STUDIES ON PEPTIDE 401 ISOLATED FROM BEE VENOM

A thesis presented to the University of London in partial fulfilment of the requirements for the degree of Doctor of Philosophy in the Faculty of Science

by

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1975

To Helen and Hugo

Abstract

In the present work an attempt was made to elucidate some aspects of the biological events involved in the anti-inflammatory activity of peptide 401. The already described biological properties of peptide 401, isolated from the venom of the common European honey bee (Apis mellifera) are its ability to degranulate mast cells and to act as a potent anti-inflammatory agent in the rat. Very little is known, however, regarding the details of its mode of action. This problem was approached in the following ways:

a) by determining if, like many of the non-steroidal anti-inflammatory agents, peptide 401 was able to inhibit prostaglandin synthesis, using for this a purified enzymatic complex, prostaglandin synthetase, obtained from sheep seminal vesicles.

b) by investigating if there was any preferential binding of peptide 401 to the rat leucocytes, which are important intermediates in the inflammatory model used to assess the anti-inflammatory activity of peptide 401. Investigation on the interactions between peptide 401 and model membrane systems (liposomes) was also carried out in order to establish what major features of these model membranes would be important for the binding of peptide 401 and its relevance to the situation in vivo.

c) by investigating if the anti-inflammatory activity of peptide 401 could in any way be explained by stimulation of synthesis and release of corticosteroids by adrenal cells of rats.

d) by studying the possible interference of peptide 401 with lymphocyte maturation or with the unspecific PHA stimulation of lymphocyte

maturation, since the lymphocytes appear to play an important role in the rat adjuvant arthritis, a model of inflammation in which peptide 401 also shows therapeutic action.

It was concluded that the anti-inflammatory activity of peptide 401 could be explained, at least partially, by inhibition of prostaglandin synthesis, since this was demonstrated in vitro. Such a conclusion was supported by the observation that a modified peptide 401 (labelled with dansyl) seems to present a certain degree of specificity concerning its binding and/or absorption by the rat polymorphonucleocytes, which are one of the major sources of prostaglandins being formed during the carrageenan oedema. Peptide 401 did not show any direct action on rat adrenal cells regarding corticosteroid production, and it did not significantly influence the maturation of rat lymphocytes.

Acknowledgements

I would like to thank Prof. C.A. Vernon for his supervision and constant encouragement during the preparation of this work. Thanks are also due to Dr. Rudolph Shipolini for his help with the chromatographic techniques and for the preparation of the dansylated peptide 401, to Dr. R.J. Flower and Dr. D. Wallach for the generous gifts of the prostaglandin synthetase complex, to Dr. J.E. Pike for the gift of prostaglandins E_1 and E_2, to Miss C.A. Morris for her technical assistance in the rat adrenal cultures, to Dr. J. Hanson for her interest in the work, to Prof. L. Wolpert for allowing me to use the facilities of the Biology Department of the Middlesex Hospital, to Mrs. V.M.B.D. Rumjanek for her help with the lymphocyte culture technique and for useful discussion and advice throughout the work and to Dr. Susan Wilkinson for her thorough revision of the text and for valuable advice during its preparation.

CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

The present work is an investigation into the mode of action of an anti-inflammatory peptide isolated from the venom of the common European honey bee (Apis mellifera). This peptide has been named peptide 401. The classification 'anti-inflammatory' derives from the observation that peptide 401 is able to reduce the swelling caused by injection of carrageenan into the foot pad of rats (rat paw oedema test) and reduces or prevents the swelling in a condition known as adjuvant arthritis.

Before the pharmacological properties of peptide 401 can be evaluated, the experimental models of inflammation must be compared with the naturally occurring inflammatory processes.

1-1 The inflammatory process

The process of inflammation consists of a series of events by means of which an organism is able to defend itself against invasion by other living forms and also to promote tissue repair following injury which can be immune, mechanical, chemical or thermal. The degree of complexity of such a fundamental process can be appreciated by considering the number of specialised systems involved and the control mechanisms necessary to coordinate them. An understanding of the phenomenon is complicated by the fact that the manifestations associated with the inflammatory process vary depending on the causal agent and on the species being studied. Thus, responses following bacterial infection, which eventually involve the immune system and lead to cytotoxic reactions, must be distinguished from cases where there is no participation of

foreign bodies such as in aseptic trauma or in some thermal lesions. Thus, despite the fact that inflammation has been investigated for many years, it is only possible to recognise some common patterns accompanying injury, such as redness (erythema), swelling (oedema), pain, fever and in some cases loss of function of the tissue affected, and some of the agents which are responsible for causing and maintaining these symptoms. Therefore, in the following summary, a sequence of events which occur in the majority of inflammatory processes will be described, from the onset of the inflammatory (acute phase), to the less understood chronic states. It must be kept in mind, however, that such a description is only a rough approximation to the in vivo situation and it does not take into account the individual cases of inflammation which diverge from the general picture.

1-1-1 Vascular events during inflammation

Following injury, the first evident responses are associated with a locally increased vascular permeability at the level of capillaries and venules of the affected tissue (107). Although the vascular response is prominent immediately after the inflammatory stimulus, the affected tissue must be considered as a whole concerning the response and it is only because the vascular manifestations are more obvious that these aspects of the inflammatory process have been described in more detail. The increased vascular permeability, which can be measured by accumulation of dyes or isotopically labelled proteins at the inflamed tissue is believed to be due to contraction of the adjacent endothelial cells of the venules, which leads to formation of gaps between the cells. As a consequence extravasation of plasma occurs and proteins escape into the interstitial space, while small

particles and cells are retained by the basement membrane (114). The pressure for this filtration is greater than the osmotic pressure of the plasma, probably due in part to the increased blood flow to the affected area as a consequence of local vasodilatation (9). The fluid which has extravasated into the inflamed area together with an obstructed venous outflow contributes to oedema formation (206). The lymphatics appear to play an important role in draining the excess of protein and fluid (107).

1-1-2 Mediators of increased vascular permeability

The immediate increase in vascular permeability which occurs within minutes and lasts for up to 1 hour is believed to be mediated by histamine, although in the rat, 5-hydroxy tryptamine (serotonin) seems also to be an important mediator (109). Local injections of histamine induce increased vascular permeability and pretreatment of the animal with anti-histamines, which block the histamine receptors suppresses this early phase of inflammation. The main sources of histamine are the tissue mast cells which are abundant around vascular and connective tissues, platelets and basophiles. Histamine, which is believed to be synthesized *in vivo* by enzymatic decarboxylation of histidine (152), is stored in the granules of the mast cells, probably complexed to heparin and a protein (190). The release of histamine occurs either as a result of degranulation without cell destruction, in which case all the contents of the granules are also released, or it can be actively and specifically secreted from the mast cells without degranulation (159, 160). Under some circumstances the contents of the granules are released into the extracellular space as a consequence of cell lysis. Such cases are denominated cytotoxic. A number of

basic peptides are able to induce degranulation of mast cells as is the synthetic polyamine called compound 48/80. Depletion of histamine by pretreatment of the animal with compound 48/80 leads to a suppression of the early phase of increased vascular permeability in the same way as pretreatment of the animal with anti-histamines. The mechanisms controlling mast cell degranulation are poorly understood but it seems that in most cases an intracellular inflow of Ca^{++} ions is a requirement (68). Likewise it is not known what factors in the inflammatory process impair further release of histamine after the acute phase. It has been shown that a mechanism of feed-back control caused by histamine itself may be important in this regulation (30). Prostaglandins may be involved in the control of immunological release of histamine (168).

The fact that anti-histamines suppress the increase in vascular permeability only during the initial phases of the inflammatory process suggests the participation of other mediators. These substances have a more prolonged effect than histamine in increasing the vascular permeability. It has been shown that the delayed phase of inflammation is associated with the kinins, of which bradykinin has been the most studied (140, 183). The kinins are polypeptides containing 9-11 amino acid residues. They are generated by proteolytic enzymes (kallikreins, trypsin and pepsin) acting on plasma glycoprotein substrates. The kinins, which are able to produce slow and prolonged contraction of smooth muscle, are quickly catabolized in vivo, which makes their isolation from inflammatory exudates very difficult. It was only after chemical synthesis of these peptides and the development of suitable experimental models of inflammation, that their role as mediators of increased vascular permeability became established.

Another group of vasoactive peptides which are believed to participate in the inflammatory process are the so-called leucokinins. These peptides are produced by proteolytic enzymes present in the leucocytes acting on a plasma protein precursor different from that of the kinins (183).

Following the kinin phase, the events occurring in the inflamed tissue appear to be mainly mediated by the prostaglandins. The participation of prostaglandins in the inflammatory process will be discussed in Chapter 3.

1-1-3 Cell migration and lysosome release

It is during the so-called prostaglandin phase that leucocyte migration to the site of inflammation occurs. The leucocytes are able to adhere to the endothelial interfaces of the small blood vessels and then migrate across the membrane into the interstitial space of the affected tissue. It is not known what factors control the sticking of leucocytes to the vascular endothelium after tissue damage or their migration across the membrane, but presumably Ca^{++} and Mg^{++} are involved (12). It is believed that prostaglandins, released at the site of inflammation may contribute to leucotaxis (leucocyte chemotaxis) (196), although this role of prostaglandins is still not certain since *in vitro* experiments have demonstrated that the leucotactic activity of prostaglandins is not very impressive when compared with other chemotactic agents such as casein (183). Although the type of leucocyte which migrates to the affected tissue has been the subject of much controversy, it is generally assumed that polymorphonucleocytes predominate in the acute cases, whereas in more chronic states the monocytes constitute the majority of cells infiltrating around the

affected area (78, 107, 127, 188). In rheumatoid arthritis, a chronic inflammation of the joints, however, the synovial fluid contains a large number of neutrophils (205). At the site of inflammation the leucocytes then release their lysosomal enzymes which are mainly hydrolytic and capable of digesting a variety of intracellular and extracellular macromolecules. Among the lysosomal enzymes released by the leucocytes are acid and neutral proteases which act on peptides, elastase, β-glucuronidases acting on the links between N-acetyl muramic acid and 2-acetamido-2-deoxy-D-glucose residues of mucopolysaccharides (common constituents of bacterial wall membranes), collagenase, acid phosphatase and α-amylase. These enzymes are responsible for the tissue damage associated with chronic inflammatory states and several of the manifestations observed during inflammation could also be attributed to the secreted products of the leucocytes. The recognized inducers of lysosome release include the immunoglobulins, a number of basic peptides and proteins (including peptides originated from the complement, called anaphylatoxins) and some proteolytic enzymes. In some cases the stimulus for lysosome release can be the interaction between the cells themselves as happens between basophiles and platelets (75, 85). In rheumatoid arthritis, the discharged lysosomal enzymes from neutrophils are believed to be associated with the phagocytosis of particulate material and immune complexes (205). The release of lysosomes can also occur as a consequence of cytotoxic processes, which are generally characteristic of antibody action against the cells, in the presence of complement. Another example of cytotoxic release of cell products is cell lysis after phagocytosis of compounds such as urate crystals, which form hydrogen bonds with the lysosomes intracellularly (187). It is possible that such is the situation

occurring in cases of gout.

The participation of monocytes is usually associated with chronic states of inflammation. These inflammatory processes can last for several months or sometimes years, and in such cases the affected tissue undergoes changes which frequently impair its normal function. Macrophages are found in great numbers around the inflamed tissue, together with connective tissue, which generally surrounds the area forming what is known as a granulomatous inflammation. The macrophages present in the granuloma are intensively engaged in phagocytosis and digestion of foreign material which can be of bacterial or parasitic origin. Materials such as silica, asbestos, beryllium and carrageenan can also induce the same process (127). In some cases, some bacterial products, and carrageenan can remain undigested inside the cytoplasm of macrophages for long periods of time.

The lymphocytes are usually involved in the inflammatory process whenever antigens are present; they can act either as direct cytotoxic agents, by means of direct cell/cell contact following antigen stimulation (34) or by releasing factors known as lymphokines when T lymphocytes are involved (thymus derived lymphocytes), or lymphokines and immunoglobulins when B lymphocytes (bone marrow derived lymphocytes) are involved. The lymphokines are able to affect a number of different systems and can cause effects such as an increase in vascular permeability, stimulation of lymphocyte mitosis, activation of macrophages and others (64). The role of lymphocytes will be discussed in more detail in Chapter 6.

1-2 Experimental models

The anti-inflammatory activity of peptide 401 was assayed in the rat paw carrageenan oedema and also in adjuvant arthritis.

1-2-1 The rat paw carrageenan oedema

The carrageenan oedema, introduced by Winter et al. (198) was the experimental model in which peptide 401 was first shown to have anti-inflammatory activity. This is a very popular assay method for anti-inflammatory drugs and is used for studies on the mediators of inflammation. It is induced by injecting carrageenan into the plantar region of the rat's hind paw. The injected paw gradually swells due to the irritant properties of carrageenan and the volume of the paw can be measured by a variety of techniques. One of the most employed uses volume displacement of mercury (198). After a given interval of time the volume of the uninjected paw is subtracted from the volume of the injected one and this value is used as the equivalent to 100% swelling; the potency of the drugs being tested is then measured as the percentage of inhibition of swelling.

Carrageenan consists of a mixture of polysaccharides (sulphated galactose units) which is obtained from the Irish sea moss (chondrus crispus), although other seaweeds contain materials with similar structural characteristics and biological properties. Two main components have been fractionated from the polysaccharide mixture: K-carrageenan and λ-carrageenan. K-carrageenan turns into a gel in the presence of potassium and λ-carrageenan is not affected. The units composing K-carrageenan are sulphated D-galactose and 3,6-anhydro-D-galactose and in λ-carrageenan there are almost only sulphated D-galactose residues. The fraction which is more effective as an

irritant is λ -carrageenan (47).

After sub-plantar injection of carrageenan into the rats, the observed gradual swelling of the rat's paw is not uniform with time, that is, more than one phase can be distinguished when the swelling of the paw is plotted against time (166). Such a multi-phase curve is an indication of the participation of more than one mediator. This is supported by the observation that several anti-inflammatory agents were able to abolish some phases of the oedema but not completely suppress it, when injected separately into the rats. The work of Di Rosa et al. (48, 49) showed that in the rat carrageenan oedema, the several observed phases of the induced inflammatory process could be correlated to the sequential release of some mediators of inflammation. Thus, by injecting antagonists of histamine and serotonin, depletion of kinins and granulocytes and injection or oral administration of inhibitors of prostaglandin biosynthesis it was possible to evaluate the contribution of each mediator to the maintenance of the oedema. According to these authors the sequence of mediator release in the carrageenan oedema is the following:

0	-	1.5 h	-	release of histamine and serotonin
1.5 h	-	2.5 h	-	kinins
2.5 h	-	6 h	-	prostaglandins.

Cell migration to the site of inflammation was shown to occur concomitantly with the appearance of prostaglandins at the site of inflammation. Although it was not possible to determine if the migrating cells were releasing the prostaglandins or if they were released by the cells of the affected tissue, it was shown (115) that polymorphonucleocytes are able to release prostaglandins during phagocytosis.

The participation of the complement system seems also to be a requirement throughout this induced inflammatory process since it was shown that in complement-depleted rats the oedema was suppressed for 6 h after injection of carrageenan (48). Although the carrageenan oedema shows many common features with inflammation of several other ethiologies, it should be pointed out that carrageenan is able to induce side effects such as hypersensitivity to cold in rats, diarrhoea, ulceration of colon and loss of weight in rabbits and guinea pigs and anti-coagulant activities (47).

1-2-2 The rat adjuvant arthritis

Peptide 401 was also shown to effectively reduce the swelling of paws of rats with induced adjuvant arthritis (27). In this model of inflammation, which is considered to be a pathological condition of immunological origin, the paws of rats are injected with killed mycobacterium tuberculosis and Freund's adjuvant. After several days a general inflammatory process takes place which has some similarities to human rheumatoid arthritis although the severity of this systemic inflammatory model is so pronounced and induces so many radical changes in the rats, such as acute loss of weight, swelling of fore paws, ears, tail and joints, together with what appears to be an infection of the respiratory tract (166, 129) that comparison is difficult. However, with the possible exception of inflammatory models where carrageenan is directly injected into the knees of rabbits (119), the rat adjuvant arthritis is considered to be the closest model available to the human rheumatoid arthritis (182).

The first lesions which can be observed are the oedemas with infiltration of monocytes, mainly lymphocytes. Elimination of or

alteration in the lymphocytes induced by means of lymphocytotoxic drugs, alleviates or prevents the arthritic syndrome (166). The implications of the role of lymphocytes in the rat adjuvant arthritis and the 'anti-arthritic' activity of peptide 401 will be considered in Chapter 6.

After the oedema formation, synovial proliferation occurs, with fibrin deposition and increased proliferation of fibroblasts and osteoblasts which actually increase the size of the bone structures. The increased proliferation of connective tissue leads to formation of granulomas, which then spread and invade the joints.

Among the common features between the induced rat adjuvant arthritis and the human rheumatoid arthritis, are the granulomatous swellings which progressively destroy the joints, monocyte infiltration and the doses of non-steroid and steroid anti-inflammatory drugs which are able to affect the course of the swelling.

1-3 Biological properties of peptide 401

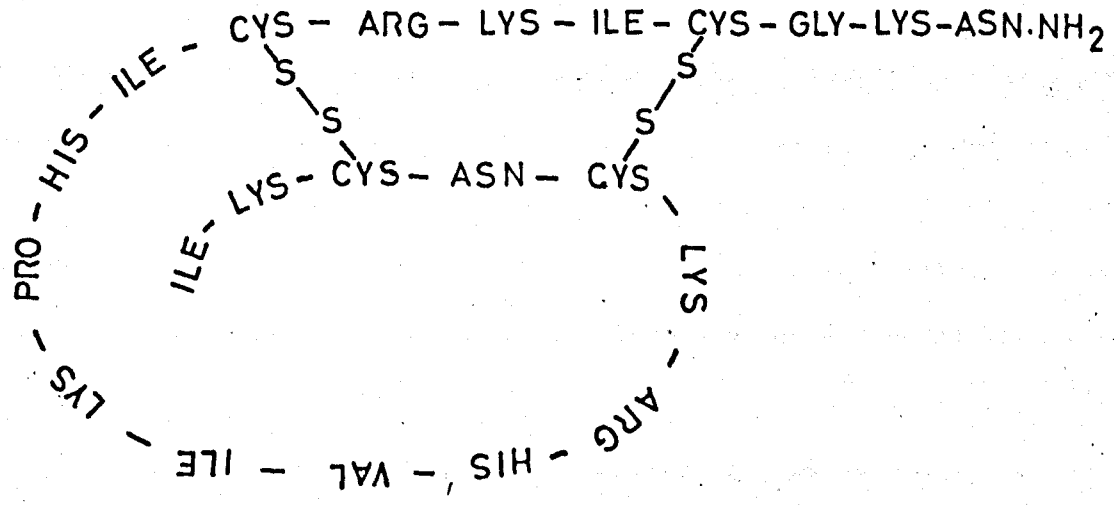
1.3.1 Mast cell degranulating activity

During the systematic investigation of the components of the venom of the honey bee (Apis mellifera), Breihaupt et al. (31) isolated a peptide which proved to be very active in degranulating mast cells. For this activity the peptide was called MCD peptide (mast cell degranulating factor). The mast cell degranulating activity of this peptide was found to be comparable with that of compound 48/80, and the observed vasodepressor response which occurred in rats after intravenous injection of MCD seemed to be reflecting an action of MCD on histamine and serotonin release by the mast cells. It was noticed that when

compound 48/80 was injected repeatedly into the rats prior to injection of MCD, the vasodepressor activity of MCD was depressed. The same effect could be obtained when the animal was pretreated with MCD followed by injections of 48/80, indicating that the vasodepressor effect was mediated by the same agents, présumably histamine and serotonin. No lytic effects of MCD on red cells could be seen.

1-3-2 Primary structure of peptide 401

Working independently, the group of Vernon et al. (177) isolated a peptide from a preparation of crude venom of the same species of bee. This peptide was called peptide 401. Upon analysis of the amino acid sequence, the primary structure of peptide 401 was found to be identical with that of MCD, as established by Haux (82). The primary sequence of peptide 401 is the following:



corresponding to a molecular weight of 2628. It contains no acidic amino acids and 9 basic amino acids and thus is a highly basic peptide and will be strongly positively charged under physiological conditions. The position of the S-S bridges was determined by the group of Vernon et al. (177).

1-3-3 Anti-inflammatory activity of peptide 401

When peptide 401 was tested for anti-inflammatory activity, using the rat paw carrageenan oedema as an anti-inflammatory model, it was found to have a potency comparable with that of other well known anti-inflammatory agents (27). Recently a more detailed study on the mast cell degranulating activity of peptide 401 has been carried out (13). Histamine release from mast cells by peptide 401 was found to be independent of extracellular Ca^{++}. It was temperature and pH dependent with an optimum activity at the physiological conditions. Cytotoxic effects on mast cells, as shown by the presence of LDH in the supernatant (lactodehydrogenase is a cytoplasmic enzyme and its release into the extracellular space indicates structural changes on the membrane, usually when lytic processes are occurring), were only perceptible at peptide 401 concentrations of 0.1 mg/ml. Peptide 401 is able to induce mast cell degranulation at concentrations of 0.001 mg/ml. Peptide 401 was also able to induce histamine release from rat leucocytes although with much less potency than its action on mast cells. No histamine release from human leucocytes could be observed. These results together with the ion requirement for histamine release from mast cells led to the suggestion that peptide 401 and compound 48/80 share a similar mechanism of action on the mast cells (11, 13).

It was suggested that the ability of peptide 401 to degranulate mast cells was not connected to the anti-inflammatory effects since compound 48/80 and melittin, another basic peptide isolated from bee venom, which are able to degranulate mast cells did not have any anti-inflammatory activity (27). Furthermore it was also established (27) that the anti-inflammatory activity of peptide 401 was not affected when the rats were pretreated with antagonists of histamine and serotonin.

The mast cell degranulating activity of peptide 401 does in fact give it a pro-inflammatory action which is reflected by the increased vascular permeability observed locally after intradermal injection of peptide 401 in the rats. The anti-inflammatory activity of peptide 401 does not seem to be dependent on production and release of corticosteroids or catecholamines from the adrenal glands, since it persists, although somewhat reduced in adrenalectomized animals (27). The possible involvement of corticosteroids in the anti-inflammatory activity of peptide 401 will be considered in Chapter 5.

Further work on the anti-inflammatory properties of peptide 401 carried out by the group of Hanson et al. (81), established the following points:

1 - Peptide 401 was able to suppress the local increased vascular permeability induced by histamine, bradykinin, serotonin and prostaglandins, as measured by accumulation of labelled albumin at the site of inflammation.

2 - Antagonists of histamine and serotonin were able to block the local inflammatory action of 401 which is presumably due to an indirect action of 401 on the mast cells, but the anti-inflammatory activity of 401 was not affected by pre-treatment of the animal with those antagonists.

3 - The anti-inflammatory activity of 401 was not suppressed by local denervation and did not seem to be mediated by catecholamines and corticosteroids.

4 - No effects of peptide 401 on tissue perfusion could be observed as shown by intravenous injection of ^{86}Rb followed by collection and counting of various tissues, measurement of skin temperature and blood flow in the perfused mesentery.

Although there was no indication that peptide 401 has any vascular effects, the authors concluded that the anti-inflammatory activity of peptide 401 could be explained by an action on the rat blood vessels. By somehow interacting with the blood vessels, peptide 401 would be rendering them unresponsive to inflammatory agents liberated during the carrageenan oedema. However, this suggestion, besides implying a local action of peptide 401 at the level of the rat's capillaries and venules either around the inflamed tissue or systemically, which is unlikely, cannot account for the therapeutic action of peptide 401 in the adjuvant arthritis condition, since in this inflammatory model the lesions involved must certainly transcend the vascular level.

1-4 Aims of the present work

The experiments described in the present work were attempts to investigate possible mechanisms of action of peptide 401. Peptide 401 and melittin were first purified from the crude bee venom, as described in Chapter 2. Melittin was purified and used as a control in some experiments since it is also a very basic peptide with a molecular weight close to that of peptide 401. Melittin shows no anti-inflammatory activity although it shares with peptide 401 the ability to degranulate mast cells.

Since many non-steroid anti-inflammatory agents have been found to inhibit the synthesis of prostaglandins, it was reasoned that peptide 401 could exert its anti-inflammatory effects by means of a similar mechanism. These experiments are described in Chapter 3. In Chapter 4, the possibility that peptide 401 could interact specifically with the rat leucocytes was considered, and the experiments done in order to test this involved the use of a modified peptide 401 (dansylated).

Model membrane systems (liposomes) were also used in order to establish if the lipid composition of the membranes was important for the binding and consequent action of peptide 401. The experiments described in Chapter 5, were carried out to test if peptide 401 could directly stimulate the synthesis and release of corticosterone by the adrenal glands of rats. The last chapter describes the experiments in which peptide 401 was investigated for its possible influence on the activation of lymphocytes, an effect that would be relevant to the therapeutic action of peptide 401 on the more chronic conditions of inflammation such as in the induced adjuvant arthritis.

CHAPTER 2

PURIFICATION OF PEPTIDE 401 AND MELITTIN

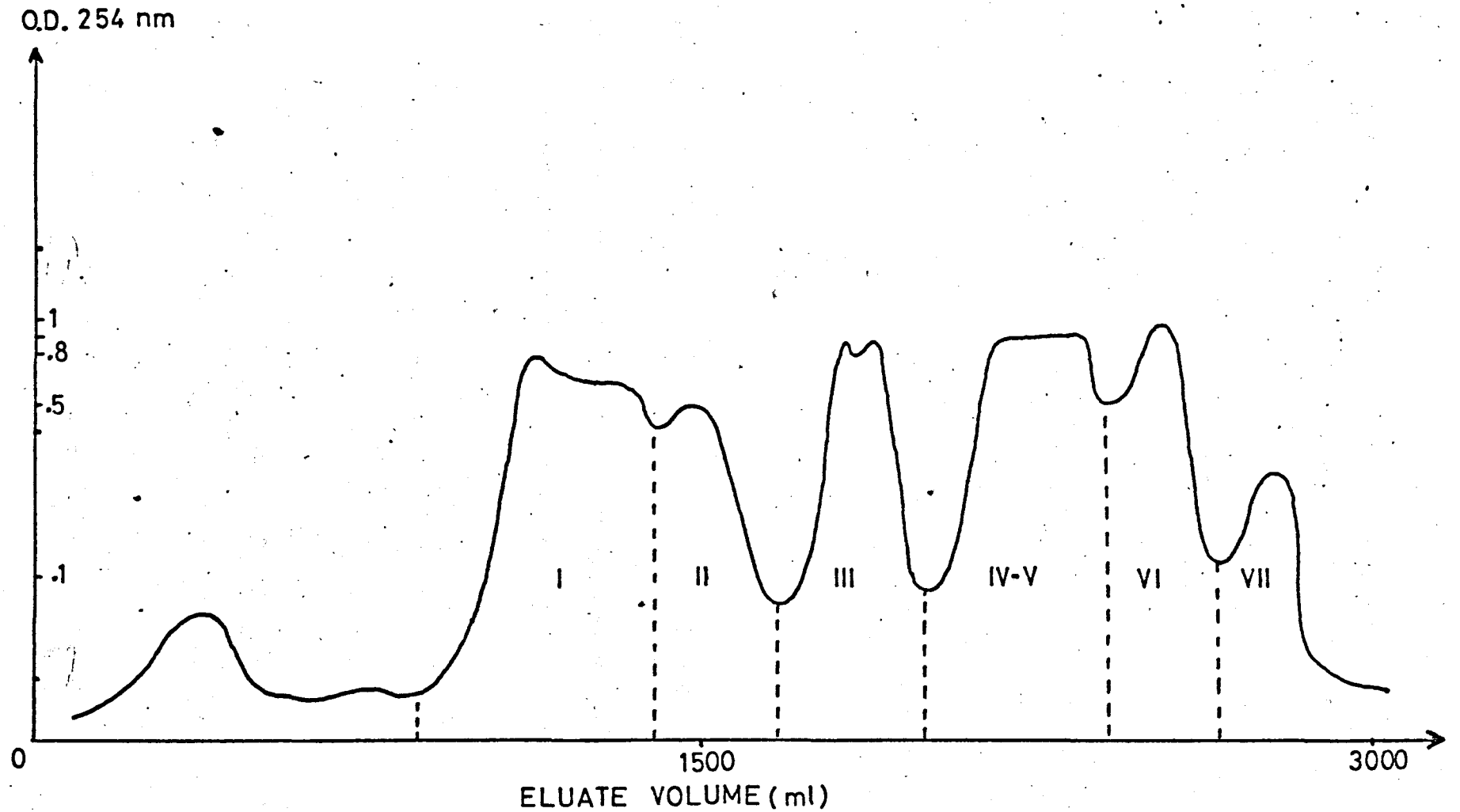
2-1 Peptide 401

The original bee venom as obtained from Bulgaria was processed there in the following way: the venom, collected on a glass plate was dried in the sun and scraped off. This powder was dissolved in water and filtered through filter paper. The filtrate was then freeze-dried and the resulting powder was dissolved in 0.1 M formic acid and subjected to force dialysis through cellophane tubing (8/32 Visking) for 72 h at 4°C. The solution outside the dialysis tubing was freeze-dried and used as the starting material for the purification of peptide 401 and melittin.

The first stage of separation was done in this laboratory by means of two K 50/100 columns, packed with Sephadex G 50 SF, equilibrated with 2% acetic acid (v/v). The gel was washed several times with 2% acetic acid prior to packing. A flow of 100 ml/h was maintained by means of a peristaltic pump for at least 24 h before application of the samples. The samples applied on the columns consisted of 10% (w/v) solutions of the starting material dissolved in 2% acetic acid. Samples of 3-5 g were applied at each time, amounting to a total of 15 g.

The elution pattern obtained after this first step is depicted in Fig. 2-1. The separation of the eluate as described in Fig. 2-1 was based on the spectra of the several peaks, taken on a Pye Unicam SP 800 ultraviolet spectrophotometer. The spectrum of fraction II indicated the absence of aromatic groups and tryptophan. Fraction

FIG 2-1 - Elution of the crude venom



II was then evaporated to about 3-4 % of its original volume, using a Büchl Rotavapor-R system. The concentrated solution was then freeze-dried, yielding a white powder weighing 1.12 g. This corresponded to 8% of the original weight applied to the Sephadex G 50 columns.

Fraction II was dissolved in 10 ml of 0.2 M Na^+ acetate buffer pH 4.2 and applied on a column of Sephadex SP G 25, a cation exchanger, which was equilibrated with the Na^+ acetate buffer. Prior to use the Sephadex gel was washed several times with the starting buffer and packed into a column measuring 24 x 40 mm. The buffer was pumped through the column until the pH of the effluent solution was equal to that of the buffer. The flow rate was 50 ml/h. After the sample had been applied, at least one column volume of the buffer was pumped through before the NaCl gradient started. The system used is described in Fig. 2-2. Beakers A and B were identical in size and volume. Vessel A contained a 1 M solution of NaCl in the 0.2 M Na^+ acetate buffer pH 4.2. Vessel B contained the 0.2 M Na^+ acetate buffer. Both containers were connected by means of thick tubing filled with the dilute buffer solution (0.2 M Na^+ acetate pH 4.2). Fig. 2-3 shows the elution pattern thus obtained.

The above fractions were collected and roto-evaporated. Each of the concentrated solutions obtained after evaporation was then applied to a column (24 x 60 mm) packed with Sephadex G 25 F, equilibrated with 5% acetic acid, for desalting. The presence of Cl^- ions in the eluates of the G 25 F column was detected by addition of drops of a $AgNO_3$ solution to aliquots of the fractions and observing for precipitates. The Cl^- free fractions were then roto-evaporated and freeze-dried. The weights of the freeze-dried fractions were: IIa - 0.376 g, IIb - 0.061 g, IIc - 0.0866 g, IId - 0.0297 g, IIe -

FIG 2-2 - Gradient system used for the isolation of peptide 401

1-magnetic stirrer
2-column
3-pump
4-recorder
5-fraction collector

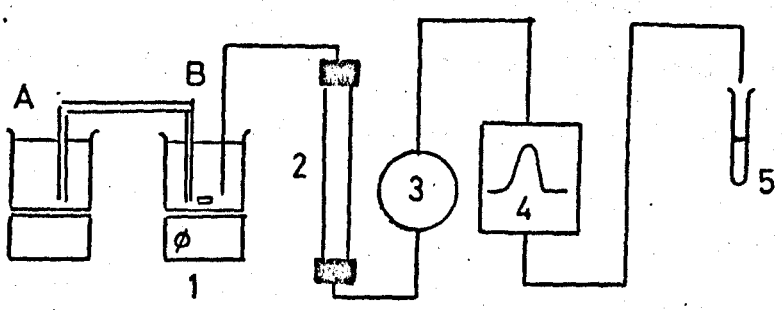
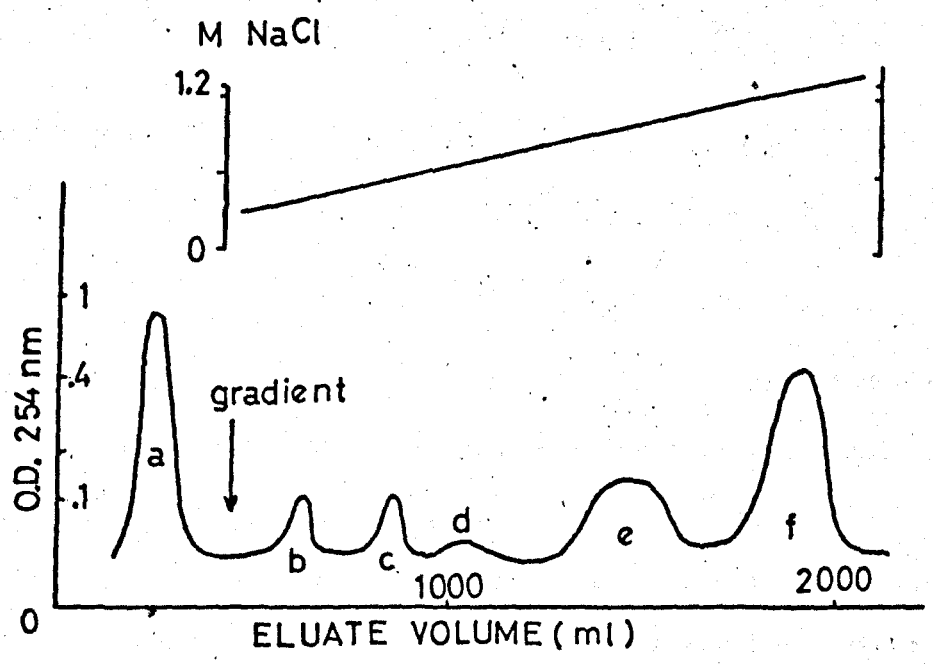


FIG 2-3 - Elution pattern of fraction II

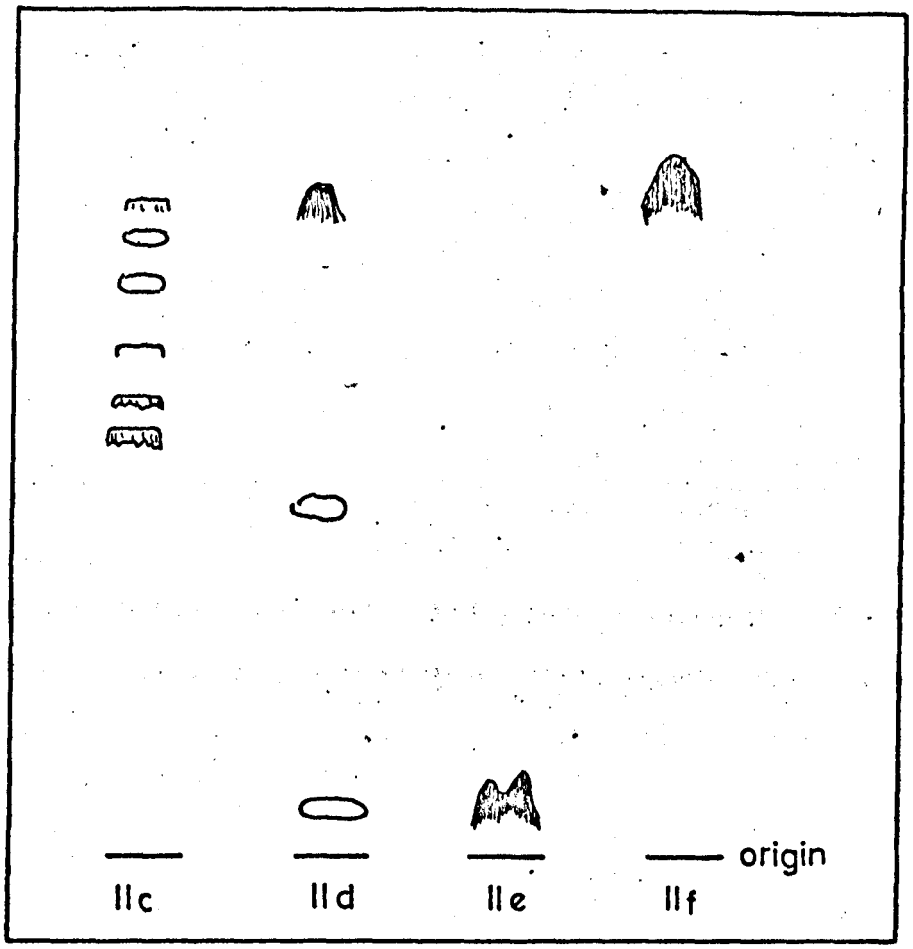


0.642 g, IIf - 0.234 g.

A small sample of the above fractions was diluted in water to a concentration of 0.1 mg/µl and applied to cellulose TLC plates. The plates were developed overnight in a solvent system consisting of n-butanol : pyridine : acetic acid : water (90:60:18:72 v/v). The plates were then dried at room temperature, sprayed with a ninhydrin solution and placed in an oven for 5 min at 75°C. Fig. 2-4 shows the separation obtained. Fraction IIa was discarded since it did not bind to the Sephadex SP C 25 gel; fraction IIb was also discarded because it was not ninhydrin positive.

Fractions IIc - IIf were analysed for N-terminal amino acids, using the dansyl chloride method, as follows: to samples of the peptides dissolved in water, 10 µl of a 0.2 N Na_2CO_3 solution was added; this solution was then evaporated to dryness. Afterwards 7 µl of water and 7 µl of an acetone solution of dansyl chloride (final concentration of 2.5 mg/ml) was added and left to stand for 1 h at room temperature. The contents of the tubes were dried and 50 µl of 6N HCl was added to hydrolyse the dansyl-peptides. The tubes were sealed and placed in an oven at 105°C overnight and then 5 µl of 50% pyridine (v/v) was added and from this solution 0.25 µl was applied to polyamide TLC sheets. The sheets were first developed using a solvent system consisting of 1.5% formic acid (w/v) in water. The second run was made by turning the sheet through 90° and developing it in a solvent system consisting of benzene : acetic acid (9:1 v/v) followed by ethyl acetate : methanol : acetic acid (20:1:1 v/v). Standard dansyl-amino acids were also developed in the same way. The sheets were then observed under UV light. The results were the following: Fraction IIc - Tyr, fraction IId - GLY, VAL, fraction IIe - ILE, and fraction IIf - GLY.

FIG 2-4 - Thin layer chromatography of samples from fraction II



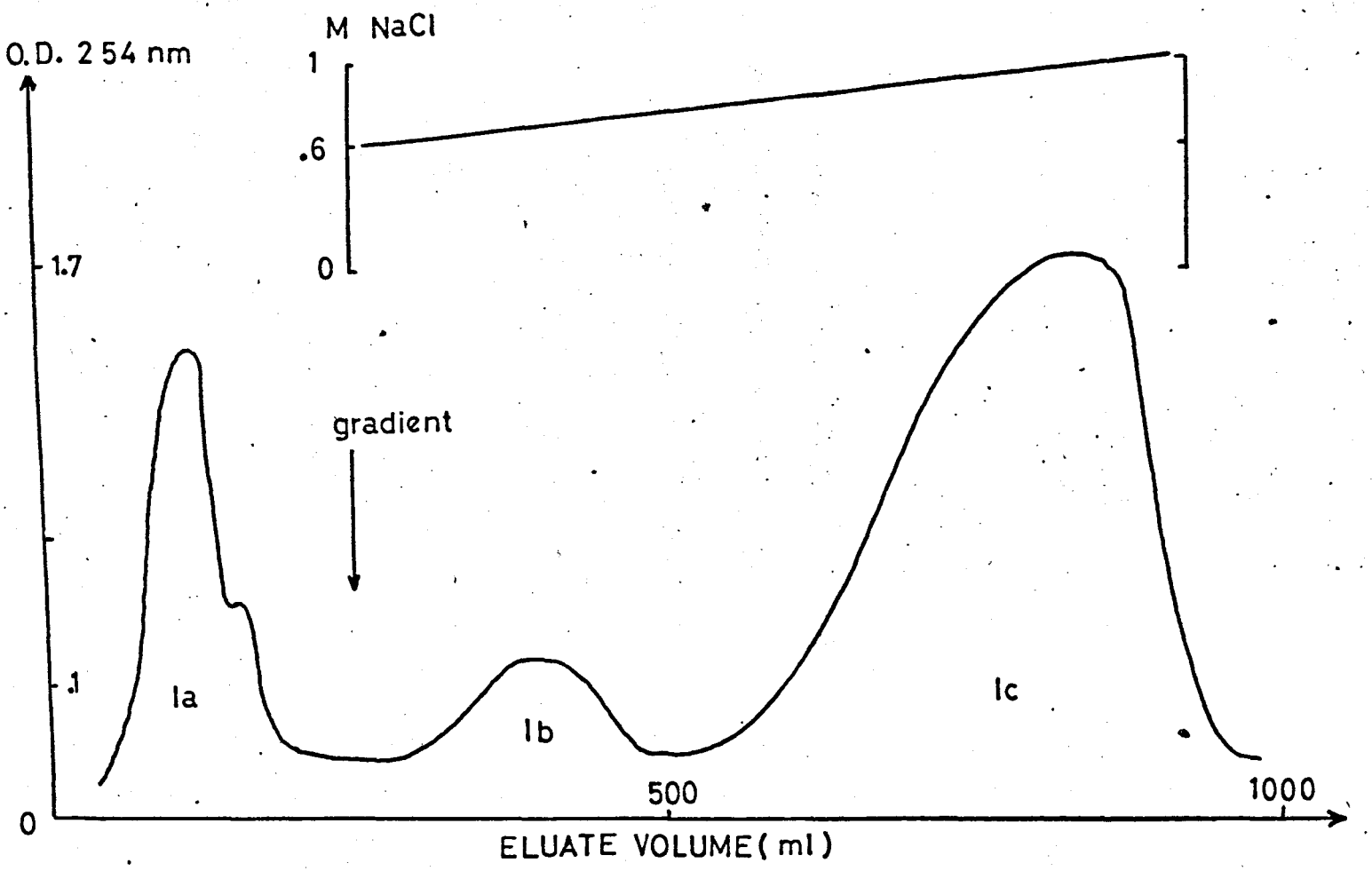
Fraction IIe was tested in the rat paw carrageenan oedema assay and shown to have a potent anti-inflammatory activity. When fraction IIe was incubated with rat mast cells it produced intense degranulation. Therefore fraction IIe consisted of peptide 401.

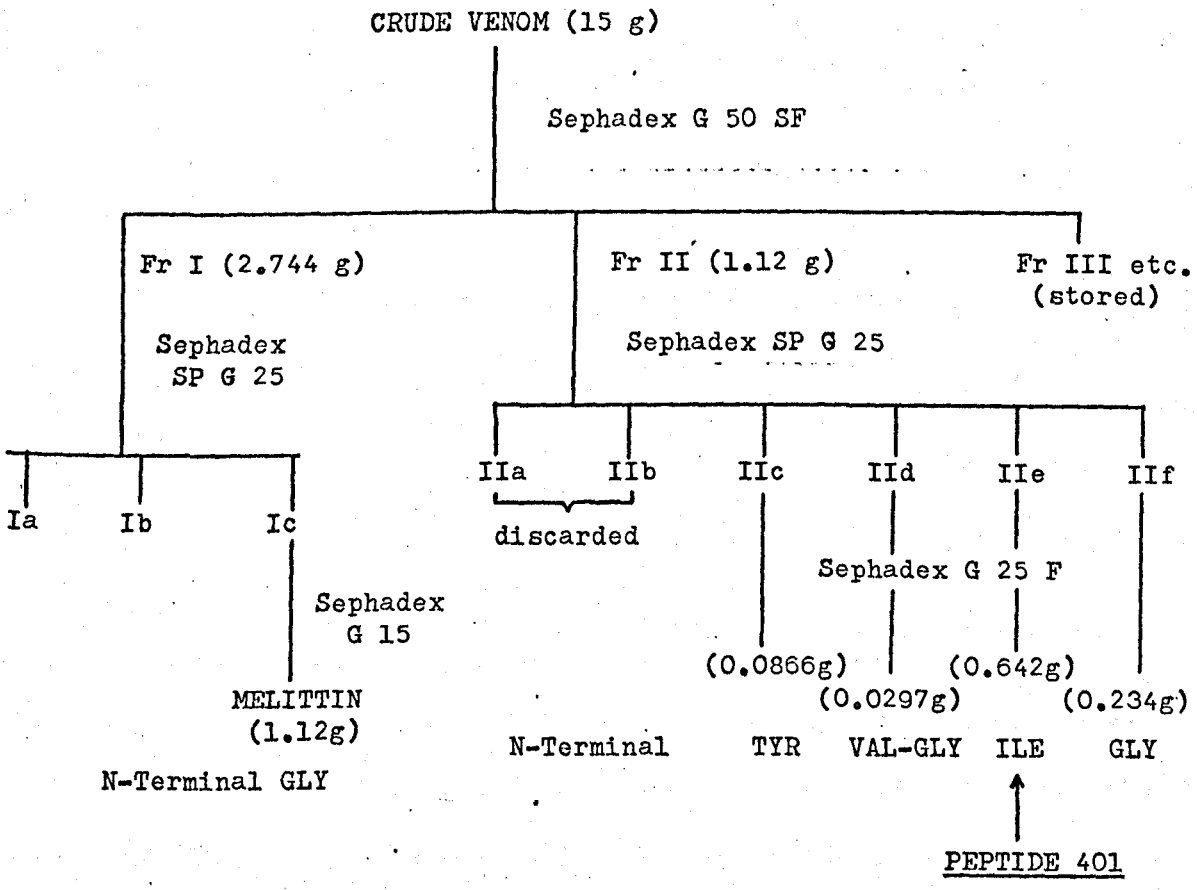
2-2 Melittin

Fraction I (see Fig. 2-1), weighing 2.644 g, was applied to a K 50/100 column packed with Sephadex G 50 SF equilibrated with 2% acetic acid. The fractions which were eluted did not separate very well and so they were recombined, roto-evaporated and applied on a Sephadex SP C 25 column equilibrated with 0.6 M Na^+ acetate buffer pH 4.2. A gradient of NaCl was applied up to a concentration of 1.05 M, using the same system described before. The elution pattern is presented in Fig. 2-5. Fraction Ic was roto-evaporated and desalted using a column of Sephadex G 15. After roto-evaporation and freeze-drying the weight of fraction Ic was 1.12 g. Thin layer chromatography on cellulose plates revealed a single spot and the N-terminal amino acid was glycine. This fraction consisted of melittin.

The diagram below summarizes the purification of peptide 401 and melittin.

FIG 2-5 - Elution of fraction I (purification of melittin)




CRUDE VENOM (15 g)
Sephadex G 50 SF
Fr I (2.744 g)
Fr II (1.12 g)
Fr III etc.
(stored)
Sephadex
SP G 25
Sephadex SP G 25
Ia
Ib
Ic
IIa
IIb
IIc
IId
IIe
IIf
discarded
Sephadex G 25 F
Sephadex
G 15
(0.0866g)
(0.0297g)
(0.642g)
(0.234g)
MELITTIN
(1.12g)
N-Terminal GLY
N-Terminal
TYR
VAL-GLY
ILE
GLY
PEPTIDE 401

CHAPTER 3

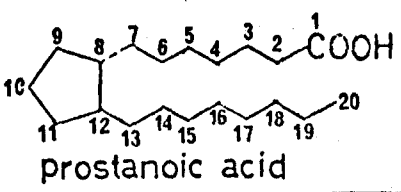
PEPTIDE 401 AND INHIBITION OF PROSTAGLANDIN SYNTHESIS

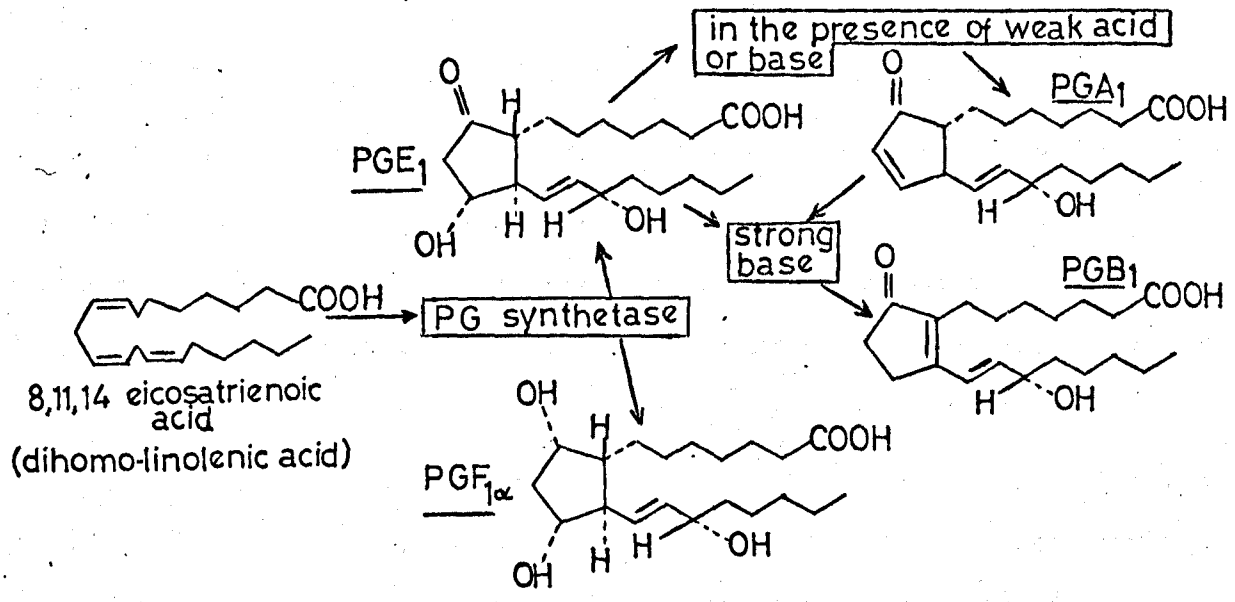
3-1 Introduction

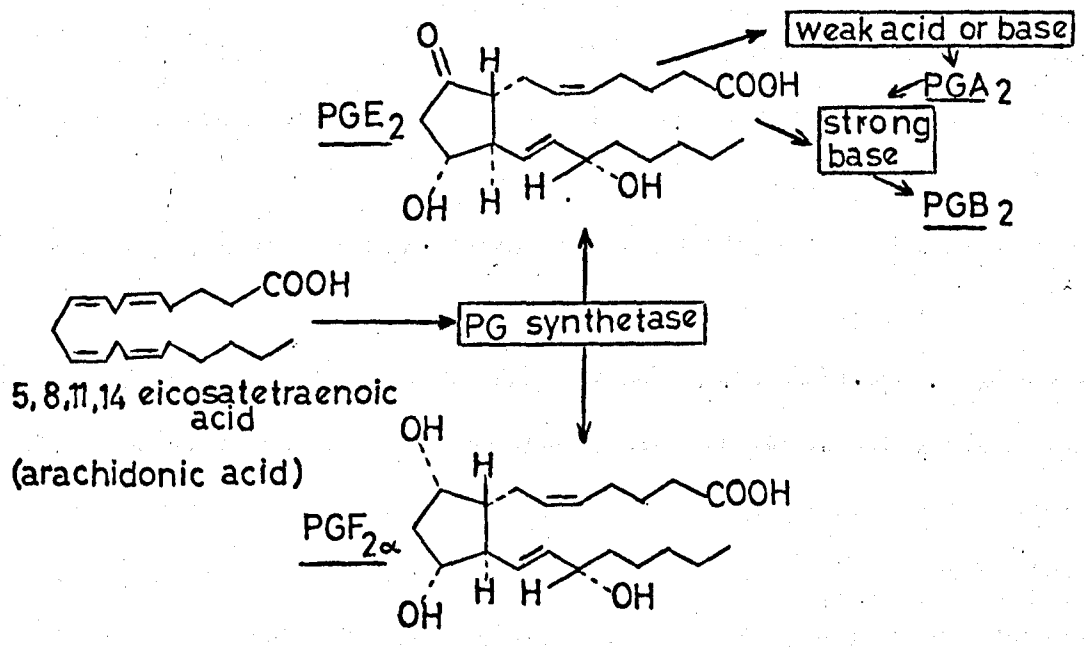
In this introduction, prostaglandins in general will be reviewed briefly, with more emphasis given to prostaglandin E_2, since the experiments described in this chapter are mostly concerned with the participation of this prostaglandin in the inflammatory process.

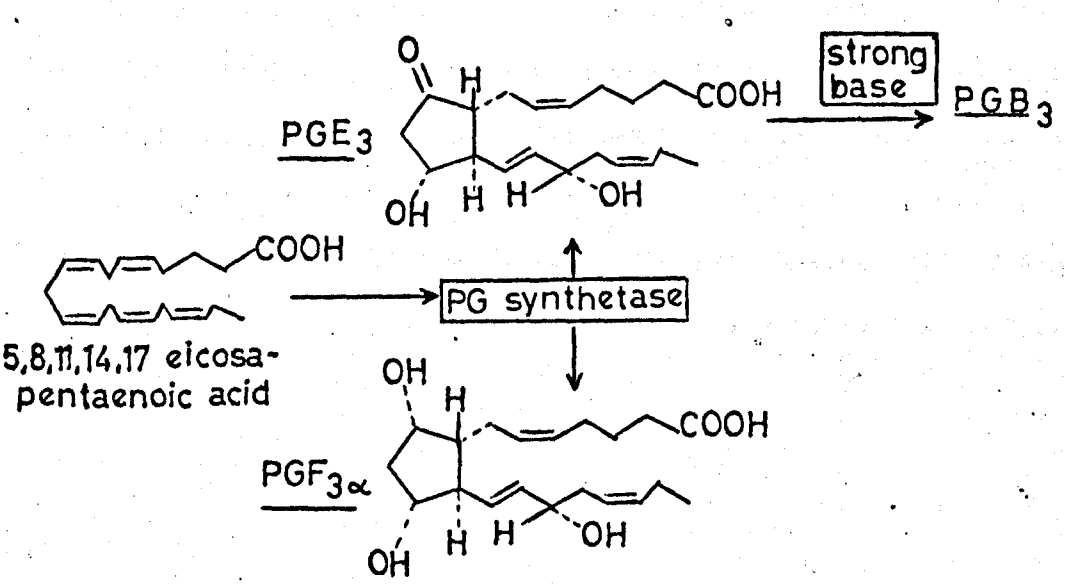
3-1-1 Isolation and structure of prostaglandins

The prostaglandins, discovered and described by Von Euler (55) and independently by Goldblatt (74), are poly unsaturated, cyclic, hydroxy fatty acids (although C-19 fatty acids can generate prostaglandins, the most common percursors are the C-20 fatty acids). The following diagram shows the main prostaglandins together with their precursors. The structure of prostaglandins is based on that of prostanoic acid. The subscript numerals denote the number of unsaturated bonds. Prostaglandins are divided into two main groups, namely E and F. The E series is characterized by the ketone group at C-9 and is thus named because during the isolation procedures, it was found to be more soluble in the ether phase, while the prostaglandins F, with an hydroxyl group at C-9, were more soluble in the phosphate buffer phase (25, 24). Prostaglandins A and B can be obtained by alkaline treatment of prostaglandins E, and both occur naturally. Thus it is believed that the A and B compounds are probably derived from prostaglandins E by dehydration reactions occurring in vivo, but whether they display well defined physiological actions or are simply catabolic products of PGE's is not certain (65). It has been suggested, however, that PGA_2 may be involved in the control of Na^+ excretion (108).


prostanoic acid


in the presence of weak acid or base
PGE1
PGA1
strong base
PGB1
PG synthetase
8,11,14 eicosatrienoic acid
(dihomo-linolenic acid)
PGF1α


weak acid or base
PGE2
PGA2
strong base
PGB2
PG synthetase
5,8,11,14 eicosatetraenoic acid
(arachidonic acid)
PGF2α


strong base
PGE3
PGB3
PG synthetase
5,8,11,14,17 eicosa-pentaenoic acid
PGF3α

3-1-2 Physiological actions and occurrence of PGs

Prostaglandins have been demonstrated to participate in a number of different biological processes such as contraction of gastrointestinal smooth muscle (22), inhibition of gastric secretion (139), contraction of reproductive smooth muscle (54, 93), relaxation of respiratory smooth muscle (113), effects on the cardiovascular system (92), blood platelet aggregation (102), diuresis and natriuresis (172, 108), luteolysis (134), actions on the nervous system (91), intraocular pressure (179), adipose tissue (165) and the endocrine system (90).

Prostaglandins were shown to be present in almost all mammalian tissues (97, 98), in corals (185) and in insects (46). However, the amount of prostaglandins which can be isolated from each tissue varies, and usually the human seminal fluid and seminal vesicles are the richest sources of prostaglandins (80, 32, 50). Because of their widespread occurrence and functional diversity, it has proven difficult to ascribe a specific physiological role to the prostaglandins. The accepted idea is that prostaglandins are modulators of hormone activity since many of the physiological actions of prostaglandins appear to be mediated through the adenyl and guanyl cyclase systems (89, 138, 200). The intracellular concentrations of cyclic AMP and GMP seem to be the controlling factors for the release of pro-inflammatory agents such as histamine and lysosomal enzymes (94). In some cases, however, prostaglandins can also be considered as hormones in the classical sense, since they can be released into the circulation and affect target cells distant from the site of release. This was observed with PGA's and $PGF_{2\alpha}$. PGE's are usually catabolized very quickly (62, 173).

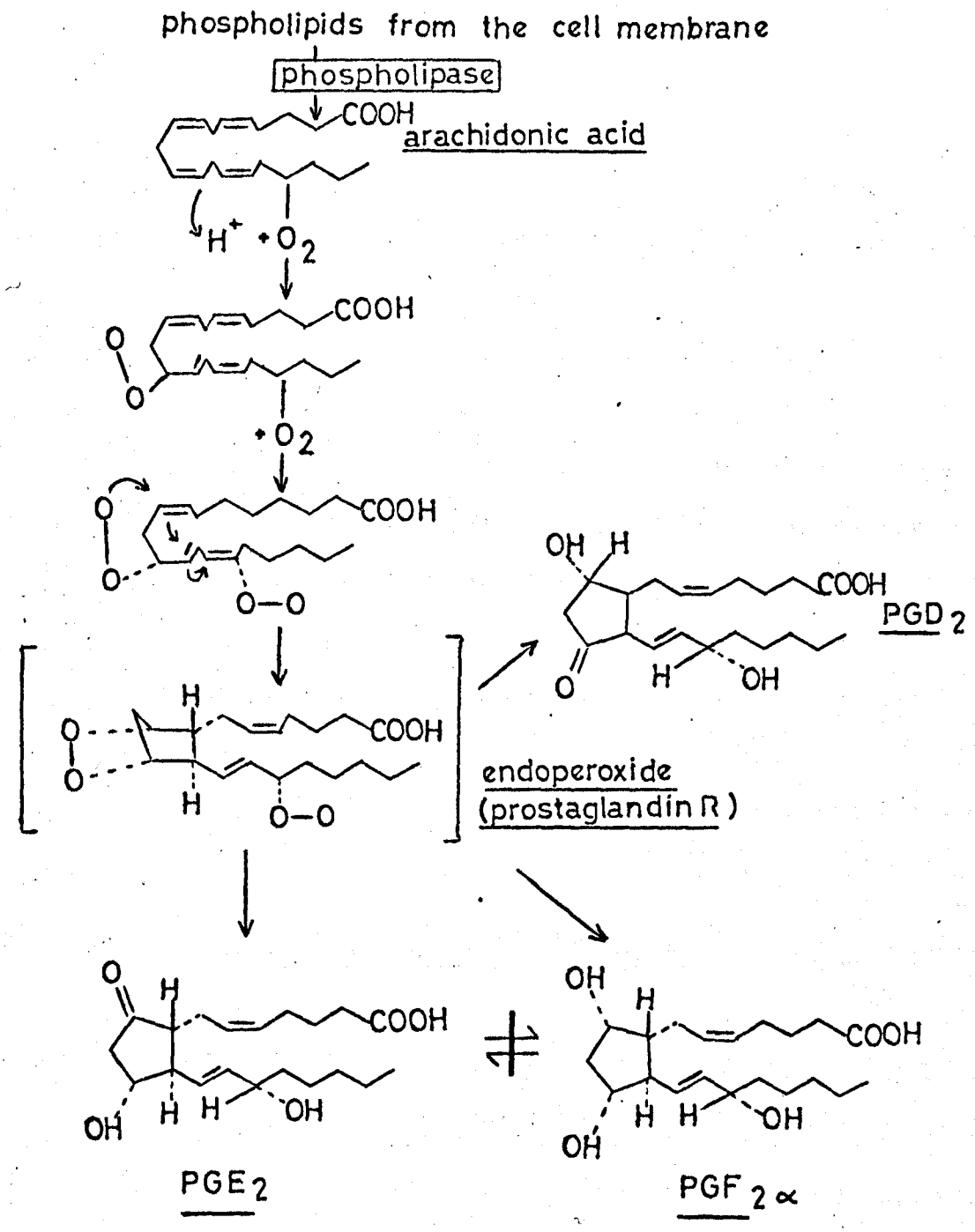
3-1-3 Precursors of prostaglandins

The essential fatty acids which are the precursors of prostaglandins do not seem to be available in a free form in the serum. It is believed that the major sources of arachidonic acid and dihomolynolenic acid are the phospholipids associated with the cell membranes (106). It is assumed that phospholipases play an important role in providing the substrates for the prostaglandin synthetases (103). The results of Bartels et al. (17) showed that injection of phospholipase A into intestines of frogs led to formation of prostaglandins, probably due to an increased amount of available precursors.

3-1-4 Biosynthesis of prostaglandins

The enzymatic complex, responsible for the synthesis of prostaglandins, generically called prostaglandin synthetase /1.14.99.1 - 8,11,14 - eicosatrienoate, hydrogen donor: oxygen oxidoreductase - in the case of PGE_1 synthesis, when 8,11,14 eicosatrienoic acid is the precursor/, is also present in most of the tissues investigated (88, 137).

Prostaglandin E_2 was found to be formed by enzymatic cyclization of arachidonic acid (20, 51). The results of studies on the mechanism of biosynthesis of prostaglandin E_2 (101, 125, 144, 147), are summarized below. There is no reason, however, to believe that the biosynthetic pathway of the other prostaglandins is very different from that of PGE_2 (19).



These *in vitro* studies on the mechanism of synthesis of prostaglandins showed that PGE_2 and $PGF_{2\alpha}$ were not interconvertible although it is possible that *in vivo* there are enzymes which can reduce PGE_1 and PGE_2 to $PGF_{1\alpha}$ and $PGF_{2\alpha}$ respectively.

Details regarding the factors regulating which specific prostaglandin is to be formed by the enzymatic complex are not completely

understood. In the work of Lands et al. (104) it was found that glutathione, one of the cofactors used for the in vitro studies of the prostaglandin synthetase, favoured the formation of PGE's, whereas the presence of copper prevented the formation of PGE's and allowed reduction of the intermediate endoperoxide to form PGF's.

Prostaglandin synthetase from sheep seminal vesicles has been partially purified (148, 149). It was found to be insoluble and associated with the microsomal fraction. Experiments in vitro demonstrated that the enzymatic complex requires glutathione and hydroquinone or adrenaline as cofactors (52). During the isolation procedure it was found that a heat stable soluble cofactor was necessary for activity. This heat stable cofactor presumably plays the same role as the cofactors added to the medium in the in vitro experiments (149).

3-1-5 Fate and release of prostaglandins

Once formed prostaglandins seem to be catabolized very quickly by a prostaglandin dehydrogenase. Prostaglandin dehydrogenase is present in practically all tissues investigated, but the lungs are considered to have the highest activity (6, 150). Until quite recently it was assumed that prostaglandins were not stored in the cells, but synthetized and released at the time of stimulus (135) since the amount of prostaglandins which could be extracted from the 'resting' tissues investigated was always very small. However, a recent paper suggests that prostaglandins can be stored in adipose tissue (110) and that corticosteroids are able to inhibit their release but not their synthesis. The results of Higgs and Youlten (87) showed that 'resting' rabbit polymorphonucleocytes contain some amount of stored prostaglandins, although this could be reflecting some biosynthesis of prostaglandins. The control of

prostaglandin synthesis is so far not yet understood. The release of prostaglandins from the tissues appears to occur following mechanical, hormonal and neurological stimuli (135).

3-1-6 Prostaglandins in the inflammatory process

Of all the reported biological actions of prostaglandins, their participation as mediators of the inflammatory process, is one of the most well documented. Although there is plenty of evidence available demonstrating direct and indirect effects of prostaglandins in inflammatory states, the mode of action of these compounds is not yet clear. This situation is made even more complicated since in many cases the same prostaglandins can have antagonistic effects and the amount and type of prostaglandins present in an inflammatory exudate depends on the kind of inflammatory model used, and on the species being studied (200, 166). For example there are some models of experimental adjuvant polyarthritis where PGE_1 and PGE_2 were shown to have anti-arthritic activities in the rat (10, 203). These prostaglandins were also able to reduce the levels of lysosomal enzymes such as β-glucuronidases (201) and to inhibit phagocytosis by guinea pig polymorphonucleocytes (39). These effects would seem to confer to the prostaglandins of the E series, anti-inflammatory activities. However, in most models of acute inflammation, prostaglandins of the E series were shown to act as potent pro-inflammatory agents, causing erythema, oedema, increased vascular permeability and pain, whereas prostaglandins of the F series were generally anti-inflammatory, especially $PGF_{2\alpha}$ (41, 69, 196, 176). The reason for such conflicting results is not clear, but certainly predictable on the basis of the multiplicity of functions of prostaglandins, on their widespread occurrence, as well as the apparent non-specificity of stimulus

required for their synthesis and release. Another point to consider is the different nature of the inflammatory models employed in these studies. An adjuvant arthritis condition must differ considerably from the acute carrageenan oedema, whether considering the cellular aspects (in chronic inflammatory states, there is a big proportion of monocytes circulating while in the more acute cases such as the carrageenan oedema, polymorphonucleocytes predominate in the early stages) (188), or the general chemical environment, that is, the participation of different components in the two different models which could act as blockers or potentiators for prostaglandin action. The local concentration of prostaglandins also appears to be an important factor determining their final effect on the inflammatory state. In the adjuvant arthritis experiments, where PGE_1 and PGE_2 were found to be anti-inflammatory relatively high concentrations (200-500 µg per rat injected at each time) were used, which produced side effects such as somnolence and occasional diarrhoea (10, 203). The results of Glenn et al. (72, 73) showed that PGE_1 and PGE_2 were able to produce inflammatory oedemas in rats, mice, gerbils and hamsters when injected into these animals. When PGE_1 was injected locally into croton oil induced granulomas, it had a potent pro-inflammatory effect, but higher dosages of this prostaglandin (0.5 - 4 mg/kg) inhibited the granulomatous reaction. Again such pharmacological doses which were required to produce anti-inflammatory and anti-arthritic effects caused adrenal hyperplasia, prostration and diarrhoea. Another instance of the dose dependent action of prostaglandins is the lymphocyte transformation. Lymphocyte transformation into blast cells is inhibited by several prostaglandins (162, 163), but higher concentrations of PGE_1 are able to stimulate lymphoblast transformation (189).

Dose dependent biphasic effects are known to occur with other mediators of the inflammatory process. Histamine, which is responsible for the early stages of the acute inflammation is also able to inhibit further release of histamine from mast cells when its concentration rises to a certain level (30). The idea that prostaglandins can antagonize their own actions depending on the concentration is likewise compatible with a 'feed-back' control with the purpose of avoiding perpetuation of the inflammatory condition.

Prostaglandins are able to reproduce many of the observed manifestations associated with inflammation. Vasodilatation was observed in the frog by Von Euler (55). Peripheral vasodilatation was obtained in man when prostaglandins were injected intravenously (21). Intradermal injections of PGE_1 and PGE_2 caused oedema and erythema (3, 4). Prostaglandins induced local increased vascular permeability in the rat and man (42, 96) and potentiated the actions of histamine and bradykinin in increasing local vascular permeability (192, 59) and increase in arterial pressure (61). Prostaglandins, mainly PGE_2, were isolated from inflammatory exudates in rat and in man (41, 8, 194, 193) and appear to be released during the so-called second phase of inflammation or the 'prostaglandin phase' (48, 49). This release of prostaglandins in the carrageenan oedema was correlated with leucocyte migration to the site of inflammation (48, 49, 5), but whether the invading leucocytes were responsible for the synthesis and release of the prostaglandins was not certain (175), since there are cases when prostaglandins are released locally during the inflammatory stimulus and are able to induce increased vascular permeability in the absence of polymorphonucleocytes (195). However, it is known that rabbit polymorphonucleocytes are able to synthetize prostaglandins, mainly

PGE_1 and that this synthesis can be stimulated during phagocytosis of bacteria (87, 86). It was suggested that the phospholipases of the leucocytes, which are normally stored in the lysosomes, are released during the endocytosis of carrageenan, which then, by acting on the phospholipids of the membranes provide the precursors for the synthesis of prostaglandins (5). This suggestion was based on the observation that other lysosomal enzymes such as β-glucuronidase and acid phosphatase are released concomitantly with the prostaglandins in the carrageenan oedema.

Prostaglandins E_1 and E_2 were shown to have chemotactic activities for leucocytes (95, 116) which led to the suggestion that this effect could be part of a physiological mechanism of attraction of leucocytes to the site of inflammation for as long as phagocytosis was occurring (86, 115). Such a process, in an uncontrollable way, could be occurring in autoaggressive pathological states such as rheumatoid arthritis (86). In chronic states of inflammation, the participation of PGE_1 was also associated with stimulation of collagen biosynthesis and thus abscess formation (7, 28).

In a recent report (29) the carrageenan oedema was induced in rats lacking the dietary essential fatty acids, which are the precursors of prostaglandins. It was found that the rat paws did not swell as much as the controls, but that arachidonic acid restored the swelling to the control levels. In these animals, the kinin phase of the carrageenan oedema is unaltered. Interestingly enough, if the oedema is induced with chicken egg white, instead of carrageenan, no difference in the swelling of paws can be observed between the essential fatty acid deficient rats and controls, showing once more the different characteristics of different inflammatory models.

Prostaglandins E_1 and E_2 are able to produce fever when injected directly in the brains of cats and rabbits (117, 57) and have also been shown to be associated with pain, acting directly at nerve endings (59) and possibly potentiating the effects of bradykinin and histamine (63).

3-1-7 Inhibition of PG synthesis by anti-inflammatory agents

Recently, strong support to the idea that prostaglandins are mediators of the secondary phase of the inflammatory process was given by the results of Vane *et al.* (174, 161, 60), which showed that non steroidal anti-inflammatory agents such as aspirin and indomethacin are inhibitors of prostaglandin synthesis *in vitro* and *in vivo*. These inhibitors do not interfere with the early development of the carrageenan oedema in the rat, which is believed to be mediated by histamine, serotonin and bradykinin (49). *In vivo* experiments showed that the observed anti-inflammatory activity of these non steroidal compounds occurred mainly during the prostaglandin phase of the carrageenan oedema and that the non steroidal anti-inflammatory agents were also able to inhibit migration of monocytes to the site of inflammation (49).

The following *in vitro* experiments were carried out in order to establish if the anti-inflammatory activity of peptide 401 could in any way be correlated with inhibition of PGE_2 synthesis, using an enzymatic complex obtained from sheep seminal vesicles.

3-2 Experimental

3-2-1 Materials

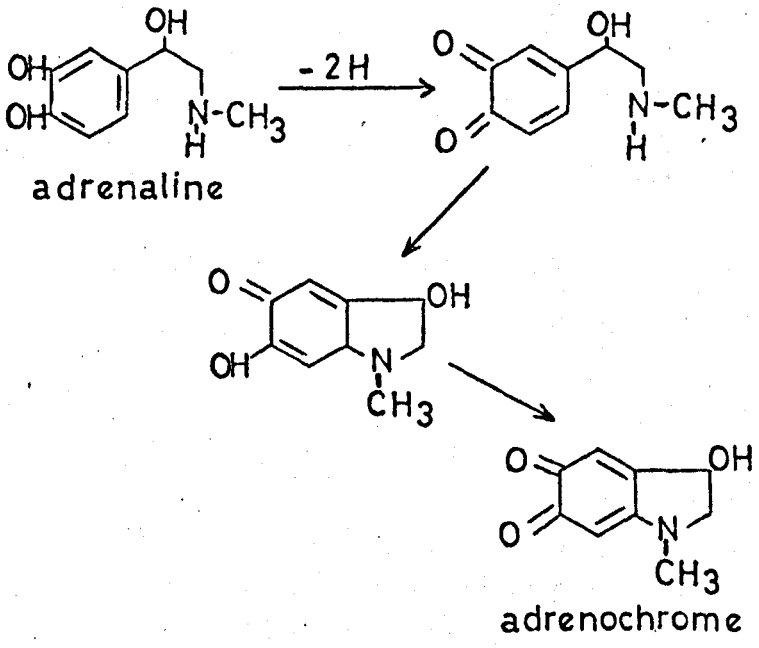
The two batches of the prostaglandin synthetase complex used were obtained from sheep seminal vesicles and were gifts from Dr. D. Wallach (The Upjohn Co.) and Dr. R.J. Flower (The Wellcome Research Laboratories). Arachidonic acid, glutathione (reduced) and trizma base were purchased from Sigma Chemical Co.; L-epinephrine from Koch-Light Laboratories; acetyl salicylic acid from Aldrich Chemical Co.; arachidonic-1-^{14}C (58 mci/mM) from the Radiochemical Centre, Amersham; silica gel plastic backed thin layer plates from Merck. Indomethacin was a gift from Dr. J. Hanson (The Kennedy Institute) and PGE_2 in crystalline form from Dr. J.E. Pike (The Upjohn Co.).

All the chemicals were used with no further purification. The labelled and unlabelled arachidonic acid were checked for purity by applying them onto a silica gel thin layer plate which was developed in a solvent system consisting of ethyl acetate : water : trimethyl pentane : acetic acid (11 : 10 : 5 : 2 v/v). The front was allowed to run for 17 cm and the plate was stained under iodine vapour, presenting no signs of impurities.

3-2-2 Spectrophotometric assay of prostaglandin synthesis

The first spectrophotometric assay tried was that of Takegushi *et al.* (167). This method consisted in measuring the formation of adrenochrome during the incubation of the enzymatic system with arachidonic acid and adrenaline in tris buffer pH 8.3 at 30°C. Adrenochrome is an oxidative product of adrenaline (L-epinephrine). The oxidation of adrenaline takes place at the same time as arachidonic acid undergoes the oxidative cyclization to form PGE_2. The following sequence

of reactions is believed to occur:

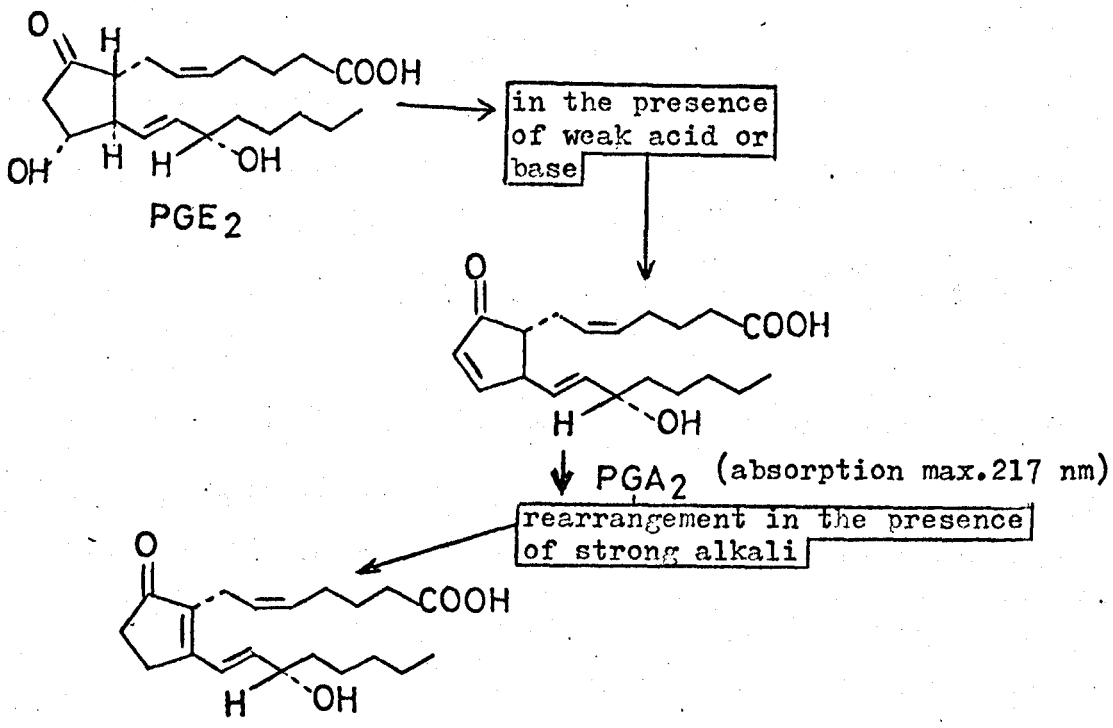


The adrenochrome formed is then estimated by spectrophotometry since it absorbs light at 480 mμ. The results however were not satisfactory, probably due to the instability of adrenochrome in the alkaline pH of the incubation medium. In this alkaline pH, adrenochrome oxidises forming melanin, a brown pigment with a different absorption maximum (83). As the results obtained by incubation of the enzymatic system with known inhibitors of prostaglandin synthesis, such as aspirin and indomethacin, were not in agreement with the results reported in the literature, and as there was much variation within a single set of experiments, this method was abandoned.

3-2-3 PGB_2 method

This method is based on the alkaline conversion of PGE_2 to PGB_2. When prostaglandins of the E series are incubated in an alkaline medium, the so called PGB's are formed which absorb ultraviolet light

at 278 mμ (18). The sequence of reactions involved is the following:



The experimental procedure, based on the method of Wallach *et al.* (181), consisted in incubating 10 mg of the acetone-pentane powder containing the prostaglandin synthetase with 0.273 mM arachidonic acid, 3.6 mM adrenaline in 0.05 M Tris buffer pH 8.3 for 5 min at 30°C with constant agitation. The final volume of the incubation medium was 3 ml. Arachidonic acid, which was kept in a stock benzene solution (50 mg / 10 ml), was added to a 10 ml volumetric flask and the benzene was evaporated under a stream of N_2. The reaction started with the addition of the incubation medium containing the enzymatic complex to the flask containing the dried arachidonic acid, and after 5 min it was stopped by adding 0.25 ml of 2 M citric acid. The inhibitors tested were usually

pre-incubated with the enzymatic suspension for 5 min before the reaction started. Indomethacin, which is not soluble in aqueous solutions, was dissolved in a few drops of ethanol (absolute) and then the buffer was added to the required volume. It was found that the small amount of ethanol in the incubation medium did not interfere in any way with the activity of the enzyme. The three blanks consisted of pre-incubating the enzyme with citric acid before adding it to the substrate flask, incubating the enzymatic complex in the absence of arachidonic acid and pre-heating the enzyme at 60°C for 5 min, cooling it to 30°C and then incubating it in the normal way.

Immediately after the reaction was stopped, the incubation medium was transferred to a 100 ml separating funnel and the prostaglandins were directly extracted with 20 ml of dichloromethane. The lower phase was collected in a 100 ml pear-shaped flask and the remaining upper phase was transferred to another separating funnel in order to obtain more of the dichloromethane extract.

The extract thus obtained was dried under vacuum, using a roto evaporator and the residue diluted in 3 ml of methanol (sol. A). From solution A, an aliquot of 1 ml was further diluted with 3 ml of methanol and the absorbancy at 278 mμ was measured using a Unicam SP 500 spectrophotometer. Another 1 ml aliquot from solution A was incubated with 0.2 ml of a 3 M methanolic solution of KOH, diluted to a total volume of 4 ml with methanol and incubated at 50°C for 30 min, to convert all the PGE_2 formed during the enzymatic incubation into PGB_2. After the alkaline treatment, the absorbancy of this solution was measured at 278 mμ, and the amount of chromophore formed under alkali treatment is given by the difference between the two readings. This value was then used to calculate the original concentration of PGE_2, using a standard

concentration-absorbancy (278 mμ) curve for known amounts of PGE_2 which had been subjected to the alkali treatment.

3-2-4 Radiochemical method

This method was conducted essentially as described by Flower et al. (66). The enzymatic complex used in this set of experiments came from another batch, but was also obtained from sheep seminal vesicles.

The enzymatic complex was incubated at a concentration of 4 mg/ml, for 10 min at 37°C with constant shaking, in a medium containing 1 mM arachidonic acid (0.08 μci of 1-^{14}C-arachidonic acid was added to the benzene solution of the cold arachidonic acid and the solvent was removed by evaporation under N_2), 5 mM reduced glutathione and 5 mM adrenaline, in a 100 mM Tris buffer solution, pH 8.2. The total volume of the incubation medium was 0.5 ml. The inhibitors were incubated with the enzymatic suspension for 5 min prior to their addition to the substrate. The blanks consisted of incubating the enzymatic complex with 0.25 ml of 1N HCl prior to addition to the substrate.

The reaction was stopped by addition of 0.25 ml of 1N HCl and then the prostaglandins were extracted with 1.5 ml of ethyl acetate. During extraction, the tubes were stirred for 15 seconds on a Vortex mixer and aliquots of 1 ml were collected from the upper phase. These aliquots were taken to dryness under vacuum and the residues were redissolved in 0.05 ml of ethanol (absolute). From these solutions, aliquots of 0.02 ml were applied to the plastic backed silica gel plates using automatic pipettes, and the plates were then developed in a solvent system consisting of the upper phase of the following mixture: ethyl acetate : water : trimethylpentane : acetic acid (11 : 10 : 5 : 2

v/v). Samples of PGE_2 were also applied as standards. The front was allowed to run for 17 cm and then the plates were dried at room temperature. Prostaglandins were visualized by exposing the plates to iodine vapour and the spots corresponding to PGE_2 were cut and placed directly in scintillation vials, containing 10 ml of a solution of 4 g of 2,5-diphenylaxazole (DPO) in 1000 ml of toluene and 500 ml of the detergent Triton X-100. The vials were stirred, and then counted in a Beckman liquid scintillation spectrometer. Before every experiment, the vials containing the scintillation fluid were counted and the values obtained used as the radioactive background.

In the first experiments, the spots corresponding to $PGF_{2\alpha}$ and PGD_2 were also cut and counted, but as the inhibition found for a given concentration of peptide 401 was constant for the prostaglandins E_2, F_2 and D_2, in some experiments only PGE_2 samples were counted.

3-3 Results

3-3-1 Spectrophotometric method (PGB_2 method)

Table 3-I shows the results of submitting standard samples of known PGE_2 concentration to the alkali treatment described before. This gives the straight line calibration, shown in Fig. 3-1, the equation of the straight line being

$$y = 0.1702 + 14.659\ x$$

where y = PGE_2 concentration (μg/ml)

x = difference between absorbancies before and after alkali treatment.

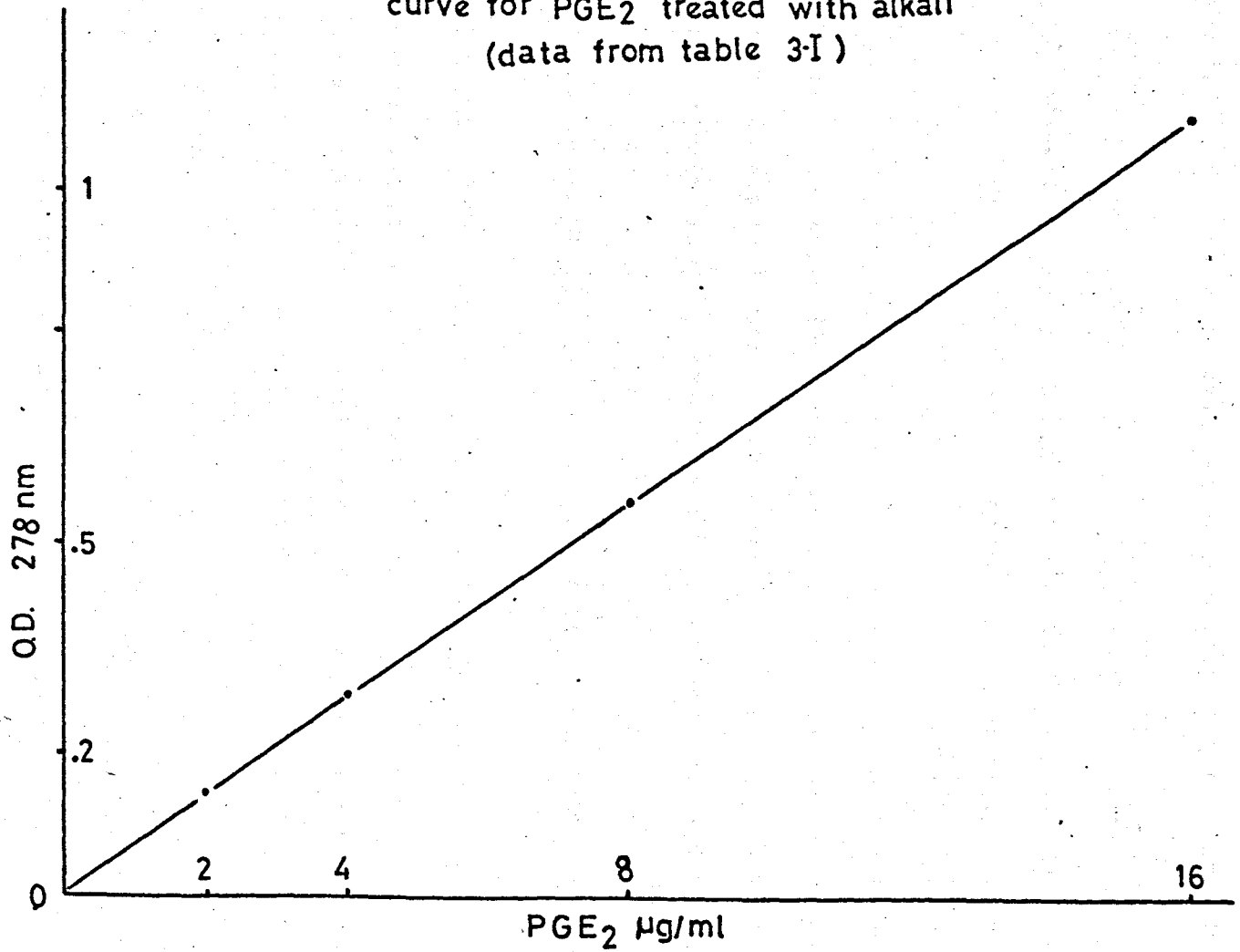
This equation was used to calculate unknown PGE_2 concentrations.

Table 3-I

Standard concentration-absorbance (278 nm) curve for PGE_2 treated with alkali

PGE_2 concentration (μg/ml)	Absorbance 278 nm	Average
16	1.083 1.124	$\bar{x}$ = 1.103
8	.556 .558	$\bar{x}$ = .557
4	.286 .289	$\bar{x}$ = .287
2	.147 .146	$\bar{x}$ = .146

FIG 3-1 Standard concentration-absorbance (278nm) curve for PGE_2 treated with alkali (data from table 3-I)



When samples of PGE_2 of known concentrations were added to a phosphate buffered saline, pH 7.4, extracted with dichloromethane and subjected to the same alkali treatment as described before, the recovery of the prostaglandin E_2 was of the order of 31% $\pm$ 2.7%.

The results of the experiments where peptide 401, indomethacin and aspirin were incubated with the prostaglandin synthetase complex, are summarized in Table 3-II and Fig. 3-2.

Although other prostaglandins which are also products of the enzymatic reaction can absorb ultraviolet light at 278 nm upon alkali treatment (18, 23), in the experiments employing the spectrophotometric method described before, no attempt was made to separate the extracted prostaglandins formed during incubation of the enzymatic complex with arachidonic acid. This is probably the reason why the values obtained employing the spectrophotometric method are higher than the ones obtained using the radiochemical method. However, it has been demonstrated that under the conditions used in the above experiments (arachidonic acid concentration of 0.273 mM), PGE_2 is the main product of the enzymatic reaction (66). Peptide 401, incubated with the enzymatic complex at a concentration of 0.6 mg/ml is able to inhibit PGE_2 formation by 41%. A concentration of peptide 401 of 0.06 mg/ml fails to show any significant inhibition of PGE_2 synthesis. Indomethacin (20 μM) and aspirin (1 mM) produced inhibitions of 71% and 73% respectively.

3-3-2 Radiochemical method

The counting efficiency of 1-^{14}C arachidonic acid was of 85%, with the scintillation fluid containing Triton X-100. Fig. 3-3 shows the quenching curve obtained by plotting the counting efficiency of 1-^{14}C-arachidonic acid, against the external standard ratio of the

Table 3-II

Effect of peptide 401, indomethacin and aspirin on PGE_2 synthesis

	Control	401 (22.8 µM 0.06 mg/ml)	401 (228 µM 0.6 mg/ml)	Indomethacin (20 µM)	Aspirin (1 mM)
µg PGE_2 in 3 ml of extract	10.08	9.33	6.62	2.34	2.46
	10.44	9.12	5.91	3.78	1.89
	9.12	8.46	5.34	3.12	1.23
	9.21	7.89	4.95	2.79	3.06
					1.74
	9.99	10.08	6.33	2.61	5.73
Mean AN S.D.	$\bar{x}$ 9.76±0.57	$\bar{x}$ 8.97±0.83	5.83±0.68	2.92±0.55	2.68±1.61

	PGE_2 (µg in 3 ml)
Enzyme pre-heated at 60°C for 5 min	0.39
Enzyme incubated without arachidonic acid	0.272

FIG 3-2 - Effect of peptide 401, indomethacin and aspirin on PGE_2 synthesis. (data from Table 3-II)

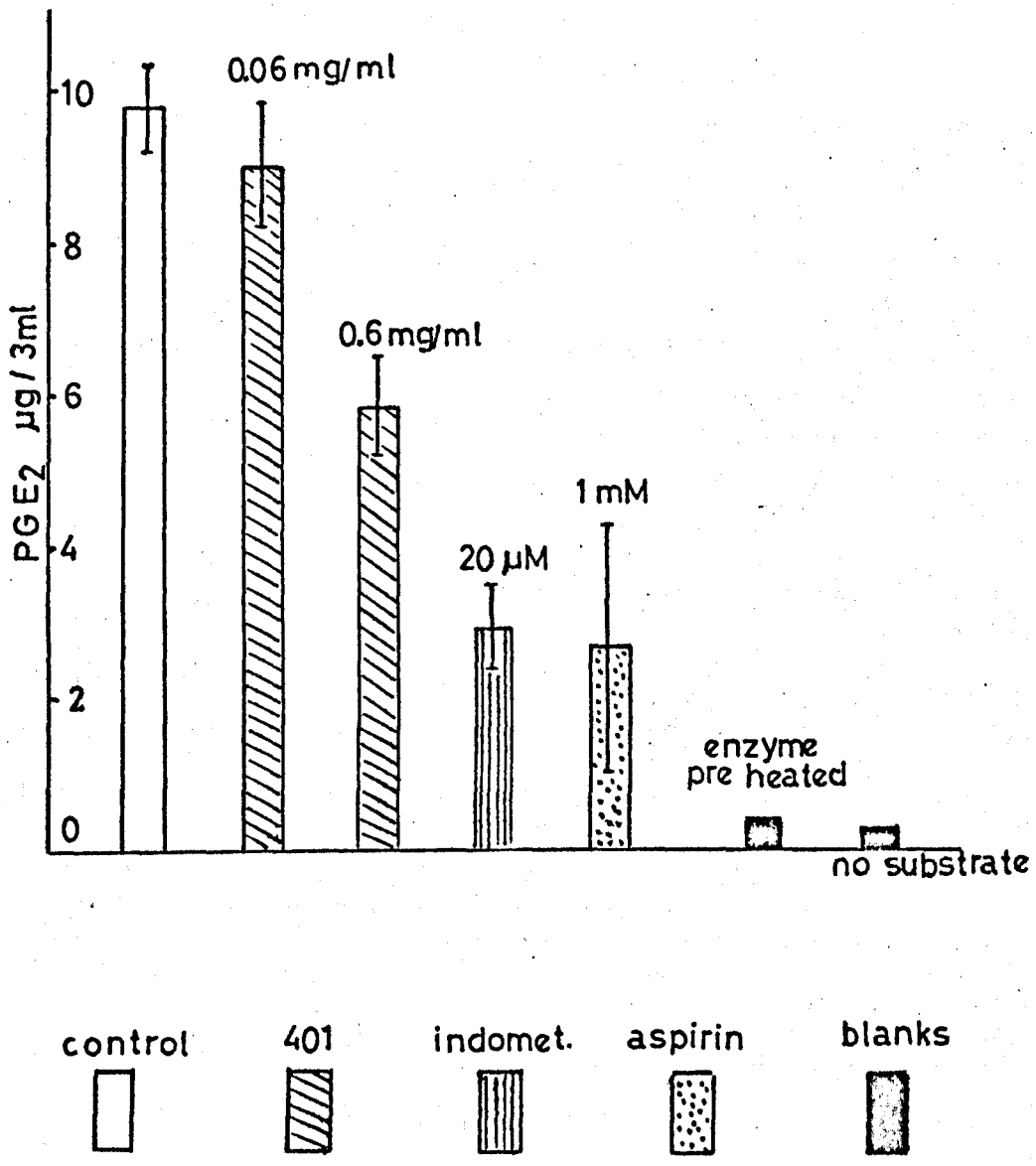
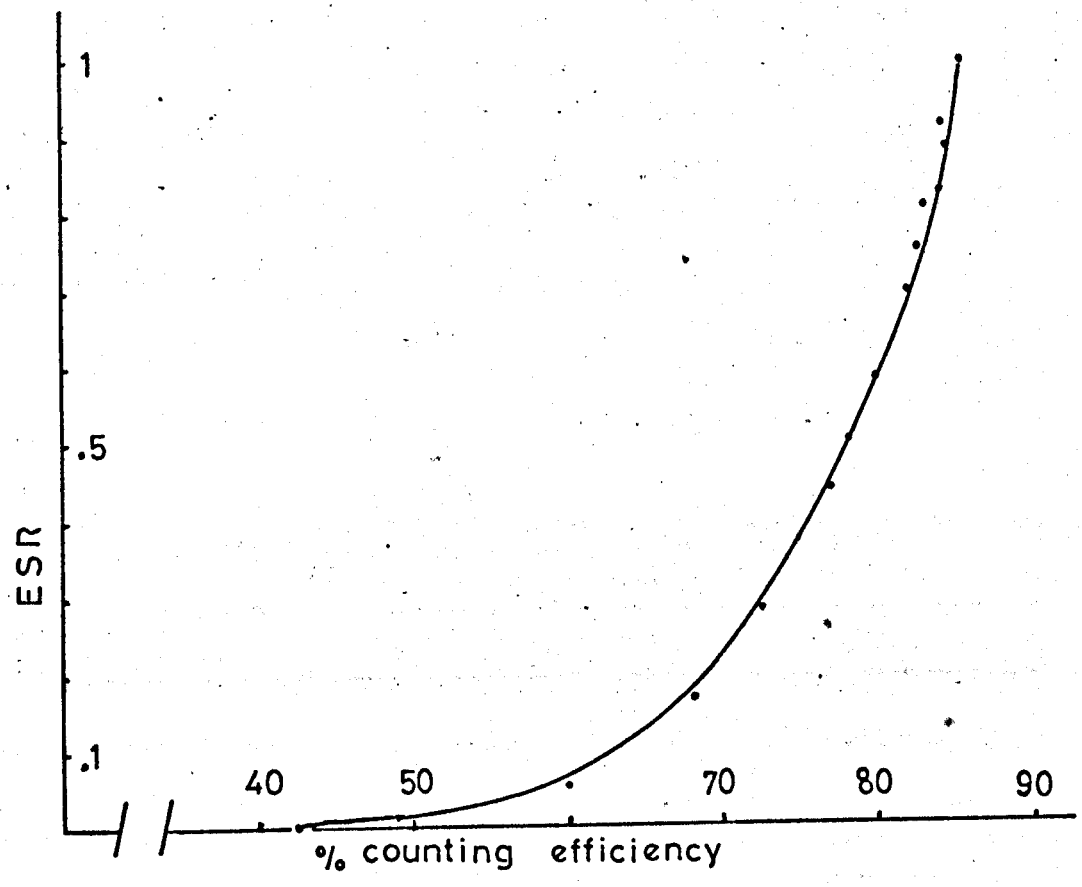


FIG 3-3 - Quenching curve for ^{14}C-Arachidonic acid.



Beckman spectrometer. Quenching was obtained by stepwise addition of chloroform to a sample of known activity.

In order to calculate the amount of prostaglandin E_2 formed in the enzymatic reaction, several dilutions of a known concentration of 1-^{14}C-arachidonic acid were made in ethanol and aliquots of these dilutions were counted in the liquid scintillation spectrometer. These results are presented in Table 3-III and Fig. 3-4. The figures on the extreme right mean the amount of PGE_2 which would be formed from the dilutions of arachidonic acid, assuming total conversion of the arachidonic acid.

Table 3-III

Correlation between several dilutions of known activities of 1-^{14}C-arachidonic acid and DPM

DPM	µg arach. acid	µg PGE_2
42670	0.101	0.116
20740	0.049	0.0558
11090	0.0262	0.0298
5630	0.0133	0.0151
2680	0.00634	0.00722

each value represents the average of two determinations

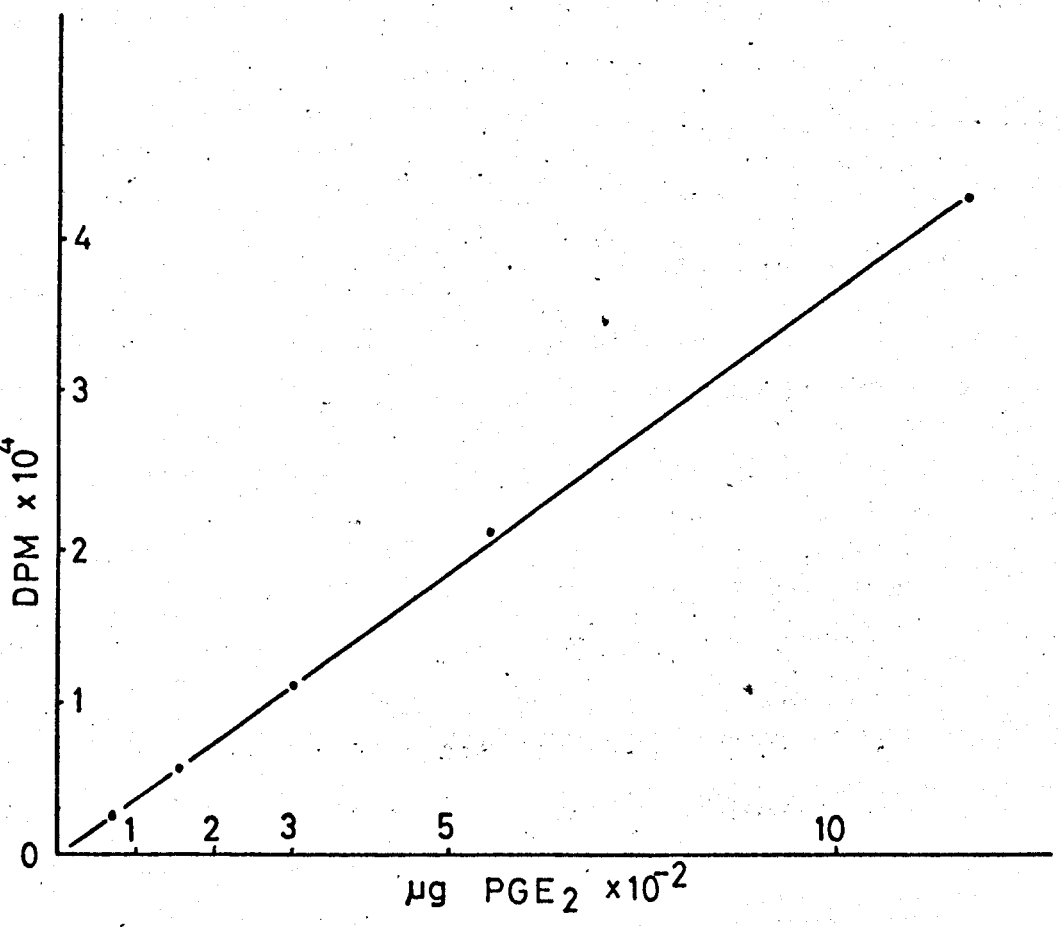
The least square straight line in Fig. 3-4 is represented by the equation

$$y = 104.654 + 367482.74x$$

where y = DPM

x = µg PGE_2.

FIG 3-4 - Standard concentration-DPM curve for PGE_2 (data from Table 3-III)



The equation used to calculate unknown concentrations of PGE_2 had to be modified to allow for dilution of the sample during the experimental procedure and for extraction losses. The dilution factor multiplied by the extraction factor was 4.38, giving a final equation: $4.38\ y = 104.654 + 367482.74\ x$ and this equation was used to calculate PGE_2 concentrations in the following experiments. The extraction factor was determined by applying a known amount of arachidonic acid to the silica gel plates, developing it and then counting the spot corresponding to arachidonic acid, as done during the experimental procedure.

The results of the experiments where the prostaglandin synthetase was incubated with several concentrations of peptide 401 and with 40 μM indomethacin and 1 mM aspirin, are presented in Table 3-IV and Fig. 3-5.

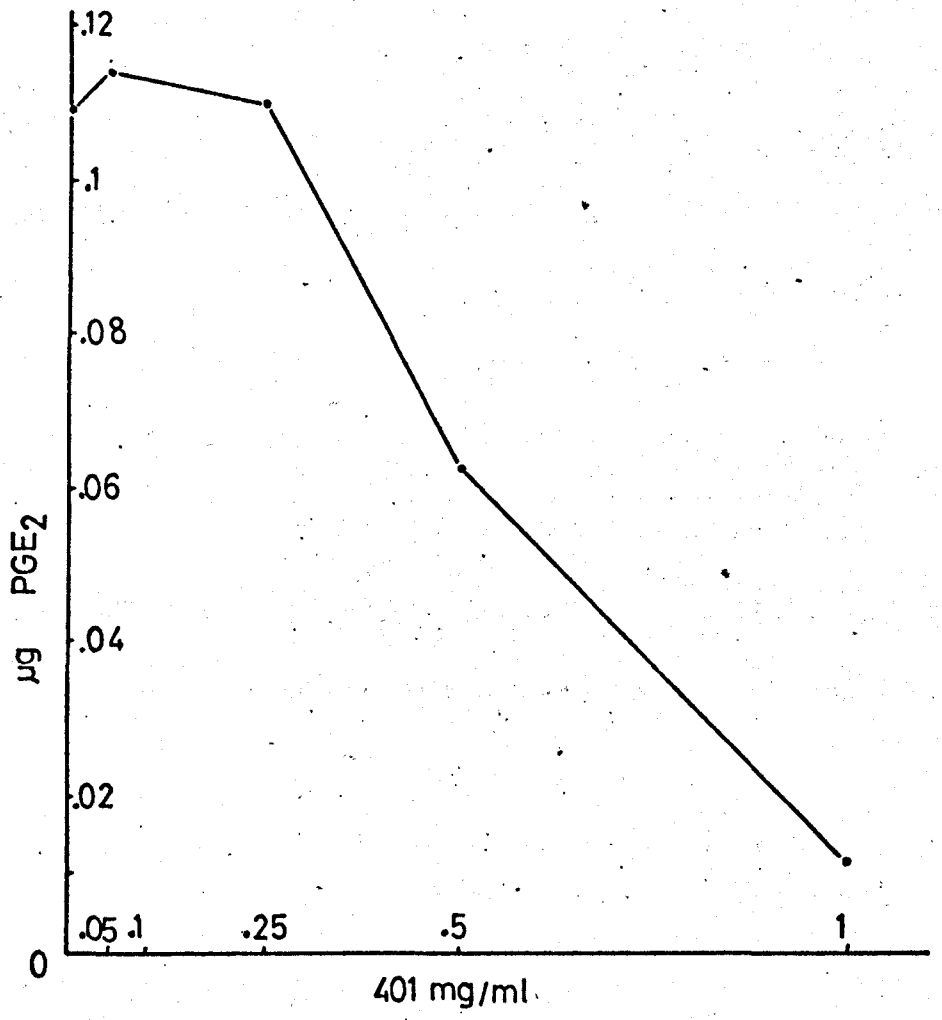
Table 3-IV

Effect of peptide 401 on prostaglandin synthesis

	μg $PGF_{2\alpha}$	μg PGE_2	μg PGD_2
Control	0.0144	0.109	0.01
401 (1 mg/ml = 380 μM)	0.00035	0.012	0.001
401 (0.5 mg/ml = 190 μM)	0.0023	0.063	0.0044
401 (0.25 mg/ml = 95 μM)	0.0087	0.111	0.0092
401 (0.05 mg/ml = 19 μM)	0.0113	0.114	0.011
401 (0.025 mg/ml = 9.5 μM)	0.01	0.097	0.013
401 (0.005 mg/ml = 1.9 μM)	0.014	0.0998	0.0148
Indomethacin (40 μM)	0.003	0.011	0.0027
Aspirin (1 mM)	0.072	0.038	0.005
Blank	0	0.00008	0.0004

each value represents the average of two determinations

FIG 3-5 - Effect of peptide 401 on PGE_2 synthesis.
(data from Table 3-IV)



Peptide 401 is able to inhibit PGE_2 formation by 43% at a concentration of 0.5 mg/ml. Smaller concentrations of peptide 401 fail to show any significant effect on PGE_2 synthesis although peptide 401 is able to inhibit $PGF_{2\alpha}$ synthesis at a concentration of 0.25 mg/ml (see Table 3-IV and Fig. 3-6).

The results of the experiments where peptide 401 was incubated in a narrower range of concentrations are presented in Table 3-V and Fig. 3-7. In Table 3-V, the results of experiments where melittin, another basic peptide isolated from bee venom (see Chapter 2), was incubated with the prostaglandin synthetase complex are also presented.

Table 3-V

Effect of peptide 401 and melittin on PGE_2 synthesis

	μg PGE_2
Control	0.072
401 (1 mg/ml)	0.0114
401 (0.75 mg/ml)	0.0149
401 (0.5 mg/ml)	0.043
401 (0.4 mg/ml)	0.058
401 (0.3 mg/ml)	0.077
Melittin (0.5 mg/ml)	0.115
Blank	0.000263

each value is the average of two determinations

This was done in order to determine if the inhibition found with peptide 401 was unspecific and simply due to the strong basicity of the molecule. It can be seen that melittin in a concentration of 0.5 mg/ml appears to

FIG 3-6- Effect of peptide 401 on $PGF_{2\alpha}$ and PGD_2 synthesis (data from Table 3-IV)

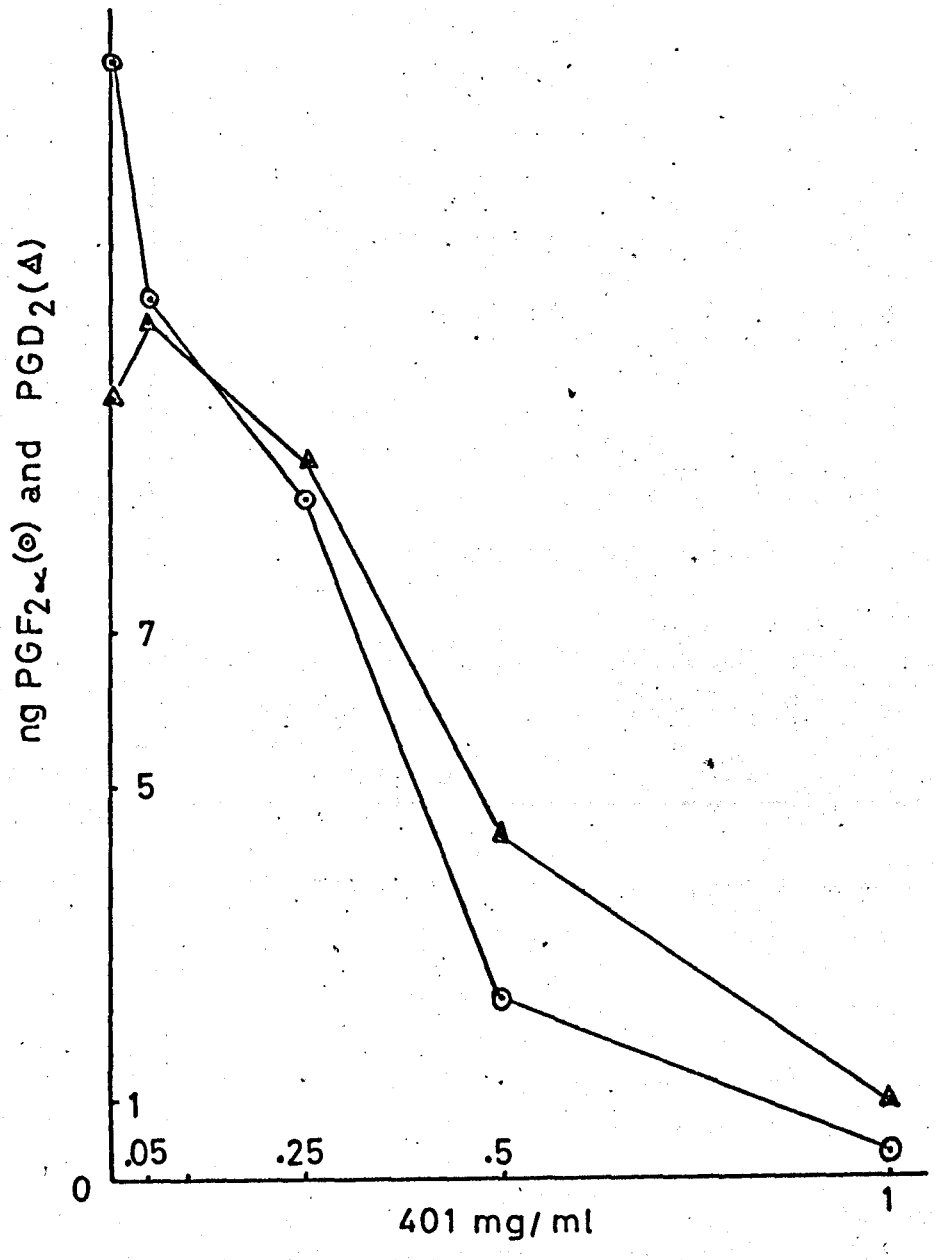
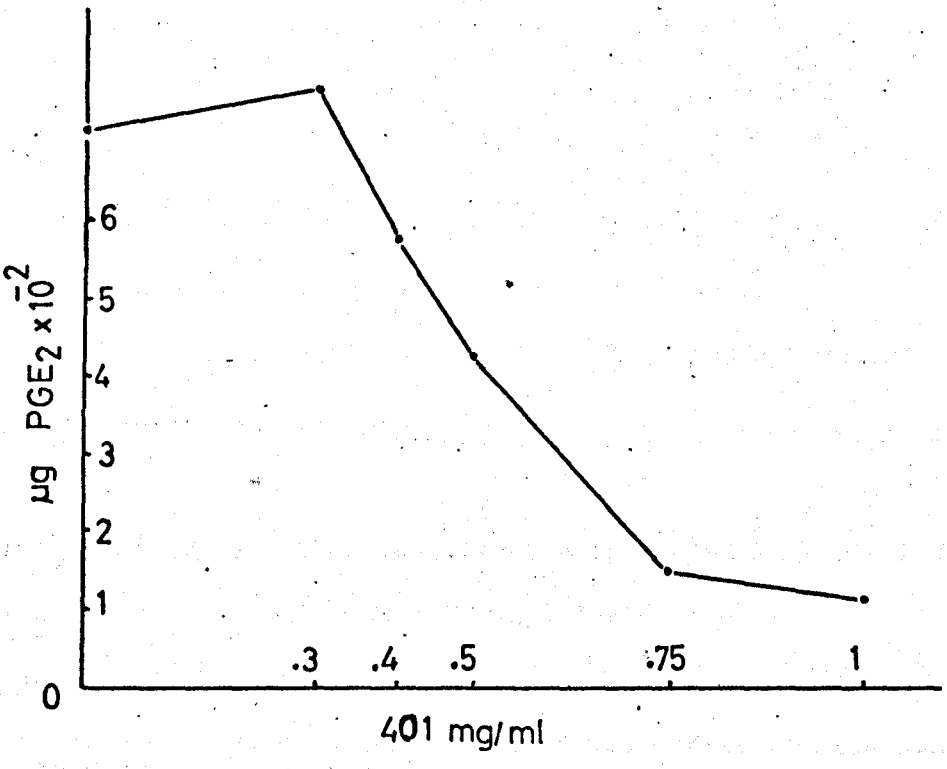


FIG 3-7- Effect of peptide 401 on PGE_2 synthesis (data from Table 3-V)



activate the formation of PGE_2. However this effect may have been reflecting a partial solubilization of the enzymatic complex, as a consequence of the detergent-like properties of melittin (154). In the above experiments only the spots corresponding to PGE_2 were collected and counted. It can be seen that peptide 401 inhibition of PGE_2 formation occurs down to a concentration of 0.4 mg/ml. Although the absolute values varied from one experiment to another, the inhibition caused by peptide 401, reflected as the ratio between the controls and a given concentration of peptide 401, was constant and reproducible in all experiments considered (1.63 $\pm$ 0.13 for 0.5 mg/ml 401).

In order to test the hypothesis that melittin was activating the synthesis of PGE_2 through partial solubilization of the enzyme, Triton X 100, a detergent, was incubated with the PG synthetase at a concentration of 0.4% (v/v). These results are presented in Table 3-VII, and it can be seen that there is only a slight difference between the Triton X-100 group and the controls.

When peptide 401, at a concentration of 0.5 mg/ml, is incubated with 3 mg of the PG synthetase, the ratio between controls and 401 groups was 1.82. The results are presented in Table 3-VI. The inhibition of 401 is more pronounced in the presence of 3 mg of the enzymatic complex, instead of the usual 2 mg per tube of the previous experiments.

Table 3-VI

Effect of peptide 401 on PGE_2 synthesis when incubated with 3 mg of the enzymatic complex

	µg PGE_2
Control	0.35
401 (0.5 mg/ml)	0.192

Although the opposite effect would be expected, the limited amount of the enzymatic complex did not allow tests using different amounts of the enzymatic complex, incubated with a constant amount of 401 and arachidonic acid. The ratio between the controls and 401 groups was not very far from the ratio obtained in experiments where 2 mg of the enzymatic complex were incubated with peptide 401 and it is not therefore possible to draw conclusions about the direct action of peptide on the enzymatic complex.

The results of experiments where fixed amounts of peptide 401 were incubated with the enzymatic complex and varying concentrations of arachidonic acid, are presented in Table 3-VII and Figs 3-8 and 3-9. Table 3-VII shows also the results of incubation of the enzymatic complex with a 0.4% (v/v) solution of Triton X-100, at a substrate concentration of 1 mM.

Table 3-VII

Effect of peptide 401 on prostaglandin synthesis at several substrate concentrations

	CONTROL			401 (0.5 mg/ml)		
	µg PGF_2	µg PGE_2	µg PGD_2	µg PGF_2	µg PGE_2	µg PGD_2
Arach.Acid						
0.1 mM	0.000585	0.0356	0.00051	0.000323	0.028	0.00022
0.5 mM	0.00383	0.122	0.0054	0.0021	0.056	0.0022
1 mM	0.0165	0.114	0.0124	0.007	0.077	0.0076
2 mM	0.027	0.071	0.028	0.025	0.129	0.029

	µg PGF_2	µg PGE_2	µg PGD_2
Triton X-100 (0.4%) 1 mM arachidonic acid	0.0055	0.121	0.008

each value is the average of two determinations

FIG 3-8 -Effect of peptide 401 on PGE_2 synthesis at several substrate concentrations.(data from Table 3-VII)

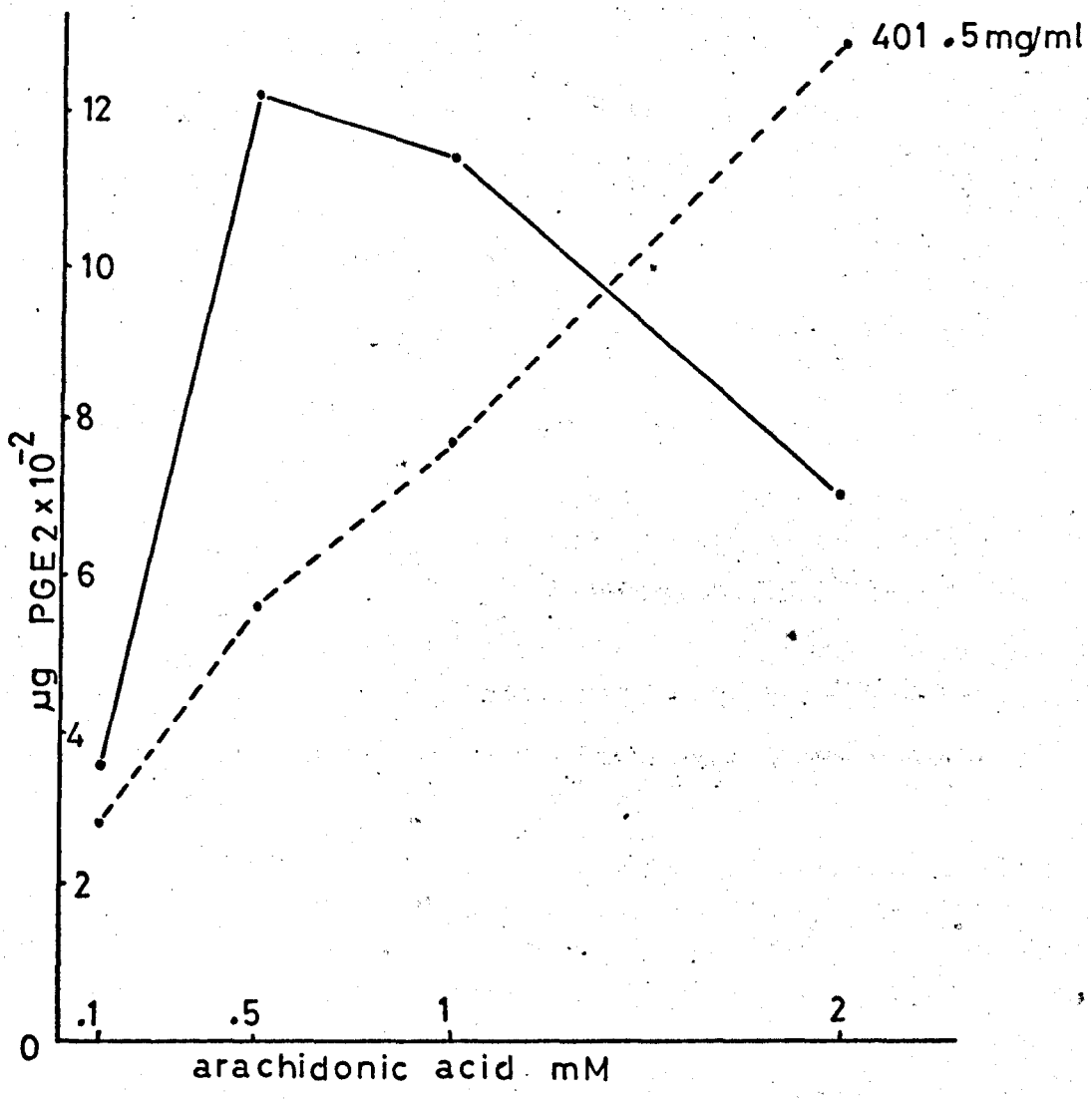
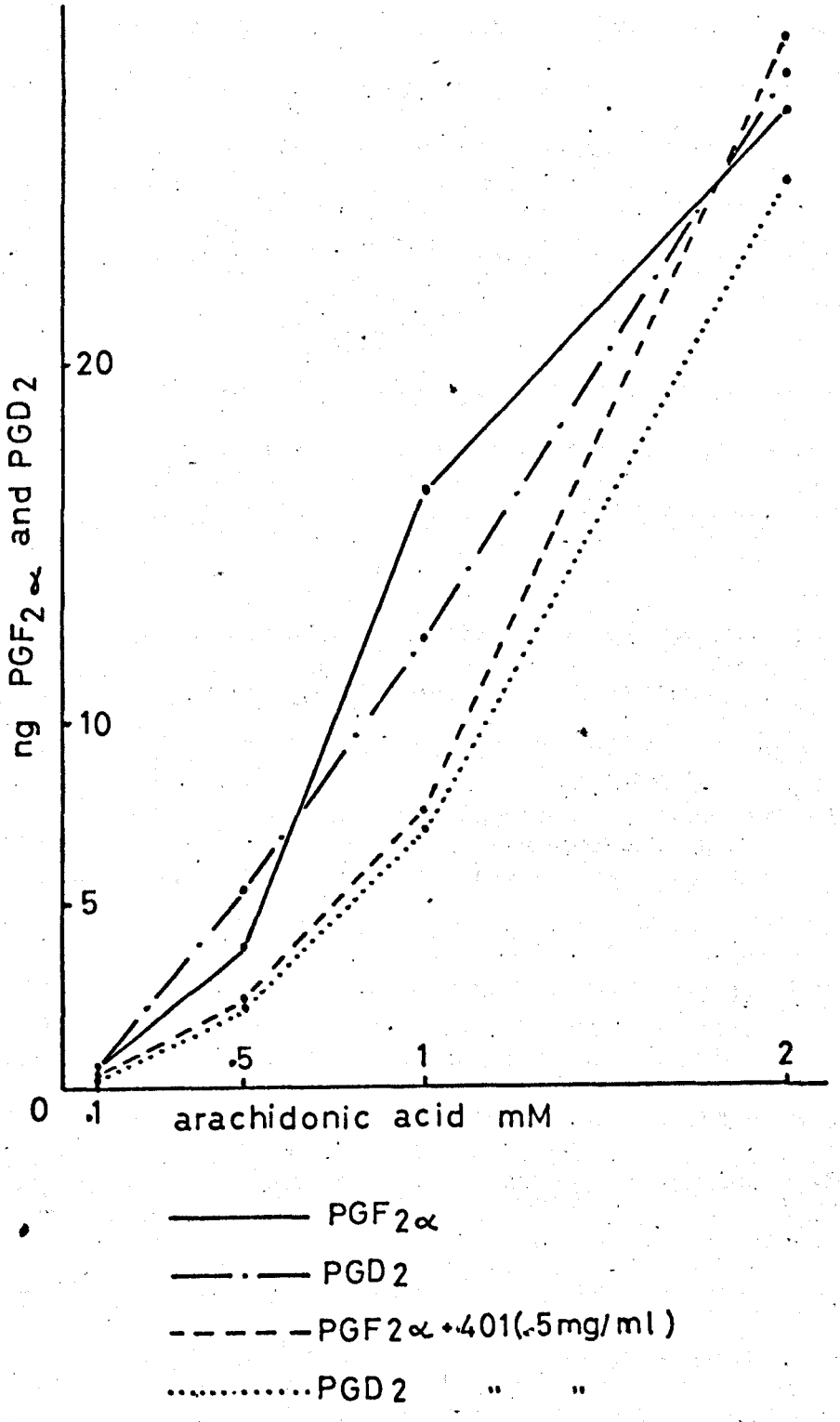


FIG 3-9 - Effect of peptide 401 on PGF_2 and PGD_2 synthesis at several substrate concentrations. (data from Table 3-VII)



It can be seen that the inhibition caused by peptide 401 on the synthesis of PGE_2, $PGF_{2\alpha}$ and PGD_2 is dependent on the substrate concentration. Inhibition by peptide 401 (0.5 mg/ml) is observed up to a substrate concentration of 1 mM. The inhibition of PGE_2 formation found when the substrate concentration was increased, is in agreement with the results of Flower et al. (66). However, due to the insoluble character of both arachidonic acid and the enzymatic complex, this effect is difficult to interpret. It should also be kept in mind that the usage of the term 'concentration' is not strictly correct under these circumstances. The interaction between arachidonic acid and the enzymatic system must be similar to the known complex formation which occurs between long chain fatty acids and proteins present in the serum (especially albumin). The insolubility of the prostaglandin synthetase complex in aqueous solutions must be reflecting a highly hydrophobic structure which presumably favours even more the interaction with arachidonic acid.

In these conditions (of increased substrate 'concentration') other products of the enzymatic reaction are being formed at higher rates than at lower substrate concentration. It is not known however, what controls these mechanisms and possibly the activation of PGE_2 formation induced by peptide 401 at substrate concentrations of 2 mM (see Fig. 3-8), could be a consequence of an inhibitory action of peptide 401 on the synthesis of prostaglandins other than PGE_2. The inhibition caused by peptide 401 was not so pronounced in the case of $PGF_{2\alpha}$ and PGD_2 formation (Fig. 3-9).

These results indicate also that some complexing between peptide 401 and arachidonic acid could be occurring. Such a complexing would be favoured by the opposite charges of those two compounds at the

pH of the incubation medium, although it can be seen in Table 3-V that melittin, which is more basic than 401, did not show any inhibition of prostaglandin synthesis but instead an activation.

3-3-3 Tests for complex formation between peptide 401 and arachidonic acid

In order to test if any complexing was occurring between peptide 401 and arachidonic acid, which would explain the inhibition of prostaglandin synthesis caused by peptide 401, the following experiments were carried out:

a) Peptide 401 to a concentration of 0.5 mg/ml was dissolved in the incubation medium (0.5 ml) and this was added to 0.1 ml of a benzene solution containing arachidonic acid (1 mM and with the same ratio of 'cold' arachidonic acid to hot arachidonic acid as used in the previous experiments). In the control tubes, buffer alone was incubated with the benzene solution of arachidonic acid. The tubes were shaken and incubated for 30 min at room temperature. After this incubation period, the tubes were centrifuged in a bench centrifuge and the upper phase, containing the arachidonic acid was withdrawn by means of a capillary tube and pipetted into another tube. The benzene was removed under a stream of N_2 and the usual incubation medium was added to those tubes, followed by the enzymatic suspension. The tubes were then incubated for 10 min at 37°C and the usual extraction procedure was carried out together with the thin layer chromatography. This was an attempt to investigate if peptide 401 would favour an increased solubility of the arachidonic acid in the buffer phase. The results are presented in Table 3-VIII.

Table 3-VIII

Effect of peptide 401 on the solubility of arachidonic acid in the buffer phase

	µg PGE_2
Control	0.0378
Substrate pre-inc. with 401 (0.5 mg/ml)	0.0383

each value represents the average of two determinations

This experiment shows that there was no difference between the amount of arachidonic acid which could be extracted after incubation of arachidonic acid with peptide 401 and the buffer alone.

b) In this experiment 0.25 mg and 0.5 mg of peptide 401 were each dissolved in 1 ml of ethanol (absolute) (to make final concentrations of 0.25 mg/ml and 0.5 mg/ml respectively). These solutions were added to tubes containing 0.05 ml of a benzene solution of arachidonic acid (same concentration and specific activity as in experiment a). The contents of the tubes were then dried and redissolved in 0.05 ml of ethanol. From these solutions, 0.02 ml was applied on the plastic backed silica gel thin layer plates and developed in a solvent system consisting of: n-butanol : pyridine : acetic acid : water (15 : 10 : 3 : 12 v/v). The plates were stained with iodine vapour followed by ninhydrin, and the spots corresponding to arachidonic acid and peptide 401 were cut and counted as described before. The results are presented in Table 3-IX.

Table 3-IX

Effect of TLC solvent system on complex formation between peptide 401 and arachidonic acid

		µg arachidonic acid
Ninhydrin positive spots	Point of application of sample without 401	0.00236
	401 (0.25 mg/ml)	0.000186
	401 (0.5 mg/ml)	0.000106
Iodine vapour positive spots	Arachidonic acid (0.25mg/ml)	0.346
	Arachidonic acid (0.5 mg/ml)	0.39

each value represents the average of two determinations

The above results show that there was no complex formation between peptide 401 and arachidonic acid in the TLC conditions.

c) Peptide 401 was dissolved in 0.05 M Tris buffer pH 8.2 to make a solution of 2 mg in 0.16 ml. From this solution 0.02 ml and 0.04 ml aliquots were added to tubes containing dried arachidonic acid (same specific activity and amount as before) and those were incubated for 10 min at 37°C. After incubation the tubes were dried and redissolved in 0.05 ml of absolute ethanol. From these solutions, aliquots of 0.02 ml were applied on the TLC plates and developed in the same solvent system as in experiment b. The plates were stained with iodine vapour and ninhydrin and the spots corresponding to peptide 401 and arachidonic acid were cut and counted as before. The results are presented in Table 3-X.

Table 3-X

Complex formation between peptide 401
and arachidonic acid in the buffer solution

		µg Arach. Acid
Control (buffer alone + arach.acid)	Ninhydrin Positive Spot (at the same R_F of 401)	0
	Iodine Vapour Positive Spot	0.367
401 0.25 mg	Ninhydrin Positive Spot	0.0019
	Iodine Vapour Positive Spot	0.393
401 0.5 mg	Ninhydrin Positive Spot	0.0033
	Iodine Vapour Positive Spot	0.35

each value represents the average of two determinations

d) Peptide 401 was dissolved in 0.05M Tris buffer pH 8.2 to make a solution of 2 mg in 0.16 ml. From this solution an aliquot of 0.02 ml was added to a tube containing the dried arachidonic acid (same amount and specific activity as before) plus 0.48 ml of the Tris buffer solution. Another aliquot of 0.04 ml, from the 401 solution was added to another tube containing the same amount of dry arachidonic acid plus 0.46 ml of the Tris buffer solution. The tubes were then stirred for 1 min on the Vortex mixer and then arachidonic acid was extracted in each tube with 1 ml of ethyl acetate. The tubes were again stirred for 15 seconds on the Vortex mixer and centrifuged on a bench centrifuge. The supernatants were decanted as quantitatively as possible and transferred to other tubes. The extraction of arachidonic acid from the buffer phase was repeated once with 1 ml of ethyl acetate. Aliquots of 0.05 ml were then collected from the buffer phase and counted. The ethyl acetate

phase was dried and the residue redissolved in 0.5 ml of absolute ethanol. From this solution aliquots of 0.05 ml were collected for counting. The results are presented in Table 3-XI.

Table 3-XI

Effect of peptide 401 on the solubility of arachidonic acid in the buffer phase

		Total µg Arach. Acid
Buffer phase	Control (buffer alone)	0.0004
	401 0.5 mg/ml	0.00134
	401 1 mg/ml	0.0016
Ethyl acetate phase	Control	0.464
	401 0.5 mg/ml	0.460
	401 1 mg/ml	0.44

each value represents the average of two determinations

The results presented in Table 3-X indicate that some complexing between peptide 401 and arachidonic acid is occurring. The results of Table 3-XI show also some complexing but not so pronounced. However the complexing observed is negligible in terms of substrate depletion as an explanation for the inhibition of PGE_2 synthesis by peptide 401. The results in Table 3-VII and Fig. 3-8 show that only below a substrate concentration of 0.5 mM would the synthesis of prostaglandin start to decrease. In the experimental conditions employed in the present work, the results in Tables 3-X and 3-XI show that the amount of uncomplexed arachidonic acid is still in a large excess over the complexed arachidonic acid.

Those results show that at an arachidonic acid concentration of 1 mM, the concentration of the free acid available as substrate for the enzymatic reaction would be approximately 0.54 mM. It should also be added that in the experiments employing the spectrophotometric method, where the substrate concentration was 0.273 mM and where peptide 401 was at a concentration of 0.6 mg/ml, the results in Table 3-II and Fig. 3-2 show that the inhibition by peptide 401 is comparable with the inhibition found using the radiochemical method (41% in the spectrophotometric method and 42% in the radiochemical method). If complexing between arachidonic acid and peptide 401 was occurring the degree of inhibition at a substrate concentration of 0.273 mM should be greater than at 1 mM. Furthermore in Table 3-V it can be seen that melittin at a concentration of 0.5 mg/ml did not cause any inhibition of PGE_2 synthesis, but in fact a slight activation. These results, together with the results in Table 3-VII and Fig. 3-8, suggest that peptide 401 is inhibiting the enzymatic complex rather than depleting the substrate.

Finally, in Table 3-XII the results of experiments where peptide 401 was incubated with a prostaglandin synthetase complex obtained from bovine seminal vesicles, are presented. The method employed was exactly the same. In these experiments, two different batches of peptide 401 were tested, as obtained from two different isolation procedures. Both batches of 401 were able to inhibit PGE_2 synthesis by approximately 97% at concentrations of 2 mg/ml.

Table 3-XII

Effects of two different batches of 401 on PGE_2 synthesis by bovine prostaglandin synthetase

		µg PGE_2
Control		0.0474
Peptide 401 (2 mg/ml)	BATCH I	0.0011
Peptide 401 (2 mg/ml)	BATCH II	0.00085

3-4 Discussion

The experiments described in this chapter demonstrated that peptide 401 is able to inhibit PGE_2 synthesis *in vitro*, when incubated with prostaglandin synthetases obtained from sheep and bovine seminal vesicles. A concentration of 401 of 1 mg/ml is able to inhibit PGE_2 synthesis by 87% and inhibition can be observed down to concentrations of 0.4 mg/ml. These results were obtained using two different assay systems for prostaglandins and employing two different batches of the enzymatic complex from sheep seminal vesicles. It was not possible to conclude whether this inhibitory effect of 401 reflects an action at the substrate site or at the cofactor site on the enzymatic complex. Likewise it was not possible to determine if any allosteric effects caused by 401 on the enzymatic complex were occurring, due to the limited amount of the acetone-pentane powder containing the prostaglandin synthetase. It was found that some complexing was occurring between peptide 401 and arachidonic acid, but the degree of complexing was only

residual and could not account for the inhibition observed. Melittin, which is another basic peptide from the bee venom, and which shares with peptide 401 the ability to degranulate mast cells, although it has no anti-inflammatory activity in the rat, did not inhibit the prostaglandin synthetase. The possibility that the anti-inflammatory activity of 401 could be explained by stimulation of synthesis of $PGF_{2\alpha}$, which in the rat carrageenan oedema has an anti-inflammatory activity, was ruled out since the synthesis of this prostaglandin was also inhibited by peptide 401. This is in agreement with the results of Hanson et al. (~~104~~ 81) who showed that in the carrageenan oedema, injections of $PGF_{2\alpha}$ in the rats up to concentrations of 50 µg/kg did not cause an appreciable anti-inflammatory effect.

A large number of compounds have been reported which inhibit prostaglandin synthesis in vitro and in vivo (65). However, the variety of the chemical nature of these compounds, together with the lack of structural data about the multi-enzymatic system responsible for the synthesis of prostaglandins, makes it difficult to propose a mechanism by means of which these inhibitors are effective. It is simpler to do so in the case of analogues of the essential fatty acids which are precursors of prostaglandins. Several of these analogues are inhibitors of prostaglandin synthesis either by acting directly on the enzymatic complex or by preventing the hydroxylation reaction on the precursors (1). The direct inhibitory action of these acetylenic analogues of arachidonic acid on the enzymatic complex occurs presumably by destruction of the catalytic site on the enzyme (105). Another group of inhibitors are the so called 'aspirin-like' drugs, which are thus named because they share the same pharmacological actions. These compounds show different degrees of inhibition depending on the experimental system employed,

that is, whether acetone powders containing the PG synthetase, slices of organs or in vivo experiments in the whole animal are used. Differences in inhibitory potency can also vary from tissue to tissue for the same inhibitor (65). In this group compounds such as aspirin, indomethacin, paracetamol, phenylbutazone, meclofenamic acid, niflumic acid etc., are included. Most of these aspirin-like drugs are organic acids but whether the acid function is necessary for their activity is not certain since there are non-acidic aspirin-like inhibitors of prostaglandin synthesis. The inhibition of PG synthesis found with these compounds is assumed to be of a competitive nature, either for the substrate and/or cofactor sites of the enzymatic complex (65). Up to date there has been no report of in vitro experiments where basic peptides or proteins were shown to inhibit the synthesis of prostaglandins directly.

It should now be considered whether the observed in vitro inhibition of PGE_2 synthesis by peptide 401 bears any relation to the anti-inflammatory activity of this peptide in the rat carrageenan oedema. Studies in the rat showed that peptide 401 injected subcutaneously or intravenously at concentrations of 0.25 mg/kg is able to suppress the local increased vascular permeability induced by carrageenan (81). Other reports (156) showed that peptide 401 at concentrations of 0.7 mg/kg is able to reduce by 30% the oedema caused by carrageenan, although such concentrations of 401 were shown to have some systemic inflammatory effects (~~104~~ 81). It should be kept in mind, however, that the anti-inflammatory activity of peptide 401 varies from one strain of rats to another (~~110~~ 26).

Thus the in vivo doses of peptide 401 which are able to produce anti-inflammatory effects are smaller than the in vitro concentrations of

401, which were found to inhibit PGE_2 synthesis in the present work. This difference, however, does not necessarily exclude the possibility that the anti-inflammatory activity of peptide 401 could be associated with inhibition of prostaglandin synthesis in the rat. It is known that different preparations of prostaglandin synthetase complexes show different sensitivities for a given inhibitor and that in the same animal the amount of the enzymatic system responsible for prostaglandin synthesis can vary from one tissue to another (65). There is no data available which would allow one to establish an equivalence between the amount of the enzyme used in the present work and that which would be found in the rat. According to the classification of Christ and Van Dorp (35) the enzyme obtained from sheep seminal vesicles has comparatively a very high activity. Thus if a sufficient amount of prostaglandin synthetase could be isolated from the rat tissues it is possible that peptide 401 would effectively inhibit prostaglandin synthesis at concentrations which produce anti-inflammatory responses in the rat carrageenan oedema. It is interesting to add that in the guinea pig, prostaglandins E_1 and E_2 lack the same inflammatory potency they have in the rat, and in the same animal, peptide 401 injected in concentrations up to 2 mg/kg fails to have any anti-inflammatory activity in the carrageenan oedema, although it still retains its ability to induce local increased vascular permeability, probably due to degranulation of mast cells (192, 81).

Alternatively we can assume that peptide 401 is interacting specifically with a certain tissue. The consequence of such specific tissular interaction would be an increased local concentration of peptide 401. If the tissue in question is involved in the inflammatory process and is responsible for the synthesis and release of prostaglandins at

the site of inflammation (since prostaglandins seem to be metabolized very quickly once they are released), one would have a situation where injections of relatively low doses of 401 could block this source of prostaglandins and in this way significantly alter the course of the inflammatory process. So far the only suggestion concerning specific tissular interaction of peptide 401 comes from the work of Hanson et al. (81) who observed a hypotensive effect of peptide 401 when injected intravenously in the rat. Although this hypotensive effect of peptide 401 could be blocked by mepyramine and methylsergide, it was found that even in the presence of these blockers of histamine and serotonin, the anti-inflammatory activity of 401 persisted. No vasoconstriction effects caused by peptide 401 could be detected with doses of 401 up to 1 mg and likewise no selective vasoconstriction in the skin was obtained with peptide 401. It was suggested then that probably 401 was exerting its anti-inflammatory activity by interacting with the blood vessels and rendering them unresponsive to inflammatory agents, but again, as mentioned before, it was demonstrated in the same work that the anti-inflammatory effects of peptide 401 do not seem to be dependent on any vascular action, since the course of the inflammatory process proceeds even in the presence of antagonists of histamine and serotonin and suppression of inflammation by 401 also occurs in the presence of these antagonists. Furthermore, such an effect of peptide 401 on the vascular endothelium would require either that some kind of modification of the vascular tissue, induced perhaps by the inflammatory process, would favour this interaction or else that peptide 401 is evenly distributed throughout the vascular system of the rat. Both possibilities, however, would appear unlikely, as mentioned above. It appears that the only actions of peptide 401 on the vascular system are indirect ones; the

local increase in vascular permeability induced by 401 and by melittin is probably simply reflecting the release of histamine due to degranulation of mast cells by these two compounds.

On the other hand, considering that peptide 401 is able to degranulate mast cells in concentrations as low as 0.001 mg/ml (see following chapter),it is reasonable to assume that there is a high degree of interaction between peptide 401 and mast cells, which is probably also reflecting a binding pattern due to characteristic features on the membranes of these cells. It is also noteworthy that peptide 401 at a concentration of 0.4 mg/ml shows some chemotactic activity for human neutrophils but none for monocytes. The chemotactic activity for neutrophils could be observed down to concentrations of 401 of 0.05 mg/ml. However, much lower concentrations of peptide 401 (0.01 µg/ml) were able to produce chemotactic activity for human monocytes (191), again suggesting a high degree of interaction with a particular group of cells. These considerations indicate the possibility of peptide 401 interacting specifically with leucocytes, which are not only ubiquitous, but which also migrate to the site of inflammation in the carrageenan oedema and there display an important role as secretory cells, releasing histamine, serotonin, hyaluronidases, proteases and prostaglandins. These features make leucocytes very probably target cells for peptide 401. Thus, the anti-inflammatory activity of peptide 401 could be explained at least partially as a consequence of inhibition of prostaglandin synthesis by the leucocytes. Attempts to investigate the effect of peptide 401 on prostaglandin synthesis by rat leucocytes obtained from peritoneal exudates, failed since the cells did not produce any measurable amount of prostaglandins, even in the presence of killed B. pertussis, which induces intensive phagocytosis and hence prostaglandin formation and release.

Concerning the possible interaction between peptide 401 and the rat leucocytes with a resulting inhibition of prostaglandin synthesis, it was observed (26, 156) that the maximum anti-inflammatory activity of peptide 401 in the carrageenan oedema is observed between 3-5 h after the injection of carrageenan, which according to Di Rosa et al. (49) is the phase when cell migration to the site of inflammation is taking place, concomitantly with prostaglandin release. Higher doses of 401 (>0.25 mg/kg) injected into the rats would counterbalance the anti-inflammatory effects by causing more release of histamine due to degranulation of mast cells and therefore masking the effects of inhibition of prostaglandin synthesis. This seems to be occurring locally after subcutaneous injections of peptide 401, when the local concentration of 401 is of such an order that only the inflammatory effects due to degranulation of mast cells are observed (81).

If the idea of a specific interaction between peptide 401 and leucocytes, with subsequent inhibition of prostaglandin synthesis is correct, what structural features of the membranes of the leucocytes would be directing this interaction? The experiments described in the next chapter were carried out in order to establish the binding characteristics of peptide 401 to model membrane systems and to determine if it is possible to extrapolate these conditions to the in vivo situation.

CHAPTER 4

INTERACTIONS BETWEEN PEPTIDE 401, LEUCOCYTES AND LIPOSOMES

4-1 Introduction

4-1-1 Interaction between basic peptides and cell membranes

In the last chapter it was suggested that peptide 401 could be exerting its anti-inflammatory activity by interacting specifically with leucocytes and inhibiting prostaglandin synthesis by these cells.

It is known that several basic peptides and proteins are able to induce pinocytosis in a number of different cells (145, 122, 70) and it was suggested that such effects were the consequence of an imbalance in the repulsive forces between the mobile units of the biological membrane (70). These sub-units are generally negatively charged and their aggregation appears to be a preliminary stage in the pinocytic process.

In some cases, the binding of basic peptides to the cell membranes is a consequence of electrostatic interactions. It is believed that the presence of sialic acid on the membrane is a requirement for binding since these acid groups are largely responsible for the negative charge of the membrane (37). This seems to be the case for tuftsin, a tetrapeptide obtained by cleavage of the last 4 amino acid residues of leukokinin (36, 122), which binds specifically to blood polymorphonucleocytes and stimulates their phagocytic activity.

Although there is no data available about the spatial organisation of the peptide 401 molecule, which would allow comparison with other basic peptides, very possibly similar conditions may be

occurring which would then increase the probability of interaction between peptide 401 and the leucocytes.

4-1-2 Labelling of peptide 401

It would be of interest to study the fate of peptide 401 when injected into the rat. However, the problem concerning studies on the interactions between peptide 401 and the tissues resides in the difficulty of labelling peptide 401 in such a way as to preserve the biological activity. Several attempts to synthesize peptide 401 have been made but success has not yet been achieved (199). Synthesis of peptide 401 would allow the introduction of labelled amino acids into the molecule. The absence of tyrosine residues in the molecule of 401 eliminates introduction of ^{131}I. Moreover the integrity of the histidine residues which could also be labelled with ^{131}I seems to be important for the anti-inflammatory activity, since experiments involving photo-oxidation of the peptide (156) demonstrated that anti-inflammatory activity is lost after exposure to light from a 100 W bulb for 1 h, in the presence of rose bengal (4,5,6,7-tetrachloro-2',4',5',7'-tetraiiodofluorescein) as a sensitizer. Introduction of compounds which react with the free amino groups of the lysine residues of 401, such as treatment with maleic acid anhydride and acetylation (partial or total) or reactions which destroy the disulphide bridges of 401 such as trifluoroacetylation, aminoethylation, and carboxymethylation all produce derivatives which are inactive with respect to anti-inflammatory activity (156, 27).

4-1-2-1 Dansylation of peptide 401

Treatment of peptide 401 with 1-N,N-dimethylaminonaphtalene-5-sulphonyl (chloride) (dansyl) yielded a derivative which retained some of

its ability to degranulate mast cells (see results). The dansyl-401 peptide was able to reduce the carrageenan oedema in the rat by 35% when injected at a concentration of 3.5 mg/kg. Concentrations of 0.7 mg/kg produced 16% reduction of the oedema (156). The same amount of untreated 401 should produce approximately 50% of reduction according to previous results on the anti-inflammatory activity of peptide 401. So, although the dansyl-401 peptide is about three times less potent in its anti-inflammatory activity, the nearly unaffected ability to degranulate mast cells indicates that the binding of d-401 to the membranes of mast cells is not drastically altered.

The product of dansylation of peptide 401 in a molar ratio 1:1 was examined by TLC as described in Chapter 2 (N-terminal analysis), and found to be a mixture of different ninhydrin positive spots. This indicated that the dansyl groups had reacted unselectively with different amino groups of the molecule of peptide 401, and also that there could be molecules of peptide 401 which had reacted with more than one dansyl group. However, although this would imply that some unreacted peptide 401 must be present, the TLC plates showed no ninhydrin positive spot which was not also associated with fluorescence. Therefore, the amount of unchanged peptide 401 must have been fairly small. Although some of the anti-inflammatory effects of d-401 could be due to the presence of trace amounts of unchanged peptide 401, it can be seen (Table 4-I) that the mast cell degranulating activity of peptide 401 was little affected by treatment with dansyl. These results would then suggest that most of the observed anti-inflammatory effects obtained with the d-401 preparation were due to the dansylated products.

The main advantage of using the dansyl labelled peptide 401 lies in the simplicity of studying its binding to the cell membranes,

at very low concentrations, by means of UV microscopy. In the first part of the following experiments the dansyl-401 peptide (d-401) was incubated *in vitro* with cell populations obtained from rat and human blood, as well as cells obtained from rat peritoneum, in order to establish if any preferential binding between d-401 and a particular group of cells was occurring. No attempts were made to study interactions between d-401 and other tissues, since the dilution (after injection of d-401 into rats) would be too big to allow the visual detection of the peptide and also because the histological techniques involved in obtaining samples of the various tissues investigated would make this scanning impractical.

4-1-3 Interaction between peptide 401 and different cell populations

With the purpose of obtaining information about the interaction between the natural peptide 401 and the cell membranes the following approaches were considered: a) It was reasoned that by treating blood cells, as well as cells obtained from the rat peritoneum, with peptide 401, some alterations on the membranes of the cells binding 401 would occur and possibly this would be reflected on the charge of these cells. By using the technique of cell electrophoresis the differences in the charges of the cells affected by peptide 401 would become apparent and if any preferential binding of 401 occurred with a particular group of cells, the electrophoretic mobilities of these cells should be more altered than unaffected cells. b) By treating latex beads, which at pH 7 are slightly negatively charged, with peptide 401, some amount of peptide 401 would adsorb to the surface of the latex beads and after removing the unadsorbed peptide 401, it should be possible to see which

group of cells would bind more latex beads. As the results of both sets of experiments were inconclusive, they will not be quoted in this chapter.

4-1-4 Liposomes

As an alternative, the effects of peptide 401 on model membrane systems, consisting of dispersions of lipids in aqueous solutions were studied. Aqueous dispersions of lipids also known as smectic mesophases, Bangosomes or liposomes consist of lipids, which can be isolated from the natural membranes and which, in contact with dilute aqueous solutions, spontaneously form closed multilammellar vesicles of concentric bilayers, containing aqueous spaces between them (16). If liposomes thus prepared are sonicated, closed vesicles presenting a single lipid bilayer can be obtained. The aqueous spaces of the liposomes can be made to contain the solutes of the aqueous solutions in which the lipids were dispersed, and the permeability rates of these trapped solutes can be easily measured by a number of different techniques. Permeability rates for several solutes as obtained from studies employing liposomes revealed that there is a striking similarity as compared to the same parameter in the biological membranes (16) and as the physical forces directing the structural association of the lipids in liposomes do not seem to differ radically from the ones in the cell membranes, as reflected by a number of common features such as shape, thickness and some electrical properties, it is possible to investigate many basic processes inherent to the cell membranes employing liposomes, without being too far from reality. Such processes include adhesion phenomena, diffusion of solutes across membranes, interactions of proteins and peptides and antibiotics, structural organization of lipids and proteins on the

membranes and antigen-antibody-complement reactions (14). It should be kept in mind, however, that the degree of complexity of the cell membranes, due to the presence of proteins, is obviously far greater than in the liposomes.

4-1-5 Interaction between peptide 401 and liposomes

In the work described here, the effects of peptide 401 on the permeability rates for glucose, as a non polar solute, and for $^{22}Na^{+}$ of liposomes prepared with the phospholipids phosphatidyl choline (lecithin), phosphatidyl serine and phosphatidic acid together with cholesterol, were studied. The aim of such experiments was to determine if any effects of peptide 401 on permeability rates to glucose and $^{22}Na^{+}$ could be correlated with the lipid composition of the liposomes and consequently charge and hydrophobicity of these membranes, hoping that parallels could be drawn with the in vivo situation. Thus the model membrane system employed in this work should give an indication whether the binding of peptide 401 to the cell membranes is simply due to non-specific electrostatic forces as a consequence of the basicity of the molecule of 401, or if there are any other factors involved directing the binding of 401, which if displayed by a tissue in vivo, could favour specific interaction.

4-2 Materials and methods

4-2-1 Experiments with dansyl-401

The dansyl-401 peptide was prepared by Dr. R.A. Shipolini from this department following the method described in Chapter 2. Peptide 401 was treated with dansyl in molar ratios of 1:1 and 1:2 (401:dansyl). Rat mast cells were obtained by injection of 10 ml of phosphate buffered

isotonic saline containing 50 µg/ml of heparin into the peritoneum of rats (killed either with a blow on the head or with ether). After massaging for a few minutes, the peritoneum was opened and the exudate was collected by means of a Pasteur pipette. The cell population thus obtained presented a proportion of 9 mast cells for every other 80 cells, which included lymphocytes and macrophages.

Rat leucocytes were obtained by injecting 100 i.u. of heparin into the peritoneum of the rats and after 3 min the rats were decapitated and the blood was collected in a beaker containing 200 i.u. of heparin. To the total blood, plasmagel (Laboratoire Roger Bellon) was added in a ratio 3/4, v/v (blood/plasmagel). The blood was then left to sediment at room temperature for 20 min and the supernatant (containing the leucocytes and red cells too) was then collected and washed in isotonic saline, using a bench centrifuge.

Human blood was collected with heparin by venous puncture.

4-2-1-1 Assay for degranulation of mast cells

To test the ability of the modified 401 to degranulate mast cells, wistar rats, weighing about 200 g were injected i.p. with peptide d-401 dissolved in the phosphate buffered saline, pH 7.4 (8.1 g NaCl, 2.272 g Na_2HPO_4, 0.544 g KH_2PO_4 in 1000 ml H_2O). After 15 min the rats were killed and pieces of the mesenteric fat were spread on microscope slides, fixed and stained with 0.2% toluidine blue in 10% formaldehyde, 5% acetic acid and 50% ethanol. Intact and degranulated mast cells were then counted.

The anti-inflammatory activity of the d-401 peptide was assayed using the rat carrageenan oedema.

4-2-1-2 Interaction between d-401 and cells

To detect if any interaction was occurring with the d-401 peptide, the cells obtained from the different sources were incubated with d-401, dissolved in the PBS buffer pH 7.4 for 10 min at 37°C in centrifuge tubes. The cells were then centrifuged in a bench centrifuge at low speed, resuspended in a small amount of buffer and observed in a Wild microscope equipped with an HBO 200 mercury lamp, using a GG 13 filter for ultraviolet fluorescence.

When measuring the interaction of human red cells with d-401, several dilutions of the blood were incubated with d-401 dissolved in isotonic saline for 15 min at room temperature. The blood suspension was then centrifuged for 5 min in a bench centrifuge and the supernatants were collected and kept in ice. The precipitate containing the red cells was washed once with 5 ml of saline and also kept in ice until used.

The fluoresence intensity of the supernatant solutions and the precipitate was then measured in a Perkin-Elmer fluorescence spectrophotometer, model MPF-3, using an excitation wavelength of 332 mμ and emission of 520 mμ, which corresponds to the fluorescence peak obtained for d-401 alone.

4-2-2 Experiments using liposomes

All the materials used in the present work were used without further purification and were of the highest purity available.

D(+) glucose grade III, Trizma base, glucose-6-phosphate dehydrogenase (type XV) from baker's yeast (300 units/mg protein), ATP, NADP and cholesterol were purchased from Sigma Chemical Co. Hexokinase, phosphatidyl choline, phosphatidic acid and phosphatidyl serine were purchased from Koch-Light Laboratories. Phosphatidyl choline (lecithin) in a

concentration of 0.1 g/ml, and phosphatidic acid (10 mg/ml) were stored under nitrogen in sealed ampoules at -20°C, in aliquots of 0.05 ml. For each preparation of liposomes a fresh ampoule was used. $^{22}Na^{+}$ in the form of sodium chloride in aqueous solution (100 mci/mg Na) was purchased from The Radiochemical Centre, Amersham, and Triton X-100 (iso-octyl-phenoxypolyethoxy ethanol containing 10 mols of ethylene oxide) was obtained from BDH Chemicals Ltd. Dialysis membranes (Visking) were obtained from Medicell International. Before every experiment, the dialysis membranes were soaked overnight in the solution used for dialysis.

All aqueous solutions were made with either triple distilled water or with deionized water which was subsequently distilled in an all glass apparatus.

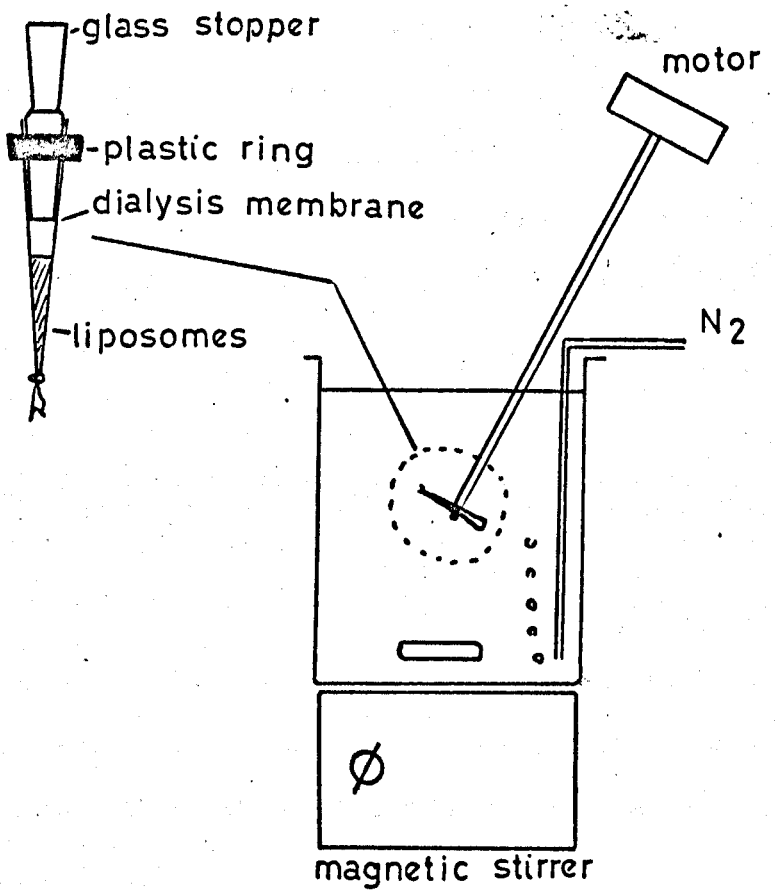
4-2-2-1 Preparation of glucose liposomes

The method for preparing the glucose liposomes was based on that described by Kinsky et al. (45, 100). Aliquots of the lipids in either chloroform or ethanol solutions were collected to make the desired molar concentration of the lipids in the aqueous solutions. The lipid solutions were pipetted into 5 ml round bottom flasks and the solvents were removed under a stream of N_2 while the flask revolved, connected to the roto evaporator. This produced a film of the lipids adhering to the walls of the flask. Traces of the solvents were then removed under vacuum for 10 min. To the dry film of lipids, 0.5 ml of a 0.3 M glucose solution was pipetted into the flask, which was then transferred to a water bath at 40°C. The flask was turned manually until all the lipids were dispersed and then it was stirred for 30 secs on a Vortex mixer. The liposomes prepared in this way will be referred

to as mechanically dispersed. After stirring, the liposomes were left standing at room temperature, with occasional stirring, for 1.5 h and then dialyzed for 1 h against 1500 ml of an isotonic solution containing 0.075 M KCl and 0.075 M NaCl. The system used for dialysis is described in Fig. 4-1. Dialysis was carried out at room temperature with the dialysis bag being turned very slowly with an electric motor ($2\frac{1}{2}$ RPM) in such a way as to allow an air bubble inside the bag to stir the liposomes gently. During dialysis the isotonic solution was constantly bubbled with nitrogen. It was found that when synthetic phospholipids were used, apart from having to introduce glass beads into the round bottom flasks and stir it on the Vortex mixer in order to disperse the lipid film, very little glucose was trapped inside the liposomes, probably due to the presence of saturated fatty acids on the phospholipid molecule (45).

After dialysis, the leakage of glucose from the liposomes was either measured directly inside a spectrophotometric cell or by means of equilibrium dialysis. The direct method consisted of adding to a spectrophotometric cell (quartz, 10 mm light path) the following solutions: 0.28 ml of 0.1 M Tris buffer pH 8, 0.5 ml of a solution containing 0.15 M KCl and 0.15 M NaCl in Tris buffer pH 8, 0.1 ml of 0.02 M magnesium acetate, 0.05 ml of 0.02 M ATP, 0.1 ml of 0.01 M NADP, 0.005 ml of hexokinase (6.7 mg/ml) and 0.005 ml of glucose-6-phosphate dehydrogenase (3.4 mg/ml). In the reference cell only NADP was omitted. The reaction was started by addition of 0.005 ml of the liposomes to the cells and the glucose leakage was measured by following the increase in absorbancy at 340 mμ using a Unicam SP 500 spectrophotometer. The increase in absorbancy is due to the formation of NADPH resulting from the following sequence of reactions:

FIG 4-1 -Dialysis system used for the glucose liposomes



$$\text{GLUCOSE} + \text{ATP} \xrightarrow{\text{HEXOKINASE}} \text{GLUCOSE-6-PHOSPHATE}$$

$$\text{GLUCOSE-6-PHOSPHATE} + \text{NADP} \xrightarrow{\text{GLUCOSE-6-P. DEHYDROGENASE}} \text{6-PHOSPHOGLUCONATE} + \text{NADPH} + \text{H}^+$$

When peptide 401 was tested for its effect on glucose permeability, it was either pre-incubated with the liposomes for 15 min at room temperature and then the liposomes were added directly to the spectrophotometric cells as described, or else a solution of 401 was added directly to the spectrophotometric cells containing the liposomes after the initial glucose leakage had been measured for a given interval of time. To estimate the total glucose content of the liposomes, 0.1 ml of a 10% solution (v/v) of Triton-X-100 was added to the cells; this was sufficient to break the liposomes and thus release all the glucose trapped.

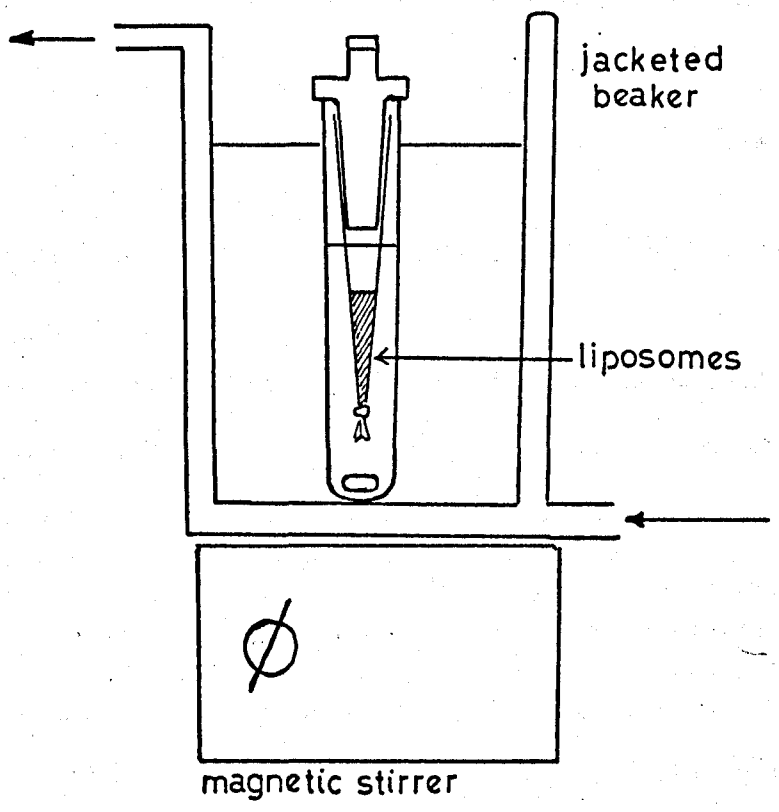
The equilibrium dialysis method consisted of pipetting 0.1 ml aliquots of the liposomes into dialysis bags which were then placed inside test tubes filled with 3 ml of the isotonic solution (0.075 M NaCl + 0.075 M KCl) containing the substance to be tested. The tubes (see Fig. 4-2) were then incubated at 40°C for 1 h and then aliquots of the solution bathing the dialysis bags were assayed for glucose as described before.

4-2-2-2 Preparation of $^{22}Na^+$ liposomes

The preparation of the $^{22}Na^+$ liposomes was conducted essentially as described for the glucose liposomes, except for the solutions used in the dialysis step and the dialysis system itself.

After obtaining the dry film of lipids 5-10 μci of $^{22}Na^+$ were added to the round bottom flask together with a known volume of the

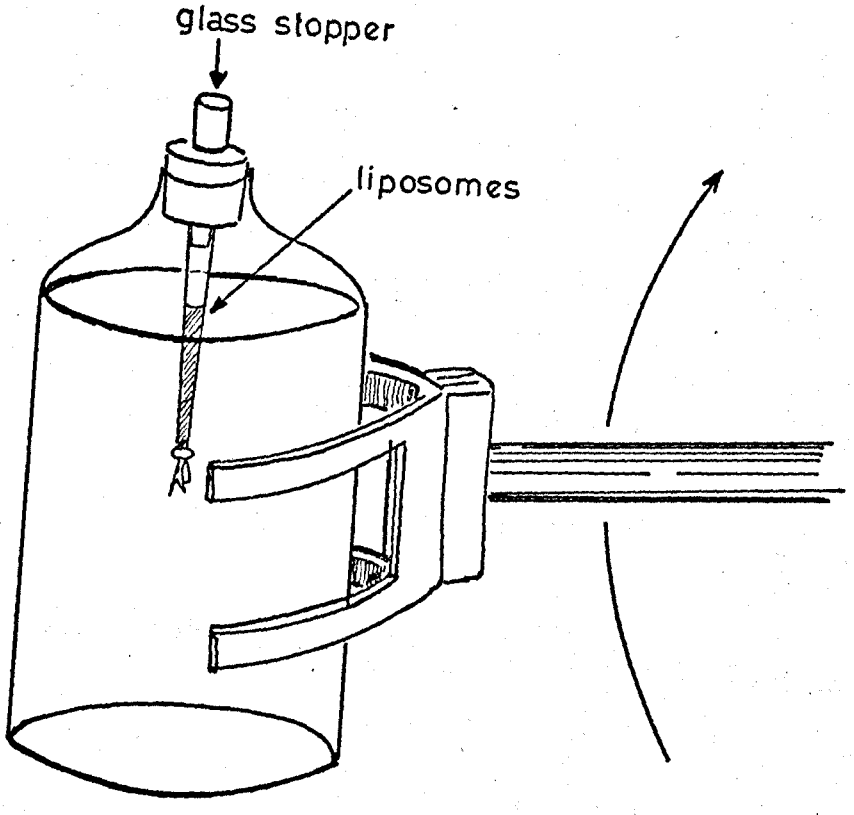
FIG 4-2 - System used to measure the permeability rates of solutes trapped in the liposomes



aqueous solutions (the volume depended on the amount of liposomes needed for a particular experiment or on the desired molar ratio of the lipids) and dispersed at 37°C as described before. In some cases the liposomes were sonicated using a bath type sonicator (American Beauty S 30) in plastic test tubes, until the dispersion became clear and slightly bluish. The aqueous solutions used for the dispersion and dialysis of the liposomes were either a TES buffer pH 7.4, made of 100 mM NaCl, 2 mM TES (N-Tris hydroxymethyl-2 amino ethane sulphonic acid), and 2 mM histidine, or a phosphate buffered saline pH 7.4. The liposomes were dialysed against the solutions in which they were dispersed using the system described in Fig. 4-3.

The plastic bottle contained 150 ml of buffer and was rotated for 15 min - 30 min each time. The solution was then replaced by a fresh one and the process was repeated. For each liposome preparation 4 changes of the solution were made. The liposomes prepared in this way were incubated inside dialysis bags as described for the glucose liposomes. Incubation was carried out for 3 h at 37°C. Aliquots of the solutions bathing the dialysis bags were then collected (0.05 ml) for counting in a Beckman liquid scintillation spectrometer, using the same scintillation fluid described in Chapter 3. The $^{22}Na^{+}$ was counted using the ^{32}P channel of the spectrometer which gave a counting efficiency of 49%. The total amount of $^{22}Na^{+}$ trapped in the liposomes was estimated by counting an aliquot of the liposomes after dialysis, added directly to the scintillation vials.

FIG 4-3 - Dialysis system used for the $^{22}Na^{+}$ liposomes



4-3 Results

4-3-1 Mast cell degranulation

The results of the tests on the ability of d-401 to degranulate mast cells are presented in Table 4-I.

Table 4-I

Effect of untreated 401 and d-401 on mast cell degranulation

	% degranulated mast cells	Mean & S.D.
Control (no injection)	0 0 1	0.3 %
401 (untreated) 0.1 µg/ml	2 0 1	1 %
401 (untreated) 1 µg/ml	66 89 82	79 ± 11 %
401 (untreated) 10 µg/ml	98 97 99	98 ± 1 %
401 (treated with 1:1 mol of dansyl) 1 µg/ml	68 64 25	52 ± 23 %
401 (treated with 1:2 mols of dansyl) 1 µg/ml	30 41 29	33 ± 6 %

As the peptide treated with 2 mols of dansyl presented less activity in degranulating mast cells than the 1:1 treated peptide 401, only the latter was used in the interaction studies with leucocytes and red cells.

4-3-2 Interaction between d-401 with cells obtained from the rat peritoneum

Plate 4-1 shows mast cells obtained from rat peritoneum which have been incubated with d-401 in a concentration of 0.1 mg/ml, and photographed under UV light. It can be seen that although the mast cells

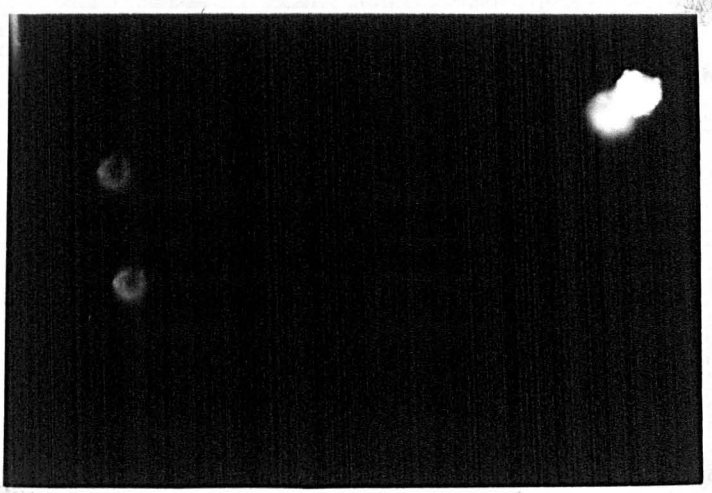


Plate 4-I - Leucocytes from rat peritoneum treated with d-401

were fluorescing much more intensely, the lymphocytes present in the same cell population and appearing on the left, were also binding some d-401. It was observed in the same experiment that addition of 1 ml of a fixative consisting of 50% ethanol, 10% formaline, and 5% acetic acid in distilled water, after incubation with d-401, did not interfere with the binding of d-401 to the cells, as judged by the unaltered fluorescence of the cells.

4-3-3 Interactions between d-401 and rat blood cells

Plates 4-2 and 4-3 show a population of leucocytes and red cells obtained from rat blood, which were incubated with d-401 at a

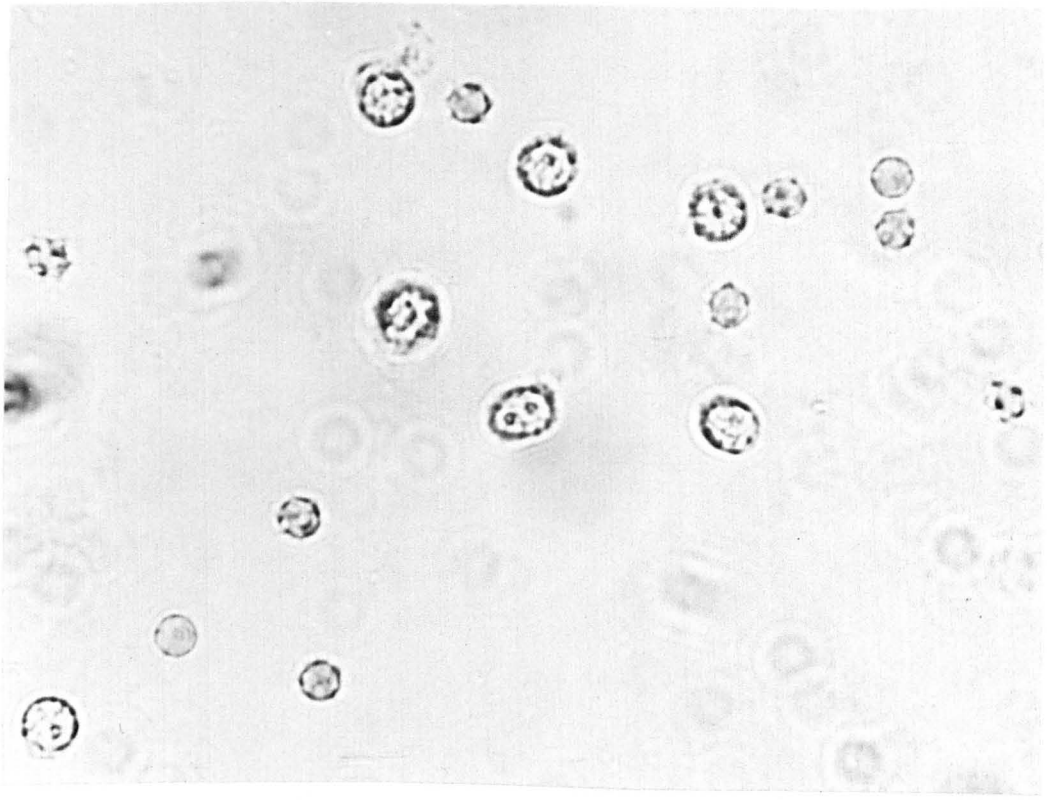


Plate 4-2 - Rat leucocytes and red cells treated with d-401;the photograph was taken with the light from a tungsten lamp

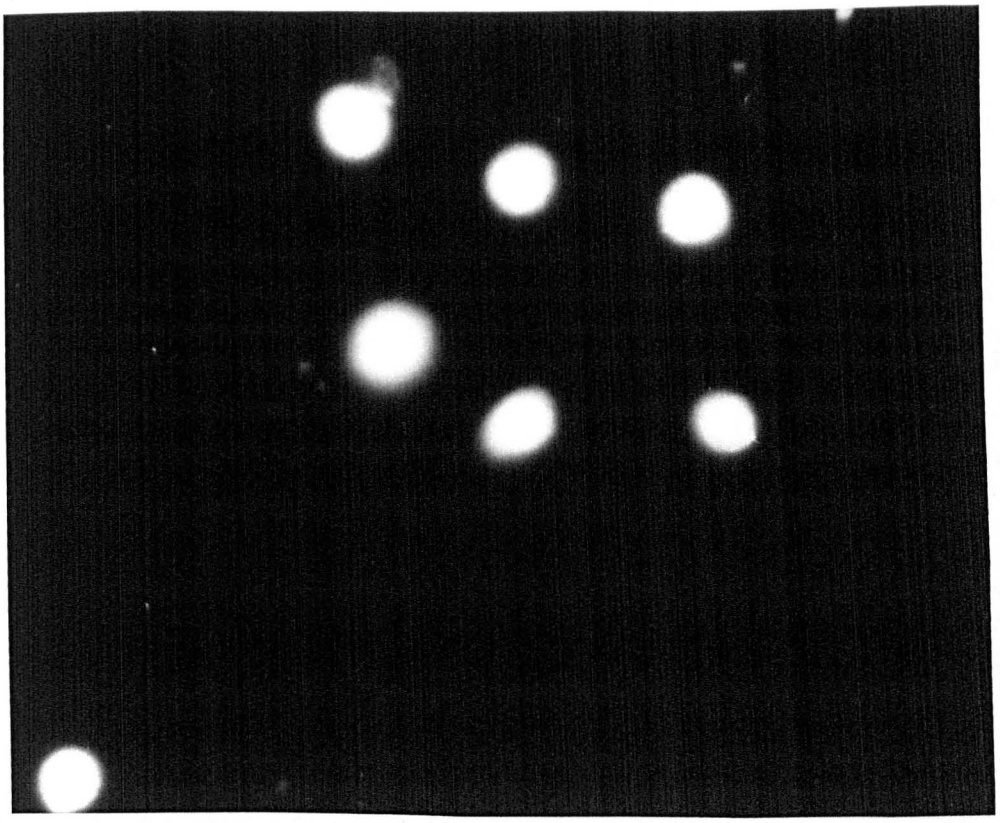


Plate 4-3 - Same as above but with photograph taken with U.V. light

concentration of 0.5 mg/ml at 37°C. In this preparation there were 24 x 10^5 leucocytes present (as counted by means of the stain crystal blue 1%, which lyses the red cells), of which polymorphonucleocytes comprised about 70%, the rest being monocytes and cells which could not be identified. The photograph in Plate 4-2 was taken with visible light and Plate 4-3 is the same field but with the photograph taken under UV light. It can be seen that only the leucocytes present fluoresce, although it is not possible to distinguish which among the leucocytes are binding d-401. In all the fields examined the red cells were either not fluorescing at all or presenting only a very faint fluorescence.

4-3-4 Interaction between d-401 and human red cells

The results of experiments where the binding between d-401 and human red cells was investigated are presented in Table 4-II.

Table 4-II

Binding of d-401 to several dilutions of human red blood cells

Supernatant Dilution	Fluorescence Intensity
0.1 / 10	55
0.5 / 10	65
1 / 10	64
2 / 10	56

In the above experiments, 0.1, 0.5, 1 and 2 ml of total blood was diluted to 10 ml with an isotonic NaCl solution, containing 0.5 mg of

d-401 and incubated for 15 min at room temperature. The cells were then centrifuged and the fluorescence intensity of the supernatant was measured as described before. The results in Table 4-II indicate that the red cells did not bind d-401 since all the fluorescence due to d-401 seemed to be present in the supernatant. This was calculated based on a standard curve made with several dilutions of d-401 in plasma and with the same dilutions of the isotonic saline as for the experiments with the red cells. The curve thus obtained is presented in Table 4-III.

Table 4-III

Standard concentration-fluorescence curve for d-401 diluted in plasma

d-401 (μg/ml)	F.I.
2	4.5
10	12.5
20	21.5
40	39.5

The precipitate containing the red cells showed only a residual fluorescence, which was probably due to scattering effects. In order to decrease the scattering, the cells were lysed with Triton X-100 and this solution presented no fluorescence at all. Although the released haemoglobin could have produced a quenching of the emitted light, or even of the exciting light, the results in Table 4-II indicate that the fluorescence found in the supernatant accounted for the whole amount of d-401 incubated with the cells, based on the curve presented in Table 4-III.

4-3-5 Liposomes

Plate 4-4 shows a preparation of liposomes made of lecithin and phosphatidic acid (8.6 mM and 0.45 mM respectively). Liposomes made of other lipids, or prepared with different molar ratios of the phospholipids do not show any apparent morphological differences at the low magnification at which the photograph in Plate 4-4 was taken, although liposomes containing cholesterol appeared to be smaller than the others. Plate 4-5 shows the aggregation caused by peptide 401 on the same preparation of liposomes. Aggregation caused by peptide 401 could be observed in all preparations of liposomes whether they contained phosphatidic acid, phosphatidyl serine or not, indicating that the presence of negative charges was not necessary for this effect. Aggregation caused by peptide 401 could be observed down to concentrations of 0.2 mg/ml. Possibly this effect of peptide 401, which could also be induced with polylysine is reflecting a disruption of the colloidal equilibrium, either due to blocking of charges (in the case of liposomes bearing negative charges) or to some kind of bridging between liposomes. However, it is not obvious why this should happen with liposomes made of neutral lipids.

4-3-5-1 Glucose liposomes

The results of adding several known concentrations of glucose to the medium containing all the components of the coupled enzymatic assay system for glucose, on the absorbance at 340 nm are presented in Table 4-IV. These results were used as the standard curve from where the glucose leakage from liposomes was calculated. Glucose leakage from the liposomes is expressed as the percentage of the total amount of glucose (in μ moles) trapped in the liposomes, which permeated across

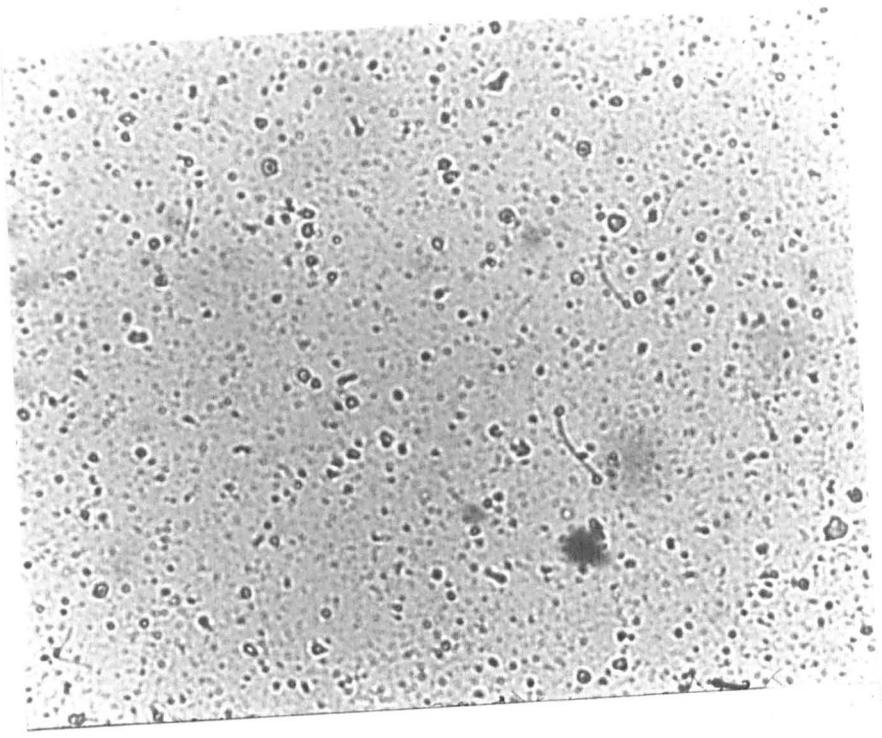


Plate 4-4-Liposomes made of lecithin and phosphatidic acid

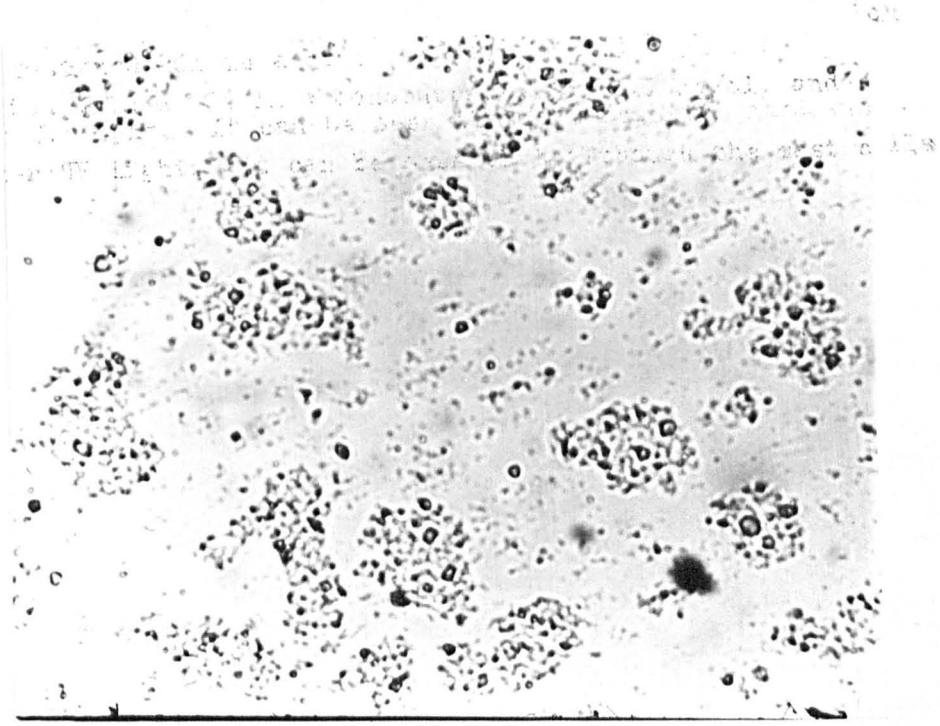


Plate 4-5-Same preparation of liposomes as above,but treated with peptide 401

the liposomes in 5 minutes.

Table 4-IV

Calibration of the glucose enzymatic assay

Glucose concentration (mM)		Absorbance 340 nm
(n = 2)	0.143	.414
(n = 3)	0.286	.810
(n = 3)	0.429	1.239

Table 4-V presents the results of experiments in which peptide 401 was pre-incubated with the liposomes (lecithin 8.6 mM and phosphatidic acid 0.45 mM) for 15 min at room temperature before the glucose assay.

Table 4-V

Effect of peptide 401 on glucose permeability

	Control (n=6) Mean and S.D.	76.1 µM 401(0.2mg/ml) Mean and S.D.
Total amount trapped in the liposomes (µmols)	177 ± 20	179 ± 29
Percentage of glucose leakage in 5 min	1.63 ± 0.5	1.97 ± 0.78

each value represents the average of three determinations

It can be seen that there are no significant differences between glucose leakage of the two groups.

Fig. 4-4 shows the results of adding peptide 401 directly to the spectrophotometric cell. These liposomes were made of lecithin 8.6 mM and phosphatidic acid 0.45 mM. Peptide 401 final concentration was 0.02 mg/1.1 ml. A very slight decrease in the permeability rate for glucose can be seen after addition of peptide 401. The decrease in O.D. immediately after addition of the test substances is an artifact caused by dilution. The experiment presented in Fig. 4-4 was repeated three more times with similar results and no apparent effect on the permeability rate for glucose due to 401 could be observed.

Table 4-VI shows the results of experiments where liposomes made of lecithin 8.6 mM and phosphatidic acid 0.45 mM, were incubated with peptide 401 in a concentration of 0.16 mg/ml, using the equilibrium dialysis system.

Table 4-VI

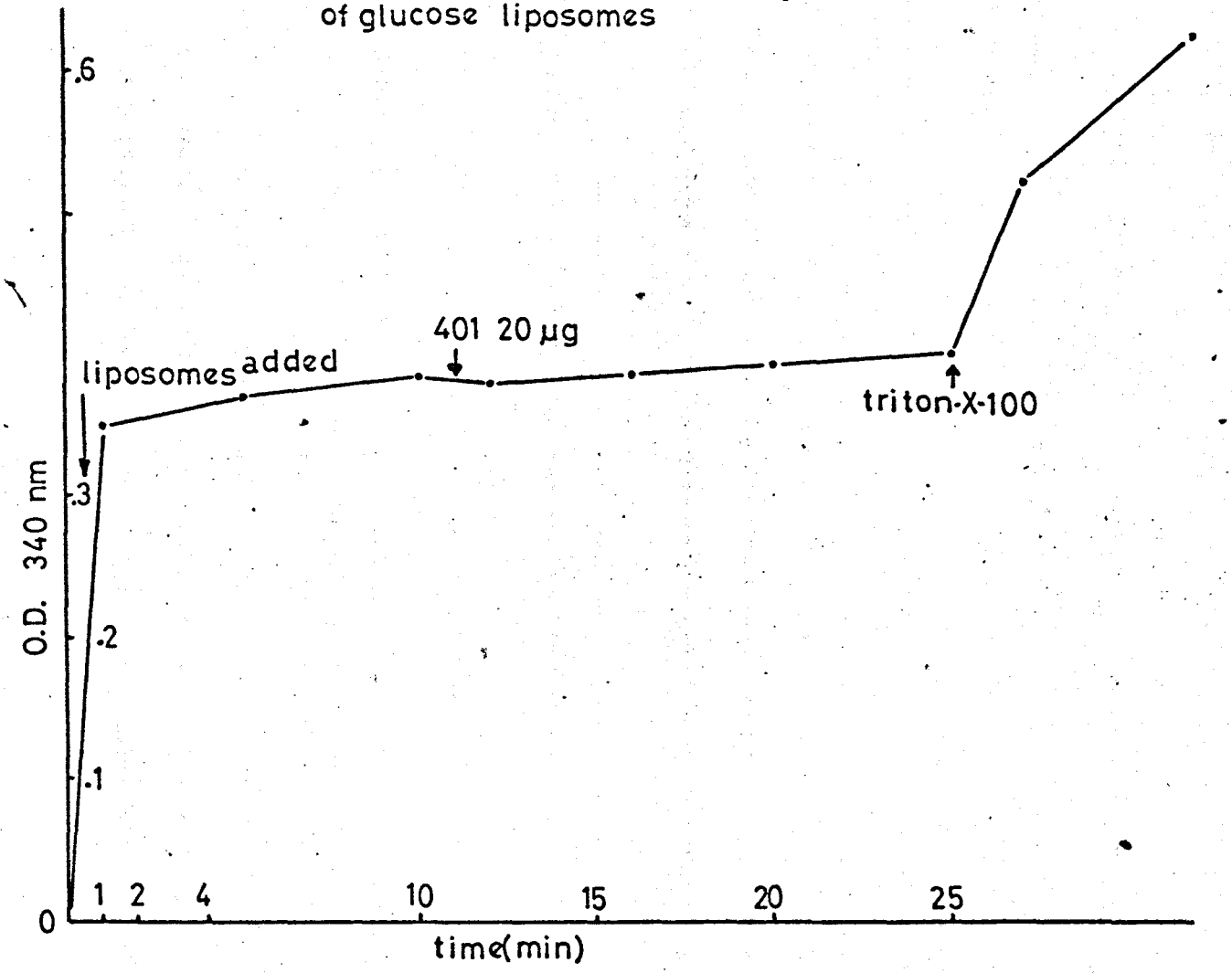
Effect of peptide 401 on glucose permeability using the equilibrium dialysis system

	μ Mols glucose/h
Control	105 ± 11
401 (0.16 mg/ml = 60.8 μM)	103 ± 10

each value represents the average of 5 determinations

The results are expressed as μ mols of glucose present in the isotonic solution (0.075 M NaCl + 0.075 M KCl) bathing the dialysis bags, after 1 hour of incubation at 40°C. The results of the experiments measuring glucose leakage across mechanically dispersed liposomes, show that

FIG 4-4 Effect of 401 on permeability rates of glucose liposomes



peptide 401 at concentration ranges of 0.16 mg/ml - 0.2 mg/ml does not affect permeability rates for glucose of liposomes made of 8.6 mM lecithin and 0.45 mM phosphatidic acid.

4-3-5-2 $^{22}Na^{+}$ liposomes

The counting efficiency for $^{22}Na^{+}$ in the scintillation fluid containing Triton X-100 was 48.9%. The quenching data presented in Table 4-VII was obtained by plotting the counting efficiencies against the external standard ratio of the Beckman spectrometer. Quenching was obtained by means of stepwise addition of chloroform to a sample of known activity.

Table 4-VII

Quenching curve for $^{22}Na^{+}$

Counting Efficiency	ESR (external standard ratio)
48.86	0.966
49.18	0.914
49.29	0.874
49.52	0.834
49.67	0.755
49.51	0.700
49.31	0.643
49.13	0.528
48.98	0.432
48.37	0.187
48.12	0.110
47.33	0.026
44.66	0

The results of the experiments measuring $^{22}Na^+$ permeability are expressed as the percentage of the total amount of $^{22}Na^+$ (in CPM) trapped in the liposomes after dialysis, which permeated across the liposomes after 3 hours incubation at 37°C. The results in Table 4-VIII were obtained after incubating liposomes made of 5.37 mM lecithin and 0.28 mM phosphatidic acid with several concentrations of peptide 401. The liposomes were dispersed in the TES buffer pH 7.4 which contained 5 µci of $^{22}Na^+$.

Table 4-VIII

Effect of peptide 401 on $^{22}Na^+$ leakage from liposomes made of lecithin (5.37 mM) and phosphatidic acid (0.28 mM)

	% Total $^{22}Na^+$
Control	5.13 ± 1.84
401 (0.1 mg/ml = 38 µM)	8.3 ± 2.3
401 (0.02 mg/ml = 7.6 µM)	5.8 ± 0.69
401 (0.004 mg/ml = 1.52 µM)	5.9 ± 0.19
401 (0.0008 mg/ml = 0.3 µM)	7.36 ± 0.88
401 (0.00016 mg/ml = 0.06 µM)	5.8 ± 1.2
Triton X-100 (0.4%)	75 %
Total amount of $^{22}Na^+$ trapped in the liposomes	12551 DPM

each value is the average of three determinations

In these experiments it can be seen that concentrations of 401 of 0.1 mg/ml and 0.0008 mg/ml were able to produce an increase in the

$^{22}Na^{+}$ permeability. Although the variation observed in the results of this particular set of experiments made the interpretation somewhat difficult, such effects could be reproduced, as shown in Fig. 4-5. Moreover, it has been shown that similar effects occur when very low concentrations of polylysine were incubated with liposomes (99). It remains unclear why such low doses of a basic peptide are more effective in increasing the $^{22}Na^{+}$ permeability than high ones. In the case of polylysine, and possibly in the case of peptide 401 too, very low concentrations may be interacting with the phospholipids in such a way as to rearrange them causing the formation of sodium'channels'. Higher concentrations, on the other hand, would have lytic effects on the membrane.

It is believed that polylysine is able to disrupt the liposomes at relatively high concentrations by inducing a concentration of charges on the surface of the liposomes (77). However, the results with liposomes in which glucose was trapped, did not indicate that peptide 401 was causing any lytic effects.

Table 4-IX and Fig. 4-5 show the results of experiments where liposomes made of lecithin 5.37 mM and phosphatidic acid 0.28 mM dispersed in the TES buffer pH 7.4 containing 10 µci of $^{22}Na^{+}$, were incubated with peptide 401 in the same range of concentrations as in the experiments presented in Table 4-VIII. In these experiments, however, part of the liposomes were submitted to sonication for 30 min at 50-55 k Hz yielding a dispersion which was clear and bluish. This indicated the presence of much smaller liposomes containing single bilayers of lipids instead of the multilamellar structure of the mechanically dispersed liposomes.

Table 4-IX

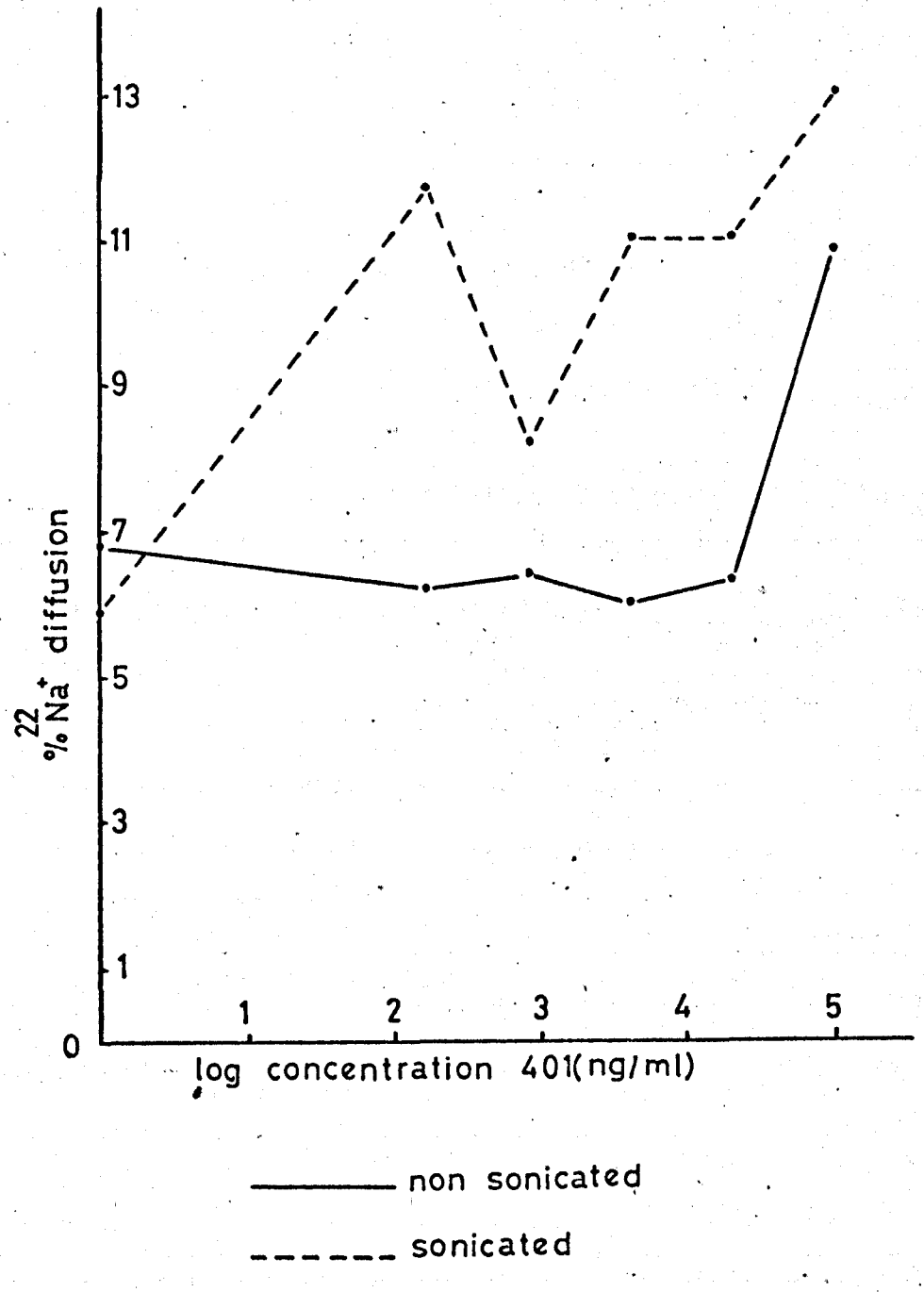
Effect of peptide 401 on sonicated and non-sonicated liposomes

	% of $^{22}Na^+$	
	Non-sonicated	Sonicated
Control	6.8	5.9
401 (0.1 mg/ml)	10.8	13
401 (0.02 mg/ml)	6.3	11
401 (0.004 mg/ml)	6	11
401 (0.0008 mg/ml)	6.4	8.2
401 (0.00016 mg/ml)	6.21	11.7
TX-100 (0.4% v/v)	78 %	91
Total amount of $^{22}Na^+$ trapped in the liposomes	15555 DPM	3045 DPM

each value is the average of two determinations

Concentrations of peptide 401 of 0.1 mg/ml are able to increase the permeability to $^{22}Na^+$ in the non-sonicated and sonicated liposomes, although the sonicated liposomes appear to be more sensitive to 401 since the whole range of concentrations of 401 is able to induce an increased $^{22}Na^+$ permeability in those liposomes. This fact indicates that the effective concentrations of 401 in the mechanically dispersed liposomes, with respect to increased $^{22}Na^+$ permeability, may be smaller than the ones reported, since the observed effect on 401 on the mechanically dispersed liposomes may be apparent only after 401 has allowed leakage of $^{22}Na^+$ from several adjacent compartments of the multilamellar structure. Thus, for the sake of interaction, the sonicated liposomes reveal that 401 is able to produce an increased

FIG 4-5 -Effect of peptide 401 on permeability rates of sonicated and non-sonicated liposomes containing $^{22}Na^{+}$. (data from Table 4-IX)



$^{22}Na^{+}$ permeability at very low concentrations, and there is no reason to assume that the lipid distribution in the sonicated liposomes is different from the mechanically dispersed ones.

Table 4-X shows the results of incubating liposomes made of lecithin 5.37 mM and phosphatidic acid 0.28 mM and also made of lecithin 5.37 mM and cholesterol 2.15 mM, both dispersed mechanically in the TES buffer pH 7.4, with several concentrations of peptide 401.

Table 4-X

Effect of peptide 401 on $^{22}Na^{+}$ leakage from liposomes containing or not cholesterol

	% $^{22}Na^{+}$ (Mean and S.D.)	
	Cholesterol(2.15mM) Lecithin (5.37mM)	Lecithin (5.37 mM) Phosphatidic Acid (0.28 mM)
Control	15.6 ± 4.2	6.19 ± 2.22
401 (0.2 mg/ml = 76 μM)	16.6 ± 2.17	14.36 ± 6
401 (0.1 mg/ml = 38 μM)	10.6 ± 0.4	8.5 ± 1.6
401 (0.05 mg/ml = 18 μM)	9.64 ± 2.3	8.8 ± 0.42
401 (0.01 mg/ml = 3.6 μM)	11.8 ± 2.9	7.2 ± 0.7
Triton X-100 (0.4%)	46	63
Total amount of $^{22}Na^{+}$ trapped	2985 DPM	28192 DPM

each value is the average of three determinations

The effect of peptide 401 in increasing $^{22}Na^{+}$ permeability can only be observed in the liposomes containing phosphatidic acid, indicating that

the presence of negative charges favours interaction between peptide 401 and the membranes. The liposomes containing cholesterol trapped much less $^{22}Na^{+}$ than the lecithin-phosphatidic acid liposomes. It is possible that because the liposomes containing cholesterol are neutral at the pH of the experiments, Na^{+} ions diffuse more freely. Although cholesterol liposomes appeared to be smaller than the phosphatidic acid-lecithin liposomes upon microscopic observation, and therefore contained less $^{22}Na^{+}$, the values presented in Table 4-X show that the control liposomes containing cholesterol, in spite of having trapped less $^{22}Na^{+}$, showed a higher permeability rate for $^{22}Na^{+}$ than the control liposomes made of lecithin and phosphatidic acid.

In order to test if the charge alone of the liposomes was important for the binding and the increased $^{22}Na^{+}$ permeability effect of 401, experiments were made in which phosphatidic acid was replaced by phosphatidyl serine, another acidic phospholipid.

Table 4-XI and Fig. 4-6 show the results of incubating liposomes made of lecithin 5.9 mM and phosphatidyl serine (PS) 9.12 mM and also liposomes made of lecithin 5.9 mM, PS 9.12 mM and cholesterol 2.35 mM with peptide 401 in concentrations of 0.2 mg/ml and 0.1 mg/ml. The liposomes were dispersed mechanically in TES buffer pH 7.4.

Table 4-XI

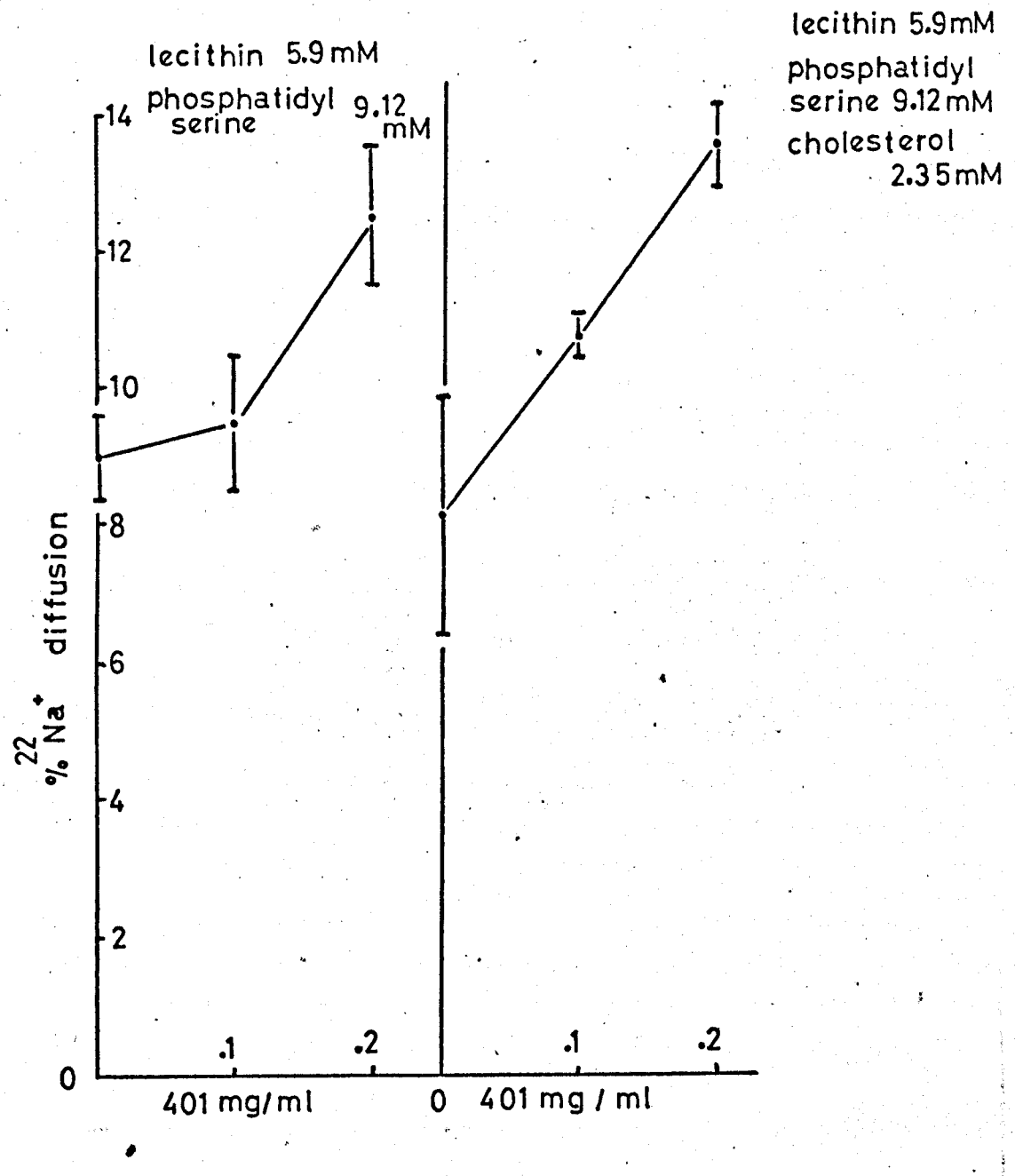
Effect of peptide 401 on $^{22}Na^{+}$ leakage from liposomes made of lecithin 5.9 mM, phosphatidyl serine 9.12 mM and containing or not cholesterol 2.35 mM

		% $^{22}Na^{+}$ leakage in 3 h
Lecithin 5.9 mM PS - 9.12 mM	Control	8.98 ± 0.6
	401 (0.2 mg/ml)	12.49 ± 1
	401 (0.1 mg/ml)	9.48 ± 1
	TX-100 (2.5%)	34.3 ± 5.9
	Total amount of $^{22}Na^{+}$ trapped	172 215 DPM
		% $^{22}Na^{+}$ leakage in 3h
Lecithin 5.9 mM PS - 9.12 mM Chol - 2.35 mM	Control	8.11 ± 1.69
	401 (0.2 mg/ml)	13.51 ± 0.6
	401 (0.1 mg/ml)	10.7 ± 0.25
	TX-100 (2.5%)	37.5 ± 2.75
	Total amount of $^{22}Na^{+}$ trapped	160 486 DPM

each value is the average of four determinations

The increased $^{22}Na^{+}$ permeability effect of 401 can be observed in both liposome preparations with approximately the same degree. The same experiment was then repeated, but with a different ratio between phosphatidyl serine and cholesterol. It was reasoned that in the previous experiment, the negative charges due to phosphatidyl serine were in a large excess and would thus be masking any effect of cholesterol on the binding of 401 to the liposomes. The results of this experiment are presented in Table 4-XII and Fig. 4-7. The liposomes were prepared with

FIG 4-6 - Effect of peptide 401 on $^{22}Na^{+}$ permeability rates of liposomes made of several lipids.
(data from Table 4-XI)



lecithin 5.9 mM and PS 1.82 mM and also with lecithin 5.9 mM, PS 1.82 mM and cholesterol 2.58 mM.

Table 4-XII

Effect of 401 on $^{22}Na^{+}$ leakage from liposomes made of lecithin 5.9 mM, phosphatidyl serine 1.82 mM containing or not cholesterol 2.58 mM

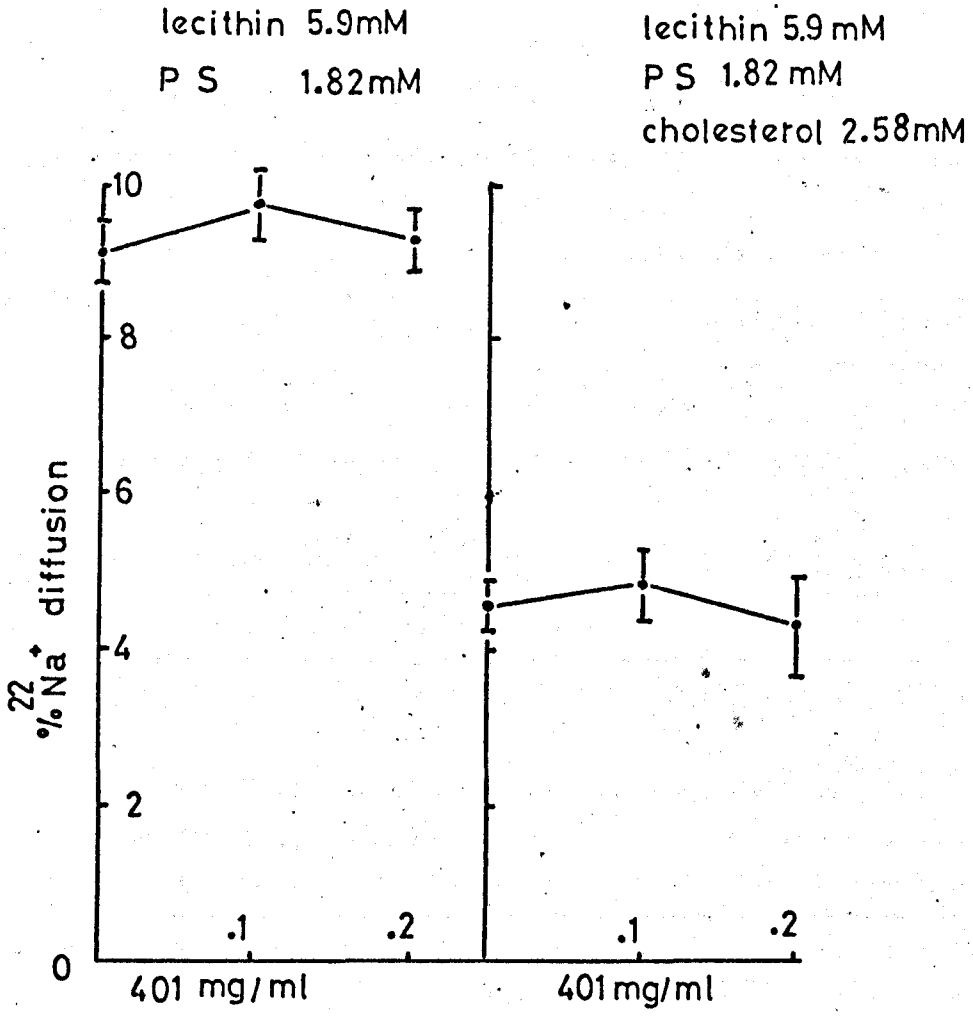
		% $^{22}Na^{+}$ leakage in 3h
5.9 LEC 1.82 PS	Control	9.16 ± 0.37
	401 (0.2 mg/ml = 76 µM)	9.28 ± 0.34
	401 (0.1 mg/ml = 38 µM)	9.75 ± 0.43
	Triton X-100 (2.5% v/v)	67 ± 15.6
	Total amount of $^{22}Na^{+}$ trapped	25 536 DPM
		% $^{22}Na^{+}$ leakage in 3h
5.9 LEC 1.82 PS 2.58 CHOL	Control	4.55 ± 0.27
	401 (0.2 mg/ml)	4.35 ± 0.6
	401 (0.1 mg/ml)	4.85 ± 0.41
	Triton X-100 (2.5% v/v)	52.3 ± 11.1
	Total amount of $^{22}Na^{+}$ trapped	36 250 DPM

each value is the average of four determinations

It can be seen that no effect of 401 in increasing the $^{22}Na^{+}$ permeability occurred in either group of liposomes, although both preparations were visibly aggregated indicating that some binding of 401 to the liposomes took place. A decrease in $^{22}Na^{+}$ permeability in the liposomes containing cholesterol can also be seen, although these liposomes trapped more $^{22}Na^{+}$ than the lecithin-PS liposomes.

Table 4-XIII and Fig. 4-8 show the results of experiments where

FIG 4-7 - Effect of peptide 401 on $^{22}Na^{+}$ permeability rates of liposomes with different lipid composition (data from Table 4-XII)



liposomes made of 5.4 mM PS alone, PS 5.4 mM + lecithin 5.25 mM and PS 5.4 mM + cholesterol 5.96 mM dispersed mechanically in a phosphate buffered saline pH 7.4 were incubated with peptide 401 in concentrations of 0.2 mg/ml and 0.1 mg/ml.

Table 4-XIII

Effect of peptide 401 on $^{22}Na^{+}$ leakage from liposomes made of phosphatidyl serine (5.4 mM) alone, phosphatidyl serine (5.4 mM) + cholesterol (5.96 mM) and phosphatidyl serine (5.4 mM) + lecithin (5.92 mM)

		% $^{22}Na^{+}$ leakage in 3h
5.4 mM PS	Control	25.3 ± 5.8
	401 (0.2 mg/ml)	37.5 ± 2.83
	401 (0.1 mg/ml)	35.8 ± 1.84
	TX-100 (2.5%)	83.6 ± 3.88
	Total amount of $^{22}Na^{+}$ trapped	13 037 DPM
5.4 mM PS 5.96 mM CHOL	Control	4.6 ± 0.51
	401 (0.2 mg/ml)	12.3 ± 2.5
	401 (0.1 mg/ml)	7 ± 0.45
	TX-100 (2.5%)	66.7 ± 8.4
	Total amount of $^{22}Na^{+}$ trapped	35 605 DPM
5.4 mM PS 5.92 mM LEC	Control	12.6 ± 1.59
	401 (0.2 mg/ml)	17.62 ± 1.6
	401 (0.1 mg/ml)	17 ± 0.8
	TX-100 (2.5%)	54.6 ± 8.8
	Total amount of $^{22}Na^{+}$ trapped	21 339 DPM

each value is the average of three determinations

FIG 4-8 - Effect of peptide 401 on $^{22}Na^{+}$ permeability rates of liposomes with different lipid composition (data from Table 4-XIII)

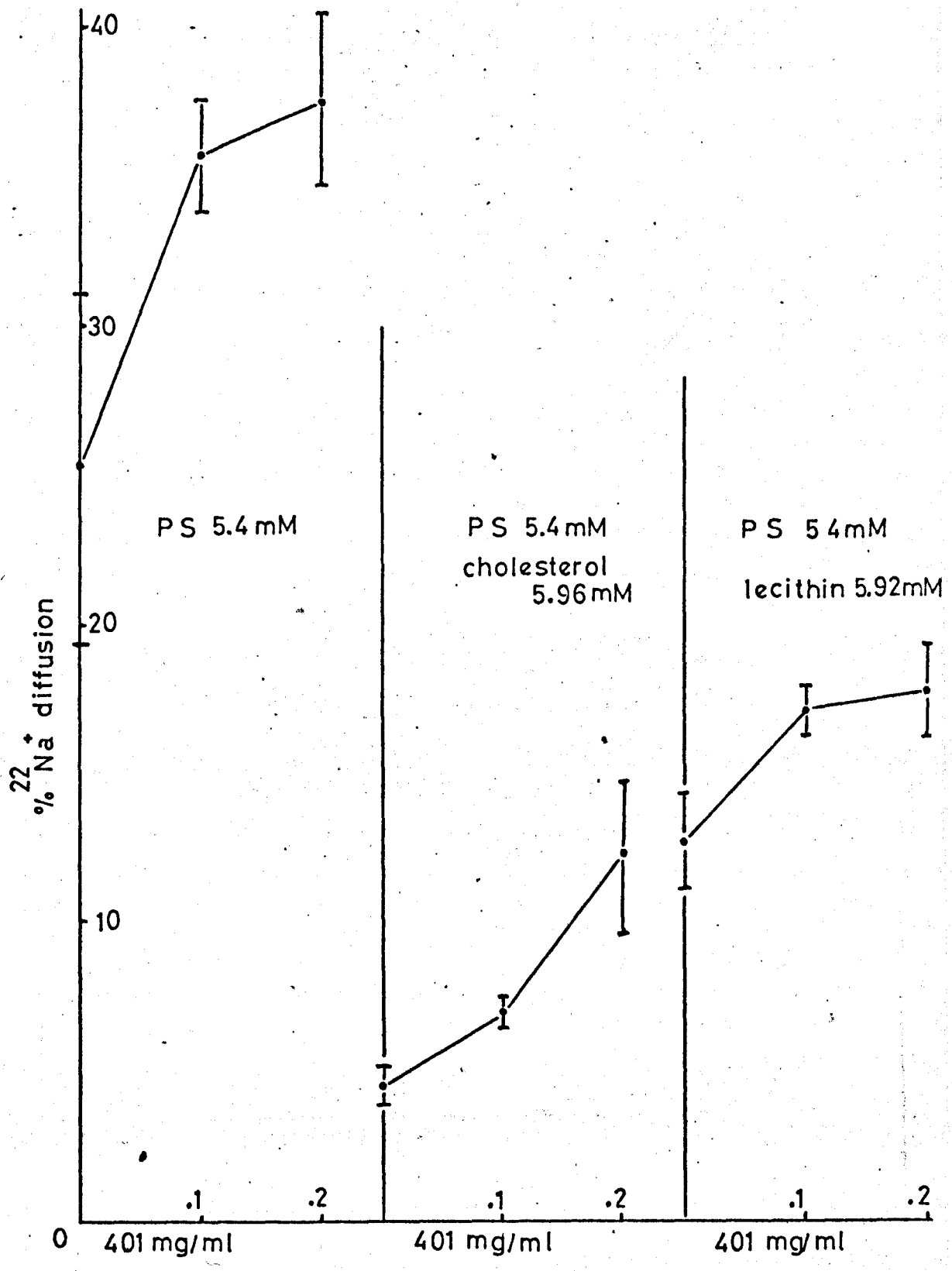
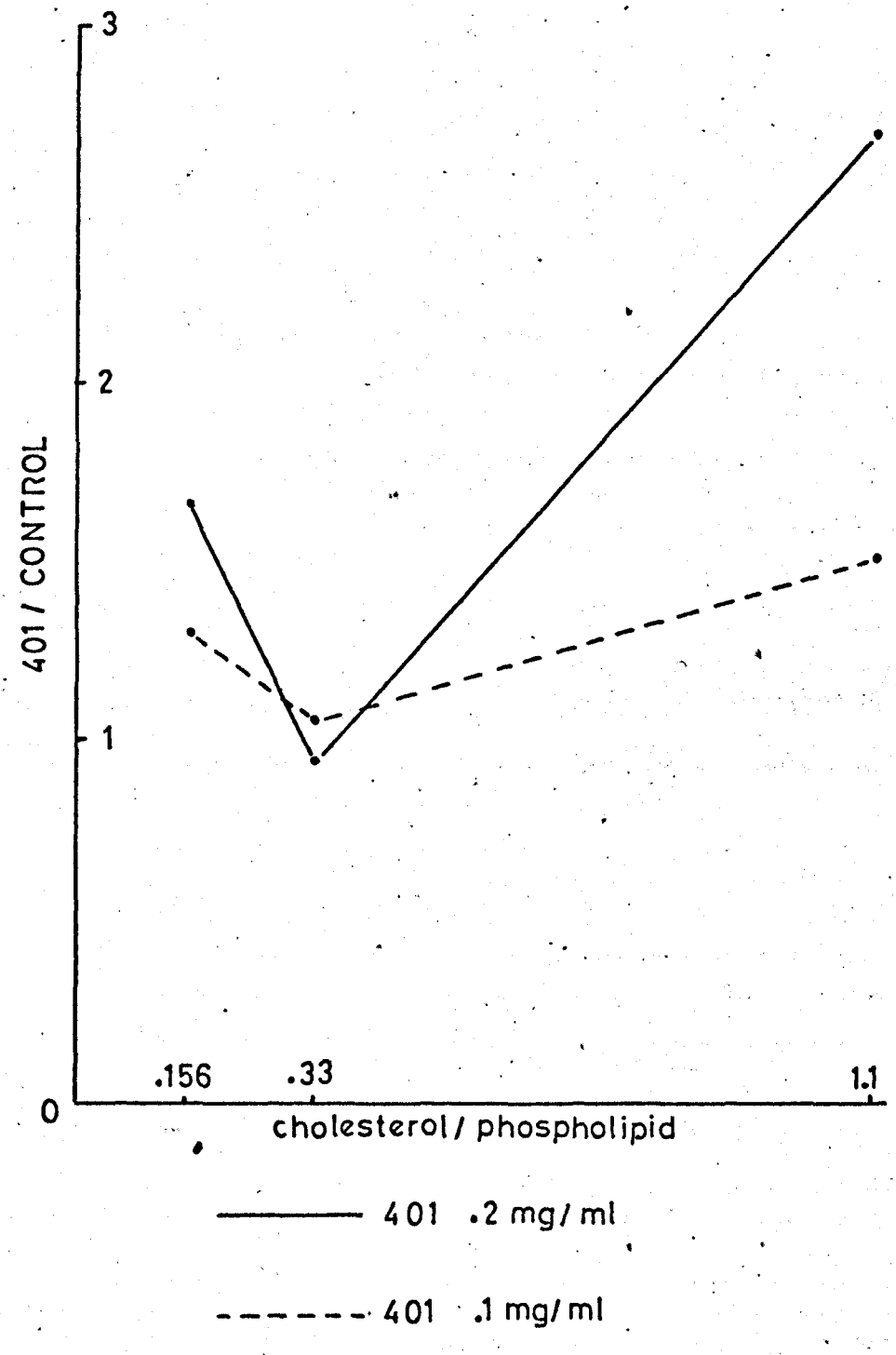


FIG 4-9 - Effect of peptide 401 on $^{22}Na^{+}$ permeability rates as a function of the cholesterol/phospholipid ratio of the liposomes



Although the liposomes made of PS alone were much more permeable to $^{22}Na^{+}$, the effect of peptide 401 was more pronounced in the liposomes containing cholesterol, showing that besides the charge of the liposomes, the nature and the ratio between the lipids forming the liposomes seems to be important for the effect of peptide 401, concerning the increase in permeability for $^{22}Na^{+}$ and possibly its binding too. In Fig. 4-9 the ratios between cholesterol and the phospholipids present in a preparation of liposomes were plotted against the ratios between the values of the 401 groups and controls of the experiments from Tables 4-XI - 4-XIII and Figs. 4-6 - 4-8. This curve shows that there seems to be a correlation between the ratio cholesterol/phospholipid and the effect of peptide 401 in increasing the permeability to $^{22}Na^{+}$. The highest effect is observed at a ratio 1.1, which according to Mahendra et al. (112) is when cholesterol is producing a maximum in 'packing' between the molecules forming the membranes, as reflected by a decrease in water permeability.

4-4 Discussion

The experiments carried out to investigate the binding of dansyl-401 to a population of human and rat blood cells demonstrated that red cells did not seem to interact with dansyl-401, whereas the leucocytes present in the blood did bind dansyl-401 as judged by intensity of fluorescence when observed under a UV microscope. It was not possible to distinguish among the leucocytes which cell type was binding d-401 more strongly. However, in a population of cells obtained from the rat peritoneum, it could be seen that among mast cells, lymphocytes and macrophages, the affinity to d-401 was mast cells > lymphocytes > macrophages (see Plate 4-1). This is in agreement with the reported

effect of peptide 401 on degranulation of mast cells (31) and also with the results of G. Atkinson (11, 13) on histamine release induced by peptide 401, showing that rat mast cells are more sensitive to peptide 401 than the rat polymorphonucleocytes. The results of the present experiments with d-401 support the suggestion put forward in the previous chapter on the possibility that peptide 401 was interacting preferentially with the rat leucocytes and inhibiting prostaglandin synthesis by those cells. However, it must be kept in mind that the alterations introduced in the molecule of peptide 401 by the dansyl groups may be changing its in vivo properties. It was shown that the anti-inflammatory activity of peptide 401 treated with a 1:1 mol ratio 401:dansyl, was reduced by approximately 1/3 (156) although its ability to degranulate mast cells was not very much altered. The experiments employing liposomes as model membrane systems were attempts to determine if a preferential binding between peptide 401 and the leucocytes could be explained by other features of the membranes besides electric charge, since in vivo most tissues have a net negative charge at the physiological pH, due mainly to the sialic acid groups of the membrane glycoproteins. With this experimental model it was also possible to study such interactions using the intact molecule of 401. It was shown that no permeability effects due to 401 could be observed when glucose was trapped in liposomes made from lecithin and phosphatidic acid. On the other hand when liposomes in which $^{22}Na^{+}$ was trapped, were incubated with peptide 401 in concentrations of 0.1 mg/ml, an increased permeability rate for $^{22}Na^{+}$ was induced. This effect could be enhanced by varying the ratio cholesterol/phospholipid of the liposomes. The lack of lytic effects on glucose liposomes with the same lipid composition as the $^{22}Na^{+}$ liposomes, together with an effect dependent on the cholesterol/

phospholipid ratio, makes it improbable that the ability of peptide 401 to increase the permeability to $^{22}Na^{+}$ derives from lytic effects on the membranes, as is shown to occur with melittin and polylysine (77, 154).

Similar results have been reported for two brain specific proteins, which only affect the permeability rates to cations but not to glucose (33). In the present work, it could be argued that because glucose, an apolar solute, is able to permeate freely across the multilamellar structure of the liposomes, and $^{22}Na^{+}$, on the other hand, does not diffuse so easily, the permeability effect caused by peptide 401 in the $^{22}Na^{+}$ liposomes was not necessarily due to any specific formation of "sodium channels". It is possible that in the case of the $^{22}Na^{+}$ liposomes, peptide 401 was simply neutralizing negative charges on the membrane and thus decreasing the opposing electrostatic barrier to $^{22}Na^{+}$. However, the permeability effects of peptide 401 on the $^{22}Na^{+}$ liposomes were shown to be dependent on the lipid composition of the membranes. When liposomes were made from nearly equimolecular concentrations of cholesterol and phosphatidyl serine this effect was more pronounced. Replacement of cholesterol by equal amounts of lecithin decreased the peptide 401 induced $^{22}Na^{+}$ permeability, showing that this was not simply due to the presence of a neutral lipid component on the membrane. Moreover, liposomes prepared with phosphatidyl serine alone showed a much greater spontaneous diffusion of $^{22}Na^{+}$, with a permeability effect caused by peptide 401 comparable with the ones found for liposomes with a phosphatidyl serine/lecithin ratio of 1.54. Although the 401 induced $^{22}Na^{+}$ permeability could be partially explained by a simple decrease in the electrostatic barrier for $^{22}Na^{+}$ diffusion in some cases, as shown by the results in Table 4-X, this clearly does not apply to other experiments with $^{22}Na^{+}$ liposomes. It appears then that the nature of the lipids, as

well as the ratios between the phospholipids and cholesterol of these model membranes are important for the $^{22}Na^{+}$ permeability effects of peptide 401. This effect of peptide 401 could have some implications in the mast cell degranulating activity of peptide 401. It was shown that degranulation of rat mast cells could be induced by means of ionophores which facilitate the penetration of Ca^{++} ions into the mast cells (68). As peptide 401 was shown to be able to degranulate mast cells in the absence of Ca^{++} ions, it was suggested that the histamine release by mast cells, caused by peptide 401, could be mediated through an exchange of Na^{+} or other cations (118, 13). In this case peptide 401 would be behaving as a Na^{+} ionophore.

The results of the present experiments indicate that the binding and $^{22}Na^{+}$ permeability effect of 401 on liposomes may be directed by the cholesterol/phospholipid ratio of these membranes. If these results bear any relevance to the <u>in vivo</u> effects of peptide 401, how does cholesterol fit in this problem? The role of cholesterol in artificial membranes has been intensively studied recently. A number of experiments employing liposomes have shown that the presence of cholesterol is able to decrease the interaction between many cationic proteins and peptides with the liposomes (45, 128) and it has been suggested that cholesterol is able to do so by decreasing the 'fluidity' of the membrane. The concept of membrane fluidity (158) as applied to the cell membrane proposes that the protein sub-units as well as phospholipids are able to move freely on the two dimensional lipid fluid moiety of the membrane, the protein sub-units being hydrophobically bound to the lipids. The observed phenomenon of 'capping' (136) or migration of protein sub-units to a certain point on the membrane prior to pinocytosis or mitosis is one example of this hypothesis. The role of cholesterol would be that

of decreasing the superficial motion of sub-units, and consequently causing a redistribution of charges on the cell membrane. The accepted idea is that cholesterol promotes a tighter packing of the phospholipids which increases the stability of the membrane probably by means of an increased interaction (hydrophobic) between the constituent lipids on the membrane (45). A number of metabolic activities inherent to the cell membrane seem to be dependent on the fluidity of the membrane (157). It has been reported recently that macrophages of rats fed on a cholesterol rich diet were not able to phagocyte as efficiently as the macrophages from control rats (58). The effect of cholesterol in decreasing membrane fluidity has led to the observation that the less metabolically active tissues seem to present a higher concentration of cholesterol as a membrane constituent (128). A similar situation appears to be occurring with the liposomes, as reflected by the few parameters which can be measured in these model membrane systems. Thus permeability rates for solutes, water permeability, and binding of basic proteins and peptides to liposomes are all decreased by incorporation of cholesterol in the membranes.

In the present work, however, the permeability effects of peptide 401 on the liposomes were increased as a consequence of cholesterol incorporation in the membranes, in a ratio 1.1 cholesterol/phospholipid. The only reported case of similar effects caused by a basic protein was that of a basic myelin protein (128) which is able to induce increased solute permeability in liposomes independently of the cholesterol content of their membranes. It was suggested that such an effect was due to the 'hydrophobicity' of this protein which has a high content of apolar amino acids in its molecule. Although peptide 401 does contain some apolar amino acids, it cannot be classified as a hydrophobic peptide. Moreover,

red cells from a number of different species have cholesterol/phospholipid ratios which are close to the value 0.9 (184, 121) and in the present work, liposomes with a cholesterol/phospholipid ratio of 1.1 were the most affected by peptide 401 although Plates 2 and 3 and Table 4-II show that there was no binding between d-401 and the rat and human red cells.

It has been concluded that apart from the lipids in the cell membranes the role of the proteins must be considered as well with respect to the binding between peptide 401 and the leucocytes. It is possible that the binding of 401 to the rat leucocytes depends on the sialic acid content of the membranes of these cells, in a way similar to that of the tetrapeptide tuftsin (36). The sialic acid content and the amount of cholesterol could then be determining the structural features of the membranes on which the interaction of peptide 401 would be dependent. Differences in the sialic acid content of the several tissues of the rat are to be expected, although there are no data on the sialic acid composition of the rat polymorphonucleocytes (120).

CHAPTER 5

PEPTIDE 401 AND CORTICOSTERONE SYNTHESIS AND RELEASE

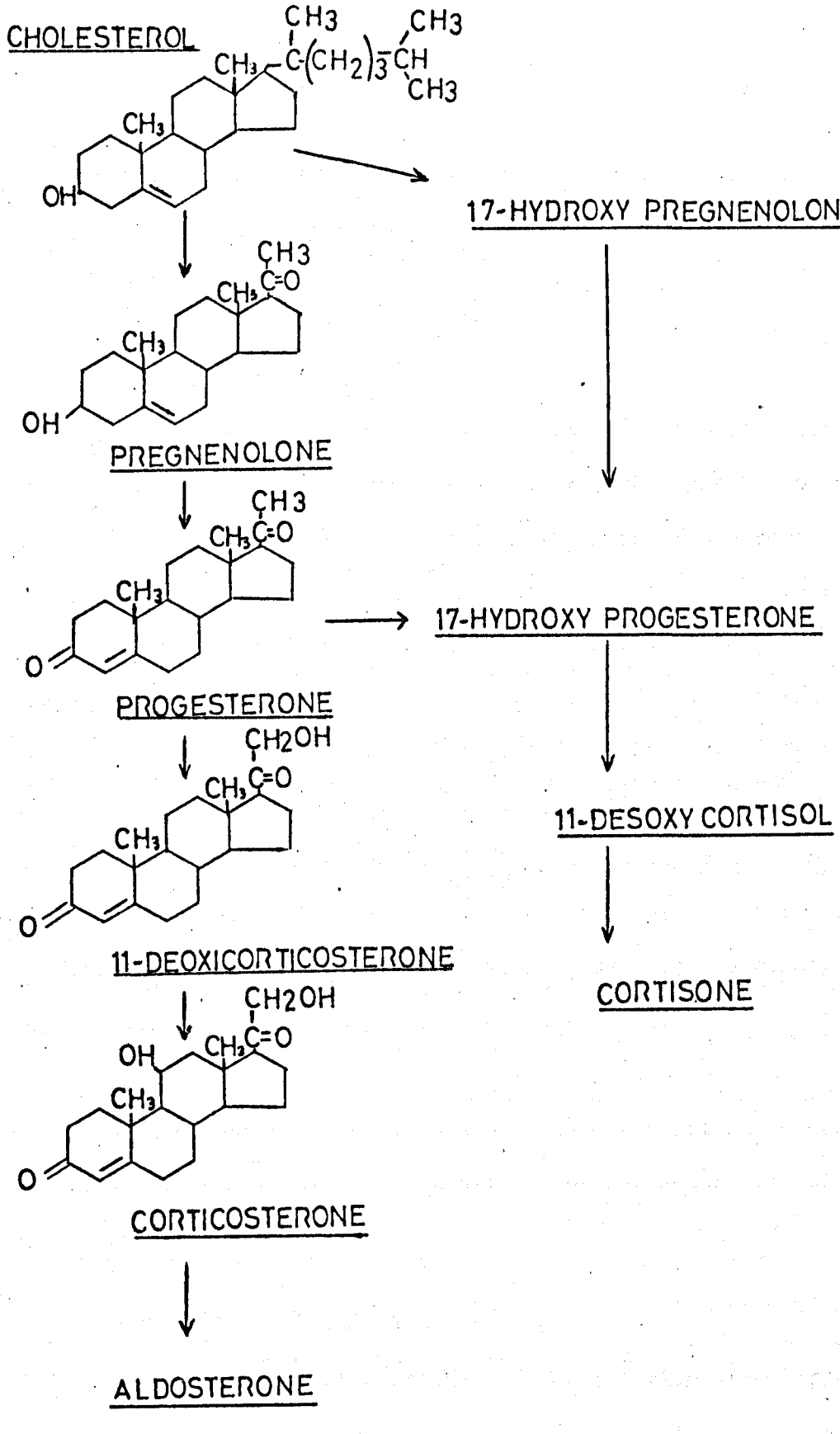
5-1 Introduction

5-1-1 Occurrence and biosynthesis

The adrenal steroid hormones are synthesized and secreted by the cells of the zona glomerulosa, fasciculata and reticularis of the adrenal cortex. The main precursor of the adrenal corticosteroid hormones is cholesterol. The biosynthetic pathway of some of the corticosteroids is summarized below. The steroid hormones are synthesized and released as a consequence of the action of ACTH (adrenocorticotropic hormone). ACTH is a polypeptide hormone synthesized by the adenohypophysis. The adrenal cortex is not innervated and ACTH is believed to be the major controlling agent for synthesis and release of the steroid hormones by the adrenal cortex. The steroid hormones are not stored in the cells and so ACTH action must involve de novo synthesis of the hormones (170). It is believed that the corticosteroid-genesis induced by ACTH is mediated by stimulation of adenyl cyclase and hence intracellular formation of cyclic AMP (153).

5-1-2 Hormonal action of corticosteroids

The corticosteroid hormones are classified according to their physiological role. Thus the mineralocortocoids influence the electrolyte balance of the organism, and the glucocorticoids control most reactions of the carbohydrate and protein metabolism. Another class of adrenal corticosteroids include the sex hormones, which control the secondary


CHOLESTEROL
17-HYDROXY PREGNENOLONE
PREGNENOLONE
17-HYDROXY PROGESTERONE
PROGESTERONE
11-DESOXY CORTISOL
11-DEOXICORTICOSTERONE
CORTISONE
CORTICOSTERONE
ALDOSTERONE

sexual characters, but the adrenal glands are not very important concerning the production of these hormones.

5-1-3 Anti-inflammatory activity

Apart from their actions on the carbohydrate and protein metabolism the glucocorticoids are very potent anti-inflammatory agents (84). The glucocorticoids are able to act either locally or systemically suppressing erythema, oedema, heat and local increased vascular permeability. However as anti-inflammatory agents their therepeutic action is limited due to the number of side effects produced. Thus a long-term treatment with glucocorticoids can lead to interference with the pituitary-adrenal relationship, imbalances on the electrolyte composition of the several fluids, osteoporosis and decreased resistance to infections since glucocorticoids have immunosuppressive effects (170), i.e. they inhibit antibody production,apart from causing the disappearance of cells whichare normally engaged in phagocytosis and digestion of foreign compounds and bacteria. The anti-inflammatory activity of the glucocorticoids is poorly understood. There have been attempts to establish a direct correlation between the action of the glucocorticoids on carbohydrate metabolism and their anti-inflammatory effects, but no clear picture has emerged (71).

5-1-4 Action on membranes

It has been proposed that the anti-inflammatory effects of steroid hormones are a result of their direct action on the membranes of lysosomes (186), making them more stable, i.e., not susceptible to lysis. Lysosomal enzymes which are usually responsible for many of the manifestations of inflammation are consequently not released. The work of Baugham *et al.* (15) using liposomes as model membranes supported this

view since steroid hormones and cholesterol have stabilizing effects on the lipid membranes (see also Chapter 4). However, recent work on the anti-inflammatory activity of glucocorticosteroids suggests that inhibitory effects on the metabolism of leucocytes may be more important than a direct action on the lysosomal membranes. Thus, by depressing several metabolic pathways the stimuli for lysosome fusion with the cytoplasm membrane would be blocked (132).

5-1-5 Action on cell migration

The anti-inflammatory action of glucocorticoids can also be related to the disappearance of cells that normally participate in the inflammatory process. It has been shown that glucocorticoids decrease the number of monocytes present in the circulation during inflammation (171), although this action is also evident in rheumatoid arthritis where neutrophiles seem to predominate. Glucocorticoids are able to decrease the sticking of leucocytes to the vascular endothelium and to decrease the migration of monocytes across the basement membrane (171). The direct cytotoxic effect of lymphocytes is also suppressed by glucocorticoids although the lymphocytes retain their ability to adhere to the foreign cells (141).

5-1-6 Peptide 401 and stimulation of corticosteroid synthesis

The possibility therefore that peptide 401 can derive part of its anti-inflammatory effect by stimulating production and release of corticosteroids has to be considered. The slight reduction in the anti-inflammatory activity of peptide 401 when injected into adrenalectomized rats, suggests that part of its activity might be mediated by the corticosteroids (~~104~~ 27, ~~130~~ 81). Furthermore, it was shown that when whole

bee venom as well as some impure fractions of the venom that might have contained peptide 401 are injected into rats, the corticosterone levels in the serum rise to values that could definitely produce anti-inflammatory effects (38, 202). It has been suggested that the therapeutic effect of the bee venom in the rat adjuvant arthritis is a consequence of this increased formation of corticosterone (111).

However, the problem of assaying corticosteroids *in vivo* is difficult because of the stress involved in the experimental conditions. Apart from the variations of the serum corticosteroids associated with the normal circadian rhythm of the animals studied, handling and injections of test substances into the animals are *per se* non-specific inducers of corticosteroid formation. For example, injection of isotonic saline into rats has been observed to increase the level of serum corticosterone. With bee venom, this problem is further complicated by the ability of several fractions of the venom to induce histamine release which itself is able to induce corticosteroid formation (38, 151). In order to avoid, therefore, the difficulties in interpreting the results of *in vivo* experiments, the present work was carried out using an *in vitro* system. In the rat corticosterone is the main steroid hormone secreted by the adrenal cortex (178).

5-2 Experimental

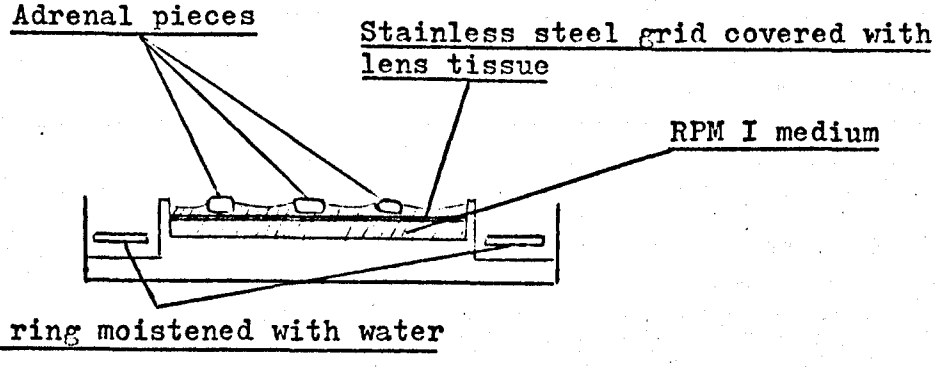
5-2-1 Materials

The incubation medium for the rat adrenal glands, RPMI 1640, foetal calf serum, glutamine, triptose phosphate broth and the plastic tissue culture dishes, were obtained from Gibco Biocult. Dichloromethane (Hopkin and Williams) was washed with 10% sulphuric acid, 4 times with distilled water, dried overnight over $CaCl_2$ and distilled through a

Vigreux column. Absolute ethanol (James Burrough Ltd.) was refluxed for 2 h with 5 g of 1,2,4-dinitrophenylhidrazine and 10 ml of concentrated HCl. It was then distilled twice through a Vigreux column. Sulphuric acid was boiled for 3 h. Anhydrous sodium sulphate was obtained from Fisons Laboratory Reagents. Corticosterone, collagenase (Type I), ACTH (adrenocorticotropic hormone, porcine, grade II, 88 i.u./mg) and deoxyribonuclease Type I were obtained from Sigma Chemical Co. All the surgical tools and glassware used for the collection and incubation of the rat adrenal glands were sterile.

5-2-2 Collection and incubation of adrenal glands

Male rats (wistar) were killed with a blow on the head and the adrenal glands removed as quickly as possible and placed in chilled PBS, pH 7.4 until used. The glands were then washed with RPMI, freed from fat and quartered using scalpels. The pieces were then placed on tissue culture dishes as described in the diagram below.



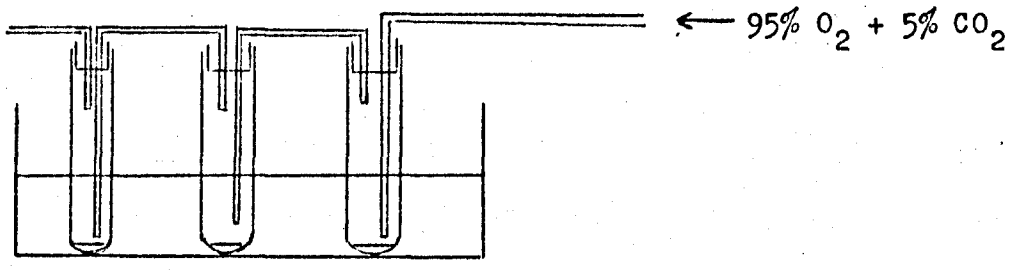
The RPMI medium with the test substances was added to the centre well until it just touched the gland pieces thus forming a film over them. The dishes were placed inside dessicators half filled with sterile distilled water and then a gas mixture consisting of 95% O_2 and 5% CO_2 was bubbled through the water for 20 min. The closed dessicators were then incubated at 37°C for the required amount of time. After incubation,

the medium in the centre well was collected by means of sterile syringes and the corticosterone present was extracted and assayed. The pieces of the adrenal glands of each dish were then immersed in acetone and in absolute ethanol, dried and weighed.

5-2-3 Cell dispersions

The method used to obtain cell dispersions from rat adrenal glands was based on that of Falke et al. (56). After killing the rats as described before, the adrenal glands were removed and placed in cold PBS, pH 7.4 until used. The glands were then trimmed of fat and each one was cut into 6-8 pieces. The pieces from every 10 glands were pooled together in a 25 ml conical flask and to this 5 ml of the RPMI medium containing 5 mg/ml collagenase and 0.5 mg/ml DNAase was added. The reason for adding DNAase was to prevent sticking of cells to DNA released from dead cells. The flasks were then placed in a water bath at 37°C and incubated for 20 min with constant agitation and gassing (95% O_2 + 5% CO_2). After this incubation the supernatant was collected with a Pasteur pipette and placed in a container at 0°C. The incubation with collagenase and DNAase was repeated two more times. After the last incubation, the remaining pieces were sucked and discharged repeatedly with a Pasteur pipette in order to disperse the remaining tissue. The bigger particles were then discarded. The cell suspension was then left to stand for 5 min at 0°C and the heavy sediment discarded too. The cell suspension was then washed three times with enzyme-free RPMI, using a refrigerated centrifuge at 100 g for 10 min each time. The cell pellet was then gently resuspended in a fresh medium (RPMI) and counted in a haemocytometer. The cell count was usually between 200,000-600,000 cells/ml. From this cell suspension, aliquots of 0.5 ml were incubated with

the substances being tested which were dissolved in 0.5 ml of the RPMI medium. Incubation was carried out as described in the diagram below for 2 h at 37°C with constant agitation.

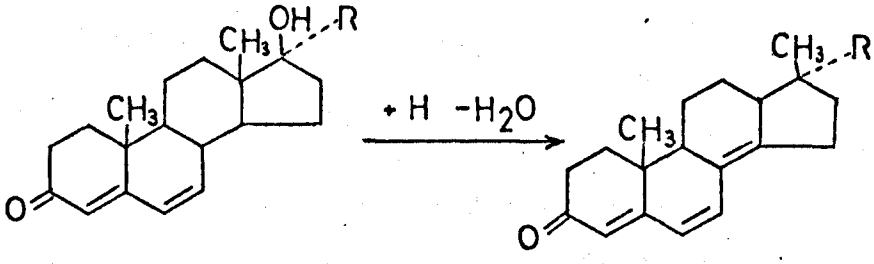


The blanks were obtained by sonicating the cells prior to incubation. After the incubation the cells were centrifuged and aliquots of 0.5 ml from the supernatant were collected and the steroids extracted and assayed. Alternatively, the total contents of the incubation tubes were extracted.

5-2-4 Extraction and assay of corticosterone

This was done according to the method of De Langen et al.(44). The aliquots obtained from both the tissue culture and cell dispersion experiments were extracted directly with 10 ml of dichloromethane. After decanting the aqueous phase, the dichloromethane was poured into tubes containing 1 g of anhydrous Na_2SO_4, stirred and left standing at 4°C overnight. Standard solutions of known amounts of corticosterone were treated in the same way. From these solutions, aliquots were collected and added to the fluorescent reagent which consisted of 25 ml of concentrated H_2SO_4 + 10 ml of 75% ethanol. The ratio between the aliquots of the extract and the fluorescent reagent was 1.5. The mechanism by which corticosteroids fluoresce in the presence of concentrated H_2SO_4 is poorly understood. When steroidal 17 , alkyl,-

17 -hydroxy-4,6,-dien-3-ones, are treated with concentrated sulphuric acid, the following reaction occurs that probably applies for corticosterone as well(146).



Ethanol is added to solubilize the steroids.

The tubes containing aliquots of the dichloromethane extracts and the fluorescent reagent were then stirred and left to stand at room temperature for 30 min. Prior to measuring the fluorescence intensity, the tubes were stirred again. A fresh fluorescent reagent was prepared for every experiment. The fluorescence intensity of the aliquots was measured in a Perkin-Elmer fluorescence spectrophotometer model MPF-3, using an excitation wavelength of 466 nm and emission wavelength of 520 nm. These two values for wavelength were obtained after scanning a sample of corticosterone prepared as described above.

5-3 Results

5-3-1 Quartered glands

Table 5-I and Fig. 5-1 show the results of incubating the quartered adrenal glands in the presence and absence of ACTH (0.1 mu/ml)

at 37°C and at 4°C, for several intervals of time. The results are expressed in ng of corticosterone produced per 100 mg of tissue. For every experiment a standard curve plotting corticosterone concentration against fluorescence intensity was made. From these curves, the values of each point were calculated.

Table 5-I

Effect of ACTH on corticosterone production by quartered adrenal glands at 37°C and 4°C

		ng corticosterone in 100 mg 37°C	ng corticosterone in 100 mg 4°C
Control	1 h	62.4	20.1
"	5 h	128.8	25.1
"	20 h	341	27.6
ACTH	1 h	69.2	28.3
"	5 h	140.1	35.8
"	20 h	450	29.9

At every time interval of the results above, the medium bathing the adrenal pieces was collected and replaced by a fresh one with the same components. The gassing was also repeated.

The results of experiments where the quartered adrenal glands were incubated for 5 h at 37°C with several concentrations of ACTH are presented in Table 5-II and Fig. 5-2.

FIG 5-1 - Effect of ACTH on corticosterone synthesis and release by quartered adrenal glands at 4°C and at 37°C (data from Table 5-I)

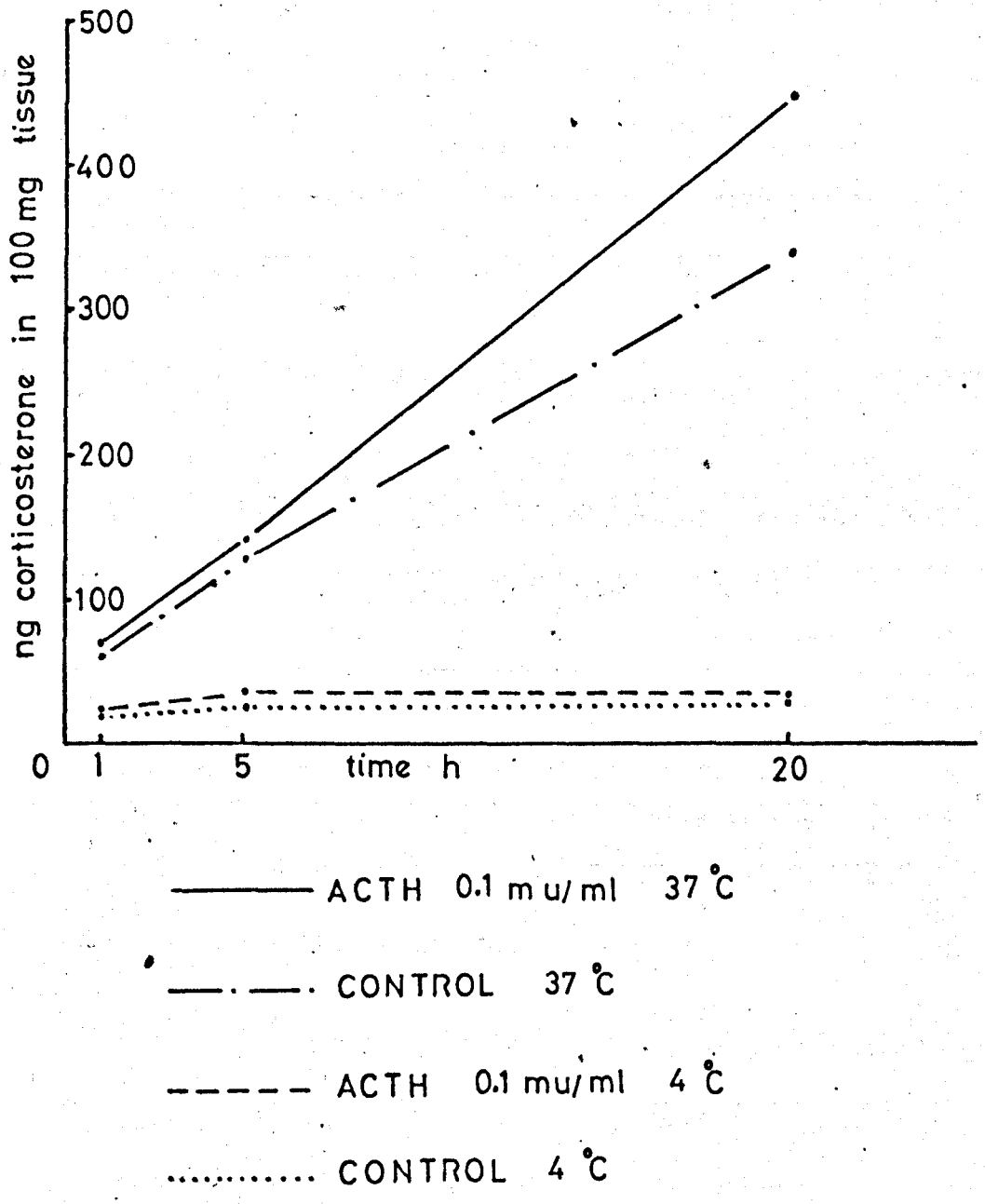


Table 5-II

Effect of several concentrations of ACTH on corticosterone production by quartered adrenal glands

	µg corticosterone in 100 mg of tissue
Control	0.293
ACTH (0.156 mu/ml)	0.782
" (0.312 mu/ml)	1.536
" (0.625 mu/ml)	1.567
" (1.25 mu/ml)	1.866

each value represents the average of two determinations

Table 5-III and Fig. 5-3 show the results of experiments where quartered adrenals were incubated for 5 h at 37°C with ACTH and peptide 401. Peptide 401 was incubated in concentrations of 1 and 10 µg/ml.

Table 5-III

Effect of peptide 401 and ACTH on corticosterone production by quartered adrenal glands

	µg corticosterone in 100 mg of tissue
Control	5.52
ACTH (.312 mu/ml)	15.75
" (2.5 mu/ml)	12.38
401 (1 µg/ml) 0.38 µM	24.39
" (10 µg/ml) 38 µM	16.7

each value is the average of two determinations

FIG 5-2 - Effect of ACTH on corticosterone synthesis and release by quartered adrenal glands. (data from Table 5-II)

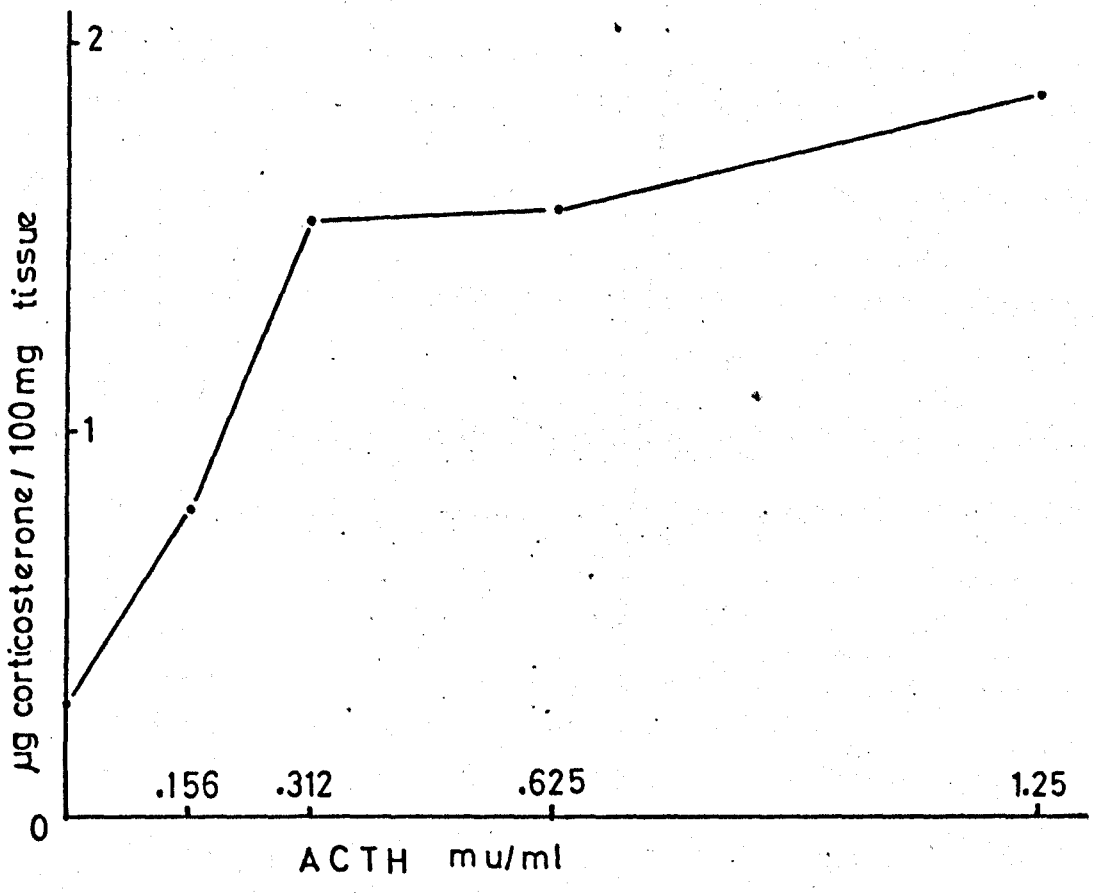
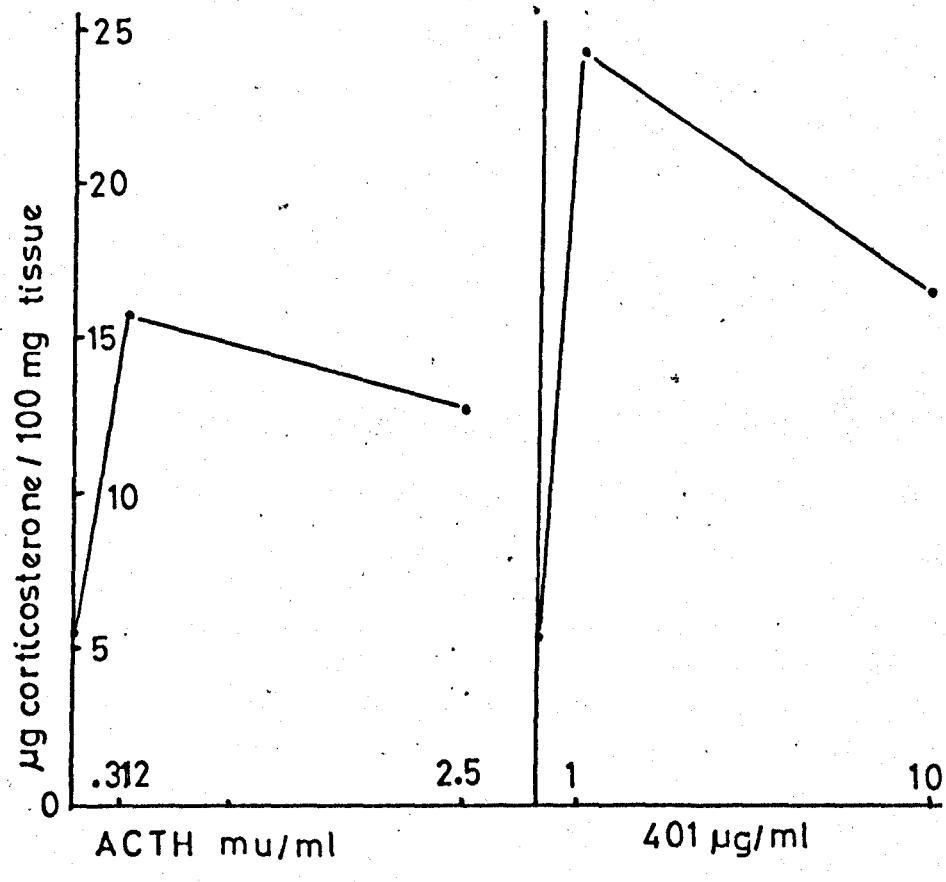


FIG 5-3 - Effect of ACTH and peptide 401 on corticosterone synthesis and release by quartered adrenal glands (data from Table 5-III)



Although the above results indicate that peptide 401 stimulates the synthesis of corticosterone, the previous control experiments (Table 5-II, Fig. 5-2) could not be reproduced. When these experimental conditions were repeated, the glands frequently did not respond to ACTH and when they did, the dose response curve was never coherent. The amount of corticosterone present in the medium bathing the glands varied too much and the irreproducibility was such that no conclusions could be drawn. This behaviour is caused by the difficulty of oxygen and the nutrients present in the medium to reach the inner regions of the glands. The adrenal cortex is normally highly vascularized and in the present culture conditions, only the outer surfaces are bathed by the medium. Likewise, the formed corticosteroids are not able to diffuse freely out of the tissue and may accumulate inside thus influencing the metabolical state of the adrenal cells (56). For this reason, the method of quartered adrenal glands was replaced by cell dispersions of adrenal glands.

5-3-2 Cell dispersions

The standard curve obtained by plotting the corticosterone concentration against the fluorescence intensity, after treatment with the fluorescent reagent is presented in Fig. 5-4. The curve in Fig. 5-4 was obtained by adding known amounts of corticosterone to a cell dispersion of rat adrenal glands. The equation derived from this curve is

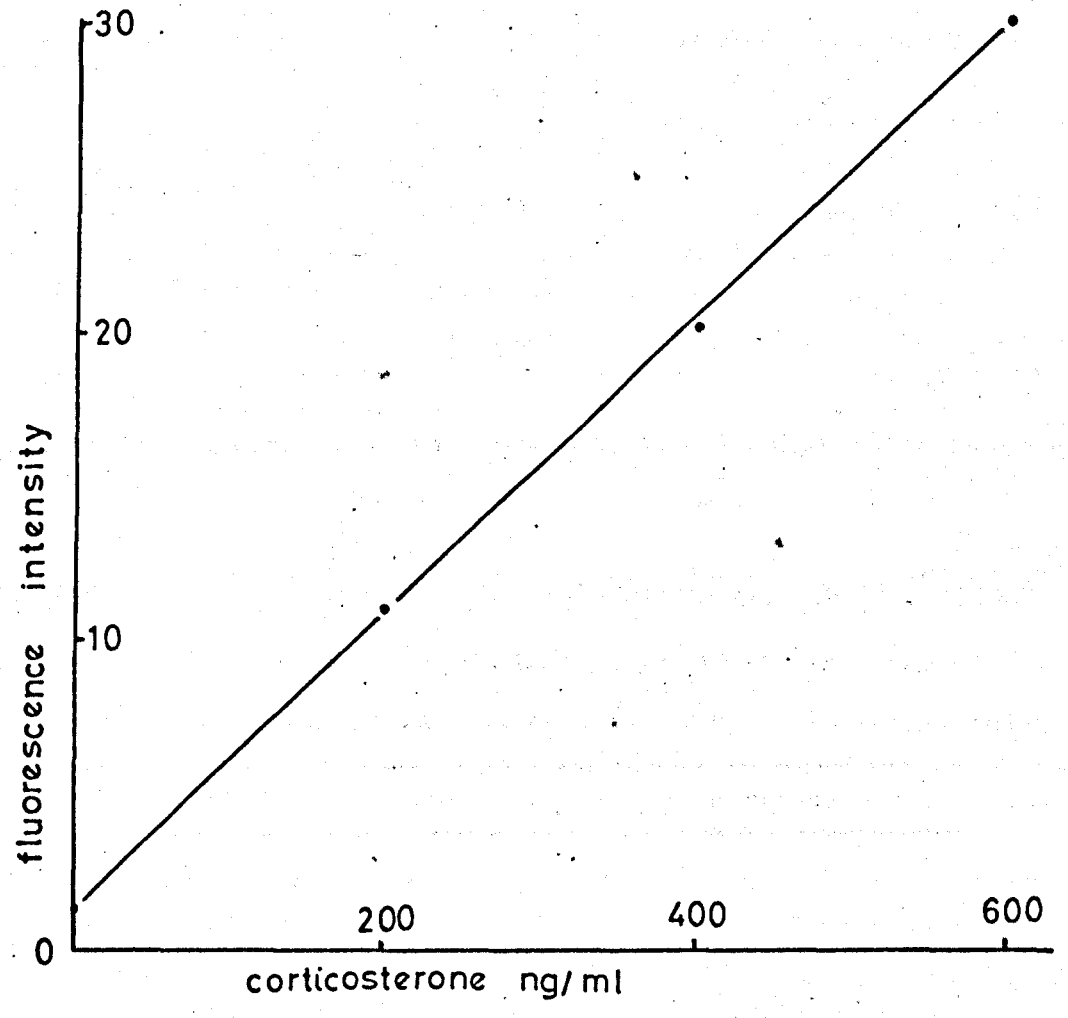
$$y = -25.75 + 20.71\,x$$

where y = ng corticosterone

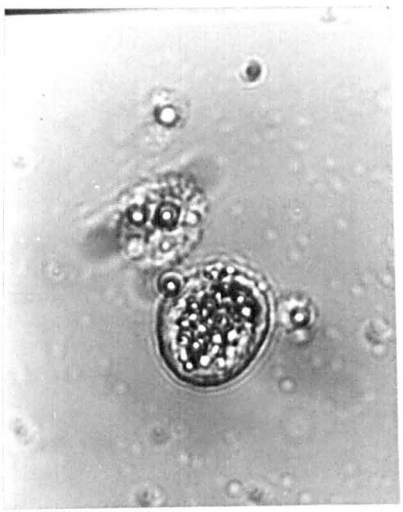
x = fluorescence intensity.

This equation was applied to calculate the concentration of corticosterone in all samples from the cell dispersion experiments.

FIG 5-4 - Standard concentration - fluorescence intensity curve for corticosterone treated with concentrated H_2SO_4



Figs. 5-5 and 5-6 show typical preparations of cells from adrenals obtained in the present work.



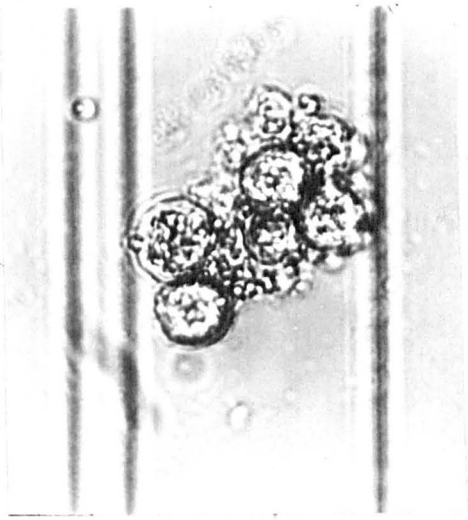


Table 5-IV shows the amount of corticosterone formed as a function of number of cells obtained from rat adrenals, incubated in the absence of ACTH.

Table 5-IV

Corticosterone production by adrenal cell dispersions in the absence of ACTH

No. cells/ml	ng corticosterone
172 500 (2)	0
195 000 (2)	1.79
416 000 (2)	12.56
660 000 (3)	14.84

The number between parenthesis indicates the number of experiments

It can be seen that although the unstimulated cells produce very little

corticosterone (which is in agreement with the results of Falke et al. (56), there is a direct correlation between the number of cells present in the incubation medium and the amount of corticosterone produced. Table 5-V shows the results of incubating the adrenal cell dispersions with several concentrations of ACTH and peptide 401. The results are expressed as ng corticosterone/ml/h.

Table 5-V

Effect of peptide 401 and ACTH on corticosterone production by adrenal cells

	Corticosterone (ng/ml/h)
Blank	0
Control	1.79
ACTH (.01 mu/ml)	0
" (.1 mu/ml)	8.42
" (1 mu/ml)	150.6
401 (.005 µg/ml) 0.0019 µM	0
" (.05 µg/ml) 0.019 µM	0
" (.5 µg/ml) 0.19 µM	3.86

each value is the average of two determinations

It can be seen that only concentrations of peptide 401 of .5 µg/ml elicit some corticosterone production; however, this amount is not much higher than the amount produced by the control cells. In another set of experiments peptide 401 was incubated with the adrenal cell dispersions in higher concentrations. The results are presented in Table 5-VI and Fig. 5-7.

Table 5-VI

Effect of peptide 401 and ACTH on corticosterone production by adrenal cell dispersions

		ng corticosterone/ml/h
Control		6.28
ACTH	.01 mu/ml	7.31
"	.1 mu/ml	20.77
"	1 mu/ml	530.5
401	.1 µg/ml 0.038 µM	2.65
401	1 µg/ml 0.38 µM	4.21
401	10 µg/ml 3.8 µM	2.65

It can be seen that although the cells were responding to ACTH, peptide 401 failed to significantly induce an increased formation of corticosterone. The results of similar experiments, but with higher concentrations of peptide 401 are presented in Table 5-VII.

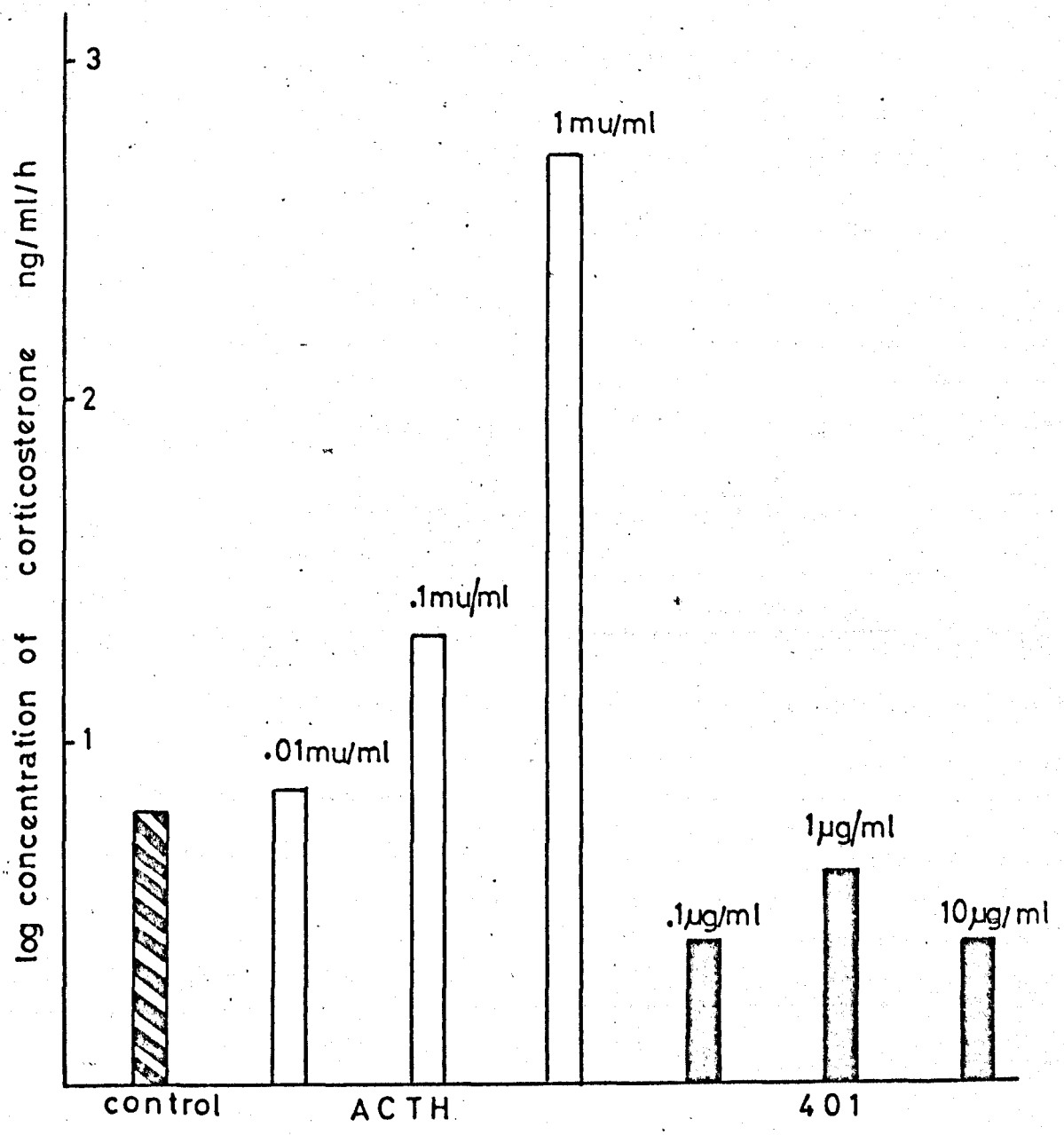
Table 5-VII

Effect of peptide 401 and ACTH on corticosterone production by adrenal cell dispersions

		ng corticosterone/ml/h
Control		7.48 ± 0.6
ACTH	1 mu/ml	361.25 ± 144
401	1 µg/ml 0.38 µM	6.44 ± 1.58
401	10 µg/ml 3.8 µM	5.75 ± 1.78
401	100 µg/ml 38 µM	5.41 ± 2.6

- each value is the average of three determinations

FIG 5-7 - Effect of ACTH and peptide 401 on corticosterone synthesis and release by adrenal cells suspensions. (data from Table 5-VI)



The above results show that adrenal cells incubated with peptide 401 up to concentrations of 100 µg/ml, fail to have any influence on the corticosterone production.

As there are a number of reports where injections of whole bee venom into rats induced an increased formation of corticosterone (38, 202), another set of experiments was carried out in which the whole venom, the fraction containing the low molecular weight components of the venom and the fraction containing the high molecular weight components were incubated with the adrenal cell dispersions. The results are presented in Table 5-VIII.

Table 5-VIII

Effect of several bee venom fractions and ACTH on corticosterone production by adrenal cells

	ng corticosterone/ml/h
Control	0
ACTH 1 mu/ml	295.1
Whole bee venom (0.1 mg/ml)	0
Low mol. weight (0.1 mg/ml)	0
High mol. weight (")	0

each value is the average of two determinations

5-4 Discussion

The present experiments show that peptide 401 in a concentration range of .005 - 100 µg/ml does not induce increased corticosterone formation when incubated with cell dispersions obtained from rat adrenal glands. When peptide 401 was incubated with quartered adrenal glands a single experiment showed that peptide 401 in a concentration of 1 µg/ml was able to induce corticosterone formation with values of approximately 5 times over the controls. This might mean that some integrity in the gland is required for peptide 401 activity. However, it is not possible to draw any conclusions from the experiments using the quartered glands since the results of several other experiments were not reproducible, with much scattering among them, and it was not possible also to establish a dose response curve for ACTH. The reasons for such erratic behaviour were already commented on in section 5-3.

Although the cell dispersions lack the tissue organization of the quartered glands, corticosteroidogenesis could be induced by ACTH in concentrations which are normally found in the rat blood after inflammatory stimuli (153), showing that the precursors for the synthesis of corticosteroids were available and that all the systems involved in the biosynthesis of the corticosteroids were intact. Thus it can be concluded that peptide 401 does not have a direct action on the adrenal cells as happens with ACTH. The other fractions present in the bee venom also failed to have any influence on corticosterone production in the single dose tested of 0.1 mg/ml. However, this does not rule out the possibility that peptide 401, like the other bee venom fractions tested *in vivo*, can stimulate corticosteroid formation indirectly. For instance, it has been shown that histamine is able to stimulate production of corticosteroids (38, 151). Since peptide 401, like other peptides

present in the bee venom, is able to induce histamine release, this could constitute a way for indirect action on the adrenals. If such was the case, however, the anti-inflammatory activity of peptide 401 would only be transient and occurring only at concentrations of peptide 401 which are able to produce systemic inflammatory effects in the rat. The optimum dose of peptide 401 for anti-inflammatory activity would be expected to induce histamine release only at the site of inoculation. Furthermore, pre-treatment of the rats with antagonists of histamine and serotonin does not suppress the anti-inflammatory activity of peptide 401 (81, 27). Presumably, the slight reduction in the anti-inflammatory activity of peptide 401 in adrenalectomized rats is reflecting precisely this histamine mediated effect.

Alternatively peptide 401 can have an in vivo stimulatory effect on the adenohypophysis with a resulting increased formation of ACTH. This seems to be occurring with the whole bee venom. The experiments of Alfano et al. (2) show that the bee venom is not able to induce corticosterone formation in hypophysectomized rats. It should be worthwhile to test the ability of peptide 401 to influence the production of ACTH using a similar experimental approach as in this chapter.

In conclusion, the fact that the anti-inflammatory activity of peptide 401 persists in adrenalectomized rats and that peptide 401 fails to induce corticosterone production by rat adrenal cell dispersions makes it improbable that the anti-inflammatory activity of peptide 401 is mediated by corticosteroids.

CHAPTER 6

PEPTIDE 401 AND MATURATION OF LYMPHOCYTES

6-1 Introduction

6-1-1 Lymphocytes in rheumatoid arthritis

Peptide 401 was shown to have anti-inflammatory activity in induced adjuvant arthritis in the rat (27). One of the characteristics of this animal model is that the chronic inflammatory arthritis can be passively transferred by viable lymphoid cells, but not by serum (131, 180). This suggests that a cell mediated immune process is involved. In man, rheumatoid arthritis was first thought to be due almost entirely to a humoral component, i.e., to the presence of immune complexes that are known to activate the complement system and produce chemotactic substances for polymorphonucleocytes (204), and probably other inflammatory cells. However, the occurrence of rheumatoid arthritis in agammaglobulinaemic patients (143) and the presence in the lesions of large numbers of lymphocytes other than plasma cells, suggested that these cells might play a role in the maintenance of this chronic inflammatory state (123).

The actions of the lymphocytes might be mediated by a direct cytotoxic effect towards the synovial cells (133). In addition, those actions might be mediated by lymphokines, which are substances synthesized by sensitized lymphocytes and capable of various activities that would help to perpetuate the inflammatory state. These activities include vasodilatation, accumulation of macrophages and proliferation and differentiation of lymphocytes (53). Supporting this notion is the

finding that lymphokine-like materials have been found in the synovial fluid of patients with rheumatoid arthritis and in some patients with other forms of chronic inflammatory arthritis (164). To stress the role played by lymphocytes, it was shown that in lymphocyte depletion experiments in man (130) and in rats (43) an amelioration of the arthritic condition could be observed.

6-1-2 Lymphocyte activation

Secretion of biologically active materials as well as transformation into an effector cell depends on lymphocyte activation. Lymphocyte activation refers to a series of morphological and biochemical changes that accompany the transformation of a small lymphocyte into a blast cell after contact with antigen. This may or may not lead to cell division, but the increased incorporation of ^{3}H-thymidine into the cell DNA has been used as one of the parameters indicating lymphocyte activation. Some of the possible actions of anti-inflammatory drugs in chronic arthritic conditions might arise from inhibition of lymphocyte migration to the site of inflammation and/or interference with lymphocyte activation resulting in a decreased production of lymphokines and cytotoxic lymphocytes. In fact, experiments with non-steroidal anti-inflammatory agents have shown that this may be the case. Aspirin and indomethacin are able to inhibit *in vitro* the amoeboid movements of lymphocytes induced by ATP (76). These movements are believed to be associated with the capacity to migrate across the endothelium of the blood vessels during inflammation. The same drugs were also shown to inhibit the direct cytotoxic effects of lymphocytes in an experimental tumour model (197). Furthermore, salicylate is capable of inhibiting *in vitro* protein synthesis and ^{3}H-thymidine incorporation by lymphocytes,

reflecting an impairment of lymphocyte activation (126, 67). Suppression of lymphocyte transformation was also observed *in vitro* after aspirin administration (40).

6-1-3 Peptide 401 and lymphocyte activation

In Chapter 4 it was shown that d-401 was able to bind to rat lymphocytes and therefore the possibility exists that peptide 401 can exert its anti-inflammatory effects in the rat adjuvant arthritis through an action on lymphocytes. This was investigated in the present experiments by measuring the influence of peptide 401 on the incorporation of ^{3}H-thymidine by lymphocytes in the presence and absence of phytohaemaglutinin (PHA). PHA is a lectin isolated from the bean *Phaseolus vulgaris* which is capable of inducing lymphocyte activation in a way which closely resembles the reaction between antigen and sensitized lymphocytes (79), a situation which supposedly occurs in the rat adjuvant arthritis.

6-2 Experimental

6-2-1 Materials

The animals used were wistar rats (3 months old) obtained from the Wright Fleming Institute, St. Mary's Hospital Medical School. The tissue culture medium, RPMI 1640 was obtained from Flow Laboratories. This medium was supplemented with HEPES buffer, 10% foetal calf serum and the antibiotics penicillin (100 µg/ml) and streptomycin (100 µg/ml), also from Flow Laboratories. The culture microtest plates, with flat bottom were obtained from NUNC, cat. no. 1480. PHA (phytohaemagglutinin), reagent grade, was obtained from Wellcome Research Laboratories, and was dissolved in 5 ml of distilled water to give a stock solution of 10 mg/ml.

This was stored at 4°C and diluted with RPMI to the required concentration when used.

Prostaglandin E_1 was a gift from Dr. J.E. Pike (The Upjohn Laboratories). PGE_1 was dissolved in a few drops of ethanol and this was diluted to the required concentration with the RPMI medium. This solution was filtered through a Milipore membrane (0.45 μ pore size) in order to sterilize it.

Peptide 401 was diluted in the RPMI medium and also filtered as described above prior to use. Methyl ^{3}H-thymidine (5 ci/mM) was obtained from the Radiochemical Centre, Amersham. Methyl ^{3}H-thymidine was diluted with RPMI to give a final concentration of 1 μci per culture. The scintillation fluid consisted of a solution of toluene containing 6 g / 100 ml PPO and 12 mg / 100 ml of POPOP. The filter paper, grade 3 MM, size 1.9 cm was obtained from Whatman.

6-2-2 Lymphocyte culture technique

The animals were killed by cervical dislocation and the mesenteric lymph nodes were used as the source of lymphocytes. The cell suspension was obtained by teasing the lymph node with syringe needles and pressing the cells through a metal sieve with the aid of a plunger obtained from sterile disposable syringes. The tissue remaining on the metal sieve was repeatedly washed with the RPMI medium during the teasing procedure. The cell clumps were allowed to sediment and the supernatant transferred to a sterile disposable universal with a conical bottom. This was then centrifuged for 5 min at 300 g in a bench type centrifuge, at room temperature. The cells were washed once, resuspended, and counted in a haemocytometer. Cell viability was assessed using phase contrast microscopy and the final cell suspension was adjusted to a

density of 5×10^6 cells/ml.

Replicate cultures were set up in the microplates with 200 µl of the cell suspension for each well. The microplates were placed inside a dessicator and gassed with 5% CO_2 in air. The dessicator was kept in an incubator at 37°C for 48 h. The drugs being tested were added at the beginning of the culture period and left during the whole incubation time.

6-2-3 Assessment of the mitogenic response

This was done through cellular incorporation of ^{3}H-thymidine into intracellular DNA precipitated by trichloroacetic acid (TCA). ^{3}H-thymidine was added to each well 24 h after the cells were put in culture conditions. After another 24 h the microplates were cooled in ice and 100 µl aliquots were collected and left to dry in filter paper discs mounted in pins. The filters were subsequently transferred to a beaker, washed three times with a cold 10% solution of TCA (w/v) and twice in cold methanol. The discs were then transferred to scintillation vials, allowed to dry and then counted in a Packard liquid scintillation spectrometer, with 5 ml of the scintillation fluid.

6-2-4 Viability test

In order to test if peptide 401 itself had any cytotoxic effects on the rat lymphocytes and on rat leucocytes obtained from a peritoneum exudate, a fluorochromatic test, based on that of Rotman et al. (142), was carried out. The method consisted of incubating the cell suspension (in the RPMI medium) with peptide 401 in several concentrations and time intervals, at 37°C and then adding to the suspension fluorescein diacetate (FDA). Esterases present on the cell membrane react with FDA

and form free fluorescein, which does not diffuse out of the cells as quickly as FDA penetrates into the cytoplasm (142). Therefore it is possible to measure the rate of increase in fluorescence intensity as a consequence of intracellular accumulation of fluorescein. The mechanism whereby fluorescein accumulates intracellularly, though not known, is dependent upon cell integrity.

In the present work, the rate of increase in fluorescence intensity was measured using a Perkin-Elmer MPF-3 fluorescence spectrophotometer equipped with a thermostated cell holder, using an excitation wavelength of 490 nm and emission of 516 nm. The cell suspensions were incubated at 37°C in the spectrofluorimeter with the compounds being tested, and after the required time FDA in a final concentration of 5 µg/ml was added to the medium and the rate of increase in fluorescence was recorded. FDA was dissolved with a few drops of acetone and then diluted to the required concentration with the RPMI medium.

6-3 Results

6-3-1 Lymphocyte activation and peptide 401

The results of the experiments measuring the effect of peptide 401 on the ^{3}H-thymidine incorporation by lymphocytes in the presence and absence of PHA are presented in Table 6-I.

Table 6-I

Effect of peptide 401 on lymphocyte activation in the absence and presence of PHA

	NO PHA		PHA (10 µg/ml)	
No. of experiments		CPM	No. of experiments	CPM
	EXP 1			
(3)	Control	1632±234	(4)	6461±851
(4)	401 (100µg/ml)	1281±190	(4)	5003±539
(4)	401 (10µg/ml)	2012±211	(4)	5688±561
(4)	401 (1µg/ml)	2181±399	(4)	5576±427
(4)	PGE_1 (10^{-4}M)	516±40	(4)	1154±63
	PGE_1 (10^{-4}M) + 401 (100 µg/ml)		(4)	1266±263
	EXP 2			
(4)	Control	848±71	(4)	3677±324
(4)	401 (100µg/ml)	640±49	(4)	2855±414
(4)	401 (1-µg/ml)	1148±111	(4)	3759±171
(4)	401 (1µg/ml)	996±78	(4)	3353±241
(4)	401 (0.5µg/ml)	1052± 275	(4)	3391±550
(4)	PGE_1 (10^{-4}M)	417±75	(4)	296±27
(4)	PGE_1(10^{-4}M)+401(100µg/ml)	346±10	(4)	336±9
(4)	PGE_1(10^{-4}M)+401(10µg/ml)	378±33	(4)	428±20
(4)	PGE_1(10^{-4}M)+401(1µg/ml)	367±133	(4)	401±8
(4)	PGE_1(10^{-4}M)+401(0.5µg/ml)	397±59	(4)	345±43

In order to establish if the effect of peptide 401 on the ^{3}H-thymidine incorporation by the lymphocytes was significant, the individual results of the experiments where peptide 401 was incubated with the cells in concentrations of 100 and 10 µg/ml were pooled and used in the students' t test. The values for P were the following:

a) differences between the results of the control group (n=7) and peptide 401 (100 µg/ml) (n=8), in the absence of PHA

$t = 2.88$, corresponding to

$.02 < P < .01$

b) differences between the values of control group (n=7) and peptide 401 (10 µg/ml) (n=8), in the absence of PHA

$t = 1.69$, corresponding to

$.2 < P < .1$

c) differences between the control group (n=8) and peptide 401 (100 µg/ml) (n=8) group, in the presence of PHA (10 µg/ml)

$t = 1.59$, corresponding to

$.2 < P < .1$

d) differences between the control group (n=8) and peptide 401 (10 µg/ml) (n=8) group in the presence of PHA (10 µg/ml)

$t = .501$, corresponding to

$.7 < P < .6$

The significance of the results of experiments where peptide 401 was incubated in concentrations smaller than 10 µg/ml was not calculated since the values of these groups were very close to the respective controls.

6-3-2 Viability tests

The results of the cytotoxicity of peptide 401 on the rat mesenteric lymphocytes are presented in Table 6-II.

Table 6-II

Effect of peptide 401 and melittin on cell viability

	4 h F.I. (2 min after adding FDA)	22 h F.I.
Control	51.5	58.5
401 (1.25 mg/ml)	61.5	36.5
401 (0.625 mg/ml)	40.5	40.5
401 (0.125 mg/ml)	55.5	33.5
401 (0.0625 mg/ml)	44.5	34
Melittin (0.5 mg/ml)	31	32
Control cells kept in ice	96	78

The results in Table 6-II show that peptide 401 in concentration of 0.125 mg/ml has some cytotoxic effects on the lymphocytes after incubation for 22 h whereas no effects are apparent after 4 h. The same test was carried out with cells obtained from the rat peritoneum exudate. The cells were pre-incubated for 1 h at 37°C with the compounds being tested and then FDA was added to the medium. The results are presented in Table 6-III.

Table 6-III

Effect of peptide 401, melittin and indomethacin on cell viability after 1 h incubation

	F.I. (3 min after adding FDA)
Control	36.7
Melittin (0.5 mg/ml)	9.5
Indomethacin (0.033 mg/ml)	34.5
401 (1.25 mg/ml)	47.5
401 (0.625 mg/ml)	33.5
401 (0.125 mg/ml)	33.5
401 (0.0625 mg/ml)	31.5
Blank (85°C for 2 min)	1.5

The results in Table 6-III show that peptide 401 has no cytotoxic effects on the cell population obtained from the rat peritoneum exudate after 1 h incubation. Melittin however seems to be much more toxic to the peritoneum cells than to the mesenteric lymphocytes.

6-4 Discussion

In the present work, experiments were carried out in order to investigate if the anti-inflammatory activity of peptide 401 in the rat adjuvant polyarthritis could be explained by a direct action on the lymphocytes, as reflected by suppression of lymphocyte activation. Peptide 401 tested in a concentration range of 0.5 - 100 µg/ml did not show any significant effects on lymphocyte activation, as shown by

^{3}H-thymidine incorporation. The observed suppression of ^{3}H-thymidine incorporation by lymphocytes incubated with peptide 401 in concentrations of 0.1 mg/ml, in the absence of PHA, $.02 < P < .01$, indicated the possibility of some cytotoxic effects, since this suppression was not as significant when the cells were stimulated by PHA. Such a conclusion is supported by the results of the cytotoxic tests carried out with the rat lymphocytes, which showed that concentrations of peptide 401 of approximately the same order as the ones that produced a reduction in ^{3}H-thymidine incorporation, were able to cause a 43% decrease in the rate of fluorescein formation by the membrane esterases of the lymphocytes. The cytotoxic effects of peptide 401 on the lymphocytes could only be observed after incubation with the cells for 22 h and as in the experiments involving ^{3}H-thymidine incorporation, peptide 401 stays in contact with the cells for at least 48 h, the reduced incorporation of ^{3}H-thymidine by the lymphocytes in the absence of PHA is presumably due to effects of peptide 401 on the membranes. Why the group of cells incubated with peptide 401 in the presence of PHA is less affected, remains unclear. It is possible that the cell agglutination caused by PHA allowed a smaller area of the membrane to be exposed to peptide 401. Experiments performed by G. Atkinson (13) using rat polymorphonucleocytes showed that peptide 401 in concentrations of 0.1 mg/ml had some cytotoxic effects (measured by LDH release). However, in the present experiments, no cytotoxic effects due to peptide 401 could be observed after 1 h incubation with a population of leucocytes obtained from the rat peritoneum.

It should be pointed out that peptide 401, in the concentration range tested, did not enhance the blastogenesis induced by PHA. This differs from the effects of other basic polypeptides which were shown to

potentiate the action of phytomitogens (124). The possibility that peptide 401 could be interfering with the inhibitory action of PGE_1, by forming complexes due to its basicity, was ruled out since the present experiments show that there is no difference between the groups incubated with PGE_1 alone and those in which PGE_1 was incubated together with peptide 401.

Considering that peptide 401 had also no effects in vitro on human lymphocyte activation by PHA, and in antibody production by mouse lymphocytes in a Jerne plaque assay (169), the possibility that the anti-inflammatory activity of peptide 401 in the rat adjuvant polyarthritis is mediated through effects on lymphocytes seems remote. However, it should be kept in mind that peptide 401 may have a different effect in vivo. Furthermore, the possibility still remains that peptide 401, like other anti-inflammatory agents (76), may act on the lymphocytes inhibiting their migration in vivo, since it was demonstrated that d-401 was able to bind to lymphocytes and that peptide 401 caused some cytotoxic effects on lymphocytes.

Summary and Conclusions

1 - Experiments in vitro showed that peptide 401 was able to inhibit the synthesis of PGE_2, using an enzymatic complex obtained from sheep seminal vesicles. As prostaglandins are important mediators of inflammation in the model used to assay the anti-inflammatory activity of peptide 401 it was concluded that inhibition of prostaglandin synthesis could explain the therapeutic action of peptide 401 in the rat carrageenan oedema.

2 - Dansylated peptide 401 was shown to bind preferentially to rat leucocytes, when incubated with a population of rat blood cells. This fact together with the notion that rat leucocytes are regarded as major sources of prostaglandins during the carrageenan oedema, and that the maximum anti-inflammatory activity of peptide 401 coincides with leucocyte migration to the site of inflammation, support the idea that peptide 401 derives its anti-inflammatory activity through inhibition of prostaglandin synthesis by the rat leucocytes.

3 - The same pharmacological effects of peptide 401 could be involved in the rat adjuvant arthritis where peptide 401 also shows anti-inflammatory activity. In this experimental model, the lymphocytes are suspected to play an important role. As it was shown that d-401 was also able to bind to lymphocytes (not so strongly as to mast cells), the possible influence of peptide 401 on the activation of lymphocytes was studied. However, the results of these experiments indicated that peptide 401 had no effects on lymphocytes, apart from slight cytotoxicity. Therefore, it is unlikely that the anti-

inflammatory activity of peptide 401, either in the acute model of the carrageenan oedema or in the adjuvant arthritis, is mediated by any effects on lymphocytes.

4 - The experiments with the liposomes were carried out in order to determine if the lipids of the membranes were important for the binding of peptide 401 to the leucocytes. It was found that the ratio between cholesterol and the phospholipids was important for the observed effects of peptide 401 in increasing the permeability rates to $^{22}Na^{+}$. The increased $^{22}Na^{+}$ permeability induced by peptide 401, which did not seem to be a consequence of lytic effects could have implications in the ability of peptide 401 to degranulate mast cells, since this could involve an exchange of cations.

5 - The possibility that the anti-inflammatory activity of peptide 401 could be mediated by a direct stimulation of corticosterone production by the rat adrenal glands was ruled out since no such effects could be detected. However, the possibility exists that peptide 401 may act on the rat adenohypophysis, stimulating the release of ACTH.

The present work consisted of a series of <u>in vitro</u> tests. These were attempts to isolate some events interconnected in the process of inflammation. However, because these isolated systems lack the degree of organization and hence the controlling factors present in the general process, it must be kept in mind that any conclusions derived from such experiments are necessarily oversimplifications.

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