MATERIALS AND METHODS
A. Human specimen.
B. Animal specimen.

A. Human specimen:

I. Selection of cases:

A total number of 120 patients were investigated for this study. Adequate clinical history was recorded in each case with careful examination which included the following information of the patients:

a) Age and civil status.

b) Parity, date of first and last pregnancies (including report of, if there had been any, recent pregnancy with information on lactation).

c) Menstrual history including menarche, cycle time, amount of bleeding and date of last menstrual period.

d) Presenting symptoms - special care was taken to record any suspicious symptoms suggesting hormonal deviation or other pathological conditions.

e) Gynaecological examination for clinical
f) Previous or current treatment specially hormonal therapy, radiotherapy and gynaecological surgery.

g) Cytological and histopathological report.

The cases were classified in different groups:

1) Pre-menopausal normal - These comprised of normal fertile women within the age group of 20 to 35 years with regular menstrual cycles. Care was taken to select normal cases without any pathology in cervix. Vaginal smear examinations were done to confirm normality of cervix.

ii) Post-menopausal normal - These cases were selected after cessation of menstruation for at least 2 years. Vaginal smear examinations were done to diagnose typical post-menopausal condition.

iii) Pre-menopausal cancer cervix - Carcinoma of the cervix uteri with clinical diagnosis of stage II and III occurring in pre-menopausal condition were selected in these groups. Concomitant histopathological examinations of punch biopsy of cervix were done. These examinations revealing epidermoid carcinoma of grade III were taken up for study.
iv) Post-menopausal cancer cervix - The diagnosis of post-menopausal cancer cervix was carefully made with respect to the age of the patient and definite periods of menopause before the onset of the disease. Histopathological examination of biopsy material confirmed the diagnosis of epidermoid carcinoma.

II. Collection of specimen:

Biopsy specimens of normal subjects, selected as described, from out-patients clinic of Chittaranjan Seva Sadan were obtained. Also operated specimens of similar subjects undergoing hysterectomy operation for gynaecological pathology other than in cervix, were collected. Specimens of cancer cervix were obtained from either biopsy material of patients attending out-patients clinic of Chittaranjan Cancer Hospital or operated material from patients undergoing radical operation for cancer. Immediately after collection, tissue pieces were transferred to a beaker placed in ice bucket to prevent decay of enzyme activity. Adequate care was taken to reject necrotic portion, blood clot etc. in case of malignant tissue collection.

A tissue piece after collection, was washed with cold distilled water and blotted with a blotting paper.
The tissue was weighed and homogenised in 0.85% cold saline with glass homogenizer. Required volume of homogenate was made with cold 0.85% NaCl. Homogenate was centrifuged in a refrigerated centrifuge at 1500 RPM at 4°C for 20 minutes. The supernatant was assayed for alkaline and acid phosphatase activities.

B. Animal specimen:

I. Biological materials:

All animal experiments have been performed on inbred Swiss female mice belonging to the age group of 3-4 weeks maintained in the animal colony of Chittaranjan National Cancer Research Centre, Calcutta.

Tumours:

Two types of transplanted tumours were selected for the present study—Ehrlich's carcinoma and Sarcoma 180, in the ascitic form, where the tumour cells are grown as uniform cell suspension in the abdominal cavity of the host.

Ehrlich's carcinoma:

This tumour was received from the Institute of pathology and Institute for Cell Research and Genetics, Karolinska Institute, Stockholm, Sweden, through the
courtesy of Dr. G. Klein and has been maintained since then in the Deptt. of Tumour Biology, Chittaranjan National Cancer Research Centre by serial transplantation in inbred Swiss mice. This tumour probably arose spontaneously in the mammary gland of a white mouse and was located by Ehrlich in 1907. The tumour grew rapidly in the solid form subcutaneously and after about 14 days of tumour development 1/3rd of the tumour became necrotic and haemorrhagic with no occurrence of metastasis. In 1932, Lowenthal and John successfully produced the ascitic form of this Ehrlich's carcinoma by intraperitoneal injection of tumour cell suspension.

The ascites develops as a milky fluid in the abdominal cavity of mouse containing usually rounded tumour cells of different sizes within 6-7 days after intraperitoneal injection of approximately 1 million cancer cells/ml.

**Sarcoma 180 ascites tumours:**

The tumour was obtained from the school of Tropical Medicine, Calcutta, through the courtesy of the Director and has been maintained by serial transplantation in inbred Swiss mice.

This tumour was originally developed from the Crocker
mouse sarcoma 180 by Charlotte Friend in 1951 by intraperitoneal injection of minced tumours into Swiss albino mice. Crocker Sarcoma 180 was discovered in the laboratory of Woglon (1914) at the Crocker laboratory in New York city (Now the Institute of Cancer Research, Columbia University) in the right axilla of a white male mouse and was identified as mammary carcinoma. During serial subcutaneous transplantation there was sarcoma-tous change of tumour connective tissue which subsequently became a sarcoma, sometime in 1919. Crocker Sarcoma 180 was solid tumour with almost no haemorrhagic or necrotic areas, growing rapidly, and metastasis occurring in the lung and subcutaneous areas adjacent to the tumour implant. The host lives upto 4 weeks or sometimes longer. Sarcoma 180 ascites tumour develops as a milky fluid within 3-5 days of transplantation and 10 days after intraperitoneal injection of about 1 million cancer cells the fluid contain about 75-150 million tumour cells/ml.

II. Oophorectomy of the female mice:

Mice belonging to the age group of 3 to 4 weeks were selected for this operation.

The animal was first anaesthetized with ether and then a mid line longitudinal incision was made extending
from the level of the urinary bladder to the level of the lower poles of the kidneys. The skin was retracted laterally towards either side and the ovary traced along the uterus. The upper horn of the uterus was ligated with silk thread. The ovary together with its surrounding fat and a small portion of the uterus excised. The procedure was followed on the other side also. Finally, the incision was stitched with silk thread. Asepsis was strictly maintained throughout the operation. Four weeks after oophorectomy the animals were grouped in three groups. In one transplantation of Ehrlich's carcinoma were done. In the second group, Sarcoma 180 were transplanted while the third group were kept as such for comparative study.

III. Tumour transplantation:

Ehrlich's carcinoma (ascites) and Sarcoma 180 (ascites) were maintained by serial transplantation from male to male and female to female Swiss mice. Strict asepsis was maintained throughout the transplantation procedure. The ascitic fluid was drawn out from donor mice carrying the tumour for 7-9 days. The freshly drawn ascitic fluid was washed in 0.21 phosphate buffer three times. After the last centrifugation, the cell pellet
was diluted in 0.2M phosphate buffer (pH 7.6). The cells were counted in a counting chamber with trypan blue to detect the viable cells and dilutions with phosphate buffer readjusted to 2 x 10^7 cells/ml. Aliquots of 0.2 to 0.5 ml of the above cell suspension were inoculated intraperitoneally in the oophorectomized mice.

Composition of 0.2M Phosphate buffer

(Lehmanus and Nowory 1964).

0.2M Na_2HPO_4, 2H_2O Solution - 86 ml
0.2M NaH_2PO_4, 2H_2O Solution - 14 ml
Total 100 ml

IV. Hormone Administration

All the three groups were sub-divided into groups of three. Two groups were taken for hormone administration while the third group were injected with the hormone solvent and served as control. The experimental design with respect to grouping of mice, oophorectomy, transplantation and hormone administration has been indicated in the Chart (page 46).

The female sex hormones injected were oestrogen and progesterone. Oestrogen (ovocyclin, Ciba) and progesterone (Luteocyclin, Ciba) were injected in a dose of 10 \( \mu g/3 \) body weight. Twenty four hours after injection, the mice were sacrificed. The control group receiving the
hormone solvent (sesame oil) were also sacrificed after twentyfour hours of injection.

ANIMAL EXPERIMENTAL DESIGN

Mice

Non-Oophorectomized (Control-without hormone injection)

Oophorectomized

Control Hormone treated

Normal Tumour bearing
1) Ehrlich's Carcinoma
2) Sarcoma 180

Normal Tumour bearing: Carcinoma
1) Ehrlich's Carcinoma
2) Sarcoma 180

Progesterone treated

Normal Tumour bearing
1) Ehrlich's Carcinoma
2) Sarcoma 180
V. Collection of materials:

Animals were sacrificed and different organs including uterus, kidney, lung, brain, liver and about 2.5 cm. of small intestine were dissected out. The specimens were washed with ice cold distilled water, blotted, weighed and preserved in the deep-freeze for biochemical analysis.

For the tumour cells, the ascitic fluid was first drawn out in a pre-weighed centrifuge tube. It was then centrifuged in a refrigerated centrifuge and the supernatant discarded. Weight of the packed cell was taken and stored in deep-freeze for enzyme assay. All these organs and tumour cells were homogenised in 0.85% NaCl in a same way as described in case of human tissues and supernatant of centrifuged homogenate was taken for assay of alkaline and acid phosphatase activities.

Assay of alkaline phosphatase activity:

Reagents:

- All the reagents used in this experiment were of analar quality. Distilled water used was double glass distilled.

1) 0.85% NaCl in distilled water.
2) Substrate solutions for Alkaline phosphatase (pH 8-10):

I. Substrate:
   a) Sodium-β-glycerophsphate 0.016M
   b) Sodium-α-glycerophosphate 0.016M
   c) Sodium-β-naphthyl phosphate 0.016M
   d) Sodium-α-naphthyl phosphate 0.016M
   e) Sodium Phenyl phosphate 0.016M
   f) Sodium diethyl barbiturate 0.016M

II. Petroleum ether about 3 ml.

3) 30% Trichloroacetic acid.

4) Aminonaphthol sulphonie acid.
   a) 15% Sodium bisulphite - 97.5 cc.
   b) 1-2-4 aminonaphthol sulphonie acid - 0.25 g
   c) 20% Sodium sulphite - 2.5 cc.

   The solution was preserved in brown glass bottle and stored in refrigerator.

5) Molybdate solution.
   a) Ammonium molybdate - 25 g
   b) 10N Sulphuric acid - 500 ml
   c) Distilled water - 500 ml
6) **Standard phosphate solution:**

   a) Monopotassium phosphate - 0.351 g
   b) 10N Sulphuric acid - 10 ml
   c) Distilled water - 990 ml

7) 5% T.C.A. solution

**Standard curve for inorganic phosphate and determination of K values:**

Different aliquots of stock solution with known quantity of inorganic phosphate was taken in different graduated 10 ml tubes. Volume was made up to 5 ml. 1 ml of molybdate solution and 0.4 ml of aminonaphthol sulfonic acid was added to it. Volume was made up to 10 ml with water. It was mixed well and made to stand for 5 minutes for the development of colour. The optical density was measured in Hilger Spectrophotometer at 660 m\(\mu\) wave length. From the spectral data, a plot of absorbency at 660 m\(\mu\) vs. concentration of inorganic phosphate yielded a straight line of gradient K. The value of K calculated from observed results were found to be equal to 12.

**Experimental Assay of Alkaline Phosphatase:**

**I. Incubation mixture:**

   Substrate solution (prewarmed at 37°C for few min.) - 4.5 ml
Supernatant (from tissue homogenate) - 0.5 ml.

II. Incubation at 37°C for one hour.

III. Enzyme activity was stopped with cold 1 ml 30% T.C.A.

Enzyme control - 1 ml 30% T.C.A. was added to the incubation mixture prior to incubation and then incubated for one hour to test the specificity of enzyme action.

IV. Both experimental and control samples were centrifuged at 1500 RPM for 15 minutes at room temperature. 4 ml of supernatant was collected in a test tube for color reaction.

V. To each test tube 0.5 ml of molybdate solution and 0.2 ml of aminonaphthol sulphonic acid was added. The volume was made up to 5 ml with distilled water.

Reagent blank - 4 ml 5% T.C.A. was taken in a test tube 0.5 ml molybdate solution and 0.2 ml aminonaphthol sulphonic acid was added.
to it. Volume was made up to 5 ml with distilled water.

VI. All test tubes were allowed to stand for 5 minutes for colour reactions. Absorbance of colour density was measured at 660 m\(\mu\) in Hilger Spectrophotometer using the reagent blank to set the spectro for 100% transmittance. The different tubes containing different substrates were treated in a similar manner. The differential affinity of alkaline phosphatase for different substrates were determined.

Assay of acid phosphatase:

Substrate solutions (pH 5.0) for acid phosphatase.

The procedure was the same as alkaline phosphatase. Only acid pH of the incubation mixture was adjusted with 1N acetic acid.