for separation of free from the bound hormone.

Collection and Storage of samples:

Blood serum was used for the assay of both FSH and LH. The animals were etherized and about 5 ml of blood was taken in clean vials by cardiac puncture. The samples were left at room temperature for nearly two hours and then kept at -4°C overnight. The samples were centrifuged at 2000-3000 r.p.m. for 20-30 minutes and the sera were transferred carefully to stoppered bottles. The samples were preserved at -20°C until the assay was done. Before aliquots were taken for analysis, samples were completely thawed and carefully mixed.

1) Radio-iodination of hormone:

The method of Greenwood et al. (1963) as modified by Midgley (1966) was used for iodination.

Reagents for iodination:

0.5M phosphate buffer, pH 7.5: It is prepared by mixing 0.5M NaH₂PO₄ with 0.5M Na₂HPO₄ to give a pH of 7.4.

0.05M phosphate buffer, pH 7.5: It was prepared by a ten fold dilution of 0.5M phosphate buffer.
Chloramine 'T': 2.5 μg/ml of chloramine 'T' in 0.05 M phosphate buffer was freshly prepared prior to use.

Sodium metabisulphate: 2.5 μg/ml of sodium metabisulphate in 0.05 M phosphate buffer was freshly prepared prior to use.

1% Gelatin - Phosphate Buffer Saline (PBS): One gm of gelatin was warmed in a small volume of PBS till it was dissolved and the volume was made up to 100 ml with PBS.

Transfer solution:

- 100 mg potassium iodide (KI, Blue)
- 1 mg of bromo-henol blue (BHBr)
- and 1.6 gm sucrose (BHBr) in 10 ml distilled water.

Preparation of Sephadex-675 Column:

Sephadex G-75 was allowed to swell overnight in PBS at room temperature. The columns were prepared by packing Sephadex into a glass column of 0.7 x 23 cm.

The column was rinsed with PBS and then filled with the same buffer to about 1/3 of its height. The sephadex slurry was poured from the top and the column was packed leaving a few centimeters blank from the top to permit the buffer to drip. The sephadex was packed to a total height of 20 cm.

The packed column was charged with 1 ml of 5% bovine serum albumin (BSA) and then washed several times with PBS.
Iodination Procedure:

1) 2.5 μg of the hormone contained in 2.5 μl of PBS were placed in a 2 ml vial.
2) 25 μl of 0.5 phosphate buffer (pH 7.5) was added to the vial and mixed.
3) 0.5 μCi of 125I was then added to the reaction vial and mixed well. The radioactivity was counted using a manual γ-ray Spectrometer (EC, India).
4) 10 μl of Chloramine T solution (2.5 mg/ml) were added to the reaction vial and the reaction was allowed to take place for 30-40 seconds by finger tapping. The reaction timing is variable for different hormones.
5) The reaction was terminated by the addition of 35 μl of sodium metabisulphate (4.5 mg/ml).
6) A few drops of transfer solution was added to the vial. The whole reaction mixture was carefully layered with the help of a Pasteur pipette over the Sephadex G-75 column to separate undamaged and damaged protein fractions from free iodide.
7) The vial was rinsed with 100 μl (3 drops) of rinse solution and this solution was transferred to the column. The residual counts in the reaction vial and Pasteur pipette were also determined.
8) The column was eluted with PBS and 1 ml fractions were collected in the tubes containing 0.5 ml of 1% gelatin-PBS. The elution was carried out until all the bromophenol
blue (which acts as a marker) was washed down.

9) The radioactivity in all the tubes and the column were counted.

Calculation of Specific activity (SA) :

\[
SA = \frac{\text{Counts incorporated into protein}}{\text{Total counts}} \times \frac{500}{2.3} = \mu \text{Ci/\mu g.}
\]

A graph was plotted using the number of eluted fraction vs radioactive counts.

Dilution of labelled hormone for experimental use :

Eluted fractions which form the descending limb of the protein peak were selected and suitably diluted with 0.1 % PBS buffer to obtain an appropriate hormone concentration of µg/100 µl.

Reagents provided by WHO :

Anti-FSH and anti-LH serum : Provided in lyophilized form. Each bottle contained 40 milli Units (mU) of the WHO International Reference Preparation of Pituitary gonadotropin for immunoassay (Code 76/549), stored at 4°C until required.

FSH and LH tracers : Provided in lyophilized forms, and stored at 4°C until needed.

Normal rabbit serum : provided in liquid form, containing 0.1 % sodium azide, stored at 4°C, or aliquotted and stored
at below -20°C until needed.

Donkey anti-rabbit serum: provided in liquid form, contained 0.1% sodium azide and stored at 4°C, or aliquotted and stored at below -20°C, until required.

Bovine serum albumin: provided in lyophilized form, stored at 4°C.

Other reagents required for the assay included:

1) Glass distilled or deionized water.
2) Buffer P

Preparation of Peptide Assay Buffer (P):

- 2.35 g sodium dihydrogen phosphate (anhydrous \( \text{NaH}_2\text{PO}_4 \)) (MW = 120)
- 11.6 g disodium hydrogen phosphate (anhydrous \( \text{Na}_2\text{HPO}_4 \)) (MW = 142)
- 2.8 g sodium chloride (NaCl)
- 0.1 g mercuric chloride (Merthiolate)
- 5.0 g bovine serum albumin (BSA)

BSA was provided by Matched Reagent Programmes.

- If the hydrated forms are used then the amounts taken must be increased in proportion to the degree of hydration. The constituents were dissolved in approximately 750 µl of distilled water and the pH was adjusted at 7.2 to 7.4. EDTA was added to a final
concentration in 1 litre of 0.025 M. (Made up to 1 litre). If necessary, adjust using concentrated NaOH or HCl.

This peptide assay buffer was used as a diluent for all reagents in peptide assay except the tracer. This buffer was stored at 4°C.

Tracer Diluent: The tracer diluent is peptide buffer (P) plus 0.5% normal rabbit serum (NRS) (e.g., 0.5 μl NRS in 100 μl buffer P). The NRS is essential for the double antibody separation stage.

Preparation of reagents:

Tracer: The contents of each vial of lyophilized tracer was dissolved with 1 ml assay buffer (P). After reconstitution this stock solution was stored at 4°C. During each assay batch 0.2 ml of this stock solution was made up to 10.5 ml with tracer diluent (i.e., buffer P, containing 0.5% NRS). The tracer should not be stored after dilution.

RFBK and MHI antiserum: Reconstituted the contents of one bottle of antiserum with 10 ml of assay buffer P. The mixture was allowed to stand for 5-10 min and then it was mixed thoroughly prior to use.

Data on 1982 Antiserum:

1. Source: Generally donated to WHO by Professor W. Butt, Birmingham, U.K. The antiserum is a different aliquot
Prepared from the same pool as that used from 1977-1981.

2. Raised in a rabbit.

3. Binding: When used as directed expected Bo 1 is 30-40 %.

4. Final dilution in assay tube: 1:2, 800,000.

5. Specificity (Cross-reactions estimated by noting relative potencies at B/Bo of 50 %.

Second Antibody Donkey anti-rabbit serum diluted to 1:40 in buffer P just prior to use.

Samples: No special sample preparation step is needed.

Standards: Before performing an assay, carefully constitute the contents of one vial of lyophilized standard with exactly one ul of assay buffer P. Re-stoppered the bottles and the mixture was allowed to stand for 5-10 minutes. The vial now contained FSH or LH standard 401 U/L (hRC I.R.P. 78/549).

This was the solution B. From this solution different concentration were taken and standard solutions were made using buffer P as diluent.

Assay procedure:

Reagents are added to the tubes as follows:

Total count tubes: Two tubes were taken for this purpose and 100 µl of working strength 125I-FSH tracer (125I-LH tracer in LH assay). Tubes containing liquid should be capped before counting.
Non-specific binding tubes: Two tubes having 100 μl of working strength 125I-FSH and 125I-LH tracer (in LH assay) and 600 μl of buffer (P). These tubes contained second antibody added and were separated in the same way as the rest of the assay.

Zero-antigen (Bo) tubes: 2 or 3 sets of tubes (two tubes each) containing 100 μl of working strength 125I-FSH and 125I-LH in LH assay, 100 μl of anti-FSH serum or anti-LH serum as the case may be and 500 μl of buffer (P).

Standards and unknown samples: The rest of the tubes contained 100 μl of working strength 125I-FSH or 125I-LH according to the assay and 100 μl of anti-FSH or anti-LH serum and 100 μl of standard or sample + 400 μl of buffer (P).

Second antibody separation:

1. The second antibody was diluted to 1:30, i.e. 330 μl to 10 μl of buffer P, as previously described and mixed thoroughly.

2. 100 μl of this was added to each tube except total count tubes.

3. The tubes were vortex mixed and incubated at 4°C overnight (18-24 hr).

4. The tubes were centrifuged for at least 45 min at a minimum of 1500 g. Failure to centrifuge at an adequate speed or for an adequate time will lead to poor
replication, particularly in the standard curve.

5. The precipitate contained the antibody bound FSH or (antibody bound LH in LH assay) was counted. The supernatant and the precipitate were separated either:
   a) By decanting, i.e. by pouring off the supernatant and drying the tubes by inversion on a pad of absorbent tissue or
   b) By careful aspiration of the supernatant by means of a water pump.

Radioactivity Counting:

The tubes were counted for one minute either by using a manual $\gamma$-ray spectrometer (at, India) or in Las wallac 1230 Ultrogamma counter.

Calculation of results:

A dose response curve was plotted. The standard curve obtained by the LH computer was counter checked by plotting it on a sheet of graph paper.

The sensitivities and inter and intra-assay variations were 8 ng and 10 and 6% respectively for the FSH assay and 8-10 and 5.6% respectively for the LH assay.

PART II

1) Studies on the effects of an aqueous extract of Vinca rosea leaves for 7 and 15 days.
2) Castration + testosterone treatment for 7 days.

Castration + testosterone + extract treatment for 7 days.

3) Extract treatment + ascorbic acid feeding for 7 and 15 days.

4) Extract treatment for 7 days + Discontinuation of treatment for one and 2g months respectively.

Normal intact nature male albino rats of proven fertility were given intra-muscular injections of aqueous extract of *Vinca rosea* leaves (*Catharanthus roseus*, Linn. belonging to the family Apocynaceae) at a dosage of 1 mg of the extract/0.2 ml/day for 7 and 15 days, so that the dosage was 5 mg/kg body weight of the animal. The experimental animals were distributed as follows:

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatment</th>
<th>No. of animals</th>
<th>Day of autopsy used</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Normal (control) rats</td>
<td>35</td>
<td>Batches of 5 sacrificed along with each group of treated rats</td>
</tr>
<tr>
<td>II</td>
<td>7 days treatment with <em>Vinca rosea</em> leaf extract</td>
<td>30</td>
<td>8th day</td>
</tr>
<tr>
<td>III</td>
<td>15 days treatment</td>
<td>30</td>
<td>10th day</td>
</tr>
<tr>
<td>IV</td>
<td>Bilaterally castrated (7 days + testosterone)</td>
<td>15</td>
<td>8th day</td>
</tr>
<tr>
<td>V</td>
<td>Bilaterally castrated (7 days + T + Vinca extract)</td>
<td>15</td>
<td>8th day</td>
</tr>
</tbody>
</table>
VI  Leaf extract + AA** feeding (7 days) 30  8th day
VII Extract + AA (15 days)  35  16th day
VIII Extract 7 days + 1 month withdrawal  35  38th day
IX  Extract 7 days + 2½ months withdrawal  40  67th to 83rd day

- Normal adult male rats were bilaterally castrated by the scrotal approach under light ether anaesthesia using sterile conditions.

** Testosterone (T) injections were given intra-muscularly at a dose of 200 μg/0.1 ml/day/rat to study the androgen replacement therapy in castrated rats and to compare the effects with castration + T + leaf extract treated animals.

Source of Testosterone (T) : Testosterone (T) was obtained from Harrington Brothers Limited, 1942, Road, Salhan, London 6 W. Known amount of testosterone was dissolved in Toluene and finally prepared in ground nut oil which was used as a vehicle.

*** Ascorbic acid (AA) was fed extraneously at a dose rate of 50/100 mg/day/rat.

Preparation of aqueous extract of Vinca rosea Leaves :

The aqueous extract of Vinca rosea leaves were prepared according to the method of Chinoy and Geetha Ranga (1983).

After the respective treatments the animals were autopsied and used for the various studies.
The following parameters were studied after the respective treatments:

(i) Body weight and organ weights were determined in all groups of animals as described in Part I "A" (1) and (2) respectively.

(ii) Sperm counts, percent motility and fertility test were performed as per the procedure given in Part I "A" (3) and (4) respectively.

(iii) Activities of succinate dehydrogenase (SDH) and acid phosphatase (ACP) were determined in all the groups as described in Part I "A" (1) and (2) respectively.

(iv) Method for total protein was similar as described under Part I "B" (3).

(v) Levels of fructose was measured as per the method given in Part I "B" (4).

(vi) Total, dehydro and reduced ascorbic acid and Glutathione (GSH): In groups I to V the method was same as given under Part I "B", (5) and (6) respectively.

(vii) Cholesterol: Method was same as described under Part I "B", (7).

(viii) Ascorbic acid: Free ascorbic acid (AA), ascorbigen (AGG), ascorbic acid utilization (AAU) and ascorbic acid macromolecule (AA-MM) complexing were assayed by the method detailed under Part I "B", (8).
(ix) Ascorbic acid free radical forming special peroxidase activity (AA-FR activity): The activity was assayed by the method of Chinoy (1973) as mentioned in Part I B, (9).

(x) Histological and Histocytometric studies: were carried out by the method described under Part I C, (1).

(xi) Scanning Electron Microscopic (SEM) study of the spermatocytes from cauda epididymis was carried out as described under Part I D.

(xii) RIA of Testosterone: Levels of testosterone in blood serum of control and treated animals were determined by radioimmunoassay according to the method of WHO, Method Manual (1982), as described under Part I E, (1).

(xiii) RIA of FSH and LH: Circulating levels of Follicular Stimulating Hormone (FSH) and Luteinizing (LH) were carried out according to the method of WHO (1982) described under Part I E, (2).

For all the analyses, a minimum of six replicates were done for each tissue and parameter. The results were analysed statistically by using the 'Student's 't' test and the level of significance was also determined by the method of distribution of probability of Fisher and Yates.