CHAPTER III

MATERIALS AND METHODS
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Adult guinea-pigs (*Cavia porcellus*) were selected as experimental animal because of their rare susceptibility to spontaneous cancer and for their inability to synthesize vitamin C. Guinea-pigs and primates are the best animals for experimentation with vitamin C, because these animals lack the enzymatic mechanism for the preparation of vitamin C in vivo. The guinea-pigs were procured from a local farm and were reared in the departmental animal house for a period of three months on a standard laboratory diet (Hawk, 1976) before they were subjected to experimental procedure. Only the male guinea-pigs were selected for the experimental purposes.

1. History & Taxonomy of the experimental subject

Guinea-pigs are extensively used in biological and medical experimentation and the earliest record concerning the biology of the guinea-pig is that of Goldsmith (1791). Lavoisier in 1780 (Lane-Petter, 1963) is commonly credited with being one of the first to use the guinea-pig in research, having used it to measure heat production. Weir (1974) reviewed the origin of the domestic guinea-pig and its interrelationships with other species of cavies.

The guinea-pig was first described scientifically by Aldrovandus and Gesner before 1607 (Wagner, 1976). Pennant (Wagner, 1976) reported 'that the coining of the term "Guinea-pig" which called this animal the "Restless cavy" is however, inaccurate, for cavies, when there is no exciting cause, such as fear or hunger, are very quiet animals, and will lie side
PLATE No. 1: The experimental animal Cavia porcellus.
by side for hours, until one or other of these disturbing causes rouses them to activity.

The term cavy, probably derives from its generic name, cavia. The origin of the name guinea-pig is indeed obscure. Most European nationalities perceived the animal as a small pig that arrived from across the sea and adopted fitting names: Meerschweinchem (little sea pig); cochon de mer (sea pig); lapin de Barbarie (Barbary rabbit). Paterson (1972) reported that the name guinea- might have come from "Guiana" or more simply that in another era may have meant "foreign". Sire (1968) suggested that porcellus may have resulted from comparing the animals' nails to small wooden shoes (Petits sabots). However, porcellus is the Latin name for "little pig". The systematic position of *Cavia porcellus* Linn. (Simpson, 1945) has been outlined below:

**Phylum** - Chordata - Animals with notochord and gills.

**Subphylum** - Craniata - Chordates with organized head region.

**Class** - Mammalia - Warm blooded craniates with hair coat and young nourished from mammary glands.

**Subclass** - Theria - Viviparous mammals.

**Infraclass** - Eutheria - Placental mammals.

**Order** - Rodentia - Small herbivorous rodents with single pair of incisors in the upper and lower jaws.

**Family** - Caviidae - More or less tailless rodents that have one pair of mammae, four digis on the forefoot and three on the hindfoot.

**Genus** - *Cavia*

**Species** - *Cavia porcellus*. 
2. Rearing of the laboratory animals

Before the onset of the experiment, guinea-pigs were reared in a wooden wire netted cage (10 feet x 6 feet) for a period of three months in room temperature. In addition to the standard diet they were supplemented with gram and green grass. The cage was cleaned every day and disinfectant was applied once a week. To start with the experiment, iron cages (1.5ft. x 1.5ft.) were cleaned properly and one animal was kept in each cage. A time gap of three months was thought to be sufficient to standardize the animals to the new environmental conditions. After the standardization of the animals, the experiment was started in the same laboratory conditions. These animals were treated with the carcinogen and the induction of cancer was studied in details. In another set of experiment, vitamin C was applied after the induction of carcinogenesis and the revival effects were studied following the same procedure as in the case of induction of experimental carcinogenesis.

3. Induction of cancerous growth and subsequent treatment with vitamin C

Three sets of male, healthy guinea-pig each consisting of 24 numbers having three replications and 4 experimental set ups totalling 288 in number were selected for the experiment. The first set was fed with standard laboratory diet (control set). The second set was treated with the hepatocarcinogen-azocarmine (Chroma, gesellschaft, West Germany) with a dose of 5 mg per day for 56 days by intramuscular injection. Azocarmine is an acid and its carcinogenic nature was proved by Yoshida (1935).
These animals were dissected at an interval of seven days regularly and the liver was examined carefully for the occurrence of induced lesion in all the treated sets. When a whitish raised lesion of one to two millimeter in diameter was observed it was taken as the biological end point of carcinogenesis to evaluate the induction effects. These lesions were recognised as premalignant followed by development of tumours with metastasis. These lesions and tumours were also diagnosed histologically showing cellular transformation and carcinogenic nodules with the continuations of treatment beyond seven days. Carcinogenesis was induced in the third set of experimental guinea-pigs in a similar way and after the induction of cancer, i.e., from seven days onwards, the guinea-pigs were fed with 75 mg of ascorbic acid (I.D.P.L., India) tablet. Ascorbic acid was fed orally for the experimental period after the induction of cancer. Ascorbic acid plays an important role in regulating optimum oxidation-reduction potential and also maintains the structural integrity of cells and tissues. The chemical structure of azocarmine and ascorbic acid is given below.

![Chemical structures of azocarmine and L-ascorbic acid](image-url)
Guinea-pigs were weighed in a spring balance, anesthetized with chloroform and was dissected to study the liver. The weight of the liver was taken in a chemical balance and the structure of the liver including the lesion or tumour formation was studied. A SLR Pentax Camera was used to take the photographs of the liver.

4. Methods of study

Attention was focussed on the carcinogenic changes of the liver and the blood picture of the different sets of guinea-pigs and their subsequent revival. The control set was maintained without any carcinogen treatment so that it could be compared with the carcinogen treated set and with the vitamin treated cancerous animals of the third set. The study was aimed at exploring the induction effect of the azo-dyes as well as its revival to the normal state after the application of vitamin C. The method of study employed mainly concerned with the induction and cytogenesis of cancer and its possible revival with the application of vitamin C at morphological, histological, cytochemical, haematological and at biochemical levels.

(i) Morphological studies

The induction of cancerous growth in guinea-pig shows a number of morphological features of the animal body as well as in the organ system. Animal, suffering from malignant growth registers a decrease in their body weight. These experimental animals were weighed in a spring balance and the weight of the animal was recorded daily. The weight of the liver in comparison
to the body weight was studied in addition to the structure of the liver and its lesion and tumour formation. The mortality rate of the animal has been found out in the three sets of the experiment.

(ii) Histological studies

The induction of cancerous growth and their subsequent revival was studied in the liver cells of guinea-pig by routine histological method. The histology of the hepatic cells affected by carcinogens producing lesions and tumours had a marked difference in the structure of the cell, its size, shape and their adhesiveness. To make a histological study the effected parts of the liver were fixed in carnoy. Sections of 6-8 µ thick were prepared following routine histological procedure. These carnoy fixed liver sections were stained with haematoxylin and eosin. Sections were dewaxed liver in xylol and degraded to water and then stained in haematoxylin (aqueous) for 30 seconds. Then it was passed through tapwater and upgraded alcohols. The slide was stained in eosin for 5 minutes and after complete dehydration it was cleared in xylol and mounted in DPX. The histology of the liver including the nuclear and cytoplasmic changes were noted. Photomicrographs were taken with the help of an Olympus (Japan) microphotographic apparatus.

(iii) Cytochemical studies

Carcinogenic changes in the liver were studied by important cytochemical technique for evaluation of the changes produced in
the cytochemical constituents of the cell, specifically in the level of deoxyribonucleic acid (DNA), ribonucleic acid (RNA), glycogen particles and ascorbic acid. The DNA, RNA, glycogen and ascorbic acid content of the three experimental sets of guinea-pig were studied and a comparative study of the three sets was made.

(a) Detection of DNA

DNA was stained with feulgen fuchsin (Gurr, 1958) stain. Feulgen fuchsin was prepared as follows. 100 ml of water was boiled and then allowed to cool to about 70°C or less before the addition of 0.5 mg of basic fuchsin and dissolving by stirring. Allowed to cool upto 25°C, then added 0.5 gm of potassium metabisulphite and 10 ml. of N/1 hydrochloric acid. The neck of the flask was plugged with cotton wool and allowed to stand for several hours, then added 1 gm of decolorizing charcoal. It was then shaked well and allowed to stand for half an hour before filtering. The finished solution was straw colored and was kept in a well closed bottle free from dust in a cool place away from light. Solution B was prepared by dissolving 0.5 mg of potassium metabisulphite and 5 ml of N/1 hydrochloric acid in 90 ml of distilled water. Carnoy fixed sections were degraded to water after dewaxing in xylol. Rinsed in cold N/1 hydrochloric acid and hydrolysed in N/1 HCl at 60°C for 5 minutes. Rinsed in N/1 HCl and distilled water. Immersed in feulgen fuchsin stain for 45 minutes and then rinsed in solution B. Counterstained in fast green for
30 seconds. Dehydrated in upgraded alcohols, cleared in xylol and mounted in DPX. DNA was seen in shades of reddish purple against a blue green background.

(b) **Differentiation of DNA and RNA**

Brachet's (1957) method for distinguishing between the two classes of nucleic acids was followed to differentiate between the DNA and RNA. Methyl green pyronin stain was prepared by dissolving 15 gm of chloroform washed and dried methyl green, 0.25 gm. of pyronin, 2.5 ml of 95% alcohol, in 97.5 ml of acetate buffer (PH 4.7). Carnoy fixed sections were dewaxed with toluene and degraded to water. Stained in methyl green pyronin for 20 minutes. Washed in distilled water and differentiated in 95% alcohol for 5-10 minutes. Dehydrated in absolute alcohol, cleared in toluene and mounted in DPX. RNA appeared as red particles in the nucleolus and in cytoplasm, while DNA as blue particles in nuclei.

(c) **Detection of glycogen**

Glycogen was stained by Bauer-Feulgen method (Gurr, 1958). Liver sections were dewaxed in xylol and degraded to distilled water. Immersed in 4% chromium trioxide solution for one hour. Washed in running tap water for 7 minutes. Stained in Feulgen fuchsin solution for 10-20 minutes. Rinsed in potassium metabisulphite solution and then washed in running water for 10 minutes. Dehydrated in upgraded alcohol, cleared in xylol and mounted in DPX. Glycogen appeared as intense reddish violet particles.
(d) Detection of ascorbic acid

The ascorbic acid content of the hepatic cells and their accumulation or scarcity in the tumor bearing host was examined cytochemically to evaluate the role played by the same in the cancerous animals as well as in the revival experiment. Liver sections were dewaxed in dioxane and downgraded to water. Immersed in 5% silver nitrate solution (aq) in dark for 45 minutes. Washed in 1% sodium thiosulfate solution (aq) and then in distilled water. Dehydrated in dioxane and mounted in DPX. Ascorbic acid appeared as black granular precipitate.

(iv) Biochemical studies

Biochemistry, as a pursuit of professional stature, was added to the oncological sciences only by about forties. The biochemical changes produced in the liver tissue by the hepatocarcinogen-azocarmine was studied quantitatively and their subsequent revival due to the application of ascorbic acid. The induction of cancer and their subsequent changes with the treatment of vitamin C was investigated at the biochemical level by quantitative estimation of DNA, RNA and ascorbic acid.

(a) Quantitative estimation of DNA and RNA

Liver extracts were prepared for the determination of DNA and RNA following the Schmidt-Thannhauser (1945) method with the following modification of Fleck and Munro (1962). To the 5 ml. extract of 1:20 homogenate of the tissue and ice-cold distilled water was added 2.5 ml. of ice-cold 0.6N HClO₄. Mixed
and kept at 0°C for ten minutes. Centrifuged, discarded the supernatant fraction and washed the precipitate with cold 0.2N HClO₄. Drained off excess acid by inverting the tube briefly over filter paper. 4 ml. of 0.3N KOH was added to the precipitate and incubated at 37°C for one hour. After incubation, cooled in ice; and protein and DNA was precipitated by adding 2.5 ml. of 1.2N HClO₄. After standing for ten minutes in the cold, centrifuged down the precipitate and the supernatant fraction was taken out (RNA fraction). Washed the precipitate twice with 5 ml. of 0.2N HClO₄ and the washings were added to the RNA fraction. Following the addition of 10 ml. of 0.6N HClO₄ to the RNA fraction and washings, this fraction was made upto 100 ml. with distilled water, giving a solution of ribonucleotides in 0.1N HClO₄, which was read at 670 nm in the spectrophotometer — Sicospec-100 (The Scientific Instrument Co., Calcutta) using pure yeast RNA (BDH, INDIA) as the standard, to give a measure of RNA content. To estimate DNA, the precipitate obtained on acidifying the alkaline digest was dissolved in 5 ml. of 0.3N KOH by warming briefly to 37°C; the solution was then made upto 50 ml., including a further 12 ml. of 0.3N KOH. Two ml. of this solution was used for DNA estimation by the method of Ceriotti (1952) at an wavelength of 490 nm of the spectrophotometer (ECI. GS 865 B) using yeast DNA (BDH-INDIA) as the standard.
(b) **Quantitative estimation of vitamin C**

Vitamin C was suspected to be an antitumour agent from long time back for its healing properties. The healing property of vitamin C might help the cancerous animals to protect themselves from the destructive forces. Cameron and Pauling (1979) found a large number of patients treated with vitamin C to extend their life span. In the present experiment, vitamin C was extracted in a 6% trichloroacetic acid (TCA) solution and was estimated following the process of Roe and Kuether (1943). 2 gms. of the wet liver tissue were homogenized in 40 ml. of TCA and 800 mg of acid washed N02H was mixed with it. Filtered through an acid washed filter paper. To 2 ml. of the filtrate was added 0.5 ml. of 2-4 dinitrophenylhydrazine reagent which was followed by 3 hours of incubation at 37°C and then added 2.5 ml. of 85% H2SO4 in an ice bath. Allowed to stand for half an hour. The blank was prepared by adding 2,4-dinitrophenylhydrazine reagent after the incubation period whereby the color forming reaction was checked. Spectrophotometric readings were taken at a maximum wavelength of 540 nm using pure ascorbic acid (BDH) as the standard.

(v) **Haematological studies**

The blood picture of an animal is an index to the body physiology. Any change of the blood corpuscles either qualitative or quantitative expresses a change in the physiology because the blood system functions as the defence mechanism of the body. Besides other specific functions white blood corpuscles (WBC)
deals with the defence of the body whereas the red blood corpuscles (RBC) are related with the oxidation of the body metabolism. Haematological studies were performed under the following heads:

(a) **Differential count of WBC**

Peripheral blood was subjected to differential count on air-dried film using Leishman stain under a compound research microscope (Olympus OIC GB). Leishman stain was prepared by dissolving 0.15 gm. of leishman stain powder in 100 ml. pure absolute methyl alcohol. The solution was then heated in a water bath and allowed to cool and then filtered. The filtrate is the leishman stain for staining the blood corpuscles. A drop of blood was placed on a clean dry slide and a film was prepared with the help of another slide. The film was air dried. One to two drops of leishman stain was added to the blood film and an equal amount of water was also added to it. After 5-10 minutes the slides were washed in running tap water and then allowed to stand. The slide was then ready for examination.

(b) **Total count of RBC and WBC**

Total count of RBC and WBC was done by using a haemocytometer (Fein-Optik, GDR). To get the total count of RBC, blood was drawn in the RBC counting pipette upto the 0.5 mark and the rest of the bulb was filled up by sucking up RBC diluting fluid upto the mark 101 of the pipette. RBC counting fluid was prepared by mixing sodium chloride 0.6%, sodium citrate 1%, formalin 1% and distilled water upto 100 ml. RBC was counted in the five big squares of the haemocytometer slide which
contained 80 small squares. The number of cells found in 80 small squares multiplied by 10,000 will give the red cell count per cubic millimeter. WBC counting fluid was prepared by mixing glacial acetic acid 1.5 ml., gentian violet 1 ml. and distilled water 98 ml. The WBC pipette was filled up to 0.5 mark and then WBC diluting fluid was sucked up to the mark 11. A drop of the mixed blood was placed in the haemocytometer slide and WBC was counted in the 16 outer big squares. The total number of WBC in all the 16 big squares multiplied by 200 will give the total WBC count.

(c) Estimation of haemoglobin

Haemoglobin was estimated in the haemometer (Wochenschr, West Germany). It contains a standard haemoglobin solution. Blood was drawn in the blood sucking pipette and was placed in the graduated tube which contained 1% hydrochloric acid up to the lowest mark. The mixture was stirred with a glass rod and to it was added 1% HCl until the color of the blood mixture matched with the standard. The reading in the graduated tube gives the haemoglobin concentration in gram percentage.

All the three sets of experiments were conducted in the normal room temperature under ambient laboratory condition and the changes in the carcinogen treated group and ascorbic acid treated group after induction of carcinogenesis were noted against the control set. The result showed definite changes in the cancerous growth that could be compared with the control
set of guinea-pig on one hand and the revival experiment of the guinea-pig treated with carcinogen and vitamin C on the other. The interaction between different components of liver cells and carcinogen and their response to the anticancerous substance was studied and a possible target of action of the carcinogen was sought at.

VI. Statistical analysis

Statistical analysis was carried out to evaluate the possible role of vitamin C in the inhibition of malignancy using the standard method of analysis of variance (Goon, Gupta and Dasgupta, 1968).