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THE EFFECTS OF CYCLIC NUCLEOTIDES AND AGENTS WHICH AFFECT THEIR INTRACELLULAR ACCUMULATION ON NEUTROPHIL MOTILITY

by



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A Thesis Submitted to the Faculty of Medicine,
University of the Witwatersrand, Johannesburg,
for the
Degree of Doctor of Philosophy.

JOHANNESBURG, 1976.

This is to certify that the Thesis "The Effects of Cyclic Nucleotides and Agents which Affect their Intracellular Accumulation on Neutrophil Motility" presented for the degree of Doctor of Philosophy (Medicine) at the University of the Witwatersrand, Johannesburg, is my own work and has not been presented at any other university.

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R O N A L D A N D E R S O N

M.Sc. (Rand)

Parts of this thesis have been published in the following papers:

1. *In vitro* stimulation of neutrophil motility by levamisole. Maintenance of cGMP levels in chemotactically stimulated levamisole treated neutrophils.
   R. Anderson, A. Glover, H.J. Koornhof and A.R. Rabson. Journal of Immunology, 117, 428, 1976.
2. The *in vitro* effects of histamine and metiamide

on neutrophil motility and their relationship to intracellular cyclic nucleotide levels.
R. Anderson, A. Glover and A.R. Rabson.
(Submitted).

3. The effect of chemotactic factors and agents which influence neutrophil movement on anaerobic glycolysis and hexose monophosphate shunt activity.
R. Anderson, A. Glover and A.R. Rabson.
(Submitted).

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4. The Research on which this thesis is based was commenced in July, 1974 and completed in October, 1976.

ACKNOWLEDGEMENTS

I thank Professor J. Metz, Director, South African Institute for Medical Research, Johannesburg, for provision of the facilities for the investigations included in this thesis. I thank Professor A.R. Rabson and Dr. R. Sher of the Department of Immunology and Professor H.J. Koornhof of the Department of Microbiology, South African Institute for Medical Research and School of Pathology, University of the Witwatersrand, Johannesburg, for their interest, encouragement and advice. My thanks also to Mrs. Annemarie Glover without whose assistance many of the experiments presented in this thesis could not have been undertaken. I am greatly indebted to Miss Elizabeth King for typing the manuscript and to Mr. and Mrs. Max Ulrich of the South African Institute for Medical Research, Photography Unit, for all the photographs in this thesis.

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SUMMARY

The cell type exclusively dealt with in this thesis is the human blood neutrophil, which is also referred to in the text as polymorphonuclear leukocyte (PMN).

The experimental work in this thesis has been accomplished using one immunological and a number of biochemical investigative techniques. The former is the Boyden technique (Boyden, 1962) for the quantitative assessment of leucocyte motility. This technique has been applied according to the method of Boyden in conjunction with the modified intra-filter procedure described by Zigmond and Hirsch (1973). Biochemical techniques have been used to evaluate neutrophil intracellular cyclic nucleotide levels, glycolysis and hexose monophosphate shunt activity (HMP or HMS).

This thesis is comprised of two main parts. The first part is a literature review consisting of three chapters. These are a brief historical review, a discussion of previously described experimental work relating to biochemical events associated with cell motility and finally, current investigative filter methodology in leucotaxis. The second part is experimental and consists of six chapters. The first chapter of part two describes the techniques and methods employed in the subsequent experimental work. The following

three chapters describe the effects of cyclic nucleotides and various agents which promote alterations in their intracellular levels on neutrophil motility. Particular attention has been devoted to histamine and levamisole since the former has been reported to mediate substantial inhibition of neutrophil chemotaxis, a finding which may have clinical significance (Hill and Quie, 1975); the latter drug has been reported by many authors to promote stimulation of various immune functions. The penultimate chapter describes the effects of the cyclic nucleotides and various drugs which enhance their intracellular accumulation, and leucoattractants on neutrophil glycolysis and HMS activity.

Using the technique of Ziamond and Hirsch (1973) which enables an analytical dissociation of random from directed movement, it has been possible to demonstrate that cyclic 3' - 5'-adenosine monophosphoric acid (cAMP) and cyclic guanosine 3' - 5'-monophosphoric acid (cGMP) *per se* have no effects on neutrophil motility. On the other hand agents which cause elevation of intracellular levels mediate profound effects on motility. cAMP elevating agents cause a stimulation of random motility and chemokinesis (stimulated random movement) with an accompanying inhibition of directional motility (chemotaxis). Drugs which cause elevation of cGMP levels are either without effect or cause stimulation of all three types of movement. Cyclic nucleotides

and the various drugs had no effect on neutrophil glycolysis. However, cAMP elevating agents all caused a marked inhibition of PMN HMS activity. cGMP elevating agents caused a variety of effects on HMS activity.

The two leucoattractants used in this study, endotoxin activated serum (EAS) and alkali hydrolysed casein exerted a dissimilar profile of effects on neutrophil biochemistry. EAS promoted an early increase in neutrophil cAMP levels and stimulation of both glycolysis and HMS activity. Casein, on the other hand, had no effects on cAMP levels and glycolysis and caused a depression of HMS activity.

Experimental results are discussed at the end of each chapter and the final chapter presents an overall discussion designed to co-ordinate the results of the previous four chapters.

For much of this presentation the term "chemotaxis" is used inaccurately. This is unavoidable since much of the literature reviewed assumes that effects observed on neutrophil motility in positive gradients can be attributed to alterations in chemotactic responsiveness, without dissection of the various types of motility which could be influenced.

P A R T I

C H A P T E R 1

Historical Background

HISTORY OF LEUCOTAXIS

The term "chemotaxis", with reference to directed movement of leucocytes, was first used by Leber in 1888 who clearly showed the "directed" movement of leucocytes towards a number of biologically derived substances, such as bacteria and fungi, animal tissue putrefaction products and water and alchoholic extracts of *Staphylococcus aureus*. Accumulation of leucocytes at the site of the chemoattractant substance was accomplished by injecting the test substance into the cornea or anterior chamber of the eyes of frogs and rabbits. In other experiments chemotactic substances were placed in capillary tubes and introduced into the anterior chamber or into the peritoneal cavity of rabbits with accompanying subsequent leucocyte accumulation.

In order to ascertain that leucocyte movement was actually directed and not a random movement, Leber injected test substances into the cornea of a guinea-pig and when some leucocyte accumulation was apparent to the naked eye the affected area was excised and examined microscopically in a moist chamber at 37°C. In this fashion continuous cell movement could be observed for some hours and Leber observed leucocyte movement towards the test substance.

Comandon (1919) devised an *in vitro* technique to demonstrate leucocyte chemotaxis. This method consisted of spreading a thin film of blood between a slide and cover-slip and recording leucocyte movement by time-lapse cinematography. Comandon demonstrated that avian

leucocytes were attracted towards avian red cells parasitized with *Haemamoeba danilewski*. The leucocytes apparently exerted pressure on the red cells until they burst and the polymorphonuclear leucocytes subsequently phagocytosed the parasite and the red cell debris. Similar behaviour was shown by human leucocytes towards red cells parasitized with *Filaria loa*. Starch graunles and a variety of bacteria such as streptococci, *Corynebacterium diptheriae* and *Bacillus anthracis* were also shown to be chemotactic for human leucocytes.

A further contribution to the *in vivo* study of chemotaxis was made by Clark and Clark (1921 and 1922). Substances under investigation with regard to leucotactic poential were introduced into the transparent tail of a tadpole either by injection as minute droplets or within small capillaries. The tadpole was anaesthetized in a special chamber which enabled continuous microscopic examination of the tail over long periods of time. Accumulation of leucocytes was observed to occur with a large variety of chemotactic substances including bacteria, starch, agar and gelatin.

Wright in 1921 introduced an *in vitro* technique designed to test chemotaxis. This method consisted of saturating a square of blotting paper with the test substance, covering it with a drop of blood and incubating for a given period of time subsequent to which the blood was washed away and the number of leucocytes which had been retained was microscopically evaluated. This

method inherently contained many variables and was considered unsatisfactory.

McCutcheon and Dixon (1936) using the cover-slip technique of Comandon tested the chemotactic response of human leucocytes towards a variety of bacteria including Gram positive, Gram negative and acid-fast bacilli and found the same intensity of response in all cases. All the leucocytes within a certain range of the bacterial clump moved directly towards it. A predominantly polymorphonuclear leucocyte response was evident.

Harris in 1952 introduced another technique for demonstrating chemotaxis. A random dispersion of variable human polymorphonuclear leucocytes was obtained by a modification of the method originally advocated by Wright. A drop of blood was placed on a cover-slip and incubated for about one and a half hours at 37°C in a moist chamber. During the incubation period a large number of the leucocytes in the blood clot adhered to the cover-slip and remained adherent subsequent to removal of red blood cells and serum by washing. Adherent leucocytes were incorporated in a thin plasma layer which was allowed to clot between the cover-slip and a microscope slide. Test substances which were solid or semi-solid were placed on the slide in contact with the plasma layer. When the test substance was a fluid it was incorporated onto a drill-hole on a perspex slide and thus came into

contact with the plasma layer. In this way test substances were localised at a given point on the plasma film. Leucocytes, when incubated at 37°C, left the glass surface to which they were previously adherent and moved freely in the plasma film. Movements of leucocytes towards test substances were recorded photomicrographically and the paths taken by moving leucocytes registered on the negative as traces.

When the leucocytes moved at random the traces were irregular and apparently non-directed. However, a chemotactic response was characterised by a convergence of traces towards the test substances. In this way various bacteria and starch grains were found to be chemotactic.

In 1953 Rebuck and Crowley described "the skin window technique". The skin of the upper forearm was abraded and overlaid with a glass coverslip. The coverslip was removed at hourly invervals and replaced by another coverslip. Each coverslip was fixed and stained and the number and type of adherent cells was enumerated microscopically. This technique gave an estimation of *in vivo* cell movement. The skin window gives an assessment of the quality of the leucocyte migration with regard to appearance and predominance of a particular cell type at a given time.

The previously described work is selective with a view to indicating work which may have been of a contributory and relevant nature prior to 1962. Harris in 1960 described the history of leucocyte chemotaxis as

being "studded with more picturesque names than solid facts". Prior to 1962 literature pertaining to leucocyte chemotaxis was more suggestive than precise. Techniques both *in vivo* and *in vitro*, although intriguing, were often highly intricate and difficult to interpret. Conclusions implicating a "chemotactic response" of certain white blood cell types to certain "chemotactic substances" could certainly be made, but a quantitative assessment of such responses was totally impossible. The aforementioned and related studies of movement of different cell types created an awareness of the phenomenon of directed cell movement and of the attractant substances capable of initiating such movement. In particular, there was no satisfactory evidence to implicate leucocyte chemotaxis as an important primary host defence mechanism.

The advent of a reliable quantitative *in vitro* technique for the evaluation of chemotaxis was considered essential to clarify an obscure situation. It was not until 1962 that Boyden described the first accurate technique for the assessment of chemotaxis. Ingenious in its conception, the actual test apparatus was fairly simple, comprising a vertically bicompartmental chamber in which the upper and lower chambers were separated by a millipore filter. The pore size of the filter is determined by cell size such that cells

could migrate through actively but not drop passively. A numerically standardized cell suspension was placed in the upper compartment of the chamber and soluble chemotactic substances placed in the lower chamber. The chambers were subsequently incubated and the cells allowed to respond chemotactically. After a suitable time interval the filter was fixed and stained and the lower surface examined microscopically. Chemotaxis was evaluated by counting those cells which had completely traversed the filter. This technique permitted quantitation of cell responsiveness to a known chemotactic factor and conversely the assessment of the chemotactic potential of a fluid by its capacity to induce chemotaxis.

In his studies Boyden investigated the ability of rabbit peritoneal polymorphonuclear leucocytes (PMN) to migrate actively towards rabbit sera which had been "activated" by immune complexes and aggregated gamma globulin.

This method, which made it possible to perform numerous assays on a given day with a fair degree of accuracy, has yielded much information on the chemotactic process. The existence of many leucoattractants and of the various leucocyte types capable of initiating a chemotactic response has been well documented.

The phenomenon of recurrent infection had not hitherto been associated with defects in leucocyte motility. The micropore filter technique has found immediate application in the study of human diseases. The category of recurrent infections of humans has come under re-investigation. Chemotactic defects have often been found and may be either humoral or cell associated.

The various modes of generation and diverse nature of chemotactic factors together with a description and classification of types of defective leucotaxis in human disease have been previously described (Anderson, 1974). For this reason they will not be discussed in this thesis.

Biochemical fractionation of complex mixtures found to induce a chemotactic response in the Boyden system has resulted in the acquisition of biochemically pure preparations of leucoattractants. *In vitro* interaction of these with the appropriate cell type, in conjunction with measurement of specific biochemical responses has been informative with regard to the cellular biochemistry associated with chemotactic stimulation.

Revitalised interest in the field of leucotaxis has occurred over the past decade, and accordingly major advances are limited to this period. For this reason it is intended to devote the remainder of this introduction to a review of the literature pertaining to the cellular biochemistry of leucotaxis subsequent to 1962.

C H A P T E R il

Biochemistry of the Neutrophil Chemotactic Response

EVENTS OCCURRING AT THE CELL MEMBRANE

a) Relationship of leucotactic potential to molecular hydrophobicity

Wilkinson (1971, 1972, 1973 and 1974) has demonstrated that increases in molecular hydrophobicity, induced by a variety of chemical treatments, correlate with enhanced leucotactic activity of such structurally altered molecules. The acquisition of such activity can be accomplished by acid-alkali denaturation and reduction-alkylation treatment of human serum albumin (HSA) (Wilkinson 1971 and 1974); by removal of the haem moieties from haemoglobin and myoglobin, a procedure which increases the net surface hydrophobicity of these molecules (Wilkinson 1973); and by conjugation of non-polar side groups to the HSA molecule (Wilkinson and MacKay, 1972). The same author postulates that molecular denaturation in aqueous solution is accompanied by a transposition of hydrophobic groups from the molecular interior to situations of increased surface exposure. Exposure of such groups would lead to an increase in the overall hydrophobic properties of the protein molecule and an increase of molecular surface activity. Such surface active proteins could be expected to manifest high affinity for cell surfaces. Schiffman *et al* (1975) have reported that certain synthetic small peptides with an N-terminal formyl methionine residue possess leucotactic activity. Showell *et al*, (1976) using a variety of such synthetic di-, tri-, and tetrapeptides with an N-terminal

formyl methionine have demonstrated that such peptides are truly chemotactic. The leucotactic activity is dependent on the nature of the amino acid components. Peptides containing non-polar amino acids possess greatest leucotactic potential. Those containing a C-terminal phenylalanine are particularly effective.

b) Cell membrane changes mediated by chemotactic factors

Gallin *et al*, (1975) have reported that certain neutrophil chemotactic factors such as C5a derived by immunological or enzymatic cleavage of the fifth component of complement (Snyderman *et al*, 1969) dialysable transfer factor (Gallin and Kirkpatrick, 1974) kallikrein and plasminogen activator Kaplan *et al*, 1972) mediate a diminution of the negative surface charge of human neutrophils as measured cytophorometrically. Such changes cannot be mediated by other proteins, which are apparently non-chemotactic. No correlation was evident between the extent of the chemotactic response and reduction of cell surface negativity between the various leucoattractants. However, marked correlation was evident in C5a dose response experiments where increasing chemotaxis was associated with decreasing cell surface negative charge. The authors suggest that the decreased surface charge enhances cell adhesion and is a prerequisite for locomotion. The authors in support, cite the work of Nagura *et al*, (1973) who showed that treatment of rat macrophages with poly-electrolytes which decrease cell surface charge,

facilitates particle attachment during phagocytosis. However, Hardy *et al*, (1976) have reported that enhanced phagocytosis is associated with increased cell surface charge. An inverse relationship between cell surface negativity and cell adhesion has been reported for amoebae by Ambrose and Forrester (1968) and for bone marrow granulocyte precursor cells by Lichtman and Weed (1972). Other authors, however, are in disagreement with these findings. Lomnitzer *et al*, (1976a) have also reported that a chemotactic factor, endotoxin activated fresh human serum (EAS) mediates a reduction of cell surface negative charge as assessed by a cytophorometric technique. These authors, however, were unable to demonstrate any detectable change in cell surface charge by agents which inhibit neutrophil glass adherence. Furthermore, leucocyte inhibitory factor (LIF) which specifically inhibits human PMN migration (Rocklin, 1974) by promoting increased cell adherence (Lomnitzer *et al*, 1976b) causes an increase in cell surface charge (Lomnitzer *et al*, 1976c). The effects of LIF on neutrophil motility are currently under investigation in several laboratories and preliminary data from this laboratory indicate that LIF may cause inhibition of cell motility.

The relationship of surface charge to cell motility is somewhat obscure. Depression of cell surface negative charge by leucoattractants is evident and may indeed be a prerequisite for chemotaxis (Gallin *et al*, 1975 and Lomnitzer *et al*, 1976a) and LIF, an agent which causes increased cell surface charge, mediates an inhibition of cell motility (Lomnitzer *et al*, 1976c). Presumably

adherence to an appropriate surface is essential for cell motility. Atherton and Born (1972), in animal experiments, have demonstrated the importance of granulocyte adhesion to the vascular endothelium for initiation of *in vivo* leucocyte emigration from venules. However, the degree of adherence in relation to the rate and extent of cell motility has not been elucidated.

c) Requirement of Divalent Cations Mg^{++} and Ca^{++} for Cell Motility

Bryant and Sutcliffe (1972) have reported that leucocyte adhesion is dependent upon the presence of Mg^{++} and can be abolished by EGTA which specifically chelates this cation. There was no requirement for adhesion of Ca^{++}, K^{+} and Na^{+}. These findings have been confirmed by Lomnitzer *et al*, (1976b). Becker and Showell (1972) demonstrated a requirement of both Ca^{++} and Mg^{++} in the external medium for a maximal chemotactic response. This has been confirmed by Gallin and Rosenthal (1974), Wilkinson (1975) and Estensen *et al*, (1976).

Johnson *et al*, (1970) have reported that divalent cation ionophores increase the permeability of cell membranes to divalent cations by forming chelate compounds of a hydrophobic nature which facilitate cation trans-membrane passage. Ionophores have been used experimentally to further elucidate the relationship of divalent cations to cell motility. Wilkinson (1975) and Estensen (1976) have reported

that the ionophore A23187 at low concentrations in the absence of Ca^{++} and Mg^{++} can mediate a return to normal neutrophil chemotactic responsiveness. Furthermore Becker (1975) has shown that A23187 in a Ca^{++} free medium causes a stimulation of random and directed motility, which has been confirmed by Estensen *et al*, (1976). Although there is agreement as to the requirement in part of external Ca^{++} and Mg^{++} the ionophore studies indicate that ion influx is of importance in cell motility. Gallin and Rosenthal (1974) have shown that various leuco-attractants, upon interaction with human neutrophils, mediate a rapid release of radiolabelled Ca^{++} from neutrophils associated with depressed Ca^{++} uptake and a shift of intra-cellular Ca^{++} out of the cytoplasm and into a granular fraction. These results are apparently contradictory to the ionophore studies. However, comparison is difficult since there are no studies pertaining to ionophore mediated radiolabelled cation fluxes in chemotactically stimulated cells. There is no evidence from the ionophore studies to suggest that Ca^{++} efflux does not occur during cell motility. Also Ca^{++} is only very slightly reduced during the first 10 minutes of chemotactic stimulation, and indeed the apparent reduction of influx could be an artifact of the experimental system since no correction has been made for simultaneous efflux of radiolabelled Ca^{++}, if this was large enough to affect results.

The monovalent cation K^{+} has been reported by Ward and Becker (1970), to be necessary for optimal neutrophil migration since ouabain which apparently blocks the potassium pump in neutrophils (Woodin and Wieneke, 1970)

causes a K^+ reversible inhibition of neutrophil chemotaxis. These findings have been disputed by Ramsey and Harris (1973) and Wilkinson (1975).

It is probable that Mg^{++} is of importance in cell adhesion and Mg^{++} and Ca^{++} may regulate intracellular processes involved in cell motility.

d) Chemotactic factor activation of membrane esterases

Becker and Ward (1976a) demonstrated that organophosphorus inhibitors of serine esterases inhibit the chemotactic response of rabbit PMN cells to chemotactic factors produced by the interaction of immune precipitates with 10% rabbit serum. The organophosphorus inhibitors employed in the demonstration of inhibition were diisopropylphosphofluoridate and a series of homologous phosphate esters which varied in alkyl nature of the first carbon atom. Two types of inhibition were demonstrable. The first was termed by the authors "cell-dependent inhibition", in which elimination of chemotactic responsiveness was attained by incubating PMN cells with a phosphate inhibitor in the absence of chemotactic factors for one hour. Cells were subsequently washed free of inhibitor and were shown to be chemotactically unresponsive. Inhibition was time dependent, being normal at one hour. Inhibitor concentrations required to eliminate cellular responsiveness varied with phosphonate ester type.

The second type of inhibition observed was termed "chemotactic factor-dependent inhibition" and was demonstrated by assessing the chemotactic responsiveness of PMN cells suspended in appropriate concentrations of a phosphonate ester. To avoid loss of chemotactic activity due to cell-dependent inhibition, the concentration of inhibitor, phosphonate type and duration of cell-inhibitor contact were manipulated to minimize this effect. Inhibition, in the presence of chemotactic factor, was obtained at concentrations of phosphonate inhibition which cause negligible or non-existent cell-dependent inhibition, whilst manifesting a high level of inhibition apparently mediated by the interaction of chemotactic factor with the PMN cell.

It was concluded that these two types of inhibition were due to the inactivation of two PMN serine esterases. The cell-dependent type was attributed to the inhibition of the "activated esterase", as termed by the authors. It is postulated that this esterase exists in or on the PMN cell in an already activated state and is therefore susceptible to phosphonate inhibition in the absence of chemotactic factor. Chemotactic factor-dependent inhibition is considered due to the inhibition of a second esterase termed the "activatable esterase". Becker and Ward speculate that this enzyme is present in or on the cell in precursor form, which is not susceptible to inactivation by the organophosphorus inhibitor. Hypothetically the chemotactic factor would cause

activation of this esterase with concomitant conversion to inhibitor susceptibility.

Subsequent work contributed to a further characterization of the two esterases. Becker and Ward (1967b) demonstrated that carboxylic acid esters afforded protection against the inhibition of the activated esterase of chemotaxis by phosphonate esters. Degree of protection was dependent upon the relative concentrations of protective ester and inhibitor. Protective esters also inhibit chemotaxis and the authors postulate that the carboxylic acid ester competes with the phosphonate for the enzymatic site of the activated esterase.

Requirement of a high degree of specificity of the structure of the protective ester was evident. The ester must be an acetate esterified to a negatively charged or neutral group. Experiments indicated that the cell-bound activated esterase is able to bind the protecting ester to the enzyme active site. That the enzyme was capable of ester hydrolysis was undetermined. Differences in inhibition profiles indicated that the activated esterase was not the known aliesterase of rabbit leucocytes.

The authors conclude "that the activated esterase of chemotaxis is an enzyme capable of being competitively inhibited by, or of hydrolyzing neutral acetate esters, and acetates bound to negatively charged groups, or of doing both". They postulate that such characteristics

are indicative of the properties of an acetylase.

Ward and Becker in 1968 first demonstrated the phenomenon of "deactivation", whilst further characterizing the activatable esterase of chemotaxis. They demonstrated that incubation of rabbit PMN cells with 30% rabbit serum for sixty minutes eliminated cell responsiveness to a chemotactic stimulus. Similar observations were made when cells were pre-incubated with "purified" $\overline{C567}$. This phenomenon of inhibition of leucotaxis by interaction of chemotactic factor with PMN cells prior to chemotactic evaluation was termed deactivation. Phosphonate esters prevented deactivation and also prevented chemotaxis. The profiles of protection against deactivation were similar to those of chemotactic-factor dependent inhibition suggesting that the activatable esterase was involved in both cases.

Acetate esters, previously shown to inhibit the action of the activated esterase, did not prevent deactivation. The only other substances found to prevent deactivation were aromatic amino acid derivatives. The protection afforded by these was specific insofar as nonaromatic amino acid compounds and simple acetate esters had no effect. In conjunction with these and previous findings, the authors suggest that "the activatable esterase of the rabbit PMN is a serine esterase with a special affinity for aromatic amino acid derivatives".

Becker and Ward (1969) demonstrated that the rabbit peritoneal PMN cell contains 3 esterases capable of hydrolyzing the aromatic amino acid ester acetyl DL-phenylalinine naphthyl ester. Two of these esterases, termed esterase 1 and 2 are inhibited by phosphonate esters. Esterase 3 is not inhibited. Using inhibition profile studies the authors concluded that esterase 1 is the activated form of the activatable esterase of chemotaxis (proesterase 1).

Becker (1972) demonstrated that C3a, C5a and $\overline{C567}$ of human origin and bacterial chemotactic factor (Ward, 1968a) show identical profiles of inhibition of chemotaxis by phosphonate esters. This apparently indicates that induction of chemotaxis by these 4 chemotactic factors involves activation of proesterase 1 of the rabbit PMN cell.

$\overline{C567}$, C5a and C3a activated proesterase 1 of rabbit peripheral blood leucocytes. No activation was observed with rabbit peritoneal PMN cells. The decreased esterase of the latter cells may be attributable to partial "deactivation". These cells had previously migrated to the peritoneum in response to glycogen stimulation and may have exhausted some of their chemotactic potential. Bacterial chemotactic factor did not activate proesterase 1 of rabbit peripheral blood PMN cells. This may indicate a difference in mechanism of the two types of chemotactic factor. The difference may however be of a quantitative nature. Bacterial chemotactic factor exhibits a much lower chemotactic activity

than C3a, C5a and $C\overline{567}$.

The author concludes that activation of proesterase 1 is a prerequisite for the chemotactic activity of rabbit PMN cells and suggests that under several different circumstances the level of chemotactic activity attained is related to the degree of such activation.

These findings have been disputed by Todd and Dowdle (1973) who failed to detect esterase activity in rabbit peritoneal PMN cells stimulated with the leucoattractant casein. The studies of Becker and Ward would be a great deal more convincing if they could assert the specificity of the esterase inhibitors. This could be accomplished by demonstrating absence of interference with neutrophil metabolic pathways essential for optimal cell motility such as glycolysis (Carruthers, 1966; Goetzl and Austen, 1974) and the hexose monophosphate shunt (HMS) (Goetzl and Austen, 1974). Indeed, Goetzl and Austen (1974) have reported that diisopropylfluorophosphate (DFP), one of the serine esterase inhibitors used by Becker and Ward (1967a), mediates substantial, progressive dose dependent inhibition of glycolysis at concentrations of 10^{-5}M upwards. In their experiments Becker and Ward (1967a) used DFP concentrations of $1 - 5 \times 10^{-3}$M to attain inhibition of chemotaxis. Such concentrations would be expected to mediate considerable inhibition of glycolysis. This possibility would cast doubt upon the validity of the conclusions of Becker and Ward (1967a) with regard to the rôle of serine esterases in neutrophil motility.

Goetzl (1975) has reported that the synthetic esterase inhibitors L-1-tosylamide-2-phenylethyl-chloromethyl ketone (TPCK) and N-α-p-tosyl-L-lysine-chloromethyl-ketone (TLCK) cause an inhibition of chemotaxis. The former also inhibits random migration and the latter affects a mild stimulation. HMS activity is unaffected by these inhibitors. However, this may not be a valid criterion by which to implicate absence of non-specific effects since HMS may play little part in directed motility. Children with chronic granulomatous disease (CGD) have normal neutrophil chemotaxis in the absence of infection. (Gallin, personal communication - 1975). (See also Chapter VIII of this thesis).

The clinical significance of these studies remains undetermined since there have been no reports of defective leucotaxis associated with diminished cell esterase activity.

Microfilaments and Microtubules

a) Microfilaments: These have been documented in many cell types and have been identified as actin-like polymers of 4-7 nm diameter. Actin-like microfilaments have been associated with many cell activities which appear to involve contraction such as membrane movements and cytoplasmic streaming. (Schroeder, 1970; Bradley, 1973; Allison *et al*, (1971); Wessels *et al*, (1971). The

functional connection between such effects and microfilaments has largely been based on experiments involving the drug cytochalasin (Carter, 1972). Since the drug appeared to interfere with cell activities associated with the presence of microfilaments it was assumed by many workers that cytochalasin was a specific inhibitor of contractile protein microfilaments. Cytochalasin in many cases does not enter the cell and the primary target may be the cell surface (Singer and Holtzer, 1972; Estensen and Plagemann, 1972). Furthermore, Forer *et al*, (1972) reported no interference *in vitro* by cytochalasin in actinomyosin interactions or transformations.

Becker *et al*, (1972) has reported that cytochalasin B caused a depression of neutrophil chemotactic responsiveness. Zigmond and Hirsch (1972a) found that the same drug mediated inhibition of random migration. These findings may implicate microfilaments in neutrophil locomotion. Zigmond and Hirsch (1972b) have also shown that cytochalasin causes inhibition of leucocyte glycolysis, which further emphasises the dubious nature of cytochalasin as an investigative tool with regard to microfilament participation in a particular cell function.

Keyserlingk (1968) has shown that the broad locomotor pseudopods associated with normal neutrophil movement contain cortical microfilaments of 6 nm diameter. Furthermore, actin (Tatsumi *et al*, 1973) and myosin (Tatsumi *et al*, 1973; Stossel and Pollard, 1973) have been isolated from neutrophils. Using the histochemical technique of

"arrowhead" formation (Ishikawa, 1969), Pollard and Weihing have demonstrated that the cortical microfilaments associated with cell motility are actin polymers. Boxer *et al*, (1974) have isolated a contractile protein of M.W. 42,000 from human neutrophils which exhibits the functional properties of actin.

Boxer *et al*, (1976) have isolated pure actin, myosin and actin binding protein (ABP) in large amounts from the leucocytes of patients with chronic myelogenous leukaemia. Gel formation and contraction was evident upon mixing of all 3 proteins in sucrose solution. Antibody to myosin caused inhibition of contraction, while antibody to ABP inhibited both gel formation and contraction.

Neutrophils incubated with antibody to myosin and antibody to ABP manifested 70% inhibition and 30% inhibition of migration respectively. Immunofluorescence studies indicate that ABP is localized on the cell membrane.

Microfilaments are membrane associated contractile elements structured from the protein actin, which probably play a major part in neutrophil locomotion, which may be accomplished by contraction and relaxation of actinomyosin. Contraction of muscle actinomyosin is dependent upon adequate concentrations of ATP. K^{+}, Ca^{++} and Mg^{++} and this would indicate the existence of at least one intracellular source of requirement of divalent cations.

b) Microtubules: These are a class of proteinaceous intracellular fibres found in a wide variety of eucaryotic cells. They were originally defined by their morphology as viewed electron microscopically (EM), appearing as tubules with an outer and inner diameter of 250A^{O} and 150A^{O} respectively. Since the widespread use of glutaraldehyde as fixative in EM their occurrence has been documented in all eucaryotic cells examined (Roberts, 1974).

Microtubules are structured from the protein tubulin (Mohri, 1968) which has been shown by Weisenberg *et al*, (1968) to be a colchicine binding protein of M.W. 120,000. The protein is a dimer of 2 monomer components both of M.W. 60,000. Shelanski and Taylor (1968) have shown that GTP stabilizes tubulin and that each tubulin dimer has 2 GTP binding sites and 1 colchicine binding site. Hydrolysis of bound GTP is probably related to polymerization since no contractile activity has been reported for tubulin.

The existence of microtubules in neutrophils has been demonstrated convincingly (Malawista and Bensch, 1967; Bensch and Malawista, 1969; Gallin and Rosenthal, 1974; Malech *et al*, 1976). Colchicine and vinblastine have been shown to mediate disruption of microtubules in neutrophils (Malawista and Bensch, 1967; Bensch and Malawista, 1969). These drugs also cause an inhibition of neutrophil chemotaxis. (Caner, 1964; Phelps, 1970). These findings

have been disputed by Baum (1975) and Snyderman (1975). Russell *et al*, (1976) have reported that colchicine causes a marked inhibition of the true chemotactic response as measured by the method of Zigmond and Hirsch (1973) but a stimulation of random migration. These findings suggest a dependence, at least of directed motility, upon the presence of intact microtubules.

Gallin and Rosenthal (1974) have reported that the leucoattractant C5a, upon interaction with human neutrophils causes a substantial increase in the number of microtubules as assessed by counting these structures on the appropriate electron-micrographs. Malech *et al*, (1976) have investigated centriole, microtubule and microfilament orientation in human neutrophils during chemotactic stimulation. This was accomplished by overlaying neutrophils on a filter of insufficient pore size (0,45μm) to allow intra-filter migration whilst permitting the establishment of a gradient of leucoattractant (EAS). Electron-micrographs revealed that those cells exposed to a gradient of leucoattractant manifested an increase in sub-membrane microfilaments and microtubules. These extend radially from centrioles to the cell periphery and into the pseudopods (in agreement with Keyserlingk, 1968). This increased cytoskeleton assembly was detectable only at the side of the cell exposed to chemotactic stimulation. No such organization was evident in neutrophils exposed to buffer only.

In vitro microtubular assembly studies using tubulin proteins isolated from brain indicate that the extent of polymerization is regulated by the level of Ca^{++} and the nucleotide triphosphates ATP and GTP with optimal polymerization occurring in a low Ca^{++} environment (Weisenberg, 1972; Berry and Shelanski, 1972; Shelanski *et al*, 1972; Fuller *et al*, 1975). Furthermore, Fuller *et al*, (1975) have reported that *in vitro* assembly and disassembly of cytoplasmic microtubules may be regulated in part through mitochondrial sequestration and release of cytoplasmic calcium. Mitochondria can take up and release calcium and probably act as the organelles which regulate the level of intracellular calcium. Neutrophils, however, show a relative paucity of mitochondria (Becker, 1971). Fuller *et al*, (1975) have reported *in vitro* stimulation of microtubule formation in intact fibroblasts mediated by dibutyryl cyclic adenosine 3^1, -5^1, - monophosphate (d'cAMP). The same authors have also reported that cAMP stimulates the calcium pump for rapid Ca^{++} release. Microtubule assembly is almost certainly under the control of cAMP and calcium ions.

The relationship between microfilaments and microtubules in neutrophil motility is uncertain. Gallin and Rosenthal have suggested that the "microtubule system may provide the net vector of motion (i.e. polarized pseudopod formation), while the microfilament system, which may require local increases in Ca^{++}, would provide the mechanical work for motion".

EFFECT OF CHEMOTACTIC STIMULATION ON NEUTROPHIL GLYCOLYSIS AND THE HEXOSE MONOPHOSPHATE SHUNT PATHWAY (HMP)

a) Glycolysis: It is unlikely that oxidative phosphorylation contributes substantially to the energy requirement of neutrophil locomotion due to the comparitively low numbers of cytoplasmic mitochondria (Becker, 1971). Furthermore, Ward (1966) and Carruthers (1967) have reported that dinitrophenol (DNP), an uncoupler of oxidative phosphorylation, exerts only slight depression of the neutrophil chemotactic response. Carruthers (1966) and Goetzl and Austen (1974) have shown that inhibitors of glycolysis (iodoacetate and iodoacetamide) show a parallel inhibition of glycolysis and chemotaxis. Iodoacetamide (Goetzl and Austen, 1974) is the more potent of the two inhibitors. Carruthers (1967) has demonstrated inhibition of chemotaxis by deoxyglucose (an inhibitor of glycolysis). This inhibition can be blocked by the addition of glucose. Goetzl and Austen (1974) have assessed the effect of the leucoattractants kallikrein and C5a on neutrophil glycolytic activity as estimated by lactate production. Both chemotactic factors induced a significant increase in lactate production (25 - 100% stimulation). Glycolysis has therefore been implicated, directly and indirectly, as being the major source of ATP production for neutrophil chemotaxis.

b) Hexose Monophosphate Shunt Pathway (HMP)

Goetzl *et al*, (1973) and Goetzl and Austen (1974) have reported that the partially purified chemotactic factors C5a and kallikrein mediate 100 - 600% increases in HMP activity above that of the corresponding unstimulated cells. The stimulation was evident at all times tested, being maximal at 60 - 120 minutes and of a similar magnitude for adherent neutrophils and neutrophils in suspension. The increase in HMP activity with time paralells the neutrophil chemotactic responsiveness. The metabolic inhibitor 6-aminoicotinamide which may mediate a selective inhibition of the HMP (Ammon and Steinke, 1972) at 10^{-5}M with only minimal inhibition of glycolysis (Goetzl and Austen, 1974) causes a concomitant inhibition of chemotaxis of 23 - 45%; the assumption being that the inhibition is HMP specific. The control systems in the experiments involving leucoattractant/HMP interactions would be of greater value if the presence of other agents in the leucoattractant preparations had been controlled for. For example, C5a was prepared by trypsin digestion of C5a for 30 minutes followed by trypsin inhibition with soya been inhibitor. The effects of C5a and the trypsin/soya bean inhibitor complex were not controlled for. Similarly, the appropriate controls were omitted for kallikrein experiments.

Goldstein *et al*, (1975) have reported that biochemically pure C5a caused a stimulation (500%) of nitroblue tetrazolium (NBT) reduction by human neutrophils with a concomitant 350% increase in HMP activity. Enhancement of NBT reduction by normal cells *in vitro* has been convincingly shown to be associated with phagocytosis (Baehner and Nathan, 1968) and neutrophil HMP activity (Karnovsky, 1968). C5a stimulation of HMP activity and NBT reduction occurs in the absence of phagocytosis. No parallel studies correlating the degree of HMP stimulation and NBT reduction with the extent of neutrophil chemotaxis were performed. These results are in agreement with those of Goetzl and Austen (1974).

Goetzl *et al*, (1974) have reported that ascorbic acid and glutathione which may enhance the oxidation of NADPH to $NADP^+$ (De Chatelet *et al*,(1972), the early rate limiting step in the HMP, promote a stimulation of neutrophil chemotaxis and random migration (100 - 300%) associated with an increase in HMP activity ($\sim$- 100%). The ascorbic acid mediated enhancement of neutrophil motility and HMS activity could be completely eliminated by 10^{-5}M 6-aminonicotinamide. No effect on glycolysis of ascorbate acid was evident. No experiments were performed to assess the extent of ascorbate stimulation in the presence of the leucoattractants C5a and kallikrein. This would appear to be an important experiment required to detect an increment in leucoattractant associated HMP activity attributable to ascorbate. The magnitude of such an increment would enable a valid assessment of

the relationship between ascorbate, HMP activity and stimulation of neutrophil chemotaxis.

The work of the above authors (Goetzl *et al*, 1973; Goetzl *et al*, 1974; Goetzl and Austen, 1974; and Goldstein *et al*,1975) has implicated HMP involvement in neutrophil motility, increased HMP activity being associated with an enhanced level of motility. However, there is evidence to suggest that stimulation of the HMP associated with neutrophil locomotion may be coincidental. Goetzl and Austen (1974) demonstrated that diisopropylfluorophosphate (DFP) which causes a suppression of neutrophil chemotaxis has no inhibitory activity on HMP activity. Gallin has also shown (personal communication, 1975) that children with CGD, a condition associated with impaired HMP activity, manifest normal leucocyte migratory responsiveness *in vitro*. The relationship of HMP activity to neutrophil motility is imprecise.

IN VITRO MODULATION OF NEUTROPHIL MOTILITY BY CYCLIC NUCLEOTIDES AND AGENTS WHICH AFFECT THEIR INTRACELLULAR ACCUMULATION

a) Cyclic nucleotides as leucoattractants

A number of authors have reported chemotactic activity for cAMP (Leahy *et al*, 1970; Ramsey, 1972; Gammow *et al*, 1971; Rivkin and Becker, 1976). On the other hand, a

number of authors (Kaley and Weiner, 1971; Wissler *et al*, 1972; and Symon *et al*, 1972) have been unable to detect such activity. It is difficult to attribute the discrepancy in results to variations in technique since a similar spectrum of neutrophil types and filter systems have been used by workers in either camp. Of those who advocate that cAMP is leucotactic, none have convincingly eliminated alternate modes of stimulation of neutrophil motility, such as enhancement of random migration, and this is one aspect which requires clarification. The cAMP controversy may also be related to the low molecular weight (351) of cAMP, which in filter systems may diffuse rapidly from the lower to the upper compartment thereby negating the positive gradient. There is a paucity of data on the rôle of cAMP as a leucoattractant.

b) The effect of c'AMP and agents which promote its intracellular accumulation on neutrophil migration *in vitro*

Tse *et al*, (1972) reported that cAMP (10^{-5}M - 10^{-3}M) mediates inhibition of both random and directed migration of human blood neutrophils. Dibutyryl cAMP and ATP were without effect. Agents which the authors claim may mediate a depression of intracellular cAMP such as epinephrine, guanosine 5'- diphosphate, imidazole and ADP all cause a stimulation of neutrophil random motility. Caffeine and 5-hydroxytryptamine which may elevate intracellular cAMP cause an inhibition of random motility. These agents were not

tested for their effects on neutrophil chemotaxis. Epinephrine has been reported to increase intracellular cAMP in neutrophils by beta adrenergic stimulation (Rivkin *et al*, 1975) although stimulation of the alpha-adrenergic type (Goldberg, 1974) may promote the effects observed by Tse and colleagues.

The same authors also investigated the effects of a series of purine and pyrimidine analogues on neutrophil random motility. They observed that the purines xanthine, hypoxanthine, adenine and guanine and uric acid, mediated enhancement whereas dimethyl xanthines (threobromine and theophyline) exerted a relatively mild stimulation. Caffeine (trimethyl xanthine) as mentioned above, caused inhibition of motility. Neutrophil motility was unaffected by the pyrimidines uracil and 1,3-dimethyl uracil, although thymine (5-methyl uracil) increased motility, indicating that a substituent at position 5 of the pyrimidine nucleus may be a prerequisite for stimulation.

Rivkin *et al*, (1975) showed that prostaglandins (PG) A_1 and E_1 which mediate a substantial increase in neutrophil intracellular cAMP, cause a decrease in chemotaxis at a concentration of 2.5×10^{-5}M. Prostaglandin F_2 was without effect on cAMP levels or chemotaxis. The effect of these agents on random motility was apparently not investigated. Epinephrine, norepinephrine and isoproterenol all promoted elevated cAMP levels without effect on chemotaxis. The authors state that the effects of these agents on chemo-

taxis and random motility are "variable" but random motility results are not shown. Theophylline at high concentrations (1 mM - 8 mM) caused enhancement of intracellular cAMP levels with concomitant inhibition of directed and spontaneous motility. Bourne *et al*, (1973) have been unable to detect increases in neutrophil cAMP levels following interaction with theophylline in the range used by Rivkin *et al*. They also reported profound inhibition of neutrophil post-phagocytic metabolism. These observations indicate that the theophylline inhibition of motility may not be related to increased intracellular levels of cAMP.

Rivkin *et al*, also showed that the effects of isoproterenol and norepinephrine on cAMP levels and of epinephrine on the inhibition of chemotaxis (where inhibition was evident) could be blocked by propanolol (beta adrenergic blocker) but not by phenoxybenzamine (alpha adrenergic blocker) indicating that these effects are mediated via the beta receptor. Cholera toxin was also shown to mediate increased neutrophil cAMP after a 30 minute lag period which paralleled its inhibitory effect on chemotaxis and random motility.

Paradoxically, the same authors, Rivkin and Becker (1976) have reported that exogenous cAMP has a variable effect on neutrophil chemotaxis but promotes a stimulation of random motility at concentrations of $2.5 - 5 \times 10^{-5}$M. Dibutyryl cAMP (5×10^{-4}M - 1×10^{-3}M) causes a considerable inhibition of chemo-

taxis, but a stimulation of random motility. Both cyclic nucleotides are chemotactic at 10^{-3}M. Henion *et al*, have reported that cAMP penetrates cell membranes poorly, if at all, compared with d'cAMP (the latter being more hydrophobic) and this may account for the variable effects of cAMP and the high concentrations required to stimulate motility. These results would appear to be contradictory to those described in the preceding report by the same authors (Rivkin *et al*, 1975).

Valone *et al* , 1974) have observed inhibition of platelet random migration by epinephrine and isoproterenol. The inhibition can be prevented by blockade of the beta receptor, but not by alpha receptor blockade. The prostaglandins PGE_1 and PGE_2 caused a stimulation of platelet random migration, which could be inhibited by indomethacin.

Hill *et al*, (1975) have reported inhibition of neutrophil chemotaxis by cAMP (maximal at 10^{-4}M). Beta adrenergic stimulation by epinephrine, norepinephrine and isoproterenol also caused inhibition of chemotaxis which was maximal at a concentration range of 10^{-4}M - 10^{-3}M for all three agents. The inhibitory effect of epinephrine could be eliminated by propanalol, although the effects of propanalol *per se* were apparently not controlled for. Phentolamine (an alpha receptor blocker) had no effect on the inhibition.

Phenylephrine, which mediates stimulation of the alpha adrenergic type, caused an enhancement of chemotaxis which could be prevented by phentolamine. The prostaglandins PGE_1 and PGE_2 (10^{-8}M - 10^{-6}M) mediated a substantial inhibition of chemotaxis whereas PGF_2, at the same concentrations, promoted stimulation. Histamine inhibition of neutrophil chemotaxis could be prevented by treatment of cells with the drug metiamide, an antihistamine which blocks the H1-receptor site, indicating that the histamine effect on chemotaxis is mediated via the H2-receptor site. Cholera enterotoxin also causes inhibition of chemotaxis which can be blocked by cholera enterotoxin. No studies were performed to determine the effects of these agents on neutrophil motility.

Despite some discrepancies there seems to be general agreement that the elevated intracellular cAMP levels promote inhibition of neutrophil chemotactic responsiveness. This can be accomplished in 5 different ways; by the addition of cAMP; by beta adrenergic stimulation; by histamine; by prostaglandins(A_1,E_1 and E_2); and by cholera enterotoxin. The effects of these agents on nuetrophil random migration is less clear since agents which can inhibit chemotaxis such as d'cAMP (Rivkin and Becker, 1976), epinephrine (Tse

et al, 1972) prostaglandins (Valone *et al*, Hill *et al*, 1975; and Shibuya *et al*, 1976) and histamine for eosinophils (Clark *et al*, 1976) can promote stimulation of random motility.

THE EFFECT OF cGMP AND AGENTS WHICH PROMOTE ITS INTRACELLULAR ACCUMULATION ON NEUTROPHIL MIGRATION *IN VITRO*

Estensen *et al*, (1973) have reported that 8 bromo cGMP and agents which promote increases in intracellular cGMP in a variety of cell types (neutrophils not included) mediate an enhancement of leucotactic responsiveness. The agents used were phorbol myristate acetate (PMA), which was particularly effective, and acetyl choline and carbamyl choline. No investigations of the effect of these drugs on random motility were performed. Valone *et al*, (1974) have reported that carbachol (a cGMP stimulant) mediates stimulation of platelet random motility.

In a continuation of this research from the same laboratory, Hill *et al*, (1975) have reported that imidazole as well as acetyl choline, carbamyl choline and PMA, promote an increase in neutrophil chemotaxis. The stimulation of chemotaxis associated with acetyl choline and carbamyl choline could be blocked by the cholinergic inhibitor, atropine. No mention is made of the effects of atropine *per se*. Cyclic GMP at concentrations of 10^{-9}M - 10^{-6}M also

promoted stimulation of chemotaxis. The authors have apparently not investigated the effects of these agents on neutrophil random motility since no data is shown.

These results indicate that agents which elevate intracellular cGMP levels cause a stimulation of neutrophil motility. The exact types of motility affected remains to be determined.

C H A P T E R III

Current filter Methodology for the *in vitro* assessment of Neutrophil Motility

MILLIPORE FILTER METHODS IN CHEMOTAXIS

1) Boyden's technique for assessing chemotaxis

Stephen Boyden in 1962 described the first accurate technique for the *in vitro* assessment of chemotaxis. This method has subsequently attained wide acceptance. Although the test apparatus has since undergone a number of modifications designed to increase its reliability, the underlying principle, i.e. the migration of chemotactically responsive cells through a micropore membrane, remains unchanged.

The "Boyden Chamber" is fairly uncomplicated and consists of a vertically bicompartmental chamber in which the upper compartment (chamber 'A') is separated from the lower chamber (chamber 'B') by a micropore membrane. The pore size of the membrane is determined by the dimensions of the cell type used in the chemotactic investigation such that cells can migrate actively through the membrane but not drop passively. The rigidity of the assay can be improved by decreasing the pore size and increasing the depth of the membrane. The micropore membrane is inserted at the junction of the two compartments and firmly held in place by a double ring of perspex. The base of the ring is lightly smeared with petroleum jelly to enhance the bicompartmental seal and so prevent interchamber leakage.

A numerically standardized cell suspension is placed in the upper compartment of the chamber and a

soluble leucoattractant in the lower compartment. The extent of leucotaxis is microscopically evaluated according to the number of cells which have migrated across the filter after a suitable time of incubation.

Chamber assembly is accomplished by the introduction of 1.5 ml of test solution into chamber B, the apparatus being tipped slightly to avoid air bubbles becoming trapped beneath the filter membrane. Cell suspension (3.6 ml) is then pipetted into the cell compartment (A) simultaneous to the addition of the remaining aliquot (1.5 ml) of test solution. The apparatus is incubated at 37^{o}C in a moist atmosphere containing 5% carbon dioxide, for 3 hours. After this time the filters are inverted and the number of cells which have completely traversed the filter in response to the leucoattractant are microscopically enumerated.

The inherent principle involved in the Boyden chamber is the establishment of a chemotactic factor concentration gradient. At the outset of an experiment the chemotactic factor may be completely localized in the lower chamber. However, with time, a positive concentration gradient develops. Chemotactic factor diffuses across the micropore filter into the cell compartment. Chemotactic factor-cell interaction occurs in this compartment and a migratory response is induced. The extent of cellular migration is dependent on the nature of the concentration gradient of leucoattractant. Optimal migration is normally induced by positive

gradients. If the gradient is negative, deactivation will occur and cell migration is reduced.

At the base of the filter, cells can migrate no further. Presumably cell retention is dependent upon the degree of cell adherence to the filter. The extent of cell migration (as measured by the filter counts) is therefore dependent upon the rate of cell migration and the level of cell adhesion.

The cell response to chemotactic factors in the Boyden chamber is not absolutely uniform, i.e. all cells in the upper chamber capable of eliciting a chemotactic response do not completely traverse the filter. Cells of the same type are seen at both the upper and lower limits of the filter and at varying intra-filter depths. This suggests a differential cellular response to chemotactic factor. Such a phenomenon may be due to variations in cellular intrinsic chemotactic potential. These may correlate with cell maturity or previous exposure to chemotactic factor, which may cause a complete or partial deactivation of the chemotactic response. This could possibly account for the various levels of cell migration observed in the filter.

Dimensional Modifications of Boyden's System

Early modifications of Boyden's technique were mainly devoted to minimizing the cellular and chemo-

tactic factor requirements demanded by this system. Upwards of 5×10^6 PMN cells in 3.5 ml of medium and 3 ml of chemotactic factor were required. Situations in which the leucoattractant potential of a fluid was under assessment, were not particularly problematical. In such cases rabbit peritoneal cells could be used and were available in large numbers. However the application of the technique to clinical situations in humans was somewhat difficult. In cases where the subject of chemotactic investigation was neutropenic or in circumstances which do not permit the withdrawal of large volumes of blood the technique was rather limited.

Subsequent modifications by various workers have reduced the size of the upper and lower chambers and the surface area of the millipore filter. Such modifications have been reported by Ward *et al*, (1966), Kay (1970), Miller (1971) Wilkinson (1971b), and Martin *et al*, (1973) and have permitted reduced cell number requirement and reduced leucoattractant volume. In most clinical investigations such modified chambers are now used.

Modifications associated with cell enumeration

1) Keller *et al*, (1971) demonstrated that cell loss from the lower side of the chemotaxis filter can and does occur. Such loss increases with time. To avoid 'cell-droppage' these authors

suggested a double filter system. Loss of cells was prevented by collecting PMN cells on a cell-impermeable filter, placed underneath the cell-permeable filter. The chemotactic index was assessed by enumerating the cell number on the lower side of the upper filter and on the upper side of the lower filter. The two figures were summed to give the chemotactic index. In this laboratory, however, all droppage from the filter after 3 hours of incubation has been shown to be insignificant as assessed by Coulter counting of cells present in the lower compartment. Furthermore, cell distribution on the lower filter was extremely variable and cell presence may only reflect those areas in which the filters were in opposition.

2) Gallin *et al*, (1973) reported an "improved" *in vitro* millipore technique for the evaluation of chemotaxis. Using this method PMN cells were radio-labelled with Chromium-51 prior to introduction into the cellular compartment of a modified Boyden chamber. A double millipore filter system as described above was used, with the exception that both filters were of 5μ pore diameter. The system was incubated for 3 hours after which time the filters were separated and the radioactivity associated with each filter was determined. Chemotaxis was assessed by the amount of radioactivity associated

with the lower filter.

This technique has certain advantages such as the elimination of staining procedures and removal of observer error. However the same criticisms mentioned above for the double filter systems may apply to this system also. This technique does not permit assessment of intra-filter events in the early stages of cell movement.

3) Zigmond and Hirsch (1973) presented a modified micropore filter technique designed to facilitate the microscopic evaluation of chemotaxis. Test solutions being investigated for chemotactic potential were placed in the lower chamber and cell suspension in the upper chamber. A negative control of a non-chemotactic solution was run in parallel. The chamber incubation period was shortened to allow cellular migration into the filter but not complete filter transpassage by the cells. After a suitable time interval filters were removed, stained and cleared as previously. However, as with previous techniques the filter was not inverted and the lower side quantitated, but was scored by measuring with the optical micrometer on the fine focus knob of the microscope, the distance from the top of the filter to the furthest focal point which contained at least two cells in focus. In positive

controls the distance migrated into the filter by the "fastest moving cell front" was much greater than that of the negative controls. With this technique, results are expressed in microns travelled and not cells per high power field.

With regard to investigations of leucotactic dysfunction, the technique of Zigmond and Hirsch may have certain limitations. Using this method cellular quantitation may be difficult since a number of cells may migrate into the filter to normal depths, thus masking the inability of others to elicit a chemotactic response. The method of Zigmond and Hirsch could be used to assess the quality of the neutrophil response and that of Boyden used quantitatively. This may be an invalid criticism.

Using this technique, Zigmond and Hirsch (1973) have shown that chemoattractants may exert a two-fold effect on neutrophil motility. Leucoattractants in the absence of a gradient (equal concentrations above and below the filter) promote an enhancement of random migration. Such a stimulation of random motility has been termed chemokinesis (Wilkinson, 1975). The extent of chemokinesis is dependent on the concentration of leucoattractant usually increasing with increasing leucoattractant concentrations. However, at high chemoattractant concentrations,

chemokinesis may diminish, presumably due to de-activation. By determining chemokinesis values for a given range of concentrations of a leucoattractant substance it is possible to evaluate the distance migrated on a directional basis. This can be achieved by calculation of the predicted distance of migration for a given positive gradient, assuming migration to be on a random basis, from chemokinesis values observed with leucoattractant concentrations at either limit of the positive gradient and intermediary (Zigmond and Hirsch, 1973). The distance migrated on a truly directional basis, true chemotaxis, is obtained by subtraction of the predicted value (based on chemokinesis) from the observed value for the positive gradient. Positive values indicate a true chemotactic response. This method can be used to evaluate cell capacity to elicit a directional response to a known chemotactic factor. Conversely, the leucotactic potential of a given substance can be investigated.

Up to this point the term chemotaxis, implying a "one-step" directional movement, has been used frequently and possibly incorrectly. It is therefore pertinent to mention that the report of Zigmond and Hirsch (1973) has demonstrated that chemoattractants may influence leucocyte motility in two ways. Apart from a true directional cellular response to a positive concentration gradient

the leucoattractants, in the absence of a gradient, may also promote increased random motility. This type of cell movement is termed chemokinesis. The process of leucocyte accumulation by artificial induction either *in vivo* or *in vitro* is therefore not a single step procedure, but rather an additive effect of chemokinesis and chemotaxis. This would appear to be a biologically sound combination. Chemotaxis should hasten the arrival of cells at an inflammatory site while the stimulated random component may facilitate the circumvention of any mechanical barriers encountered en route and enhance collision with offending particles at the destination point.

Modifications associated with cell application

Baum *et al*, (1971) and Hill *et al*, (1975b) have reported that the cytocentrifuge can be used to apply neutrophils directly to micropore filters. This eliminates abnormal or unequal settling of cells. However, time and speed of centrifugation are critical to achieve a regular distribution on the membrane surface. The author has no personal experience of this technique.

P A R T II

METHODS IN CHEMOTAXIS

1) The Chemotaxis Chamber

Due to uneconomical cell and serum requirements the Boyden technique was considered unsatisfactory. It was therefore decided to adopt a micro-chemotactic technique.

The technique chosen was that introduced by Wilkinson (1971). The chamber used in chemotaxis tests was a simplified version of Boyden's original chamber. The lower compartment was a 5 ml. glass beaker. This was filled with 4 ml. of the chemotactic substance under test. The "sawn-off" barrel of a tuberculin syringe was suspended in this fluid, and a circular filter was glued to its lower end. The type of glue used was commercially available, of the trade name of "Uhu". This glue satisfied 3 criteria (Wilkinson, personal communication, 1973):

a) It was non-toxic to PMN cells.

b) It was non-chemotactic.

c) It was methanol soluble.

Chamber modifications

A number of modifications were introduced to Wilkinson's method.

a) Alterations to the cell compartment

To facilitate filling of the inner chamber the "barrel-length" was reduced to 6 mm to allow the "layering" of cell

suspension onto the millipore filter. This modification was slightly more economical on materials as 2 chambers per syringe could be made.

b) Alterations of fluid compartment

In place of a 5 ml. beaker in which 3 cell chambers were suspended, individual chambers were made. A 5 ml. plastic test tube "push-on" cap was punched with a number 2 cork-borer, and the circular plastic disc removed. The "cell-chamber" (syringe and filter) was fitted into this and was tightly held in place, while permitting vertical adjustment. A 4 ml. autoanalyser tube substituted for the beaker to give a system consisting of one cell chamber/one fluid chamber. The inner chamber freely fitted the outer chamber. This modification permitted ease of manipulation of the inner chamber to equalize inner and outer fluid levels during chamber assembly. Facilitated manipulation of the inner chamber during cell fixation was also allowed.

c) Elimination of air bubbles

A major problem with this technique was the appearance of air bubbles, above and below the filter during chamber filling. This may be caused by air trapped within the filter. This problem was eliminated by pre-filling of the chamber with cell suspending solution. Chambers were allowed to stand for a few minutes, during which time air bubble formation occurred, then emptied and filled with

leucoattractant and cell suspension. No air bubbles formed within the experimentally assembled chambers. This modification, although slightly more time consuming, permitted closer filter-filter agreement.

A diagram of the modified chamber is presented in Figure Ia (See below):

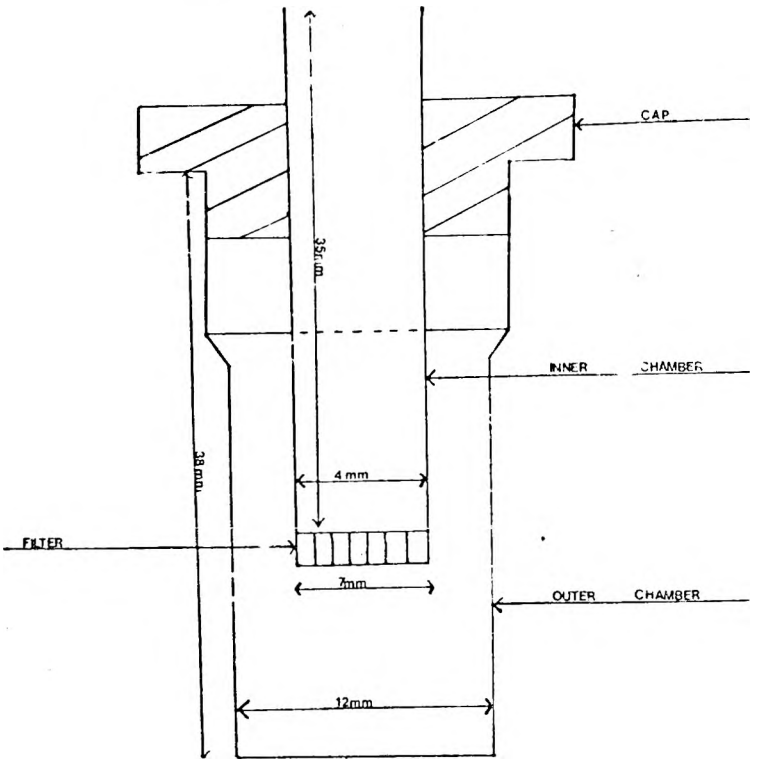


Figure Ia Diagram of the modified chemotaxis chamber.

2) Neutrophil Preparation

Heparinized human venous blood (5 units preservative free heparin, Pan Heparin, per ml. of blood) was centrifuged at 500 g for 10 minutes. The buffy layer cells present at the white-red cell interface and the first few millimetres of the red cell layer were transferred to plastic test tubes. This leucocyte-rich supernatant was sedimented at 37°C for 30 minutes to further remove red blood cells (RBC). After sedimentation the leucocyte-rich plasma was removed and diluted in equal parts with tissue culture medium (TC 199 Grand Island, New York) pH 7.2 and centrifuged for 5 minutes. The supernatant was removed and the cell pellet resuspended and washed twice with TC 199 (500 g for 5 minutes). The cell suspension was adjusted to give an appropriate concentration which was determined by the nature of the leucoattractant or volumes of various drugs added to the cell suspension.

3) Leucoattractant Preparation

Two leucoattractants have been used in this study.

a) Alkali Hydrolysed Casein

Casein was solubilized according to the method of Wilkinson (1972). Commercially available casein "Hammarsten" (Merck) was insoluble in TC 199 at pH 7.2. However, by gently increasing the pH, by drop-wise addition of 1M NaOH, to pH 12, casein solubilized.

The solution was carefully brought back to neutrality with $NaH_2PO_4.2H_2O$. The final concentration of casein was 5 mg/ml. TC 199. The casein solution was then titrated to determine the dilution which induced optimal neutrophil chemotaxis. For all studies in this presentation the same casein preparation was used at an optimal concentration of 600 µg/ml.

b) Endotoxin activated serum (EAS):

This was prepared by the interaction of fresh, normal human serum with a purified endotoxin preparation. Purified bacterial endotoxin was obtained commercially as Difco Lipopolysaccharide (*E.coli* 0127 : B8). The endotoxin was carefully weighed out prior to each experiment and chemotactic activity generated by the addition of fresh serum (500 µg endotoxin/ml. serum). The mixture was incubated at 37°C for 30 minutes to generate chemotactic activity and diluted ¼ by the addition of 3 parts of TC 199. The concentration of EAS in this system which induces optimal neutrophil chemotaxis is 25% (Anderson, 1974).

4) Chemotaxis Assay

Two types of assay were employed:

a) The Boyden assay

Assembly and Incubation of Chambers. The chemotactic chamber used for both assays is based upon the modifications introduced by Wilkinson (1971) of the Boyden

chamber. In the classical Boyden system leucoattractant substances (1 ml. volumes) were introduced to the lower chamber. Numerically standardized neutrophil suspensions were introduced into the upper chamber with a long-needled 1 ml. syringe. A cell volume of 0.2 ml. was carefully layered onto the millipore membranes of pore size 5 μ (Millipore Corp). Chambers were always pre-incubated with cell suspending medium alone, to permit formation and elimination of air bubbles. The chambers were emptied of medium, and cell suspensions and leucoattractants introduced to their respective compartments. The inner chamber (cells) was then fitted into the lower chamber (leucoattractant) and the inner and outer fluid levels equalised. Chambers were incubated at 37°C for three hours, with the exception of kinetic experiments (1-5 hours).

Filter removal and staining. Subsequent to incubation the inner chamber was carefully removed and immersed in methanol for 30 seconds. Methanol caused cell fixation and also dissolved the glue. The filter was easily removed. Filters were rinsed in distilled water and stained in haematoxylin for 1 minute. After staining, filters were rinsed in distilled water.

The subsequent stages were 1 minute in each of:

1. 75% ethanol.
2. 90% ethanol.
3. 80 : 20 ethanol: butanol.
4. 100% ethanol.
5. Xylene.

The first four stages are dehydrating steps, while the final stage causes filter clearing.

Microscopic evaluation of chemotaxis. Following staining, the filters are inverted (so that the bottom side is uppermost), placed on a glass slide, and mounted in xylene. Chemotaxis is assessed under the high power, by counting the number of cells which have completely traversed the filter. Counting is greatly facilitated by the use of an optical grid. The chemotactic index is expressed as cells (neutrophils)/ high power field (HPF). Using this technique the mean filter-filter variation is 17.2% for identical filters.

b) The Method of Zigmond and Hirsch

The modifications of the method of Boyden (1962) as introduced by Zigmond and Hirsch (1973) have been described in Chapter III (Page 41). These relate to manipulations of leucoattractant concentrations in the cell and chemoattractant chambers to discern types of motility, i.e. chemokinesis and directional migration; a shortened incubation period which also does not permit trans-filter passage of cells; and an evaluation of cell motility according to the intra-filter distance attained by the "fastest moving cell front". Chamber assembly and filter staining and processing procedures are identical to those used in Boyden's method.

Experimental Design. To determine the effects of a potential leucoattractant substance on chemokinesis and chemotaxis, dissociation of these two types of motility is necessary. This can be accomplished by the method of Zigmond and Hirsch using the experimental system referred to as "the checkerboard" (Wilkinson, 1975 - personal communication). The experimental design is shown in Table 1.

TABLE I

Experimental Design, with results of a hypothetical Checkerboard

Leucoattractant Concentration (% EAS) above the filter	Leucoattractant Concentration (% EAS) Below the filter: 3	6	12
3	55	78(61)	92(66)
6		70	82(68)
12			65

Results are expressed as microns migrated (μm). Figures within the diagonal lines are experimentally

derived values for chemokinesis (equal concentrations of leucoattractant (EAS), above and below the filter). Those above the diagonals, outside the brackets, are experimental migratory values in positive gradients (higher concentrations of EAS below the filter). Results in parenthesis are expected values for migration in positive gradients (assuming migration to be on a solely random basis) calculated by the method of Zigmond and Hirsch (1973). The extent of directional migration is calculated by subtraction of the expected value from the observed (experimental) value. Results below the diagonal (not shown) represent migratory values in negative gradients (higher concentrations of leucoattractant above the filter).

Calculation of predicted migratory values in positive gradients, using the method of Zigmond and Hirsch, from results shown in Table 1.

There are three positive gradients shown in Table 1.

Positive gradients shown in Table 1

	% EAS above filter	% EAS below filter
A	3	6
B	3	12
C	6	12

Predicted value for Gradient A

Assumptions which have to be made (Zigmond and Hirsch, 1973) are:

1) A linear gradient extends across the filter.

2) Acceleration of cells between two known velocities is constant.

3) Movement of the front two cells of the population can be considered as a single particle moving with a velocity which is a function of the concentration of stimulatory material.

Known values

Depth of filter	=	120 μ
Time of incubation (T)	=	40 minutes (1 unit time)
Velocity in 3% EAS (V_1)	=	55 μm
Velocity in 6% EAS (V_2)	=	70 μm
Velocity in 12% EAS (V_3)	=	65 um

Calculation

For this gradient, (3%/6% EAS) the distance moved will be the sum of the distance migrated in 3% EAS (5μm) plus the increment due to acceleration from 3% EAS → 6% EAS.

∴ Distance travelled

in 1 unit time	=	55 μm + extra distance due to acceleration (μm)

Extra distance migrated due to acceleration from 3% $\longrightarrow$ 6% EAS in 1 unit time

$= V_2 - V_1$

$= 70 - 55$ µm/unit time

$= 15$ µm/unit time

$\therefore$ over 55 µm the extra distance migrated due to acceleration

$= \frac{15 \times 55}{120}$ µm

$= 6.46$ µm

$\therefore$ total distance travelled in 1 unit/time from 3% $\longrightarrow$ 6% EAS

$= 61.46$ µm

Calculation of predicted value for Gradient B

This calculation is more complicated since 3 chemokinesis values must be taken into consideration. Distances migrated in 3% EAS = 55 µm, 6% EAS = 70 µm, 12% EAS = 65 µm; d_1 (the distance from the top of the filter to the place in the filter where the concentration is 6%) will be one third of the way through the filter (40 µm) since a linear gradient has been assumed. The remaining distance d_2 (80 µm), represents the distance of the 6% $\longrightarrow$ 12% EAS gradient.

Known values

d_1 = 40 μm

d_2 = 80 μm

V_1 = 55 μm/unit time

V_2 = 70 μm/unit time

V_3 = 65 μm/unit time

Required:- To calculate the total distance migrated from 3% → 12% EAS in 1 unit time.

The total distance migrated = The distance migrated from 3% ⟶ 6% EAS + extra distance migrated from 6% ⟶ 12% EAS in the time remaining.

$= d_1 + dx$ μm

$= 40 + dx$ μm

Time taken to migrate from 3% ⟶ 6% EAS (T_1) $= \dfrac{d_1}{\frac{V_1 + V_2}{2}}$ unit time

$= \dfrac{40}{62.5}$ unit time

$= 0.610$ unit time

∴ Time remaining (T_2) $= 1 - 0.610$

$= 0.390$ unit time

dx = $(V_2)\ (T_2)$ + increment due to acceleration in T_2

Distance migrated due to acceleration in 1 unit time $= V_3 - V_2$ µm

$= -5$ µm/unit time

$d_2 = 80$ µm

∴ Distance migrated due to acceleration from 6% → 12% EAS $= -5 \times \frac{80}{120}$/unit time

$= -3.33$ µm/unit time

∴ dx = 70 µm/unit time (0.390 unit time + (-3.33 µm/unit time)(0.390 unit time).

$= 27.3$ µm $- 1.3$ µm

$= 26$ µm

∴ total distance migrated $= 40 + 26$ µm

$= 66$ µm

This calculation is a slightly simplified version of the method of Zigmond and Hirsch (1973) but final results are identical.

Calculation of predicted value for Gradient C

This calculation is identical to that for Gradient A with substitution of the appropriate values.

Calculation of true chemotactic increments

This is accomplished by subtraction of the predicted value as calculated above and from the observed value. The true chemotactic increment for migration from 3% → 6% EAS is 78 μm - 61 μm = 17 μm. Similarly the respective values for 3% → 12% and 6% → 12% EAS are 26 μm and 14 μm. Using this system it is possible to assess the potency of a leucoattractant and the precise effects of inhibitors and stimulants of cell motility on random and directed locomotion. Using this technique variation between identical experimental filters is slight (8.8% mean variation).

Assessment of neutrophil random migration

Random migration was assessed in Boyden chambers using the methods of Boyden and Zigmond and Hirsch. No leucoattractants were present in this system. Medium 199 was present above and below the filter. The medium was supplemented with 0.1% bovine serum albumin to enable adequate neutrophil migration (Becker and Ward, 1969). Filter processing and counting procedures were identical to those for chemotaxis.

Intracellular cyclic nucleotide estimations

Neutrophil preparation. Human peripheral blood was collected as previously described and separated on a

Ficoll Hypaque gradient by centrifugation at 600 g for 15 minutes. Mononuclear cells were di carded and the resultant pellet resuspended in physiological saline and sedimented at 37°C for 30 minutes with a 25% volume of 3% gelatin (Difco, Detroit, Michigan). Gelatin is a biologically inert protein and was used in preference to high molecular weight dextrans which stimulate neutrophil phagocytic activity (Sher *et al*, 1974). The neutrophil-rich fraction was removed and centrifuged at 250 g for 10 minutes and the resultant cell pellet treated with 0.83% ammonium chloride at 4°C for 10 minutes to lyse residual erythrocytes. The latter procedure has no effect on neutrophil function (as assessed by effects on migration). However, centrifugation at 600 g for 30 - 40 minutes in Ficoll-Hypaque markedly reduces chemotactic responsiveness. No such effects were observed when the time of centrifugation was reduced to 15 minutes. These procedures have been adopted to minimize non-experimental alterations in cell function.

Experimental system. Subsequent to lysis the remaining cells, which consistently contained 95 - 99% viable neutrophils were washed once and resuspended to 2 x 10^7 neutrophils/ml. Cell suspensions (0.5 ml.) were treated with appropriate concentrations of the materials under investigation to give a final concentration of 10^7 neutrophils/ml.

After varying incubation times (0, 1, 3, 5, 10, 20 40 and 60 minutes) the reaction was terminated by the addition of an equal volume of cold 2% perchloric acid (PCA). Neutrophils in each tube were further disrupted in an MSE ultrasonic disintegrator for 2½ minutes at an amplitude of 30 peak to peak. The specimens were then centrifuged at 500 g for 10 minutes, the pellet discarded and the supernatant restored to pH 7.0 by the addition of 0.3N KOH. Specimens were re-centrifuged for 20 minutes, and were then freeze-dried on a Vertis lyophiliser.

Radioimmunoassay procedures. Freeze-dried specimens were reconstituted in 0.05M sodium acetate buffer, pH 6.2, and aliquots of 0.2 ml. were used for cGMP and 0.1 ml. for cyclic AMP estimations. The Schwarz/Mann cyclic AMP and GMP radioimmunoassay kits and the procedures supplied with each kit were used. Standard curves were run with each assay and were found to be consistently linear. Duplicate samples were assayed and the final results expressed as pico (p) moles/10^7 PMN (polymorphonuclear leucocytes).

The assay procedures for both cAMP and cGMP involve the competitive binding of the succinyl cAMP/cGMP tyrosine methyl ester (I^{125} labelled standard provided) and endogenous cyclic nucleotide for a limited number of binding sites on an antibody specific for cAMP or cGMP. Aliquots of the specimen

(0.2 ml. for cGMP and 0.1 ml. for cAMP) are incubated with 0.1 ml. of antibody and 0.1 ml. of radiolabelled antigen. The final reaction volume is 0.5 ml. Tests are incubated for 18-24 hours at $4^{o}C$. After incubation, a 2.5 ml. volume of 60% saturated ammonium sulphate $[(NH_4)_2SO_4]$ is added to each tube which is allowed to stand at $4^{o}C$ for 10 minutes (protein precipitation). The resultant precipitate is centrifuged at 5 000 g for 20 minutes and the supernatant discarded. The radioactivity associated with the protein precipitate is measured by solid crystal scintillation counting for 1 minute.

The concentration of cyclic nucleotide in the unknown is inversely proportional to the amount of radioactivity in the precipitate. Cyclic AMP and cyclic GMP in the specimen compete with the corresponding radiolabelled standard cyclic nucleotide for the appropriate antibody. The greater the concentration of the antigen in the sample the less radiolabelled standard will bind with the antibody. The $(NH_4)_2SO_4$ precipitates the antigen/antibody complexes, but not the non-protein antigen and the degree of radioactivity in the precipitate reflects the amount of standard (I^{125}) antigen which has been bound.

Calculation of results. For cAMP the known concentrations used for construction of the standard curve are 0.025, 0.050, 0.10, 0.25, 0.50, 1.0, 2.5, 5 and 10 p moles. The known concentrations for the cGMP standard curve are 0.1, 0.2, 0.5, 1.0, 2.0, 4.0 and 10 p moles. Calculation is as follows:

1) Duplicate background counts are averaged and subtracted from average duplicate values for each cyclic nucleotide concentration to give the corrected standard counts.

2) The standard counts are converted to a percent of the corrected trace binding counts. Trace binding samples are those containing radiolabelled cyclic nucleotide and antibody only (no unlabelled antigen present) and should represent the maximum amount of antigen (I^{125}) which can be bound. The concentration of unlabelled cAMP or cGMP is inversely proportional to the % trace binding.

$$\text{i.e. \% of trace binding} = \frac{\text{Standard Count}}{\text{Trace Binding Count}} \times 100$$

For cAMP the results are plotted as % trace binding against p mole/tube on semi-log paper with p mole/tube as the log function. For cGMP the results of the standard curve are plotted on logit-log paper with % trace binding as the logit function against p mole cGMP:tube as the log function.

For each experimental series the same batch of radioimmunoassay kits was used. Standard curves were always included whenever a radioimmunoassay was performed and results calculated from the standard curve. The profiles of the standard curves always confirmed to the specifications of the manufacturer. Standard curves for cAMP were linear for the concentration range 0.2 - 5.0 p moles. Those for cGMP were linear for the complete concentration range (0.1 - 10 p moles). Variation between duplicate counts was small and those with a difference of more than 10% were omitted.

Typical standard curves for cAMP and cGMP (from this investigation) are shown in Figures 1b and 1c respectively.

Assay of Glycolytic activity. The extent of glycolysis was measured by lactate production (Hohorst, 1962) using recognized procedures (Boehinger Mannheim, Biochemical Test Combination). Pure neutrophil suspensions (prepared as for cyclic nucleotide estimations) were used in this study and resuspended to a final concentration of 2×10^7/ml. in 0.15M phosphate buffered saline (PBS) containing 10mM glucose. Each assay tube (performed in triplicate) utilized 6×10^6 PMN (0.3 ml.) in a final volume of 0.5 ml. The remaining 0.2 ml. contained the various agents (leucoattractants or drugs) under investigation. Tubes were incubated at 37°C for various times in

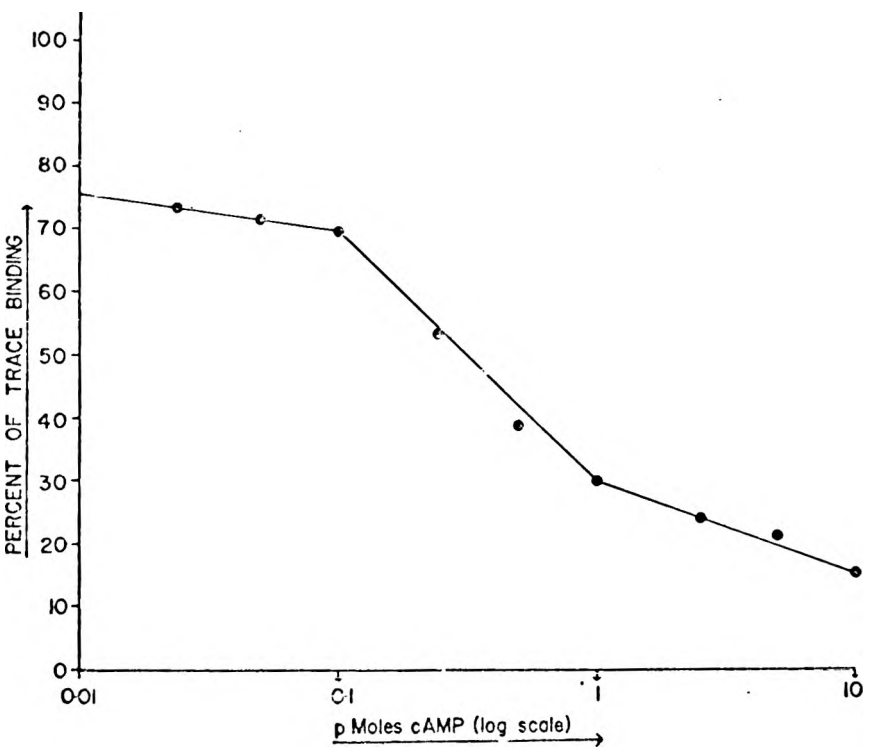


Figure 1b Typical standard curve for the estimation of cyclic AMP.

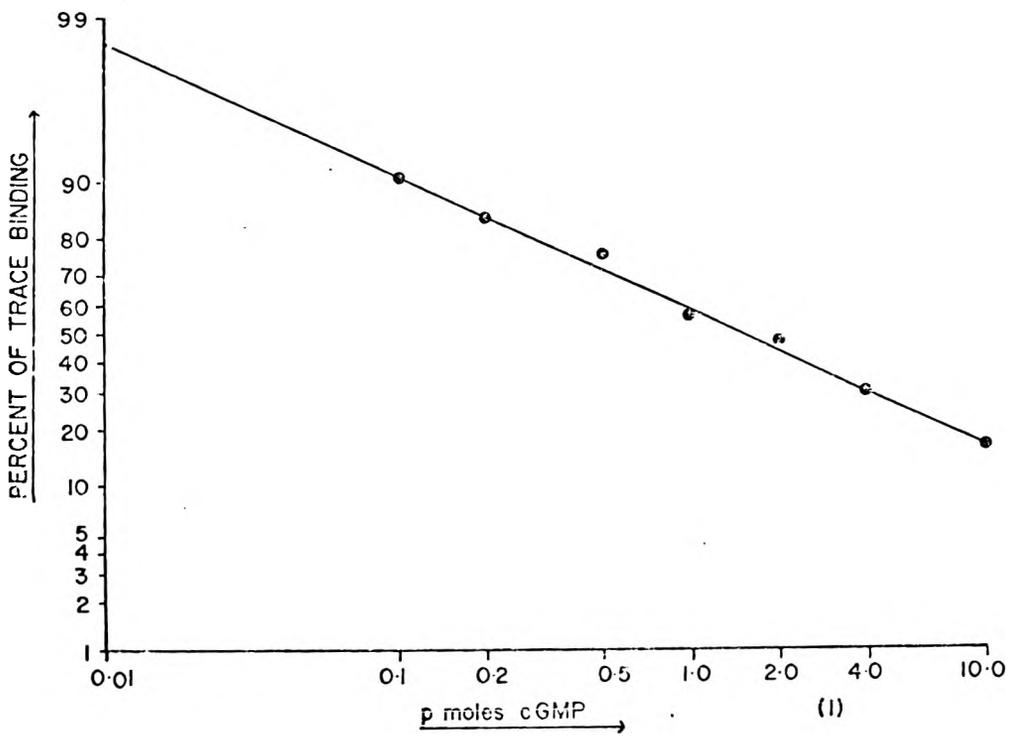


Figure 1c Typical standard curve for the estimation of cyclic GMP,

kinetic experiments (0-60 minutes) or for a fixed time (60 minutes). Following incubation the reaction was terminated and the system deproteinized by the addition of 1.0 ml. of cold 0.6N PCA. Tubes were mixed well and centrifuged at 3,000 rpm for 10 minutes. Aliquots (0.2 ml.) from each tube were assayed for lactic acid.

The principle of the assay is the spectrophotometric change accompanying the reduction of the enzyme cofactor β - nicotinamide adenine dinucleotide (NAD^+) to NADH during conversion of lactate to pyruvate by lactic dehydrogenase (LDH). Each assay tube contains the following:

1. 2.00 ml. of assay buffer (0.5M glycine buffer pH 9.0; 0.4M hydrazine).
2. 0.20 ml. of 27mM NAD.
3. 0.02 ml. of LDH (2 mg LDH/ml.)
4. 0.2 ml. of supernatant fluid after deproteinization.

For blanks 0.20 ml. of 0.6N PCA substitutes for supernatant fluid. Tubes are incubated at 25°C for 60 minutes and the change in optical density at 340nm (ΔE340nm) is assessed spectrophotometrically. Results are expressed as mg.lactate/6 x 10^6 PMN/unit time, by the conversion factor 0.25 x ΔE340 = mg lactate/0.5 ml.

The variability between identical samples was 3.1%.

Assay of Hexose Monophosphate Shunt (HMP) Activity

The extent of HMP activity was measured according to the method of Wood *et al*, (1963) by potassium hydroxide (KOH) absorbtion of $C^{14}O_2$ produced from glucose radiolabelled (C^{14}) in the C_1 position. (Obtained from New England Nuclear, Boston, Massachusetts as D-Glucose 1-^{14}C). Pure neutrophil suspensions were used for this study and resuspended to a final concentration of 2×10^7/ml. in glucose free 0.15M PBS. Each assay was performed in duplicate and utilized 4×10^6 PMN in 0.2 ml. and 0.6 ml. of glucose in PBS containing 0.06 µCi. The remaining 0.2 ml. contained the various agents (leucoattractants or drugs) under investigation..

The test apparatus was a 20 ml. glass scintillation vial (Packard) which served as the outer chamber stoppered with a tightly fitting perforated McCartney top. Placed inside the outer chamber was a 2 ml. autoanalyser cup which served as the inner chamber. The radiolabelled glucose was placed in the outer chamber and 0.6 ml. of 1N KOH in the inner chamber. The outer chamber was stoppered and the apparatus allowed to stand in a 37°C water bath for a few minutes.

The reaction was initiated by the introduction of the cell suspension to the outer chamber by injection through the cap with a long-needled syringe. For kinetic experiments the time range used was from 0-60 minutes; the reaction was terminated by the addition of 2 ml. of 2N HCl to the outer chamber through the cap with a long-needled syringe. The chambers were allowed to stand for 60 minutes to permit release of $C^{14}O_2$ and absorbtion by the KOH. 0.5 ml. volumes of KOH were then transferred to scintillation vials containing 10 ml. of acid instagel (5.5 ml. of 17N HCl/litre instagel (Packard) and speciments counted on a Tri-Carb liquid scintillator for 5 minutes. Results were expressed as corrected mean counts per minute (cpm). Background controls contained radiolabelled glucose and appropriate additives, except neutrophils, and counts obtained were subtracted from the corresponding experimental values. The variability between identical samples for this technique is 7.1%.

Semi-quantitative Nitroblue Tetrazolium Test (N.B.T.)

This test was performed according to the method of Sher *et al*, (1974). Pure neutrophils

(0.2 ml. of a suspension containing 3 x 10^6 PMN/ml.) were mixed with equal volumes of N.B.T. solution (1 mg/ml.) and the appropriate stimulant. Tubes were incubated at 37°C for 30 minutes and smears of the mixtures were made on glass slides after incubation. These were fixed in methanol and stained for 5 minutes in dilute 10% haematoxylin. Slides were evaluated microscopically and results expressed as percentage of N.B.T. (reduced) positive PMN.

C H A P T E R V

The Effects of Exogenous Cyclic Nucleotides on Neutrophil Motility *In Vitro*

INTRODUCTION

The effects of exogenous cAMP on various types of neutrophil motility has been evaluated by a number of research groups using the classical Boyden system. Reports pertaining to the leucoattractant potential of cAMP have been conflicting, although there seems to be agreement in the few reports available that exogenous cAMP mediates an inhibtion of neutrophil chemotaxis. However, effects on random migration are variable. A major criticism of these studies is that the techniques used by these groups do not permit an accurate analysis of the effects on neutrophil locomotion since there is no dissociation of random from directed motility in positive gradients. Zigmond and Hirsch (1973) have stressed the necessity of distinguishing whether altered locomotory responses to a given factor are attributable to either or both of directed and random motility. Cyclic GMP has been reported by Estensen *et al*, (1973) and Hill *et al*, (1975), in different reports from the same laboratory, to enhance neutrophil chemotaxis, but effects on random migration were not described. The aforementioned criticism is also applicable to these reports.

The present study has been motivated by the uncertainty of cyclic nucleotide effects on neutrophil motility; cAMP, d'cAMP and cGMP have been investigated using the classical Boyden technique and the method of Zigmond and Hirsch.

MATERIALS AND METHODS

a) Chemicals

Adenosine 3' : 5' - cyclic monophosphoric acid (cAMP) as the sodium salt and N^6, O^2 - dibutyryl adenosine 3' : 5' - cyclic monophosphoric (d'cAMP) as the monosodium salt were obtained from the Sigma Chemical Company (St. Louis, Missouri) and were of 98% and 97% purity respectively. Guanosine - 3' : 5' - cyclic monophosphoric acid of 98% purity (consistently unavailable from Sigma Chemicals) was supplied by Boehringer Mannheim, South Africa.

Neutrophil preparation: Neutrophils were obtained from 20 ml. of heparinized whole blood (5 units heparin/ml.) donated by healthy male and female laboratory workers. After sedimentation at 37°C the buffy layer was processed as previously described (Chapter IV, page 49).

Leucoattractants: Two chemotactic agents were used in this study.

a) Fresh serum activated by 500 g/ml. of bacterial lipopolysaccharide (E.*coli* 0127 : B8 Difco) (EAS). The mixture was incubated for 30 minutes at 37°C followed by a fourfold dilution with TC 199.

b) Denatured casein at a final concentration of 600 μg/ml. prepared by alkali hydrolysis with subsequent readjustment to pH 7.2. The final cell concentration was 3×10^6 PMN/ml. when EAS was used as leucoattractant and 5×10^6 PMN/ml. when casein was used, the latter being the weaker of the two chemoattractants.

Cell motility studies

Two types of assay were used in these studies.

1) The Boyden assay was used in a first series of experiments to assess the leucotactic potential of cAMP and cGMP. In this study neutrophils were suspended to a final concentration of 5×10^6/ml. in TC 199 supplemented with 0.1% bovine serum albumin (BSA). Cyclic AMP and cyclic GMP were dissolved in the same medium to give a final concentration range of 1×10^{-7}M - 5×10^{-3}M. Protein was added to the TC 199 since the reaction system was protein free and it has been reported by Keller (1966), that protein supplementation is necessary for adequate migration. Cell suspensions (0.2 ml.) were placed in the upper chamber and cyclic nucleotides (1 ml.) in the lower chamber. Chambers were incubated for 3 hours at 37°C. Following incubation, filters were removed, fixed, stained, dehydrated and cleared. Results are expressed as cells/HPF on the lower surface of the filter.

In the second series of experiments the effects of cAMP, d'cAMP and cGMP on neutrophil random migration and chemotaxis to EAS and casein were investigated. Neutrophils were preincubated with the cyclic nucleotides (1×10^{-7}M - 5×10^{-3}M) for twenty minutes prior to introduction to chemotactic chambers. The cells remained in contact with the drugs throughout the incubation period. Final cell concentrations were 3×10^6 PMN/ml for EAS

studies and 5 x 10^6/ml. for random migration and casein chemotaxis. TC 199 was supplemented with 0.1% EAS for random migration studies but not for chemotaxis. Chambers were incubated for 3 hours followed by filter processing and counting.

2) The method of Zigmond and Hirsch was also used to assess the leucotactic potential of cAMP and cGMP. The reaction systems were identical to those employing the Boyden technique, the exception being the time of incubation which was reduced to 40 minutes. To investigate the effects of the cyclic nucleotides on chemotaxis, two fixed positive gradients were selected. Both systems employed 3% EAS above the filter and 6% and 12% EAS for respective systems below the filter. For chemokinesis a fixed identical concentration of EAS on both sides of the filter was selected (3% EAS). In these studies, neutrophils were treated with the cyclic nucleotides for a twenty-minute preincubation period and throughout chamber incubation. EAS was added to the neutrophil-cyclic nucleotide mixture following preincubation to eliminate deactivation as a possible complicating factor.

For reaction systems in which an effect on either chemokinesis or chemotaxis was observed a checkerboard (Chapter IV, page 53) was performed to distinguish the precise types of motility affected. The checkerboard was constructed according to the method of Zigmond and Hirsch (1973). Varying concentrations of EAS (1.5%, 3%,

6%, 12%) were placed above and below the filter in all possible combinations. Chambers were incubated for 40 minutes at 37°C, processed and mounted with the top side uppermost. Neutrophil migration was expressed as the distance travelled (μm) as measured with the optical micrometer on the fine focus of the microscope.

RESULTS

Leucotactic potential of cyclic nucleotides

The results of experiments in which cAMP and cGMP were employed at varying concentrations in the leucoattractant chamber in both assay systems are shown in Table 2 (page 77) and Table 3 (page 78). Results are expressed as mean values for 6 experiments with standard deviations. For all concentrations employed of cAMP and cGMP in both assay systems no significant differences from control values were evident (Student test).

EFFECT OF cAMP, d'cAMP AND cGMP ON RANDOM MIGRATION AND CHEMOTAXIS TO EAS AND CASEIN

The effects of cAMP and cGMP on neutrophil random migration and chemotaxis to both leucoattractants in the Boyden system, and on chemotaxis and chemokinesis as assessed by the method of Zigmond and Hirsch are shown in Table 4 (page 79) and Table 5 (page 80) respectively. Results are expressed as mean values and standard deviations for 6 experiments. For all cAMP and cGMP concentrations in

random and directed motility studies utilizing both assay systems no significant differences from control values were evident. Dibutyryl cAMP appeared to have no effect on chemotaxis in both assay systems, (Table 6, page 81) however, a statistically significant stimulation of random migration (5×10^{-4}M - 2.5×10^{-3}M) and chemokinesis (5×10^{-4}M - 5×10^{-3}M) was evident. To ascertain the precise effects of d'cAMP on neutrophil motility a checkerboard was performed in the presence and absence of 10^{-3}M d'cAMP. This concentration was employed since it mediates maximal stimulation of random migration and chemokinesis ($p = < 0.001$). The results of the experiments utilizing different combinations of EAS above and below the filter are shown in Table 7. It is evident that stimulation of cell movement in the presence of d'cAMP occurs in the absence of a gradient (within the diagonal) and for negative gradients (below the diagonal), but not for positive gradients. These results indicate that d'cAMP produces stimulation of both random migration (35%) and chemokinesis (22%, 39%, 51% and 75% for 1.5%, 3%, 6% and 12% EAS above and below the filter respectively).

To evaluate the effects of d'cAMP on directed motility the true increments for corresponding gradients, in the presence and absence of d'cAMP, were compared. The true increment was taken as the difference between the observed values for positive gradients and the estimated values (in brackets) based on chemokinesis according to the formula of Zigmond and Hirsch (1973). These results are shown in

TABLE 2

Analysis of the Leucotactic Potential of Cyclic AMP (10^{-7}M - 5 x 10^{-3}M) using a) The Method of Boyden and b) The modification of Zigmond and Hirsch

Concentration of Cyclic AMP	Cells HPF/3 hours Mean and Standard Deviation	Intra-filter penetration/μm/40 minutes Mean and Standard Deviation
0	25 ± 5.7	47 ± 7.0
1 x 10^{-7}M	24 ± 6.8	46 ± 6.2
1 x 10^{-6}M	28 ± 7.5	47 ± 6.4
1 x 10^{-5}M	25 ± 6.6	52 ± 8.4
1 x 10^{-4}M	25 ± 6.8	49 ± 6.7
1 x 10^{-3}M	29 ± 8.7	48 ± 6.1
1 x 10^{-3}M	24 ± 2.8	47 ± 7.0
1 x 10^{-3}M	22 ± 1.5	44 ± 8.0

TABLE 3

Analysis of the Leucotactic Potential of Cyclic GMP (10^{-7}M - 5 x 10^{-3}M) using a) The Method of Boyden and b) The Method of Zigmond and Hirsch

Concentration of Cyclic GMP	Boyden System. Cells/HPF/3 hrs. Mean and Standard Deviation	Intra-filter penetration μm/40 minutes Mean and Standard Deviation
0	21 ± 3.8	42 ± 7.3
1 x 10^{-7}M	21 ± 3.1	42 ± 6.7
1 x 10^{-6}M	21 ± 3.1	43 ± 7.9
1 x 10^{-5}M	22 ± 5.5	42 ± 9.4
1 x 10^{-4}M	23 ± 6.8	42 ± 10.3
1 x 10^{-3}M	23 ± 6.0	44 ± 9.0
2.5 x 10^{-3}M	23 ± 6.1	44 ± 7.2
5 x 10^{-3}M	19 ± 3.1	38 ± 9.8

TABLE 4

Effects of Cyclic AMP(10^{-7}M - 5 x 10^{-3}M) on PMN Chemotaxis and Random Migration as measured in the Boyden System and Chemotaxis and Chemokinesis as measured by the Technique of Zigmond and Hirsch

DRUG CONCENTRATION (Molar)	BOYDEN SYSTEM. CELLS HPF/3 HOURS Mean and Standard Deviation			INTRA-FILTER PENETRATION µM/40 MIN. Mean and Standard Deviation		
	CHEMOTAXIS			CHEMOTAXIS		CHEMOKINESIS
	Casein	EAS	RANDOM MIGRATION	3% EAS above and 6% EAS below filter	3% EAS above and 12% EAS below filter	3% EAS above and below filter
0	144 ± 22	216 ± 28	25 ± 5	75 ± 6	81 ± 5	64 ± 6
1 x 10^{-7}M	150 ± 20	224 ± 28	24 ± 4	76 ± 5	81 ± 6	65 ± 6
1 x 10^{-6}M	151 ± 17	217 ± 40	25 ± 4	76 ± 5	82 ± 5	67 ± 7
1 x 10^{-5}M	147 ± 22	226 ± 36	26 ± 6	74 ± 5	81 ± 6	66 ± 6
1 x 10^{-4}M	154 ± 24	220 ± 18	23 ± 4	73 ± 6	81 ± 5	63 ± 5
1 x 10^{-3}M	139 ± 14	196 ± 30	30 ± 4	75 ± 3	82 ± 5	64 ± 5
1 x 10^{-3}M	145 ± 14	222 ± 44	26 ± 3	75 ± 4	80 ± 6	65 ± 5
1 x 10^{-3}M	135 ± 22	192 ± 23	24 ± 5	70 ± 5	78 ± 5	66 ± 5

TABLE 5

Effects of Cyclic GMP (10^{-7}m - 5 x 10^{-3}M) on PMN Chemotaxis and Random Migration as measured in the Boyden System and Chemotaxis and Chemokinesis as measured by the Technique of Zigmond and Hirsch

DRUG CONCENTRATION (Molar)	BOYDEN SYSTEM. CELLS HPF/3 HOURS Mean and Standard Deviation			INTRA-FILTER PENETRATION µM/40 MIN. Mean and Standard Deviation		
	CHEMOTAXIS			CHEMOTAXIS		CHEMOKINESIS
	Casein	EAS	RANDOM MIGRATION	3% EAS above and 6% EAS below filter	3% EAS above and 12% EAS below filter	3% EAS above and below filter
0	149 ± 25	233 ± 28	25 ± 3	71 ± 5	78 ± 5	63 ± 6
1 x 10^{-7}	144 ± 26	240 ± 34	27 ± 4	72 ± 4	79 ± 5	64 ± 4
1 x 10^{-6}	145 ± 12	230 ± 30	29 ± 5	71 ± 5	78 ± 5	64 ± 4
1 x 10^{-5}	148 ± 23	235 ± 31	26 ± 6	71 ± 6	79 ± 6	65 ± 5
1 x 10^{-4}	160 ± 34	230 ± 31	30 ± 5	72 ± 6	80 ± 5	66 ± 5
1 x 10^{-3}	157 ± 20	230 ± 27	27 ± 4	74 ± 5	82 ± 5	66 ± 8
2.5 x 10^{-3}	146 ± 15	234 ± 28	24 ± 3	74 ± 5	82 ± 5	64 ± 7
5 x 10^{-3}	143 ± 24	237 ± 26	23 ± 4	73 ± 7	80 ± 7	63 ± 4

TABLE 6

Effects of Dibutyryl cyclic AMP (10^{-6}M - 5 x 10^{-3}M) on PMN Chemotaxis and Random Migration as measured in the Boyden System and Chemotaxis and Chemokinesis as measured by the Technique of Zigmond and Hirsch

DRUG CONCENTRATION (Molar)	BOYDEN SYSTEM. CELLS HPF/3 HOURS Mean and Standard Deviation			INTRA-FILTER PENETRATION µM/40 MIN. Mean and Standard Deviation		
	CHEMOTAXIS			CHEMOTAXIS		CHEMOKINESIS
	Casein	EAS	RANDOM MIGRATION	3% EAS above and 6% EAS below filter	3% EAS above and 12% EAS below filter	3% EAS above and below filter
0	151 ± 22	230 ± 29	24 ± 5	71 ± 7	82 ± 6	63 ± 5
1 x 10^{-7}	143 ± 24	234 ± 38	25 ± 4	70 ± 4	81 ± 5	65 ± 5
1 x 10^{-6}	151 ± 23	244 ± 21	25 ± 6	71 ± 7	82 ± 6	65 ± 6
1 x 10^{-5}	169 ± 31	242 ± 33	28 ± 6	72 ± 4	85 ± 8	68 ± 4
1 x 10^{-4}	146 ± 31	221 ± 42	31 ± 5	75 ± 5	86 + 9	75 ± 7
1 x 10^{-3}	163 ± 27	212 ± 33	37 ± 5	74 ± 4	85 ± 5	80 ± 5
2.5 x 10^{-3}	138 ± 20	228 ± 21	39 ± 5	70 ± 7	84 + 7	78 ± 4
5 x 10^{-3}	136 ± 18	213 ± 30	31 ± 4	70 ± 5	79 + 6	72 ± 6

TABLE 7

The Effects of Dibutyryl cAMP on PMN Chemokinesis and True Chemotaxis using EAS as Leucoattractant

a) Without Dibutyryl cAMP

% EAS above the filter \ % EAS below filter	0	1.5	3	6	12
0	26				
1.5		63	66(62)	70(61)	71(61)
3		58(62)	61	69(59)	71(59)
6		50(59)	52(59)	57	63(57)
12		53(59	54(58)	55(57)	57

b) 10^{-3}M dibutyryl cAMP above and below the filter

% EAS above the filter \ % EAS below filter	0	1.5	3	6	12
0	35				
1.5		77	81(81)	79(81)	84(86)
3		76(81)	85	93(86)	91(89)
6		77(83)	86(86)	86	95(96)
12		83(90)	89(91)	91(93)	00

The effect of varying concentration gradients and absolute concentration of EAS on the motility of human neutrophils in the presence and absence of 10^{-3}M d'cAMP. Figures along the diagonal from upper left to lower right show the distance migrated (μm) in increasing concentrations of the leucoattractant in the absence of a concentration gradient. Above the diagonal, cells are moving in a positive gradient, and below the diagonal in a negative gradient. The figures in brackets are estimates of what migration would have been in each of the tests, assuming that the cells detected the absolute concentration of the chemoattractant but not the gradient.

TABLE 8

Analysis of the Effects of Dibutyryl cAMP on True Chemotaxis.

Data recorded in Table 7

Positive Gradients		TRUE CHEMOTACTIC INCREMENTS		Inhibition of True Chemotaxis %
% EAS above filter	% EAS below filter	Control	10^{-3}M Dibutyryl cAMP	
1.5	3	4	0	100
1.5	6	9	-2	100
1.5	12	10	-2	100
3	6	10	7	30
3	12	12	2	83
6	12	6	-1	100

Table 8 (page 83). Dibutyryl cAMP causes a marked reduction of directed motility. These results are highly statistically significant ($p < 0.001$ comparing all chemotactic increments for the control group with those of the d'cAMP treated group).

DISCUSSION

A number of authors have reported neutrophil chemotactic activity for cAMP (Leahy *et al*, 1970; Gammow *et al*, 1971; and Rivkin and Becker, 1976); these findings have been disputed by others. In the present study no leucotactic activity could be demonstrated for cAMP or cGMP at fairly wide concentration ranges, encompassing cAMP concentrations which have previously been reported to be chemotactic. As well as the Boyden system the method of Zigmond and Hirsch was also employed to assess leucotactic activity at a shortened time interval. The results of both techniques were confirmatory. The difference in results between this study and those in which cAMP has been reported as being chemotactic are hard to explain. Identical filter assay systems and indicator cell types have been used by groups who have reported opposite findings.

Similarly no effects on neutrophil random migration or chemotaxis to casein and EAS could be detected by treatment of neutrophils with cAMP or cGMP. The concentration ranges employed for both cyclic nucleotides included those which have been previously reported to exert effects on neutrophil motility. Inhibition of neutrophil chemotaxis by cAMP has been reported by Rivkin and Becker (1976) and Hill *et al*, (1975). (Inhibition reported by Rivkin and

Becker was variable and only evident at high concentrations; Hill *et al*, reported striking inhibition at low concentrations). Tse *et al*, (1972) have reported that cAMP mediates inhibition of random motility (d'cAMP is without effect) whereas Rivkin and Becker (1976) have reported stimulation of neutrophil random motility by both cAMP and d'cAMP. Although cAMP and cGMP were without effect in this study, d'cAMP on the other hand exerted a two-fold effect on cell motility, as indicated by checkerboard results. A marked inhibition of true chemotaxis was evident with a concomitant stimulation of random motility and chemokinesis. These results are in agreement with those of Rivkin and Becker (1976) who reported essentially similar findings for d'cAMP over an identical concentration range.

The cGMP results are at variance with those of Estensen *et al*, (1973) and Hill *et al*, (1975) who reported stimulation of neutrophil chemotaxis by cGMP.

In conclusion this study has indicated that the cyclic nucleotides cAMP and cGMP are not chemotactic, neither do they affect neutrophil random migration and chemotaxis. This may reflect absence of intracellular penetration (at least of cAMP) due to poor interaction of charged cyclic nucleotide with the hydrophobic cell membrane (Henion *et al*, 1967). Dibutyryl cAMP is a more lipid soluble congener of cAMP, and the effects of this agent may reflect a differential permeability. The effects of cAMP elevating agents on neutrophil motility

are discussed at greater length in Chapter VII and those of cGMP in Chapter VI.

Finally, the results of this study indicate the necessity of using the method of Zigmond and Hirsch to enable correct interpretation of experimental data. This is evident for d'cAMP in the Boyden system where inhibition of chemotaxis was masked by a stimulation of random migration. However, data obtained from the checkerboard experiment revealed the true situation.

C H A P T E R VI

Effects of Levamisole, Acetyl Choline and Carbamyl Choline on Neutrophil Motility *in vitro*

INTRODUCTION

Levamisole (LMS) a potent anti-helminthic agent, has been reported to possess immunostimulatory properties with regard to cell mediated immunity (Renoux and Renoux, 1972; Tripodi *et al*, 1973; and Hirshaut *et al*, 1973). In addition, stimulation of phagocytic activity of neutrophils (De Cree *et al*, 1974) and macrophages (Hoebeke and Franchi, 1973) has been reported. Preliminary data reported by Hill and Quie (1975) indicate that levamisole consistently increased the chemotactic responsiveness of neutrophils from patients with defective leucocyte motility. The same group (Estensen *et al*, 1973 and Hill *et al*, 1975) has reported that agents which promote elevation of intracellular cGMP levels, such as acetyl choline and carbamyl choline, promote increased neutrophil chemotaxis. This study examines the effect of levamisole, acetyl choline and carbamyl choline on neutrophil random migration and chemotaxis. Checkerboards, where justified, were performed to assess types of motility affected.

It has previously been reported that LMS increases intracellular levels of cGMP in murine lymphocytes and it was of interest to determine if the drug was acting in a similar way on human PMN. For this reason, intracellular cyclic nucleotide estimations have been

performed on chemotactically stimulated cells in the presence and absence of LMS.

MATERIALS AND METHODS

Levamisole (L-2,3,5,6-tetra-hydro-6-phenylimidazo (2,1-b)thiazole)was supplied by Ethnor Laboratories, Johannesburg, South Africa. Acetyl choline (99% purity) and carbamyl choline (99% purity) were obtained from the Sigma Chemical Company (St. Louis, Missouri).

Leucoattractants

Two chemotactic agents were used in this study.

a) EAS

b) Alkali hydrolysed casein (600 µg/ml)

The preparation of both leucoattractants has been described in Chapter IV (Page 49).

Cell motility studies

The experimental design was identical to that in Chapter V for both acetyl choline and carbamyl choline. Random migration and chemotaxis to EAS and casein was assessed using the method of Boyden. Chemotaxis (using two fixed positive gradients of EAS) and chemokinesis (using 1 fixed equal concentration of EAS above and below the filter) were also investigated, using the method of Zigmond and Hirsch. Preliminary levamisole

effects were explored using only the Boyden system. For levamisole studies the cell concentration used for random migration and chemotaxis to casein was adjusted to 3×10^6 PMN/ml. to permit comparison between the three experimental groups (random migration, chemotaxis to EAS and casein). The effects of levamisole on the kinetics of neutrophil chemotactic responsiveness to EAS were also performed at 1, 2, 3, 4 and 5 hours.

Three checkerboards, employing varying concentrations in all possible combinations of EAS (1.5%, 3%, 6% and 12%) above and below the filter were also performed to assess the exact effects of levamisole (10^{-3}M) on neutrophil motility.

The concentration ranges investigated were 1×10^{-7}M - 5×10^{-3}M for acetyl choline and carbamyl choline, and 1×10^{-9}M - 1×10^{-2}M for levamisole. The drugs were preincubated with neutrophils for 20 minutes prior to addition to chambers and remained in contact throughout. For checkerboard studies EAS was added to the cells prior to addition to the chambers.

Using these techniques, the normal range for chemotaxis using EAS as leucoattractant was 180 - 240 cells/HPF, using casein 60 - 100 cells/HPF and in random migration systems, 15 - 30 cells/HPF.

Pulsing experiments

A number of pulsing experiments were performed in which PMN prior to chemotactic testing, were treated with 10^{-3}M LMS for 60 minutes after which time they were washed twice, resuspended to a concentration of 3 x 10^{6}/ml and placed in the upper compartment of the chemotaxis chamber. Positive and negative controls consisted of PMN which were identically processed. Negative controls underwent no exposure to LMS whereas 10^{-3}M LMS was added to positive controls prior to chemotactic stimulation and was present throughout the incubation period.

In addition, in another series of experiments PMN were treated with levamisole + EAS (25%) or with EAS alone. After 60 minutes these cells were washed twice and tested for chemotactic responsiveness to EAS. PMN were also pulsed for 60 minutes with levamisole + 25% fresh autologous serum or with serum alone, and after washing twice were tested for chemotactic ability to EAS.

Effect of chemotactic factor with or without LMS on neutrophil intracellular cyclic nucleotide levels

To exclude the effects of endotoxin on intracellular cyclic nucleotide levels, EAS was centrifuged in an Amicon Centriflo membrane (Amicon Corporation, Lexington, Massachusetts) which retains molecules

above 50,000 molecular weight. The resultant filtrate possessed high chemotactic activity and suitable dilutions were used in subsequent experiments. Casein was not fractionated.

Peripheral blood was collected as previously described and separated in a Ficoll-Hypaque gradient. Mononuclear cells were discarded and the resultant pellet was resuspended in physiological saline and sedimented with a 25% volume of 3% gelatin (Difco Detroit, Michigan) for 45 minutes. The neutrophil-rich layer was collected, centrifuged at 250 g for 10 minutes and the resultant cell pellet treated with 0.83% ammonium chloride at 4°C for 10 minutes to lyse residual erythrocytes. The remaining cells which consistently contained greater than 90% viable PMN were washed in medium 199 and resuspended to a final volume of 2×10^7 PMN/ml. Cell suspensions (0.5 ml.) were treated with 0.5 ml. of medium 199 containing ("C5a" or casein) and were incubated in a shaking water bath at 37°C. After varying incubation times (0, 1, 3, 5, 10, 20, 30, 45 and 60 minutes) the reaction was terminated by the addition of an equal volume of cold 2% perchloric acid. PMN in each tube were were disrupted in an MSE ultrasonic disintegrator for 2½ minutes at an amplitude of 30 μ peak to peak. The specimens were then centrifuged at 5,000 g for 10 minutes, the pellet discarded and the supernatant restored to pH 7.0 by the addition of 0.3N KOH.

Specimens were dried on a Vertis lyophyliser. Specimens were reconstituted in 0.05M sodium acetate buffer, pH 6.2 and aliquots of 0.2 ml. were used for cyclic GMP and 0.1 for cyclic AMP estimations. The Schwarz/Mann cyclic AMP and GMP radioimmunoassay kits and the procedures supplied with each kit were used. Standard curves were run with each assay and were found to be consistently linear. Duplicate samples were assayed and the final results expressed as p moles/10^7 PMN.

RESULTS

As can be seen in Tables 9 and 10 (pages 97 and 98) acetyl choline and carbamyl choline at all concentrations tested in both assay systems exerted no detectable effects on neutrophil motility.

However, as can be seen in Fig.II (Page 99) parallel assessment of the effect of different concentrations of LMS on both chemotaxis and random migration indicates that the drug at concentrations of 10^{-3}M and 10^{-4}M when added to PMN in the upper compartment of the chamber consistently induced increased chemotaxis to both EAS and casein. Random migration was also increased at these concentrations. The extent of stimulation for all three groups at both LMS concentrations is statistically significant ($p = <0.001$). The mean stimulation values (%) with ranges are shown in Table II. (See page 93)

TABLE II

Mean Values and Ranges (%) of Levamisole Stimulation of Random Migration and Chemotaxis to EAS and Casein

Levamisole Concentration	% Stimulation of Chemotaxis EAS	Casein	% Stimulation of Random Migration
10^{-3}M	72(50-120)	70(41-108)	120(60-153)
10^{-4}M	37(30-65)	40(28-59)	72(46-102)

The effect of levamisole on neutrophil chemotaxis to EAS over a closer concentration range (10^{-5}M - 10^{-2}M) is shown in Figure III (page 100). As can be seen stimulation of chemotaxis is linear from 10^{-5}M - 2.5 x 10^{-3}M. As was previously observed, inhibition of motility was evident at 10^{-2}M.

The results of kinetic experiments using a fixed levamisole concentration (10^{-3}M) are shown in Figure IV (page 101). Results are expressed as mean values of triplicate filters of one experiment only. The stimulation produced by levamisole was noticeable after 1 - 2 hours and reached its maximum at 3 hours.

The results of the experiments utilising different combinations of EAS above and below the filter in the presence and absence of 10^{-3}M levamisole are shown in Tables 12a, 12b and 12c (pages 102, 103 and 104 respectively). From these results it is evident that

stimulation of cell movement in the presence of LMS occurs in both positive (above the diagonals) and negative (below the diagonals), and in the absence of a gradient (within the diagonals). The data recorded in these tables on the effects of 10^{-3}M levamisole on chemokinesis and true chemotaxis are analysed and summarized in Table 13 (Page 105). Statistical analysis of the combined increments of chemokinesis for all chemokinesis systems, and true chemotactic increments for all positive gradients indicate that the differences are highly significant ($p = < 0.001$ for both groups). The method of calculation of true chemotactic increments is shown in Chapter IV (Page 59).

Pulsing experiments

To further understand the nature of the LMS cell interaction, pulsing experiments were performed. As can be seen in Table 14 enhancement of chemotaxis was dependent upon the presence of LMS in the system containing the PMN cells during chemotactic stimulation. Preincubation of cells with LMS followed by washing after 60 minutes resulted in no subsequent stimulation. However, when the cells were pulsed (60 minutes) with LMS in the presence of a small concentration of EAS or fresh serum, subsequent chemotactic enhancement was observed. This treatment

with LMS abolished the inhibition due to "deactivation" which was noted when the cells were pulsed with EAS alone. The level of stimulation produced by pulsing PMN with LMS and EAS was not, however, as great as that which was observed when LMS alone was present throughout the incubation period ($p < 0.001$).

Effect of LMS and partially purified serum derived chemotactic factor and casein on cyclic nucleotide levels

As can be seen in Fig. V which is a mean of six similar experiments (with standard error of the mean) when purified PMN were treated with serum derived chemotactic factor an initial elevation of intracellular cAMP occurs after 1 - 3 minutes with subsequent restoration to basal levels. The regression coefficients with standard deviations in the presence and absence of LMS were $- 0.033 \pm 0.017$ and $- 0.035 \pm 0.021$ respectively, (regression analysis). These were not significantly different from each other nor were they different from zero.

Intracellular cGMP levels (Fig. V) after an initial slight depression which corresponded to the cAMP rise showed a peak of synthesis at 3 - 5 minutes and gradually diminished over 60 minutes.

Regression analysis of cGMP levels in the presence and absence of LMS yielded regression coefficients of -0.0075 ± 0.0033 and -0.0139 ± 0.0037 respectively, indicating similar rates of depletion. These values were not significantly different from each other but were significantly different from zero with p values of <0.02 for LMS and <0.001 in the absence of LMS. However the cGMP values and 5, 10, 20, 40 and 60 minutes were significantly different in the presence and absence of LMS ($p < 0.001$).

Casein, on the other hand, mediated no significant elevation of cAMP at any time interval tested (results shown in Figure VI page 108). The regression coefficients with standard deviations in the presence of levamisole were -0.0223 ± 0.014 and 0.0165 ± 0.0156 respectively. These were not significantly different from each other nor were they different from zero. Intracellular cGMP profiles resembled those of fractionated EAS, although no peak of synthesis was evident. Regression analysis of cGMP levels in the presence and absence of LMS gave regression coefficients of -0.011 ± 0.004 and -0.008 ± 0.0038 respectively, indicating similar rates of depletion. However, the cGMP values at 5, 10, 20, 40 and 60 minutes were significantly different in the presence and absence of LMS ($p < 0.001$).

TABLE 9

The Effects of Acetyl Choline on PMN Random Migration and Chemotaxis to Casein and EAS as Evaluated by the Boyden Technique and Chemotaxis and Chemokinesis as measured by the Method of Zigmond and Hirsch

Drug Concentration (Molar)	BOYDEN SYSTEM, CELLS/HPF/3 HOURS MEAN AND STANDARD DEVIATION			INTRA-FILTER PENETRATION μM/40 MIN. MEAN AND STANDARD DEVIATION		
	CHEMOTAXIS			CHEMOTAXIS		CHEMOKINESIS
	Casein	EAS	Random Migration	3% EAS above and 6% EAS below filter	3% EAS above and 12% EAS below filter	3% EAS above and below filter
0	135 ± 21	212 ± 31	28 ± 6	69 ± 8	81 ± 9	61 ± 6
1×10^{-7}	142 ± 26	206 ± 32	22 ± 7	70 ± 7	79 ± 9	61 ± 5
1×10^{-6}	129 ± 30	198 ± 27	23 ± 6	71 ± 8	78 ± 9	60 ± 7
1×10^{-5}	136 ± 21	203 ± 26	20 ± 8	72 ± 6	78 ± 8	58 ± 6
1×10^{-4}	138 ± 17	221 ± 22	25 ± 9	73 ± 8	76 ± 6	62 ± 4
1×10^{-3}	141 ± 19	219 ± 31	26 ± 5	72 ± 9	77 ± 9	63 ± 7
5×10^{-3}	129 ± 25	232 ± 29	22 ± 4	70 ± 5	79 ± 7	62 ± 6
5×10^{-3}	135 ± 32	221 ± 19	25 ± 8	70 ± 8	80 ± 9	60 ± 5

TABLE 10

The Effects of Carbamyl Choline on PMN Random Migration and Chemotaxis to Casein and EAS as Evaluated by the Boyden Technique and Chemotaxis and Chemokinesis as measured by the Method of Zigmond and Hirsch

Drug Concentration (Molar)	BOYDEN SYSTEM, CELLS/HPF/3 HOURS MEAN AND STANDARD DEVIATION CHEMOTAXIS			INTRA-FILTER PENETRATION μM/40 MIN. MEAN AND STANDARD DEVIATION CHEMOTAXIS		CHEMOKINESIS
	Casein	EAS	Random Migration	3% EAS above and 6% EAS below filter	3% EAS above and 12% EAS below filter	3% EAS above and below filter
0	161 ± 31	243 ± 31	26 ± 6	64 ± 6	70 ± 8	55 ± 7
1×10^{-7}M	150 ± 27	248 ± 36	25 ± 8	66 ± 8	71 ± 9	54 ± 5
1×10^{-6}M	163 ± 30	232 ± 28	24 ± 6	65 ± 7	69 ± 8	53 ± 4
1×10^{-5}M	162 ± 25	226 ± 24	19 ± 5	63 ± 6	62 ± 6	55 ± 6
1×10^{-4}M	148 ± 19	261 ± 25	26 ± 4	62 ± 5	68 ± 9	56 ± 7
1×10^{-3}M	156 ± 28	240 ± 26	27 ± 4	61 ± 6	69 ± 8	55 ± 5
2.5×10^{-3}M	142 ± 27	231 ± 27	28 ± 6	62 ± 6	66 ± 7	54 ± 5
5×10^{-3}M	155 ± 25	239 ± 28	25 ± 6	63 ± 6	68 ± 8	57 ± 6

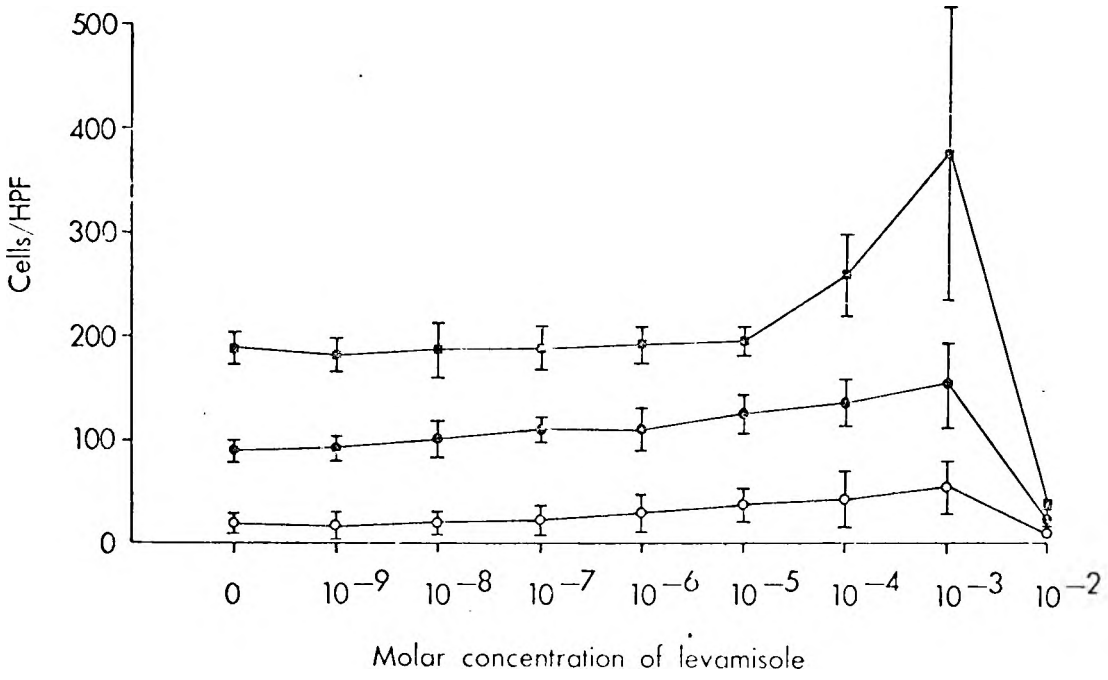


Figure II Effect of levamisole(10^{-9}M - 10^{-2}M) on neutrophil random motility (o) and chemotaxis to casein (●) and EAS (■). Results are expressed as mean and standard deviation.

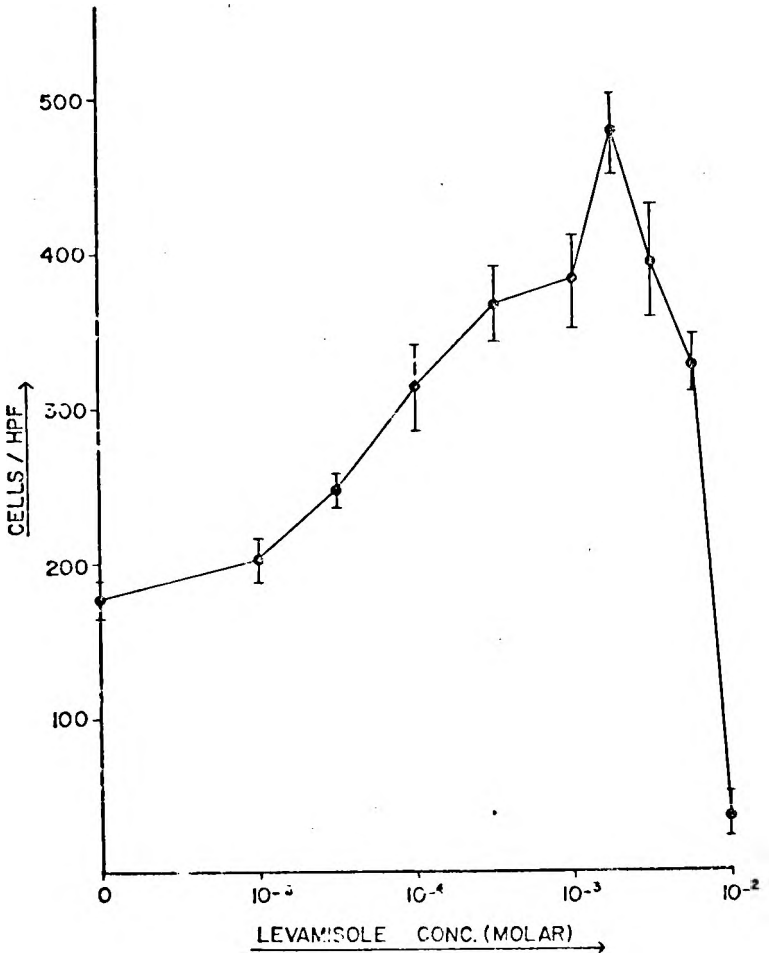


Figure III Effect of levamicole (10^{-5}M - 10^{-2}M) on neutrophil chemotaxis to EAS. Results are expressed as mean and standard error.

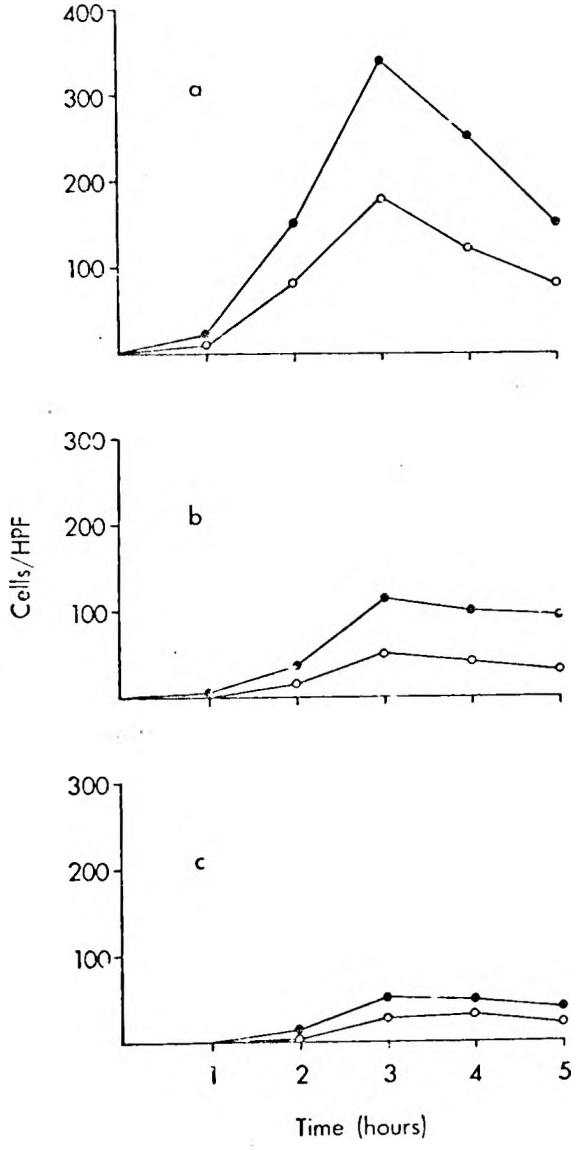


Figure IV Kinetics cf neutrophil chemotaxis to a) EAS, b) casein and c) random migration, in the presence of 10^{-3}M levamisole.

TABLE 12a

The Effects of Levamisole on PMN Random Migration, Chemokinesis and True Chemotaxis using EAS as Chemoattractant

Experiment 1

a) Control

% EAS below filter

	0	1.5	3	6	12
0	40				
1.5		56	61(57)	72(61)	79(61)
3		51(58)	60	72(66)	78(66)
6		59(64)	65(66)	71	77(72)
12		63(64)	62(70)	71(72)	72

b) 10^{-3}M levamisole on both sides of the filter

% EAS below filter

	0	1.5	3	6	12
0	54				
1.5		80	86(80)	96(80)	108(8
3		71(80)	80	105(80)	112(80
6		75(80)	75(80)	80	111(80
12		66(80)	66(80)	84(80)	80

The effect of varying concentration gradients and absolute concentration of EAS on the motility of human neutrophils in the presence and absence of 10^{-3}M levamisole. Figures along the diagonal from upper left to lower right show the distance migrated (μm) in increasing concentrations of the leucoattractant in the absence of a concentration gradient. Above the diagonal, cells are moving in a positive gradient, and below the diagonal in a negative gradient. The figures in brackets are estimates of what migration would have been in each of the tests, assuming that the cells detected the absolute concentration of the chemoattractant but not the gradient. The same applies for the following two checkerboards.

TABLE 12b

The Effects of Levamisole on PMN Random Migration, Chemokinesis and True Chemotaxis using EAS as Chemoattractant

Experiment 2

a) Control

	% EAS below filter				
	0	1,5	3	6	12
0	45				
1.5		61	74(64)	78(64)	79(64)
3		66(64)	67	72(65)	84(64)
6		57(63)	56(64)	61	67(63)
12		52(63)	56(64)	60(63)	64

b) 10^{-3}M levamisole on both sides of the filter

	% EAS below filter				
	0	1.5	3	6	12
0	56				
1.5		74	88(81)	100(80)	100(8
3		73(81)	84	92(84)	100(8
6		71(83)	80(83)	83	93(8
12		70(83)	70(83)	80(83)	83

TABLE 12c

The Effects of Levamisole on PMN Random Migration, Chemokinesis and True Chemotaxis using EAS as Chemoattractant

Experiment 3

a) Control

	% EAS below filter				
	0	1.5	3	6	12
0	30				
1.5		55	62(59)	72(60)	78(61)
3		59(59)	63	74(67)	80(68)
6		62(65)	64(67)	70	72(69)
12		60(64)	61(67)	63(68)	66

b) 10^{-3}M levamisole on both sides of the filter

	% EAS below filter				
	0	1.5	3	6	12
0	42				
1.5		72	85(75)	99(78)	102(75)
3		69(75)	77	93(83)	103(82)
6		76(83)	78(83)	86	89(81)
12		70(81)	72(76)	73(81)	78

TABLE 13

Analysis of the Effects of 10^{-3}M Levamisole on PMN Random Migration, Chemokinesis and True Chemotaxis from Data recorded in Tables 12a, 12b and 12c.

Type of Motility	Random Motility and Chemokinesis (No gradient of EAS present) % EAS above filter	% EAS below filter	% Stimulation of Control Random Motility/Chemokinesis Replicate Experiments 1	2	3	Mean Stimulation %
a) Random Migration	0	0	35	24	40	33
b) Chemokinesis	1.5	1.5	43	21	31	32
	3	3	33	25	22	27
	6	6	13	36	23	24
	12	12	11	30	18	20
	Chemotaxis (Positive EAS gradients present) % EAS above filter	% EAS below filter	% Stimulation of Control True Chemotaxis Replicate Experiment 1	2	3	Mean Stimulation %
b) True Chemotaxis	1.5	3	100	0	230	110
	1.5	6	77	33	38	49
	1.5	12	55	33	59	49
	3	6	317	0	45	121
	3	12	83	0	62	48
	6	12	520	150	100	257

TABLE 14

Results of a Series of 6 Experiments showing the Effect on PMN Chemotactic Responsiveness to EAS after pre-treating cells with LMS (10^{-3}M) and/or EAS or Serum

Upper Chamber	Mean Cells per HPF	Range	p value
PMN + M 199	131	104-153	
PMN + LMS	251	204-260	<0.001
PMN pulsed with LMS (60 min.)	130	96-158	NS
PMN pulsed with EAS (60 min.)	62	33-74	<0.001
PMN pulsed with EAS + LMS (60 min.	193	164-277	<0.001
PMN pulsed with serum (60 min.)	131	80-210	NS
PMN pulsed with serum + LMS (60 min.)	189	95-219	<0.001

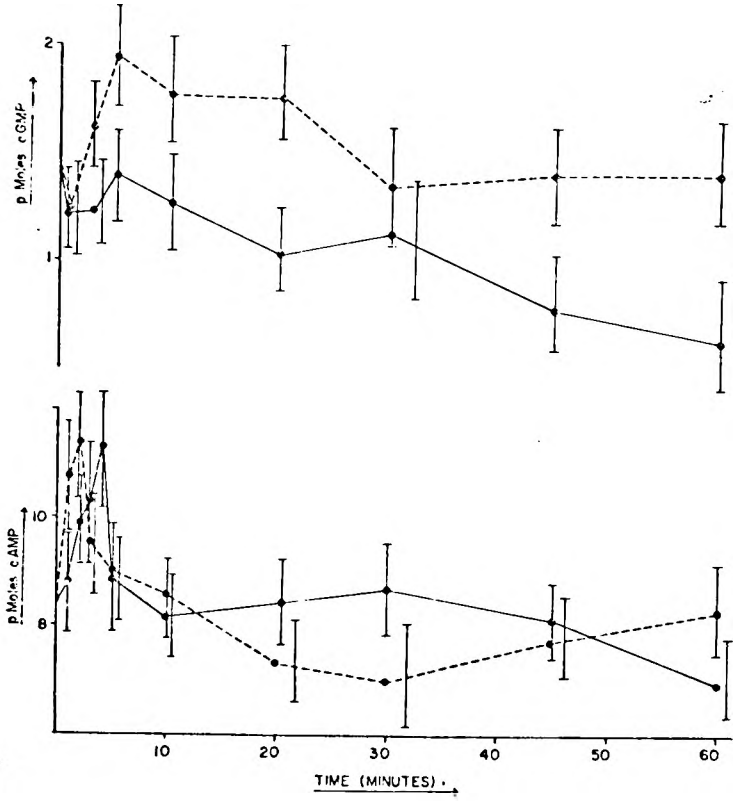


Figure V Effects of serum derived chemotactic factor (EAS) in the presence (---) and absence (——) of 10^{-3}M levamisole on neutrophil cAMP and cGMP levels. Results are expressed as mean and standard error.

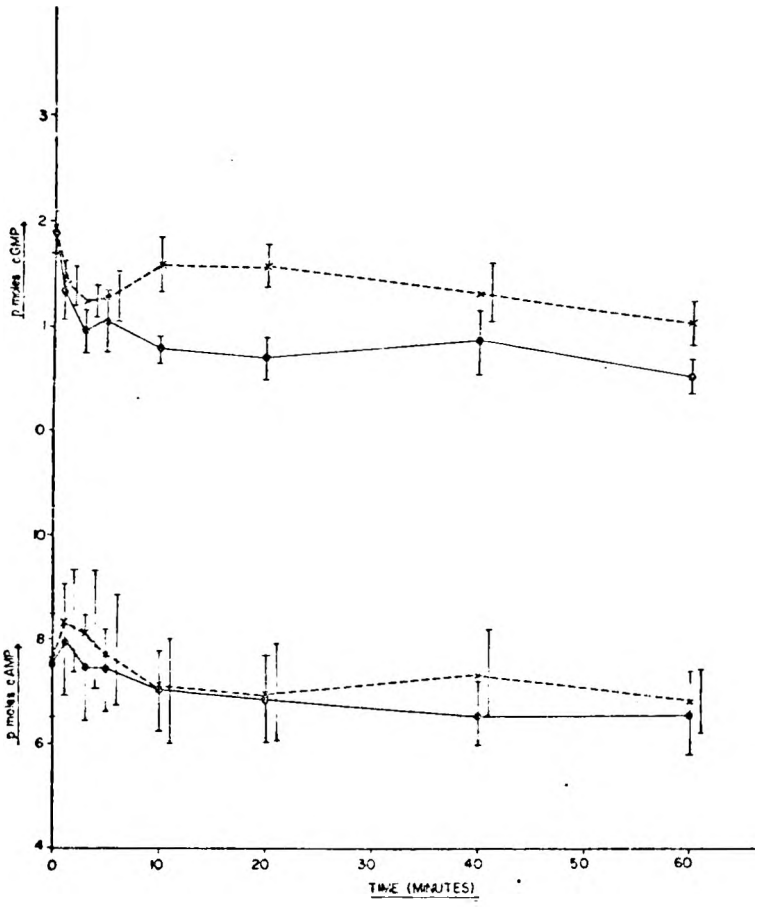


Figure VI Effect of casein in the presence (---) and absence (——) of 10^{-3}M levamisole on neutrophil cAMP and cGMP levels. Results are expressed as mean and standard error.

DISCUSSION

Contrary to the reports of Estensen *et al*, (1973) and Hill *et al*, (1975) acetyl choline and carbamyl choline had no detectable stimulatory effects on any form of neutrophil motility. The reasons for these differences are unclear since similar cell types and filter assays have been used in both studies.

Although the exact mechanism of action of LMS is unknown, it affects a number of cell functions involved in the immune and inflammatory response. The data reported here indicate that in a defined range of concentrations it consistently increases both directed and random movement of normal PMN cells in Boyden chambers. The increased chemotaxis produced by LMS was observed when either EAS or casein was used as chemoattractant. Further experiments indicated that stimulation is evident from 10^{-5}M to 7.5 x 10^{-3}M being linear in the range 10^{-5}M to 2.5 x 10^{-3}M. At a concentration of 10^{-2}M inhibition of chemotaxis occurs. When the kinetics of the stimulatory response were studied, it was observed that enhanced chemotaxis was present at all time intervals tested but was maximal at 3 - 4 hours. Similarly in random migration studies LMS at concentrations of 10^{-3}M - 10^{-6}M significantly increased non-directional movement, this effect again

being maximal at 3 hours.

Using a system which varies the concentration of leucoattractant above and below the filter (Zigmond and Hirsch, 1973) and which ascertains the influence of LMS on rate and direction of neutrophil locomotion it is evident that not only are chemotaxis and random migration increased by LMS treatment, but also stimulated random migration (chemokinesis). Stimulation of chemokinesis is greatest when lower concentrations of chemoattractant are present on either side of the filter, and presumably higher doses of EAS mediate greater "deactivation" which may counteract the LMS effect. It was unclear whether the effect on chemotaxis was any different from the effect on random migration, however using the "leading front" technique the results indicate that the stimulation of chemotaxis is not due solely to increased random migration and chemokinesis, but is due also to a true increase of directional movement. The finding that LMS stimulates all three types of neutrophil motility suggests that a common pathway may be influenced. The results may be of potential therapeutic value in the treatment of inherited or acquired conditions associated with defective PMN chemotaxis. In neonatal rats infected with *Staphylococcus aureus* far greater numbers of PMN were observed at the sites of bacterial infection in LMS-treated animals than in untreated ones, and this was associated with a markedly increased survival rate (Fisher *et al*, 1974). It is

unlikely, however, that the drug concentrations at which an effect on motility is seen *in vitro*, are attainable *in vivo*. To further understand the nature of the LMS-PMN interaction, pulsing experiments were performed using various combinations of LMS, autologous serum or EAS. These results showed that in the absence of serum the LMS effect was not evident. When PMN were pulsed with LMS in the presence of either EAS or fresh serum + levamisole however, a chemotactic stimulation was observed suggesting that serum factors contribute to the mechanism of LMS action. Pulsing with EAS alone induced an inhibition of chemotaxis. It appears therefore that LMS can eliminate the deactivation of chemotactic responsiveness observed when PMN are preincubated with chemotactic factors. The serum dependence for LMS stimulation was not apparent in serum-free random migration systems and presumably adherence of the cells to the filter may be sufficient to activate the cells and allow an LMS stimulatory effect.

It has previoulsy been reported by Rivkin *et al*, (1975) that cAMP levels of rabbit peritoneal neutrophils are unaltered by interaction with C5a and a bacterial chemotactic factor. No studies were performed on peripheral neutrophils. Likewise, no kinetic studies were carried out. The results

of the present study indicate a differential effect of two chemotactic factors on human neutrophil cAMP levels. Casein had no significant effect on intracellular cAMP whereas a chemotactic factor derived from endotoxin activated serum mediated a significant early increase (1 - 3 minutes) in cAMP. Levamisole had no effect on cAMP levels in cells treated with both leucoattractants. Furthermore both chemotactic factors mediated a depletion of cGMP levels. With casein the depletion was progressive throughout the incubation period, whereas the chemotactic factor derived from EAS caused an early decrease in cGMP levels followed by a peak of synthesis with subsequent depletion from 5 - 60 minutes. The early depression paralleled the elevation in cAMP. For both leucoattractants, levamisole promoted an early increase in cGMP levels and although depletion of cGMP occurred at similar rates in LMS treated or untreated PMN, the levels remained consistently higher in LMS treated cells.

Hadden *et al*, (1975) have demonstrated that LMS may increase intracellular cGMP levels in murine lymphocytes. Estensen *et al*, (1973) and Hill *et al*, (1975) have reported that exogenous cGMP and cholinergic agents enhance neutrophil chemotactic responsiveness to a bacterial derived chemotactic factor. However, these authors did not demonstrate that these agents

promoted elevation of intracellular cGMP levels in neutrophils and their work could not be confirmed in this laboratory. Concentrations of acetyl choline and carbamyl choline which they found to stimulate chemotaxis do mediate an elevation of neutrophil intracellular cGMP levels (see Addendum, page 114a), but this may not be sufficient to promote increased cell motility. However, the results of the present study indicate that LMS mediates the maintenance of elevated cGMP levels and LMS stimulation of motility may indeed be related to raised intracellular cGMP levels.

It has been shown that cAMP plays an important rôle in stimulating the calcium pump for rapid calcium efflux, (Fuller *et al*, 197[illegible] and Gallin and Rosenthal, 1974) have shown that interaction of PMN with chemotactic factors promotes such a calcium efflux which occurs within the first minute. These findings correlate with the initial rise in cAMP levels during exposure to chemotactic factor, described in this report. It could be hypothesised therefore that after cell-chemotactic factor interaction cAMP levels rise initially, perhaps due to adenyl cyclase activation, causing a calcium efflux which promotes optimal microtubular assembly. On the other hand, the raised cAMP levels might result from the calcium efflux as Ca^{++} ions are known to inactivate adenylate cyclase. It has also been shown that intracellular calcium levels regulate cGMP

accumulation (Goldberg, 1974) and the decline in cGMP levels noted in this study could be related to the calcium efflux. Oliver *et al*, (1974) have reported an apparent defect in microtubular assembly in Chediak-Higashi neutrophils related to inadequate intracellular cGMP levels and suggest that this cyclic nucleotide may promote microtubular polymerisation. It is likely therefore that agents such as LMS, by maintaining cGMP levels may sustain microtubular assembly thereby enhancing cell motility.

Casein on the other hand, had no effects on neutrophil intracellular cAMP levels. Other workers in this laboratory (Rabson *et al*, 1976) have suggested that the mechanisms of neutrophil chemotaxis to casein and EAS are different, due to different inhibition profiles obtained with various inhibitors of chemotaxis. Likewise, patients with impaired leucotaxis to EAS have often manifested a normal response to casein. Such a patient has been reported by Gallin (personal communication, 1976). Hydrolysed casein is a less potent leucoattractant than EAS for human blood neutrophils (Anderson, 1974) and this may reflect a lesser ability to activate neutrophils. The differences between EAS and casein are discussed at greater length in Chapter IX (page 177).

ADDENDUM

The effects of 10^{-5}M acetyl choline and carbamyl choline and 10^{-4}M propanolol, on cGMP levels of neutrophils stimulated with 10% EAS for 10 minutes are shown in Table 14a below. Results are expressed as mean values with standard deviations for 3 experiments.

Table 14a

The Effects of Acetyl Choline, Carbamyl Choline and Propanolol on Neutrophil cGMP Levels

Reaction System	p moles/cGMP/10^7PMN Mean and standard deviation
EAS alone	1.0 ± 0.3
EAS + 10^{-5}M acetyl choline	1.5 ± 0.5
EAS + 10^{-5}M carbamyl choline	1.9 ± 0.5
EAS + 10^{-4}M propanolol	3.0 ± 0.6

As can be seen, all three drugs mediate an increase of cGMP. However, the increases due to acetyl choline and carbamyl choline are slight by comparison with propanolol. The latter drug has similar effects on cell motility as levamisole (Chapter VII). It is probable that the increases in cGMP promoted by acetyl choline and carbamyl choline are insufficient to influence neutrophil locomotion.

C H A P T E R VII

The Effects of Histamine and Metiamide and other Agents which mediate Elevated Intracellular cAMP Levels on Neutrophil Motility

INTRODUCTION

It has previously been shown that a group of children with cold staphylococcal abscesses associated with hyper-immunoglobulinemia E and presumably elevated serum histamine levels, have defective polymorphonuclear leucocyte (PMN) chemotaxis (Hill and Quie, 1975). In a series of *in vitro* experiments it was demonstrated that histamine at concentrations of 10^{-8}M - 10^{-3}M exerted a progressive and profound inhibition of PMN chemotaxis, an effect which could be eliminated by an H-2 receptor antagonist (Hill and Quie, 1975).

This investigation examines in detail, the effect of histamine and metiamide, an H-2 receptor antagonist, and other agents reported to increase intracellular cAMP levels on PMN random motility, stimulated random motility (chemokinesis) and true directional motility (chemotaxis). Analysis of these effects has been made possible by the advent of the technique of Zigmond and Hirsch (1973) which employs varying concentrations of chemotactic factor above and below the filter enabling dissociation of random from truly directional motility. Employing this system, histamine, d'cAMP, prostaglandin E_1 (PGE_1) and isoproterenol, have been shown to stimulate random migration but to inhibit true chemotaxis, the net effect being one of mild stimulation as assessed in a classical Boyden system.

Because histamine has been shown to increase intra-

cellular levels of cAMP in a number of cell types (Bourne *et al*, 1973; Zurier *et al*, 1974; Beaven, 1976) the extent of cyclic nucleotide involvement has been assessed.

MATERIALS AND METHODS

Preparation of drugs Histamine (Sigma Chemical Company, St. Louis, Missouri), Metiamide (kindly supplied by Smith, Kline and French, Johannesburg) and diphenylhydramine hydrochloride obtained from Park-Davis (Johannesburg) were dissolved in medium 199 (Grand Island, New York) at pH 7.2, giving a final concentration range of 10^{-6}M - 10^{-3}M. Adenosine 3' : 5' cyclic monophosphoric acid (cAMP), N^6O^2 - dibutyryl adenosine 3' : 5' - cyclic monophosphoric acid sodium salt (d'cAMP) and L-isoproterenol hydrochloride were obtained from Sigma Chemical Company (St. Louis, Missouri). Prostaglandin E_1 (PGE_1) was kindly supplied by Dr. John Pike (Upjohn Company, Kalamazoo, Michigan) levamisole hydrochloride by Ethnor Laboratories (Johannesburg) and propanolol by I.C.I. (Johannesburg, South Africa). Drugs were dissolved in M199 and diluted according to experimental requirements.

Cell motility studies

The motility studies were of two types. The direct effects of histamine and metiamide on chemotaxis and random migration were assessed by the method of Boyden

whereby PMN which accomplish complete transfilter passage are counted microscopically. To ascertain the exact type of cell motility influenced by the drugs, the leading front method of Zigmond and Hirsch was adopted.

Neutrophils were obtained from 50 ml. of heparinized whole blood (5 units heparin/ml.) donated by healthy male and female laboratory workers. After sedimentation at 37°C, the buffy layer was centrifuged at 250 g for 10 minutes. The resultant cell pellet was washed three times with medium 199 and resuspended to a PMN concentration of 3×10^6/ml. for EAS and 5×10^6/ml. for casein.

Leucoattractants

Two chemoattractants were used in this study.

a) Fresh serum activated by 500 µg/ml. of bacterial lipopolysaccharide (*E.coli* 0127 : B8 Difco) (EAS). The mixture was incubated for 30 minutes at 37°C followed by a four-fold dilution with medium 199.

b) Denatured casein at a concentration of 600 µg/ml. prepared by alkali hydrolysis with subsequent readjustment to pH 7.2. This preparation was stored at -20°C and used for all subsequent experiments.

Chemotaxis assay

PMN chemotaxis was measured using the modified Boyden Chamber which utilizes 0.2 ml. of drug treated or control cell suspensions in the upper chamber,

separated from 1 ml. of the leucoattractant solution by a 5 μ pore size membrane filter (Millipore Corp.). Chambers were incubated at 37°C for 3 hours after which filters were removed, fixed with methanol and stained with haematoxylin. The average number of neutrophils per high power field reaching the lower surface of the filter was determined and expressed as an average for triplicate filters.

Random Migration was assessed by measuring the extent of cell migration across the 5 μ pore size filter in the absence of a leucotactic gradient. 3×10^6 cells were suspended in medium 199 containing 0.1% bovine serum albumen and a similar medium was placed in the bottom compartment, drugs being added in equal concentration to both top and bottom compartments.

Using these techniques the normal range for chemotaxis using EAS as chemoattractant was 180 - 240 cells/HPF., using casein 120 - 165 cells/HPF and in random migration systems 15 - 30 cells/HPF.

Simultaneous assessment of drug effects on different types of neutrophil motility

To ascertain the effect of histamine or metiamide on specific types of neutrophil motility, i.e. random migration, chemokinesis and true chemotaxis, PMN migration was assessed when varying concentrations of EAS (1.5%, 3%, 6% and 12%) were placed above and below the filter in all possible combinations according to the

method of Zigmond and Hirsch (1973). Systems so designed were of 2 types, without drugs and with 2.5×10^{-5}M histamine or metiamide above and below the filter. Chambers were incubated at 37°C for 40 minutes, filters were fixed, stained, dehydrated cleared and mounted with the top side uppermost. Neutrophil migration was expressed as the distance travelled (μm) as measured with the optical micrometer on the fine focus of the microscope. Results are expressed in tabular form ("checkerboard") and true chemotaxis is taken as the difference between the observed values for positive gradients (above the diagonal lines) and the estimated values based on chemokinesis (in brackets) according to the formula of Zigmond and Hirsch. The effects on chemotaxis are assessed as previously described by comparing true increments for a given gradient in the presence or absence of the drugs. Percentage stimulation or inhibition is calculated according to the formula:

$$\frac{\text{Drug value - Control value}}{\text{Control value}}$$

Dose Response Experiments

a) Chemotaxis

To assess the effects of varying concentrations of histamine and metiamide (10^{-6}M - 10^{-3}M) on true chemotaxis, a fixed positive gradient was selected (3% EAS above the filter and 12% EAS below the filter) and the true chemotactic values for each drug concentration

were calculated from the corresponding chemokinesis values as described above. Results are expressed as a percentage of the true chemotactic values observed in control systems without histamine or metiamide.

b) Chemokinesis

Using the same drug concentration range and a fixed chemokinesis system (3% EAS above and below the filter) the effects of histamine and metiamide on chemokinesis were evaluated and results were expressed according to the formula:

$$\frac{\text{Experimental value - Control value}}{\text{Control value}} \times 100$$

Drug Exclusion Experiments

a) Histamine/Metiamide

To assess the reversibility of the histamine mediated effect on true chemotaxis, cells were pre-treated with histamine (10^{-6}M - 10^{-4}M) for 20 minutes followed by exposure to metiamide (2.5×10^{-5}M). The true chemotactic increments were calculated using a fixed positive gradient (3% EAS top : 12% EAS bottom) with the corresponding chemokinesis systems. The effects of metiamide on the true chemotactic response of histamine treated PMN were evaluated by comparison of the experimental values with the corresponding negative (no histamine or metiamide) and

positive (10^{-6}M - 10^{-4}M histamine only) controls. In reverse experiments PMN were treated with metiamide (10^{-6}M - 10^{-4}M) followed by exposure to 2.5 x 10^{-5}M histamine (final concentration) and similar calculations performed.

b) Histamine or Metiamide/Levamisole

Using the same histamine and metiamide concentration ranges, PMN were treated as above and exposed to levamisole (10^{-3}M) after which they were tested for true chemotactic responsiveness. In the reverse experiments cells incubated with levamisole (10^{-5}M - 10^{-3}M) were subsequently treated with 2 x 10^{-5}M histamine or metiamide. Experimental planning was as above and results calculated accordingly.

Other Chemotactic Studies

a) Effect of Histamine on PMN motility using casein as leucoattractant

This was assessed using varying concentrations of casein (15, 30, 60 and 120 µg/ml.) above and below the filter in the absence and presence of histamine (2.5 x 10^{-5}M) on both sides of the filter.

b) Effect of drugs known to alter intracellular cAMP levels

Using predetermined concentrations based on pioneer dose response experiments, the effects of d'cAMP (10^{-3}M) isoproterenol (10^{-4}M), PGE_1 (10 µg/ml.), and propanolol (10^{-4}M), on chemokinesis and chemotaxis were

assessed in "checkerboard" systems using varying EAS concentrations (1.5%, 3%, 6% and 12%) above and below the filter.

Cyclic nucleotide studies

Peripheral blood was collected as previously described and separated on a Ficoll-Hypaque gradient. Mononuclear cells were discarded and the resultant pellet was resuspended in physiological saline and sedimented with a 25% volume of 3% gelatin (Difco, Detroit, Michigan) for 45 minutes. The neutrophil-rich layer was collected, centrifuged at 250 g for 10 minutes and the resultant cell pellet treated with 0.83% ammonium chloride at 4°C for 10 minutes to lyse residual erythrocytes. The remaining cells which consistently contained greater than 90% viable PMN were washed in medium 199 containing 5% foetal calf serum (Burroughs Wellcome) and resuspended to a final volume of 2×10^7 PMN/ml. Cell suspensions (0.05 ml.) were treated with 0.5 ml. of medium 199 containing 5×10^{-5}M histamine or metiamide and were incubated in a shaking water bath at 37°C. After varying incubation times (0, 1, 3, 5, 10, 20, 30, 45 and 60 minutes) the reaction was terminated by the addition of an equal volume of cold 2% perchloric acid. PMN in each tube were disrupted in an MSE ultrasonic disintegrator for 2½ minutes at an amplitude of 30 μ peak to peak. The specimens were then centrifuged at 5,000 g for 10 minutes, the pellet discarded and the supernatant

restored to pH 7.0 by the addition of 0.3N KOH. Specimens were re-centrifuged for 20 minutes, and were then freeze-dried on a Vertis lyophiliser. Specimens were reconstituted in 0.05 M sodium acetate buffer, pH 6.2 and aliquots of 0.2 ml. were used for cyclic GMP and 0.1 ml. for cyclic AMP estimations. The Schwarz/Mann cyclic AMP and GMP radioimmuno-assay kits and the procedures supplied with each kit were used. Standard curves were run with each assay and were found to be consistently linear. Duplicate samples were assayed and the final results expressed as p moles/10^7 PMN.

RESULTS

Parallel assessment of the effect of different concentrations of histamine on both chemotaxis and random migration using the classical Boyden technique is shown in Fig.VIIa (page 128). At concentrations of between 1 x 10^{-5}M and 5 x 10^{-5}M, histamine when added to the PMN in the upper compartment of the chamber, consistently appeared to cause a moderate increase in random migration and in chemotaxis when both EAS and alkali hydrolysed casein were used as chemoattractants ($p < 0.05$ Wilcoxon test). Likewise metiamide at equivalent concentrations (Fig.VIb) produced similar effects on both chemotaxis and random migration ($p < 0.05$).

The results of the experiments utilizing different combinations of EAS above and below the filter in the presence and absence of 2.5×10^{-5}M histamine or metiamide, these being the concentrations which exerted optimal responses in Fig. VIIa and V11b as shown in Tables 15a, 15b, 15c and 16a, 16b and 16c. It is evident that stimulation of cell movement in the presence of histamine occurs only in the absence of a gradient (within the diagonal lines) and in the presence of a negative gradient (below the diagonal). These results indicate that histamine produces stimulation of both random migration and chemokines.

To assess the effect of both these drugs on directional motility, the true increments in chemotaxis between control and drug treated cells were compared.

Results of these calculations from the data shown in the above Tables and are summarized in Tables 17 and 18. It can be seen that histamine induced a marked inhibition of true chemotaxis for all positive gradients tested ($p < 0.001$) whereas metiamide had no effect on the true chemotactic increment.

To determine whether the histamine induced inhibition of true chemotaxis was dose-dependent, the effects of the drug at a concentration range of 10^{-6}M - 10^{-3}M were assessed. As can be seen in Fig. V111 page 137) progressive inhibition was observed

from 10^{-6}M, and was maximal at 2.5 x 10^{-5}M. In further dose response experiments the parallel effects of histamine and metiamide on chemokinesis were estimated (Fig. IX). Stimulation of chemokinesis at histamine concentrations between 10^{-6}M - 10^{-4}M was observed, with optimal stimulation at 2.5 x 10^{-5}M. With metiamide a progressive increase from 10^{-6}M to 5 x 10^{-5}M was noted. The respective inhibitory and stimulatory effects of histamine on PMN chemotaxis and chemokinesis were also evident when hydrolysed casein was used in varying concentrations above and below the filter (Table 19, Page 139).

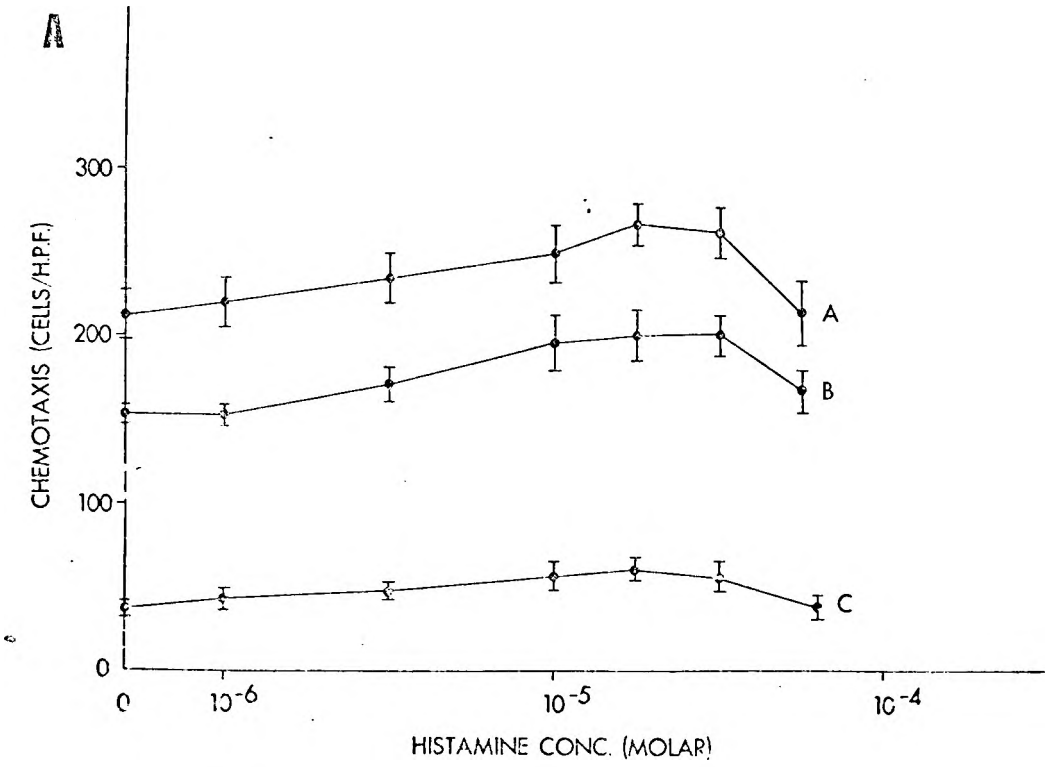
To assess whether the inhibition of chemotaxis induced by histamine treatment was reversible, a series of experiments were performed in which cells initially pre-incubated for 20 minutes with histamine at a concentration range of 10^{-6}M - 10^{-4}M, were then exposed to 2.5 x 10^{-5}M metiamide. This latter treatment had no significant effect on the extent of inhibition of true chemotaxis. (Fig. X). In the reverse experiment PMN were initially treated with a corresponding concentration range of metiamide for 20 minutes and subsequently exposed to 2.5 x 10^{-5}M histamine, a concentration which produces approximately 100% inhibition of true chemotaxis. This effect could be completely abolished at metiamide concentrations of 10^{-5}M and upwards (Fig. XI). Additive effects of

histamine and metiamide on chemokinesis were evident only at drug concentrations of less than 10^{-5}M and these were controlled for experimentally and corrected for in calculations of true chemotactic increments. In similar experiments cells pre-treated with 2.5×10^{-4}M diphenylhydramine, a concentration which has previously been shown to have no effect on neutrophil motility, manifested total inhibition of true chemotaxis upon exposure to 2.5×10^{-5}M histamine. Similarly this drug had no effect on the histamine induced stimulation of chemokinesis. Results are shown in Tables 20 and 21 (Pages 142 and 143 respectively).

In a further series of experiments PMN either prior or subsequent to histamine treatment were exposed to levamisole, an agent known to increase random migration, chemokinesis and true chemotaxis. When cells were initially treated with histamine followed by exposure to 10^{-3}M levamisole, a concentration shown to produce marked stimulation of neutrophil motility, this stimulation could be abrogated (Fig XII). However, metiamide (10^{-6}M - 10^{-4}M) pre-treatment of PMN had no effect on the levamisole stimulation. (Results not shown). Furthermore, the stimulation of chemotaxis produced by initial levamisole treatment could not be reversed by a subsequent inhibitory concentration of histamine. (Fig. XIII).

To ascertain if the histamine and metiamide effects on neutrophil motility could be attributed to changes in intracellular cyclic nucleotide levels, both intracellular cAMP and cGMP were measured 1 - 60 minutes after drug treatment. Results depicted in Fig. XIV, which is a mean of 3 experiments all having similar profiles, indicates that intracellular levels of cAMP are elevated soon after histamine treatment reaching maximal levels between 5 and 20 minutes. When PMN are treated with metiamide, an initial increase of cAMP is noted after 1 minute rapidly dropping to within normal limits for the remainder of the experiment. cGMP levels were unaffected by both drugs.

To determine whether the histamine stimulation of PMN random migration and/or the inhibition of chemotaxis was related to the increase in intracellular cAMP levels, cell movement was assessed after PMN had been treated with compounds known to increase intracellular cAMP levels. As is seen in Tables 22 and 23, d'cAMP (10^{-3}M), isoproterenol (10^{-4}M) and PGE_1 (10 µg/ml.) all produced significant increases in random migration and inhibited true chemotaxis. cAMP (5×10^{-3}M) was without effect on neutrophil motility. Propanolol, a β-blocker, actually mediated an increase in both chemokinesis (43%, 30%, 26%, 24%) and true chemotaxis (0%, 16%, 41%). (Table 24, Page 149).



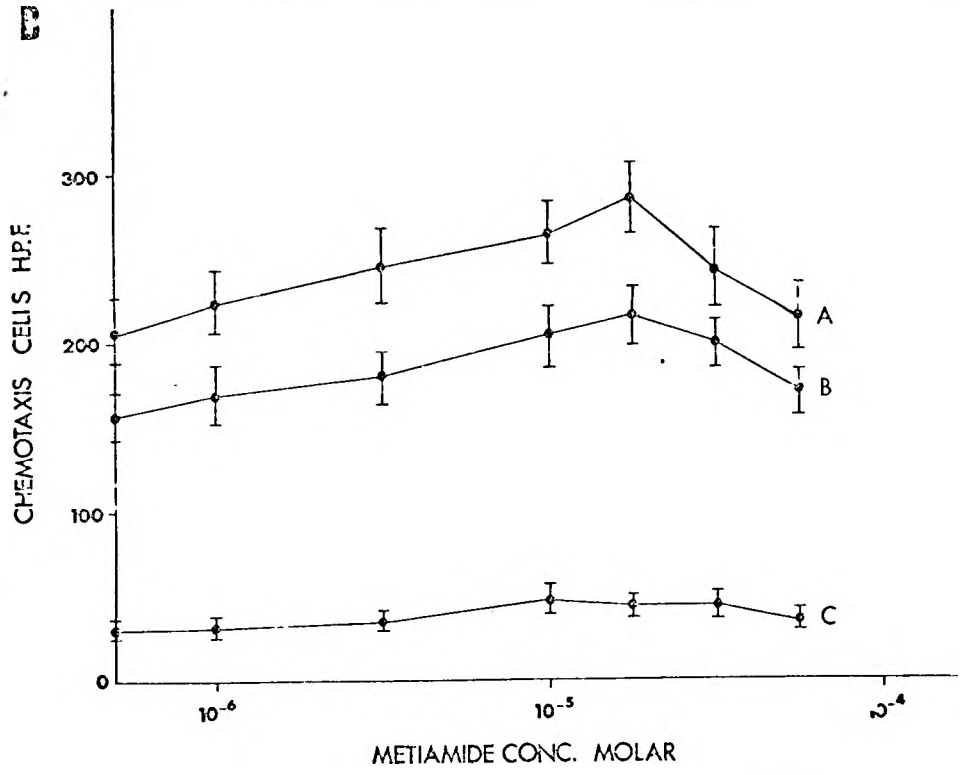


Figure V11 The effects of a) histamine and b) metiamide on PMN chemotaxis to (A) endotoxin activated serum, (B) casein and (C) in random migration systems.

TABLE 15a

Effects of Histamine on PMN Random Migration, Chemokinesis and True Chemotaxis
Experiment 1

a) Control

% EAS above filter	% EAS below filter: 0	1.5	3	6	12
0	36				
1.5		49	59(52)	63(53)	67(53)
3		52(52)	55	71(57)	81(56)
6		50(56)	53(57)	58	60(53)
12		48(54)	49(54)	51(53)	48

b) 2.5 x 10^{-5}M histamine on both sides of the filter

% EAS above filter	% EAS below filter: 0	1.5	3	6	12
0	46				
1.5		62	59(64)	63(67)	74(68)
3		65(64)	65	72(73)	72(74)
6		55(69)	62(73)	80	73(75)
12		57(71)	61(74)	62(75)	69

The effect of varying concentration gradients and absolute concentration of EAS on the motility of human neutrophils in the presence and absence of 2.5 x 10^{-5}M histamine. Figures along the diagonal from upper left to lower right show the distance migrated (μm) in increasing concentrations of the leucoattractant in the absence of a concentration gradient. Along the diagonal, cells are moving in a positive gradient, and below the diagonal in a negative gradient. The figures in brackets are estimates of what migration would have been in each of the tests, assuming that the cells detected the absolute concentration of the chemoattractant but not the gradient.

The same applies for the following two Tables

TABLE 15b

Effects of Histamine on PMN Random Migration, Chemokinesis and True Chemotaxis
Experiment 2

a) Control

% EAS below filter

	0	1.5	3	6	12
0	36				
1.5		59	63(62)	69(64)	77(64)
3		62(62)	65	81(67)	91(67)
6		60(65)	63(67)	68	70(67)
12		58(65)	61(67)	58(67)	65

b) 2.5×10^{-5} M histamine on both sides of the filter

% EAS below filter

	0	1.5	3	6	12
0	47				
1.5		70	65(68)	66(67)	71(69)
3		62(68)	66	66(66)	73(67)
6		54(67)	55(66)	65	66(70)
12		64(69)	57(68)	65(70)	75

TABLE 15c

Effects of Histamine on PMN Random Migration, Chemokinesis and True Chemotaxis
Experiment 3

a) Control

% EAS below filter

	0	1.5	3	6	12
0	30				
1.5		54	63(58)	72(59)	81(63)
3		56(58)	62	80(67)	84(67)
6		58(65)	56(67)	71	78(70)
12		54(63)	55(67)	57(69)	68

b) 2.5×10^{-5}M histamine on both sides of the filter

% EAS below filter

	0	1.5	3	6	12
0	41				
1.5		72	75(75)	79(77)	80(76)
3		69(75)	77	77(80)	81(81)
6		70(78)	71(80)	84	82(83)
12		71(77)	76(82)	75(83)	82

TABLE 16a

Effects of Metiamide on PMN Random Migration, Chemokinesis and True Chemotaxis
Experiment 1

a) Control

% EAS below filter

	0	1.5	3	6	12
0	46				
1.5		57	56(57)	58(57)	69(58)
3		52(57)	57	66(58)	70(58)
6		50(57)	51(58)	58	64(59)
12		49(58)	55(59)	50(59)	60

b) 2.5 x 10^{-5}M metiamide on both sides of the filter

% EAS below filter

	0	1.5	3	6	12
0	54				
1.5		61	63(62)	65(64)	73(64)
3		58(62)	62	72(63)	78(65)
6		50(63)	66(64)	66	73(68)
12		58(65)	59(66)	62(68)	69

The effect of varying concentration gradients and absolute concentration of EAS on the motility of human neutrophils in the presence and absence of 2.5 x 10^{-5}M metiamide. Figures along the diagonal from upper left to lower right show the distance migrated (μm) in increasing concentrations of the leucoattractant in the absence of a concentration gradient. Above the diagonal, the cells are moving in a positive gradient, and below the diagonal in a negative gradient. The figures in brackets are estimates of what migration would have been in each of the tests, assuming that the cells detected the absolute concentration of the chemoattractant but not the gradient.

The same applies for the following two Tables.

TABLE 16b

Effects of Metiamide on PMN Random Migration, Chemokinesis and True Chemotaxis

Experiment 2

a) Control

% EAS above filter	% EAS below filter				
	0	1.5	3	6	12
0	35				
1.5		55	64(57)	68(59)	70(58)
3		56(57)	58	66(61)	73(61)
6		55(59)	62(57)	63	70(62)
12		57(58)	58(57)	62(59)	61

b) 2.5 x 10^{-5}M metiamide on both sides of the filter

% EAS above filter	% EAS below filter				
	0	1.5	3	6	12
0	47				
1.5		67	76(66)	78(67)	84(69)
3		63(64)	66	75(69)	82(73)
6		66(66)	65(69)	71	81(73)
12		63(68)	67(71)	67(73)	75

TABLE 16c

Effects of Metiamide on PMN Random Migration, Chemokinesis and True Chemotaxis

Experiment 3

a) Control

% EAS above filter	% EAS below filter				
	0	1.5	3	6	12
0	43				
1.5		71	91(71)	99(73)	92(71)
3		72(71)	70	100(73)	94(73)
6		55(72)	57(73)	76	92(72)
12		46(72)	49(71)	54(72)	67

b) 2.5×10^{-5} M metiamide on both sides of the filter

% EAS above filter	% EAS below filter				
	0	1.5	3	6	12
0	50				
1.5		82	99(82)	107(82)	103(78)
3		83(82)	82	107(82)	104(76)
6		60(52)	58(82)	81	92(67)
12		57(74)	53(74)	50(77)	73

TABLE 17

Analysis of the Effects of 2.5×10^{-5}M Histamine on PMN Random Migration, Chemokinesis and Chemotaxis from Data recorded in Tables 15a, 15b and 15c

Type of Motility	Random Motility and Chemokinesis (No gradient of EAS present) % EAS above filter	% EAS below filter	% Stimulation of Control Random Migration Replicate Experiments 1	2	3	Mean Stimulation %
a) Random Migration	0	0	28	33	37	33
b) Chemotaxis	1.5	1.5	27	19	33	26
	3	3	31	2	24	19
	6	6	38	0	18	19
	12	12	44	15	21	27
	Chemotaxis (Positive EAS gradients present) % EAS above filter	% EAS below filter	% Inhibition of Control True Chemotaxis Replicate Experiments 1	2	3	Mean Inhibition %
c) True Chemotaxis	1.5	3	100	100	100	100
	1.5	6	100	100	85	95
	1.5	12	57	85	78	73
	3	6	100	100	100	100
	3	12	100	75	100	92
	6	12	100	100	100	100

TABLE 18

Analysis of the Effects of 2.5×10^{-5}M Metiamide on PMN Random Migration, Chemokinesis and Chemotaxis from Data recorded in Tables 16a, 16b and 16c

Type of Motility	No EAS gradients present (Random Motility) % EAS above filter	% EAS below filter	% Stimulation of Control Random Migration Replicate Experiments 1	2	3	Mean Stimulation %
a) Random Motility	0	0	34	17	16	22
b) Chemokinesis	1.5	1.5	22	7	15	15
	3	3	14	9	17	13
	6	6	13	14	7	11
	12	12	23	15	9	16
	Positive gradients present (Chemotaxis) % EAS above filter	% EAS below filter	% Stimulation of Control True Chemotaxis Replicate Experiments 1	2	3	Mean Stimulation %
c) True Chemotaxis	1.5	3	0	0	-15	-5
	1.5	6	22	0	0	7
	1.5	12	25	0	19	15
	3	6	0	0	-7	-2
	3	12	-8	0	33	8
	6	12	0	0	25	8

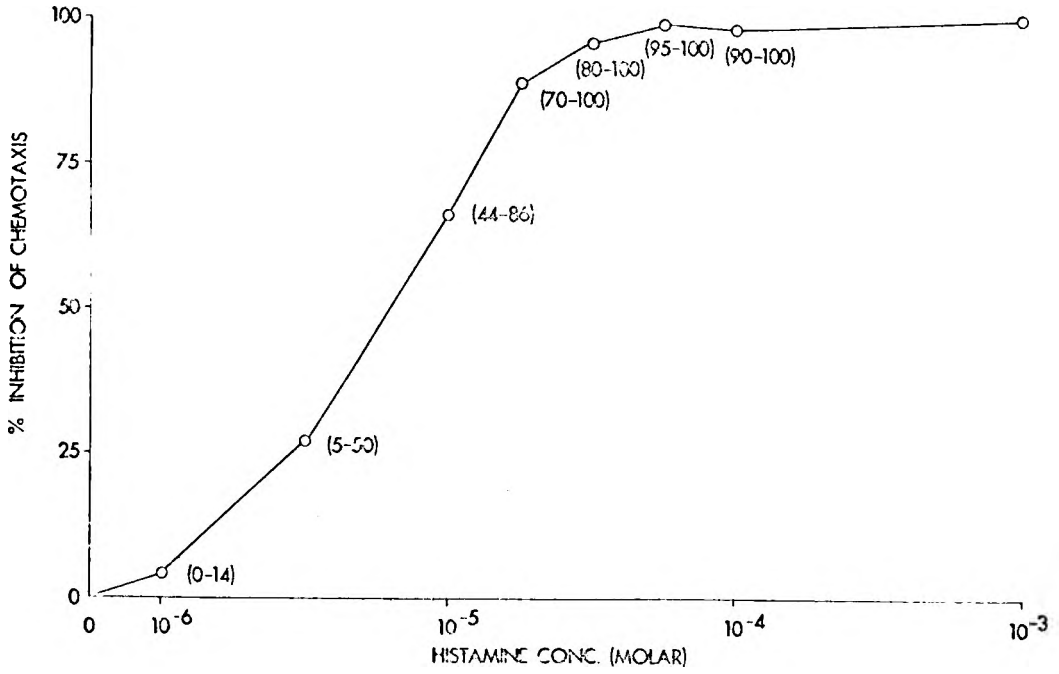


Figure V111 The inhibitory effect of increasing concentrations of histamine on true chemotaxis. To calculate these points a fixed positive gradient was used throughout (3% EAS top : 12% EAS bottom). The true chemotactic values were obtained from the difference between the observed chemotactic results and the expected values calculated from from corresponding chemokinetic results (3 : 3, 6 : 6, 12 : 12 % EAS top and bottom). The results depict mean values and the range for each concentration.

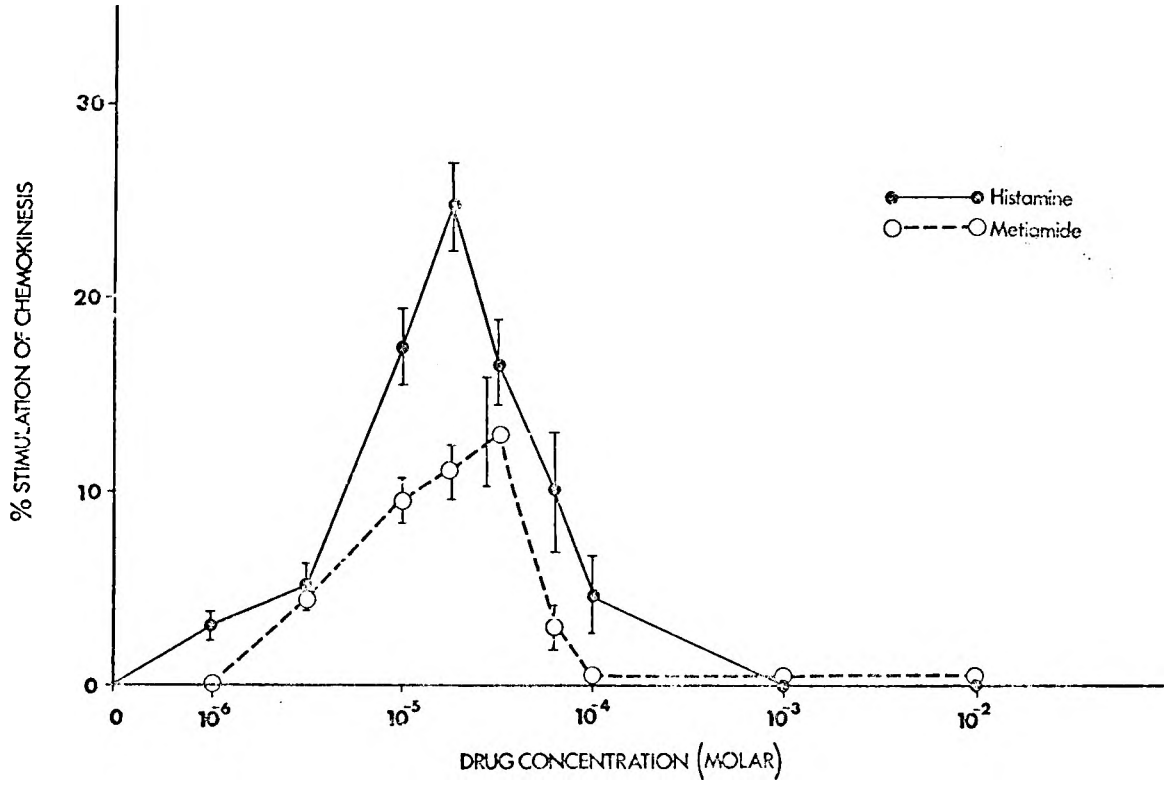


Fig. IX The stimulatory effect on chemokinesis (3% EAS above and below the filter) of increasing concentrations of histamine and metiamide. Results expressed as mean and standard error of the mean.

TABLE 19

Effects of Histamine on PMN Chemotaxis and True Chemotaxis to a Stimulus of Hydrolysed Casein

a) Without histamine

µg Casein above filter \ µg Casein below filter	15	30	60	120
15	67	75(71)	82(71)	89(72)
30		74		
60			73	
120				76

b) 2.5 x 10^{-5}M histamine above and below the filter

µg Casein above filter \ µg Casein below filter	15	30	60	120
15	81	84(83)	85(84)	84(86)
30		84		
60			91	
120				89

The effect of varying concentration gradients and absolute concentration of Casein on the motility of human neutrophils in the presence and absence of 2 x 10^{-5}M histamine. Figures along the diagonal from upper left to lower right show the distance migrated (µm) in increasing concentrations of the leucoattractant in the absence of a concentration gradient. Above the diagonal cells are moving in a positive gradient. The figures in brackets are estimates of what migration would have been in each of the tests, assuming that the cells detected the absolute concentration of the chemoattractant but not the gradient.

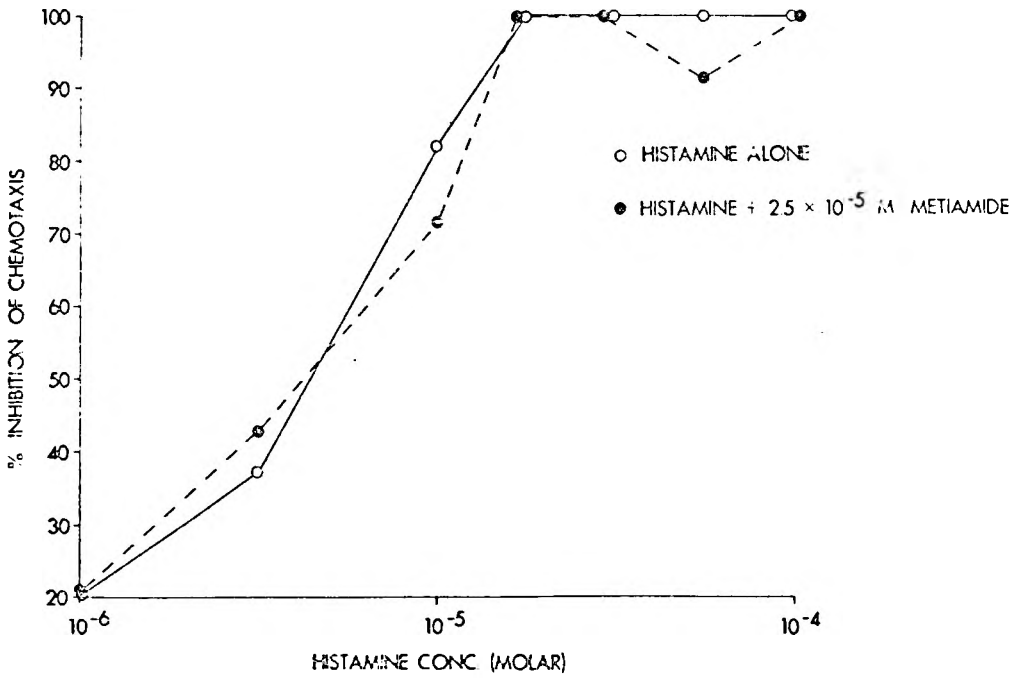


Figure X A representative experiment indicating the effect on true PMN chemotaxis of histamine alone or histamine + added metiamide.

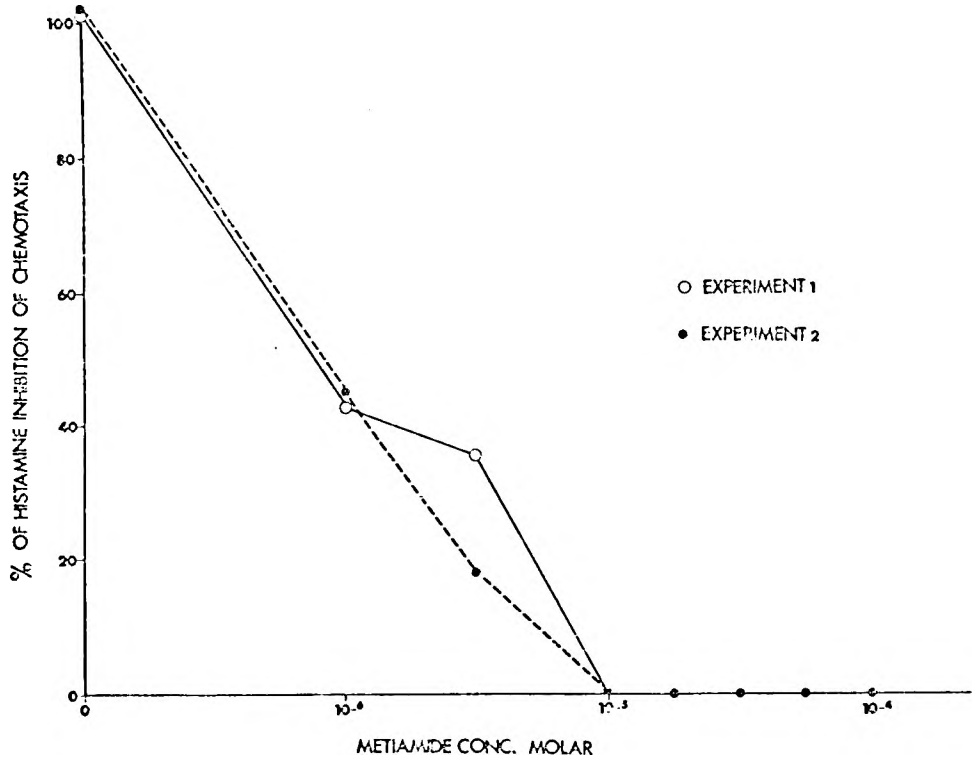


Figure XI The effect of metiamide pre-treatment on the histamine induced inhibition of true chemotaxis.

TABLE 20

a) Control

% EAS above filter \ % EAS below filter	1.5	3	6	12
1.5	64	70(65)	82(66)	91(65)
3		65		
6			72	
12				61

b) 2.5 x 10^{-4}M diphenylhydramine on both sides of the filter

% EAS above filter \ % EAS below filter	1.5	3	6	12
1.5	65	74(67)	81(68)	91(67)
3		69		
6			71	
12				63

c) 2.5 x 10^{-5}M histamine on both sides of the filter

% EAS above filter \ % EAS below filter	1.5	3	6	12
1.5	75	69(73)	79(80)	84(83)
3		80		
6			91	
12				90

d) 2.5 x 10^{-5}M histamine and 2.5 x 10^{-5}M diphenylhydramine on both sides of the filter

% EAS above filter \ % EAS below filter	1.5	3	6	12
1.5	82	71(80)	81(82)	93(83)
3		77		
6			98	
12				84

The effect of varying concentration gradients and absolute concentration of EAS on the motility of human neutrophils in a) the absence of additives; and in the presence of b) diphenylhydramine c) histamine d) diphenylhydramine and histamine. Figures along the diagonal from upper left to lower right show the distance migrated (μm) in increasing concentrations of the leucoattractant in the absence of a concentration gradient. Above the diagonal, cells are moving in a positive gradient. The figures in brackets are estimates of what migration would have been in each of the tests, assuming that the cells detected the absolute concentration of the chemoattractant but not the gradient.

TABLE 21

Effects of Diphenylhydramine on Histamine mediated stimulation of Chemokinesis and Inhibition of True Chemotaxis from Data recorded in Table 20

	CHEMOKINESIS µM					CHEMOKINESIS µM				
1. a) Control	% EAS above filter	% EAS below filter	Distance migrated (µm)	%Stimulation of Control		% EAS above filter	% EAS below filter	Distance migrated (µm)	TCV	% Inhibition of Control True Chemotaxis
	1.5	1.5	64	-	b)	1.5	3	70	5	-
	3	3	6[illegible]	-		1.5	6	82	16	-
	6	6	72	-		1.5	12	91	26	-
	12	12	61							
2. 2.5×10^{-4}M a) Diphenylhydramine	1.5	1.5	65	-	b)	1.5	3	71	7	-
	3	3	69	-		1.5	6	81	13	-
	6	6	71	-		1.5	12	91	24	-
	12	12	63							
3. 2.5×10^{-5}M a) Histamine	1.5	1.5	75	17	b)	1.5	3	69	0	100
	3	3	80	2[illegible]		1.5	6	79	0	100
	6	6	91	26		1.5	12	84	1	96
	12	12	90	[illegible]8						
4. 2.5×10^{-5}M Histamine a) 2.5×10^{-4}M Diphenylhydramine	1.5	1.5	82	28	b)	1.5	3	71	0	100
	3	3	77	18		1.5	6	81	0	100
	6	6	98	36		1.5	12	83	0	100
	12	12	84	38						

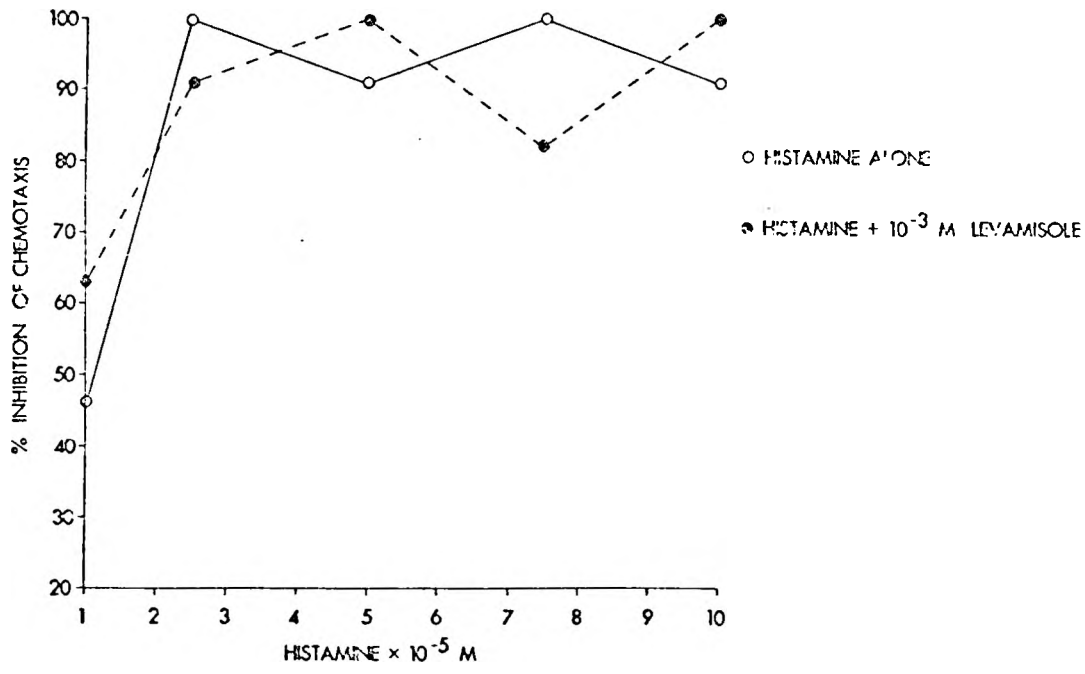


Figure XII The effect of 10^{-3}M levamisole on histamine induced inhibition of true chemotaxis.

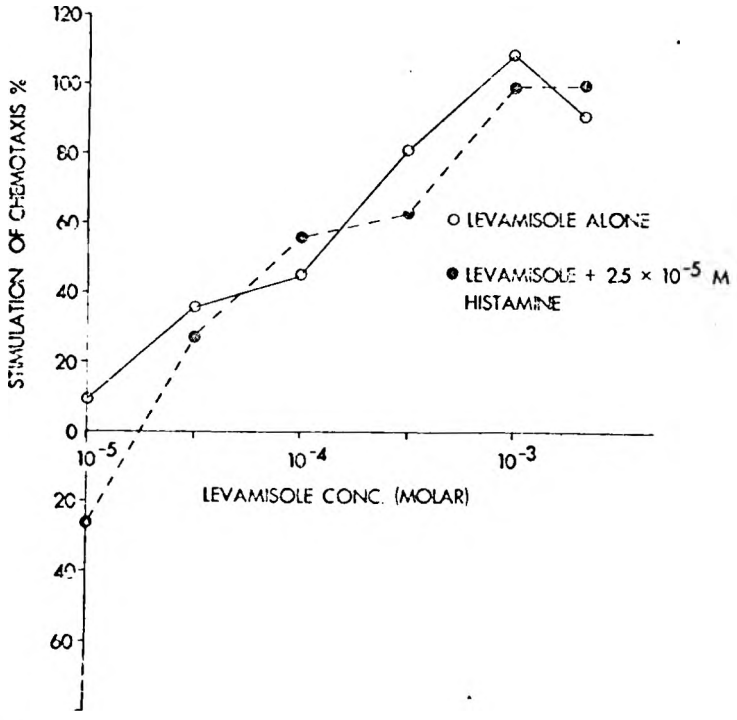


Figure XIII The effect of 2.5 x 10^{-5}M histamine on the levamisole induced stimulation of true chemotaxis.

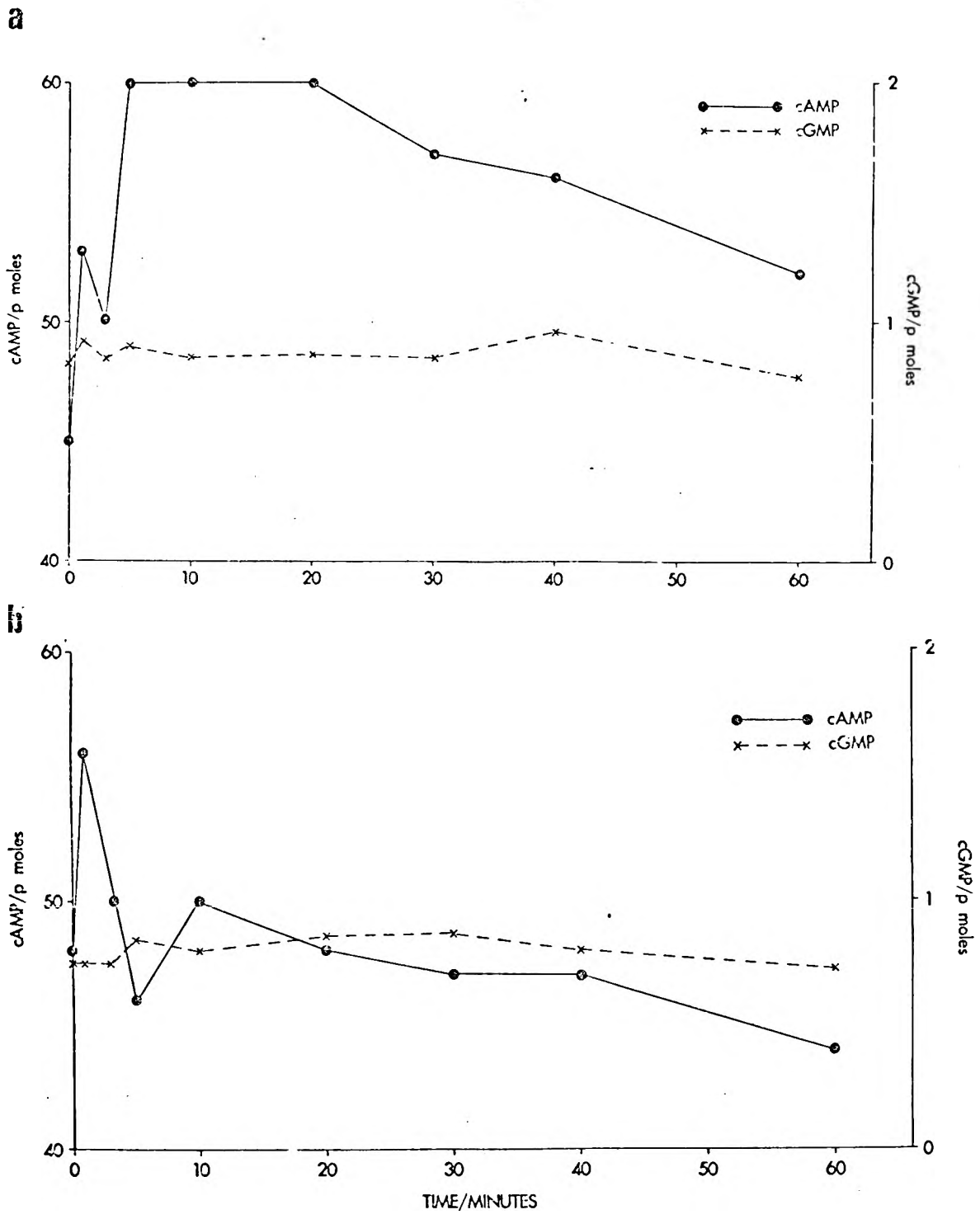


Figure XIV The effect on intracellular cAMP (——) and cGMP (----) levels after treatment with a) histamine and b) metiamide.

TABLE 22

Effects of Prostaglandin E_1 and Isoproterenol on PMN Chemokinesis and True Chemotaxis using EAS as Chemoattractant

a) Control

% EAS above filter	% EAS below filter 1.5	3	6	12
1.5	55	62(57)	67(58)	69(59)
3	59			
6		62		
12			62	

b) 10 µg Prostaglandin E_1 above and below the filter

% EAS above filter	% EAS below filter 1.5	3	6	12
1.5	73	72(75)	70(75)	70(76)
3	76			
6		78		
12			77	

c) 10^{-4}M Isoproterenol above and below the filter

% EAS above filter	% EAS below filter 1.5	3	6	12
1.5	69	61(69)	68(70)	68(71)
3	69			
6		75		
12			73	

TABLE 23

Analysis of the Effects of PGE_1, Isoproterenol and d'cAMP on Chemokinesis and True Chemotaxis, from Data recorded in Table 22

% EAS		% STIMULATION OF CHEMOKINESIS			% INHIBITION OF CHEMOTAXIS		
Above filter	Below filter	PGE_1 (10 µg)	Isoproterenol (10^{-4}M)	d'cAMP (10^{-3}M)	PGE_1 (10 µg)	Isoproterenol (10^{-4}M)	d'cAMP (10^{-3}M)
a) Chemokinesis							
1.5	1.5	33	25	21	-	-	-
3	3	29	17	40	-	-	-
6	6	26	21	51	-	-	-
12	12	24	18	75	-	-	-
b) Chemotaxis							
1.5	3	-	-	-	100	100	100
1.5	6	-	-	-	100	100	100
1.5	12	-	-	-	100	100	100

TABLE 24

Effects of Propanolol on PMN Chemokinesis and True Chemotaxis using EAS as Chemoattractant

a) Control

% EAS above filter	% EAS below filter: 1.5	3	6	12
1.5	63	76(66)	86(67)	86(69)
3		70		
6			74	
12				76

b) 10^{-4}M propanolol on both sides of the filter

% EAS above filter	% EAS below filter: 1.5	3	6	12
1.5	90	100(91)	113(91)	116(92)
3		91		
6			93	
12				94

The effect of varying concentration gradients and absolute concentration of EAS on the motility of human neutrophils in the presence and absence of 10^{-4}M propanolol. Figures along the diagonal from upper left to lower right show the distance migrated (μm) in increasing concentrations of the leucoattractant in the absence of a concentration gradient. Above the diagonal, the cells are moving in a positive gradient. The figures in brackets are estimates of what migration would have been in each of the tests, assuming that the cells detected the absolute concentration of the chemoattractant but not the gradient.

DISCUSSION

In a series of pioneer experiments using a classical Boyden technique, histamine appeared to increase both directed and random motility of PMN. To determine whether the effect on chemotaxis was any different from the effect on random migration, true chemotaxis was assessed according to the method of Zigmond and Hirsch (1973). This technique permits an accurate assessment of the degree and type of cell motility affected by agents under investigation. The results indicate that the stimulatory effect of histamine is attributable entirely to an increase of chemokinesis. Not only is there no enhancing effect on true chemotaxis but marked inhibition is evident, an effect which is masked in the classical Boyden system and which emphasises the importance of the method of Zigmond and Hirsch in enabling correct interpretation. Such a phenomenon of stimulation of chemokinesis with concomitant inhibition of true chemotactic responsiveness has been previously described in association with the effect of colchicine on lymphoblast motility, (Russell *et al*, 1975).

The histamine induced increase of chemokinesis and the inhibition of true chemotaxis was dose dependent being maximal at 2.5×10^{-5}M, and the inhibition was evident when both EAS or hydrolysed

casein were used as chemoattractants. Activation of PMN by these two chemotactic substances is probably dependent upon different mechanisms (Rabson *et al*, 1976) and the finding that histamine inhibits chemotaxis to both agents suggests that it is acting on a common pathway.

Several leucocyte functions are known to be modulated via histamine receptors, which are of two types as shown by Ash and Schild (1966). The H-1 receptor which mediates the contraction of intestinal and bronchial smooth muscle and the dilation of small venules has been shown to have no effect on histamine modulation of either eosinophil (Clark *et al*, 1975) or neutrophil (Hill and Quie, 1975) chemotaxis. The H-2 receptor, which mediates gastric acid secretion, has been observed to modulate both T-cell function (Plaut *et al*, 1975), and the histamine effects on eosinophil (Clark *et al*, 1975) and neutrophil movement (Hill and Quie, 1975; Hill *et al*, 1975). Using diphenylhydramine hydrochloride a potent H-1 receptor blocking agent, no effect on either the histamine induced stimulation of random migration or the inhibition of true chemotaxis could be demonstrated in the present study. These results are in agreement with those of Hill and Quie (1971) who showed that the histamine induced inhibition of chemotaxis was independent of the H-1 receptor site, but

could be eliminated by an H-2 receptor antagonist. Similarly Clark *et al*, (1975) have reported that eosinophil chemotaxis to excess histamine concentrations is potentiated by metiamide but not by an H-1 receptor blocker. In this study metiamide, an H-2 receptor antagonist had no detectable effect *per se* on true PMN chemotaxis although it did stimulate random migration, indicating that it was able to mimic (albeit weakly) the histamine effect on this latter function. These results suggest that random migration is enhanced by occupation of the H-2 receptor site. Because metiamide alone increased chemokinesis, its effects on the previously noted histamine stimulation of chemokinesis were not investigated. The H-2 receptor antagonist was, however, capable of blocking the histamine induced inhibition of true chemotaxis, a phenomenon which was irreversible and which was dependent upon pre-treating the cells with metiamide.

The diverse effects of histamine and metiamide on PMN directed motility suggests that metiamide weakly simulates histamine to an extent sufficent to exert a stimulatory effect on chemokinesis but insufficient to affect true chemotaxis. These results indicate that the two-fold effect on neutrophil motility induced by histamine may be mediated via the H-2 receptor site.

To assess whether levamisole, a potent stimulator of all forms of neutrophil movement mediates its effects via the H-2 receptor site, cells were pre-treated with metiamide and exposed to levamisole. Results indicate that metiamide is unable to block levamisole induced stimulation of chemotaxis, which probably acts independently of the H-2 receptor. Histamine pre-treatment of neutrophils, however, excluded levamisole stimulation, whereas levamisole pre-treatment prevented histamine induced inhibition of chemotaxis. The opposite effects of histamine and levamisole, unrelated to the H-2 receptor site, may pertain to their diverse influence on intracellular cyclic nucleotide levels (see later).

Histamine has been reported to increase intracellular levels of cAMP. More specifically, increases have been noted in human lung slices and basophil preparations (Beaven, 1976) mononuclear cells (Bourne *et al*, 1973) and neutrophils (Zurier *et al*, 1974). Cyclic nucleotide estimations in this report indicate that PMN cAMP levels are significantly increased after histamine treatment reaching maximum levels within 5 minutes and persisting for the remainder of the experiment. Cells treated with metiamide, however, showed an initial slight elevation of cAMP which soon returned to normal levels. Intracellular cGMP levels were unaltered by exposure to either histamine or metiamide.

Agents which promote fluctuations in intracellular cyclic nucleotide levels have previously been shown to modulate neutrophil movement. Hill *et al*, (1975) have demonstrated that histamine and other agents which increase intracellular levels of cAMP inhibit PMN chemotaxis, their results being similar to those of Rivkin *et al*, (1975) and Tse *et al*, (1972). Rivkin and Becker (1976) have also reported that d'cAMP stimulates random migration but inhibits chemotaxis, and these agents induce enhanced PMN migration from capillary tubes (Lomnitzer *et al*, 1976). In the present study, PGE_1, isoproterenol and d'cAMP, agents known to increase intracellular levels of cAMP all simulated the effects of histamine in stimulating chemokinesis but inhibiting true chemotaxis. cAMP, an agent which does not enter cells (Henion *et al*, 1967) had no effect on either of these functions. Although these results suggest a relationship between increased cyclic AMP levels and enhanced random motility, propanolol, a β-blocker also stimulates chemokinesis and true chemotaxis, as does levamisole, an agent which does not affect cAMP but which increases leucocyte intracellular cGMP levels (Hadden *et al*, 1975). This might indicate the existence of at least 2 distinct modes of stimulation of random motility. The first being related to increased levels of cAMP which may mediate stimulation by decreasing cell adherence (Lomnitzer *et al*, 1976) and the second by agents which do not elevate cAMP

levels (Hadden *et al*, 1975 and Haddock *et al*, 1975), and have no effect on adhesion. The mechanism of stimulation by the latter is unknown. These results also indicate that the Yin-Yang hypothesis of biologic control (Goldberg *et al*, 1974) may not apply to neutrophil random motility.

As regards true PMN chemotaxis, however, it does appear that agents which increase intracellular levels of cAMP will inhibit this function whereas levamisole and propanolol which increase cGMP levels, also promote enhancement of directed motility.

C H A P T E R VIII

Effects of Leucoattractants (Casein and EAS) and Drugs which alter Intracellular Cyclic Nucleotide Levels on Neutrophil Glycolysis and Hexose Monophosphate Shunt Activity

INTRODUCTION

The results documented in the previous three chapters have indicated that drug-induced increases in intracellular cyclic nucleotide levels promote altered PMN locomotory responses. Neutrophil chemotaxis is a glycolysis dependent phenomenon (Carruthers, 1966; Goetzl and Austen, 1974) and hexose monophosphate shunt activity has also been implicated in cell motility (Goetzl *et al*, 1974; Goetzl and Austen, 1974). To assess whether the observed changes in cell motility mediated by cAMP and cGMP were related to changes in glycolytic or HMS activity the effects on these pathways of drugs which cause elevation of intracellular cyclic nucleotides have been investigated in neutrophils stimulated by leucoattractants. To evaluate functional specificity the effects of these drugs, and also of the chemoattractants EAC and casein on HMS activity following phagocytosis of *Candida albicans*, have been determined.

MATERIALS AND METHODS

Chemicals: The drugs used in the present study were cAMP, d'cAMP, isoproterenol, histamine, prostaglandins E_1 and A_1, cGMP, levamisole, propanolol, acetyl choline and carbamyl choline. The commercial sources of these drugs have been indicated in previous chapters. Ascorbic acid was obtained from Sigma Chemicals (St. Louis, Missouri, U.S.A.).

Neutrophil preparation: For all investigations described in this study pure neutrophil suspensions were used. Heparinized venous blood was obtained from healthy donors and neutrophils were prepared according to the procedures described in Chapter IV (page 49).

Glycolysis assay: Glycolysis was assessed according to the extent of lactate production by 6 x 10^6 PMN in the presence of leucoattractants and the various drugs. Experiments were of two types. Firstly kinetic experiments were performed at 1, 3, 5, 10, 20, 40 and 60 minutes to investigate the effects on glycolysis of casein (at a final concentration of 100 μg/0.5 ml.), serum (6%) and EAS (6%). The control system was PMN in PBS alone. The effects of d'cAMP histamine, levamisole and propanolol on EAS stimulated glycolysis were also investigated at the same time intervals. In a further series of experiments the effects of the leucoattractants and drugs at a fixed time interval (60 minutes) were assessed. Drug effects on glycolysis were investigated in the presence and absence of 5% EAS.

Hexose Monophosphate Shunt Assay: HMS activity of 4 x 10^6 neutrophils was assessed by measurement of the amount of radiolabelled CO_2 (1 - $^{14}CO_2$) released from glucose labelled in the C-1 position. The $^{14}CO_2$ released during incubation was absorbed in 1N KOH. The amount of absorbed $^{14}CO_2$ is proportional to the

extent of HMS activity. The HMS assay procedure is described in Chapter IV(page 68).

In an initial series of experiments the effects of chemoattractant stimulation (casein and EAS at final concentration of 100 µg and 10% respectively) and phagocytosis (by the ingestion of *C. albicans*) were assessed at varying time intervals (1, 3, 5, 10, 20, 40 and 60 minutes). In dose response experiments a fixed time interval (60 minutes) was selected and the effects of d'cAMP (10^{-5}M - 10^{-2}M), histamine (10^{-6}M - 10^{-3}M), levamisole (10^{-6}M - 2.5×10^{-3}M) and propanolol (10^{-6}M - 10^{-3}M) on EAS stimulated neutrophils, were investigated. In further kinetic experiments the effects of the same drugs i.e. d'cAMP, histamine, levamisole, propanolol and also ascorbic acid were assessed at varying time intervals at fixed concentrations of 10^{-3}M, 2.5×10^{-5}M, 10^{-3}M and 10^{-4}M respectively. The effects of the leucoattractants, cyclic nucleotides and various drugs were also tested at fixed time intervals (60 minutes) and at fixed concentrations. Cyclic nucleotide and drug effects were evaluated in the presence of 10% EAS. A similar series of experiments were performed to assess the effects of the same agents on neutrophil phagocytosis. Phagocytosis was initiated by the addition of 0.1 ml. *C. albicans* containing 8×10^{6} organisms/ml. to give a final cell : particle ratio of 1 : 2. For opsonization

0.1 ml. of autologous serum was added to the reaction mixture. Using this system 90% - 100% ingestion is evident at 20 minutes (Rabson *et al*, 1974).

Nitroblue Tetrazolium Reduction: N.B.T. reduction was measured by a semi-quantitative slide technique which has been described in Chapter IV (page 69). The effects of the leucoattractants casein and EAS at final concentrations of 100 µg and 10% respectively were assessed.

RESULTS

Glycolysis: The effects of EAS, serum and casein on the kinetics of lactate production are shown in Fig. XV (page 162). Results are expressed as mean and standard error of the mean values for five separate experiments. As can be seen, EAS causes a marked stimulation of glycolysis at all time intervals whereas no such stimulation is mediated by serum alone or casein. Dibutyryl cAMP, histamine, levamisole and propanolol were without effect on glycolysis at all time intervals tested (results not shown). The results of experiments investigating the effects of the leucoattractants, cyclic nucleotides and various drugs are shown in Table 25 (Page 163). These results are expressed as mean values with standard deviations of six experiments. As can be seen only EAS mediated a stimulation of glycolysis. Cyclic nucleotides and drugs had no effect on the EAS stimulation of glycolysis.

HMS: The effects of phagocytic and chemoattractant stimulation on HMS activity are shown in Figure XVI (page 164). Results are expressed as mean values and standard error of the mean for three separate experiments. Phagocytosis causes a considerable increase in HMS activity (400% at 60 minutes) whereas the effects of chemotactic stimulation vary with the leucoattractant. EAS promotes a delayed stimulation which is only evident at 60 minutes, whereas casein mediates considerable inhibition of the HMS which is present at all time intervals tested. Results of experiments utilising serum have been corrected for serum inhibition. Serum alone promotes an inhibition of HMS activity which is presumably due to a competitive effect of serum glucose. It is unlikely that the inhibition is mediated by inhibition of adherence due to high protein concentrations since media supplemented with equivalent concentrations of non-chemotactic bovine serum albumin has no inhibitory effect. Furthermore, siliconisation of the cell containing chamber had no inhibitory effects, which would indicate that HMS activity is probably not adherence dependent.

The results of six separate (mean and standard error) dose-response experiments for d'cAMP and histamine are shown in Figure XVII (page 165) and of levamisole and propanolol in Fig. XVIII (page 166). As

can be seen all four agents mediate a progressive dose dependent inhibition of HMS activity. Figures XIX, XX, XXI and XXII on pages 167, 168, 169 and 170 respectively, show the kinetics of EAS associated HMS activity in the presence and absence of the same four drugs. Inhibition is evident throughout, and is greatest at 60 minutes. These results have not been corrected for serum inhibition, since the magnitude of inhibition is unaltered. The effects of ascorbate (5×10^{-3}M) on HMS activity in the absence of EAS or phagocytosis are shown in Figure XXIII (page 171). The results are expressed as mean values for triplicate samples of one experiment. Ascorbate promotes considerable stimulation of HMS activity (300% at 60 minutes) which is evident at all time intervals. Table 26 (page 172) shows the results of drug effects on EAS associated HMS activity after 60 minutes incubation. Those which mediate elevated intracellular cAMP cause a depression of HMS activity. Levamisole and propanolol mediate similar effects whereas cGMP, acetyl choline and carbamyl choline have no effect. Ascorbate promotes enhancement of HMS activity in the presence of EAS.

The effects of the leucoattractants casein and EAS, and of certain drugs which mediate depression of the HMS, on HMS associated phagocytosis are shown in Table 27 (page 173). Casein and EAS have no effects on neutrophil HMS activity associated with phagocytosis whereas all other agents tested cause inhibition.

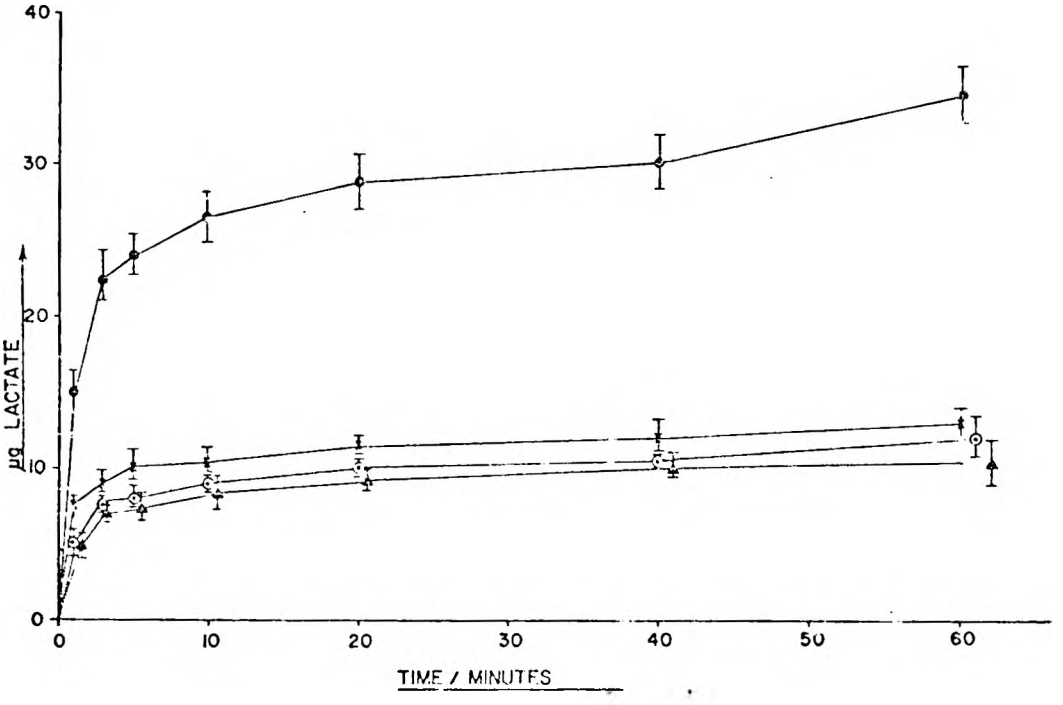


Figure XV The effects of PBS (▲), Casein (⊙), Serum (x) and EAS (o) on the kinetics of neutrophil glycolysis.

TABLE 25

Effects of 1) Casein, EAS and Autologous Serum and 2) EAS in the Presence of Various Drugs on Neutrophil Glycolysis

Final Concentrations of Drugs and Leucoattractants added to 6×10^{-6} PMN	Mean Lactate Production µg/6×10^{6} PMN/60 Min. with Standard Deviation
PBS only	9.8 ± 1.2
Casein (100 µg/ml.)	10.2 ± 0.98
Serum (6%)	12.2 ± 1.3
EAS (6%)	39.3 ± 4.1
EAS + 10^{-3}M d'cAMP	38.6 ± 3.3
EAS + 10^{-3}M cAMP	39.1 ± 3.6
EAS + 10^{-3}M cGMP	40.2 ± 4.0
EAS + 10^{-3}M isoproterenol	40.1 ± 3.2
EAS + 2.5×10^{-5}M histamine	38.2 ± 3.6
EAS + 10 µg PGE_1	41.2 ± 3.5
EAS + 10 µg PGA_1	40.0 ± 4.1
EAS + 10^{-3}M levamisole	39.6 ± 3.7
EAS + 10^{-4}M propanolol	38.3 ± 4.0
EAS + 10^{-3}M acetyl choline	39.6 ± 3.8
EAS + 10^{-3}M carbamyl choline	40.1 ± 3.6

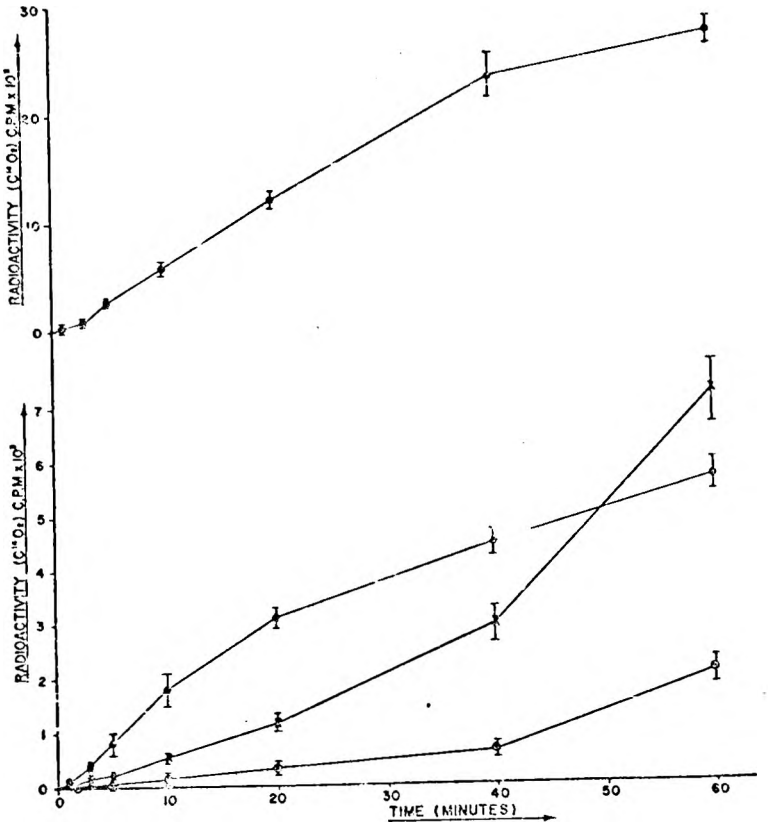


Figure XVI The effects of chemotactic and phagocytic stimulation on neutrophil HMS activity. Resting (⊕), Casein (⊙) and EAS effects (x) are shown in the lower graph and phagocytosis (⊕) in the upper graph.

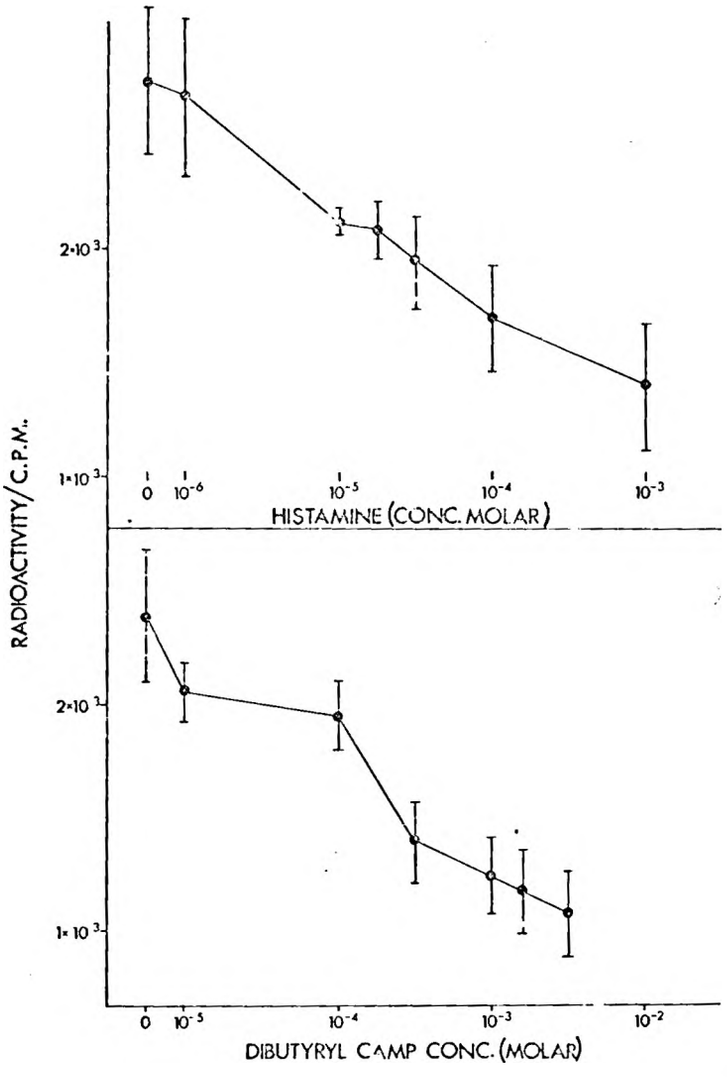


Figure XVll The effects of varying dibutyryl cAMP and histamine concentrations on neutrophil HMS activity.

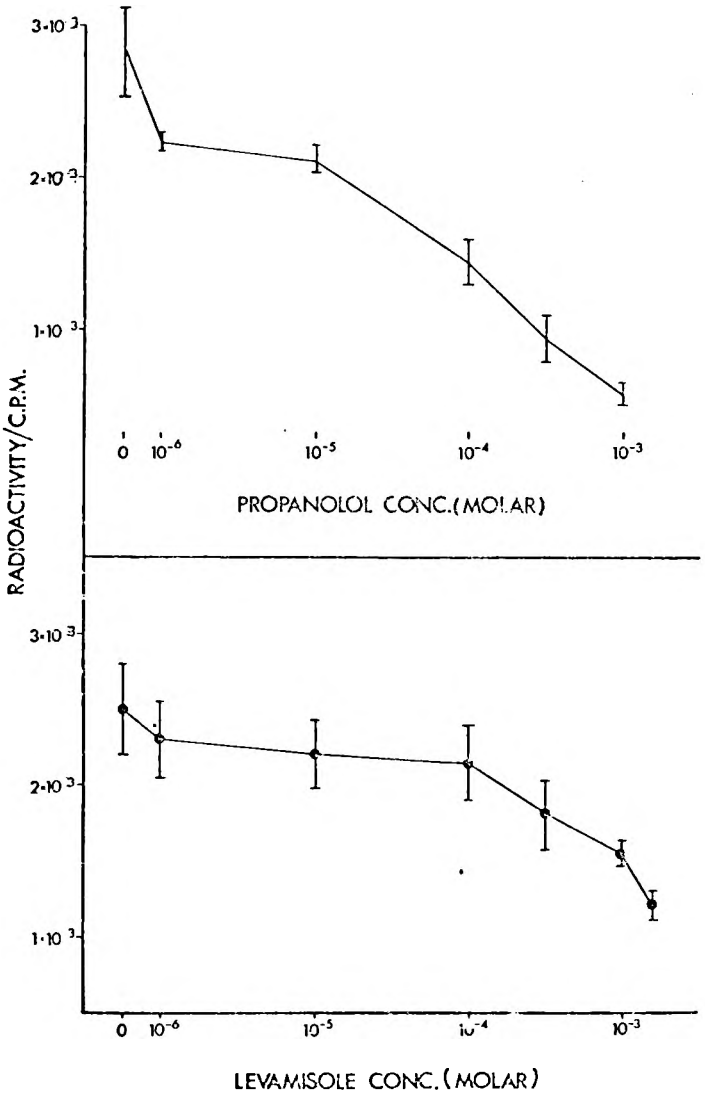


Figure XVlll The effects of varying concentrations of levamisole and propanolol on neutrophil HMS activity.

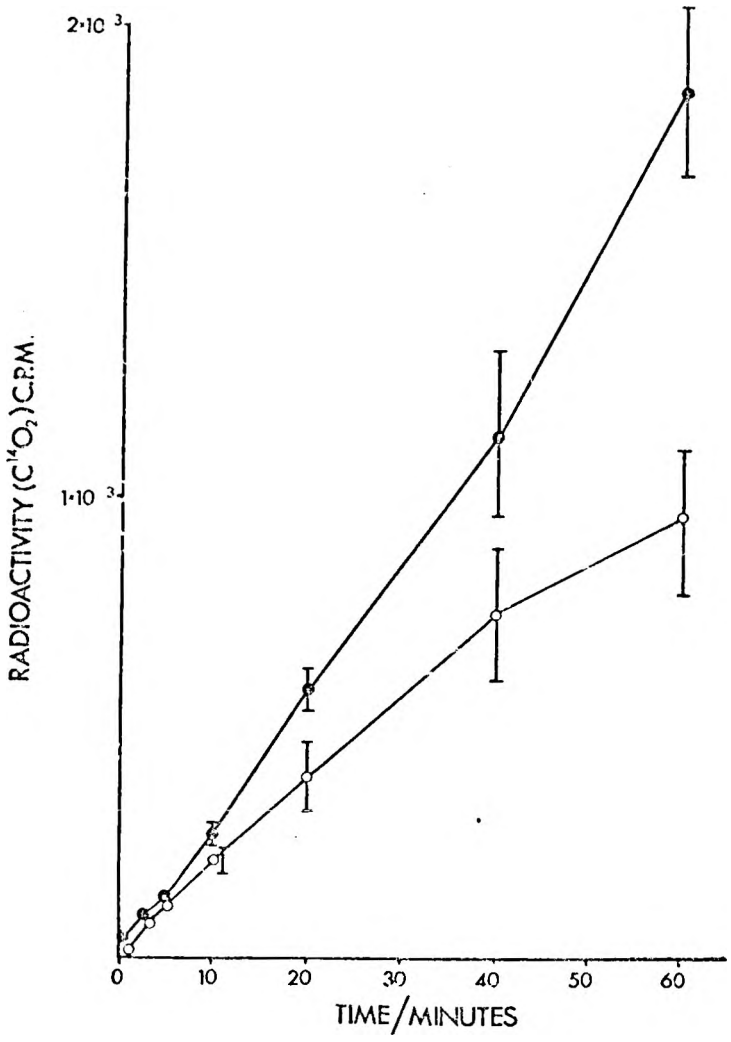


Figure XIX The effects of 10^{-3}M dibutyryl cAMP (o) on the kinetics of EAS associated HMS activity(● control).

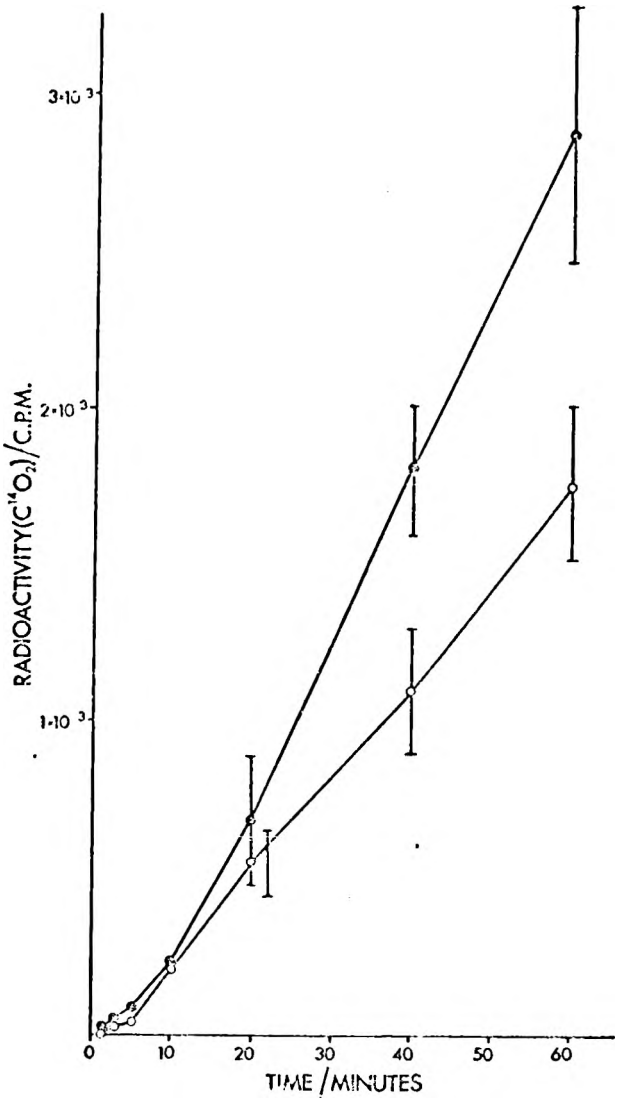


Figure XX The effects of 2.5 x 10^{-5}M histamine (o) on the kinetics of EAS associated HMS activity, (● control).

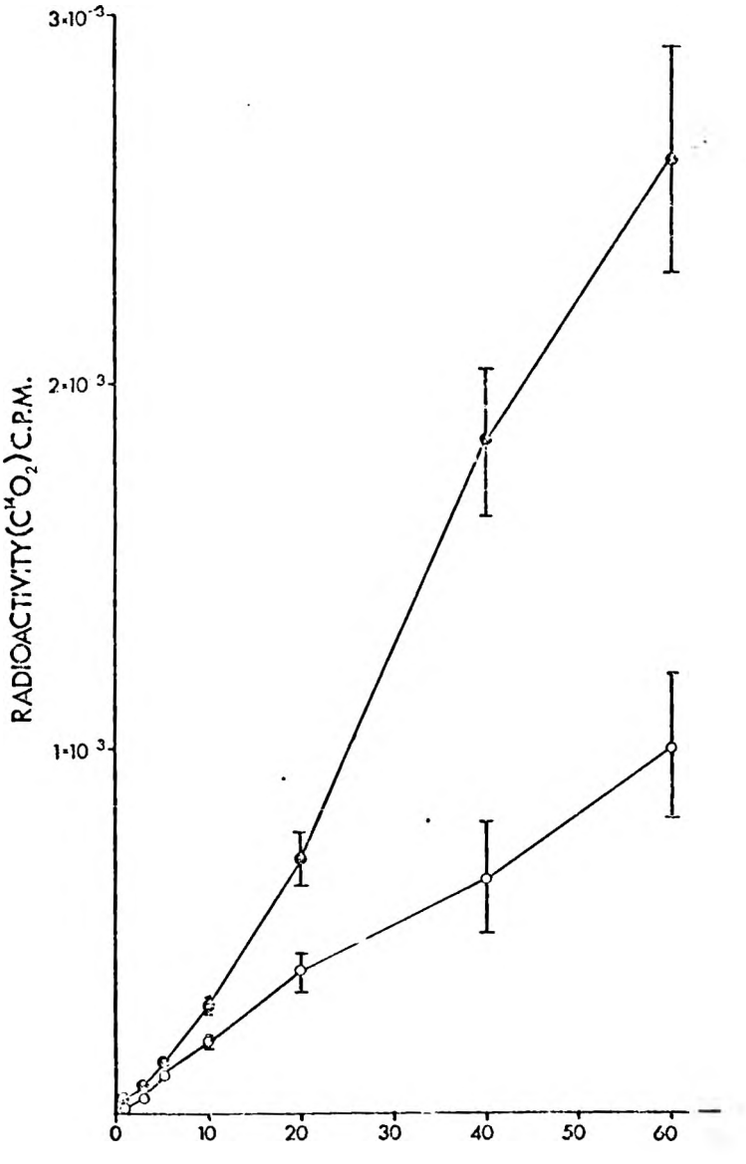


Figure XXI The effects of 10^{-3}M levamisole (o) on the kinetics of EAS associated HMS activity, (● control).

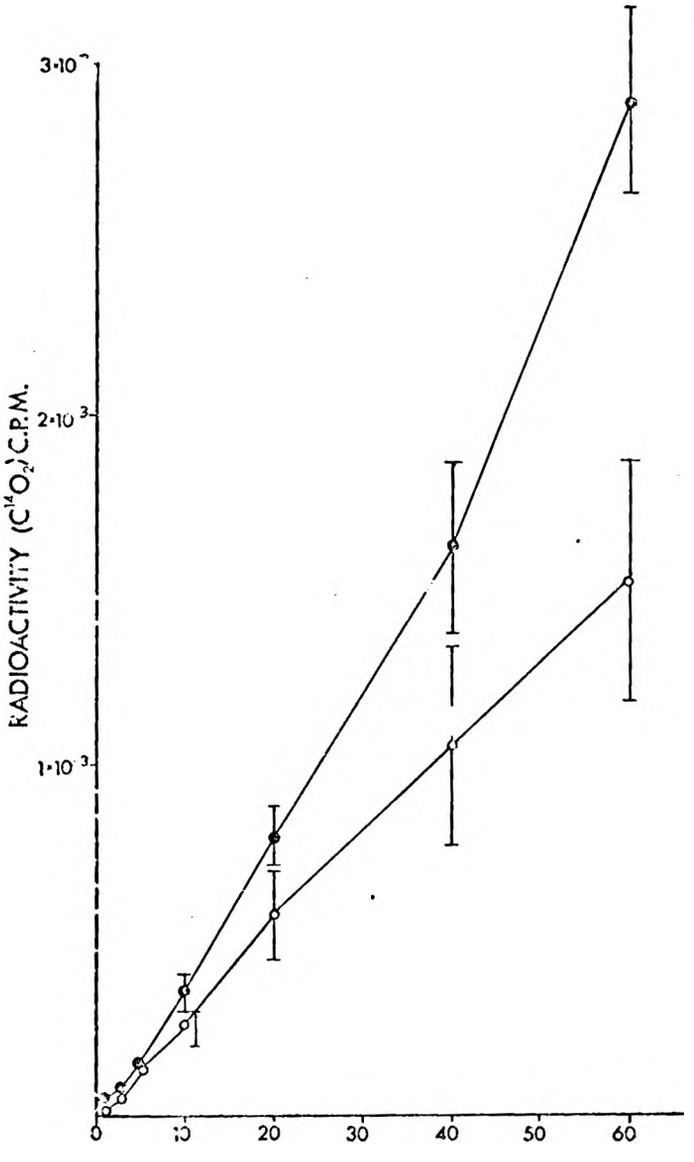


Figure XXII The effects of 10^{-4}M propanolol (o) on the kinetics of EAS associated HMS activity, (● control).

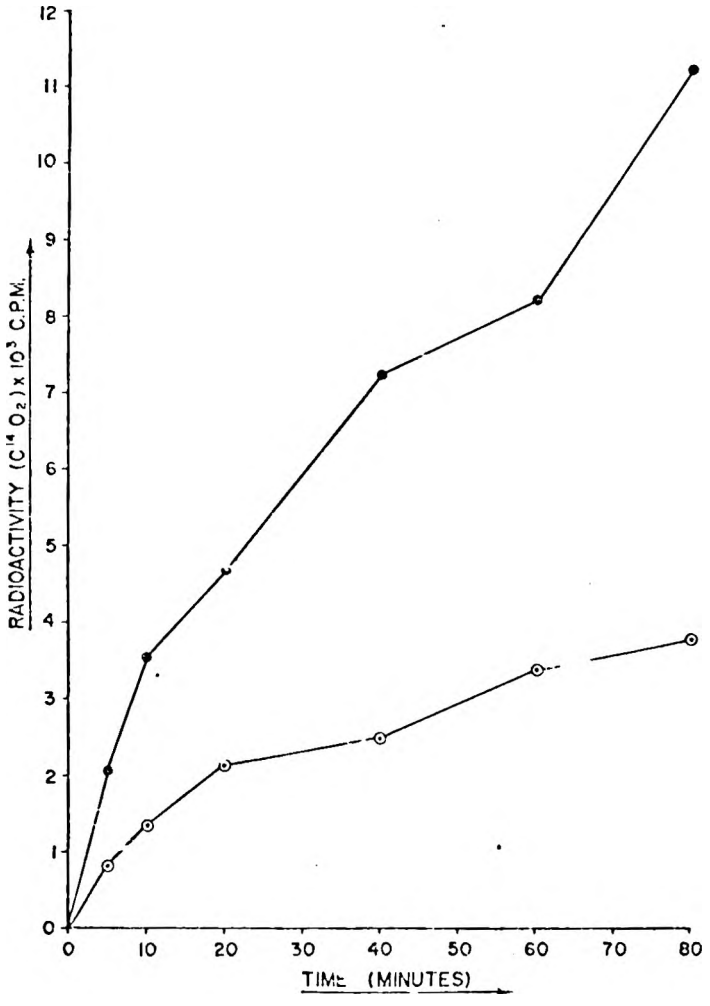


Figure XXIII The effects of 5 x 10^{-3}M ascorbate on neutrophil resting HMS activity.

TABLE 26

Effects of Various Drugs which promote Elevation of Intracellular Cyclic Nucleotide Levels on EAS Stimulated HMS Activity

Drug	Concentration	Control Mean and Standard Deviation	Experimental Mean and Standard Deviation	% Inhibition of Control	p Value
cAMP	1×10^{-3}M	2913 ± 708	2698 ± 589	7.3	NS
d'cAMP	1×10^{-3}M	2032 ± 449	1073 ± 135	47	p <0.001
Histamine	2.5×10^{-5}M	2193 ± 708	1929 ± 515	34	p <0.001
Isoproterenol	1×10^{-4}M	2193 ± 708	2079 ± 508	29	p <0.01
Prostaglandin A_1	10 µg/ml.	2193 ± 708	1462 ± 612	50	p <0.001
Prostaglandin E_1	10 µg/ml.	2193 ± 708	1642 ± 416	44	p <0.001
cGMP	1×10^{-3}M	2193 ± 708	2779 ± 769	4.6	NS
Acetyl choline	1×10^{-3}M	2193 ± 708	2727 ± 506	6.3	NS
Carbamyl choline	1×10^{-3}M	2193 ± 708	2898 ± 750	-	NS
Levamisole	1×10^{-3}M	2608 ± 626	1367 ± 425	48	p <0.001
Propanolol	5×10^{-4}M	3123 ± 395	1380 ± 675	56	p <0.001
Ascorbic Acid	5×10^{-3}M	2203 ± 442	3312 ± 500	+50 *	p <0.001

*(Stimulation)

TABLE 27

Effects of Casein, EAS, d'cAMP, Histamine, Isoproterenol, Levamisole and Propanolol on HMS Activity Associated with Phagocytosis

Leucoattractant or Drug	Concentration	Control Mean and Standard Deviation	Experimental Mean and Standard Deviation	% Inhibition of Control	p value
Casein	100 µg/ml.	28306 ± 3331	30220 ± 6395	0	NS
EAS	10%	28306 ± 3331	28262 ± 2418	0	NS
d'cAMP	1×10^{-3}M	33579 ± 5557	17223 ± 4187	49	$p < 0.001$
Histamine	2.5×10^{-3}M	33579 ± 5557	20156 ± 3618	40	$p < 0.001$
Isoproterenol	1×10^{-4}M	33579 ± 5557	27555 ± 3217	18	$p < 0.05$
Levamisole	1×10^{-3}M	32946 ± 5839	22892 ± 2611	31	$p < 0.01$
Propanolol	1×10^{-4}M	33579 ± 5557	20054 ± 3061	40	$p < 0.001$

The effects of the leucoattractants casein and EAS on N.B.T. reduction, are shown below in Table 28.

TABLE 28

The Effects of Casein and EAS on Neutrophil Nitroblue Tetrazolium Reduction

CASEIN STUDY		EAS STUDY		
Control	Casein 100 µg	Control	Endotoxin and Heat inactivated Serum	Endotoxin and Normal Serum (EAS)
1.2 ± 1.5	0.9 ± 1	1.7 ± 1.5	19 ± 3	31 ± 8

Casein has no statistically significant effect on N.B.T. reduction whereas EAS mediates a significant increase above the control which contains serum and endotoxin which had been inactivated by heating at 56°C for 40 minutes. ($p < 0.01$).

DISCUSSION

Glycolysis has been implicated directly and indirectly, as being the major source of adenosine triphosphate (ATP) production for neutrophil chemotaxis. However, casein, another potent chemoattractant, had no effect on glycolysis over a one-hour period, indicating that although glycolysis *per se* might be essential for optimal leucotaxis, stimulation of this pathway is not a pre-requisite for cell motility. These different effects on glycolysis could reflect different activities and modes of action of the two chemoattractants used, EAS being the more potent of the two attractants.

Drugs known to increase neutrophil random motility and chemotaxis, such as levamisole and propanolol had no effect on glycolysis, the results being similar to those described for ascorbic acid. Histamine, d'cAMP, prostaglandin E_1 and isoproterenol, agents which increase random motility but which inhibit true chemotaxis also had no effect on this pathway, these results indicating that enhancement of cell motility, either directed or random, is not dependent upon stimulation of glycolysis. Goetzl and Austen (1974) have reported that the partially purified chemotactic factors C5a and kallikrein, mediate 100 - 600% increases in HMS activity above that of the corresponding unstimulated cells. The increase in HMS activity with time parallels the

neutrophil chemotactic responsiveness and similar results were obtained in this study using EAS.

Goetzl *et al*, (1974) have reported that ascorbic acid and glutahione which may enhance the oxidation of NADPH to $NADP^+$ (De Chatelet *et al*, 1972) the early rate limiting step in the HMS promote a stimulation of neutrophil chemotaxis and random migration.

The work of these authors has implicated HMS involvement in neutrophil motility, increased HMS activity being associated with an enhanced level of motility. However, there is evidence to suggest that stimulation of the HMS associated with neutrophil locomotion may be coincidental. It has been demonstrated that DFP (Carruthers, 1966) and neutrophil-immobilising factor (Goetzl *et al*, 1973) which cause suppression of neutrophil chemotaxis have no inhibitory effects on HMS activity. Furthermore although EAS has been shown to stimulate HMS activity, as does kallikrein and pure C5a, casein promotes a consistent depression of resting HMS activity confirming that HMS stimulation is not essential for cell movement. Goldstein *et al*, (1975) have reported that biochemically pure C5a caused a stimulation (500%) of nitroblue tetrazolium (N.B.T.) reduction by human neutrophils with a concomitant 350% increase in HMS activity.

The divergent effects of the two chemotactic factors, EAS and casein, were further confirmed using N.B.T. reduction. Wherease EAS consistently stimulated N.B.T. reduction, casein had no effect on this function. Furthermore although ascorbic acid which increases all forms of cell movement stimulates HMS activity, other drugs with similar effects on cell motility such as levamisole and propanolol decreased HMS activity. In addition, drugs which apparently raise intracellular cAMP levels (acetyl choline, carbamyl choli. : and cGMP) have been reported to alter cell motility (Estensen *et al*, 1973)(results which could not be confirmed in this laboratory) also had no effect on HMS activity. Drugs which cause increased intracellular accumulation of cAMP also had no stimulatory effect on HMS activity and in fact decreased the HMS pathway. Furthermore, PGA_1 at a concentration known to inhibit all forms of neutrophil movement (Rabson *et al*, 1974) also depressed HMS activity, indicating a lack of relationship between stimulation and inhibition of neutrophil movement and HMS activity.

It was of interest that although casein depressed HMS activity in resting cells, it was unable to inhibit the metabolic burst associated with phagocytosis, EAS likewise had no effect. On the other hand levamisole, propanolol and drugs

known to increase intracellular cAMP levels were able to inhibit the post-phagocytic increase in HMS activity.

The results described in this paper indicate that although an intact glycolytic pathway is probably necessary for cell motility, since inhibitors of this pathway cause depressed cell motility, stimulation of glycolysis is not a prerequisite for cell motility. Furthermore alterations in HMS activity are unrelated to the effects on cell motility since agents which induce inhibition (levamisole and propanolol) have the same effects on locomotion as ascorbic acid which causes stimulation of the HMS. In addition casein, a potent chemoattractant, did not stimulate HMS activity, this depressive effect being specific for resting cells since HMS activity associated with phagocytosis is unaffected by casein. Finally, this study has indicated that the two chemoattractants, EAS and casein, have different effects on cellular biochemistry.

C H A P T E R IX

Discussion

The results presented in this thesis indicate that agents which promote changes in neutrophil intracellular cyclic nucleotide levels can also modulate cell motility. Exogenous cAMP and cGMP are without effect on motility, presumably due to poor intracellular passage. Various agents which promote elevated cAMP levels by different modes of stimulation such as d'cAMP, histamine, prostaglandin E_1 and isoproterenol, all mediate a dose dependent stimulation of random motility and chemokinesis with a concomitant inhibition of true directed motility. Microtubular synthesis, which is probably essential for chemotaxis, is a cAMP dependent process (Fuller *et al*, 1975). However, the maintenance of artifically high levels of intracellular cAMP may mediate inhibition of microtubule synthesis. On the other hand cAMP may inhibit some other cell function necessary for chemotaxis. A possible contender for this role is cell adherence. Certain of the above drugs (d'cAMP and prostaglandins) have been reported to inhibit PMN adherence *in vitro* (Lomnitzer *et al*, 1976a), with an accompanying increase in the area migrated from capillary tubes. It is possible, therefore, that the increased random motility caused by these agents is mediated by a decreased adherence to the substratum thereby facilitating such movement.

Directional movement on the other hand, may require adherence for cell orientation and agents which inhibit this function could be expected to interfere with chemotaxis.

The effects obtained with agents which promote elevation of intracellular cGMP levels are somewhat different. Although the results of Estensen *et al*, (1973) and Hill *et al*, (1975) could not be confirmed, two other agents which can promote elevated cGMP levels, levamisole and propanolol, exerted profound effects on cell motility. Both drugs caused a marked stimulation of random motility and chemokinesis. However, no inhibition of true directed motility was observed, rather stimulation was evident. The mechanism of stimulation of random motility by these drugs is probably different to that of cAMP stimulants since the effects on chemotaxis are different and also levamisole and propanolol have no effects on neutrophil adherence (Lomnitzer, 1976, unpublished observations). It is, therefore, likely that the effect of cGMP is due to a stimulation of a common component in the machinery of random migration, chemokinesis and chemotaxis. Furthermore ascorbic acid which has been reported to increase neutrophil random migration and chemotaxis (Goetzl *et al*, 1974) has also been reported to increase neutrophil intracellular cGMP levels (Gallin and Wolff, 1975). The effects of ascorbate on neutrophil migration have been confirmed by the author in this laboratory, although the precise types of motility affected have not been studied. The altered cell motility due to cGMP

can probably be considered as a direct positive stimulation whereas that mediated by cAMP on random motility and chemotaxis is secondary and indirect since the primary effect is probably on cell adherence.

Casein and EAS showed a different spectrum of effects on HMS and glycolytic activities and on cyclic nucleotide profiles. EAS promoted an early increase in cAMP levels, and a stimulation of glycolysis, the HMS and N.B.T. reduction. Casein, however, caused no stimulation of either intracellular cAMP, glycolysis or N.B.T. reduction and actually mediated a depression of HMS activity. These results would indicate that an early elevation of cAMP and stimulation of glycolysis and the HMS are not prerequisites for cell motility. Intact glycolysis is probably necessary for cell motility since inhibitors of this pathway cause depressed cell motility. Furthermore the capacity of a leucoattractant to activate glycolysis may correlate with leucotactic activity. This is indeed the case with EAS and casein, the former being the more potent of the two leucoattractants. Chemoattractants may, therefore, be of two types; firstly those which mediate little or no change in cyclic nucleotide levels and glycolysis and HMS activity; and secondly those which promote changes in all 3 functions. The former may be considered as "passive" and the latter as "active" leucoattractants. The relationship of the early increase

in cAMP, mediated by EAS, to glycolysis is obscure. Although the increase in glycolysis parallels the early peak of cAMP synthesis, agents which promote elevated cAMP levels in neutrophils have no effect on glycolysis.

On the other hand agents which stimulate random motility caused a depression of HMS activity. The exception to this finding is ascorbic acid which promotes a stimulation. These findings would indicate that alterations in HMS activity are unrelated to the effects on cell motility since agents which induce inhibition (levamisole and propanolol) have the same effects on locomotion as ascorbic acid which causes stimulation of the HMS. The depressive effect of casein on the HMS was specific for resting cells since HMS activity associated with phagocytosis is unaffected by this leucoattractant. Agents which promote effects on intracellular cAMP levels and levamisole and propanolol which increase cGMP levels cause a functionally non-specific inhibition of HMS activity since chemotaxis and phagocytosis are both affected.

In conclusion cell motility can be modulated by agents which mediate alterations in intracellular cyclic nucleotide levels. Elevated cAMP and cGMP levels promote enhanced random motility which is probably a similar manifestation of different effects. The two cyclic nuclectides mediate opposing effects on directed motility;

cAMP causes inhibition and cGMP stimulation. The effects of the cyclic nucleotides are probably unrelated to glycolysis and HMS activity. The two leucoattractants used in this study have different effects on cellular biochemistry. Although the mechanics of movement are probably the same the respective capacities to mobilize intracellular energy sources are different.

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ADDENDUM

A criticism of experiments presented in Chapters VI and VII of this thesis is the absence of control experiments to investigate the leucotactic potential of the drugs (D'cAMP, histamine, isoproterenol, levamisole and propranolol) at concentrations which stimulate neutrophil chemokinesis in the absence of any protein leucoattractant. This criticism is motivated by the possibility that the various drugs at the concentrations used may produce their observed effects by a combined action on leucocyte chemotaxis and chemokinesis. This criticism is invalid for the drugs levamisole and propranolol for the following reasons:

1) In the checkerboard systems used to dissociate effects on random and directed movement the various drugs were present in equimolor concentrations on both sides of the filter for all systems investigated, i.e. random migration, chemotaxis, chemokinesis and motility in negative gradients. At no time was a drug gradient present, therefore chemotactic effects can be excluded.

2) Levamisole and propranolol in equimolar concentrations on both sides of the filter actually enhance directed motility in positive gradients of EAS. This excludes the possibility that these drugs are themselves

chemotactic since no increment in directional motility should occur if they were attractants. In fact the extent of migration should not increase, but should remain the same or diminish due to a deactivation effect if the drugs themselves were chemotactic. On the other hand the drugs which increase intracellular cAMP levels cause an inhibition of the neutrophil directional response to EAS and casein when present on both sides of the filter. The criticisms presented may be valid for these drugs since deactivation of directional motility could be explained if D'cAMP, histamine and isoproterenol were chemotactic. Their presence on the upper side of the chamber may eliminate neutrophil chemotactic responsiveness. However a more detailed examination of the checker-board results indicates it is unlikely that these drugs have leucotactic potential due to their stimulation of motility in negative gradients of EAS. Likewise in chemokinesis systems the stimulation of random motility is equal to or greater than that of the corresponding positive gradients. For both negative gradients and chemokinesis systems it could be expected that a reduction in motility due to the combined "deactivating" effects of EAS and "chemotactic" drug would occur. This was not observed and indicates the improbability that cAMP elevating drugs are chemotactic.

Early experiments using the Boyden system indicated that the drugs levamisole, D'cAMP and histamine were not chemotactic. These results were not included in this thesis since in the opinion of the author, all necessary information on the mode of action of these agents on cell motility could be obtained from data contained in the checkerboard systems. However, to clarify this matter and to conclusively answer criticisms of this investigation, these experiments have been repeated using levamisole, D'cAMP, histamine, isoproterenol and propranolol at concentrations used in the checkerboard systems and which have been shown to have optimal effects on neutrophil motility. By placing each drug in the upper compartment only and the lower compartment only and in both compartments simultaneously, the chemotactic activity of the drugs can be assessed. Should a given drug be chemotactic the extent of migration in the positive gradient should exceed that in the absence of a gradient and in the corresponding negative gradient. If the drug possesses only chemokinetic activity the extent of migration should be equal in all three systems or greater in the absence of a positive gradient. The method of Zigmond and Hirsch (1973) has been used to investigate these effects.

MATERIALS:

The drugs used were levamisole, propranolol, D'cAMP, histamine and isoproterenol.

CHEMOTAXIS ASSAY:

The method used was that of Zigmond and Hirsch (1973) as described in Chapter V page 72. Drugs used were placed in appropriate concentrations on either or both sides of the Boyden chamber. Neutrophils were not preincubated with drugs. No added protein was present in the system since the purpose of the study was to determine the leucotactic potential of the various drugs.

RESULTS:

These are shown in Table 29, page 200. Neutrophil migration is expressed as mean and standard deviation for 3 experiments each with triplicate filters. A chemokinetic effect was observed for all drugs tested. The chemokinetic effect was independent of the chamber placing of the drug. The extent of neutrophil migration when drugs were placed in the lower chamber only did not exceed that observed when the drug was present in the upper chamber only or in both the upper and lower chambers. This was true for all drugs tested and shows convincingly that none of these drugs are leucotactic.

DISCUSSION:

Drugs investigated in this thesis which mediate their effects on leucocyte motility by increasing intracellular cyclic nucleotide levels are not leucotactic. Although the conclusions in this thesis pertaining to levamisole and propranolol effects on leucocyte motility are valid without further experiment the conclusions contained in Chapter VII on the effects of cAMP elevating agents on leucocyte motility could be invalidated if these drugs are chemotactic. However the results contained in the checkerboards indicate that this is unlikely due to stimulation of chemokinesis and motility in negative gradients. Furthermore the experiments contained in the section confirm that none of the drugs tested are chemotactic and that the conclusions are accurate and valid.

TABLE 29

ASSESSMENT OF THE LEUCOTACTIC POTENTIAL OF DRUGS WHICH STIMULATE NEUTROPHIL CHEMOKINESIS

Drug	Concentration	DISTANCE MIGRATED (μm) WITH DRUG IN: 1 Lower Chamber only	2 Upper Chamber only	3 Both upper and lower chamber
D'cAMP	2.5×10^{-3}M	38.8 ± 5.1*	40.1 ± 4.0	41.3 ± 6.2
Histamine	2.5×10^{-5}M	38.3 ± 2.96	43.3 ± 5.4	42.8 ± 5.7
Isoproterenol	1.0×10^{-3}M	36.3 ± 4.5	37.2 ± 5.1	38.2 ± 4.2
Levamisole	1.0×10^{-3}M	46.6 ± 4.6	46.5 ± 6.7	45.6 ± 4.3
Propranolol	1.0×10^{-4}M	42.0 ± 7.4	43.2 ± 6.5	44.1 ± 7.1

* Results are expressed as mean value with standard deviation. The mean value for the control (no drugs) experiments is 28.3 ± 7.4.

ERRATUM : REFERENCES OMITTED

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