MATERIAL AND METHOD
1. Animals and Tissue

Male Haffkine strain guinea-pigs (Cavia porcellus) bred in the departmental animal house and weighing 350 - 500 grams were used in the study. The isolated tracheal tissue was used both in vitro as well as after pretreatment of the animals in vivo.

Equipment used.

i) Assembly for mounting the tissue.

A two-unit isolated bath assembly (INCO) consisting of a tissue bath of 10 ml capacity, mounted vertically inside a water bath was used.

ii. Recording Assembly.

The assembly for measuring the response of the muscle isometrically include the Encardio-rite Polygraph Model No.434 serial No.0043 Manufactured by M/s. Encardio-rite Electronics, Lucknow,

3. Composition of the Physiological Salt solution.

The physiological salt solution used in the study consisted of Kreb-Hensleitt solution of the following composition in gms.litre⁻¹ NaCl: 6.9, KCl: 0.35, CaCl₂: 0.28, MgSO₄ 7 H₂O: 0.28,

NaHCO₃: 0.21, KH₂PO₄:0.16, Glucose: 1.5 Ascorbic Acid 0.2.

All chemicals used were of analytical reagent grade.

4. Drug solutions used in the study.

The following drugs were used in the study the drug solutions were freshly prepared on the day of study using double distilled water.

i) Carbamylcholine chloride (Carbachol) E.Merck.

An aqueous solution of Carbachol containing 1 mg.ml⁻¹ was prepared and subsequently diluted as required.
ii) Isoproterenol Hydrochloride (Sterling Winthrop)

A 1 mg.ml⁻¹ was prepared in distilled water containing 17 micrograms.ml⁻¹ of ascorbic acid and diluted as required.

iii) Salbutamol Sulphate (Cipla)

The drug was prepared freshly in 0.9% NaCl and diluted as required.

iv) Terbutaline Sulphate (Astra IDL)

The drug was freshly prepared in 0.9% NaCl and diluted as required.

v) Hydrocortisone sodium Succinate (Allenbury/Lyka)

Vial containing Hydrocortisone 100 mg was diluted as required.

vi) Indomethacin (Sigma)

A 1 mg.ml⁻¹ solution was prepared using sodium carbonate 0.5% solution and subsequently diluted.

vii) Theophylline (Sigma) was dissolved in 0.5 M NaOH and diluted in 0.9% NaCl.

viii) Phen tolamine HCl (Ciba) Dissolved in alcohol and diluted in 0.9% NaCl.

ix) Reserpine (Sigma) Reserpine was dissolved using glacial acetic acid and diluted as required.

x) Pyrogallol (E. Merck) Dissolved in distilled water.

Isolation and mounting of Guinea-pig trachea:

Guinea-pigs were stunned and exsanguinated and trachea was dissected free. Tracheal spirals were prepared according to constantine (1965). From each guinea-pig two or three pieces of trachea were obtained. The response to different segments is not variable as indicated by the study of Hanna and Roth (1978). The tracheal strips were equilibrated for 90 minutes under initial tension of 8 gms in Krebs-Hensleitt solution at 37°C aerated with an air pump. The high initial tension
was needed to ensure that resting tension at the end of equilibrium period was between 4 to 6 gms. Under these conditions the responses to agonists are reproducible since the muscle is near its maximal length (Stephen, 1970) One end of the tracheal spiral was tied to the tissue hook and the other end tied to a force displacement transducer FT 0.03 (Grass) and isometric response was obtained using an Encardiorite polygraph. It has been shown by Armour et al (1988) that isometric measurements may provide a more accurate representation of smooth muscle changes in response to agonists in vitro. The instrument was calibrated using 2 gm weight and at the end of the equilibration period a resting tension equivalent to 2 gms was provided to the tissue with the precalibrated instrument. The response was recorded with a sensitivity of 0.1 - 0.2mV.cm⁻¹

**Standardisation of Technique:**

The technique was standardised with each drug with respect to the dose and time required for the response to be recorded. A dose of carbachol producing submaximal contraction was chosen to increase the tone of the preparation and response was taken for 10 mintues.

**Response with isoproterenol / salbutamol / terbutaline:**

Relaxant responses with the above beta agonists were taken in tissues precontracted with carbachol. The drugs were added in a cumulative manner as per the method of Van-Rossum (1963). Two minutes was allowed for each dose to produce its response. Following the cumulative dose response to each beta agonist the tissue was washed repeatedly. Similar contractile response to carbachol was ensured before obtaining the next cumulative dose response. The above responses were obtained before and after incubation with various drugs for specified periods.
Plan of study. The process of desensitization was studied by determining the change in affinity of the beta adrenoceptor to various beta-agonists and their change in potency in guinea-pig trachea. Deesensitization was produced both in vitro and in vivo.

A salient feature of the methodological approach was the study of auto-desensitization which involves desensitisation to and challenge with the same beta-agonist. Three beta agonists were studied, isoproterenol a non-selective catecholamine, salbutamol a beta - 2 selective saligenin derivative, and terbutaline a beta-2 selective resorcinol derivative, the last two beta-2 agonists being the most frequently used bronchodilators in asthma. The mechanism of desensitization and its modulation by pre-treatment with hydrocortisone, indomethacin and theophylline has been investigated.

Since it was not technically feasible to measure affinity of the agonist directly, the study utilised two indirect indices to reflect the same (1) relaxing potency(pD₂) of the beta-agonist: Cumulative dose response curves were obtained as per the method of Van-Rossum (1963) to beta-agonists using guinea-pig tracheal spirals either pretreated in vitro or obtained from guinea-pigs pretreated in vivo. The pD₂ values for the beta agonists were calculated in isolated guinea-pig trachea in (1) controls (2) desensitized tracheas and (3) tracheas treated with hydrocortisone, indomethacin or theophylline prior to desensitization. Similarly pD₂ values were calculated using isolated guinea-pig treacheas obtained from in vivo vehicle treated guinea-pigs, desensitized guinea- pigs and guinea pigs subjected to desensitization after pretreatment with hydrocortisone, indomethacin or theophylline. EC₅₀ was calculated and relaxant potency PD₂ was calculated from the formula $pD_2 = -\log EC_{50}$.
(ii) Apparent dissociation constant ($K_B$, Value) was the second index employed. It represents the affinity of the beta receptor antagonist for the beta receptor (Furchgott 1955). The $K_B$ value is computed by the equation

$$K_B = \frac{[A][B]}{[A'] - [A]}$$

Where $K_B =$ dissociation constant for the antagonist receptor complex, $[B]$ is the concentration of the antagonist added to the bathing medium in the organ bath, $[A]$ is the concentration of the agonist needed to produce a given magnitude of response in the absence of antagonist and $[A']$ is the concentration of the agonist needed to produce the same magnitude of response in the presence of the antagonist. The other details of the procedure were according to method of Lin et al (1977).

I. In vitro production of Desensitization.

Dose response curves for the relaxant beta agonists isoproterenol, salbutamol and terbutaline were plotted according to the cumulative method of Van-Rossum (1963) using guinea-pig trachea precontracted with $(5 \times 10^{-7} \text{M})$ carbachol. Higher concentration of carbachol blocks the relaxant effect of beta agonists completely (Van Den Brink, 1973).

The $EC_{50}$ to the beta agonist was then calculated. The trachea was then incubated for 30 minutes with a concentration of two hundred time the $EC_{50}$ of the respective beta agonist to produce desensitization as per the method of Watanabe et al (1976). The tissue was washed at intervals of 10 minutes for 30 minutes the $EC_{50}$ for the relaxant effect of the beta agonists was then calculated in the desensitized trachea as well in guinea-pig tracheas where desenstization was proceeded by
treatment with hydrocortisone \((4 \times 10^{-5} \text{M (30 minutes)})\) / indomethacin \(1.7 \times 10^{-6} \text{M (30 minutes)}\) or theophylline \(5.6 \times 10^{-4} \text{M (20 minutes)}\). The relaxant potency \(pD_2\) value of the agonist was then obtained. Similarly the apparent disassociation constant \(K_B\) value for propranolol was calculated as per method of Lin et al (1977) for normal and desensitized guinea-pig tracheas pretreated with hydrocortisone / indomethacin/ theophylline prior to desensitization. The concentration of the beta antagonist used (propranolol) was \(1 \times 10^{-5} \text{M}\).

Certain precautions were taken while preparing the isolated trachea for the in vitro experiments to impede processes which could influence the observed effect of beta receptor agonists as per the method of Buckner & Saini (1975) All tissues were taken from guinea pigs which had been pretreated with reserpine \((5 \text{ mg.kg}^{-1} \text{ I.P})\) 24 hours previously in order to minimise release of endogenous catecholamine during the experiment the alpha antagonist phentolamine was added in a concentration of \(10^{-5} \text{M}\) 30 minutes prior to experiment and was present throughout. Pyrogallol, a catechol o-methyl transferase inhibitor was added 45 minutes prior to the experiment in a concentration of \(3 \times 10^{-5} \text{M}\) and was present throughout the experiment as it has been shown that O-methylated metabolites of catecholamines are weak beta-adrenoceptor antagonists (Brine et al 1979; Goldie & Patterson, 1982) and may thus reduce tissue sensitivity to beta-agonists (Kenakin. 1980).

II) In vivo production of desensitization
i) Control group: The guinea-pigs in this group were pre-treated with 0.9% NaCl containing 20 microgram.ml\(^{-1}\) of ascorbic acid by intramuscular injection at intervals of 20 mintues for five hours. The animals were sacrificed at seven hours that is two hours after the last injection and trachea isolated and subjected to the study.
ii) Desensitization group. The animals in this group were desensitized as per the method used by Conolly et al (1971) using one of the beta agonists isoproterenol (4 microgram.kg⁻¹), Salbutamol (4 microgram.kg⁻¹) or terbutaline (20 microgram.kg⁻¹). The animal was injected intramuscularly any one of the above agonists at twenty minute intervals for five hours and sacrificed at the end of the seven hours. The tracheas were taken out and pD₂ and Kᵦ values were calculated as described earlier.

iii) Desensitization proceeded by treatment.

The animal was treated prior to desensitization with anyone of the following drugs; hydrocortisone 50 mg.Kg⁻¹ I.M. eighteen hours prior to desensitization as per Brink et al (1977), indomethacin 20 mg.Kg⁻¹ IP 4 hours prior to desensitization as per method of Chandra (1986), or theophylline 45 mg.kg⁻¹ IP at the beginning of desensitization procedure as per the method of Madsen and Ribel (1981). Following treatment, desensitization in vivo was carried out as described above pD₂ and Kᵦ values were calculated.

Analysis of Results.

The results obtained in the studies were evaluated statistically using either paired or unpaired two tailed T tests.