EXPERIMENTAL
The present study of long acting progestagen contraception was carried out in the Obstetrics and Gynecological Department of Seth Sukhlal Karnani Memorial Hospital during the period of more than two years and covers about 150 women, who when offered a choice for variable methods of contraception gave their preference for a long acting injectable hormonal contraceptive to be used for at least two years.

1. Subjects:

Volunteers for this study were healthy women, who had demonstrated their fertility by having had a previous pregnancy. Subjects accepted were also free of endocrine or gynecologic diseases. All volunteers prior to entrance were aware of the investigational nature of the study, the possibility of developing amenorrhea, irregular unpredictable vaginal bleeding and prolonged infertility due to the drug. Women having past history of suffering from diabetes, jaundice, hypertension, bleeding disorders or eclampsia were excluded from the study group.

Participants in the study were instructed to keep a daily record of bleeding and were interviewed at three monthly intervals regarding side effects during the previous two months. Body weight and blood pressure were recorded at each follow up visit. They received 150 mg of Depot medroxy progesterone acetate (DMPA) within 5 days of last menstrual period or abortion or within 6 weeks of child birth, subsequent injections were given at three monthly intervals. Data pertinent to the study were
recorded for each patient according to the following scheme:

Name:
Age:
Husband's name:
Address:
Educational status (Both husband & wife):
Occupation (Both husband & wife):
Social status:

Obstetrical history:
  a) No. of pregnancies:
     1) Live birth: Boys -
     Girls -
     2) Abortion
     3) Still birth
  b) Mode of termination of last pregnancy:
  c) Age of the youngest child:
  d) Previous contraception used with duration:
  e) Menstrual history:

Pelvic examinations were done prior to injection and were repeated after 3 months interval.

Besides these, several biochemical investigations were done to ascertain the effects of the drug on metabolism.

In order to know the dropout rates, each dropout reason was assigned to one of five dropout categories.
(1) Failure :
(2) Planned pregnancy :
(3) Protocol completed :
(4) Medical reasons or side effects :
(5) Other reasons.

The patients were subjected to vaginal cytological studies and biopsy procedure prior to injection and at each 90 day.

**Blood collection :**

Fasting blood samples (10 ml) were drawn from each subject prior to injection of the drug and the same quantity of blood was taken during each follow up. Chemical cleanliness of all equipments (i.e. syringe, needle and the specimen container) used was a prime requisite.

In this study serum, plasma and erythrocytes were used for different investigations. When either serum or plasma is to be obtained from blood, hemolysis must be avoided. This can be minimised by using a dry syringe and slowly ejecting the blood from the syringe with a 25 gauge needle (170).

When the plasma was required chemical anticoagulants were routinely used. The anticoagulant used in this study was EDTA (Ethylene diaminetetraacetic acid). The residual part of plasma was used as the source of erythrocytes.

**Processing of the blood :**

For collection of serum the blood was allowed to clot in
a slanting position and centrifuged in International Refrigerated Centrifuge (Model B-20) at 1,500 g for 15 minutes.

The blood samples, collected in tube containing anticoagulant were centrifuged in International Refrigerated Centrifuge (Model B-20) at 1,500 g for 15 minutes. Plasma was separated as supernatant and the erythrocytes were collected as pellets. The red cells were washed repeatedly with normal saline and then hemolysed with fresh distilled water for enzyme assay.

Collection of rat tissues:

All the animals were sacrificed by decapitation. Liver and uterus were removed immediately, trimmed off the adhering fat material, weighed and kept into crushed ice for further processing.

Endometrial biopsy:

The biopsy specimens of normal healthy women were taken on the date of each follow up before injection, by means of curetting the uterine cavity with the help of Shorman's curette. The tissues were preserved in formalin solution.

Vaginal cytology:

Materials were taken on the same day as endometrial biopsy. The sterile swab stick was used for this purpose and swab was taken from the lateral wall as well as the posterior wall of the vagina; spread on a clean sterile glass slide; dropped immediately into fixative solution containing equal amount of alcohol and ether.
2. Animal:

Mature parous rats of Charles Foster strain weighing between 150-200 g were used in these experiments. The animals were maintained on laboratory stock diet of following composition:

- Atta: 72.5%
- Milk powder: 12.5%
- Casein: 6%
- Yeast powder: 3%
- Groundnut oil: 2%
- Minerals: 4%

Minerals were supplied as Hegsted salt mixture No.4 (171).

3. Hormone:

Depot medroxy progesterone acetate (DMPA, Depo-provera) (1 ml = 150 mg) were obtained from Upjohn Company, U.S.A., as a gift.

4. Administration of Hormones:

In case of human 150 mg/ml of Depo-provera was injected intramuscularly. Injections were given within 5 days of the last menstrual period or after 1 week of abortion or within 6 weeks of childbirth and repeated every 3 months for 2 years.

The rats were divided into 4 groups:

Gr. I. Control treated with 1 ml of vehicle.

Gr. II. 0.3 mg/ml of Depoprovera was injected intramuscularly at proestrus stage and sacrificed after 7 days of the injection.
Gr. III. Rats were injected 0.3 mg/ml of Depoprovera/week for 2 weeks and sacrificed 7 days after the last injection.

Gr. IV. Rats were injected 0.3 mg/ml of Depoprovera/week for 3 weeks and sacrificed 7 days after the last injection.

5. **Common Laboratory Chemicals**:

Common laboratory chemicals used in the present experiments were of analytical reagent grade purchased either from E. Merck, B.D.H. or Sarabhai M. Chemicals.

6. **Fine chemicals**:

Fine chemicals, such as, standard cholesterol, triolene, chromotropic acid, p-nitrophenyl phosphate, D-L Aspartic acid, α-ketoglutarate, alanine, standard calf thymus DNA, pure yeast RNA, acetyl thiocholine chloride, dithio-cis-nitro benzoic acid (DTNB), standard cysteine etc. were purchased from Sigma Chemical Co., St. Louis, U.S.A.

7. **Buffers**:

Buffers were prepared according to the methods as described in "Methods in enzymology".

**Brief description of the procedures adopted in this study**:

**Endometrial Histology**: After proper processing the tissues were mounted on the slide, stained with Weigert's Iron
Haematoxyline stain and examined under microscope.

**Vaginal Cytology**: The slides were stained with Papanicolaou stain and examined under microscope.

**Preparation of rat tissues for the estimation of lipids**:
The lipid materials were extracted and purified from liver and uterus by the method of Folch *et al.* (172).

**Estimation of total cholesterol**: Total cholesterol was estimated from human serum and rat tissues according to the method of Abell *et al.* (173). The cholesterol esters were saponified by incubation with alcoholic KOH. Free cholesterol was then extracted with petroleum ether. An aliquot of the extract was dried and cholesterol was determined photometrically by a modified Liebermann-Burchard reaction.

**Estimation of triglycerides**: Triglycerides were extracted from human serum or rat tissues (liver and uterus) with chloroform methanol mixture following the method of Van Handel and Zilversmit (174). Phospholipids were removed from the extract by adsorption on silicic acid. Determination of triglycerides were then made through estimation of glycerol content according to the procedure of Lambert and Neish (175) after saponification with alcoholic KOH. The method involved oxidation of glycerol by periodic acid to formaldehyde. The formaldehyde was determined photometrically by reaction with chromotropic acid. The major absorption peak of the coloured product was at 564 nm.
Determination of serum phospholipid: Serum phospholipid was determined according to the method of Bauman (176) using method of Dryer et al. (177) for phosphate. Lipids were extracted and serum proteins precipitated by ethanol-ether. Total phosphorus (P) in the extract was determined photometrically by the ammonium molybdate and p-semidine reagent, following oxidation of phospholipid (P) to inorganic phosphate by digestion with sulfuric acid and hydrogen peroxide. The P can be exposed as phospholipid by multiplying by a factor of 25 (178).

Estimation of rat tissue phospholipid: The lipid extract was digested with perchloric acid and sulfuric acid and the inorganic phosphate liberated from the phospholipid was measured by Fiske and Subbarow method (179) using amino naphthol sulphonic acid reagent.

Estimation of plasma protein: Plasma total protein, albumin, globulin, and A.G. ratio were estimated by Biuret method (180). The colour developed by biuret reagent was measured at 540 nm.

Estimation of protein in the rat tissues: The estimation of protein in tissues (liver, uterus) was carried out according to the method of Gornall et al. (182). The biuret method was occasionally checked by the modified micro-Kjeldahl method of Ma and Zuazaga (183). Protein in the subcellular fractions was estimated by the method of Lowry et al. (184).
Analytical Disc Gel Electrophoresis:

Disc electrophoresis (discontinuous electrophoresis) has attracted the attention of many investigators because of its well-defined, sharp separation boundaries, which can be achieved with only microgram quantities of protein. The theoretical basis for this method, which combines the principles of electrophoresis and gel filtration, is the Kohlrausch regulation principle. During electrophoresis a specific combination of ions forms a moving front of the "leading" ion followed by the "trailing" ion, thereby bracketing the sample protein between them. This permits concentration of the protein sample into an extremely sharp layer at the origin and yields the sharp resolution of individual boundaries. A further advantage of the method is offered by the use of polyacrylamide gel as anticonvection medium where acrylamide (CH$_2$ = CHCONH$_2$) is polymerized with the bifunctional molecule, N, N'-methylene bisacrylamide (CH$_2$ = CHCONH)$_2$.CH$_2$ to result in a clear and transparent gel. The qualitative and quantitative selection of these two monomers permits choice of a wide range of properties for the supporting medium. Thus, it is possible to anticipate the specific conditions for the best possible separation by proper selection of the gel components.

Application of Disc Gel Electrophoresis: There are numerous applications of disc electrophoresis in medicine and clinical biochemistry and one of such example is the analysis of serum proteins for the presence of abnormal constituents. In addition, it is a powerful tool in establishing the homogeneity of a protein and for determination of its molecular weight and size.
It has also been applied with success to the characterisation of mixtures of nucleic acids over a considerable molecular range extending up to about $10^6$. The isozyme pattern of an enzyme and their quantitative evaluation can also be achieved by this technique.

**General description of electrophoretic procedure**:

The polyacrylamide gels (5%) were prepared in tubes at pH 9.5 according to the method of Davis (181). 24 mM Tris Glycine Buffer of pH 8.3 was used for both upper and lower buffer. The electrophoresis was carried out for 1.5 hr at 25°C with a current of 2 mA per tube. In each tube 100 µg of protein containing 10% glycerol and 0.05% bromophenol blue as an indicator was charged. Sample movement was to the anode. After electrophoresis the gels were stained with Coomassie brilliant blue (0.25% w/v), amide black (0.1%, w/v) and methanol (20%, w/v) in 7% w/v acetic acid. After one hour the reaction was stopped by immersing the stained gels in a 7.5% acetic acid solution.

**Estimation of Nucleic acids**:

Nucleic acids were extracted from the tissue by the method of Schneider (185); DNA and RNA were estimated from this nucleic acid extract.

(a) **Estimation of DNA**:

DNA was estimated by the modified Burton's method (186) as devised by Croft and Lubran (187). 1 ml of nucleic acid extract was mixed with 2 ml diphenylamine reagent and incubated at 37°C for 48 hours. The intensity of the blue colour was read at 600 nm in a Beckman model DU spectrophotometer.
Amounts were obtained from a calibration curve made with pure calf thymus DNA.

(b) Estimation of RNA: RNA was estimated by the orcinol method of Meijbum (188). 1 ml nucleic acid extract was treated with 1 ml orcinol reagent for 20 minutes in boiling water bath, cooled in ice, then brought to room temperature and volume made upto 4 ml with water. Optical density was measured at 600 and 660 nm. Calibration curve using pure yeast RNA was prepared.

Estimation of serum sialic acid content:

Serum sialic acid was estimated according to the method of Warren, 1959 (189) by using thioarbituric acid (TBA) after acid hydrolysis of serum proteins.

For the formation of colour periodate oxidation was performed followed by addition of sodium arsenite solution and the mixture was kept in a boiling water bath for 30 minutes with TBA. Colours were extracted with cyclohexanone and read spectrophotometrically at 550 and 530 nm.

Estimation of Acid and Alkaline phosphatase:

Phosphatase activity was measured by the method of Michell et al. (190) which was based on the liberation of p-nitrophenol from the substrate p-nitrophenyl phosphate.

The assay system contained .02 M p-nitrophenyl phosphate, .1 M acetate buffer, pH 5.0 for acid phosphatase and .02 M p-nitrophenyl phosphate and .1 M glycine buffer, pH 10.0 for
alkaline phosphatase. The incubations were carried out at 37°C for 30 minutes, proper control being run to exclude nonenzymatic cleavage of the substrate. The reactions were stopped with 4 ml 0.1 M NaOH solution. The amount of enzyme added was 100 µg. The absorbance was read at 420 nm in spectronic 20 spectrophotometer. Specific activity was expressed in mmoles of p-nitrophenol liberated per µg of protein per hour in case of rat. In case of human serum, acid and alkaline phosphatase the activities were expressed in mmoles of p-NPP liberated/min/100 ml of serum.

Estimation of SGOT and SGPT:

Transaminases were measured by the method of Reitman and Frankel (191).

The pyruvate produced by the transamination by GPT (glutamic-pyruvic transaminase of alanine amino transferase) reacts with 2,4 DNPH (2,4-Dinitrophenyl hydrazine) to give a brown coloured hydrazone, which was measured at 510 nm. The oxaloacetate formed in the reaction with GOT (Glutamine oxaloacetic transaminase or Aspartate amino transferase) decarboxylates spontaneously to pyruvate, which was again measured by hydrazone formation.

The assay system contained the substrate, which was 200 mM D-L aspartic acid and 2 mM α-keto glutarate in case of GOT and 200 mM alanine and 2 mM α-keto glutarate in case of GPT. pH 7.4 phosphate buffer was used. The amount of enzyme added was 0.1 ml of human serum or approximately, 100 µg of protein in case of rat. The incubations were carried out at 37°C for 1 hour in case of GOT and 30 minutes in case of GPT. Proper controls were taken.
with each set. After incubation the colour was developed by 2,4-DNPH and sodium hydroxide.

The activities were expressed in μmoles of pyruvate formed per minute/litre of serum (in case of human) and μmoles of pyruvate liberated per mg of protein per hour (in case of rat).

**Estimation of Acetyl choline esterase activity:**

Acetyl choline esterase (AchE) activity in red cells was determined by Stahl's method (192), using acetyl thiocholin chloride as substrate and acetyl cholinesterase specific reversible inhibitor (anticholinesterase), thiocholin liberated was estimated spectrophotometrically using dithio-bis-nitrobenzoic acid (DTNB).

The assay system contained 100 mM tris HCl (pH 8.0) 50 mM acetyl thiocholine chloride and 100 μg of enzyme. Incubations were carried out at 37°C for 1 hour and the reaction was stopped by PCA. The colour was developed by adding dithiocisnitrobenzoic acid and sodium bicarbonate to the supernatant and O.D. was read in 1-2 minutes at 412 nm.

Activities were expressed in μmoles of thiocholin/mg of protein/hour.

**Enzyme source:**

The source of enzyme was human serum or subcellular fractions of rat tissue or human erythrocytes.
Enzyme preparation (from rat tissues)

Step 1. Cellular disruption of a 10% (w/v) organ suspension was carried out at 0-4°C in ice cold 0.32 (M) sucrose using glass homogenizer.

Step 2. Cell debris and nucleus were removed by centrifugation at 700 g for 10 minutes in a Sorvall Superspeed Centrifuge at 0-4°C.

Step 3. Post nuclear fraction was centrifuged at 14,000 g for 20 minutes at 0-4°C in Sorvall Superspeed centrifuge. The pellet was resuspended in ice cold (0.32 M) sucrose and was served as the source of mitochondria.

Step 4. Post mitochondrial fraction was centrifuged at 20,000 g for 30 minutes at 0-4°C in the same instrument. The pellet was resuspended in ice cold (0.32 M) sucrose and was served as the source of lysosome.

Results of biochemical parameters have been expressed generally in mg per mg tissue or mg per 100 mg tissue in case of rat. In human serum these are expressed as mg/100 ml of serum.

Enzyme activities have been expressed as specific activity.

Statistical Analysis:

Data were treated statistically using the student's t test. The variability of the data was presented as mean ± SEM. p < 0.001 were taken as highly significant; p < 0.05 significant and p > 0.05 non-significant.