

STUDIES WITH ANTIBODIES TO 5-HT

THESIS SUBMITTED TO
UNIVERSITY OF BOMBAY

FOR THE DEGREE OF
MASTER OF PHARMACY
IN
PHARMACOLOGY

SUBMITTED BY
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UNDER THE GUIDANCE OF
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DEPARTMENT OF PHARMACOLOGY
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BOMBAY

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Rajkimar M. Gupta

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Submitted To
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FACULTY OF TECHNOLOGY
In Pharmacology

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DEPARTMENT of PHARMACOLOGY
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A C K N O W L E D G E M E N T S

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S E C T I O N I

PRODUCTION OF THE ANTIBODIES

1.1 INTRODUCTION

The advent of preparing antibodies against drugs and physiological mediators, has provided with extremely specific and sensitive pharmacological agents for research into mechanisms of the physiological processes¹. Nevertheless, the present research tools such as receptor antagonists and depletors of the storage sites due to which we owe existing knowledge of physiology and pharmacology, are efficient ones but not the absolute. Specificity and sensitivity of these agents may be a limiting factor for the precise studies. Moreover, due to the very limited knowledge of the receptors via which they act, their own mechanisms of actions are not precisely understood. On the other hand, antibodies are highly precise in their action and function simply by sequestration of the molecules in question.

Until last decade, very few scientific reports were available to dispel the extreme importance of antibodies as pharmacological agents. These substances (drugs and physiological mediators) having low molecular weights, are not immunogenic as such and demand special treatment (conjugation with protein carriers) to elicit immune

response specific to them². At present antibodies have been prepared to a large number of drugs and physiological mediators. Table 1 lists all such substances for which antibodies have been reported.

The most significant contribution in this field is - digoxin specific antibodies³. The Fab fragments of the antibody preparation has made a record at Massachusetts hospital, U.S.A., by successfully treating a patient suffering from advanced digoxin intoxication⁴. In addition antibodies to the autocooids such as serotonin and prostaglandins have contributed quite a lot in elucidating physiological processes mediated by them^{5,6}.

In the present work, a fairly controversial physiological mediator viz. serotonin was selected for the study. The aim of the work was to prepare antibodies specific to serotonin and study its role in experimental inflammatory processes with the aid of them.

The indole-alkylamine, serotonin, is ubiquitous in mammalian tissues and is most abundantly localized in brain, enterochromaffin cells, blood platelets and mast cells. In rodents, it is considered as a potent mediator of the inflammatory reactions⁷.

TABLE -1 : Physiological Mediators and Drugs for which Antibodies have been reported.

DRUGS AND MEDIATORS		Ref. No.
<u>MEDIATORS :-</u>		
Catecholamines		8, 9
Serotonin		10-13
Melatonin		14
Histamine		15
Prostaglandins		16, 17
Bradykinin		18
Angiotensin		19
Substance P		20
<u>DRUGS :-</u>		
Analgesics :		
Aspirin		21
Codone		22
Btorphine		23
Fentanyl		24
Hydromorphone		25
Methadone		26
Morphine		27
Pentazocine		28

Contd.....

TABLE -1 : (Continued....)

DRUGS AND MEDIATORS	Ref. No.
---------------------	----------

Antibiotics :

Adriamycin	29
Anthracin	30
Chloramphenicol	31
Daunomycin	29
Gentamicin	32
Isontazid	33
Penicillins	34, 35
Sulphonamides	36
Tetracycline	37

Anticonvulsants :

Phenytoin	38
Sulthiame	39

Antidiabetic Agents :

Glibenclamide	40
---------------	----

Antihypertensives :

Salalasin	41
-----------	----

Antineoplastic Agents :

Methotrexate	42
--------------	----

Barbiturates :

Barbitone	43
Pentobarbitone	44
Phenobarbitone	45

Contd.....

TABLE-1 : (Continued....)

DRUGS AND MEDIATORS	Ref. No.
<i>Cardiac depressants :</i>	
Procaineamide	46
<i>Cardiac glycosides :</i>	
Digitoxin	47
Digoxin	48
Gitoxin	49
Ouabain	50
<i>Hallucinogens :</i>	
Cannabinoids	51
Lysergide	52
Mescaline	53
<i>Insecticides :</i>	
Aldrin	54
DDT	55
Dieldrin	54
Malathion	55
<i>Muscle relaxants :</i>	
Tubocurarine	56
<i>Steroids :</i>	
Corticosteroids	57
Dexamethasone	58
Ethinylestradiol	59
Mestranol	59
Methylprednisolone	60
Methisterone	61
Mestrol	62
Prednisone	63

 Contd...

TABLE - 1: (Continued...)

DRUGS AND MEDIATORS	Ref. No.
Stimulants :	
Amphetamine	64
Strychnine	65
Tobacco alkaloids :	
Cotinine	66
Nicotine	67
Tranquillizers :	
Chlordiazepoxide	68
Chlorpromazine	69
Diazepam	70

1.2 LITERATURE REVIEW :

The efficacy of antibodies to drugs as pharmacological agents depends on their sensitivity and specificity towards the drugs in question. Consequently, the most important aspect is the production of a suitable antiserum. This is dictated largely by chance, but number of factors such as immunogen, adjuvant, immunization schedule influence the success.

1.2.1 Basic concepts :

Antigens and the immunogens : An antigen is defined as a substance which will combine with a specific antibody while immunogen is one that will provoke an immune response. Most peptide hormones are both antigens and immunogens. By contrast serotonin is an antigen, since it can be bound by an antibody, but is not itself an immunogen unless coupled to a larger molecule (antigens of this type are usually referred as haptens)¹³.

The parts of an antigenic molecule which combine with the binding sites of the antibodies are referred as 'antigenic determinants'. In the case of peptide hormones each determinant consists of a sequence of 5 to 8 amino acids, having a diameter less than 15° A. units. The particular amino acid residues which constitute each a group have

unique three dimensional configuration and a difference of even one amino acid residue may distort the arrangement and impair its binding to the specific antibody. Even a small molecule such as ACTH with 39 amino acids may have more than one such groups, while larger peptides such as human growth hormone with 188 amino acids have several.

The immunogenicity of a substance depends on both its size and its chemical composition. Although most large polypeptides are quite powerful immunogens, other compounds such as steroids, biogenic amines, are non-immunogenic in their native state and must be conjugated to larger molecules before they will stimulate an immune response. A variety of chemical procedures are available for the covalent linkage of such substances to larger molecules. The method selected should not distort the structure of the hapten, since the antibodies produced will reflect the structure of the prosthetic group as it exists in the conjugate, but eventually be required to react with the original molecule. An homologous protein (such as rabbit serum albumin for the rabbit) or synthetic straight chain polymers (poly-L-lysine), neither of which are themselves immunogenic, may be chosen as the carrier. Alternatively, proteins that are themselves immunogenic (such as bovine serum albumin for rabbits) may be used with equal expectation of the success.

The immunogenicity of a peptide is related to its molecular weight, the rigidity of the structure as imposed, its susceptibility to the enzymatic degradation, the accessibility of the determinant groups, its content of certain amino acids including phenylalanine, tryptophan, glutamic acid, lysine and especially tyrosine, and its structural relationship to any corresponding peptide in the species under immunization.

Adjuvants : Adjuvants are the substances when injected with antigens (usually mixed with them but some times given prior to the antigen) nonspecifically enhances or modifies the immune response to that antigen .

The most widely used adjuvant is that introduced by Freund⁷¹. The adjuvant consists of a neutral detergent (Arlacel A), paraffin oil and (in the complete form) killed mycobacteria. The aqueous solution of the antigen is mixed with equal part of the adjuvant so as to form stable emulsion.

One of the ways in which this adjuvant is believed to work is by releasing the immunogen slowly over the course of weeks, these by preventing its rapid uptake into the circulation. This avoids rapid degradation of the immunogen by circulating enzymes and in the case of certain

hormones such as insulin, minimizes any direct physiological effect of the immunogen on the recipient. The adjuvant also facilitates phagocytosis of the immunogen by macrophages which is an initial step for antibody production. Thirdly, it stimulates the formation of local granulomatous lesion which may act as a focus of antibody production. Finally, the adjuvant causes both local and general stimulation of the reticuloendothelial system with the multiplication of macrophages and of immunologically competent lymphoid cells.

The other adjuvant procedures which may be employed in conjunction with Freund's⁷¹ is the adsorption of immunogen on to micro-particulate materials such as carbon (lamp black), polystyrene latex or polyacrylamide beads or on to inorganic salts, such as aluminium hydroxide or phosphate.

Antibodies : Antibodies are gamma-globulins having capacity to combine with specific antigenic determinants. Structurally and functionally, its principal members are distinguished as IgG, IgA, IgM and IgE. All of these four types consist of both heavy and light chains, containing some 450 and 200 amino acids respectively. Antibodies of the IgM class (19 S macroglobulin) are the largest in size, with the molecular weight approaching 1,000,000. In most mammalian species, the IgG group is

quantitatively and qualitatively (functionally) the most important and is the one usually involved in immune response to haptens. The molecules of this group have a molecular weight of some 160,000 and circulate at a concentration of 10-15 mg/ml. They have general structure as illustrated in Figure I.

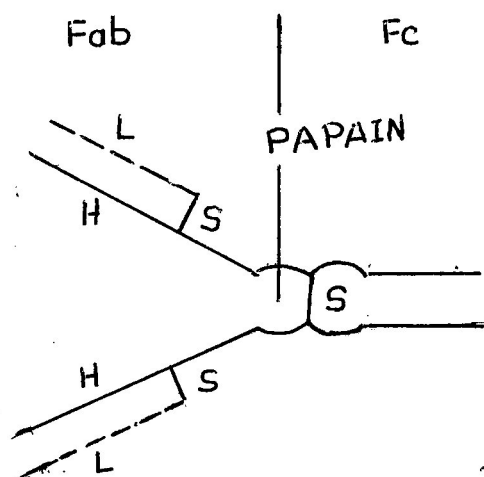


Fig. 1.

Rabbit IgG antibody molecule is comprised of two light (L) and two heavy (H) chains connected by sulphhydryl (S) bonds. Cleavage by papain results in the degradation of the molecules into the three fragments, two of which (Fab) are still able to combine with homologous antigen while the third (Fc) is not.

Each IgG molecule has two combining sites, each consisting of the N terminal sequence of one light and one heavy chain. Since the most immunogens contain several

different determinants, any antiserum is likely to contain several different population of antibody molecules within the IgG class, each directed against one determinant. Furthermore, antibodies raised against a single determinant, such as a hapten, are also expected to show small differences according to the line of cells from which they originate.

The great specificity of these molecules is probably explained by close physicochemical fit of their combining sites to the corresponding antigenic determinant (the term 'avidity' is used to denote the energy of the combining sites of an antibody and its specific antigen).

Mechanisms of antibody production : The term 'reticuloendothelial system' is a functional designation for a population of cells which have ability to phagocytose foreign elements. Each consists of a network of a heterogeneous population of cells of which the largest (macrophages) remain fixed while others (histiocytes) can migrate. They are found at numerous sites including the spleen, liver, bone marrow, lungs and lymphnodes. The immunogen is trapped by these cells and then broken down by the high concentration of proteolytic enzymes present, yielding fragments which probably present the antigenic determinants. The fragments are finally bound to the nucleic acid or other

cellular constituents and exist as cytoplasmic granules within the phagocytes, where they may persist for several months.

While the immunogen is initially taken up and processed by the phagocytes, it is the cells of lymphoid series (found in the reticuloendothelial system), that actually synthesize antibody. The lymphoid series include plasma cells, large and small lymphocytes and primitive types known as blast cells. It is believed that immunologically competent small lymphocytes receive material from adjacent macrophages which enables them to synthesize specific antibody. The small lymphocytes develop into plasma cells which are the main antibody producers and have life time of only few days.

Following the first injection of an immunogen in Freund's adjuvant, there is a delay of some 7 to 8 days before circulating antibodies are detected. Maximum levels are achieved after several weeks and then fall slowly. The antibody response to a second injection differs from the primary response in a number of ways. The lag period is reduced to about 4 days, the maximal rate of antibody production is much greater and the peak of production is achieved within 10 days, the antibody formed is almost entirely of IgG class.

1.2.2 Methods of conjugation :

Conjugation methods are essentially the chemical procedures employed to link a hapten to a protein carrier via co-valent bonds. The selection of a particular method is determined by the physico-chemical characteristics of the hapten and the carrier protein, and the ingenuity of the investigator.

Choice of the carrier : Generally, the proteins such as albumins, globulin fractions, hemocyanin, fibrinogen are employed as the carriers for the hapten. Out of all these bovine serum albumin, which is comparatively more soluble in aqueous media and highly antigenic in rabbits, is the protein carrier of the choice. It lends various functional groups for the coupling. The most available groups are ϵ ^{amino} groups of lysine. In addition, there are alpha amino groups, phenolic hydroxyl groups of tyrosine, sulphhydryl groups of cysteine, imidazole groups of histidine, the carboxyl groups of dicarboxylic amino acids and hydroxyl groups of serine. One can also substitute small molecules directly on the rings of tyrosine, tryptophan or histidine by diazotization.

Nature of the hapten :

a) Haptens with free carboxyl groups such as angiotensin can be conjugated with proteins via peptide synthesis at epsilon amino groups of lysine residues¹⁸.

The reactions are performed using either mixed anhydride or carbodiimide procedure. Mixed anhydride is a simple procedure and does not require isolation of an active derivative⁷³.

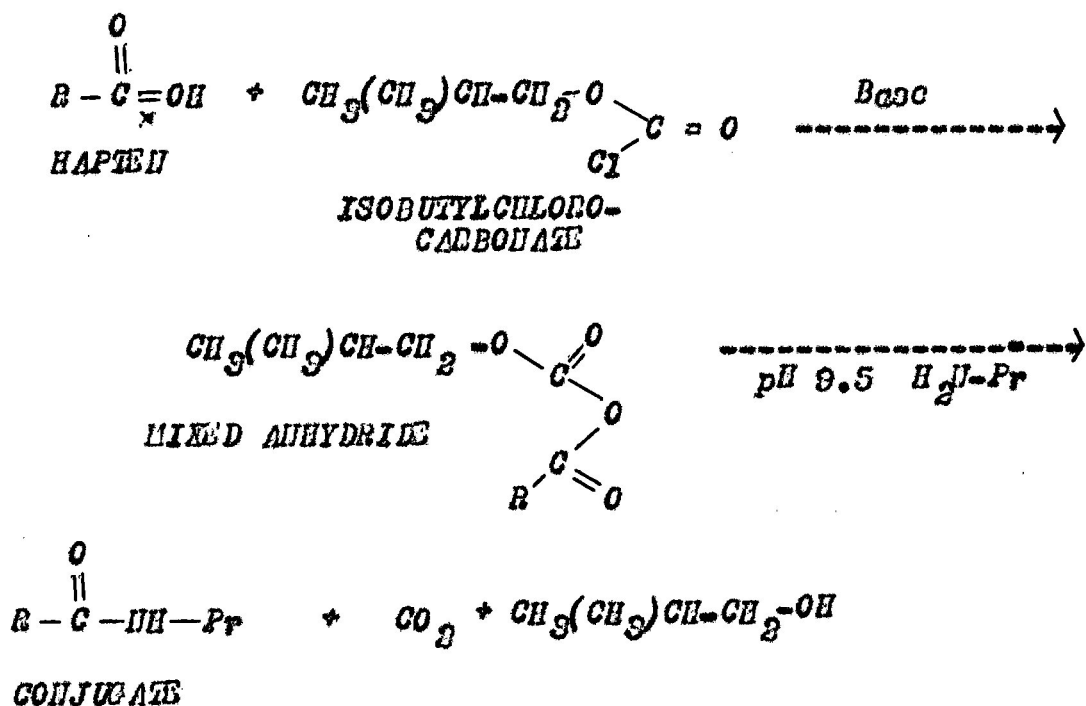


Fig. 2 : MIXED ANHYDRIDE PROCEDURE

Carbodiimides present a another direct method⁷³.

Unlike mixed anhydride procedure, coupling reaction can be performed in aqueous solution using water soluble carbodiimide such as ethyl carbodiimide⁷⁴. The conditions of the reaction are very simple. The carrier, excess of the hapten and the reagent are simply stirred together for suitable time length.

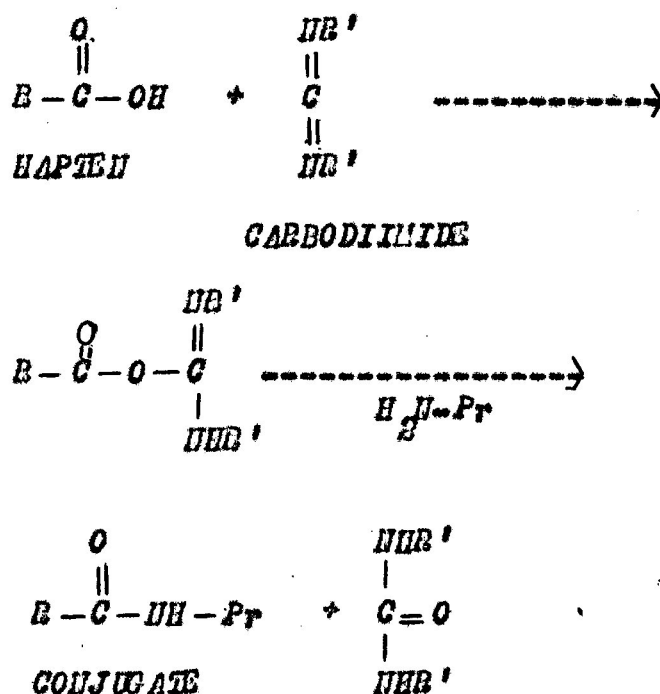


Fig. 3 : THE REACTION OF CARBODIIMIDES WITH HAPTENS HAVING FREE CARBOXYL GROUP.

Folic acid⁷⁵, digitoxigenin⁴⁷, have been conjugated to protein carriers with carbodiimides.

b) Haptens with aromatic amino groups can be conjugated to proteins by the classical diazotization procedures of Landsteiner⁷⁶. An interesting application of this procedure is the preparation of chloramphenicol protein conjugate which elicit antibodies specific to chloramphenicol⁷⁷.

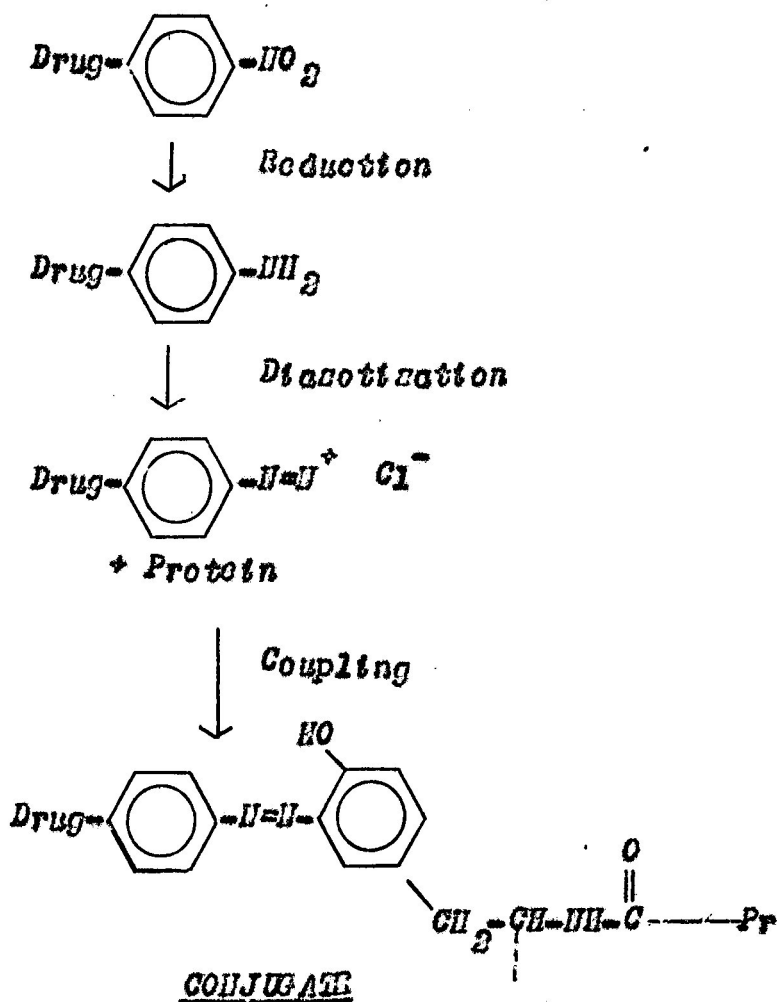


Fig. 4 : METHOD FOR CONJUGATING HAPTENS WITH AROMATIC AMINO GROUPS TO PROTEIN BY THE FORMATION OF DIAZONIUM SALTS.

c) Haptens with aliphatic amines can be reacted with proteins using various reagents such as carbodiimides, p-nitrobenzoyl chloride, toluene 2,4-diisocyanate, glutaraldehyde, formaldehyde, etc.⁷³. Among the haptenic groups conjugated using such reagents are :- Serotonin¹¹, Catecholamines⁸, histamine¹⁵, etc.

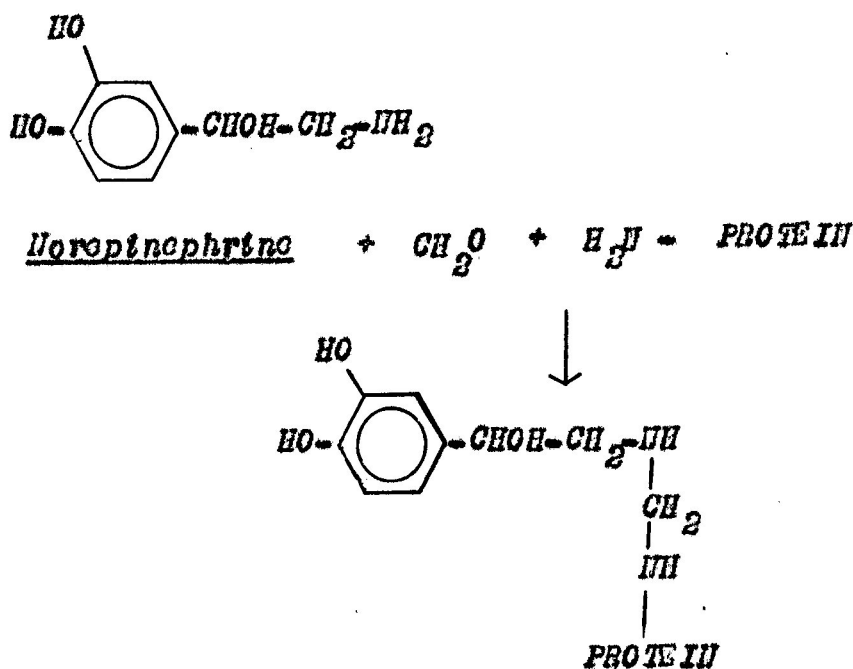


Fig. 5: PREPARATION OF NOREPINEPHRINE PROTEIN CONJUGATE USING FORMALDEHYDE

d) For the haptens containing free hydroxyl groups (alcohols, phenols, sugar, polysaccharides, nucleosides, etc.), first a derivative of the compound must be made so as to introduce functional groups capable of reacting with proteins.

Succinic acid for introducing free carboxyl group⁷⁹, phosgene for introducing reactive chlorocarbonate⁸⁰, p-aminobenzoic acid to provide aromatic carboxyl group,⁸¹ periodic acid for oxidation of sugars to dialdehydes⁸², are some of the most widely used reagents to synthesize reactive derivatives of the hapten. The classical procedure for coupling digoxin to albumin involves oxidation of vicinal hydroxyl groups on the digitoxose to dialdehydes and then direct reaction with amino groups of the protein³.

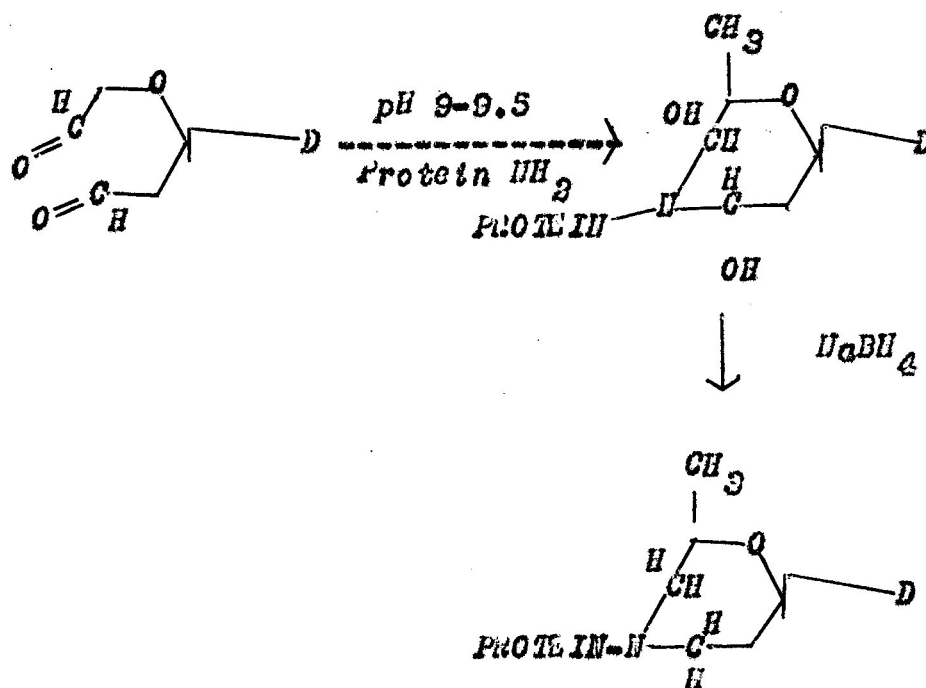
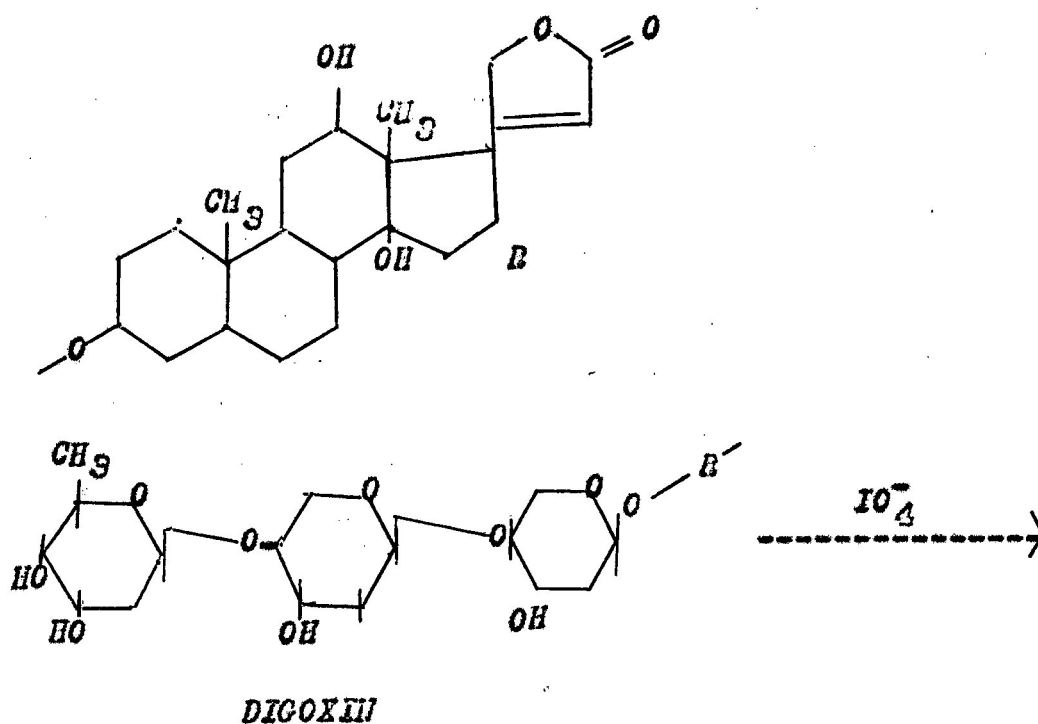


Fig. 6: METHOD OF CONJUGATION OF DIGOXIN TO PROTEIN.

c) For the haptens containing aldehyde or ketonic groups, first an oxime derivative is prepared⁸³. The free carboxyl group introduced is then reacted with proteins by the methods as described earlier.

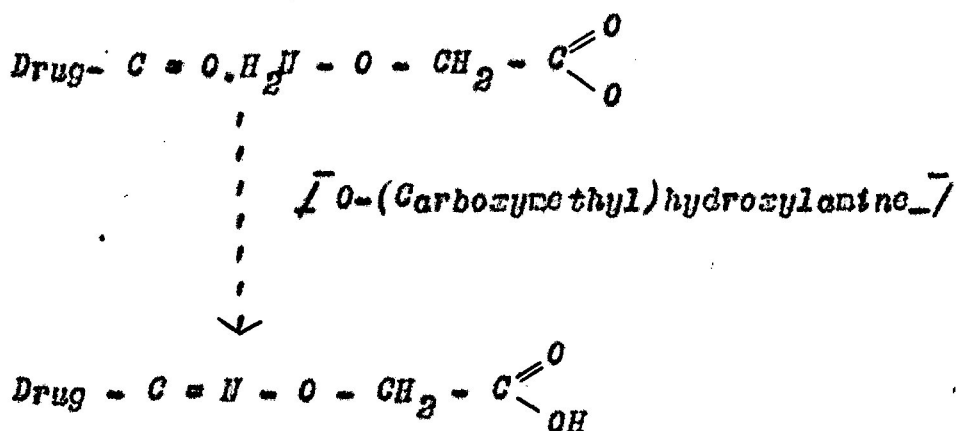


Fig. 7 : FORMATION OF AN OXIME FROM THE REACTION OF A CARBOXYMETHYL HYDROXYLAMINE.

Bis-diazotized benzidine (BDB) can be used as a bridging reagent between proteins and haptens containing aromatic rings that react with diazonium compounds⁸⁴.

f) The procedures for the conjugation of serotonin to protein have been described by various authors. Kanadive and Sheon¹² have used Hannich's reaction to couple first position of indole nucleus of serotonin to epsilon amino groups on protein. In an another procedure described by Fillip¹⁰, serotonin was conjugated by forming azo derivative

of it with *p*-aminoacetanilide.

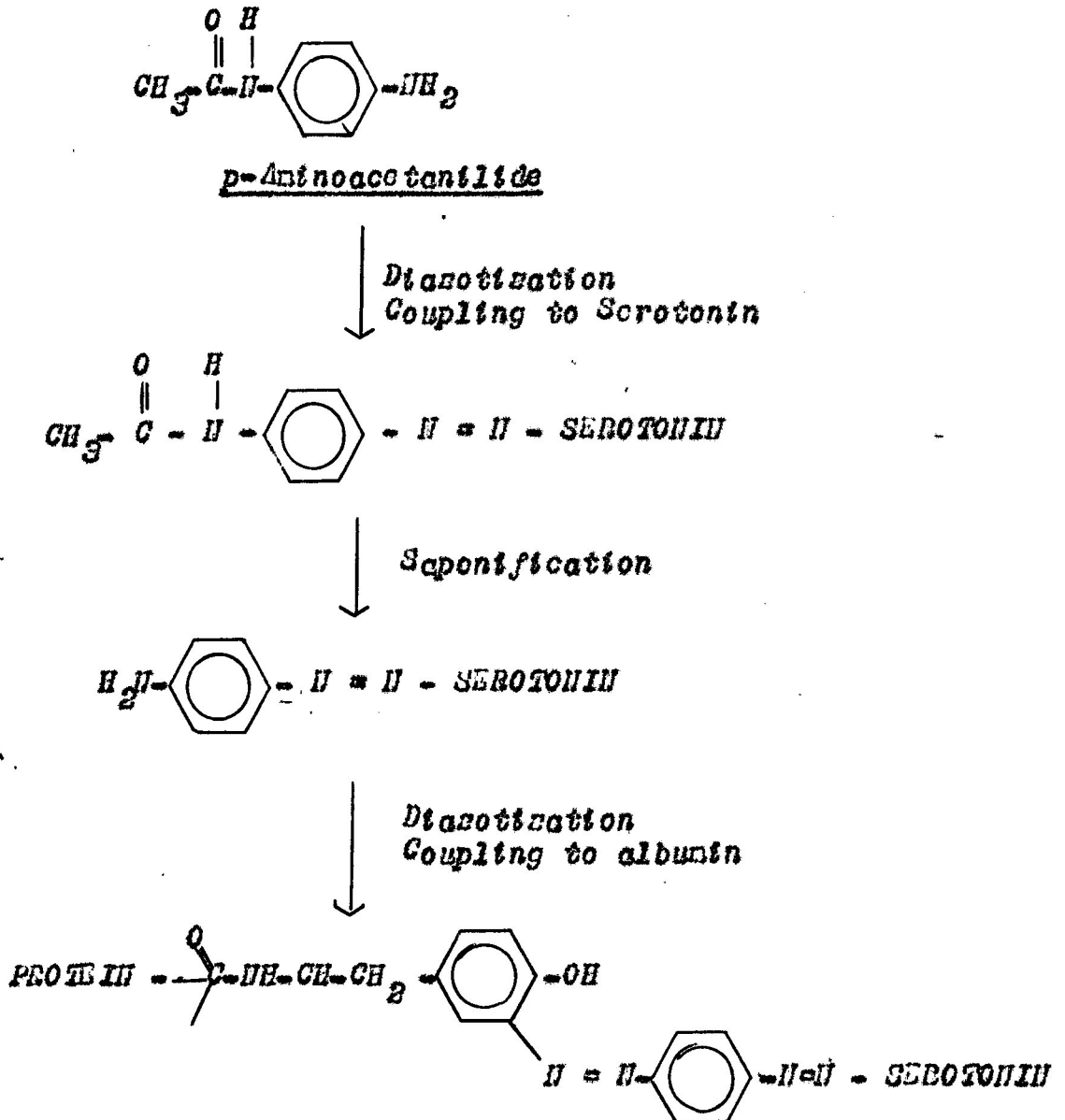


Fig. 8: CONJUGATION OF SEROTONIN TO ALBUMIN BY FILLIP'S METHOD.

The conjugation reaction recently reported by Peskar & Spector¹³ involves the following steps :-

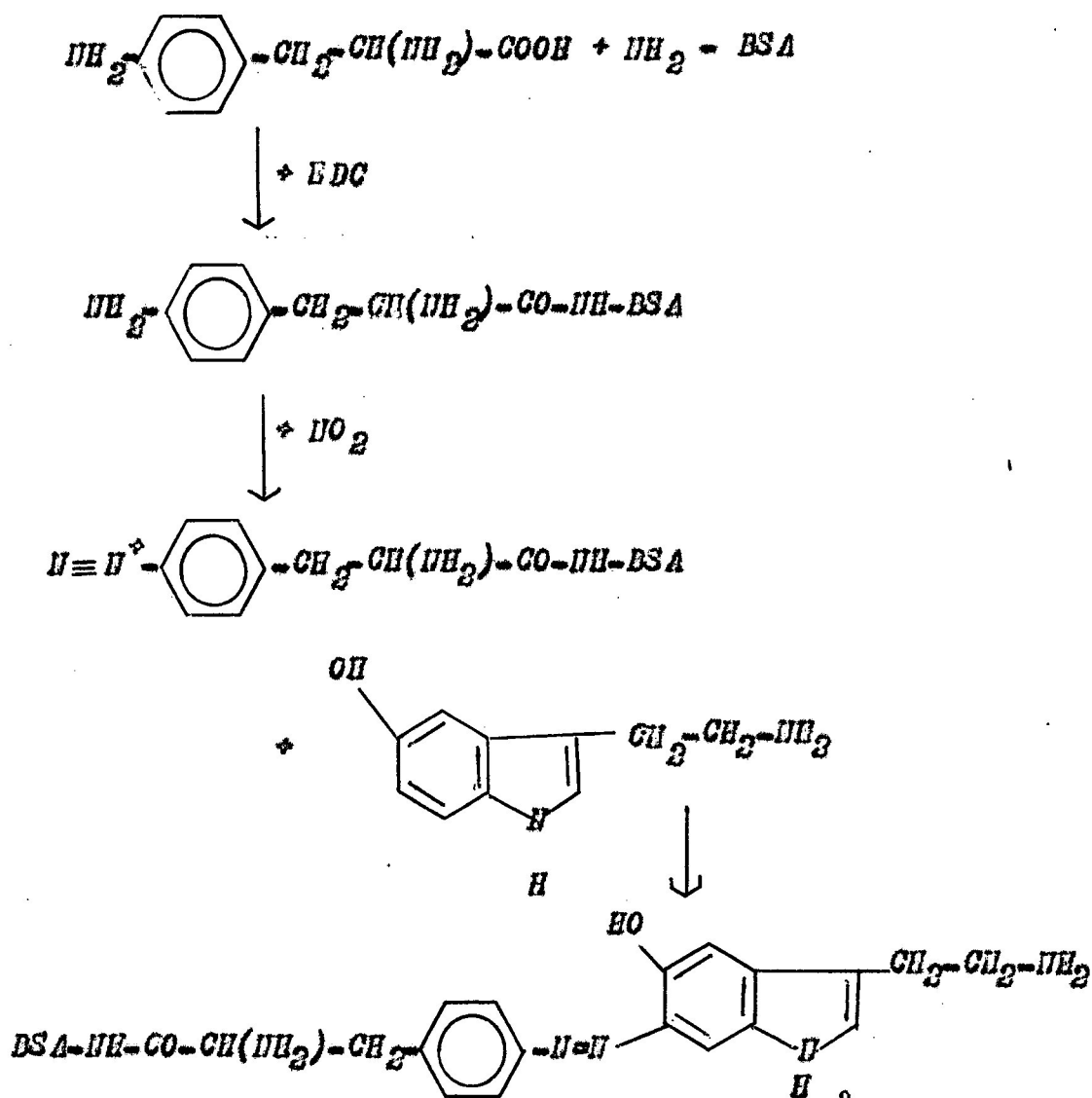


Fig. 9: CONJUGATION OF SEROTONIN TO PROTEIN, SPECTOR'S METHOD

Other workers who have reported antibodies to serotonin, have followed these reactions with minor modifications.

Purification of the Conjugates : The procedures used for coupling hapten to protein, leave behind many undesired chemical agents. These agents are mainly unreacted hapten or the reagents, and by-products of the reaction. Although the presence of these do not harm the desired immune response, characterization of the conjugates become a problem. Generally, these undesired elements are eliminated from the final reaction mixture by exhaustive dialysis in cold.

Characterization of the Conjugates : One of the major findings by Landsteiner⁷⁰, pioneer of this field, relates degree of haptenic substitution to immune response. His work established that too much or too little hapten leads to poor response and with serum albumin as a carrier 20-40 haptenic groups are optimal⁸⁵.

Number of methods are available to characterize the conjugates for the content of haptenic groups. The most widely adopted procedure is ultra violet spectrophotometric analysis. The ultra violet spectra of the hapten, the protein carrier used and of the conjugate, are recorded. The results are interpreted by assuming hapten-protein conjugate as binary mixture of the protein and the hapten.

The shift in the absorbance values of the conjugates as compared to the absorbance values of protein carrier, are attributed to the presence of haptenic groups. The number of haptenic groups per molecule of the carrier are estimated with the aid of the formula proposed by Freedman⁶⁶.

$$H = \frac{\overline{I}(OD \ C_a) (\epsilon \ P_b) - \overline{I}(OD \ C_b) (\epsilon \ P_a)}{\overline{I}(OD \ C_b) (\epsilon \ H_a) - \overline{I}(OD \ C_a) (\epsilon \ H_b)}$$

where OD = Optical density.

ϵ = Molar Extinction Coefficient.

C = Conjugated Product.

P = Protein.

H = Hapten.

a, b = Two different wavelengths.

However, these calculations are regarded approximate as it is assumed that spectral properties of the protein and the hapten remain unchanged after conjugation.

The most of the conjugation reactions are of the substitution type. Qualitatively, one or more functional groups of the protein carrier are substituted by haptenic groups. Eventually, the degree of substitution can be determined by analysing carrier protein and its conjugate for the functional groups involved in the reaction. For the

analysis of free amino groups, ninhydrin⁸⁷ and trinitrobenzene sulfonic acid⁸⁸ are used as reagents.

A more convenient and direct procedure for such estimations, is Radiotracer technique of Abraham⁸⁹. A radioactive hapten is incorporated in the conjugation procedure and the direct estimation of the degree of substitution is made by counting undialyzable radioactive material.

Banadive and Sheon have employed ultra-violet spectrophotometric and ninhydrin reaction¹² for the characterization of serotonin-albumin conjugates. Recently, Peskar & Spector have described radiotracer technique for the estimation of serotonin moles on the coupled protein¹³.

1.2.3 Immunisation and collection of the antisera :

Choice of the Animal Species : Immune response depends both on the responsiveness of the animal and properties of the injected material. The responsiveness to each immunogen is genetically determined, probably by a single gene, and is transmitted as a Mendelian dominant⁹⁰. Certain inbred strains of a species may produce antibodies to a particular antigenic determinant while other strains do not⁹¹. Consequently, it is best to use random bred

animals unless a particular strain is known to respond well to the immunogen under consideration.

The choice of species for immunization is usually restricted to rabbits and guinea-pigs. Rabbits are easier to handle, are more hardy and 30-40 ml blood can be taken from an ear vein at frequent intervals. Guinea-pigs are less hardy and blood must be obtained by cardiac puncture, with its attendant risks. Nevertheless, they are economic (initial cost and maintenance) and need slightly less immunogen, enabling larger number to be used.

A logical policy is to carry out an adequate trial in the most convenient species and then switch to another if the first is unproductive. To minimize the number of animals being kept one time, it is best to dispose off any that have completely failed to respond after about 3 months immunization. If neither rabbit nor guinea-pigs respond well to the particular antigen after an adequate trial, chickens may be tried. These are hardy and easy to bleed, but there are difference in physicochemical properties of their gamma-globulins, when compared to mammalian globulins⁹².

Adjuvant and the Dosage of Immunogen : The most potent antisera, are produced with the aid of Freund's

Complete Adjuvant⁷¹. Traditionally, equal volumes of antigen and adjuvant are mixed together, but experience shows that emulsions with a water phase content of 20-30% are produced more easily, are less viscous and somewhat more immunogenic.

Other adjuvant procedures that may be employed in conjunction with Freund's are adsorption of immunogen on to micro-particulate materials such as carbon, polystyrene latex and aluminium hydroxide. In all cases the effect is probably mediated by phagocytosis of immunogen-laden solid material while, with some of the more easily degraded antigens, protection may be given against enzymatic destruction in vivo.

Antibody response is virtually independent of immunogen dosage over a wide range, once a certain minimum threshold dose is exceeded. The minimum effective quantity varies from one substance to another. With the use of Freund's Adjuvant⁷¹, satisfactory response can be obtained with primary dose of 100 ug/rabbit and booster doses of half the primary dose, when serum albumin is used as an antigen. If the immunogen is freely available, and if it is unlikely to have any toxic or undesirable action on the injected animal, doses of 0.5 to 5 mg for primary inoculation and 0.25 to 2 mg subsequently (depending on purity)

are most likely to be effective. Scarce, materials or substances that may have undesirable side effects (such as insulin/ACTH), may be given in lower dosages. Unwanted biological effects of later injections will be neutralized by circulating antibodies.

Route of Administration : Although the immune response is a systemic reaction, the site at which the immunogen is administered may influence the predominant site of antibody production. With subcutaneous and intramuscular injections, the highest concentration of immunogen is found in local granulomata and in regional lymph-nodes (e.g., the popliteal gland following foot pad injection); after intraperitoneal injections in the mesenteric nodes; and following intravenous administration, in the spleen and lungs.

Although few critical studies have been made of the effect of route of administration on the immune response to poor immunogens, available evidence suggests that the sites may be ranked in decreasing order of effectiveness as follows :- Lymph nodes, intra-articular (usually into knee joints), intradermal, intramuscular, intraperitoneal, subcutaneous, and finally, intravenous⁹³. As with other variables, the sensible course is to select a route of

injection to begin with and change to another route later if unsuccessful.

For the sake of simplicity the intramuscular route may be used first. When rabbits are immunized, 0.5 to 1 ml of emulsion should be administered into each thigh and upper foreleg. Guinea-pigs should be injected with 0.25 to 5 ml of emulsion subcutaneously in the abdominal wall. Injections into the foot-pads (or anywhere in the feet) have often been recommended but are to be avoided except as a measure of last resort, since the animals invariably get very swollen, painful feet and are in obvious distress for many days or weeks as a result. Attempts to inject oily emulsions into any part of the vascular system are usually rapidly lethal.

Timing of Injections and Bleeding^{94,95} Timing of injections is important in order to obtain the maximum response with minimum effort. Since one of the properties of an oily emulsion is to allow a sustained slow release of antigen over a period of several weeks, the practice of administering injections at weekly intervals is without value when Freund's adjuvant is used. Antibody levels rise relatively slowly after a primary injection reaching a peak 6 weeks later. Booster doses should not be given

earlier than a week after the first injection not more frequently than once each month thereafter. Antibody titre rise to a maximum about 10 days after each booster injection and animals should be bled between 7th and 14th days, several bleeds may be taken during this time without affecting the amount or quality of the antibody.

With many of the better immunogens, two injections with Freund's adjuvant may yield maximum titres of antibody, however, affinity often continues to increase with further injections administered at monthly intervals. With any system, there is a belief based on experiences that the most potent antisera may be obtained by giving a course of several injections followed by a rest for 3-6 months prior to a final booster injection.

Storage of Antisera : Generally, native antisera are stored at -20 to -80°C , except the ones that are immediately under test, of which a small aliquot, with preservative is stored temporarily at 4°C . WHO specifies that precious antisera may be stored at -80°C in liquid carbon dioxide. Nevertheless, for general handling of antisera following points may be kept in mind.

- 1) Never freeze an antiserum containing cresol as a bacteriostatic agent as the protein will be badly damaged ;

- ii) Rapid freezing is to be preferred. This can be best achieved by freezing of a serum in a glass vessel using CO_2 , alcohol bath.
- iii) Repeated freezing and thawing should be avoided.
- iv) Electrical refrigerator operating at low temperature tend to unreliable and induce an element of insecurity.
- v) Coupling antisera to solid phases such as cyanogen bromide, improve the stability of the antibodies.

• However, storing antisera in the lyophilized state is much better than freezing at -60°C . Properly frozen and dried antisera (i.e., with a residual moisture less than 2%) can retain activity for extremely long periods.

1.3.4 Assessment of the Antibodies :

An antibody is an immunoglobulin molecule secreted into the tissue fluids from lymphoid cells which have been exposed to a foreign substances, antigens. The presence of several different antigenic determinants in most immunogens results in the production of several antibody populations, each originating from a single clone of cells and directed against a single determinant. Hence, every serum including each bleed from an individual animal, must be assessed

carefully. Antibodies in the serum are usually detected by antigen-antibody reactions.

Farr's Test⁹⁶ : The test uses antigen labelled with ¹³¹Iodine that is mixed with antibody containing serum and left to equilibrate. Ammonium sulphate solution is then added to the mixture resulting in the precipitation of antibody together with any labelled antigen bound to it. The unbound antigen remains in solution. The quantity of isotope labelled antigen bound to the salt precipitated antibody is then estimated by placing the washed precipitate in radioactive counting equipment. This test is the measure of the capacity of antiserum to bind antigen.

Precipitin Test⁹⁷ : If soluble antigen reacts to form an insoluble antigen-antibody complex, the reaction is indicated by a precipitate. This reaction may be carried out in a aqueous solution or gel. For the precipitin reaction in gel, wells are cut in a layer of agar in a petri dish. Antiserum and antigen solutions are placed opposite each other in the wells and after allowing a few days for diffusion to take place precipitin bands will form where antigen and antibody meet in suitable proportion. The value of the method is that it is much more simple to perform.

Agglutination reaction⁹⁸ : In this reaction the antigen is part of the surface of some particulate material such as a red cell, bacterium or perhaps an inorganic particle (e.g. polystyrene latex) which has been coated with antigen. Antibody added to a suspension of such particles combines with the surface antigens and links them together to form clearly-visible aggregates or agglutinates. This test is much more sensitive than the precipitation test.

Complement fixation⁹⁹ : The fact that antibody, once it combines with antigen, is able to activate the complement system is used as a way of showing the presence of a particular antibody in a serum.

In a test the antibody, complement and antigen are first mixed together and after a period of incubation the indicator system, antibody-coated sheep cells, are added.

If the reaction

Antigen + antibody + complement $\rightarrow$

Absorption of complement on antigen-antibody complex has already taken place, it will prevent the reaction:-

RBC + anti-RBC serum + complement $\rightarrow$ hemolysis.

Thus, a positive complement fixation test (presence of antibodies) is indicated by absence of lysis.

These tests are rather complex to perform but they are applicable to all kinds of antigens soluble or insoluble.

Neutralisation Test : Another immunological test used only occasionally by the biochemist is the "neutralisation test". It is applicable only to biologically active antigens, such as toxins, hormones, and drugs and requires an anti-serum that reacts with, and so neutralizes, the biologically active part of the molecule.

Antibody Titre : The titre of the antibodies is generally determined by quantitative precipitation test¹⁰⁰. In the test series of tubes are set up, each containing a constant amount of antiserum, and decreasing amount of antigen. The precipitation occurs in each tube. The protein content of the precipitate at optimal proportions of antigen-antibody, is estimated.

1.3 MATERIALS AND METHODS :

1.3.1 Materials :

1. Chemicals :

- i) Serotonin : E. Light & Co. Ltd., England.
- ii) Bovine serum albumin : Sigma Chemical Co., U.S.A.
- iii) Formaldehyde : Merck, Bombay.
- iv) Glutaraldehyde : Fluka Chemical Co.
- v) Benzidine : Sigma Chemical Co.
- vi) Toluene, 2,4-dinitrobenzene :
- vii) Ethyl carbodiimide : Sigma Chemical Co., U.S.A.
- viii) Folin Ciocalteu reagent : Centron Res. Lab., India.
- ix) Agar : Oxoid Ltd., London.
- x) Freund's complete adjuvant : Difco, U.S.A.
- xi) Silicon solution : HICO Products Pvt. Bombay.

All other routine chemicals used were of A. R. grade.

2. Glassware : All glassware used for collection of plasma were silicized.

3. Animals : Albino rabbits : Norwegian strain.

4. Others : Dialyzing membrane (seamless cellulose membranes, average pore size - 2×10^6) : Union Carbide, USA.

1.3.2 METHODS

1.3.2.1 Conjugation of Serotonin to a Protein :

Coupling Procedures :

i) Formaldehyde condensation : Formaldehyde

condensation was carried out as described by Banadiv and Sheon¹², with minor modification. To 100 mg of bovine serum albumin in 3.0 ml of water, were added 2.0 ml of 5% sodium acetate, 2.0 ml of 5% formaldehyde, and 3.0 ml of aqueous solution containing 20 mg of serotonin creatinine sulphate. The mixture was stirred at room temperature until a yellow precipitate started to separate. The reaction was stopped at this point by dialysing in neutral phosphate buffer at 15-20°C for 3 days. During this operation the mixture got turbid. It was clarified by centrifugation. Figure - 10 illustrates the presumed structure of the conjugate.

ii) Glutaraldehyde condensation : The general method described by Habesh¹⁰² was used to conjugate serotonin to bovine serum albumin via glutaraldehyde. Twenty mg of bovine serum albumin and 5 mg of serotonin creatinine sulfate were dissolved in 2 ml of phosphate buffer, pH 7.0. A 50% solution of glutaraldehyde was diluted 1:115 with distilled water, and 1 ml of it was added to above albumin-serotonin mixture, dropwise with continuous stirring. The mixture

was incubated at room temperature for an hour, during which it turned yellow. Following this the reaction was terminated by the addition of 0.1 ml of 1 molar lysine. The reaction mixture was further incubated for half an hour, and then dialyzed against phosphate buffer, at 15-20°C for three days. Figure-11 illustrates the method of conjugation.

iii) Azo-linking using bio-diazotized benzdine⁸⁴

a) Preparation of bio-diazotized benzdine :

14.66 mg of benzdine (0.079 moles) was dissolved in 10 ml of distilled water containing 0.9 ml of 2N HCl. The solution was cooled to 2°C, and to this 1 ml of sodium nitrite solution was added (11 mg, 0.25 moles) dropwise with constant stirring. A yellow colour developed immediately. The reaction was allowed to continue for an hour with intermittent stirring. Aliquots of the reagent were stored at -50°C.

b) Preparation of the serotonin conjugate :

2.25 ml of bio-diazotized benzdine, prepared as above, was added to 1 ml solution of sodium acetate cooled to 2°C. 15 mg of serotonin and 50 mg of bovine serum albumin were dissolved in 10 ml of borate buffer pH 9.0, and cooled to 2°C. The bio-diazotized solution in acetate was then added dropwise to the above albumin serotonin mixture. The pH was maintained

0.1 ml of toluene 2,4-diisocyanate was added to it. The reaction mixture was stirred at 2°C for half an hour, and then centrifuged to sediment the unreacted diisocyanate. The supernatant was carefully removed to avoid contamination with the diisocyanate in the film at meniscus. It was then allowed to stand for an additional hour at 2°C to permit any dissolved toluene 2,4-diisocyanate to react.

b) Conjugation of serotonin to di-isocyanate

treated protein : The supernatant from the previous step was added to the equal volume of borate buffer (pH 9.0) containing 15 mg of serotonin. The resultant mixture had pH 8.7. It was stirred at 37°C for one hour, and then dialyzed against 0.1 M ammonium carbonate at $15-20^{\circ}\text{C}$ for 24 hours, to destroy any unreacted isocyanate groups on the conjugate. The solution of the conjugate was subsequently dialyzed against a neutral buffer at $15-20^{\circ}\text{C}$ for 2 days. Figure-14 illustrates the method of synthesis and the presumed structure of the immunogen.

The five conjugates thus prepared were designated as shown below :-

<u>Reaction</u>	<u>Conjugate</u>
1. Formaldehyde condensation	5-HT-F-BSA
2. Glutaraldehyde condensation	5-HT-G-BSA
3. Azo linking using benzidine	5-HT-BDB-BSA
4. Peptide synthesis using ethyl carbodiimide	5-HT-ECDI-BSA
5. Cross linking using toluene-2,4-diisocyanate	5-HT-TDIC-BSA

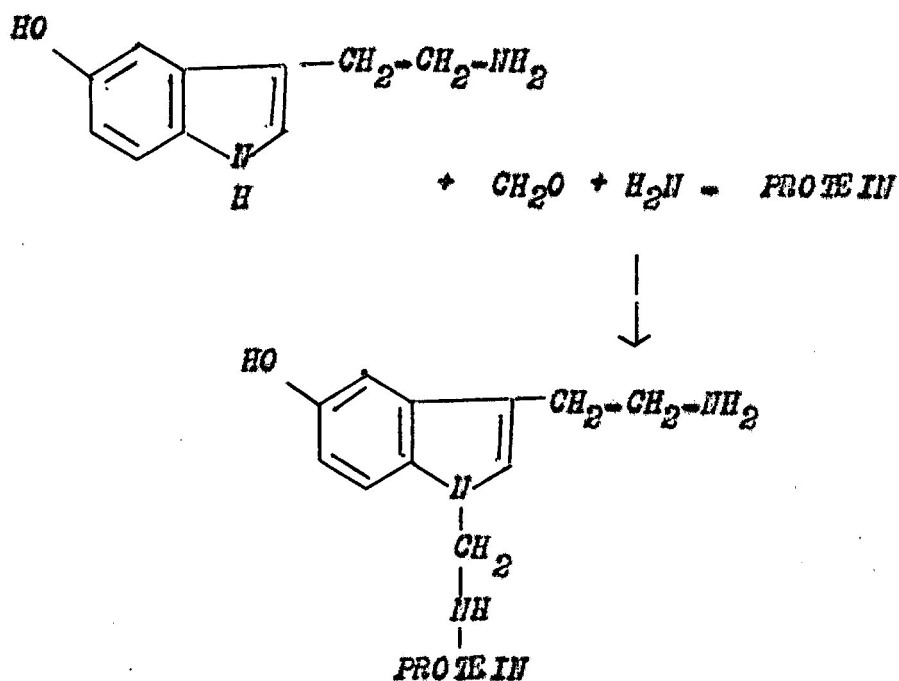


Fig. 10 : PREPARATION OF SEROTONIN ANTIGEN USING FORMALDEHYDE.

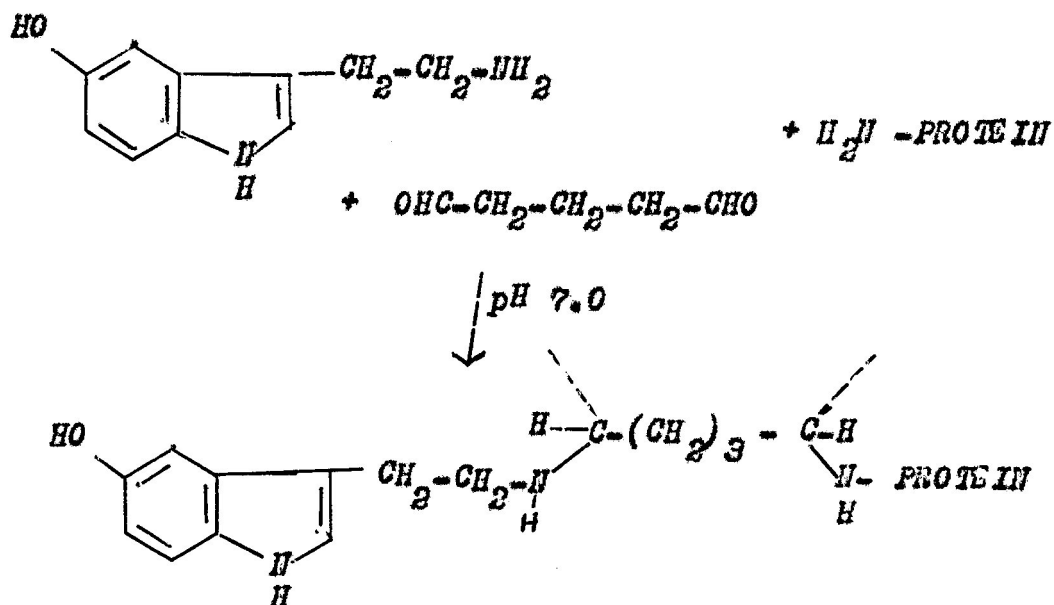
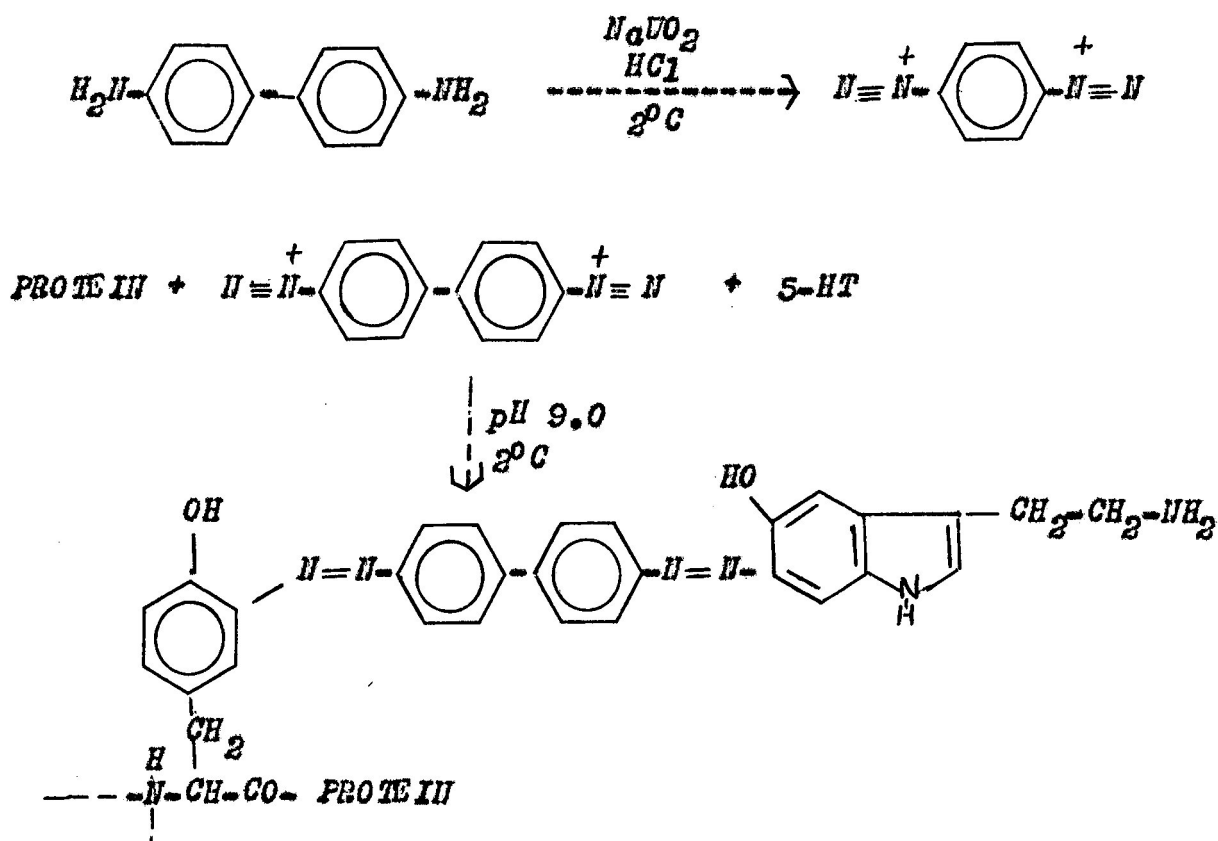
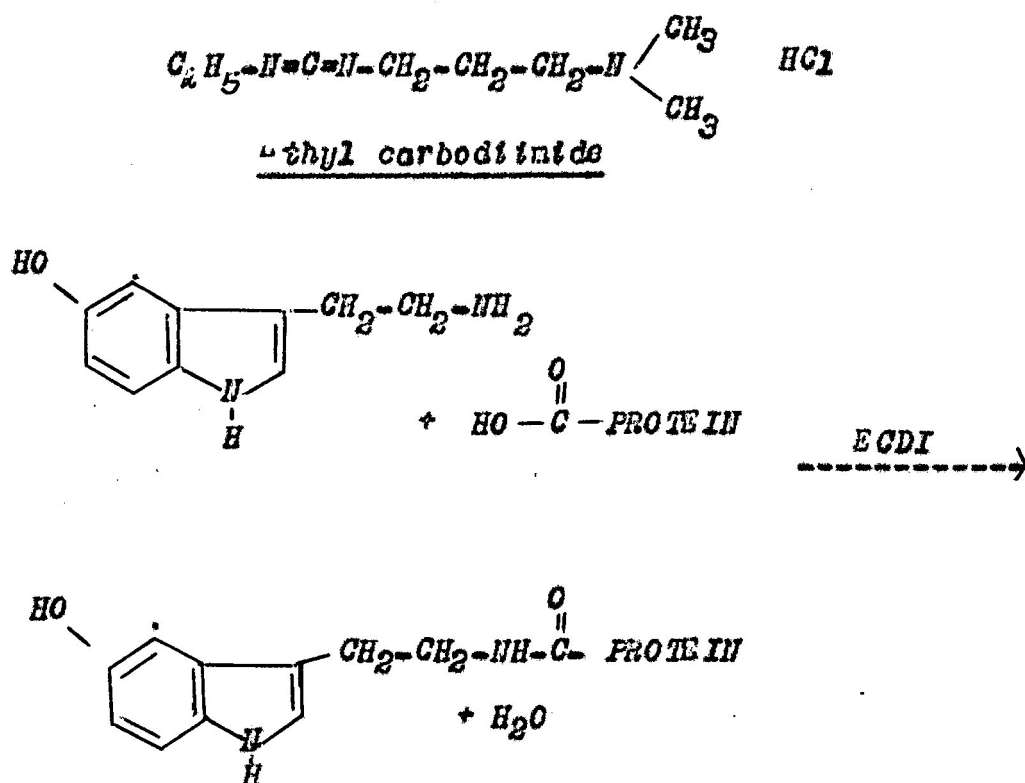


Fig. 11 : PREPARATION OF 5-HT ALBUMIN CONJUGATE USING GLUTARALDEHYDE



**Fig. 12 : PREPARATION OF SEROTONIN PROTEIN CONJUGATE
BIS-DIAZOTIZED BENZIDINE AS BRIDGING AGENT**



**Fig. 13 : PREPARATION OF SEROTONIN-ALBUMIN CONJUGATE
via PEPTIDE BOND FORMATION.**

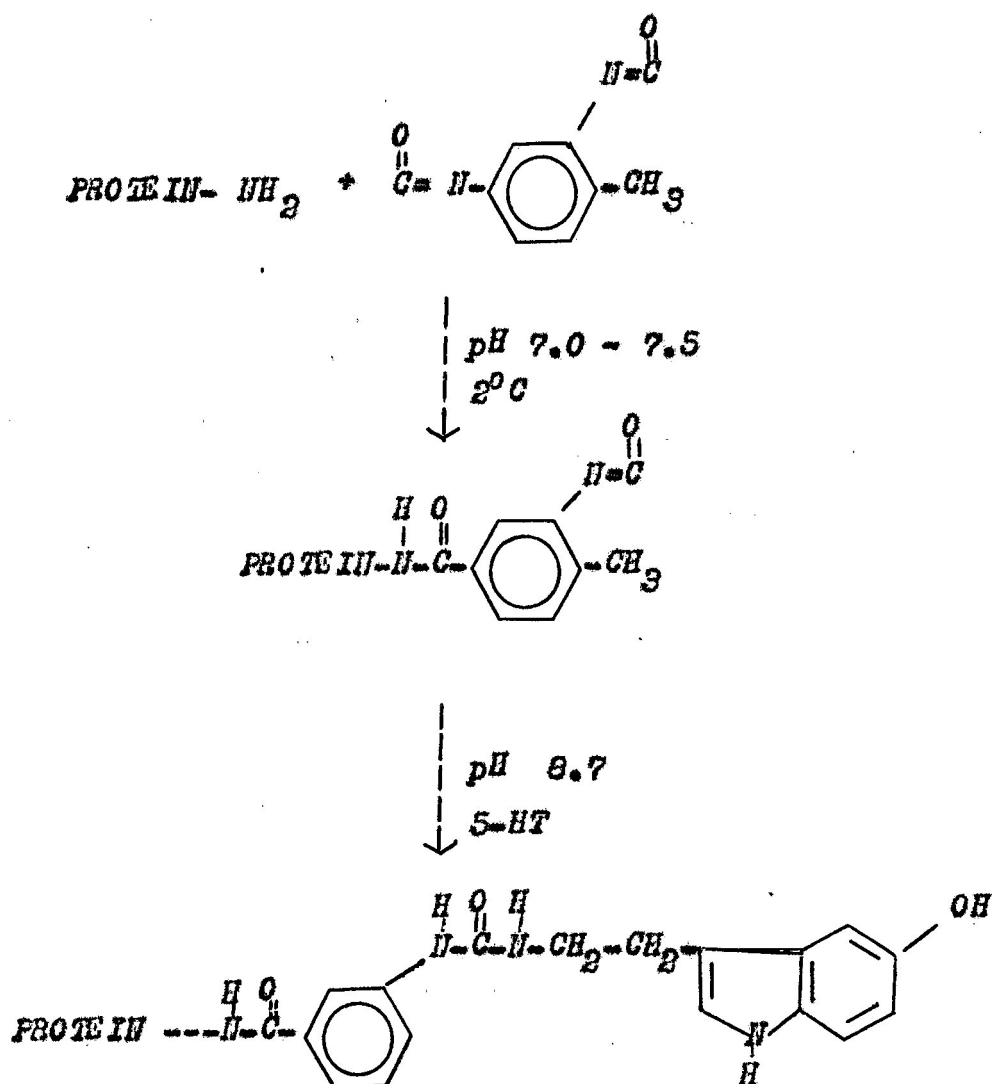


Fig. 14 : REACTIONS INVOLVED IN THE PREPARATION OF
SEROTONIN ANTIGEN USING TOLUENE 2,4-DIISOCYANATE.

Characterization of the conjugates :

i) Protein estimation of the reaction mixtures : This was carried out by Lowry's method¹⁷⁸. The method is based on the following principle :- A copper tartrate complex is allowed to react with the proteins in an alkaline solution. The protein-copper complex then reduces phosphomolybdate to form a blue substance with a broad absorption peak around 750 nm.

Procedure : The following reagents were used :

- (a) 2% sodium carbonate in 0.4% sodium hydroxide ;
- (b) 0.5 % Copper sulphate in 1.0% sodium tartrate.

Reagent A : 1 ml of (b) + 50 ml of (a) ;

Reagent B : Diluted Folin Ciocalteu reagent to make 1.0 N
(Commercial sample is 1.8 N).

To a protein sample containing about 100 micrograms protein (in 0.2 ml volume), 1.0 ml of reagent A was added. It was mixed and allowed to stand for 10 minutes or more. To it 0.1 ml of reagent B was added and mixed rapidly. The mixture was then allowed to stand for 30 minutes and the extinction measured at 750 nm. For confirmation a series of concentrations of proteins within limit of 5 to 100 ng was prepared.

For comparison, a standard protein bovine serum albumin was taken and a calibration curve was computed plotting extinction vs. protein concentration. The protein concentration of the sample was detected from the calibration curve.

(i) Fluorometric analysis of the unreacted serotonin

Unreacted serotonin was extracted from the final reaction mixture using the method of ^{Lovenberg &}Garlick¹⁰⁴. To 10 ml of reaction mixture, 1 ml of 10% zinc sulphate was added, mixed and then 0.5 ml of 1M sodium hydroxide was added. The precipitate of the conjugate was sedimented by centrifugation and the supernatant solution containing the unreacted serotonin was separated¹⁰⁴. The serotonin in the supernatant was assayed fluorometrically. The fluorescence of all supernatants was measured directly by setting the Spectrophotofluorometer at 0 and 100 units of intensity, using the blank solution at λ max. 295 and 330 mμ. The concentration of 5-HT in the samples was determined from a standard graph.

Molar ratio of serotonin to albumin was calculated using the formula

$$R = \frac{(S_0 - S_e) \times (A)}{A \times (S)}$$

Where ,

- R = Molar ratio of serotonin to albumin.
- S₀ = Original amount of 5-HT taken.
- S_e = Estimated amount of unconjugated 5-HT.
- (A) = Molecular weight of BSA.
- (S) = Molecular weight of 5-HT.
- A = Original amount of BSA.

iii) Spectrophotometric analysis :

Spectrophotometric studies were made as described by Freedman *et al.*⁸⁶. The extinction coefficient of the conjugate and control protein was determined at 278 mμ. and 295 mμ. using a spectrophotometer. All solutions were prepared in borate buffer, pH 8.0.

The relative concentrations of protein and serotonin residues in the conjugate were calculated from the formula proposed by Freedman *et al.*⁸⁶, taking $E_{1\text{ cm}}^{1\%}$ of 5-HT, and BSA at 278 mμ as 142, 6.8 and at 295 mμ as 114 and 1.43, respectively.

1.3.2.2 Immunization into experimental animals :

Immunization :

Antibodies were produced in male healthy rabbits weighing 2 kg, using three serotonin-albumin conjugates, namely, 5-HT-BDB-BSA, 5-HT-G. BSA and 5-HT-F. BSA. Eight rabbits were used for each antigen. In all instances, primary injections contained 1 mg and booster doses 500 μg of each of the appropriate immunogen. Aqueous solution of immunogens were emulsified with equal volume of complete Freund's adjuvant, prior to the injection. Primary injections consisted of 2 ml and booster injections of 1 ml of the immunogen emulsion. Following procedures were

adopted for inoculation of the immunogens.

1) Multiple intradermal inoculation¹⁰⁵ :

Prior to the injection, the backs of the animals were shaved and disinfected using 70% alcohol. Using a tuberculin syringe and a rigid needle of 21 gauge, 10 injections each of 0.1 ml were made in the skin over the whole shaved area of the back.

2) Foot-pad inoculation¹⁰⁶ :

Using tuberculin syringe and a fine needle of 26 gauge, 4 injections of 0.25 ml were made into all four foot-pads.

Foot-pad inoculation along with multiple intradermal inoculation was used for primary immunization in all cases.

3) Intramuscular injection¹⁰⁶ :

Thigh muscles of hind limbs were employed for all booster injections. A suitable area of thigh muscle was shaved and disinfected prior to the injection. 0.5 ml of the emulsion containing the desired amount of conjugate was injected into the muscles of each hind limb.

Booster injections at monthly intervals were given until a high titre of antibodies was attained.

Collection of immune plasma¹⁰⁷,

Animals were bled 10-14 days after booster injections. Following methods were used for the collection of blood from immunized animals.

1. Ear vein puncture : The lateral margin of a ear was dry shaved, cleaned with 70% alcohol, coated with vaselline and wiped free of excess with clean gauze. Heat was applied locally on the ear by means of incandescent lamp. A longitudinal incision of 2 mm was made into one of the marginal veins. Venous return proximal to the cut was compressed to assist flow of the blood. The blood was collected in 1% EDTA solution. The method was employed for small trial bleedings of 5-10 ml.

2. Heart puncture : A animal was restrained on a table with the flanks exposed. The thoracic area was cleaned with 70% alcohol, the excess being removed with sterile gauze. The heart beats were palpated with middle finger wetted with alcohol. The cage was penetrated at to the site where heart beats were felt most prominently. The needle was advanced in the cage about 3 mm until it transmitted the heart beats and the blood was removed by applying slight negative pressure. The blood was collected in siliconized glass bottles containing 1% EDTA to a final

concentration of 1 mg/ml. The method was used for occasional bleedings of 40-50 ml.

3. Terminal bleeding : The animal was anaesthetized by injecting sodium pentobarbitone 30 mg/kg body weight, intraperitoneally. The anaesthetized animal was attached to a animal operation table and the throat region was shaved free from hair. Stretching the skin side ways, a incision was made to the left of the trachea. Fascia was carefully cut open and muscles were separated to visualize the carotid artery. The artery was clamped and cannulated with siliconized glass artery canula. When the clamp was released, the blood streamed out from the open end of the canula. The blood was collected in EDTA solution until the flow was ceased. A maximum of 120 ml of the blood was collected from each animal. The method was used for final collection of blood from the animals which had attained high titre of antibodies.

Separation of Plasma¹⁰⁸ :

The blood obtained was cooled immediately and centrifuged at 4°C in plastic tubes at 1000 r.p.m. for 15 minutes. The supernatant was transferred to new tubes, and the thrombocytes were separated from plasma by centrifugation at 7000 r.p.m., for 30 minutes in a refrigerated centrifuge. The entire process of plasma separation was

performed using siliconized laboratory glasswares. The plasma thus obtained was stored at -20°C until use.

1.3.2.3 Characterization of the Antibodies :

Detection : Antibodies in the plasma were detected by agar gel diffusion method as described by Ouchterlony¹⁰⁹.

Principle : When an antigen and its specific antibody are allowed to diffuse towards each other in a gellified medium, precipitin line is formed.

Method : 1% agar was prepared by heating it in normal saline. The agar was filtered hot through moist cotton and to it sodium azide in final concentration of 0.1% was added as preservative. The 3 ml of the agar while still in liquid condition was layered carefully on cleaned dried microscopic slides. The agar was allowed to set at 10°C and wells were cut in it using a standard template. The wells had constant diameter of 3.0 mm and inter-bore gap of 3.0 mm. Using a fine capillary pipette native immune plasma was placed in the central well and 0.1 % solution of antigen in phosphate buffer pH 7.4, in the surrounding wells. The slides thus prepared were incubated for 24 hours in a petri dish containing balls of moist cotton to facilitate diffusion of antigen and antibodies.

After full development of precipitin lines, the agar was washed for 3-4 days with normal saline to remove unreacted antigen and antibodies from the preparation. Finally, the gel was washed with distilled water and dried to a thin film in hot air-oven. The precipitin lines were then stained with 0.5 % Amido black 10B in methanol-acetic acid-water (40:10:50) for 10 minutes¹¹⁰. Excess stain was removed by washing with 1% acetic acid solution. Final washing was done with 5% glycerol which prevents agar from cracking.

The precipitin reaction in agar gel was performed for following studies :-

a) Control and experimental screening of plasma for the presence of antibodies. For experimental screening, plasma from individual immunized rabbits and corresponding conjugates used for immunization was used. Control studies were performed using plasma obtained from normal rabbits.

b) Cross reaction of the immune plasma with the carrier protein, bovine serum albumin.

The native immune plasma was placed in the central well and the bovine serum albumin as 0.1% solution in phosphate buffer, pH 7.4, in the surrounding wells.

c) To study specificity of the antibodies for in the immune plasma for serotonin

Principle : The antibody population raised to the carrier hapten conjugate can be rendered specific to the hapten if antibodies to carrier which cross react, are absorbed out.

Method : The antibodies to the carrier, bovine serum albumin was absorbed using the following method¹¹¹.

To a series of tubes containing 0.5 ml of undiluted plasma, 0.5 ml of two-fold serially diluted 0.1 % bovine serum albumin solution in borate buffer pH 8.0 was added. The tubes were then incubated for 1 hour at 37°C and for 24 hours at 4°C. Resulting precipitates were centrifuged and supernatants tested for specificity to the conjugate along on the agar gel.

Antibody titre : The absolute amount of the antibodies in the plasma was determined by quantitative precipitation reaction¹⁰⁰.

Principle : When antigen and antibody are present in optimal proportions, all of the antibodies capable of forming precipitate get flocculated.

Method : To a 0.5 ml of undiluted plasma in a series of tubes was added 0.5 ml of two-fold dilutions of the conjugate 5-HT-BDB-DSA. The tubes were then incubated at 37°C for one hour and at 4°C for 2 days. The tube showing maximum precipitation was taken optimal for the precipitation test. The precipitate was centrifuged out and washed with phosphate buffered saline twice to eliminate serum proteins trapped in antigen-antibody aggregate. The washed precipitate was analysed for nitrogen content by Kjeldahl's method¹¹².

The precipitate was transferred to a Kjeldahl's flask and to it was added a pinch of digestion mixture, 3 ml concentrated sulphuric acid and some pumice stone. The flask was heated on a low flame until the mixture turned yellow and then heating was continued on high flame until the mixture was almost colourless. The flask was cooled and the contents were diluted to 50 ml. Two ml of the diluted mixture was heated with 10 ml of 10% NaOH in a round bottom flask. Ammonia evolved was absorbed in 5 ml of $\text{N}/70 \text{H}_2\text{SO}_4$. The excess of acid was then back titrated using $\text{N}/70 \text{NaOH}$ and Thastiro indicator. End point was change in colour from orange to green. Initially distilled water and sodium hydroxide used in the method were assured free from nitrogen impurity using the same procedure. Two ml of ammonium sulphate solution (0.2 mg N/ml) was used as a standard for comparison.

1.4 RESULTS

1. The Conjugates :

a) Colour, Solubility, etc. : The conjugates of albumin to serotonin prepared by using bis-diazotized benzidine as a reagent, were dark brown in colour. After dialysis and lyophilization when the conjugated product was resolubilized in normal saline, it exhibited slight turbidity.

The conjugates obtained by glutaraldehyde and formaldehyde reactions were light yellow in colour. After lyophilization the conjugates could be completely resolubilized in normal saline.

The conjugates obtained using ethyl carbodiimide and toluene-2,4-diisocyanate as reagents were insoluble in normal saline and were not used for immunization.

b) Protein content : During conjugation reactions and the purification procedure there was loss in the protein content of the reaction mixtures (Table 2). The highest yield of the soluble conjugated product was obtained with bis-diazotized benzidine cross linking reaction. The estimated protein concentration in the purified BDB reaction product was 97.5% of the original protein taken.

c) Serotonin-protein molar ratio : From ultra-violet absorption data of BSA-G-5-HT and BSA-F-5-HT conjugates, it was concluded that coupling of serotonin to the protein carrier had occurred. The optical density of both the conjugates were in excess to the optical density of bovine serum albumin at 278 mμ and 295 mμ (Table 3). This shift in absorbancy for the conjugates was considered to indicate that serotonin had been covalently bound to the carrier protein. The number of serotonin groups per molecule of bovine serum albumin as determined by the formula of Freedman *et al.*⁶⁰, were 32 and 35 for 5-HT-G-BSA and 5-HT-F-BSA conjugates respectively.

The original concentration of serotonin in BDB reaction mixture was 15 ng. At the end of the reaction when unreacted serotonin was measured spectrofluorometrically, only 4.25 ng of it was recovered. From this, it was assumed that the unrecovered amount of serotonin had got conjugated with the protein bovine serum albumin. Based on this assumption calculated molar ratio of serotonin to albumin was 36:1.

2. Immunization and collection of the blood :

During the course of immunization the rabbits developed expected adjuvant arthritis (inflammatory lesions

in joints) and the adjuvant granuloma at all sites where adjuvant-antigen mixture was injected. Unfortunately, in four cases out of twenty-four, clinical abnormalities during the immunization period were so much advanced that the animals got to be sacrificed.

Following completion of immunization schedule, individual bleedings from the rest 18 rabbits were tested for the presence of antibodies to the appropriate immunogens. Agar gel diffusion method as described by Ouchterlony was used. Precipitin lines were developed in all the cases. Normal rabbit plasma taken as control showed no precipitin lines for the conjugates. From this, it was concluded that all the rabbits immunized with serotonin-albumin conjugates have produced antibodies to it.

The plasma obtained from rabbits immunized with BDB conjugate, exhibited the most significant precipitin reaction. The precipitin lines on the agar gel containing undiluted BDB plasma and 0.1 % solution of BDB conjugate in adjacent wells, could be observed within 30-90 minutes. This indicated that comparative antibody titres were highest in the rabbits immunized with BDB conjugate. Based on this observation only the rabbits immunized with BDB conjugate were bled for the want of antibodies.

(i) Number of rabbits bled	...	7
(ii) Average bleed from each rabbit		120 ml
(iii) Total volume of immune plasma obtained	...	...
		400 ml

5. The Antibodies :

a) Specificity to serotonin : Absorption of the antiserotonin plasma with the carrier protein, bovine serum albumin, resulted in the total removal of antibodies to the carrier. As shown in the figure 15-16, the absorbed antiserotonin plasma exhibited specificity for serotonin on reaction with 5-HT-BDB-BSA conjugate and did not react with the carrier protein.

b) Absolute antibody titre : The quantitative precipitation test when performed using 0.5 ml of neat antiserotonin plasma and two-fold serial dilutions of 0.1 % solution of 5-HT-BDB-BSA conjugate, exhibited visible turbidity/flocculation in all the tubes. The tube containing 0.5 ml of plasma and 1:1 dilution of the conjugate showed the maximum amount of the flocculation. The flocculated material from this tube when analysed for nitrogen content by Kjeldahl's method showed the nitrogen value of 1.14 ng/ml. The antibody content of the plasma calculated from these results was 6.2 ng/ml expressed as protein.

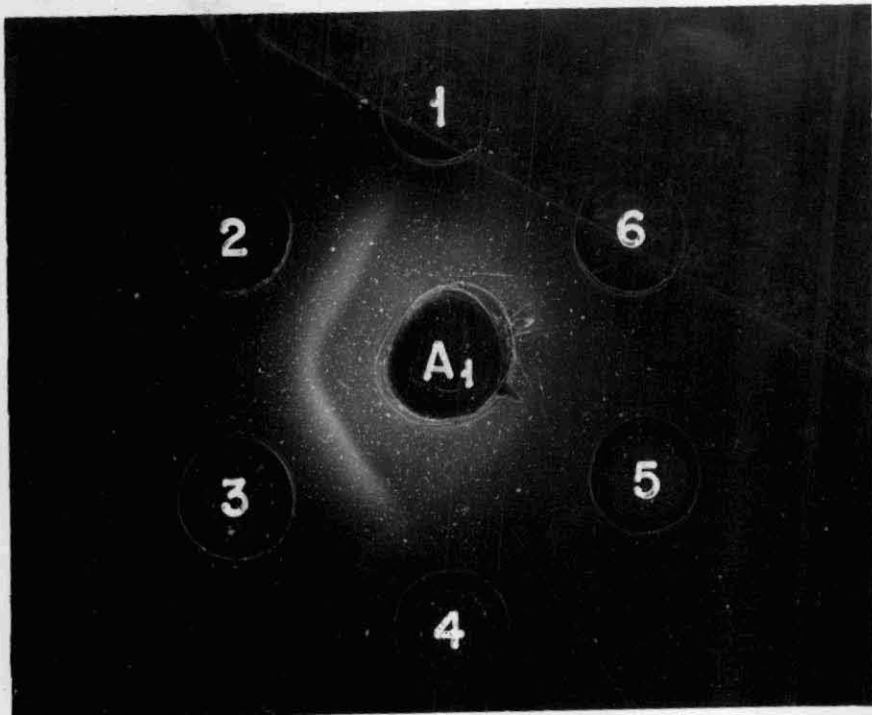


Fig. 15: Detection of antibodies to 5-HT by agar gel diffusion test (I).

A_1 = Immune plasma ; 1,4 = Normal saline ;
 2,3 = 5-HT, protein conjugate;
 5,6 = Bovine serum albumin; = Precipitin lines.

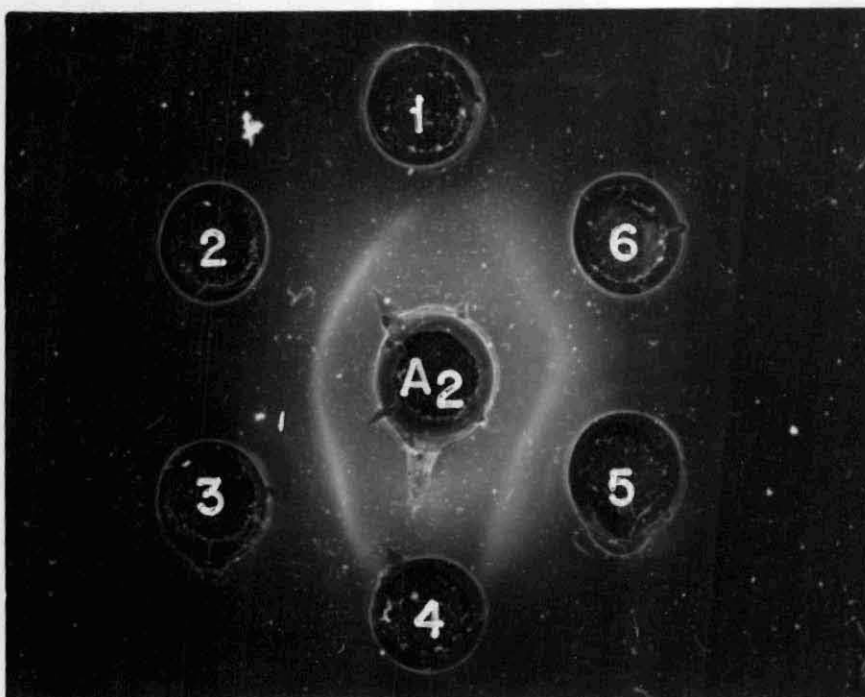


Fig. 16 : Detection of antibodies to 5-HT by agar gel diffusion test (II).

A_2 = Absorbed immune plasma; 1,4 = Normal saline;
 2,3 = 5-HT, protein conjugate; 5,6 = Bovine serum albumin;
 = Precipitin Lines.

TABLE- 2 : Conjugation of serotonin to albumin, original and estimated protein in purified hapten protein conjugates.

Sr.No.	CONJUGATE	Original protein conc. in reaction mixture	Estimated protein conc. in purified conjugated product.	% loss
		ng/ml	ng/ml	
1	5-HT-F-BSA	5.0	1.20	76
2	5-HT-G-BSA	1.0	0.350	65
3	5-HT-DDB-BSA	2.0	1.95	2.5

TABLE- 3: Conjugation of serotonin to albumin, estimate of serotonin-albumin molar ratio from spectral analysis .

Conjugate	Optical density 278 mμ.	Optical density 295 mμ	Moles of serotonin per mole of albumin
BSA-F-5-HT	0.230	0.165	35.26
BSA-G-5-HT	0.230	0.150	32.23
Bovine serum albumin	0.05	-	-

1.5 DISCUSSION

Importance of ultraviolet absorption spectra in determining the concentration and structure of chemical compounds is quite a much. Molar absorptivity is a characteristic of a compound. Any shift in its value indicates modification in the structure of the compound¹¹². From the ultraviolet absorption value of the bovine serum albumin and its serotonin conjugates (5-HT-F-BSA and 5-HT-G-BSA), it was concluded that coupling of the serotonin to the carrier protein had occurred. Thus, whereas, the optical density of BSA at 278 mμ was 0.05, the optical density of its conjugates was 0.150 to 0.165. This shift in the absorbancy of the protein preparation was considered to be indicative of the serotonin molecules had been covalently bound to the albumin.

Number of moles of 5-HT attached per molecule of albumin were calculated considering the conjugates as binary mixture of albumin and 5-HT. Calculations done using the formula of Freeman⁸⁶ revealed 32-36 moles of 5-HT per molecule of albumin. However, these calculations were considered approximate as they were based on the assumption that spectral properties of albumin and 5-HT had not changed during conjugation.

Similarly reduced amounts of 5-HT recovered from bis-diazotized benzdine reaction, indicated that reaction of 5-HT with albumin had occurred. The calculated ratio of serotonin to albumin was 36:1. For the calculation purpose it was assumed that all unrecovered serotonin had coupled to the protein.

Frackel-Conrat et al.^{113, 114} have studied extensively the reaction of proteins with formaldehyde. They observed that the first step in the reaction involves the free amino groups with the formation of amino methylol groups which then condense with other functional groups (e.g., phenol, imidazole and indole groups) to form methylene bridges. Hence it was assumed that in formaldehyde condensation reaction the point of condensation of formaldehyde is N of the indole nucleus of serotonin (Figure-10) .

It has been shown that diazonium derivative of benzdine predominantly reacts with aromatic compounds containing powerful electron releasing groups¹¹⁵. The coupling takes place para to electron releasing group via electrophillic attack. On this basis it was presumed that tyrosine and histidine amino acids of protein and sixth position of the aromatic ring of serotonin were involved in bis-diazotized benzdine reaction (Figure-12, p. 41).

The reaction had the advantage that main metabolic sites of serotonin such as phenolic hydroxy group and aliphatic amino group, were possibly not distorted by the reagent. This aspect is important because the resultant antibodies are usually directed against the most exposed part of the molecule and it is essential to keep exposed these groups to develop antibodies specific to 5-HT.

Glutaraldehyde is an excellent cross linking agent¹¹⁵. Its mechanism of reaction is analogous with formaldehyde. Habeeb *et al.*¹⁰² have shown that it is very reactive towards the N-terminal amino groups of peptides as well as the α -amino groups of amino acids. The sulfhydryl group of cysteine is also very reactive with glutaraldehyde. This suggested that in glutaraldehyde condensation reaction, reaction had occurred predominantly at α -amino groups of lysine and aliphatic amino groups of serotonin (Figure-11, p.40).

Extensive loss of protein in formaldehyde and glutaraldehyde reactions can be attributed to -

(i) Precipitation due to extensive polymerization of the bovine serum albumin ; (ii) Denaturation under experimental conditions and (iii) Loss by hydrolysis during reaction as well as during dialysis.

All the three conjugates used for immunization elicited production of antibodies in the rabbits, as borne out by the results of immuno-diffusion on agar gel. This indicated conjugation procedure, immunization schedule, animal species chosen, adjuvant used were appropriate and complementary to each other for production of the antibodies.

The immune plasma was rendered specific to serotonin by simple absorption with the carrier protein. As expected the absorbed plasma exhibited specificity only for hapten-albumin conjugate. This obviously indicated that immune plasma contains antibodies specific to serotonin.

Antibody titre is the indication of the amount of antibodies as moles or ng. H or ng protein present per ml. of original serum. The value is indicative of the immune response of the host producing antibodies. The best method to determine is to precipitate the antibodies in a serum under optimal conditions. Quantitative precipitation test is used for the purpose. Antibody titre for immune plasma to 5-HT, as determined by quantitative precipitation test was 6.2 ng/ml expressed as protein. However, this represented combined value of antibodies to the carrier and antibodies to the hapten.

Antibodies to serotonin have been produced by others. These authors have conjugated the hapten to antigenic protein utilizing mainly aromatic ring and aliphatic amino group of serotonin. (Figure-8, p.21 ; Figure-9, p.22).

S E C T I O N II

PHARMACOLOGICAL STUDIES

2.1 INTRODUCTION

The advent of preparing antibodies to various drugs and biological substances has led to extremely specific and sensitive quantitation of these substances. However, the usefulness of these antibodies should not end with assay methods. With digoxin antibodies, Smith *et al.*⁴ have demonstrated that antibodies can be used clinically, to reverse the severe life threatening intoxication of the drug. The analgesic effect of morphine can be decreased by active immunization of mice against morphine¹¹⁷. Ferretra *et al.*⁶ have shown that antibodies directed against prostaglandin can inhibit the inflammatory reactions in which they are said to be involved.

The specificity conferred on these proteins makes them invaluable pharmacological agents for research into the mechanisms of action of physiological and pharmacological substances. Preparation of antibodies to 5-HT and their use for immunopharmacological studies have been described by various authors^{5,118}. The possibility that some of the experimental inflammatory conditions in which involvement of 5-HT is unclear, can be clarified using antibodies to 5-HT as pharmacological agent promoted us for the following work :-

2.2 LITERATURE REVIEW :

2.2.1 Physiological role of serotonin :

The indolealkylamine, serotonin, has been a favoured biogenic mediator for research in recent years. This is largely due to its ubiquitous distribution in nature and due to its involvement in various vital physiological processes of mammalian tissues. In the brain it is considered as putative neurotransmitter¹¹⁹. An association has been made between serotonin and emotional stability¹²⁰. Jouvet¹²¹ has assembled considerable evidence that it is the determining transmitter involved in slow wave and paradoxical sleep. In gastro-intestinal tract it is believed to be involved in physiological mechanisms of the peristaltic reflexes¹²². Due to the fact that during clotting there is release of serotonin, it has been suggested that it has role in various aspects of coagulation¹²³. Erspaner¹²⁴ has suggested that it should be considered as a renal hormone important for regulation of urine flow in normal and pathological conditions. But the most important is its role in inflammatory and anaphylactic reactions. Parrat^{125,126} and Rowley¹²⁷ have assembled considerable evidence that it is the determining mediator involved in inflammatory and anaphylactic reactions in rodents. Much confusion remains regarding the relationship

of it with histamine and prostaglandins which are also released during inflammatory reactions. The studies have been made utilizing pharmacological agents such as methysergide, 2-Bromo-LSD, Compound 48/80, Reserpine. Unfortunately, these agents are considered nonspecific and there is no validity of the conclusions reached. The recent advancement in studying its role in physiological processes, is to use antiserotonin antibodies as specific pharmacological agent. Its physiological role in central nervous system⁵ and in gastro-intestinal organs¹¹⁸ is being under study with antibodies specific to it. Here, attempt has been made to study involvement of serotonin in acute inflammatory conditions in rodents, using antiserotonin antibodies.

2.2.2 Inflammation : Basic concepts :

(i) Acute and chronic inflammation : The term inflammation is derived from a latin verb 'inflamare' which means 'to burn'. As early as 38 A.D., Celsus, first explained four cardinal signs of inflammation as local redness, heat, swelling and pain. In the recent times, inflammation is defined as the response of living tissue (micro-circulation) to local injury which leads to the local accumulation of blood cells and fluid. The overall process, evaluated against the broad perspective of evolution, is a useful one. It has a primary significance of defence against

bacterial invasion and injury.

Broadly, the phenomenon of inflammation can be differentiated as acute and chronic with the following salient features :

- a) The acute response can be brought about by an immunological mechanism, but its principal characteristic is that it can be elicited by any injurious agent.
- b) Although the acute response is, basically, stereotyped a slight modulation, dependent upon the injurious agent may be expected.
- c) The chronic form represents, in most cases, an immune response with lymphocytes and plasma cell predominating in the inflammatory reaction. In some instances when the irritant is non-antigenic such as lipids or an insoluble foreign body macrophages predominate.
- d) Generally, polymorphonuclear leukocytes and mononuclear leucocytes are observed to be associated with acute and chronic forms respectively.

(ii) Oedematous agents and Inflammatory agents :

The irritants which only elicit exudation of oedema fluid but no cellular infiltration are designated as

oedematous agents. On the other hand the irritants which elicit both mobilization of inflammatory cells and tissue oedema are called inflammatory agents.

Oedema resulting from the injection of oedematous agents is specifically inhibited by their respective antagonist (e.g. serotonin oedema by methysergide) but not by antiinflammatory agents. Conversely, oedema induced by inflammatory agents is inhibited by antiinflammatory agents (e.g. carrageenan oedema by salicylates or indomethacin).

Finally, in defining agents as being oedemagenic or inflammogens it is imperative that the species and site of irritation be specified since, for example, biogenic amine serotonin is a powerful oedemogen in rats whereas in rabbits it is ten-times less active.

A wide variety of the substances such as carrageenan, kaolin, dextran, have been described as inflammogen of the rat hind limb and pleural cavity^{123,129}. It is the algal polysaccharide, carrageenan which is most widely employed as inflammogen for pharmacological investigations¹³⁰. The oedema produced by this agent in rat hind paw is biphasic and responds to standard antiinflammatory agents. The mechanisms for its phlogistic effects are not well understood.

The mediators such as histamine, serotonin, bradykinin and prostaglandins have been attributed roles for the effects in question¹²⁹. Whether these mediators have individual, sequential or potentiative effect, is under investigations.

(iii) Acute Anaphylaxis (as inflammatory reaction) :

The deposition of antigen-antibody complexes (immune complexes) on mast cells in the body is associated with an inflammatory reaction ranging from a mere increase in vascular permeability to severe haemorrhagic and necrotizing lesion. This allergic reaction so called acute anaphylaxis is the type of acute inflammatory process. The mechanism underlying the process is as follows :-

When an immune system of a individual is exposed to a massive dose of antigen, it produces antibodies. Among the large population of antibodies, some belong to IgE class (reaginic antibodies). The Fc portion of these globulins have affinity for tissue mast cells and following contact, the globulins get fixed on them. When the individual is re-exposed to the same antigen, interaction of it with reaginic antibodies at mast cells result in an anaphylactic reaction. The process is facilitated by release of mediators such as histamine, serotonin, bradykinin, SRS-A. In rodents,

predominant among these mediators are histamine and serotonin. These amines induce transient enhancement of vascular permeability and cellular (smooth muscle) contraction, leading to vascular leakage, haemorrhage and thrombosis.

(iv) Mediators :

Endogenous chemical agents which are

- a) derived from the injured tissues ;
- b) demonstrably present during inflammatory reaction and absent when the reaction has subsided; and
- c) can be inhibited by a specific antagonist, leading to diminution of the aspect of inflammatory reaction for which it is responsible

termed as mediators. The depletion of tissues for the particular mediator leads to diminution of inflammatory response.

Mediators of the acute inflammatory reactions are classified into two major groups - those derived from plasma and those derived from tissues. In plasma there are two major mediator producing systems - a) the kinin system ; b) complement system. Predominant mediators of the kinin system are - bradykinin and kallikrein. On the other hand, major mediators of complement system are C_3 and C_5 fragments.

The important mediators derived from tissues are - vaso-
active amines (histamine, serotonin), acidic lipids (slow
reacting substances of anaphylaxis, prostaglandins), products
of lymphocytes, substance P, and cyclic AMP.

Among these mediators histamine and prostaglandins
are potentially responsible for increased vascular permeabi-
lity and chemotaxis of leukocytes. Bradykinin, substance P
and serotonin are considered selective mediators for
vascular permeability effects. The complement components
 C_3 and C_5 are selective potent chemotactic factors. The
recent report suggests that biogenic amine serotonin has
chemotactic activity too.¹⁹¹

2.2.3 Inflammation : Histopathological aspects :

The process of acute inflammatory reactions is
characterized by the events such as vascular leakage of the
oedema fluid and infiltration of the leukocytes at the
affected sites. Although the whole process is, basically,
stereotyped, a slight degree of modulation occurs in these
events, depending on the injurious agent. The process is
initiated by injury such as (i) physical stress (heat,
trauma); (ii) chemical irritants (acids, alkali, turpentine
oil); (iii) bacterial and viral invasion; (iv) immunolo-
gical reactions (antigen-antibody reactions at mast cells).

1. Vascular leakage :

It is the exudation of the plasma from inflamed microscopic vessels which become leaky without being totally disrupted. The plasma seeped out contains much of the blood components except R.B.C., W.B.C., platelets, colloidal material and large molecules as that of lipoproteins. The main permeability barrier is the endothelium. Among the cellular mechanisms that may cause the endothelial layer to leak out, followings have been suggested.

(i) Formation of intracellular gaps by endothelial contraction : The biogenic mediators such as serotonin, histamine, induce ultrastructural changes leading to contraction of endothelial cells of microcirculation¹³²⁻¹³³. The process of this phenomenon can be described as :
 (a) bulging of the cell body into the lumen , (b) a change in the shape of nucleus from oval to rounded, (c) appearance of multiple folds in the nuclear and cell membrane and (d) contraction of the myofilaments and appearance of an inter-cellular gaps on one or both sides.

(ii) Transcellular leakage : It appears that there exist small canals in normal endothelium¹³⁴. Under pathological conditions their size and/or number increases and as a consequence leakage of plasma follows.

(iii) Unzipping of the endothelial junctions : When tissues are exposed to relatively mild stress, as produced by heat or trauma, leaks often appear between endothelial cells¹³⁵. The process is explained as physical (mechanical) rupture of the delicate endothelial junctions leading to detachment of the cells.

The other very important facet of vascular leakage is the functional pattern e.g., its development with time. In some cases there is a burst of the exudation fluid immediately following to the injurious agent and lasts for a short period. Its average duration is of the order of 15-30 minutes and structurally it exhibits high specificity for venules (100 μ diameter). The response is claimed by the autocooids such as serotonin and histamine liberated in the injured area¹³⁶.

In some other cases where phlogistic agents such as carrageenan¹³⁷, turpentine¹³⁸ and kaolin¹³⁹ are employed, delayed prolonged leakage (of onset of few hours and duration from few hours to days together) is observed. The leakage is predominantly from the capillaries of the injured area. The theories explaining the phenomenon are still immature, but it is believed that prostaglandins are possible mediators of the process.

2. Cellular Infiltration :

The important histopathological feature of inflammation is infiltration of leucocytes. In the early stage the predominant cells are neutrophils while in the later stage it is monocytes. Broadly the process involves two steps :

i) Pavementation - It is the initial step and only includes adherence of the leucocytes around the endothelial layer in the lumen. The phenomenon is explained by (a) physical entrapment by glue-like substance present on the luminal surface;¹³⁹ (b) weak chemical attraction via calcium ions on the luminal surface¹⁴⁰.

ii) Emigration of the cells : Chemotactic factors are chemical substance which have property to enhance migration of cells towards them. They are generally derived from C_3 and C_5 fragments of the complement, neutrophils and collagen.

Emigration of the pavementing leucocytes is facilitated by these chemotactic principles which seep into the interendothelial junctions and thereby attract leucocytes. The mechanisms involving chemotaxis responsiveness are controversial. The recent data¹⁴¹ suggests that

the cells have surface receptors for the chemo-attractants. Leucocytes can sense a difference in concentration gradient of the chemotactic molecules across its own dimension to follow directional movements.

3. Phagocytosis :

The most important task for infiltrated neutrophils and monocytes is ingestion and disposal of foreign (inflammatory) particles. Cytoplasmic granules of these cells are rich in the enzymes such as peroxidases, lysozymes and alkaline phosphatases. It is believed that during the process chemotactic factors and vasoactive agents such as prostaglandins are generated¹⁴². These substances boost the leakage of more leucocytes which assist the process of phagocytosis.

2.2.4 Inflammation : Mediators :

1. Serotonin :

Distribution : Serotonin has been found in most tissues and physiological fluids, and its wide distribution make it ideally suited as a mediator of acute inflammation. Enterochromaffin cells of gastro-intestinal tract, blood platelets and the tissue mast cells are particularly rich in this amine. Platelets and mast cells are generally believed to constitute the major source of serotonin

released by injury or anaphylaxis.

Synthesis, storage, release and metabolism : Serotonin is synthesized from the amino acid tryptophan by the action of the enzymes tryptophan hydroxylase and 5-HTP decarboxylase. Following synthesis it is stored in various cells such as mast cells, enterochromaffin cells of gastro-intestinal tract, and platelets in an inactive form. The mechanism of serotonin release is a subject that has attracted much speculations and which remains obscure. There are two main hypotheses - i) the splitting of a hypothetical bond linking serotonin to a protein and ii) leaking of serotonin through a cellular or subcellular membrane rendered permeable or destroyed.

There seems little doubt that during the initial phase of acute inflammation the release of serotonin is from mast cells, which may be considered as self contained pharmacological laboratory. Evans¹⁴³ has recently listed the biological active substances within these cells as follows :

5-HT, histamine, acid phosphatase, ATPase, dopa-decarboxylase, histidine decarboxylase, 5-HTP decarboxylase and heparin forming enzymes. In the immediate reaction to the injury the mast cells degranulate explosively and discharge their contents. The most important components

from the vascular permeability aspect being serotonin and histamine.

The amine is metabolized by the enzyme mono-amino oxidase to form 5-hydroxyindole acetic acid.

Serotonin and vascular permeability : In rodents, this amine is a powerful dilator of the capillaries. This action appears to be directly on the contractile mechanism and is independent of the nerve supply. In rat this amine is approximately ten-times more active than histamine in increasing vascular permeability, whereas in the guinea-pig or rabbit it is ten-times less active. Its action in increasing vascular permeability are complicated, due to dual effect that may be elicited according to the concentration of serotonin injected. Thus, injection of a concentration below 20 ug/ml in rat leads to vasodilation and increased vascular permeability. In higher dosage no leakage of plasma protein occurs, indeed a vasoconstriction response is elicited. As with histamine this amine exerts its permeability enhancing effect by causing spaces to appear between endothelial cells. Its duration of action following a single intradermal injection is between 20-30 minutes.

It is only weakly active in causing leucocyte emigration¹³¹ although its effect is greater than that of histamine.

Evidence for the involvement of serotonin in inflammatory conditions : Originally, Rowley¹³⁷ postulated serotonin as a mediator of vascular injury produced by agents which damage mast cells in rats. In 1957, Spector et al.¹⁴⁴ showed the presence of it in pleural exudates collected from rats injected with .1 ml of turpentine intrapleurally. In the same year, Farrat et al.¹²⁶ showed involvement of serotonin in anaphylactoid reactions in rodents. At present considerable evidence is available to show that serotonin is released after chemical and physical injury. When small quantity of turpentine is injected into pleural cavity of rats and the resultant exudate is analysed, fairly high concentration of serotonin is found in first three hours¹⁴⁵. Further evidence obtained with depletion studies in turpentine induced pleurisy suggest that 5-HT act in a sequential fashion to histamine as a mediator.

Similarly, it was found that irradiation of the rat intestine, leads to a profound depletion of serotonin content of the bowel walls. The loss in serotonin levels

was most striking between 24-48 hours¹⁴⁶.

Evidences also indicate that 5-HT is involved in immunological type of injuries¹⁴⁷. It has been shown to be released from sensitized platelets of rabbits, dogs and guinea-pigs following the addition of antigen in the presence of specific antibodies. Small amounts of serotonin has been found in the circulation of rabbits during hypersensitivity reactions¹⁴⁸. Inhibition studies with 2-bromo lysergic acid show involvement of serotonin in Arthus and Schultz-Dale reactions. Recently it has been shown that in acute pinnal anaphylaxis serotonin is released in sequential fashion to histamine as a mediator¹⁴⁹.

Controversies : The concept of serotonin as a mediator of acute inflammatory reactions is still premature as the evidences available are not adequate and are controversial. There exist a fair amount of confusion pertaining to the role of it in chemically induced oedema in rat pleural cavity. While, a large body of reports^{129, 144, 150} have suggested involvement of serotonin in the inflammatory reaction in this cavity, the recent report imposes only prostaglandins as causative mediators¹⁵¹. Although serotonin is ubiquitiously distributed in cutaneous mast cells of rodents, inhibition studies with 5-HT antagonists in animals exposed to chemical injury have been shown to be

ineffective¹⁵². There are controversial reports with reference to chemotactic activity of 5-HT during acute inflammatory reactions^{131, 153}.

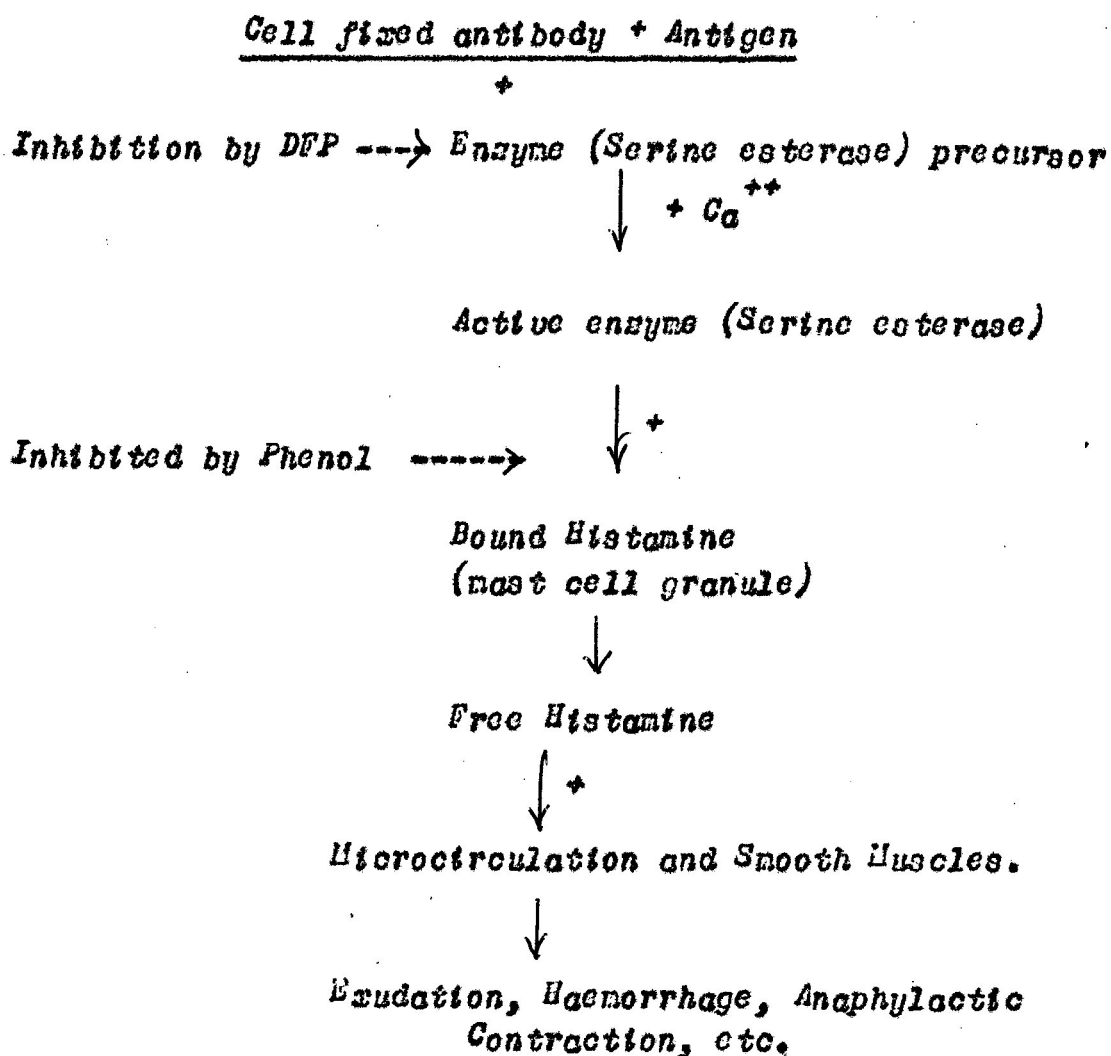
The much argument remains concerning to its release kinetics and relation with other mediators such as histamine, bradykinin and prostaglandins. Recently, in rats prostaglandins have been shown to potentiate the effect of serotonin on vascular permeability¹⁵⁴.

2. Histamine as a mediator :

Histamine, an amine found in mast cells (basophils), in platelets, and in parietal region of the stomach, is widely known as one of the important mediator of acute inflammatory reactions. Abundance of evidence is available to show that histamine is released following burns, chemical injury, X-ray injury and bacterial invasion.¹⁵⁵ In anaphylactic injury its release is fully documented and raised levels have been demonstrated in Schultz-Dale reaction¹⁵⁶.

The mechanisms involved in the release of histamine from mast cells and platelets have been intensively studied. Of particular interest is the role of homocytotropic antibody in release reactions in various systems.

Following is the scheme of biochemical events leading to release of histamine during anaphylactic reactions.



Recently, it has been reported that histamine have chemotactic activity also and following release from mast cells it causes the influx and localization of eosinophils at the inflammatory site¹⁵⁷.

3. Prostaglandins as Mediator :

These are long chain C_{20} compounds synthesized in cells from polyunsaturated fatty acids. They can be classified into various groups (e.g. E, F, A and B) on the chemical basis of their structure. They appear to be present in practically every tissue of the body but the factors involved in their synthesis and release during inflammatory reactions are not yet clearly defined.

PGE_1 and PGE_2 evoke a local increased vascular permeability into humans or rat skin¹⁵⁸. Inhibition studies with indomethacin (a prostaglandin synthetase inhibitor) and cortical steroids (inhibitors for prostaglandin release) have indicated the involvement of it in inflammatory processes elicited by chemical irritants¹⁵⁹. Recently, studies with antibodies to prostaglandin (E_1) have demonstrated that following chemical injury in rodents (hind paw) inflammatory reaction is partly mediated by prostaglandins⁶. The steps covering the synthesis and release of prostaglandins during inflammatory reactions is defined as follows¹²⁸ :-

1. Injection of the irritant.
2. Generation of neutrophil chemotactic factor.
3. Release of neutrophil chemotactic factor.
4. Pavementation of neutrophils.
5. Extravascular infiltration of neutrophils.

6. Neutrophil-irritant interaction.
7. Neutrophil-lysosomal enzyme release.
8. Activation of prostaglandin biosynthetic pathway.
9. Synthesis of vasoactive prostaglandin intermediate
--- Production of prostaglandin.
10. Release of vasoactive prostaglandin intermediate.
11. Increased capillary permeability.
12. Oedema formation.

Prostaglandin may not only be the mediators but may also inhibit certain of the processes involved in inflammation¹⁶⁰. Such action may result from the ability of certain of these compounds (PGE_1) to stimulate accumulation of cyclic AMP inside the cells (by activating adenyl cyclase). This process could lead to inhibition of phagocytic activity and inhibition of mediator release from mast cells.

2.2.5 Inflammation : Experimental Models :

Inflammation is made up of various events occurring in an orderly sequence, though not necessarily dependent upon each other for their development. In carrying out laboratory investigations it is necessary to select experimental model which will facilitate the study of the desired components of the inflammatory reactions.

t) Rat hind paw oedema¹⁶¹ :

This is one of the most widely used model to study acute vascular injury as a function of time. The technique is to inject aqueous solution of the irritant in subplanter region of hind paw of rats. Oedema produced is then measured as change in volume of the paw using simple plethysmographic apparatus.

The algal polysaccharide, carrageenan, is a potent inflammogen in this system and elicits biphasic response, as described below :

Acute phase - It follows immediately after injection of the irritant and lasts for a hour. The principal mediators are histamine and serotonin.

Delayed phase - It develops gradually at the end of first phase and lasts for several hours. Bradykinin and prostaglandins have been demonstrated the possible mediators for this phase.

The drawback of the method is that it does not permit collection of oedema fluid for histological and pharmacological studies.

ii) Rat pleural oedema¹⁶² :

It is a most suitable model for studying histopathological and pharmacological features of inflammatory reactions induced by inflammogen such as carrageenan, turpentine oil and silver nitrate. The system permits quantitative recovery of the exudates for identification of the mediators and enumeration of cellular contents. With this system it is wise to perform all studies by inducing moderate injury and by ignoring all results in which exudates are blood stained. In rodents, the mechanism of inflammation in this cavity is attributed to neutrophil chemotactic factors and mediators such as serotonin, histamine and prostaglandins.

iii) Rat granuloma pouch¹⁶³ :

It is a model utilized for chronic inflammatory studies. Briefly, the method consists of raising an air filled sac in the dorsal subcutaneous tissue of the rat by injecting 25 ml of air. The sac is injected with small volume of chemical irritant usually turpentine, resulting in formation of sterile abscess. The ensuing inflammation is studied 4-14 days later by measuring the thickness of the wall of the granulation that forms around the abscess, or the weight of the pouch or the volume of the fluid within the pouch.

iv) Ultra-violet Erythema¹⁶⁴ :

The standard technique is to give an appropriate dose of ultra violet radiation to the depilated skin of the guinea-pig for 20 seconds and to grade the resultant erythema by eye, expressing the results in arbitrary units. However, the model is not considered to be precise and is only used for preliminary screening of the drug.

v) Cotton pellet granuloma¹⁶⁵ :

This is, perhaps, the most widely used experimental model for studying proliferative components of the inflammatory process. In a standard procedure sterilized cotton pellets weighing 10-20 mg and of uniform surface area are implanted in subcutaneous sites along the flanks or in the axillae of a rat. The duration of implantation is varied from one to fourteen days. The inflammatory response elicited by the cotton pellets is described as follows :-

- a) Transient vascular leakage : It is a leakage of inflammatory fluid over a period of 20-30 minutes.
- b) Delayed vascular leakage : It is a leakage of inflammatory fluid and infiltration of leucocytes over a period of 3-72 hours.
- c) Proliferation : It is a proliferative phase and corresponds to the appearance of collagen in the granuloma. For the assessment of this stage pellet are recovered and dry wt. of the granuloma in relation to the body weight, is determined.

2.3 MATERIALS AND METHODS :

2.3.1 Materials :

(1) Chemicals :

- i) Serotonin (Creatinine sulfate) : L. Light & Co. England.
- ii) Histamine : Ward Blenkinson & Co. Ltd., England.
- iii) Prostaglandin $F_{2\alpha}$.
- iv) Carrageenan : Sigma Chemical Co.
- v) Silver nitrate : E. Merck, Germany.
- vi) Turpentine oil : Ranban Patent Depot, Bombay.
- vii) Evan's blue : E. Merck, Germany.
- viii) Methysergide bimalate : Sandoz.
- ix) Atropine sulfate : B.D.H.
- x) Mepyramine maleate : M & B. (India).

(2) Animals :

- Albino rats : Haffkine strain
 Albino mice : Haffkine strain
 Guinea-pigs : Haffkine strain

2.3.2 M E T H O D S

2.3.2.1 Studies on Acute Inflammation :

i) Serotonin induced rat hind paw oedema¹⁶⁶ : Non-fasting male albino rats of Haffkine strain weighing 140-150 gms., were used. They were assigned randomly between two groups of ten animals each. The first group was treated with immune plasma intravenously through tail vein and the second group with normal plasma as a control in the same way. The animals were rested for half an hour following which oedema was induced in right hind paw by injecting in subplanter region 1 μ g of serotonin in 0.1 ml of normal saline, pH 7.4. The left hind paw was injected with 0.1 ml of normal saline as a control to the right paw. The oedema induced by serotonin was measured as a change in the volume of the paw at 30 minutes when the swelling had reached the maximum.

A plethysmographic apparatus was used for measuring the paw volume. The rat was held firmly (unanaesthetized) and the paw was immersed into the pool of mercury upto the ankle joint. The increase in the mercury level in graduated tube was taken equivalent to the volume of the hind paw immersed.

The inhibitory effect of antiserotonin antibodies on hind paw swelling was studied using 0.5 ml and 1.0 ml of immune plasma. The plasma was filtered freshly before intravenous administration. The subplanter dose of serotonin (1 ug/paw) was kept constant.

ii) Carrageenan induced hind paw oedema¹⁶¹ : 1% mixture

of carrageenan was prepared by mixing the carrageenan with normal saline, pH 7.4. The male albino rats of Haffkine strain weighing 140-150 gms were selected for the experiment. They were assigned in four groups of 10 animals each. Immune and normal plasma was administered intravenously through tail vein as per the following scheme :-

- Group I : 0.5 ml immune plasma per animal.
- Group II : 1.0 ml immune plasma per animal.
- Group III : 0.5 ml normal plasma per animal.
- Group IV : 1.0 ml normal plasma per animal.

Animals were rested for half an hour following which 0.1 ml of 1% mixture of carrageenan in normal saline was injected in subplanter region of the left hind paw. The right hind paw was injected with normal saline as a control. The volume of the injected paw was measured at 30 minutes using plethysmographic apparatus as described earlier.

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iii) Carrageenan induced rat pleural exudation :

Male albino rats weighing 140-150 gms. were used. They were assigned in two groups of 10 animals each. Pleural exudation was induced by injecting 0.25 ml of 1% mixture of carrageenan in the left lower portion of the cavity. The injection was made between 7th and 8th rib using tuberculin syringe and a 5.0 mm needle of 26 gauge. The animals were given light ether anaesthesia to facilitate the careful injection of the substance.

Normal and Immune plasma in a dose of 1 ml were administered intravenously through tail vein half an hour prior to the carrageenan treatment.

Group I : Normal plasma

Group II : Immune plasma.

After three hours of the carrageenan treatment animals were sacrificed using light ether anaesthesia. Pleural cavity was carefully opened. The exudation was collected using a clean pasteur pipette. The individual collection was measured using tuberculin syringe. The exudates were immediately centrifuged and the clear supernatant was stored at -20°C for further studies.

iv) Turpentine induced rat pleural exudation :

The experiment was performed as per the procedure

described for the preceding experiment. In place of carrageenan, turpentine oil in a dose of 0.06 ml per animal was used to induce pleural exudation. One ml of immune plasma was used to study the inhibitory effect of antibodies to 5-HT, on the 3 hour pleural exudation.

v) Silver nitrate induced rat pleural exudation :

The experiment was performed as per the method used for carrageenan induced rat pleural exudation. Each rat was injected intrapleurally 0.15 ml of 0.4 % solution of silver nitrate in distilled water. One ml of immune plasma was administered intravenously through tail vein half an hour prior to the injection of silver nitrate. The exudation was collected at 3 hours following the injection of the irritant.

vi) Estimation of histamine release¹⁶⁷ :

Histamine levels in the pleural exudates obtained from the previous experiments, were estimated by bioassay on guinea-pig ileum preparation as follows :-

A guinea-pig weighing about 400-500 gms. was starved for 24 hours. It was killed by stunning on the head and cutting through its throat. Immediately, the abdomen was cut opened to expose intestinal tract. The 10 cm. portion of the terminal ileum was removed and placed

in a shallow dish containing warm (37°C) Tyrode solution (The composition is given in Appendix). The lumen was gently irrigated with warm Tyrode solution so as to dislodge faecal residues, if any. A segment measuring 2-3 cm was isolated from the piece of ileum cleaned as above. A thread was attached at both ends and the preparation was mounted in the organ bath containing Tyrode solution. A thread attached to the upper end of the tissue was connected to a frontal writing lever and contractions were recorded. The tyrode solution used contained antagonists for acetylcholine and serotonin.

Atropine sulphate (for ACh) .. 100 mcg/litre
 Methysergide bimalate (for 5-HT) .. 100 mcg/litre.

Dose response curve for histamine was recorded. Pleural exudates were diluted suitably to get contraction within the range of standard dose response curve. Following the experiment, the dose response curve was repeated and any changes noted.

The weight applied : 0.5 gm.
 Magnification : 10 to 12 times.

The contractions were recorded for 30 seconds and the duration of the complete cycle was 2½ minutes.

viii) Estimation of serotonin release¹⁶⁸ :

Virgin rats about two months old, weighing 100-130 gms. were used. They were killed by sharp blow on the head and cutting through the throat. The abdomen was cut opened and the uterus was dissected out by clearing the adhering tissues. It was kept in a De Jalon solution. A thread was attached at both ends of a horn of the uterus. The preparation was then suspended in a organ bath containing De Jalon solution kept at 28°C and aerated with air. In order to decrease the great sensitivity of this preparation to acetylcholine, atropine sulphate in a concentration of 100 mcg/litre was added to the stock solution. Dose response curve for serotonin were recorded using doses of serotonin 1.0, 2.0 and 4.0 ng/ml. A cycle of 5 minutes was maintained. Drug was kept in contact until the maximum response was obtained. Pleural exudates suitably diluted with De Jalon solution were added in small amounts to give contraction within the range of standard responses. Finally, the dose response curve was repeated to assure that the sensitivity of the preparation was not altered.

viii) Estimation of prostaglandin release¹⁶⁹ :

Prostaglandin levels in the pleural exudates obtained from the previous experiments, were estimated by bioassay on isolated rat fundus preparation.

Fundus strips were obtained from adult male albino rats having had no food for 48 hours but water ad libitum. After killing a rat by a blow on the head, the fundus part of the stomach was carefully dissected out and was cleaned with Tyrode solution at 37°C. The fundus was then stretched on cotton pad moistened with Tyrode solution and was cut in the direction of longitudinal fibres. The strip thus obtained was the maximal straight stretches of longitudinal muscles that can be obtained from the fundus. A length of 3 cms. of it was suspended in 10 ml. organ bath containing Tyrode solution at 37°C and aerated with oxygen. The strip was allowed to acclimatized for one hour before starting with experiments and during this period Tyrode solution was refreshed every 15 minutes. Frontal writing lever loaded with 0.5 g weight and giving magnification about 20 times was used for recording the contractions. Contractions were obtained for 90 seconds on a slowly rotating sooted drum of a kymograph. A cycle of 8 minutes was followed for individual response.

The Tyrode solution contained atropine sulphate, mepyramine maleate and methysergide as antagonists to acetylcholine, histamine and serotonin respectively.

Mepyramine maleate	50 mcg/litre
Methysergide bimalate	100 mcg/litre
Atropine sulphate	100 mcg/litre.

Dose response curve to prostaglandin $F_{2\alpha}$ was recorded for 1.0, 2.0 and 4.0 ng/ml. Pleural exudate were added to the bath to give contractions in the range of standard responses. Finally, dose response curve to prostaglandin was repeated.

2.3.2.2 Studies on Acute anaphylaxis :

Acute pinna anaphylaxis : Albino mice weighing about 18-20 gms. were selected and distributed in two groups of 20 mice each. They were sensitized to normal horse serum by injecting 0.1 ml of 1:10 dilution, subcutaneously. A booster dose of 0.1 ml of 1:50 dilution of normal horse serum was made after 15 days of the primary immunization. Following 6 to 8 days of the booster treatment animals were challenged with the horse serum on the pinna.

Immune plasma in a dose of 0.2 ml and Evan's blue 0.15 ml of 0.5% solution in saline were administered intravenously through the tail vein respectively half an hour and a minute prior to the challenging reaction.

For the challenged reaction animals were lightly anaesthetized with ether, a drop of neat horse serum was placed on one of the pinna and a needle was pierced through it. On the other pinna control reaction was carried

out using normal saline instead of horse serum. The reaction was observed for 30 minutes when a coloured wheal appeared around the challenged site. The reaction on the pinna was evaluated by measuring the diameter of the wheal using vernier callipers. Effect of immune plasma on the anaphylactic reaction was expressed as percentage inhibition of the control response obtained using normal plasma.

2.4 R E S U L T S

(1) Inhibition of serotonin induced hind paw oedema :

5-HT when injected in the subplanter region of the rat hind paw, elicited dose dependent increase in the paw volume (Table - 4). The reaction when studied over a period of an hour at the intervals of 10 minutes, showed maximum swelling at 30 minutes. A dose of 1 μ g of serotonin which elicited swelling of the magnitude 22.6 ± 0.54 (volume in arbitrary units) was used for studying the inhibitory effect of 5-HT antibodies. The results summarised in Table - 5, demonstrates that passive immunisation of the rats with 0.5 ml and 1.0 ml of immune plasma inhibits the oedematous effect of serotonin. The inhibition was observed to be dose dependent.

(2) Inhibition of hind paw oedema to carrageenan :

Carrageenan when injected in the subplanter region of rat hind paw in a dose of 0.1 ml of 1% solution in normal saline, elicited paw swelling of the order 29.0 ± 0.745 (volume in arbitrary units). The swelling was measured at half an hour after the injection of the irritant. When the effect of 5-HT antibodies was studied on this preparation, dose dependent inhibition in the hind paw oedema was observed (Table - 6).

(3) Inhibition of rat pleural exudation :

Rat pleural exudation was induced using three different irritants -

- i) Carrageenan ... 0.25 ml of 1% solution in normal saline;
- ii) Turpentine oil ... 0.06 ml (undiluted);
- iii) Silver nitrate ... 0.15 ml of .4% solution,

injected intrapleurally per animal.

The following facts were observed as the effect of these irritants on pleural exudation.

- a) Significant amount of exudates were collected in the pleural cavity at 3 hours following the injection of the irritants.
- b) The 3 hour exudate volume for these irritants varied as follow :-
Silver nitrate > Turpentine oil > Carrageenan.
- c) All exudates were turbid but free from blood stains.

The effect of 5-HT antibodies when administered intravenously half an hour prior to an injection of these phlogistic agents in question, was inhibitory (Table-7).

The maximum inhibition of 51.54% was observed in carrageenan induced pleural exudation. The turpentine and silver nitrate induced pleural exudation was observed to be inhibited by 28.44% and 29.4%, respectively by 1.0 ml of immune plasma.

(4) Inhibition of the mediator release :

1) Estimation of histamine content : As evident from the figures 17 and 18, the isolated guinea pig ileum preparation used to estimate histamine content of the pleural exudates, was sensitive to either 1 or 10 ng amount of histamine.

- a) All the control exudates recovered from the inflamed pleural cavity showed the presence of histamine (Table-8).
 - b) All the pleural exudates obtained from the animals treated with immune plasma prior to the injection of the irritant, showed reduced histamine content (Table-8). Inhibition was not uniform. It varied for the different irritants as follows :
- Turpentine > Silver nitrate > Carrageenan

ii) Estimation of prostaglandin content : As

evident from the figures 19 and 20 , the isolated rat fundus preparation used to estimate prostaglandin content of the pleural exudates was sensitive to 1-10 ng amount of PG-F_{2α} .

- a) All the control pleural exudates when tested on this preparation showed the prostaglandin like activity (Table-9).
- b) All the pleural exudates obtained from animals treated with immune plasma prior to the injection of irritants, showed reduction in prostaglandin content (Table-9).

iii) Estimation of serotonin content : The

isolated rat uterus preparation used to estimate serotonin content of pleural exudates, showed sensitivity to 1-10 ng amounts of serotonin (Figures-21 and 22).

- a) All control pleural exudates when tested on this preparation showed presence of serotonin (Table-10).
- b) Pleural exudates obtained from antibody treated animals failed to show serotonin like activity on this preparation (Figures- 21 & 22). The exudates were added undiluted upto the volume of 0.5 ml in 10 ml bath.

(5) Inhibition of mice pinna-anaphylactic reaction :

Acute anaphylactic reaction on mice pinna was provoked using normal horse serum as sensitizing and challenging antigen. The reaction was evaluated by measuring the diameter of the wheal produced at 30 minutes. It was observed that 5-HT antibodies when given 1/2 an hour prior to the challenge reaction, inhibits the development of wheal by 32.8 % (Table-11).

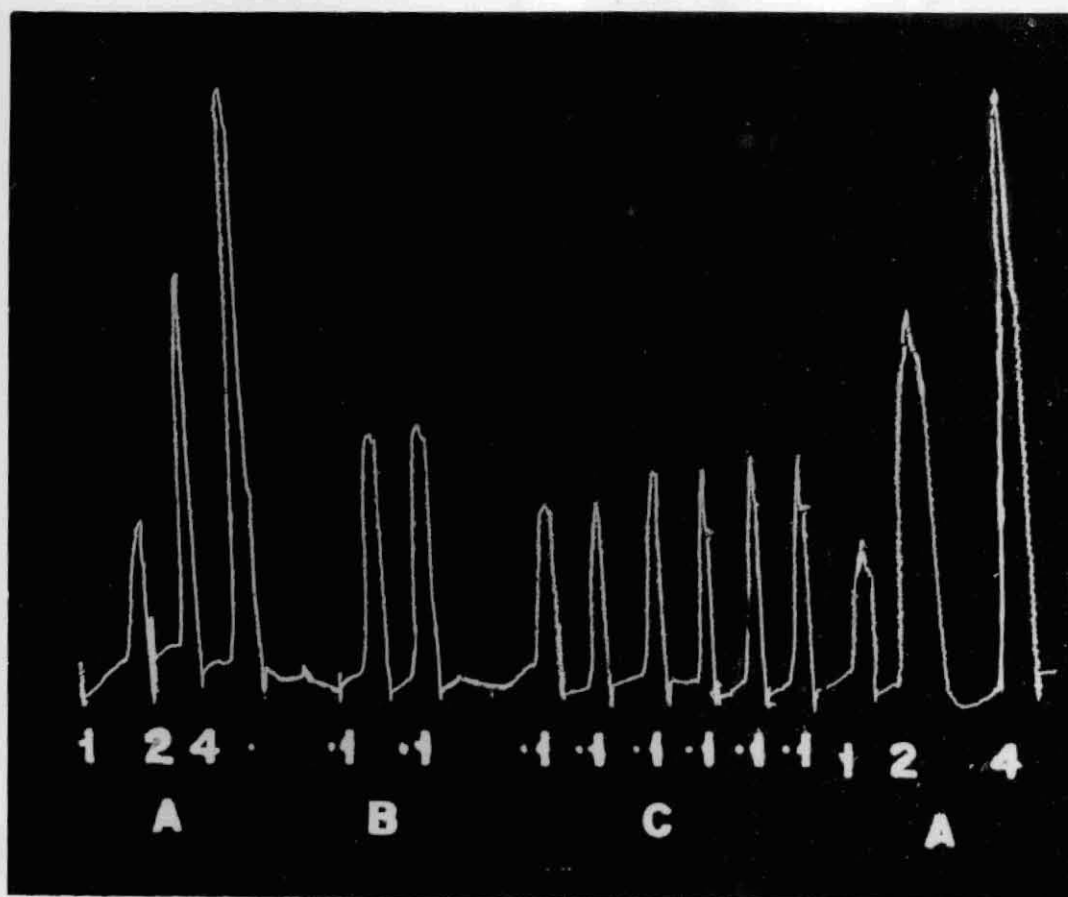


Fig.17 : Assay of Histamine content, of Carrageenan induced pleural exudates on isolated guinea-pig ileum.

A = Histamine ng/ml.

B = Control (Normal Plasma Treated) Pleurisy fluid (1:100 dil.) in ml.

C = Experimental (Immune plasma treated) pleurisy fluid (1:100 dil.) in ml.

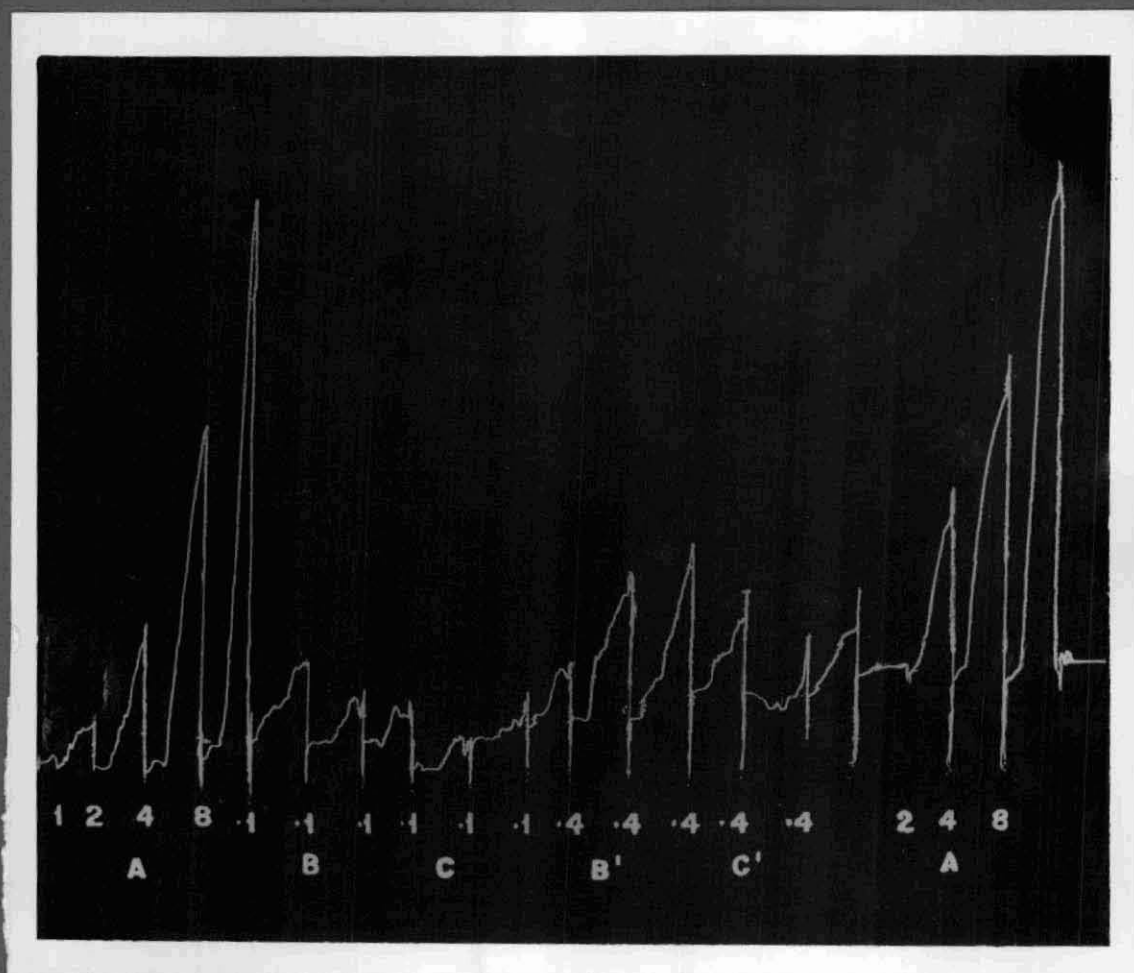


Fig. 19 : Assay of Prostaglandin content of Carrageenan induced rat pleural exudate on isolated rat fundus preparation.

- A = Prostaglandin $F_{2\alpha}$ ng/ml.
 B, B' = Control Pleural Exudates in ml.
 C, C+ = Experimental Pleural Exudates in ml.

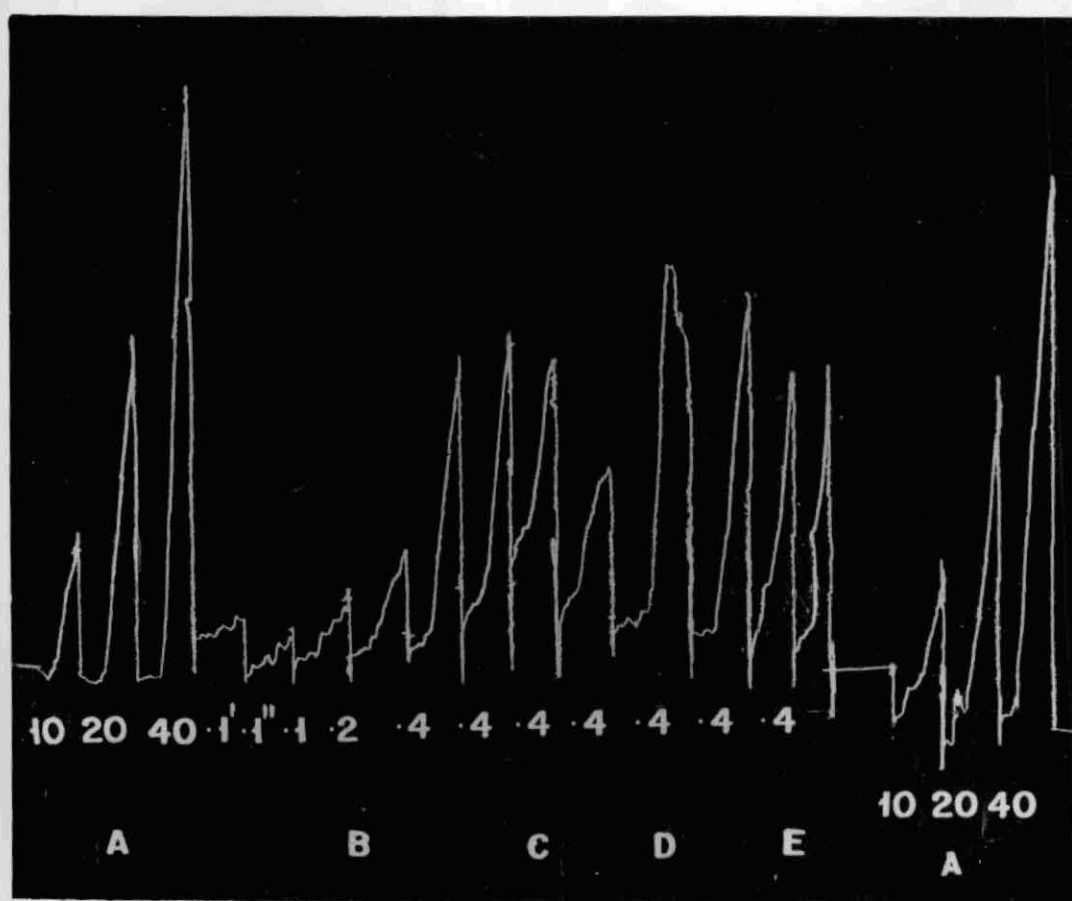


Fig. 20 : Assay of Prostaglandin Content of Turpentine and Silver nitrate induced rat pleural exudate on isolated rat fundus preparation.

- A = Prostaglandin $F_{2\alpha}$ ng/ml.
- B = Turpentine induced Control pleural exudate in ml.
(0.1' - 1:25 dilution; 0.1'' - 1:5 dilution rest undiluted).
- C = Turpentine induced experimental pleural exudates
- D = Control pleural fluid (Silver nitrate) in ml.
- E = Experiment pleural fluid (Silver Nitrate) in ml.

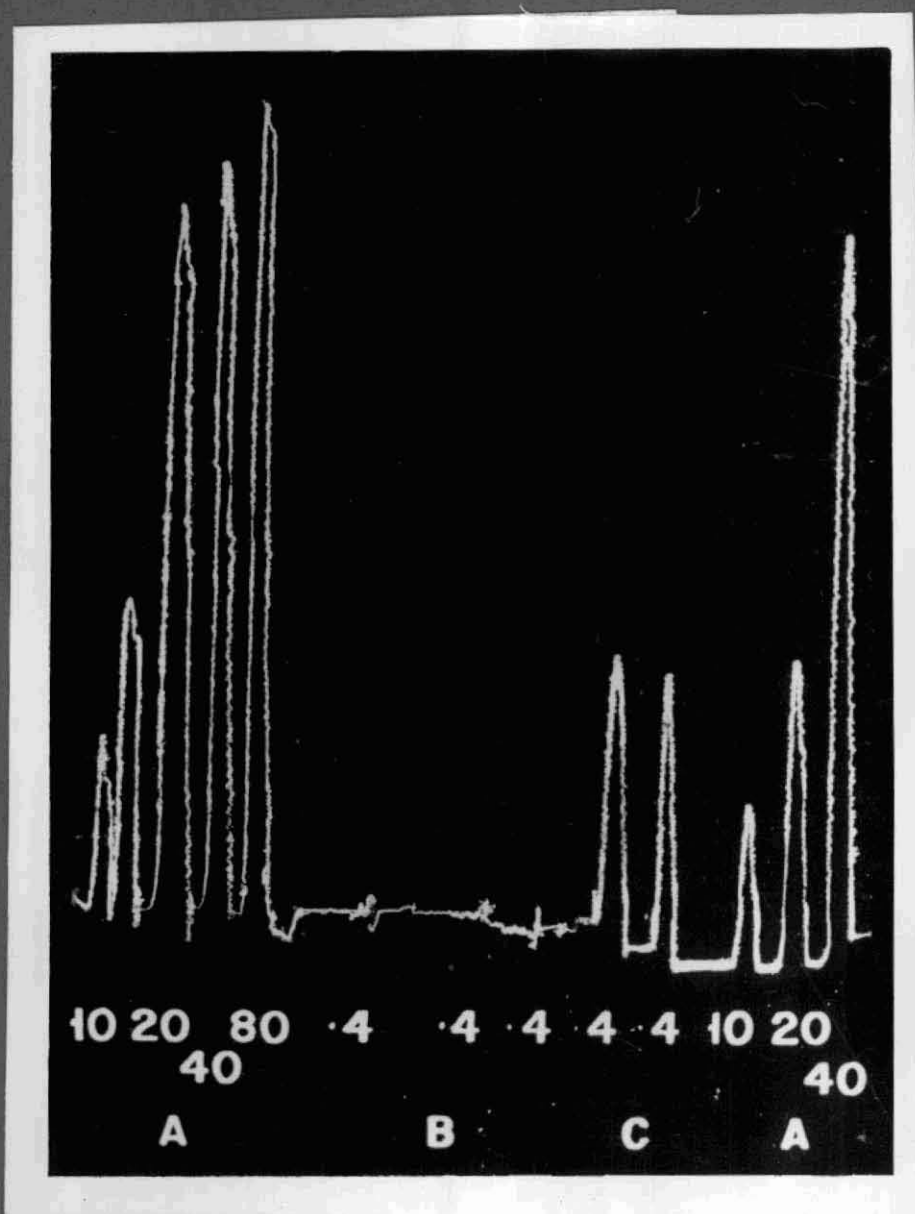
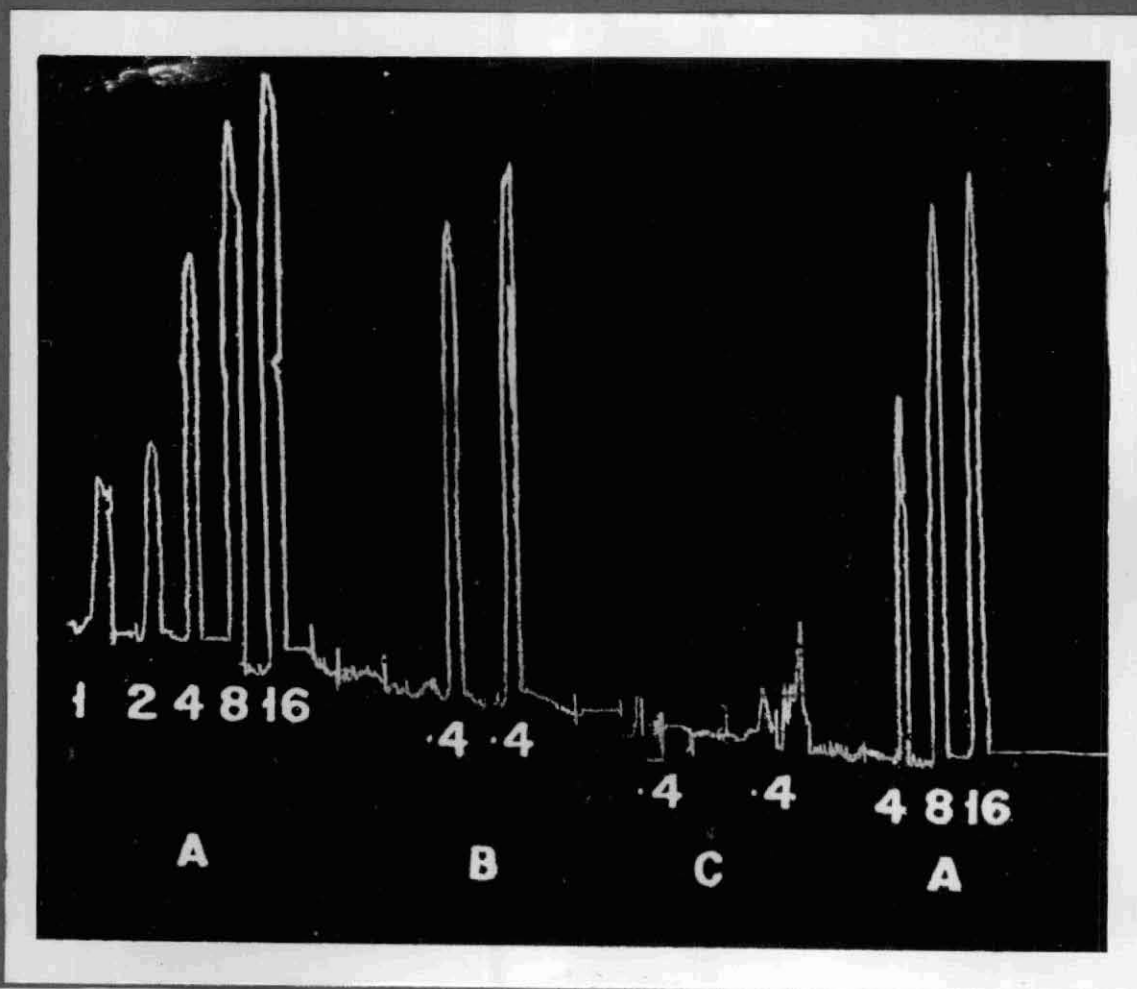


Fig.21 : Assay of Serotonin Content of Carrageenan induced rat pleural exudate on isolated rat uterus preparation.

A = 5-HT ng/ml.

B = Control pleurisy fluid in ml.

C = Experimental Pleurisy fluid in ml.



*Fig. 22: Assay of serotonin content of turpentine-induced
Pleural Exudates on isolated rat uterus.*

A = 5-HT ng/ml.

B = Control Pleurisy fluid in ml.

C = Experimental Pleurisy fluid in ml.

Table - 4Effect of 5-HT on rat hind paw

No.	Dose (ug/paw)	Average oedema volume at 30 mins.		
		a. u.	±	S. E.
1	0.1	5.0	±	0.547
2	1.0	22.6	±	0.748
3	10.0	35.4	±	0.872
4	100.0	49.2	±	0.735

a. u. = Arbitrary Units.

Table - 5The effect of 5-HT antibodies on serotonin oedema
in the rat hind paw.

(Serotonin - 1 ug/paw)

Substance	Dose ml/animal (i/v)	Oedema volume		% Inhi- bition
		a.u.	± S.E.	
Control plasma	0.5	22.60	± 0.54	-
Control plasma	1.0	22.90	± 0.60	-
Immune plasma	0.5	15.6	± 0.40	30.97
Immune plasma	1.0	9.9	± 0.55	57.20

a.u. = Arbitrary Units.

Table - 6

The effect of 5-HT antibodies on rat hind paw oedema induced by carrageenan.

Substance	Dose (ml/animal) i/v	Oedema volume a.u. $\pm$ S.E.	% Inhibition
Control plasma	0.5	29.00 $\pm$ 0.745	-
Control plasma	1.0	28.4 $\pm$ 0.670	-
Immune plasma	0.5	21.1 $\pm$ 0.566	27.24
Immune plasma	1.0	14.5 $\pm$ 0.500	49.94

a.u. = Arbitrary units.

Table - 7

The effect of 5-HT antibodies (1 ml immune plasma) on rat pleural exudation induced by various chemical agents.

No.	Plasma	Pleural irritant	Oedema volume ml $\pm$ S.E.	% Inhibition
1.	Control	Carrageenan	0.712 $\pm$ 0.041	-
2.	Immune	Carrageenan	0.345 $\pm$ 0.021	51.54
3.	Control	Turpentine	1.09 $\pm$ 0.041	-
4.	Immune	Turpentine	0.78 $\pm$ 0.025	28.44
5.	Control	Silver nitrate	1.87 $\pm$ 0.062	-
6.	Immune	Silver nitrate	1.32 $\pm$ 0.039	29.41

Table - 8

The effect of 5-HT antibodies (1 ml immune plasma) on
histamine release provoked by various chemical
agents in rat pleural cavity.

No.	Plasma	Pleural irritant	Release/animal	% Inhibition
1.	Control	Carrageenan	1.006 ug	-
2.	Immune	"	0.836 ug	16.90
3.	Control	Turpentine	434.91 ng	-
4.	Immune	"	156.00 ng	64.12
5.	Control	Silver nitrate	374.00 ng	-
6.	Immune	" "	240.76 ng	35.61

Table - 9

The effect of 5-HT antibodies (1.0 ml immune plasma) on
prostaglandin release provoked by various chemical
agents (in rat pleural cavity)

No.	Plasma	Pleural irritant	Release/animal	% inhibition
1.	Control	Carrageenan	3.718 ng	-
2.	Immune	"	2.632 ng	29.19
3.	Control	Turpentine oil	45.24 ng	-
4.	Immune	" "	20.90 ng	53.80
5.	Control	Silver nitrate	96.49 ng	-
6.	Immune	" "	31.30 ng	67.72

Table - 10

The effect of 5-HT antibodies (1.0 ml immune plasma)
on Serotonin release provoked by various chemical
agents (in rat pleural cavity).

No.	Plasma	Pleural irritant	Release/animal	% Inhibition
1.	Control	Carrageenan	30.94 ng	-
2.	Immune	"	not detected	100 % P
3.	Control	Turpentine	20.20 ng	-
4.	Immune	"	not detected	100 % P
5.	Control	Silver nitrate	18.20 ng	-
6.	Immune	" "	not detected	100 % P

Table - 11

The effect of 5-HT antibodies (0.2 ml immune plasma)
on acute pinna anaphylactic reaction in
mice.

Substance	Wheal Diameter mm $\pm$ S.E.	% inhibition
1. Control plasma	2.98 $\pm$ 0.069	-
2. Immune plasma	2.00 $\pm$ 0.078	32.8

2.5 DISCUSSION

The inhibition of serotonin induced rat hind paw oedema by immune plasma offered the evidence that antiserotonin antibodies are able to combine and sequester free serotonin and thereby diminish its effect on receptor sites. The inhibition was dose dependent. Inhibition values for 0.5 ml and 1.0 ml of immune plasma were 30.97% and 57.80% respectively, against oedema induced employing 1 μ g of 5-HT.

The antibodies were also effective in carrageenan induced oedema of hind paw. The release of serotonin as one of the mediators of acute carrageenan induced oedema of hind paw, has been documented by number of investigators. DiRosa and Giroud¹²⁹ have studied rat hind paw oedema in great detail. They have shown that carrageenan oedema is biphasic and the early phase involves release of serotonin and histamine subsequently. DiRosa and Padanilam have confirmed the said fact. They have shown striking reduction of the oedema following depletion of biogenic amines from the rat hind paw using compound 48/80¹⁷⁰. Above results in concomitant to the finding of DiRosa suggested that antibody preparation is capable of

neutralizing the effect of endogenously released serotonin also.

These experiments, in conjunction with the several reports available on serotonin antibodies, suggested that the antibodies can be a mean for elucidating mechanisms of acute inflammatory reactions in which serotonin is said to be involved.

The inhibition of pleural exudation and the inhibition of release of prostaglandins and histamine when serotonin antibody preparation precedes the injection of irritants in the pleural cavity clarifies a number of important points concerning the role of serotonin in acute pleural inflammation in rodents.

1) As originally proposed by Spector et al.¹⁴⁵ 5-HT is one of the mediators of chemically induced inflammation of the rat pleural cavity. It is released at an early period following the exposure of tissues to the irritant. As suggested by Evans¹⁴⁹, the release occurs from the mast cells present in the tissue surrounding the blood vessels. With the studies performed it is difficult to comment on the precise mechanism by which it induces the inflammatory response. Hajno^{132,133} suggested that its predominant action is on vascular bed. It causes leakage

of blood vessels by contractions of the endothelial cells.

2) In addition to serotonin, prostaglandins and histamine are other mediators of inflammatory reactions in pleural cavity of the rodents. The release of histamine and prostaglandin is determined by 5-HT. Here, it is difficult to comment on the mechanism by virtue of which 5-HT may be doing so. But the fact has significance that it adds a new dimension to the studies on mediators of inflammation. It is the first time when such relation among serotonin and other mediators of inflammation have been shown.

The fact that different irritants used had provoked varied amounts of pleural exudate volume and varied amounts of mediators release (release of 5-HT, histamine and prostaglandins) suggest that the basic mechanism in chemically induced acute inflammation is same but the intensity varies with the irritant.

Acute anaphylactic reaction is a type of acute inflammation. The important aspect of the reaction is that it mediated by an immune injury - antigen-antibody reaction. The role of chemical mediators in the anaphylactic reaction in mice has not been studied in detail. It is doubtful that histamine plays a significant role in bringing out

the reaction as mice are extremely insensitive to histamine¹⁷¹. Recently, attention has been diverted towards serotonin. There is a considerable evidence to implicate 5-HT as a causative mediator. Fink¹⁷² has proposed that 5-HT is the mediator of Schultz-Dale reaction in the mouse uterus. The author observed that uterus of both sensitized and normal mice are 1000 times more sensitive to 5-HT than to histamine. Moreover, the sensitivity of uterus to 5-HT and to Schultz-Dale reaction was abolished by 5-HT antagonists. Fox and co-workers¹⁷³ subsequently reported that the 5-HT antagonists chlorpromazine, promazine, reserpine and LSD were effective in preventing anaphylaxis in actively sensitized mice. Our results on acute pinna anaphylaxis confirm the above findings and it can be said that, 5-HT has significant role in acute anaphylaxis in mice.

S E C T I O N III

3.1 GENERAL DISCUSSION

3.2 SUMMARY AND CONCLUSION

3.1 GENERAL DISCUSSION :

The indole alkylamine, serotonin has very broad distribution among mammalian tissues. Mast cells, blood platelets, enterochromaffin cells of gastro-intestinal tract and pineal gland in the brain are particularly rich in this amine. In mast cells it is stored in the granular fraction along with other mediators including histamine. Rat mast cells contain 5-HT about $1 \mu\text{g}/10^6$ cells.

In rodents, 5-HT is considered to be a potent inflammogen. Significantly high levels of it have been demonstrated in hind paw and pleural cavity of rats following localized chemical injury. A study has been made by Capasso *et al.*¹⁵⁰ on/of the exudate provoked by intrapleural injection of carrageenan in rats. The authors found an early release of serotonin in the exudate. At three hours, 5-HT levels as estimated by pharmacological assay, were 75 ng/ml. Similarly, Di Rosa¹²⁹ has demonstrated serotonin in exudate provoked by turpentine injected intrapleurally. Sanyal and Uest have shown that 5-HT and histamine content of injured skin in the rat increases following mechanical injury. They have also shown that healing of the injured area is greatly delayed in rat by

depletion of skin histamine and 5-HT. In these studies serotonin has been shown to be released either concomitantly or sequentially to histamine.

Such studies concerning role of 5-HT in inflammation have been made utilizing variety of methods of quantitation and scores of blocking agents. Storage site depleters such as compound 48/80, biogenic amine releasers such as reserpine and receptor antagonists such as cyproheptadine, methysergide, 2-bromo LSD, have been used to single out its mechanisms of action. Unfortunately, in most instances, these agents interact with other biogenic amines. Compound 48/80 has been shown to deplete both serotonin and histamine from mast cells, while reserpine releases all biogenic amines including catecholamines from their storage sites. Cyproheptadine has strong interaction with histamine. The advent of interest in production of antibodies to drugs and biogenic amines has brought a new dimension of specificity to pharmacological agents.

Utilizing antibodies specific to serotonin, we undertook to study the role of serotonin in acute inflammatory and anaphylactic reactions. The first step for this work was to prepare antibodies specific to 5-HT.

The immunogenicity of a substance depends on both its size and its chemical composition. Serotonin is a small molecule (MW 405.00) and is not antigenic as such. Antigenicity was conferred over it by conjugating it to a larger molecule, bovine serum albumin. Five different chemical reactions were employed to serotonin to bovine serum albumin. Overall aim of the conjugate reactions were -

- i) to have conjugates of high solubility in aqueous media.
- ii) to achieve molar ratio of serotonin to albumin, 30-40:1.
- iii) to utilize a position of serotonin molecule other than phenolic hydroxyl group and aliphatic amino group, for conjugation. These groups are metabolic parts of serotonin molecule. For preparing antibodies specific to serotonin, they require exposure to antibody forming system in the body.
- iv) to evolve a relatively simple procedure for conjugation. The procedures involving reaction at high temperature, and pH (alkaline) are likely to distort molecular configuration of serotonin.

The conjugated product obtained using diazonium derivative of benzidine was the most satisfactory. The

conjugate obtained was soluble in normal saline.

Fluorometric analysis revealed 36 groups of serotonin per molecule of albumin. Most importantly, the point of attachment on serotonin molecule was not phenolic hydroxyl or aliphatic amino groups but aromatic ring at 6-position. The reaction procedure was simple and there was no significant loss in protein content.

Rabbits when immunized with the conjugates, such as 5-HT-BDE-BSA, 5-HT-G-BSA and 5-HT-F-BSA, produced antibodies, as borne out from the results of agar gel diffusion. This indicated that conjugation procedures used, animal species selected, adjuvant used, time interval between booster doses were satisfactory for antibody production. Similar method may be used to produce antibodies to other biogenic amines and drugs.

5-HT content of the rabbit platelets is very high. O'Brien et al. ¹⁷⁵ have reported 101.1 ± 0.5 nM of serotonin per milligram platelet protein. Normally, serotonin exists in bound form in the platelets and is released during physical stress and clotting process. It is expected if serum is collected antibody molecules would have their binding sites occupied with serotonin released from platelets. Hence, instead of serum plasma was

collected from the blood obtained from immunized animals. Moreover, entire separation procedure was performed at low temperature using siliconized laboratory glasswares.

It has been shown that immunization with hapten protein conjugate leads to production of antibodies both for hapten and the carrier. Hence, to detect the antibodies against hapten, it is critical to separate out antibodies to the carrier. In the present case antibodies to the carrier were precipitated out by incubating the immune plasma with carrier protein, bovine serum albumin, in optimal proportion. Absorbed plasma when tested on agar gel showed precipitin lines for the conjugate but not for the carrier protein. The structural difference between conjugate and the carrier protein is only the presence of 5-HT molecules on the conjugate. Hence it is evident that antibodies specific to 5-HT are present in the absorbed immune plasma.

For pharmacological studies with antibodies to serotonin, it is necessary that antibody preparation have capacity to neutralize its biological effects. Serotonin is a potent oedemagen of rat hind paw. One microgram of it, when injected in subplanter region, elicits significant rise in paw volume. Antibody preparation when tested on

This model showed dose dependent inhibition of half an hour oedema volume. Immune plasma was administered half an hour prior to the injection of serotonin, to facilitate diffusion of antibodies in the hind paw. Similarly, with antibody preparation there was dose dependent inhibition of carrageenan induced oedema of rat paw. Serotonin release in early phase of carrageenan induced oedema, has been documented by various authors. Dillson et al.^{139,170} have documented early release of 5-HT in rat hind paw following chemical injury. They have shown that 5-HT is released along with histamine following the injection of carrageenan in the subplantar region of the rat. The above results in concordance to the findings of Dillson indicate that serotonin antibodies can sequester 5-HT in vivo and can be used as pharmacological agents for studies on acute inflammation.

To study the involvement of a mediator in inflammatory reactions - i) it must be demonstrably present in the inflamed tissues and ii) its action could be inhibited by the use of specific antagonist.

To study the role of serotonin in acute inflammation rat pleural exudation model was selected. This model have advantage that pleural fluids can be quantitatively recovered and can be studied for --

- (i) Type and number of inflammatory cells ;
- (ii) Type and concentration of mediators of inflammation.

Three different irritants - carrageenan, turpentine and silver nitrate - were used to induce pleural exudation. Three hour pleural exudates obtained from control animals exhibited simultaneous presence of histamine, serotonin and prostaglandins. As suggested by Uvans¹⁴³ the 5-HT and histamine may be from the mast cells present in the connective tissue adjacent to the blood capillaries in the pleural cavity. The author has shown release of both of these amines from mast cells following chemical or physical injury. The existence of prostaglandin in inflammatory fluid can be explained by the theory proposed by Vinegar *et al.*¹²⁸. The authors have proposed that prostaglandins are synthesised by the damaged tissue during an inflammatory reaction. Lysosomal enzymes released during phagocytosis are the main activator for prostaglandin biosynthetic pathway. The lysosomal enzyme such as phospholipase A₂ liberates arachidonic acid from the phospholipids of tissue membrane which then forms prostaglandin via the prostaglandin synthetase chain in the tissue¹⁴².

When 5-HT antibodies were given preceding the injection of these irritants, there was inhibition in three hour exudate volume. Serotonin levels could not be detected in the exudate when tested on isolated rat uterus preparation sensitive to one nanogram amount of 5-HT. Both of these facts taken together confirm that 5-HT has a definite role in inflammatory reactions in the pleural cavity of rats.

Further studies on pleural exudate obtained from 5-HT antibody treated rats showed inhibition in the release of histamine and prostaglandins. Inhibition in histamine release varied from 16.9 to 64.12 %. While prostaglandin release/formation was inhibited by 29.19 to 67.72 %. To our knowledge this is the first report of this type on the role of serotonin in acute inflammation. With the studies performed it is difficult to comment on the mechanism by which serotonin inhibits the release of histamine and prostaglandins during such reactions. Recently Miller *et al.*¹⁴⁹ have proposed that inflammatory process is controlled by the sequential release of mediators. In an inflammatory process induced by immune injury, authors have demonstrated sequential release of histamine and serotonin as follows :-

0 to 10 minutes		Histamine
10 to 20 minutes		Serotonin
20 to 30 minutes	--...	Histamine.

Our results in concomitant with the authors' result suggest that inflammatory process is controlled by the mediators such as serotonin, histamine and prostaglandins. These mediators are released sequentially. Release of one mediator is controlled by the other. In rat pleural cavity serotonin controls the release of histamine and prostaglandins, during inflammatory process.

The mechanism that underlies anaphylactic shock in mice has not as yet been determined. The organ or organs concerned are not known; nor has there been a definite identification of a chemical mediator. It is doubtful that histamine plays a significant role in bringing about the reaction. Mice are extremely insensitive to histamine¹⁷¹. In addition, antihistamine drugs, which completely protect guinea-pigs, do not protect the mouse from anaphylactic shock¹⁷⁶. Much attention has recently been directed to the possibility that 5-HT might mediate the reaction. In vitro studies, using the Schultz-Dale reaction as an indicator of anaphylaxis, have provided some of the evidence

implicating serotonin as a mediator. The mouse uterus is much more sensitive to serotonin than to histamine, and the contraction that usually follows administration of antigen is blocked by antiserotonins of serotonin, such as lysergic acid di-ethylamide and reserpine¹⁷². Serotonin administration to mice mimics anaphylaxis¹⁷⁷. Our observation, inhibition in the development of acute mouse pinna anaphylactic reaction as a effect of 5-HT antibodies, confirms that 5-HT is also an important mediator inducing inflammation by immune injury.

In conclusion it can be said

(i) 5-HT plays important role in chemically and immunologically induced inflammation in rodents.

(ii) In rat pleural cavity during an inflammatory process, 5-HT controls the release of histamine and prostaglandins.

3.2 SUMMARY AND CONCLUSION :

5-HT was coupled to a protein carrier, bovine serum albumin, using different chemical reactions. The conjugates prepared were purified by dialysis and were characterized by ultraviolet spectrophotometric and spectrofluorometric studies. 32-36 molecules of 5-HT were found coupled to per molecule of albumin. Antibodies to 5-HT were produced by immunizing the rabbits with these conjugates. Plasma was collected. Antibodies were detected by agar gel diffusion test. The results revealed presence of antibodies specific to 5-HT in the plasma. The antibodies were evaluated for their anti-5-HT effects on rat hind paw preparation. Dose dependent inhibition in half an hour oedema volume was observed. Similarly, dose dependent inhibition of carrageenan induced hind paw oedema was also observed.

The role of endogenously released 5-HT in acute inflammation was studied using immune plasma. Inflammation was induced by injecting chemical irritants such as carrageenan, silver nitrate and turpentine oil in the rat pleural cavity. Animals passively treated with immune plasma exhibited inhibition in 3 hour exudate volume.

Inhibition was 51.54%, 29.41 % and 28.44% respectively for carrageenan, silver nitrate and turpentine pleural exudation. Further analysis of the exudates on isolated tissue preparations revealed inhibition in histamine and prostaglandin release. Serotonin levels were reduced to undetectable amounts.

In an anaphylactic reaction elicited on pinna of the mouse sensitized with horse serum, when immune plasma was administered prior to the challenge reaction, there was inhibition in half an hour wheal diameter.

From the results it can be concluded :

- i) Antibodies to 5-HT can be prepared by immunizing the experimental animals with 5-HT-protein conjugate.
- ii) 5-HT plays a significant role in chemically induced inflammation of rat pleural cavity. The other mediators released are histamine and prostaglandins. During the course of inflammation the release of both histamine and prostaglandin is mediated via serotonin.
- iii) 5-HT also plays significant role in an anaphylactic reaction in mice.

SECTION IV

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A P P E N D I XConstitution of Physiological solutions :Tyrode Solution

No.	Ingredients	Quantity in g/litre
1.	Sodium chloride	8.00
2.	Sodium bicarbonate	1.00
3.	Glucose	2.00
4.	Potassium chloride	0.20
5.	Calcium chloride	0.20
6.	Sodium dihydrogen phosphate	0.05
7.	Magnesium chloride	0.01

DeJalon's Solution :

No.	Ingredients	Quantity in g/litre
1.	Sodium chloride	9.0
2.	Potassium chloride	0.42
3.	Calcium chloride	0.06
4.	Sodium bicarbonate	0.50
5.	Glucose	0.50

S Y N O P S I S

S Y N O P S I S

STUDIES WITH ANTIBODIES TO SEROTONIN

The advent of preparing antibodies against drugs and physiological mediators, has provided with extremely specific and sensitive pharmacological agents for research into mechanisms of the physiological processes (1). Nevertheless, the present research tools such as receptor antagonists and depleters of the storage sites due to which we owe existing knowledge of physiology and pharmacology, are efficient ones but not the absolute. Specificity and sensitivity of these agents may be a limiting factor for the precise studies. Moreover, due to the very limited knowledge of the receptors via which they act, their own mechanisms of actions are not precisely understood. On the other hand, antibodies are highly precise in their action and function simply by sequestration of the molecules in question.

Until last decade, very few scientific reports were available to dispel the extreme importance of antibodies as pharmacological agents. These substances (drugs and physiological mediators) having low molecular weights, are not immunogenic as such and demand special treatment (conjugation with protein carriers) to elicit immune response.

specific to them. At present antibodies have been prepared to a large number of drugs and physiological mediators e.g. digoxin, barbiturates, morphine (drugs), serotonin, histamine catecholamines, prostaglandins (mediators).

The most significant contribution in this field is - digoxin specific antibodies. The Fab fragments of the antibody preparation has made a record at Massachusetts Hospital, U.S.A., by successfully treating a patient suffering from advanced digoxin intoxication (2). In addition antibodies to the autocooids such as serotonin and prostaglandins have contributed quite a lot in elucidating physiological processes mediated by them (3,4).

In the present work, a fairly controversial physiological mediator viz., serotonin was selected for the study. The aim of the work was to prepare antibodies specific to serotonin and study its role in experimental inflammatory processes with the aid of them.

The indolealkylamine, serotonin, is ubiquitous in mammalian tissues and is most abundantly localized in brain, enterochromaffin cells, blood platelets and mast cells. In rodents, it is considered as a potent mediator of the inflammatory reactions.

Production of the antibodies to serotonin :

To induce immune response to serotonin, the first approach was to prepare artificial serotonin antigen. For this purpose serotonin was conjugated as a hapten to a protein carrier, bovine serum albumin. Following five chemical procedures were adopted (5) :-

1. Formaldehyde condensation;
2. Glutaraldehyde condensation,
3. Bis-diazotized benzidine, cross linkage;
4. Toluene-diisocyanate cross linking; and
5. Ethyl carbodiimide coupling.

All conjugated products were purified by exhaustive dialysis. The dialysed material was lyophilised and used for immunization. Out of these five conjugates only three were selected for further work.

The immunogenicity of the haptenic determinants is reported to be the function of molar ratio of the hapten to the carrier protein. Twenty to forty haptenic groups per molecule of carrier protein are considered to be optimum. Hence the conjugates were assessed for molar ratio of the serotonin to bovine serum albumin. Following chemical procedures were adopted :-

1. UV spectroscopy,

2. Fluorometric estimation of residual serotonin.

From UV absorption spectra, it was concluded that coupling of serotonin to albumin has occurred. With the formula proposed by Freedman (6), the number of serotonin molecules, ranged 30-40/molecule of the albumin.

Rabbits were immunized using complete Freund's Adjuvant. The aqueous solution of the antigen was suspended in the adjuvant and the mixture was inoculated at multiple intradermal sites and thigh muscles. Following the primary injection, booster injections were given at monthly intervals for four months. Animals were bled 10-14 days following the last injection. Blood was collected by cardiac puncture. Following assessment of the antibody titre, the entire blood was withdrawn via intra-arterial cannulation.

As the serotonin content of the rabbit platelets, is very high instead of the serum, plasma was collected. All bleedings were taken into EDTA solution. The clear plasma was obtained by centrifugation. To avoid the release of serotonin from platelets the entire process of the blood collection and separation of the plasma was carried out at low temperature using siliconized glassware. The plasma thus obtained was stored at -20°C .

Antibodies in the plasma were detected by Ouchterlony's method i.e. double diffusion in agar (7). The precipitin bands were formed both for conjugate and the carrier protein. Absorption of the antisera with the carrier protein resulted in total removal of the antibodies to the carrier. This plasma exhibited specificity for the conjugate alone. From these results it was interpreted that antibodies to serotonin are produced.

Absolute amount of antibodies in the plasma were determined by quantitative precipitin test. The precipitates obtained were analysed by Kjeldahl's method. The final pooled plasma contained 6.2 ng/ml of the antibodies expressed as proteins.

Biological Studies :

For the biological studies with antiserotonin antibodies it is critical to see that the antibody preparation have capacity to neutralize pharmacological actions of serotonin. Serotonin is a potent phlogistic agent in the rat hind limbs. Hence this preparation was selected for the biological assessment of the antibodies. Oedema was induced in hind paws of the rats by injecting serotonin in the sub-planter region. Using simple plethysmographic apparatus oedema was measured as change in volume of the paw. It was

observed that when the rats are passively immunized with immune plasma there is protection from serotonin effect. The protection was found to be dose dependent. To account for the nonspecific effects of the proteins and other constituents of the plasma, animals passively immunized with normal plasma were employed as control. To ensure distribution of the antibodies at reaction sites the plasma was administered intravenously half an hour prior to the injection of serotonin. The results indicated that immune plasma can be employed as a pharmacological agent.

The next approach was to use the immune plasma to study acute inflammatory reactions in rodents using various phlogistic agents. Initially, rat hind paw preparation (8) was selected as a model for acute inflammation. Carrageenan was used as a phlogistic agent. The normal plasma was employed as a control as described earlier. It was observed that when the animals are passively immunized with immune plasma (half hour before the carrageenan) there is dose dependent inhibition in the paw volume. The inhibition was 50 % when 1 ml of the immune plasma was used. This indicated that serotonin has a role in the carrageenan induced oedema in rats.

As it was not possible to quantitatively recover the exudate and identify the mediators by using this

Preparation, rat pleural exudation model was selected for detailed studies. Pleural exudation was induced by injecting carrageenan in the lower portion of the pleural cavity (9). Normal and immune plasma were administered half an hour before the carrageenan treatment. Animals were sacrificed after 3 hours and the pleural fluid was collected. One ml of the immune plasma caused 50% inhibition in 3 hour pleural exudate volume. This indicated that serotonin has a role in pleural inflammation.

Histamine and prostaglandins are also known as the mediators of acute inflammatory reactions. It has been reported that there exists some relation for their release during inflammatory reactions. To test this hypothesis the pleural exudates obtained were evaluated for the content of these mediators.

Histamine levels were measured by bioassay employing guinea pig ileum preparation bathed in Tyrode solution containing methysergide and atropine as antagonists to serotonin and acetylcholine respectively.

Rat fundus preparation was selected for estimating prostaglandin like activity of the exudates. PG-F_{2a} was used as standard for comparison. Methysergide, atropine

and mepyramine were used to block the effects of serotonin, histamine and acetylcholine respectively.

Serotonin levels were estimated by bioassay on rat uterus preparation. DeJelon solution containing atropine was used.

Carrageenan pleural exudate from animals passively immunized with immune plasma revealed the following facts :

1. Undetectable levels of serotonin unlike control exudates. This can be explained as an effect of 5-HT antibodies.
2. Reduction in prostaglandin and histamine levels. This may be explained as a sequel of serotonin blockade.

Turpentine oil and silver nitrate solutions have been described as potent phlogistic agents in rats (10). It has also been said that serotonin plays a role in inflammation induced by these agents. Hence to test this hypothesis and to compare them with previous findings, pleural exudation was studied employing these irritants. It was observed that there was reduction in 3 hour exudate volume and in histamine and prostaglandin levels. Comparison of these findings with carrageenan induced pleuritis indicated

that the basic mechanism of inflammatory reactions in pleural cavity is the same but the intensity changes with the irritant.

Finally immune plasma was employed to study the role of serotonin in anaphylactic reaction. Mice pinna anaphylaxis was selected as an experimental model (11). Animals were sensitized with horse serum. Immune plasma and evan's blue dye were administered respectively half an hour and a minute prior to the challenge reaction. Reactions on the pinna were evaluated by measuring the diameter of the wheals. Animals passively treated with normal plasma were used as control. It was observed that treatment with immune plasma inhibits an anaphylactic reaction by 30% indicating the role of serotonin in it.

S u m m a r y

Antibodies to serotonin were prepared via conjugation to a protein carrier. The antibodies thus prepared were evaluated for their antiserotonin effect on the rat hind paw oedema. In carrageenan induced oedema of the rat hind limbs, antibodies offered partial protection. In the rat pleural inflammation pretreatment of the animals with antibodies resulted in suppression of the 3 hour exudate volume. At the same time there was partial inhibition in prostaglandin and histamine release. In anaphylactic

reaction induced in mice, antibodies offered partial protection. From these results it can be said that serotonin play role in inflammatory reactions in rodents.

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A A A A A A A


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Statement Under University Rule Nos. 0.412 & 0.413.

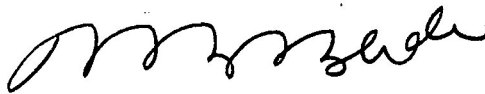
As required by the University Rule No. 0.412 and 0.413, I state that the work presented in this thesis is of original in nature and so far to the best of my knowledge has not been submitted for any degree of this or any other University.

Whenever reference has been made to the work of others, it has been clearly indicated along with the source of information in the "References".


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