Investigation of vascular permeability changes induced by ultra-violet irradiation in laboratory animals

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INVESTIGATION OF VASCULAR PERMEABILITY CHANGES INDUCED BY ULTRA-VIOLET IRRADIATION IN LABORATORY ANIMALS

A thesis submitted to the University of New South Wales

by

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SUMMARY

The inflammatory reaction induced by ultra-violet radiation has been investigated in the skin of guinea pigs, rats and rabbits. The source of radiation was a high pressure mercury arc in quartz. Particular attention has been paid to the increase in vascular permeability indicated by the exudation of plasma protein "labelled" with Evans blue. The time-courses of superficial erythema and tissue leucocytosis have been correlated with the permeability changes.

In all three species, ultra-violet injury induces a diphasic increase in vascular permeability. In both guinea pigs and rats, the early phase of the permeability response lasts about 15 min. and is inhibited by antagonists of histamine in the guinea pig, by antagonists of 5-hydroxytryptamine-(5-HT) in the rat. In the rabbit the early response is slightly more prolonged and is insusceptible to histamine antagonists.

The late or principal phase of increased vascular permeability matures 20 - 24 hr. after irradiation in guinea pigs and rabbits and approximately 50 hr. after irradiation in rats. In guinea pigs the late permeability response is insusceptible to antagonists of histamine and in rats is insusceptible to antagonists of both histamine and 5-HT. The mediation of the late response has been studied in some detail in guinea pigs. It is unaffected by protease inhibitors such as the trypsin inhibitors from soy bean, lima bean, potato, ovomucoid and

bovine parotid and also by ε-aminocaproic acid which is an inhibitor of the fibrinolytic system. The response is also unaffacted by inhibitors of lecithinase activity, by α-amylase, 2-deoxy-(D)-glucose or sodium α-naphthyl acetate.

Neither erythema nor tissue leucocytosis appear to be consistently related to the permeability response. In the rat erythema is absent and in the guinea pig experimental neutropenia does not influence the permeability response.

The results of this investigation strongly suggest that none of the known endogenous, permeability factors induces the major phase of vascular permeability in ultra-violet injury.

In the light of the above results and other recent work, it seems that further progress in this field is most likely to result from a correlation of ultrastructural studies with biochemical and biophysical investigations.

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Chapter 1.

HISTORICAL INTRODUCTION.

The modern study of inflammation arose from the realisation by John Hunter, the 18th century English surgeon and experimentalist, that ".... inflammation, in itself, is not to be considered as a disease, but as a salutary operation, consequent either to some violence or disease, arising from very different causes, and often without apparent cause, and that its operations are far more extensive than simply the act of producing union in parts divided by violence" (Hunter, 1828).

Besides introducing the concept of inflammation as a useful and purposive process, Hunter postulated that the fundamental and earliest changes occurred in the small blood vessels. The role of the blood vessels he described as follows:

"The very first act of the vessels when the stimulus which excites inflammation is applied is, I believe, exactly similar to a blush.... Inflammation sets out from that point, and that afterwards a new action begins, which is probably first a separation of the coagulating lymph and the throwing it out of the vessels."

The notion of the small vessels as the site of the earliest changes in inflammation was not completely new since Boerhave, in Holland, in the late 17th and early 18th centuries, had considered that inflammation arose from "a conglomeration of the blood, determined by the construction of the small

arteries and a change in the constitution of the plasma" (Castiglioni, 1958).

Enlarging upon the foundation laid by Hunter, Addison (1843) and Waller (1846 a) observed the results of chemical irritation on the circulation of the tongue of the frog,noting the stasis of blood flow occurring in capillaries some time after tissue injury. They also described the margination of leucocytes in inflammation, Waller concluding that the "pus corpuscles" were identical with the colourless corpuscles of the blood (Waller, 1846 b) and assuming that they passed "de toute pièce" through the walls of capillaries leaving no hole behind.

These observations were further extended by Wharton Jones (1851) and Lister (1858), the latter, particularly emphasising the prolonged nature of the inflammatory reaction elicited by minimal exposure to irritants, and insisting upon the need for studying the <u>early</u> changes of inflammation before ".... the copious exudation of lymph, suppuration, ulceration or gangrene...." occurred (Lister, 1858).

Employing mild stimuli to elicit the inflammatory response, Lister perceived for the first time that inflammation could resolve without necessarily progressing to suppuration or necrosis.

"But the most important lesson to be learnt from these simple experiments", he says, "is that the tissues possess.... an intrinsic power of recovery from irritation when it has not been carried beyond a certain point; a principal of fundamental

importance, which has never, so far as I am aware, been established or conjectured."

Cohnheim, (1889), in his "Lectures on General Pathology", embodied the results of his own experiments with those of earlier workers and presented the first comprehensive study of the vascular changes in inflammation.

Cohnheim believed that a "molecular change" occurred in the walls of blood vessels which led to the events of the inflammatory reaction, and proposed "that it is only and solely the vessel wall which is responsible for the entire series of events."

He attempted to explain the four classical signs of Celsus in terms of the vascular reactions which he observed. Thus, an inflamed part is <u>red</u> due to the overloading of its vessels; <u>swollen</u>, not only "because of the increased vascular fullness but especially because of the great increase of transudation"; <u>painful</u>, due to tension on the nerves of sensation; and <u>warmer to the touch</u> (if superficial) due to the increased blood supply.

Cohnheim proposed that increased transudation was due to both a qualitative and quantitative change in the filtering capacity of the vessel walls leading to a greatly augmented flow of protein-rich lymph. In Cohnheim's own words "... the result is an increase of transudation, and of the albuminous contents, as well as the admixture of the colourless and red blood corpuscles with the transuded fluid."

Arnold (1876) considered that there was a tenacious cement substance between the endothelial cells whose coherence became less, and permeability greater in inflammation. Cohnheim was less committal in proposing that the endothelium of the inflamed vessels underwent a "change of a chemical nature", but at the same time he suggested that increased vascular permeability in inflammation might be simply an accentuation of the normal physiological process of permeability. Thus he wrote:

"Between the normal circulation and the inflammatory circulatory disturbance, there are all imaginable gradations. Not only does the quantity and concentration of the fluid transuded show such gradual increase that it is altogether arbitrary whether certain degrees should be referred to as inflammation or recognised as within the limits of health; the extravasation of the corpuscular elements of the blood presents the like gradations."

Cohnheim agreed with Hunter that diverse types of stimuli elicited similar vascular reactions, and that inflammation was invariably associated with healing; and with Lister that suppuration was <u>not</u> an essential part of true inflammation.

In 1893 Metchnikoff published his "Comparative Pathology of Inflammation". His comparison of tissue reactions to injury in lower forms of life with those in mammals led to his concept of inflammation "... as a phagocytic reaction on the

part of the organism against irritants. This reaction is carried out by the mobile phagocytes, sometimes alone, sometimes with the help of the vascular phagocytes of the nervous system."

Metchnikoff attached little importance to the increased vascular permeability and exudation so much stressed by Cohnheim. In Metchnikoff's own words, "... the exsudation of a serous fluid in inflammation cannot be regarded as a means which the organism may make use of to destroy the pathogenic microbes, this service being performed especially by the phagocytes. Since, however, the poisonous chemical products of the bacteria are the most important agencies in producing that general intoxication that we know as disease, it is possible that the serous exsudation may serve to attenuate or modify the action of these products."

That the "poisonous chemical products of the bacteria" could indeed induce inflammatory changes in the absence of their parent organism had been shown by Massart and Bordet (1891), who induced suppurative inflammation in experimental animals by the subcutaneous injection of bacterial culture fluids free of organisms.

Grawitz (1886, 1887) and Scheurlen (1887) had shown earlier that suppuration could be induced in the absence of living micro-organisms by the injection of such simple chemical substance as putrescine and cadaverine, the structures of which are as follows:

$NH_2.CH_2.CH_2.CH_2.CH_2.NH_2$ $NH_2.CH_2.CH_2.CH_2.CH_2.CH_2.NH_2$

Putrescine Cadaverine

Thus, by the end of the 19th century, inflammation had come to be regarded as a process capable of being initiated by chemical agents as well as organisms, but which ultimately was "salutary" or "health promoting"; a process by which noxious agents were overcome and the process of repair initiated.

However, Portier and Richet (1902) and Arthus (1921) showed that agents which themselves were non-toxic, e.g. heterologous serum, could induce an inflammatory response. Such discoveries led Opie (1910) to define inflammation as "... the process by which the cells and exudate accumulate in irritated tissues and usually tend to protect them from further injury."

The advantages of increased permeability in inflammation are generally considered to be twofold.

Firstly, as suggested by Metchnikoff, the outpouring of fluid from the intra-vascular space may ameliorate the effects of irritant or noxious substances simply by diluting them.

Secondly, antibody proteins, among the proteins passing across the capillary membrane, may enable the host to neutralise specifically an irritant or toxic material present.

A third function, however, is also served by the exudate in that invading bacteria become coated with protein or "opsonin" (Wright and Douglas, 1903-04), a process "which

promotes phagocytosis by making them (bacteria) less offensive to the leucocytes" (Payling Wright, 1956).

The accumulation of antibodies in inflamed areas was demonstrated by Rigdon (1939). He injected staphylococcus anti-toxin or diphtheria anti-toxin intravenously immediately after inducing inflammation by the application of xylol to the skin of rabbits (i.e. during the period of increased vascular permeability) and observed that the anti-toxins localised in the inflamed areas. Therapeutic substances, e.g. sulphonamides, accumulated similarly.

Though, in general, the process of inflammation may be beneficial, its clinical effects both immediate and remote, e.g. pain, swelling, immobility, fibrosis and deformity, may be far from beneficial. Clinicians, therefore, have sought ways of modifying the response so that desirable and beneficial effects might be retained, but undesirable sequelae eliminated.

The beneficial effects of analgesics and narcotics have been known empirically to many generations of clinicians. Major advances in rational therapy, however, are more likely to result from investigations based on precise knowledge of the physio-pathological processes involved. This thesis reports an investigation which attempts to contribute to the growing basic knowledge of this subject.

Chapter 2

PHYSIOLOGICAL AND ANATOMICAL FEATURES OF VASCULAR PERMEABILITY

Claude Bernard (1879) was one of the earliest workers to draw attention to the fundamental importance in mammals of the precise regulation of the body fluids' composition - a process made possible by the movement of substantial amounts of water, electrolytes and other substances between the blood vascular system and the tissue fluid-lymph system. The main site of this exchange is the capillary wall.

Some idea of the magnitude of the capillary bed is given by Krogh's (1929) estimate that in a man whose muscles weigh 50 kg. with 2,000 capillaries per sq. mm. of muscle, the total surface area of the capillary bed is about 6,300 sq. metres; and from Zweifach's (1961) calculation that though one ml. of blood would take 5 - 7 hours to traverse a single capillary, the capillary bed is so large that the entire blood volume (approximately 5,000 ml. in the adult) can be circulated through the capillary system in a few minutes. As Zweifach has emphasised, "... the minute vessels represent the focal point around which the entire operation of the cardiovascular system is organised" (Zweifach, 1961).

Our knowledge of the organisation of the micro-circulation largely depends upon the work of Chambers and Zweifach (1947) and Zweifach (1961). During periods of inactivity, blood flow through a tissue is restricted mainly to preferential or "thoroughfare" channels joining arterioles and venules. From

the proximal, metarteriolar portions of the thoroughfare channels, capillary offshoots arise at acute angles, each branch having its own precapillary sphincter. As tissue metabolism is increased, more capillaries open up and become perfused with blood.

Starling (1895), in his classic paper "On the Absorption of Fluids from the Connective Tissue Spaces", observed that the capillary walls were freely permeable to water and crystalloids but almost impermeable to proteins. Furthermore, the injection of protein into the tissue fluid space in a concentration approaching that in plasma prevented the absorption of tissue fluid into the blood capillaries.

"The importance of these measurements", wrote Starling, "lies in the fact that although the osmotic pressure of the proteids of the plasma is so insignificant, it is of an order of magnitude comparable to that of the capillary pressures; and, whereas capillary pressure determines transudation, the osmotic pressure of the proteids of the serum determines absorption." Starling's hypothesis may be stated thus, where filtration and reabsorption are in equilibrium:

$$CP + OP_{e.f.} = TP + OP_{plasma}$$

where CP = the effective blood pressure within the capillary

$OP_{e.f.}$ = the osmotic pressure of the extracellular fluid

TP = tissue turgor pressure

OP_{plasma} = the osmotic pressure of the plasma in the capillary.

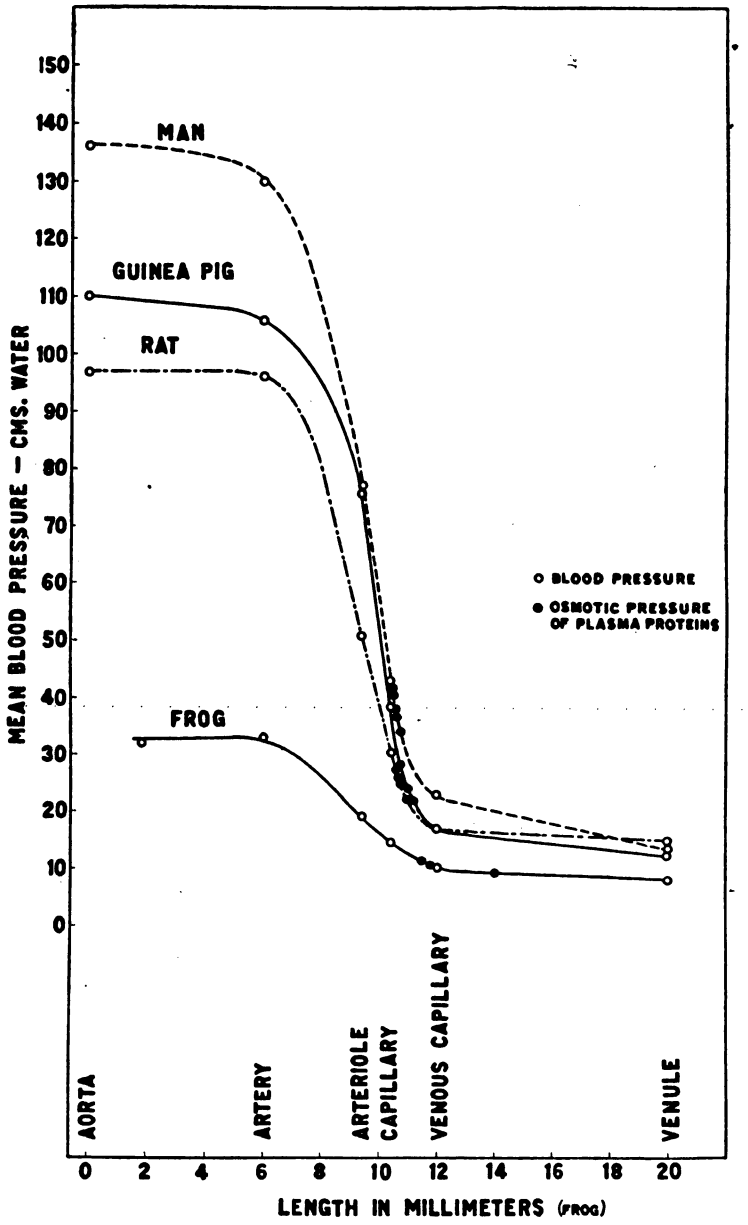


Fig. 1. Chart showing relation between capillary blood pressure and the osmotic pressure of the plasma proteins in four species. (Landis, 1934)

The Measurement of Capillary Pressure

Starling's hypothesis was supported by the work of Landis who, by direct cannulation, measured the intra-capillary pressure in the mesentery of the frog (Landis, 1925-26; 1927 a and b), rat and guinea pig (Landis, 1930a), in the muscle and skin of the frog (Landis, 1931), and in the skin of man (Landis, 1930b).

Landis observed a falling gradient of hydrostatic pressure from the arteriolar to the venular side of the capillaries. At the arterial end, the pressure is greater than the colloid osmotic pressure of the plasma proteins while on the venous side it tends to be a little less (see Fig.1).

Thus at the arteriolar end conditions should favour fluid filtration out of the vascular bed, while at the venous end fluid absorption should predominate.

Since the flow through the thoroughfare channels usually is rapid with less fall in hydrostatic pressure than in the true capillary, Chambers and Zweifach (1947) considered these channels to be the main site of outward filtration in the resting state. Absorption was regarded as the main function of the true capillary.

Increased arterial pressure, Landis (1934) noted, usually but not always resulted in increased fluid filtration. Increased venous pressure on the other hand invariably resulted in increased filtration.

The osmotic pressure of interstitial fluid (2-3 mm.Hg.

in man; Payling Wright, 1956) and the tissue turgor pressure (2-3 mm. in man), appear to have little importance in the physiological control of fluid movement.

Permeability of the capillary wall to water

Leathes (1895) observed that large volumes of water moved rapidly from the extravascular compartment after the intravenous injection of hypertonic solutions.

Rapid movement of water in the opposite direction was recorded by Hevesy and Jacobsen (1940) who reported that, 30 seconds after the intravenous injection of heavy water, it was diluted by a volume of fluid equal to the expected volume of extracellular fluid. In man and the guinea pig, 105 - 140 per cent of the water in blood is exchanged per minute with extravascular water (Flexner, Gellhorn and Merrell, 1942; Flexner, Cowie and Vosburgh, 1948). The rapidity of fluid exchange was also noted by Pappenheimer (1953) who observed that water diffused back and forth across the capillary wall 80 times faster than it was brought to the site by the incoming blood.

Flexner, Cowie and Vosburgh (1948) confirmed the essential relation between colloid osmotic pressure and capillary pressure proposed by Starling, but disagreed with the conventional interpretation of fluid exchange by alternate bulk filtration and reabsorption. They considered that "diffusion ..... is the essential process by which the exchange of water and solutes takes place between plasma and extravascular fluid".

Permeability of the capillary wall to electrolytes and small molecules.

The rate of exchange of ions and small molecules is variable and does not necessarily parallel that of water in rate or direction. For example, in the guinea pig, 60 per cent of the sodium and chloride in plasma is exchanged per minute with that in the extra-vascular space whereas plasma water exchange is 2.3 times more rapid than either sodium or chloride. Calculations from the data of Ray and Burch (1959) indicate that, in man, the absolute amounts of sodium chloride and potassium ion exchanged per minute are about 19,000g. and 600g. respectively.

While agreeing that water and dissolved gases might diffuse through the entire capillary membrane, including the endothelial cell, Chambers and Zweifach (1947) suggested that the transfer of ionic and other lipid insoluble substances is controlled by filtration through pores in the cement substance between endothelial cells. They proposed that small molecules such as water might diffuse freely through the pores but that large molecules would be held up by a process of "molecular sieving", a procedure which might be further enhanced by the adsorption of protein which partially "clogged" the pores.

The results of Pappenheimer, Renkin and Borrero (1951) appeared to confirm the "pore theory" of Chambers and Zweifach. They concluded that uniform pores 60 - 90 Å in diameter or rectangular slits 37 Å wide, occupying a minute fraction of the

total capillary surface, would account for their findings. Pappenheimer (1953) later estimated the population density of the pores at 1-2 x 10^9 per sq.cm. of capillary wall.

The permeability of the capillary wall to protein and lipid

Although only 5 per cent of the plasma proteins escape through the capillary wall, all the plasma proteins are represented in this fraction (Yoffey and Courtice, 1956). In general, the rate of passage of protein molecules across the capillary membrane varies inversely with their molecular size so that interstitial fluid and lymph contain proportionately more albumin than globulin (Loewen, Field and Drinker, 1931).

As with proteins, all the lipid-protein complexes in plasma have been identified in lymph and hence must have crossed the capillary membrane (Page, Lewis and Plahl, 1953; Courtice and Morris, 1955). The larger β -lipoproteins leave the circulation more slowly than the smaller α -lipoproteins. However, some of the β -lipoprotein complex does escape despite the fact that its molecule is approximately twice as large as the pores proposed by Pappenheimer (1953).

In the post-absorptive state in cats which have been fed fat, the peripheral lymph contains chylomicrons which appear to have come from the blood since the chylomicron counts of peripheral lymph may vary directly with those of the blood (Yoffey and Courtice, 1956). However, the clearance of chylomicrons from the plasma of fat-fed cats is more rapid than can be explained by the breakdown of chylomicrons into smaller compo-

nents and subsequent trans-capillary transport as lipid-protein complexes. Morris and Courtice (1956) have suggested that some chylomicrons escape unaltered through the intact capillary wall.

The structure of the capillary as revealed by electron microscopy

Electron microscopic studies reveal three structural components in the capillary wall - viz. an inner layer of endothelial cells, a basement membrane and an outer, discontinuous layer of cells and fibres comprising the adventitia.

In the common type of capillary, for example, in skeletal muscle, heart, intestines and pancreas, the endothelium is a continuous layer without fenestrations or pores (Palade, 1953; Odland, 1961). Numerous small vesicles 200-600 Å in diameter are concentrated along both the inner and outer cell borders. Such vesicles have been cited as evidence of the intra-vesicular transport of water and solutes - so called "pinocytosis" (Palade, 1956) or "cytopempsis" (Moore and Ruska, 1957).

Alksne (1959) reported similar appearances in normal capillaries in mouse's skin. In addition, he has demonstrated increased numbers of endothelial vesicles during the period of increased vascular permeability induced by histamine, and also the intravesicular transport of dense particulate matter (cf. Palade, 1961; Jennings, Marchesi and Florey, 1962).

In electron micrographs the basement membrane usually appears as a thin, irregular layer 200-500 Å thick, surrounding

the endothelium. It consists of a feltwork of fine fibrils about 100 Å in diameter (Palade, 1953) enmeshed in a homogeneous matrix of unknown composition. No pores have been demonstrated in the basement membrane, despite acceptable limits of resolution in the instruments employed. In fact, the basement membrane appears to be a significant barrier to the passage of particles comparable in size to protein molecules and hence must be considered jointly with the endothelial cells as part of the "endothelial barrier". Palade (1961) suggests that the basement membrane is a "yielding gel" through which moving particles make temporary channels as they pass.

In summary, at least two factors may be involved in the transfer of lipid insoluble substances across the capillary wall:

(a) transport across the endothelial cell within membranous vesicles,

(b) diffusion across the basement membrane.

Evidence for these processes, however, has largely been obtained using particulate materials like mercuric sulphide and clearly more information is required concerning the passage of natural proteins.

Chapter 3.

INCREASED VASCULAR PERMEABILITY IN INFLAMMATION.

The major, proximate benefits of inflammation mentioned in Chapter 1 result from the accumulation in the injured tissue of a relatively large amount of protein-rich exudate due to increased permeability of the small vessels. Since the escape of protein through vessel walls can be detected fairly easily by labelling with certain dyes, particular attention has been paid to the mechanism by which increased vascular permeability to protein develops. In this thesis the term increased vascular permeability will be used in this sense.

Permeability may be increased by simple elevation of the capillary pressure, as in exercise, without any intrinsic change in the endothelium-basement membrane complex. However, increased capillary pressure alone is not adequate to account for the large increase in permeability of the vascular wall to protein occurring in inflammation.

The greater importance of some intrinsic change in the endothelial barrier as proposed by Cohnheim (1889) was shown by Landis (1934) who, after damaging a capillary in the exposed frog's mesentery by gentle pressure with a glass rod, observed a greatly increased rate of passage of colloidal dye through the damaged section compared with the adjacent, undamaged portion, despite the similar hydrostatic pressure throughout the vessel.

The concept of biochemical mediation of increased vascular permeability in inflammation

Cohnheim (1889) was one of the first to postulate that the essential factor underlying increased permeability in inflammation was a "chemical" or "molecular" change in the walls of small blood vessels. Its exact nature, however, still remains obscure.

The concept that this "chemical" or "molecular" change might be mediated by chemical substances released or activated in injury largely depends upon the fact that certain agents which increase vascular permeability can be isolated from both normal and inflamed tissues.

Of the various permeability factors (PF's) which have been proposed as natural mediators, those which appear to merit greatest consideration fall into three main groups:

(a) Amines, such as histamine and 5-hydroxytryptamine.

(b) Proteases, such as kallikrein, globulin PF, and plasmin; and polypeptides like leucotaxine, bradykinin and kallidin.

(c) Lecithinases, which are the active, permeability-increasing substances from certain clostridial toxins.

Histamine was discovered by Windaus and Vogt (1907) and was later shown by Eppinger (1913) and Sollman and Pilcher (1917) to be capable of increasing capillary permeability. By comparing the vascular reactions after various types of mild

injury with the changes occurring after the application of histamine to human skin, Lewis (1927) concluded that either histamine or a "histamine-like" substance was liberated "in vivo" after injury due to e.g. mild heat, freezing, pricking, galvanic current, light stroking or even after more severe injury such as that resulting from anaphylaxis, bacterial toxins or the application of mustard gas.

5-hydroxytryptamine (serotonin) was identified by Rapport, Green and Page (1948) and later was shown to induce markedly increased vascular permeability in the skin of rats (Rowley and Benditt, 1956), but to have little effect in the skin of guinea pigs and rabbits (Sparrow and Wilhelm, 1957).

Polypeptides were first proposed as mediators of increased vascular permeability in inflammation after Menkin (1936) found, in inflammatory exudates induced by chemical, bacterial and thermal injury, a freely diffusible compound which "manifested no property in common with histamine". He later claimed to have purified and identified this fraction as a relatively simple polypeptide "leucotaxine" (Menkin, 1938).

Polypeptides which increase permeability were isolated by Duthie and Chain (1939) and Cullumbine and Rydon (1946) from tryptic and peptic digests of plasma proteins (particularly fibrin and albumin) and were also demonstrated by Cullumbine and Rydon (1946) in the fluid of blisters occurring 2-4 hours after thermal or chemical irritation. Spector (1951) also obtained polypeptides by tryptic and peptic digestion of human fibrin and

reported that polypeptides containing 5-14 amino acids were capable of inducing increased permeability and emigration of leucocytes.

The presence, in inflamed tissues, of PF's which result from proteolytic activity suggested the participation in the inflammatory response of endogenous proteases activated by tissue injury. Early presumptive evidence of this was produced by Beloff and Peters (1945) who isolated from burnt rats' skin a protease which was later shown to yield permeability-increasing material when allowed to act upon fibrin under appropriate conditions (Cullumbine and Rydon, 1946).

In 1928 Frey and Kraut described the presence, in the urine of dogs, of a hypotensive substance kallikrein, which, they believed, originated in the pancreas. Kallikrein has subsequently been shown to be present in other tissues including blood (Werle and von Roden, 1936; Frey, Kraut and Werle, 1950; Hilton and Lewis, 1957; Chapman and Wolff, 1958; Fox and Hilton, 1958; Lewis, 1959).

Pharmacologically active peptides produced by the action of various enzymes "in vivo" are collectively known as kinins. Two such kinins result from the action of human urinary kallikrein on acid treated human plasma - kallidin I and kallidin II. Kallidin II is a decapeptide differing structurally from kallidin I only in having a terminal lysine grouping. Kallidin I is a nonapeptide indistinguishable from bradykinin (Pierce and Webster, 1961).

Bradykinin, originally discovered as a product of the action of trypsin or snake venom on bovine pseudoglobulin (Rocha e Silva, Beraldo and Rosenfeld, 1949), has been shown to be a nonapeptide (Elliott, Horton and Lewis, 1961) and has recently been synthesised (Boissonas, Guttman, Jacquenoud, Konzett and Stürmer, 1960). It is one of the most potent permeability factors known (Logan, G.G. and Wilhelm, D.L., cited by Wilhelm, 1962).

Plasma kinins are also formed by the action of bovine and human plasmin on plasma proteins (Beraldo, 1950; Schachter, 1956; Lewis and Work, 1957). Plasmin, a proteolytic enzyme present in blood as an inactive precursor, plasminogen, (Christenson and McLeod, 1945) and responsible for the process of fibrinolysis (MacFarlane and Biggs, 1948; Astrup, 1956), can be activated by various kinases, e.g. bacterial (Garner and Tillet, 1934; Milstone, 1941) and tissue kinase released after cell damage (Astrup and Permin, 1948).

The plasma kinin formed by human plasmin "in vitro" is indistinguishable from bradykinin (Lewis, 1958). Its rate of formation differs from that of the kinins formed by the action of kallikrein on plasma globulin. Maximal kinin formation by salivary kallikrein occurs within a few minutes, whereas with plasmin, 20-30 minutes elapse before maximal kinin formation occurs (Lewis, 1959).

Back, Guth and Munson (1963), investigating plasmin induced hypotension in dogs, were unable to demonstrate

significant kinin formation by plasmin "in vivo". However, their findings led them to suggest that plasmin may release kallikrein as part of the hypotensive response.

The lack of "in vivo" kinin formation described above is consistent with the almost complete absence of PF activity of both human and guinea pig plasmin in the guinea pig (Miles and Wilhelm, 1960; Logan and Wilhelm, unpublished).

The α_2-globulins of guinea pig serum contain a component of high molecular weight which can be activated by dilution to increase vascular permeability in the homologous species (Mackay, Miles, Schachter and Wilhelm, 1953; Miles and Wilhelm, 1955; Wilhelm, Miles and Mackay, 1955; Wilhelm, Mill and Miles, 1957). Both the precursor of the PF and its inhibitor are also present in tissue fluid and lymph (Miles and Wilhelm, 1958).

Similar activable precursors of globulin PF's have been demonstrated in the sera of rats, rabbits and man (Wilhelm, 1956; Wilhelm, Mill, Sparrow, Mackay and Miles, 1958; Elder and Wilhelm, 1958; Mill, Elder, Miles and Wilhelm, 1958).

Guinea pig globulin PF, though considered to be a protease (Wilhelm, 1962), probably owes most of its permeability increasing potency to the activation of a kininogenase rather than direct kinin formation itself (Mason and Miles, 1962).

Thus, of the three proteolytic enzymes considered as mediators in this thesis, only kallikrein has been shown to

be a kininogenase, plasmin and guinea pig globulin PF apparently owing at least part of their activity to the intermediary release or activation of a kininogenase.

Lewis (1963) attempted to deduce an important sequential relationship between the two principal groups of mediators so far discussed in suggesting that histamine, liberated during various kinds of injury in the hind limb of the dog, might be responsible for the increased concentration in efferent lymph of the enzyme which forms plasma kinins.

All the above PF's with the exception of globulin PF in the rabbit resemble histamine in the brevity of their action. Elder and Miles (1957) reported that certain clostridial toxins induced prolonged, and in some cases, diphasic increases in vascular permeability when injected in sub-necrotising doses into the skin of guinea pigs. In the case of Clostridium welchii, the PF resides in the α -toxin which is a lecithinase (Macfarlane and Knight, 1941). This appears to have been the only instance so far reported in which prolonged increases in vascular permeability have been induced by specific substances. The α - and β -toxins of Cl. oedematiens also contain lecithinases which as yet have not been characterised.

Despite extensive experimental observations on the above PF's, with few exceptions their natural role in injury has not been clearly established.

The established role of chemical mediators in various types of injury.

Prolonged increases in vascular permeability have been reported in the inflammatory response to mild thermal injury (Sevitt, 1958; Spector and Willoughby, 1958b, 1959b; Wilhelm and Mason, 1958, 1960), bacterial injury (Burke and Miles, 1958), certain forms of chemical injury, e.g. turpentine-induced pleurisy in the rat (Spector and Willoughby, 1957, 1958a, 1959a), U-V radiation injury (Logan and Wilhelm, 1963), roentgen radiation injury (Willoughby, 1960; Jolles, Remington and Simon-Reuss, 1961) and ultra-sonic injury (J.F. Burke, unpublished; cited by Wilhelm, 1962).

The various responses appear to differ mainly in the length of the second or delayed phase, varying from a few hours in the case of bacterial and thermal injury (Burke and Miles, 1958; Wilhelm and Mason, 1960), to more than 23 days in the case of roentgen radiation (Jolles, Remington and Simon-Reuss, 1961).

Chemical injury to the skin, induced for example by the application of xylol in guinea pigs and rats, appears to cause only a monophasic permeability response, though there may be two super-imposed components (Wilhelm, 1962).

In turpentine-induced pleurisy and thermal and U-V irradiation injury to skin the early phase can be suppressed by the use of antihistamines in the guinea pig, and antagonists of 5-HT in the rat. In guinea pigs antihistamines also cause minor inhibition of the permeability response to chemical injury to the skin (Wilhelm, 1962).

However, in none of these cases, with the particular exception of Clostridium welchii infections, has satisfactory evidence of the biochemical mediation of the principal phase of increased vascular permeability been adduced.

Chapter 4.

THE EXPERIMENTAL USE OF ULTRA-VIOLET RADIATION AND ITS BIOLOGICAL EFFECTS.

Increased vascular permeability induced by mild thermal injury is relatively shortlived, lasting only a few hours (Wilhelm and Mason, 1960) and hence the opportunity for the administration of potential biochemical inhibitors is rather restricted.

Accordingly, it was decided to study, as an experimental model, a type of injury whose main permeability response develops more slowly and lasts longer. This technique offered the theoretical advantage that locally or systemically administered inhibitors might have a more decisive effect on the maturation of the response.

Since ultra-violet irradiation induces a permeability response in the guinea pig that matures approximately 18-21 hr. after irradiation, it seemed a convenient technique for the present work.

In this study the identification of natural PF's has been attempted by the inhibition technique of Wilhelm and Mason (1960) rather than by procedures such as chemical isolation of PF's from inflamed tissues or attempts to deplete test animals of endogenous PF's.

The chemical extraction of PF's from injured tissues may lead to seriously misleading results as artefactual degradation compounds may contaminate the final product.

In any case, chemical isolation alone is inadequate proof of activity "in vivo". Also, substances such as histamine or 5-HT are readily liberated by minor trauma to the skin and the possibility remains that their presence is incidental to, rather than the result of, injury.

Various workers have attempted to deplete experimental animals of potential PF's. The skin of rats can be depleted of histamine by repeated injections of compound 48/80 (Feldberg and Talesnik, 1953; Brocklehurst, Humphrey and Perry, 1955; Spector and Willoughby, 1957a); of polymyxin B (Parratt and West, 1957); and of 5-HT by the use of reserpine (Erspamer, 1956; Parratt and West, 1957). In rats depleted of histamine, the permeability response to antigen-antibody reactions (Feldberg and Talesnik, 1953) and chemical injury (Spector and Willoughby, 1958a; 1959a) is diminished.

However, depletion of PF's by these procedures is never complete and partial and often equivocal inhibition is the usual effect. Such results are unconvincing as the depletory agents themselves may cause severe toxic (Feldberg and Talesnik, 1953) or other non-specific effects (Spector and Willoughby, 1959a; Wilhelm et al., 1958).

Satisfactory depletion of such PF's as globulin PF and blood kallikrein clearly is impossible without impairing the health of animals to such an extent that they are unsuitable for experimental use.

Inhibition techniques lack the above disadvantages.

Provided that reasonably specific inhibitors are used, suppression of the permeability response is strong evidence of the "in vivo" action of the PF in question.

In such studies the use of minimal injury is essential in order to minimise the release of non-specific factors such as intracellular enzymes and other cell constituents which result from cell destruction, (cf. Lister, 1858).

The biological effects of ultra-violet radiation

The gross effects of ultra-violet radiation result from intracellular biochemical changes. The absorption of U-V energy causes a disturbance of biochemical equilibrium, the degree of which is, in general, proportional to the amount of energy involved (Glasser, 1950).

Proteins and nucleic acids probably are the most important "target portions" in the cell, proteins showing maximum absorption at 2,800 Å and nucleic acids at about 2,600 Å (Ely and Ross, 1949).

U-V radiation may affect enzymes within the cell or in the cell wall. Thus both human erythrocytes in blood (Koeppe, 1926; Eidenow, 1930) and frog erythrocytes in Ringer's solution (Maroney, 1960) are haemolysed by U-V radiation.

Koeppe (1926) suggested, in the case of human erythrocytes, that this was due to "the activation of a catalase enzyme in the fatty stroma of the cell wall" (cf. Hogberg and Uvnas, 1957 on the mechanism of disruption of mast cells by compound 48/80).

In the case of frogs' erythrocytes in Ringer's solution, however, the swelling and subsequent lysis seem to be osmotic effects due to the increased transfer of cations such as Na^+ and K^+ across the cell membrane (Green, 1956).

Investigation of the effects of U-V irradiation on non-human cellular systems has shown that irradiation of yeast cells causes the release of intra-cellular materials including the amino-acids arginine, histidine, threonine, alanine, tryptophane and tyrosine which originate either from the cell proteins (Loofbourow, 1947) or the intra-cellular pool of free amino-acids (Taylor, 1947; Halvorsen and Spiegelman, 1953).

This process appears to be selective and is dependent upon energy provided by glucose metabolism (Swenson and Dott, 1961).

U-V irradiation of Amoeba proteus at 2,537 Å increases the permeability of the cell to fluid by inducing the formation of pinocytotic vesicles. This appears to be purely a cytoplasmic effect as identical changes can be induced both in enucleated fragments and in the intact organism (Rinaldi, 1959 a & b).

Experimental evidence from the latter system, however, though interesting and possibly illustrative of general cellular processes, must be interpreted with caution in relation to the behaviour of cells in higher animals.

The Effect of U-V Radiation on Skin.

The greatest biological effects of U-V radiation are obtained with wave lengths 2,000 Å - 4,000 Å, and for the most

part such effects are limited to the integument which, in man and other animals, is screened by the horny layer of the epidermis or by a covering of hair or fur.

In man, little radiation of wave-length shorter than 3,200 Å penetrates below the epidermis, most being absorbed by the stratum corneum and the stratum granulosum (Bachem, 1929; Takahashi, 1930-31). No radiation at all below 2,400 Å reaches the living part of the epidermis, all being absorbed in the dead, horny layer of the skin (Bachem and Kunz, 1929).

In the guinea pig, the stratum corneum is thick and, as in man, little U-V radiation appears to penetrate beyond the epidermis since increased permeability and leucocytic emigration occur only in the tissue immediately subjacent to the epidermis (see below).

The rat, on the other hand, has a thinner epidermis which seems to permit greater penetration of U-V radiation (Hueper, 1941).

In the rabbit, calculations based on photographic measurements of the transmission of U-V radiation by the intact skin of the ear (thicker than 0.23 mm.) indicate that only 1/48,000th of the incident radiation at wave-length 3,025 Å is actually transmitted by the skin (Takahashi, 1930-31).

The classical reaction of U-V irradiated skin is familiar as sunburn. Erythema, beginning in a few hours and becoming maximal in 12-24 hr., is accompanied by pain and oedema. Finally the skin exhibits pigmentation and/or

epithelial desquamation in 48-72 hr. The longest wave-length of U-V radiation that induces a "burn" in man is 3,150 Å (Laurens, 1938). Below this, there is maximal effect at 3,000 Å and at 2,500 Å with a pronounced minimum at 2,800 Å (see Bachem, 1932), corresponding to maximum absorption by the proteins of the epidermis.

Erythema results from vaso-dilatation and engorgement of the superficial blood vessels of the dermis.

The mechanism of induction of erythema is uncertain. It seems noteworthy that practically all radiation of wave length less than 3,200 Å is screened out by the epidermis (see above) and that therefore the vascular changes cannot be attributed to direct radiation effects upon the dermal vessels.

Lewis and Zotterman (1926) proposed that a histamine-like substance is released, whereas Blum (1945) considered the mediator was some other diffusible substance. Klouwen and Mighorst (1957) suggested that erythema may be mediated by hydroxylated aromatic acids whose formation from proteins is induced by U-V radiation.

Whatever the mechanism, however, studies in the rabbit's ear indicate that an intact sensory nerve supply is necessary, because the appearance of erythema can be suppressed greatly or even eliminated by the use of local analgesia or section of the main sensory nerve trunks (Cluzot, Cardot and Kofman, 1928).

In man, paradoxically, sunburn erythema can be suppressed

by high doses of long wave length U-V (greater than 2,800 Å). Such suppression, however, is accompanied by more, not less, damage to the skin (Blum and Terus, 1946a).

Using histochemical techniques, Daniels, Brophy and Lobitz (1961) investigated some of the biological processes in irradiated skin. Glycogen was found to accumulate in the cells of the basal layer of the epidermis, the concentration becoming maximal 12 hours after irradiation when 60-70 per cent of basal cells contained increased amounts of glycogen. In the next 24 hours, the percentage slowly decreased. Up to 24 hours after irradiation, when morphological changes occurred in the upper third of the prickle cell layer, no change was demonstrated in either the succinic dehydrogenase or alkaline phoshatase systems, nor was increased staining for disulphide or sulphhydryl bonds observed.

More sensitive, radio-isotopic investigations of biochemical activity reveal that in the mouse, irradiated skin shows increased incorporation of ^{32}P into phospholipid as measured 24 hours after irradiation (Johnson and Mier, 1962).

Similar incorporation of ^{32}P into acidic phospatides has been reported in actively secreting cells of pancreas (Hokin and Hokin, 1958), adenohypophysis (Hokin, Hokin, Saffran, Schally and Zimmerman, 1958), sub-maxillary and parotid glands (Eggman and Hokin, 1960; Hokin and Sherwin, 1957) and in polymorphonuclear leucocytes phagocytosing

starch particles (Karnowsky, 1962).

The latter author has drawn attention to the fact that "transport of substances across cell membranes in an outward or inward direction over a wide range of particles from that of a sodium ion to that of a starch particles more than 1 μ in diameter" is associated with an increased labelling of specific phosphatides of cells with ^{32}P. He suggests that this may be interpreted tentatively as indicating "the involvement of acidic phosphatides in membrane functions .... which could conceivably underlie the active passage of substances through membranes over the whole size spectrum from the transport of a sodium ion to the formation of a micropinocytotic vesicle (Palade, 1953; 1956; 1959) .... to the formation of a phagocytic vesicle (Goodman and Moore, 1956; Goodman, Moore and Baker, 1956; Essner, 1960)".

However, the demonstration of the exact significance of the increased ^{32}P labelling of phosphatides in U-V irradiated mouse skin requires further elucidation, particularly in relation to the events of the inflammatory reaction.

Chapter 5

MATERIALS AND METHODS

Animals

Albino guinea pigs (450-800 g.), rats (200-250 g.) and rabbits (2.2-3.8 kg.) were used. Although smaller guinea pigs (450-550 g.) were preferred for testing permeability factors, larger ones (600-800 g.) were found more satisfactory for experiments with ultra-violet injury.

In all three species, animals of both sexes were used for testing PF's, though the cleaner skins of female guinea pigs and rats were preferred. In fact, female rats only were used for ultra-violet experiments.

For tests with permeability factors, a similar depilation procedure was used in all species. The hair on the animal's back was closely clipped over an area extending from the superior borders of the scapulae in front to the level of the iliac crests behind, and laterally to about the mid-axillary line. The stubble was then removed with grey barium sulphide paste (Miles and Miles, 1952), after which the animal was washed with tepid water and soap, and dried with a soft towel.

The fur of depilated guinea pigs grows sufficiently in 24 hr. to interfere with ultra-violet radiation; and in 48 hr. the skin of chemically depilated rats develops a dermatitis, becoming brown and scaly. In the rabbit also, unless the paste used is very thin and its application very brief,

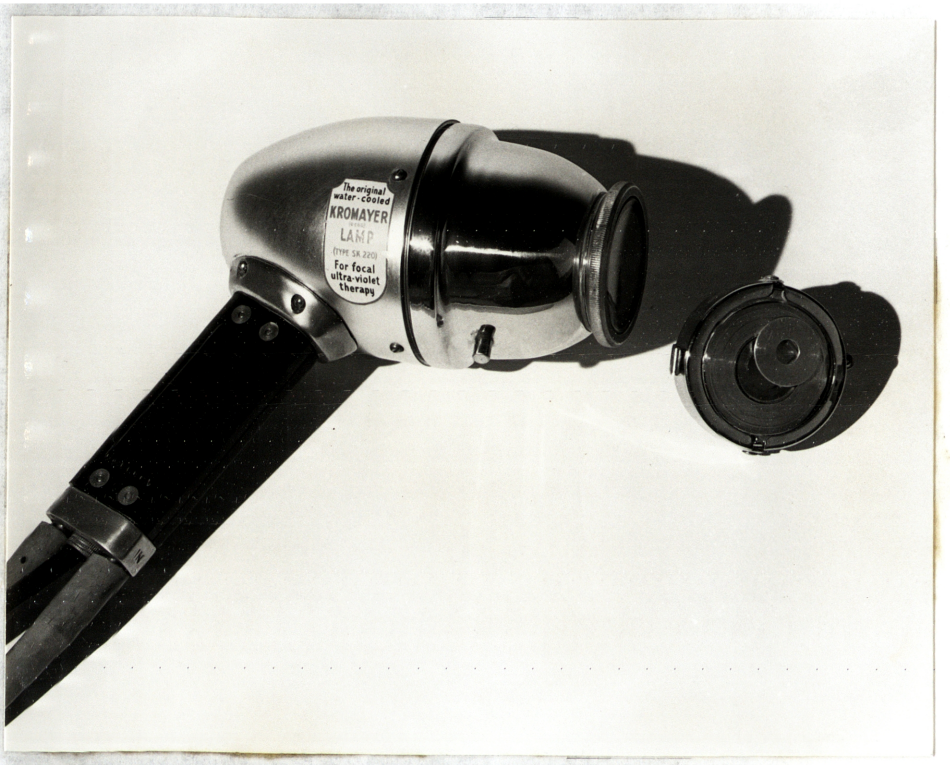


Fig. 2 The Kromayer lamp modified for experimental use by the insertion in the lamp face of an aluminium disc with a central aperture 8 mm. in diameter

depilation is followed within 48-72 hrs. by a severe chemical dermatitis.

Thus in experiments lasting longer than 12 hr. the required sites were depilated freshly each morning - in guinea pigs and rabbits with barium sulphide, in rats with an electric shaver.

The Source of Ultra-violet Radiation

A Kromayer Model II lamp was used as a source of U-V radiation. It consists of a water-cooled, high pressure mercury arc in quartz, housed in a mobile head (Fig. 2). The emission spectrum consists of a series of intense spectral lines of wave lengths between 2,570 Å and 3,650 Å, superimposed on a faint continuous spectrum extending from 1,850 Å, through the visible into the infra red. The passage of the radiation through 22mm. of constantly circulating cold water absorbs practically all the infra-red emission, leaving only one band at 10,140 Å. The rated energy output at the face of the lamp shield is approximately 522,000 ergs./sq.cm. of radiation up to 3,132 Å.

Animals were irradiated by applying the 8 mm. aperture in the head directly to the animal's skin.

Although almost all the infra-red radiation is removed by filtration through circulating cold water, the possibility of mild thermal injury occurring during prolonged irradiation, particularly, was considered.

A thermocouple, made in the School of Physics, University

of New South Wales, by soft-soldering insulated Cupron wire inside a $\frac{5}{4}$ inch stainless steel needle of the size used for intra-cutaneous injection (25 gauge), was inserted into the skin of irradiated sites in guinea pigs. The maximum temperature rise during and after irradiation was measured.

After irradiation for up to 120 sec., maximum increases of only 1.2 degrees centigrade were recorded.

Estimation of increased vascular permeability.

Increased vascular permeability was detected by the extra-vascular leakage of Evans Blue which becomes firmly found to the plasma albumin (Rawson, 1943).

Pontamine sky blue 6BX (Miles and Miles, 1952) proved toxic in our guinea pigs, especially during the winter. However, satisfactory results were obtained with Evans Blue and this was subsequently used in all three species.

All three species received Evans Blue 30 mg. per kg., with a maximum dose of 18 mg. for guinea pigs weighing 600 g. or more. Rabbits and guinea pigs were injected with a 2.5 per cent solution in 0.425 per cent saline, rats with a 1.25 per cent solution in 0.64 per cent saline.

The testing and evaluation of permeability factors was carried out by the methods of Miles and Miles (1952) and Wilhelm et al. (1958). Comparative tests in guinea pigs showed that the diameters of lesions induced by several PF's in animals injected with Evans Blue were slightly larger than those injected with Pontamine Blue. Despite this, the relative

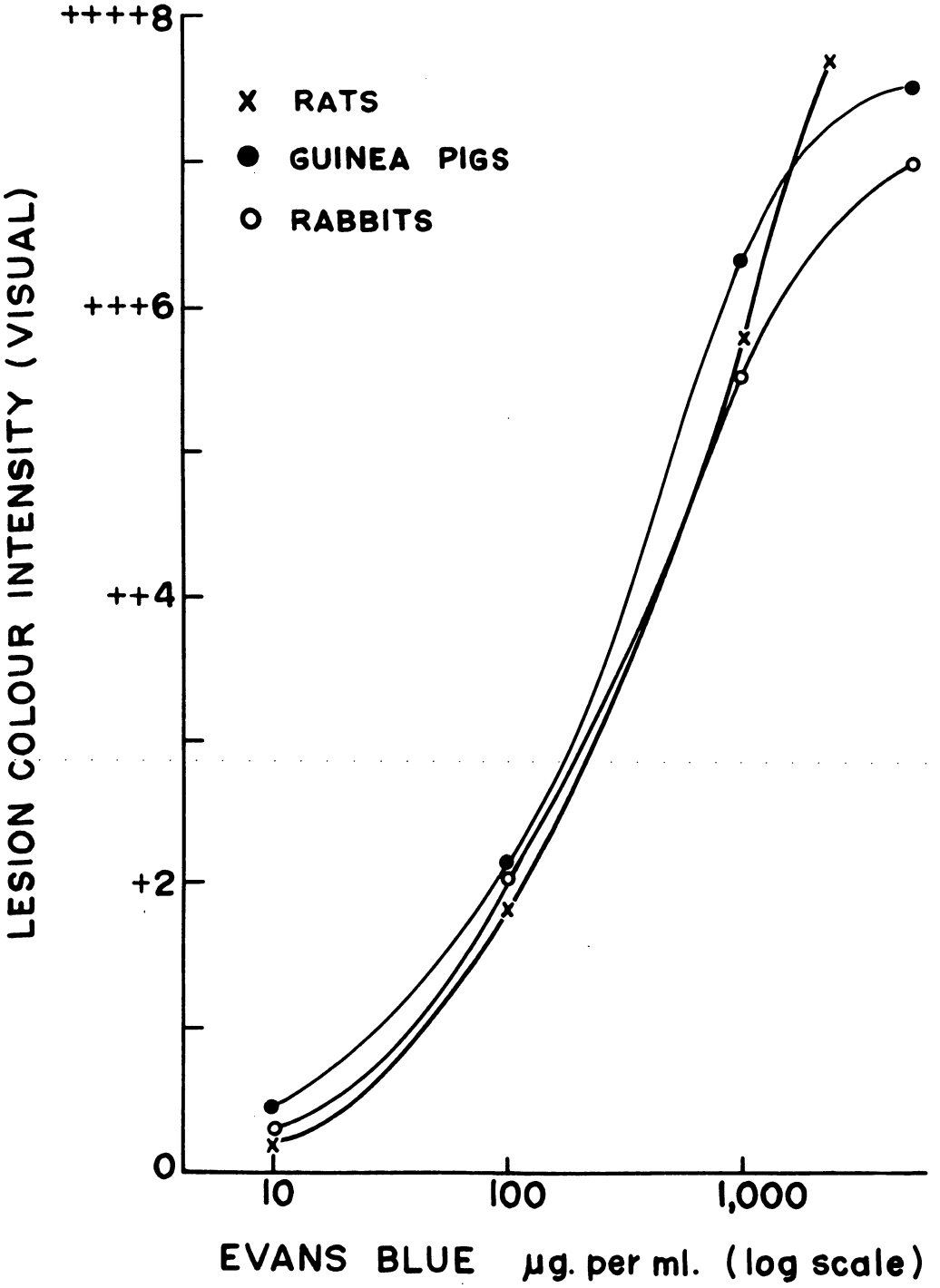


Fig. 3 Colour reference curves

potencies of the PF's in our animals were similar to those reported elsewhere using Pontamine Blue (Miles and Wilhelm, 1960).

In experiments with ultra-violet injury, the use of a standard injury over a constant area necessitated the measurement of differences in permeability response by the assessment of differences in the colour intensities of the lesions, rather than by measuring lesion-diameter as in testing permeability factors. To do this, colour reference curves were obtained as follows.

Graded concentrations of Evans Blue dissolved initially in serum and then diluted with 0.85 per cent saline were injected intra-dermally in each species in killed, "blued" animals. Lesions were graded visually and assigned a numerical colour value, mean results being plotted as illustrated in Fig.3. The concentrations of dye in solution, which produced lesions of colour intensity +++ and + were calculated from the figure.

In all subsequent U-V experiments, 0.05 ml. of each of the concentrations yielding lesions of intensity +++ and + was injected in each animal. The U-V lesions were then assigned a numerical colour value by reference to the standard lesions and the results expressed as dye equivalents by calculation from the colour reference curves (Fig. 3). The dye equivalent of a lesion is the concentration of Evans Blue in solution which produces a lesion of the appropriate depth of colour when injected intra-dermally in a volume of 0.05 ml.

Homogeneity of response

In the guinea pig, the test area was bounded in front by lines joining the upper borders of the scapulae, and behind by a line joining the iliac crests. Avoiding the skin 1 cm. on either side of the mid-line, the test area extended laterally for 3.5 cm. from the mid-line. A vertical row of 5 - 6 lesions was induced on each side of the back.

In general, the intensity of the early and late responses tended to be slightly greater in the upper thoracic than in the lumbar regions. This variation was countered by duplicating all lesions in each animal, siting one lesion nearer the neck on one side and the duplicate lesion nearer the lumbar end on the other side. All results were expressed as means.

Drugs

Histamine was used as the acid phosphate (British Drug Houses) and 5-hydroxytryptamine (5-HT) as the creatinine sulphate (Upjohn Co., Kalamazoo, U.S.A.).

The antihistamines triprolidine (Burroughs Wellcome and Co.) and promethazine (May and Baker Pty. Ltd.) were used as hydrochloride, chlorprophenpyridamine (Allen and Hanburys) and mepyramine (May and Baker) as maleate. The weights of histamine, 5-HT and antihistamines are cited as base. The 5-HT antagonist, 2-brom-D-lysergic acid diethylamide (BOL 148), was supplied by Sandoz Ltd.; compound 48/80 by the Wellcome Research Laboratories, Beckenham, England; and Polymyxin B sulphate by Burroughs Wellcome and Co.

Globulin permeability factor (globulin PF) from guinea pig serum was a pooled preparation containing fractions G_2 and G_2/1R prepared according to Mackay (1955) and Wilhelm et al. (1957) at the Lister Institute of Preventive Medicine, London.

Kallikrein, prepared from hog pancreas, was supplied by Winthrop Laboratories, Sydney; crystallised trypsin (salt free, 2,500 Armour units per mg.) prepared from bovine pancreas, by Armour Pharmaceutical Co. Ltd., England; human fibrinolysin by the Ortho Pharmaceutical Corp., Raritan, New Jersey, U.S.A. ("Actase") and by A.B. Kabi, Stockholm, Sweden ("Kabi 1127"). Preparations of streptokinase were supplied by Lederle Laboratories Division, American Cyanamid Co., Pearl River, New York, U.S.A. ("Varidase") and by A.B. Kabi, Stockholm, Sweden ("Kabikinase").

Soya bean trypsin inhibitor (SBTI) was supplied by Worthington Biochemical Sales Co., U.S.A.; lima bean trypsin inhibitor (LBTI) by Nutritional Biochemical Corporation, U.S.A.; and potato trypsin inhibitor (PoTI, fraction 4/ITS_1) by Dr. J. Hladovec, Pharmaceutical and Biochemical Research Institute, Prague, Czechoslavakia. Ovomucoid trypsin inhibitor was prepared by Dr. P.J. Mill at the Lister Institute of Preventive Medicine, London, according to Lineweaver and Murray (1947). The trypsin-kallikrein inactivator from bovine parotid gland ("Trasylol", 1,000 KIU per ml.) was supplied by Farbenfrabiken Bayer, Leverkusen, Germany.

SBTI was prepared for use by dissolving approximately

5 mg. in 1 ml. phosphate buffer pH 8, I = 0.2, and then made up in 0.85 per cent saline to the required concentration. Unless specified, all the other preparations for tests in guinea pigs and rabbits were dissolved in saline, for tests in rats in Locke's solution.

The inhibitor of the fibrinolytic system, ε-amino caproic acid (ε-ACA) and the glucose analogue 2-deoxy-(D)-glucose, were supplied by the California Corporation for Biochemical Research, U.S.A. A second preparation of ε-ACA was obtained from Lederle Laboratories through Professor C. de Gruchy, Melbourne, Australia.

α-amylase was supplied by the Rystan Co., Mt. Vernon, N.Y., U.S.A., and sodium α-naphtyl acetate by Carnegies of Welwyn Ltd., Herts., England.

A freeze dried preparation of the α-toxin of Cl. welchii (prep. 345/Ia) was supplied by Dr. M.G. Macfarlane, Lister Institute of Preventive Medicine, London. For each experiment, fresh samples of toxin were dissolved in 0.85 per cent saline, held at $4^{\circ}C$ and discarded when older than 6 hours (see Elder and Miles, 1957).

The saturated calcium salt of ethylenediaminetetraacetic acid (EDTA-Ca_2) was prepared as follows (Blaker, R.G., personal communication). To an isotonic aqueous solution of EDTA-Na_2 excess $CaCO_3$ (approx. 70 g.) was added. The carbon dioxide was allowed to escape and the chalk to settle overnight. The supernatant 13.25 per cent EDTA-Ca_2 was diluted to a 5 per cent solution with 0.85 per cent saline.

The lecithin analogue DL-2,3-Distearoyloxypropyl-(dimethyl) -β-hydroxyethylammonium acetate was supplied by Dr. R.P. Geyer, Harvard Medical School, Boston, Mass., U.S.A. (see Rosenthal and Geyer, 1960). Dispersions were prepared by dissolving a known amount of solid in boiling acetone and then cooling to 37°C. A sufficient volume of 0.85 per cent saline containing 0.1 per cent guinea pig albumin was slowly added to the dissolved analogue so that the final concentration of analogue was approx. 1 mg. per ml. The acetone was then evaporated under reduced pressure at 37°C. At all stages of preparation, the solution was constantly stirred. The final volume of the preparation was measured to determine the precise concentration of dispersed analogue. Subsequent dilutions were made with 0.85 per cent saline.

Nitrogen mustard (Mustine HN_2) was supplied by Boots Pure Drug Co. It was administered intravenously to guinea pigs anaesthetised by the intraperitoneal injection of sodium pentobarbitone 40 mg. per kg.

Microscopic Sections.

Guinea pigs' and rabbits' skin was fixed in buffered 10 per cent formalin and sections 5μ thick stained with haematoxylin and eosin, or with haematoxylin and carbol chromotrope for eosinophils (Lendrum, 1944).

The investigation of histological changes in rats' skin included the effects of irradiation on mast cells. For this purpose, the skin sites from rats were fixed in 7.2 per cent

mercuric chloride in 80 per cent ethanol (Kelsall and Crabbe, 1959) and sections 5μ thick were stained with buffered toluidine blue, 0.5 per cent, pH 4.0 (Culling, 1957). Pilot studies indicated little effect of irradiation on mast cells and were not pursued; but the same blocks of embedded skin were used for the preparation of sections stained with haematoxylin and eosin.

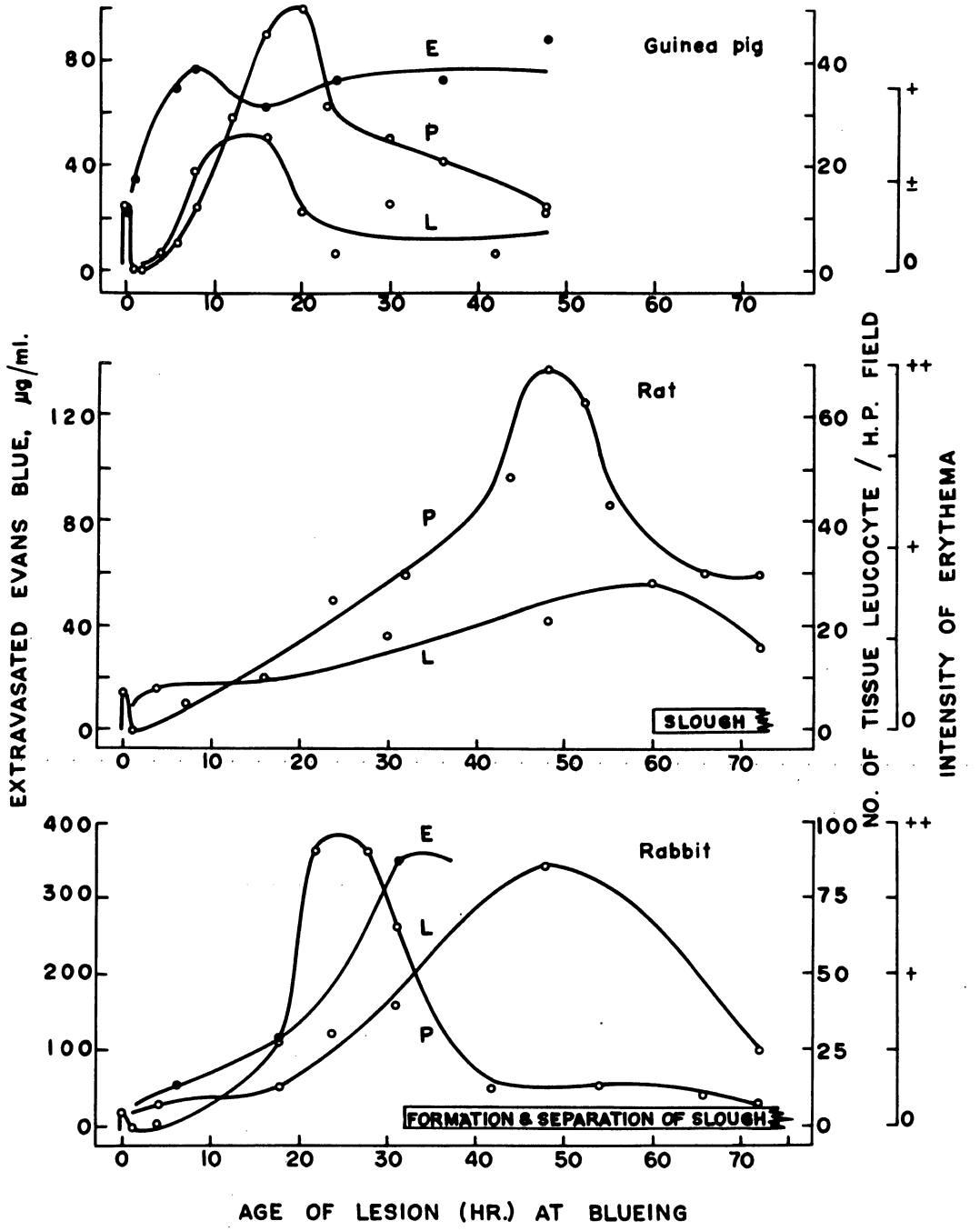


Fig. 4 The inflammatory response in ultra-violet injury in guinea pigs, rats and rabbits

E - Erythema P - Permeability L - Leucocytosis

Chapter 6

THE INFLAMMATORY REACTION IN ULTRA-VIOLET INJURY

Ultra-violet irradiation of the skin of guinea pigs, rats and rabbits induces an inflammatory response consisting of a diphasic increase in vascular permeability, a single wave of tissue leucocytosis and, except in the rat, erythema.

However, there are certain notable differences between the species in the rate of development of the permeability response, as well as in the intensity of tissue leucocytosis and its time relationship to the permeability response.

Ultra-violet Injury in Guinea Pigs

Irradiation of guinea pigs' skin for 15 sec. induces a diphasic increase in erythema (vasodilatation and increased blood flow), a diphasic increase in vascular permeability, and a monophasic tissue leucocytosis occurring a few hours in advance of the late phase of increased permeability (Fig.4, top block).

Erythema

A pale, fleeting erythema which disappears within 30 sec. is seen in about half the test animals on completion of 20 sec. irradiation.

In all the animals, a later principal phase of erythema begins after 30 min., being pale initially but increasing in depth and intensity of colour during the 3rd and 4th hours to become an obvious reddish pink of moderate intensity during the 6th to 8th hours. Its intensity varies little over the ensuing 66 hr., though oedema of the irradiated sites at 24 hr. makes the

erythema more apparent.

Irradiation for 120 sec. consistently induces an early erythema which is deeper in colour than that seen after 15 sec. exposure. After 15-20 min. the initial erythema fades markedly without disappearing. It then increases in intensity during the first hour and follows a subsequent time course similar to that of the later phase after 15 sec. exposure. After 72 hr. the lesions are brown and ecchymotic.

Increased vascular permeability.

The permeability response to U-V irradiation is diphasic.

The early response. - The early response is short-lived. After 15-20 sec. irradiation, the earliest traces of dye appear just as the initial erythema is fading. Permeability is greatest about 7-8 min. after irradiation and then declines, having disappeared in 30 min.

Such a short exposure induces the early response inconsistently and the lesions are never more than pale blue in intensity (dye equivalent 25-75 µg. per ml.).

After 120 sec. exposure, which regularly evokes a prominent early response, the lesions are brighter and correspond to a maximal dye equivalent of about 250 µg. per ml. (range 75-500 µg. per ml.). The duration of the early response corresponds very closely with that of the increased permeability induced by injected histamine (Miles and Miles, 1952).

The late response. - The early response to 15-20 sec. irradiation is followed by a latent period of near normal low permea-

bility lasting about 2 hr. This is succeeded by a period of increasing vascular permeability which is easily detectable by 8-12 hr. and maximal in 18-21 hr. (dye equivalent at maximal permeability 100 µg. per ml.; range 80-180 µg. per ml.). The degree of permeability then declines rapidly in the next 6 hr. and then more slowly over the ensuing 24-48 hr.

Histological changes.

Histological changes in the dermis and epidermis appear very rapidly after injury.

Within 30 min. of irradiation, intra-nuclear vacuolation appears in the epidermis, the vacuoles continuing to enlarge so that in some cases the nucleus becomes reduced to a mere crescent.

Eight hr. after irradiation the epithelial cells have begun to "round up" and become discrete, and their normally basophilic cytoplasm has become progressively more eosinophilic. Small intercellular spaces appear in the prickle cell layer and obvious inter-cellular oedema is present by 16-18 hr.

At this stage there are occasional microscopic areas of necrosis of the full thickness of the epithelium, with nuclear fragmentation. In these areas the epithelium takes on a homogeneous eosinophilic colour and microscopic areas of slough formation are seen.

Thirty hr. after irradiation mitotic figures appear in the basal cells of the epithelium of the skin and hair follicles, particularly in those parts adjacent to areas of greatest

epithelial damage.

The superficial dermis towards the centre of the lesion is also damaged and the collagen fibres swell and stain homogeneously with eosin. In addition, they lose their bright refractility in polarised light.

In 48 hr. the multi-layered squamous epithelium is reconstituted in all but the most severely damaged parts, and the necrotic epithelium is shed in keratin-like, eosinophilic plaques.

Tissue leucocytosis.

Skin from depilated control sites in guinea pigs contains only occasional neutrophils in the superficial dermis. Ultra-violet irradiation induces mild cellular infiltration of the upper third of the dermis. The cells are predominantly neutrophil leucocytes and up to 5 neutrophils per high power field (HPF) appear within ½-1 hr. By 8 hr. the numbers increase to 15-20 per HPF and reach a maximal 20-30 per HPF 16 hr. after irradiation. After this, the number of leucocytes declines slightly to about 10 per HPF at 24 hr. and then remains fairly constant for a further 24 hr. Eosinophils and lymphocytes appear in small numbers beyond 12 hr.

Tissue leucocytosis in guinea pigs is always relatively slight, even at its maximum, and is considerably less than that seen in the rabbit (see below).

Ultra-Violet Injury in Rats.

Irradiation of rats' skin for 10-30 sec. elicits res-

ponses generally comparable to those resulting from 15-20 sec. exposure in the guinea pig. In rats, however, erythema is inconspicuous or absent, and the late permeability response matures more slowly (Fig. 4, middle block).

Erythema

The rat is peculiar among the three species tested, in that skin sites irradiated for 10 sec. display erythema at no stage. In fact, in "unblued" animals, the injured sites are indistinguishable from the surrounding skin until 24-30 hr. after irradiation when they become brown and scaly. Increasing the time of irradiation to 30 sec. only slightly enhances the development of erythema. About 2 min. after 30 sec. irradiation, a faint brownish erythema appears, becoming maximal in 5 min. Thereafter its intensity declines and, while it remains fairly easily detectable for 30 min., it has disappeared by 45 min. No erythema is subsequently detectable and after 30 hr. the lesions become brown and scaly as after 10 sec. irradiation.

In lesions older than 24-30 hr. after either exposure, the dermal vessels are very fragile and light stroking or brushing against the affected sites is sufficient to cause marked local ecchymosis.

Increased vascular permeability

The early response. - The early response to 10-30 sec. irradiation in rats is similar in development and duration to that in guinea pigs, although it is induced inconsistently by the shorter exposure. After 30 sec. exposure in rats,

increased vascular permeability occurs in 2-3 mins. and is maximal in 7-9 min. (dye equiv. 150 µg. per ml.).

The earlypermeability changes coincide with the onset of obvious local oedema. Both oedema and increased permeability begin to decrease after 10-15 min. and while permeability has returned to near normal levels in 30 min., slight oedema is still detectable.

The late response. - The second or late phase of the response in rats can be elicited maximally by only 10 sec. irradiation. The response becomes maximal in 48-54 hrs. (dye equiv. 140 µg. per ml.), compared with 18-21 hr. in the guinea pig (cf. delayed permeability response in rats after bacterial injury; cited by Wilhelm, 1962).

Histological Changes.

Moderate oedema of the dermis and epidermis can be seen within 30 min. of irradiation, when there is already some evidence of epithelial damage. By 1½-4 hrs. the epithelium of the centre of some lesions has become thinned and eosinophilic, with pyknotic nuclei. Epithelial degenerative changes are much more pronounced by 8-12 hr. after irradiation when most lesions show intra-epithelial vesiculation and a deposit of brown granular material in the superficial layers of the epidermis. Occasional lesions, particularly after 30 sec. irradiation, appear brown and scaly to the naked eye as early as 7-8 hr. after irradiation.

In 24 hr., the epithelium is a thin, relatively

structureless layer, and there are patchy areas of leucocytic infiltration into the underlying dermis.

Between 30-48 hr. after irradiation the leucocytic infiltration of the dermis, which up to this stage has been only slight, becomes more marked - being most prominent along the line of future sloughing of the necrotic superficial dermis and epidermis. The slough shows evidence of partial separation 60-72 hr. after irradiation, by which time all lesions are brown and scaly to the naked eye. The epithelium has been reconstituted as a single layer in most parts, and the number of neutrophils present in the dermis decreases rapidly.

At all stages the leucocytic infiltration is relatively slight and there is considerably more variation in the degree of tissue leucocytosis between individual animals than occurs in guinea pigs.

Ultra-Violet Injury in Rabbits

Whereas 15-20 sec. irradiation in the guinea pig and 10 sec. in the rat consistently induces a delayed permeability response with minimal associated histological damage, irradiation for only 5 sec. is sufficient to elicit a similar permeability reaction in the rabbit (Fig. 4, bottom block). Short though this exposure is, however, it causes marked histological damage, the irradiated areas in 72 hrs. being small brown sloughs "punched out" of the surrounding skin.

Erythema

Whereas, in the guinea pig, maximal erythema occurs in

8-10 hr., in the rabbit, erythema in lesions induced by 5 sec. irradiation is detectable in 6 hr. but does not reach its maximal intensity until 22-26 hr. The lesions at all times lack the deep pink colour that occurs in the guinea pig and have a more orange hue. After 30 hr., the lesions become more orange-brown, and after 42 hr. all become brownish and scaly.

Exposure for 120 sec. evokes a similar erythematous response, but the erythema is fainter than after 5 sec. irradiation. This recalls the observation of Blum and Terus (1946) that erythema in human sunburn can be suppressed by large doses of ultra-violet irradiation of long wave length (2,800Å), but only at the cost of increased necrosis of the skin.

Increased vascular permeability.

While 5 sec. irradiation consistently induces a late permeability response in rabbits, it inconsistently induces an early response. Even when present, the early response is often so feeble that its limits are often difficult to determine.

After 120 sec. irradiation, the early permeability response occurs more uniformly. Even so, the response tends to be erratic and to show great variation between individual animals.

Mean results obtained from multiple experiments in individual animals indicate that a permeability response begins to develop 2-3 min. after the completion of irradiation, showing an upward trend in the first ½ hr., downward in the second. But the lowest level of permeability following 120 sec.

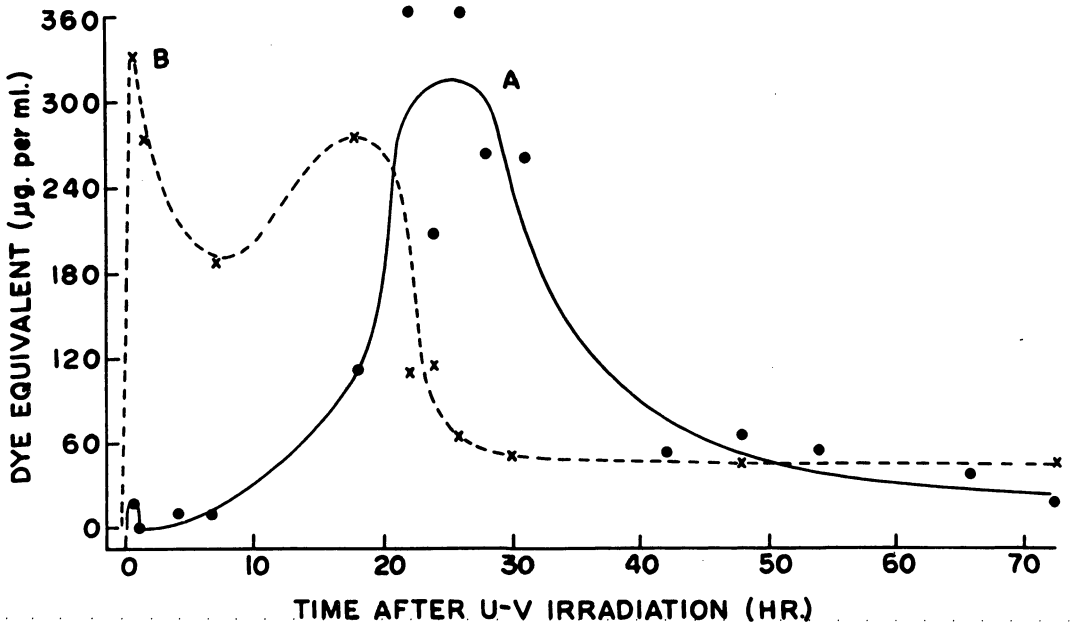


Fig. 5 The accelerated permeability response in rabbits to prolonged U-V irradiation

A = 5 sec. irradiation, B = 120 sec. irradiation

irradiation is never as low as that after 5 sec. irradiation. This apparent failure of the early response to disappear entirely probably represents the accelerated appearance (Fig.5) of the later response (cf. thermal injury; Wilhelm and Mason, 1960).

After 5 sec. irradiation, normal low permeability is regained at the end of 1 hr. Thereafter, a mild increase in vascular permeability is apparent by 4-8hr., the rate of increase accelerating rapidly thereafter, to reach maximal permeability 22-26 hr. after irradiation. At peak permeability, the lesions have a dye equivalent of 360 µg. per ml. After 26 hr. permeability decreases fairly rapidly until the lesions are 45 hr. old, when the rate of decrease slows and the response line shows a plateau formation (Fig.4). Beyond 70 hr. permeability is minimal.

Histological Changes

In sections from lesions induced immediately after "blueing" (i.e. those aged ½ hr. when the animal was killed), mild oedema of the superficial dermis is the earliest histological change noted. This is succeeded in lesions 1-4 hr. old by intercellular oedema of the epidermis and the appearance of nuclear vacuolation and other degenerative changes signifying incipient epithelial necrosis.

Between 4-6 hr., the epidermis and superficial dermis are thrown up into papillary folds and evidence of karyorrhexis is seen. Cellular infiltration of the superficial dermis has

begun and neutrophils and "pseudo-eosinophils" are prominent. "Pseudo-eosinophils" are neutrophils which, in the rabbit, have a "predilection for acid dyes" (see Maximow and Bloom, 1957).

By 18-24 hr., most lesions show necrosis of the full thickness of the epithelium and the presence of a great amount of protein-rich, oedema fluid. It should be noted that this time interval coincides with the period of greatest vascular permeability as measured by the dye method and also with the presence of palpable oedema (18-28 hr.). In lesions aged 18-24 hr., a much more intense neutrophil infiltration occurs than is seen in either the rat or the guinea pig.

The inflammatory response to minimal U-V injury in the rabbit is complicated by a severe degree of skin damage. Leucocytosis seems to occur principally in relation to tissue necrosis rather than to the other components of the inflammatory response.

Chapter 7

THE EFFECT OF ANTAGONISTS OF HISTAMINE AND 5-HYDROXYTRYPTAMINE ON THE PERMEABILITY RESPONSE TO U-V INJURY

Since Lewis (1929) first postulated the release of a "histamine-like" substance in various types of injury of human skin, histamine has been considered as a natural mediator of increased vascular permeability on three principal grounds: (i) its ubiquitous distribution in animal tissues, (ii) its proven ability to increase vascular permeability when injected into the skin of man and experimental animals, and (iii) its ready isolation from both normal and injured tissues. However, this evidence alone is inadequate proof that histamine is the natural mediator of the vascular permeability changes consequent on injury. Further information concerning the role of histamine has been sought by tests with a potent and specific antihistamine (triprolidine) in an attempt to demonstrate conclusively the role of histamine in the various phases of the permeability response in U-V injury.

Guinea Pigs

The potency and duration of action of antihistamines in the guinea pig

Of various antihistamines tested in the guinea pig by Wilhelm and Mason (1960), the four most potent were selected for use, namely, triprolidine, chlorprophenpyridamine, mepyramine and promethazine.

Each drug was injected intravenously in a dose of 0.1 mg. per kg. body weight and its effects tested on histamine and

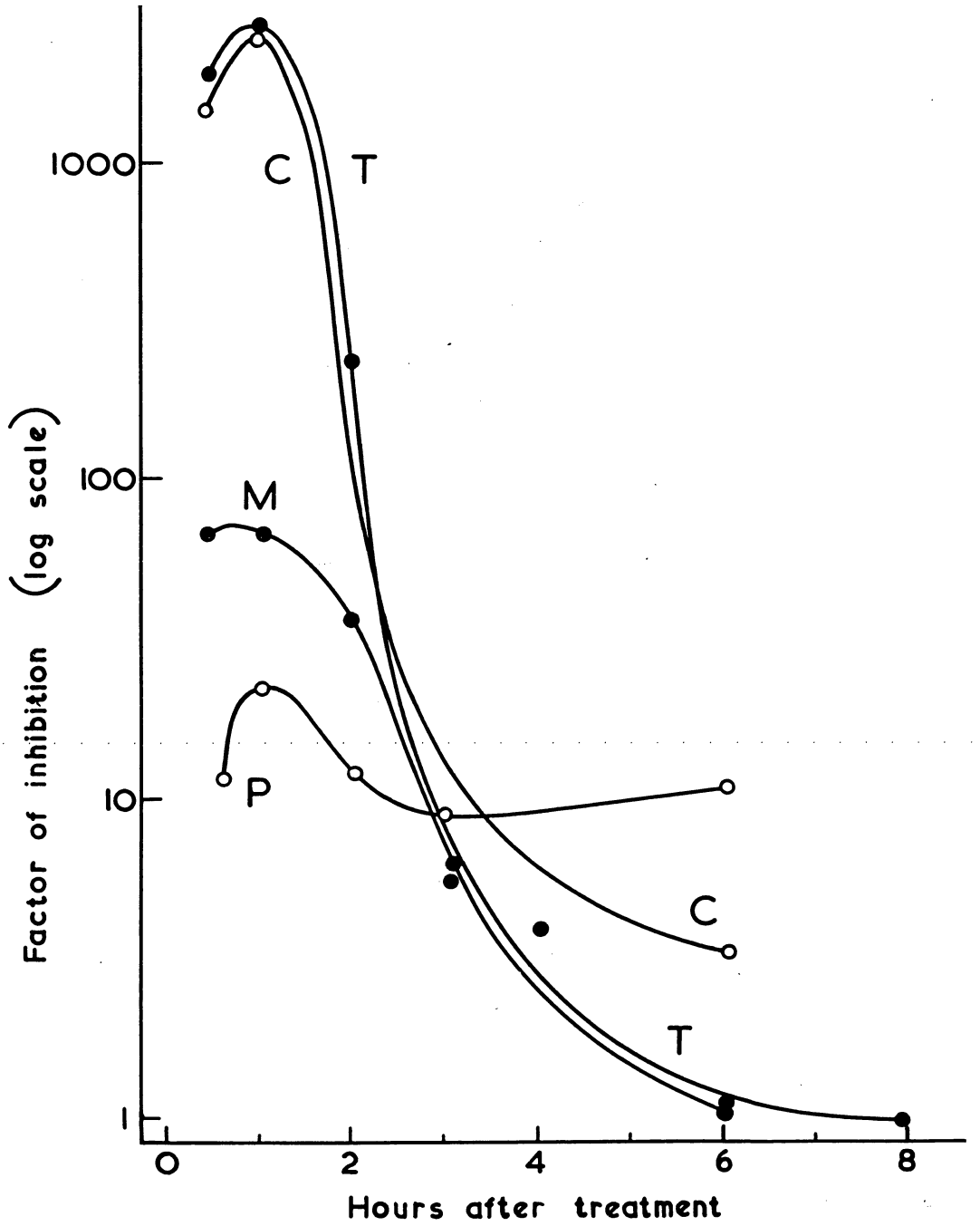


Fig. 6 The potency and duration of action of four antihistamines in the guinea pig

C - Chlorprophenpyridamine
M - Mepyramine
T - Triprolidine
P - Promethazine

other PF's injected intracutaneously in different groups of animals at intervals ranging from ½ - 24 hr. after the injection of antihistamine.

The responses for the same PF's were also tested at similar intervals in control animals injected intravenously with 0.85 per cent saline. Inhibition of the various PF's at different intervals was then calculated by the method of Miles and Miles (1952) and Sparrow and Wilhelm (1957), inhibition being expressed as the factor by which the PF potency of histamine (or the other PF's) in control animals exceeded that in antihistamine-treated animals.

The results are summarised in Fig. 6. Triprolidine and chlorprophenpyridamine are the most effective antagonists, and both suppress the histamine response over 1,000-fold for 1½ hr. after their intravenous administration. The degree of inhibition declines rapidly during the third hr. but is still approximately 10-fold during the fourth hr. No inhibition is apparent 6 hr. after treatment.

On the basis of the above results, triprolidine was selected as a potent inhibitor of histamine for tests on the permeability response to U-V radiation.

<u>The effect of triprolidine on the early permeability response to U-V injury</u>

The early permeability response to 120 sec. irradiation appears within 1 - 2 min., is maximal in 7 - 8 min. and disappears in 15 - 20 min. This time course is similar to that

occurring in mild thermal injury (Wilhelm and Mason, 1960) and is also remarkably similar to that of the permeability response to histamine injected intracutaneously (Miles and Miles, 1952).

In untreated control animals, the colour intensity of lesions induced by 120 sec. irradiation is equivalent to that produced by the intradermal injection of 0.05 ml. of Evans blue solution containing 250 µg. dye per ml.

Triprolidine, injected intravenously in a dose of 0.1 mg. per kg. immediately prior to 120 sec. irradiation reduced the dye equivalent of irradiated sites to approximately 10 µg. per ml. In one experiment as little as 0.01 mg. per kg. of triprolidine was sufficient to reduce the dye equivalent of lesions to 40 µg. per ml. In other tests, doses of 1.0 mg. triprolidine per kg. reduced permeability to such an extent that the dye equivalents of the lesions could not be assessed from the colour reference curves.

The early permeability response in guinea pigs is clearly very susceptible to antihistamine. This result strongly suggests that the early response is mediated by endogeneous histamine.

<u>The effect of triprolidine on the late permeability response to U-V injury</u>

The influence of triprolidine on the late permeability response was tested by injecting the antihistamine systemically (either intravenously or intraperitoneally) in a dose of 1 mg. per kg.

(i) 5 - 15 min. before irradiation (to eliminate the early response)

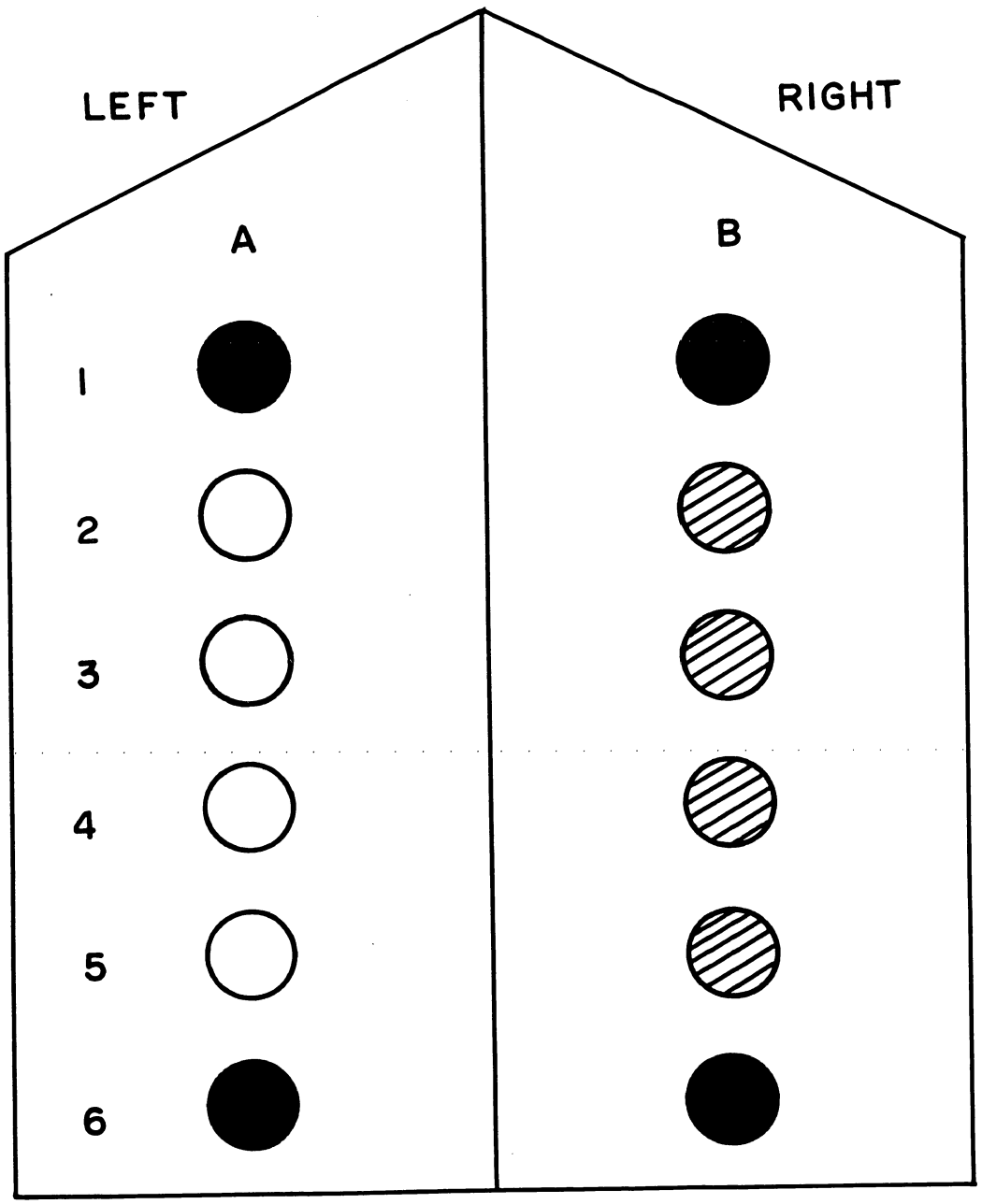


Fig. 7 Plan of lesion sites for the testing of inhibitors by local, intracutaneous injection

(ii) 3 hr. after irradiation (at the commencement of the late response)

(iii) ½ - 1 hr. before "blueing" (at the time of maximal permeability).

In one experiment, the first dose was given intravenously and the second and third intraperitoneally; in a second experiment the procedure was reversed, the first and second doses being given intraperitoneally, the third intravenously. All animals were "blued" 22 hr. after irradiation and the lesions examined ½ hr. later.

In both tests, the lesions in test animals had colour intensities equal to, or slightly greater than those in control animals similarly injected with 0.85 per cent saline.

The effect of antihistamine given by local, intracutaneous injection was also tested at similar intervals. The details of the experimental plan are illustrated in Fig. 7. A row of six skin sites was irradiated on either side of the back. For convenience the left side is referred to as side A, the right as side B. The sites are numbered 1 - 6 from the cervical toward the lumbar region.

Sites A_1, B_1, A_6 and B_6 were left as untreated control sites. Triprolidine (10 µg. in 0.1 ml. of 0.85 per cent caline) was injected 5 - 15 min. before irradiation in, for example, site A_2; and further injections made 3, 20 and 21½ hr. after irradiation in sites A_3, A_4 and A_5 respectively. At similar intervals, the corresponding sites B_2-B_5 were injected with 0.1 ml. of physiological saline. The animals were "blued" 22 hr.

after irradiation and examined 30 min. later. Variation over the test area was minimised by using groups of 3 - 4 animals in each experiment and varying the sides of the animals on which the inhibitor was injected and also the location of the treated sites within the areas A and B_{2-5}.

Two antihistamines, triprolidine and mepyramine, were tested by local injection. In neither case was there any inhibition of erythema or the late permeability response. In summary, the failure of antihistamines to suppress the late permeability response suggests that histamine plays no part in the development or maintenance of the late response. Furthermore, the early response which is mediated by histamine does not appear essential for the development of the later events.

Rats.

The effect of antagonists of 5-H-T and histamine on the early permeability response in rats

In the rat, 5-hydroxytryptamine (5-HT) induces a time course of vascular permeability closely resembling that of histamine in the guinea pig. Furthermore, the 5-HT response also resembles the early permeability response induced in the same species by mild thermal injury (Wilhelm and Mason, 1960) or 30 sec. U-V irradiation (Logan and Wilhelm, 1965a). In fact, 5-HT contributes to the mediation of the early thermal response in the rat (Wilhelm and Mason, 1960); and accordingly, the 5-HT antagonist, BOL-148 was tested against both the early and late phases of the permeability response in U-V radiation.

When given to rats immediately prior to 30 sec. U-V irradiation, BOL-148, 1 mg. per kg., strongly suppresses the early response. Whereas, in control animals the dye equivalent of the lesions is 60 - 120 µg. per ml., in treated animals it is less than 10 µg. per ml. Even a dose of 0.1 mg. per kg. of BOL-148 is sufficient to reduce the colour intensity of the lesions to an equivalent of 16 - 38 µg. dye per ml.

Although triprolidine in doses of up to 1 mg. per kg. intravenously inhibits the effect of intracutaneous histamine 30 to 50-fold in rats, it has no effect on 5-HT similarly injected and does not inhibit the early U-V permeability response.

The effect of antagonists of 5-HT and histamine on the late permeability response

In one experiment the effect of the above antagonists on the late response in rats was tested by giving a single intravenous dose of inhibitor (1 mg. per kg.) immediately before "blueing" animals bearing lesions 0 - 72 hr. old.

In another experiment, batches of animals each bearing eight U-V lesions, of uniform age, were injected intraperitoneally with multiple doses of BOL-148, triprolidine and Locke's solution respectively. In each case, the test preparations were given immediately prior to irradiation, then 5, 24 and finally 51 hr. after irradiation. The last dose preceded "blueing" of the animals at the period of maximal permeability.

In each of the above experiments, triprolidine decreased

the amount of dye exudation in treated animals as compared with controls. Nevertheless, the results were not conclusive since the colour intensities of lesions in treated animals still fell within the lower limits of tests in other control animals.

In one experiment, BOL-148 gave similar though less marked inhibition. However, these 5-HT results must be qualified in the same way as those for histamine, and hence no conclusive evidence was found to implicate either 5-HT or histamine as mediators of the delayed permeability response to U-V radiation in rats.

Rabbits

In the rabbit, histamine has approximately the same PF potency as in the guinea pig, but 5-HT is relatively ineffective (Sparrow and Wilhelm, 1957). These observations are also applicable to the animals used in this study (unpublished). In view of these results, triprolidine alone was tested in the rabbit; and since histamine antagonists did not affect the late permeability response in guinea pigs, triprolidine was tested solely on the early response in rabbits.

The early response to 120 sec. irradiation in rabbits differs from that in other species in being more prolonged and lasting at least 60 min. Maximal permeability is attained within 25 - 30 min., after which a slight but progressive decline occurs (see Ch. 6).

In the rabbit, the time course of the early U-V response also closely resembles the time course of the permeability

effects of intracutaneous histamine, 0.6 µg. per lesion.

Although the response to injected histamine is substantially inhibited (approximately 35-fold) by intravenous triprolidine, 0.1 mg. per kg., the early permeability response in U-V irradiation is entirely unaffected.

Chapter 8

THE EFFECT OF PROTEASE INHIBITORS

Some evidence was produced in earlier chapters to suggest that certain proteolytic enzymes participate either directly, or indirectly as kininogenases in the permeability response in inflammation. If such is the case, agents which inhibit the proteolytic or kinin-forming activity of these enzymes should reduce vascular permeability in injury.

Accordingly trypsin inhibitors from several sources and epsilon-aminocaproic acid (ε-ACA), an inhibitor of the fibrinolytic system, were tested in guinea pigs.

The effect of the trypsin inhibitors from soy bean, potato, lima bean and ovomucoid

The trypsin inhibitors from soy bean (SBTI) and potato (PoTI) when mixed with guinea pig globulin PF in final concentrations of 100 µg. per ml. prior to intracutaneous injection into "blued" guinea pigs, substantially reduce its permeability increasing effect (approx. 100-fold). In the same concentrations, lima bean trypsin inhibitor (LBTI) and ovomucoid trypsin inhibitor (OvTI) are ineffective.

SBTI in a concentration of 100 µg. per ml. completely inhibits the kinin-forming activity of plasmin in diluted human plasma (Lewis, 1958). The permeability increasing effect of plasma kallikrein is also inhibited by SBTI (Mason, Brenda and Sparrow, Elizabeth, unpublished; cited by Mason and Miles, 1962).

SBTI and PoTI appeared to have equal potency against the

PF effects of globulin PF and trypsin in preparatory experiments. However, "in vitro" tests suggested that protease inhibitors might have varying potencies against different proteases and particularly against kallikrein from different sources (Webster and Pierce, 1960). Hence all four protease inhibitors, SBTI, PoTI, LBTI and OvTI, were tested in guinea pigs against the permeability response to U-V irradiation.

The above inhibitors were not given systemically since relatively small doses (3.3 mg. per kg.) given intravenously in guinea pigs have been alleged to have non-specific effects (Wilhelm and Mason, 1960), and optimally effective doses in rabbits (25 - 30 mg. per kg.) are toxic (Zweifach, Nagler and Troll, 1961).

Protease inhibitors were injected locally according to the scheme outlined for the testing of antihistamines (see p.55, Fig. 7). While in individual experiments there was some suggestion of slight inhibition of permeability by both SBTI and OvTI injected locally 3 hr. and 20 hr. after irradiation, in all cases the mean values in duplicate experiments were within the range of readings recorded for control sites and are therefore not regarded as significant. Indeed, in some instances treated sites showed greater permeability than control sites injected with 0.85 per cent saline.

The effect of ε-aminocaproic acid

Human and guinea pig plasmin are stated to have little permeability increasing effect when injected intracutaneously

in "blued" guinea pigs (Wilhelm, Miles and Mackay, 1955). These results need careful appraisal in view of the fact that the preparation of human plasmin used in the present experiments (Actase) appeared to be markedly lacking in active principle as judged by its activity against fibrin heated at 60°C for 20 min. When tested against a 0.2 per cent solution of fibrinogen in veronal buffer pH 7.2 (the same test system used by Wilhelm, Miles and Mackay, 1955) it became apparent that the substance owed a substantial part of its effect to a high concentration of streptokinase acting on the plasminogen contaminating the fibrinogen substrate (Logan, G., unpublished).

ε-aminocaproic acid in a concentration of 1×10^{-4} to 1×10^{-2} M is a potent inhibitor <u>in vitro</u> of the activation of plasminogen to plasmin (Ablondi, Hagan, Philips and de Renzo, 1959; Alkjaersig, Fletcher and Sherry, 1959; Fukutake, Shida, Arakawa and Kato, 1960), but has little effect upon pre-formed plasmin. In fact, in concentrations greater than 10^{-2}M, ε-ACA enhances the proteolytic activity of plasmin (Alkjaersig, Fletcher and Sherry, 1959).

ε-ACA was used as an inhibitor to assess the possible role of plasmin as a mediator of permeability in injury. The acid is a very soluble compound and is rapidly absorbed and excreted when given parenterally. In the rabbit, 75 per cent of a single, rapid intravenous dose of 1.0 g. per kg. is cleared from the blood within 15 min. (Flaum, 1960). In man, because of the rapidity of excretion, doses of approximately

100 mg. per kg. are required for therapeutic effect (Nilsson, Sjoerdsma and Waldenström, 1960).

During preliminary standardisation, ε-ACA was first tested by local injection in "blued" guinea pigs against standard PF's such as histamine, compound 48/80 and GP globulin PF exemplifying short chain amines, histamine liberators and proteases respectively.

ε-ACA was "non-blueing" in concentrations of up to 162 µg. per ml. in 0.85 per cent saline. When mixed with globulin PF in final concentrations of 100 µg. per ml. (i.e., less than 10^{-3}M) or 10 µg. per ml. (less than 10^{-4}M) no inhibition of the globulin PF occurred. Similar concentrations, however, induced up to 3-fold inhibition of the histamine response in "blued" guinea pigs.

ε-ACA was tested locally, according to the schedule outlined for the testing of antihistamines (see p.55, Fig. 7). Concentrations of 0.13 mg. per ml. (10^{-3}M) or 13.1 mg. per ml. (10^{-1}M) injected into separate sites 15 min. before irradiation and 3 hr., 20 hr. or 21½ hr. after irradiation, caused no decrease in either erythema or permeability when compared with untreated control lesions or control lesions injected with equivalent volumes of 0.85 per cent saline. In fact, permeability tended to be slightly greater in the ε-ACA treated lesions than in the controls.

In view of its effect on the histamine permeability response, ε-ACA was tested by local injection against the early

permeability response induced by 120 sec. U-V irradiation in the guinea pig.

Two pilot experiments in single animals revealed no decrease in permeability below control levels in lesions injected with ε-ACA at concentrations of 10^{-3}M or 10^{-1}M respectively.

The effect of Trasylol

A polypeptide inhibitor of trypsin and kallikrein isolated from cattle parotid glands (Frey, Kraut and Werle, 1950; Werle, Maier and Ringelmann, 1952; Werle and Appel, 1958) and made available commercially as "Trasylol" (Farbenfabriken Bayer) was tested in guinea pigs. When injected intravenously in relatively small doses (500 units per kg. body weight), Trasylol did not inhibit PF's such as histamine, globulin PF, trypsin, kallikrein or synthetic bradykinin. In fact, the effects of all PF's were slightly enhanced in comparison with the effect of the same PF's in control animals injected intravenously with saline.

A 10-fold increase in dose was required before any suppression was obtained. Even so, after a dose of 5,000 units Trasylol per kg., the maximum inhibition of trypsin and globulin PF was only 2- and 3-fold respectively.

The effect of Trasylol on the permeability response to U-V injury was first tested by the method outlined for the testing of antihistamines (p. 55, Fig. 7). Skin sites bearing lesions induced by 20 sec. irradiation were injected with 10 units Trasylol 15 min. before irradiation, and 3 hr., 18 hr. and 19½ hr. after irradiation respectively. The animals were

"blued" 20 hr. after irradiation. All treated lesions were compared with untreated control lesions and control lesions locally injected with 0.85 per cent saline.

Some slight inhibition of both permeability and erythema was noted in these animals. Control lesions (both saline-injected and untreated) had dye equivalents of 91 - 138 µg. per ml., while that of the treated lesions was 66 µg. per ml. Inhibition was greatest in those lesions injected up to 2 hr. before "blueing" (i.e. 18 and 19½ hr. after irradiation). Even at maximal inhibition, however, the degree of permeability in inhibitor-treated lesions was always within the range of control values in other experiments and thus does not represent conclusive evidence of inhibition.

An alternative method of testing was devised to assess the effect of Trasylol in the developing stage of the delayed permeability response to U-V injury in guinea pigs in the period 0 - 12 hr. after irradiation. Multiple 20 sec. exposures were made on the skin of the back in two groups of three guinea pigs so that lesions aged 12, 8, 4, 2, 1 and 0 hr. were present at the time of "blueing". Immediately before making the first exposures in the test batch of animals, Trasylol was injected intravenously in a dose of 500 units per kg. Further doses of Trasylol were given intraperitoneally 3½ hr., 7½ hr. and 11½ hr. after the first irradiation. A second control group of animals was irradiated in similar manner but received 0.85 per cent saline instead of Trasylol. All animals were "blued" 12 hr. after

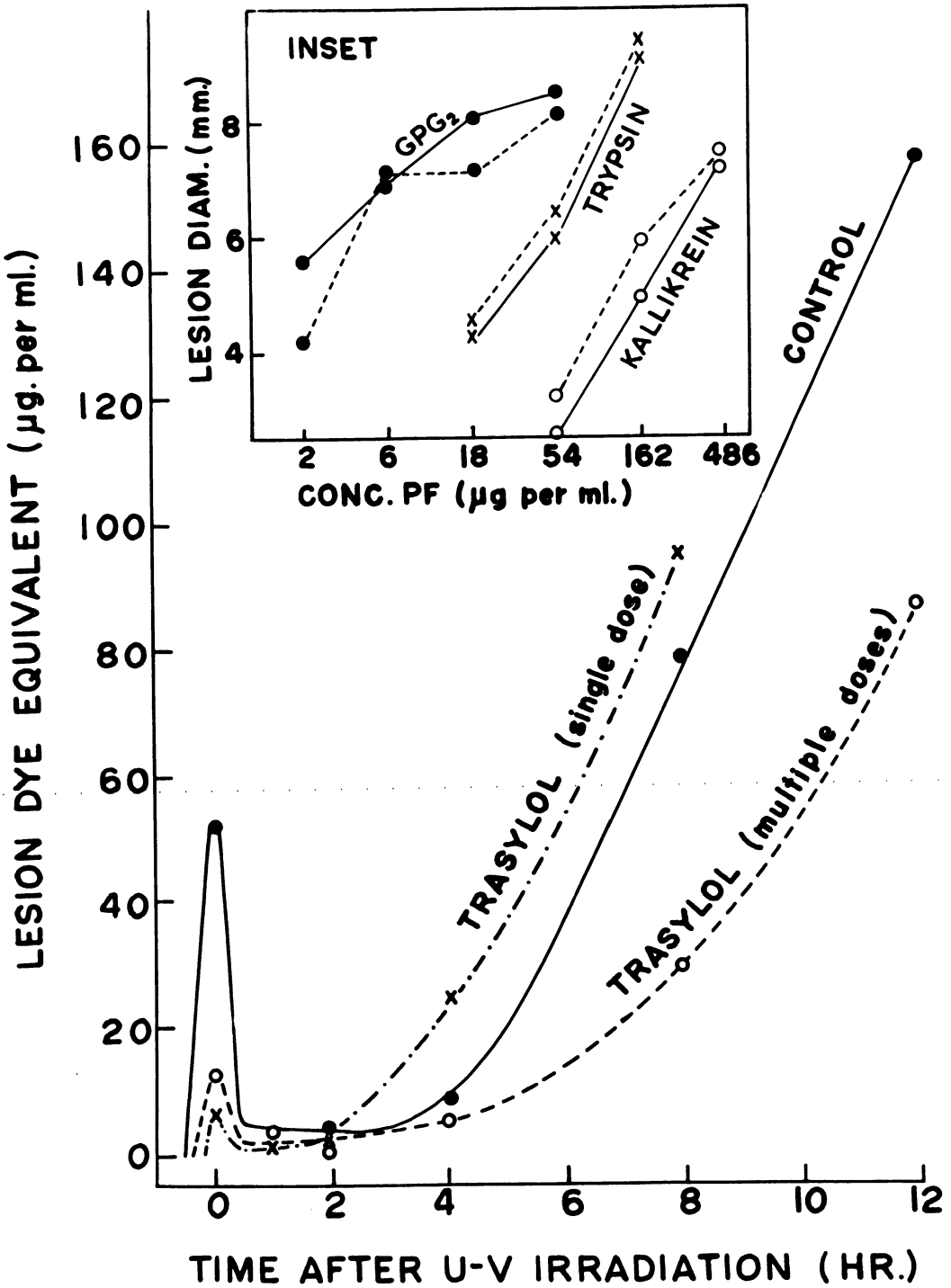


Fig. 8 The effect of Trasylol on the late permeability response to U-V injury

Inset - The effect of Trasylol on guinea pig globulin PF, trypsin and kallikrein

the first exposure, lesion colour intensity estimated, and a response-line constructed from the means of several readings for each lesion. In two such experiments (Fig. 8) the delayed permeability response appeared slightly reduced in its early developing period. However, the effect was slight, and again results obtained in the treated groups lie within the range of normal control variation.

On the other hand, the early response was more convincingly suppressed by Trasylol, maximal permeability being reduced from a dye equivalent of 56 µg. per ml. to 14 µg. per ml. However, while quite marked reduction of the early response to 20 sec. irradiation is effected by a dose of 500 units of Trasylol per kg., a 10-fold increase in dose produces negligible effect on the early response to 120 sec. irradiation. Trasylol has no effect on erythema in irradiated skin sites.

The effect of G_1S/P.

Fraction G_1S/P of guinea pig serum, a preparation rich in the natural inhibitor of the globulin PF (Wilhelm et al., 1955) was also tested against the late permeability response to U-V injury.

G_1S/P pre-mixed with globulin PF for 90 min. in a "non-blueing" concentration of 0.05 per cent caused slight inhibition only (approx. 2.5-fold) of the effect of globulin PF.

When tested against the delayed response to U-V injury by the usual routine for locally administered preparations (Fig. 7),

the response was slightly decreased 3 hr. and 19½ hr. after irradiation as compared with saline injected controls. These results parallel those with SBTI, but again are similarly inconclusive since the results from the treated animals in this particular experiment fall within the range of control readings from other experiments.

In summary, various protease inhibitors appeared to suppress mildly the late permeability response in comparison with control sites in particular experiments. The suppression was always slight, but it occurred repeatedly with various preparations. On the other hand, the treated lesions yielded results that fell within the range of results in control animals in other experiments. The present work therefore does not permit firm conclusions concerning the role of proteases, and the problem requires further investigation by another technique.

Chapter 9

THE EFFECT OF INHIBITORS OF LECITHINASE ENZYMES

The only PF's known to have prolonged, local effects comparable with those in ultra-violet injury are the α-toxins of certain clostridial organisms (Elder and Miles, 1957), and rabbit globulin PF (Wilhelm et al., 1958). The clostridial toxins are effective in various species, but the rabbit PF is peculiar in that its PF effects are prolonged only when tested in the homologous species.

The α-toxin of Cl. welchii induces a diphasic permeability response consisting of a short-lived early response followed by a delayed phase in which permeability is maximal during the third and fourth hours and has declined in six hours. The Cl. septicum response is also diphasic but the delayed phase appears earlier (within $\frac{3}{4}$ hr.), and lasts longer (about 6 hr.) than with Cl. welchii α-toxin.

With the α-toxin of Cl. oedematiens, only a late phase is evoked. It begins in 6 hr., becoming maximal in 24 hr. and lasting for at least a further 24 - 48 hr. The α-toxin of Cl. welchii is a lecithinase, but the α-toxins of Cl. septicum and Cl. oedematiens have not been characterised.

There is a remarkable similarity, firstly, between the time course of the late permeability response to U-V injury in guinea pigs (Logan and Wilhelm, 1963) and that to the α-toxin of Cl. oedematiens; and secondly, between the time course of late permeability in mild thermal injury (Wilhelm and Mason,

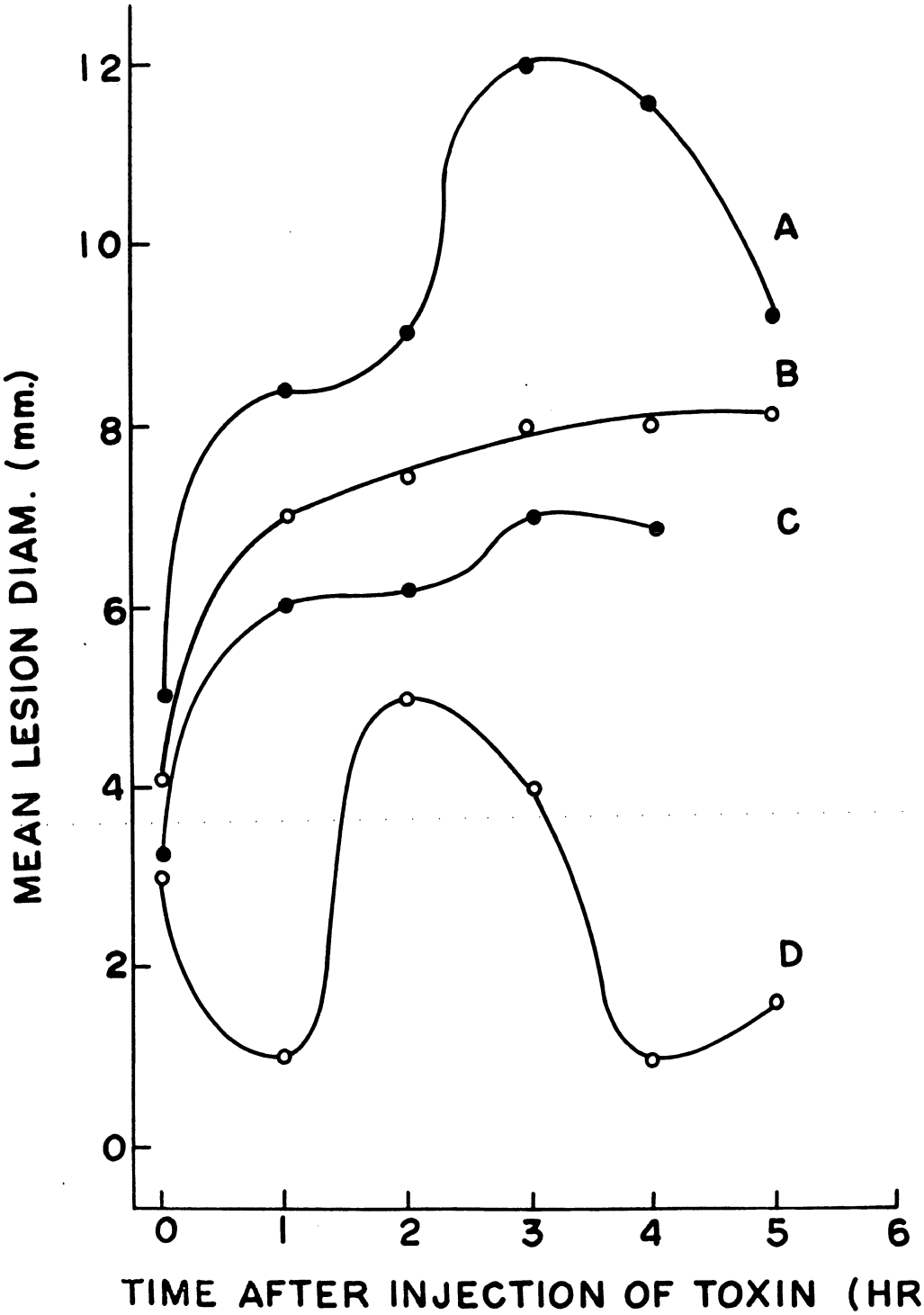


Fig. 9 The time course of increased vascular permeability after the injection of Cl. welchii α-toxin

A = 54 μg. B = 18 μg. C = 9 μg. D = 2 μg. per lesion

1960) and that of the α-toxin of Cl. welchii. Accordingly, inhibitors of the α-toxin of Cl. welchii were tested for their effect on the delayed permeability response to U-V injury in guinea pigs.

In the present work, the intracutaneous injection of 2 - 54 µg. purified Cl. welchii toxin at hourly intervals for 4 hr. prior to "blueing" induced permeability responses (see Fig.9) which were similar to the results obtained by Elder and Miles (1957).

The lethal and permeability increasing effects both reside in the α-toxin. The lethal effect can be suppressed by the previous systemic administration of ethylenediamine tetra-acetic acid (EDTA; Moskowitz, Deverell and McKinney, 1956) or its calcium salt (EDTA-Ca; Moskowitz, 1956), the latter compound having the advantage of being less toxic and hence permitting larger systemic dosage. EDTA-Ca also reduces toxin-induced permeability in guinea pigs (Blaker, R.G., personal communication). Accordingly the sodium and calcium salts of EDTA were tested as inhibitors of the permeability responses to both Cl. welchii α-toxin and U-V irradiation.

In preliminary experiments, EDTA-Na_2 (0.1 - 100 µg. per ml.) mixed with the α-toxin prior to intracutaneous injection, caused very slight inhibition only of the 3 hr. response. Since EDTA-Na_2 is too toxic for systemic use in guinea pigs, on the advice of Dr. R.G. Blaker, Department of Microbiology and Immunology, State University of New York, the saturated calcium salt was used (EDTA-Ca_2).

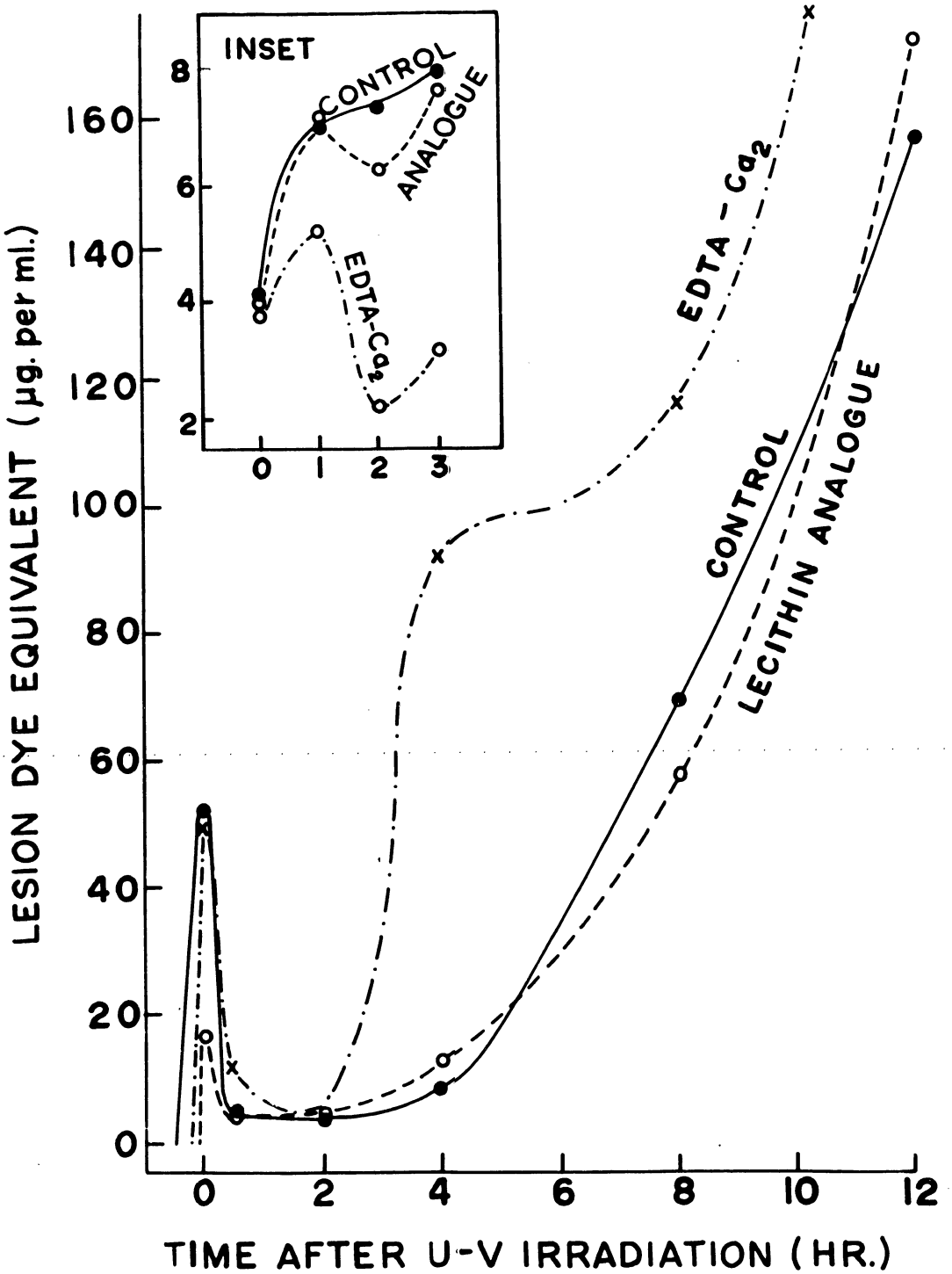


Fig. 10 The effect of lecithinase inhibitors on the late permeability response to U-V injury

Inset - The effect of the inhibitors on the permeability response to injected Cl. welchii α-toxin 18 µg. per lesion

Doses of 150 mg. per kg. of EDTA-Ca_2 caused moderate suppression of the permeability response to Cl. welchii α-toxin. A single intravenous dose has maximal effects in ¼ - 3¼ hr., whereas a single intraperitoneal dose has maximal effect in the period 2 - 5 hr. after injection.

The degree of suppression is markedly increased by giving repeated doses of EDTA-Ca_2 (see below). The effect appears to be specific in that PF's like histamine, compound 48/80 and globulin PF are not antagonised.

The effect of EDTA-Ca_2 on the permeability response to U-V injury was tested as follows. In two batches of guinea pigs, skin sites were irradiated 12, 8, 4, 2, ½ and 0 hr. before "blueing". One batch of animals received intraperitoneal EDTA-Ca_2, 150 mg. per kg. 12½, 8 and 3 hr. before "blueing". The second control batch was given the same volumes of 0.85 per cent saline at similar intervals.

As control measures, hourly intracutaneous injections of Cl. welchii α-toxin, 18 and 9 µg. were made for the last 3 hr. before "blueing", in sites adjacent to the test area. Immediately after "blueing", injections of histamine, 48/80 and globulin PF were made in both control and treated animals.

While the permeability response to Cl. welchii α-toxin was profoundly suppressed in the test animals, the developing late permeability response to U-V irradiation was unaffected (Fig. 10). The responses to injected histamine, compound 48/80 and globulin PF were also within normal limits.

Effect of a lecithin analogue

A synthetic lecithin analogue, 2, 3-Distearoyloxypropyl (dimethyl)-β-hydroxyethylammonium acetate, inhibits "in vitro" the lecithinase A activity of moccasin venom and also the lecithinase D activity of Cl. welchii α-toxin (Rosenthal and Geyer, 1960, 1962). In each case, effective inhibition occurred only when the enzyme and inhibitor were mixed before adding the substrate.

In addition, doses of 2 - 10 mg. of the analogue gave good protection against the lethal effects of Cl. welchii α-toxin, when mixed with the toxin prior to its intravenous or intraperitoneal injection. A dose of 2 mg. given intravenously, soon after the intravenous administration of toxin, afforded little protection (Rosenthal and Geyer, 1962).

In the present work, a suspension of analogue in 0.85 per cent saline containing 0.1 per cent purified guinea pig albumin did not, itself, increase permeability in doses up to 16 µg. per lesion. When tested locally, the analogue, in doses up to 10 µg., did not inhibit the permeability effect of the toxin. The lack of effect was noted both when the analogue was injected premixed with toxin, and when injected alone 15 min. prior to the toxin.

On the other hand, a single dose of lecithin analogue, 5 mg. per kg., given intraperitoneally 3½ hr. before "blueing", moderately suppressed the toxin response in lesions 3 hr. old, but the inhibitory effect declined in the third hour.

Unlike EDTA-Ca_2, multiple doses of the analogue have no more inhibitory effect than a single dose. Like EDTA-Ca_2, however, repeated doses of the analogue have no effect whatever on the permeability responses to U-V injury (Fig. 10) or control injections of histamine, compound 48/80 and globulin PF, though moderate inhibition of the toxin response occurs, (Inset, Fig.10)

The analogue also has no effect on the U-V permeability response when tested by local, intracutaneous injection $\frac{1}{2}$ - 22$\frac{1}{4}$ hr. before "blueing".

Comparison of EDTA and certain antihistamines

Macfarlane and Knight (1941) have suggested that positively charged metallic ions are essential for the lecithinase activity of Cl. welchii toxin "in vitro". Dawson and Bangham (1961) have also shown that lecithin is hydrolysed by the lecithinase D of Cl. welchii α-toxin only when the substrate possesses a positive electrical charge. Metallic ions such as Ca^{++} or long chain bases, e.g. stearylamine, appeared to be equally effective in achieving the optimal electrokinetic potential of the substrate.

Since the permeability effect of the toxin "in vivo" can be inhibited by a chelating agent which is already fully saturated with Ca^{++}, (see p.39), it is unlikely that inhibition is due to a calcium-binding effect. This suggested that EDTA-Ca_2 might exert its effect by competition with shorter chain endogenous amines like histamine or 5-HT.

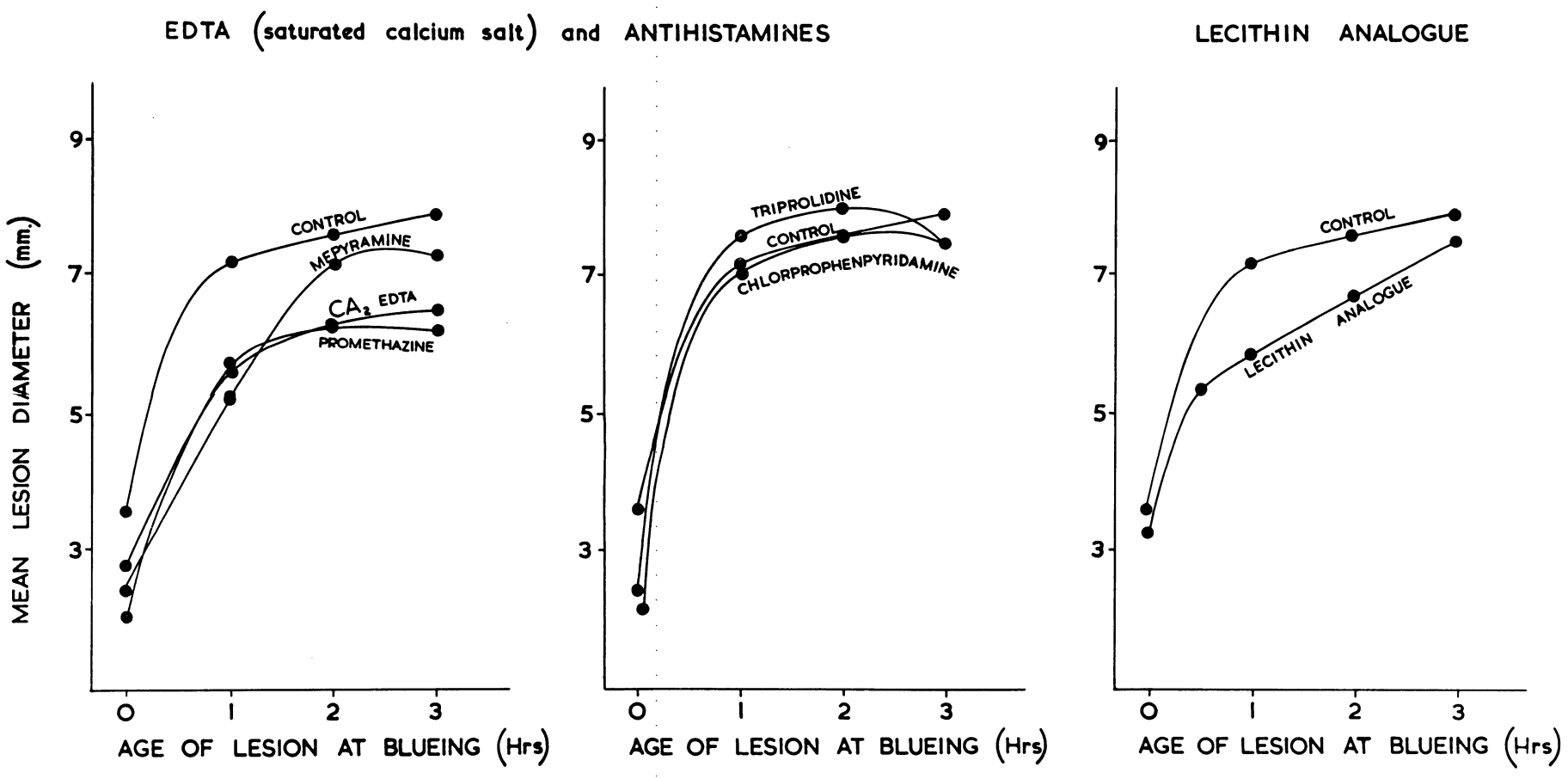


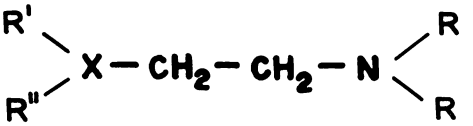
Fig. 11 The effect of antihistamines, EDTA-Ca_2, and a lecithin analogue on the permeability response to injected Cl. welchii α-toxin

Accordingly four antihistamines, Triprolidine, Chlorprophenpyridamine, Mepyramine and Promethazine, were tested against the PF effect of α-toxin in guinea pigs. Each drug was given in a dose of 0.1 mg. per kg. intravenously in different batches of guinea pigs. Time courses of increased permeability induced by toxin were obtained for treated animals by making intracutaneous injections of 18 µg. toxin at hourly intervals over 3 hr. and "blueing" the animals immediately before the last injection.

Triprolidine or chlorprophenpyridamine did not affect the time course of permeability due to Cl. welchii toxin, but mepyramine gave slight inhibition and promethazine moderate inhibition (Fig.11).

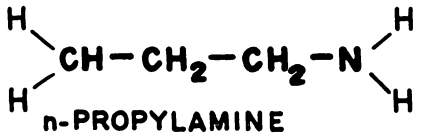
The results indicate an interesting chemical structural specificity. The antihistamines triprolidine and chlorprophenpyridamine which in guinea pigs had the greatest and most specific activity against the PF effect of histamine, can be regarded as substituted aliphatic mono-amines derived from n-propylamine (Fig.12). Mepyramine and promethazine, on the other hand, which were less effective against the PF effect of injected histamine, can be regarded as substituted di-amines derived from ethylenediamine (Fig.12), as can EDTA. On a molar basis, promethazine has 1,500 times more inhibitor potency than EDTA-Ca_2.

The significance of the above observations is at present obscure, but the results suggest that endogenous basic organic substances other than histamine might be considered as electro-

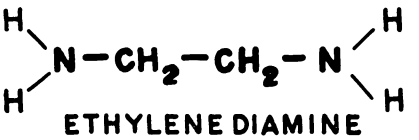


GENERAL FORMULA OF ANTIHISTAMINES

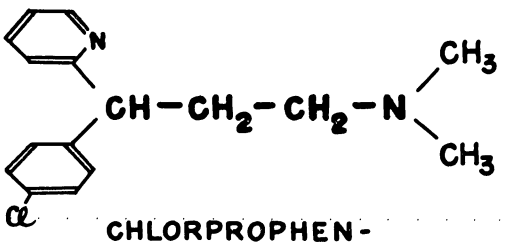
X = C, N or O. (AFTER GOODMAN AND GILMAN, 1958)



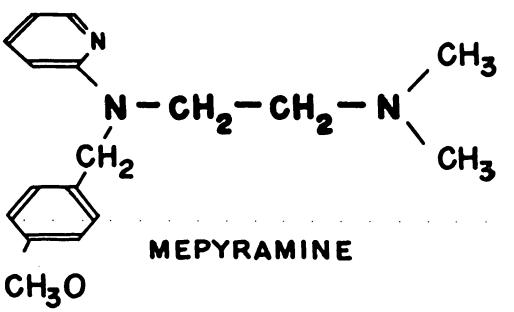
n-PROPYLAMINE



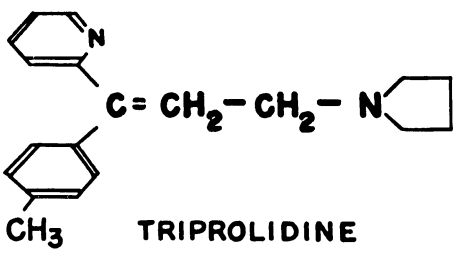
ETHYLENE DIAMINE



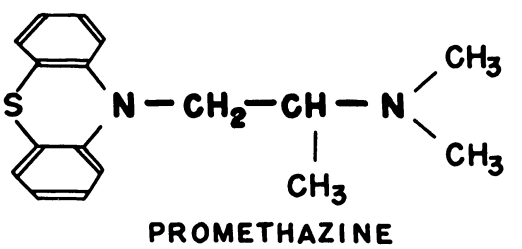
CHLORPROPHEN-PYRIDAMINE



MEPYRAMINE



TRIPROLIDINE



PROMETHAZINE

Fig. 12 The structural formulae of four antihistamines

kinetic activators in biological processes.

Chapter 10

THE EFFECT OF VARIOUS OTHER CHEMICAL PREPARATIONS

Sodium α-naphthylacetate has been reported to suppress oedema induced by the injection of formalin in the rat's paw, i.e. in severe chemical injury (Holler, Lindner and Stoklaska, 1958).

When given to guinea pigs either intravenously (10 mg. per kg. body wt.) or subcutaneously (100 mg. per kg. body wt.), sodium α-naphthylacetate failed to inhibit the permeability effect of histamine, globulin PF and synthetic bradykinin, and slightly and consistently enhanced (up to 3-fold) the PF effect of the histamine liberator, compound 48/80. Sodium α-naphthylacetate also had no effect in guinea pig skin on the permeability response to 18 μg. Cl. welchii α-toxin.

When given by local intracutaneous injection in amounts up to 100 μg., sodium α-naphthylacetate caused no more "blueing" than control injections of saline. However, this dose also failed to inhibit the late U-V permeability response when tested locally by intracutaneous injection according to the schedule illustrated in Fig. 7.

The effect of 2-deoxy-(D)-glucose

An analogue of glucose, 2-deoxy-(D)-glucose was found by Goth (1959) to inhibit the anaphylactoid reaction of rats to intravenous dextran or ovomucoid, but did not affect the reaction to compound 48/80 injected intravenously.

In preliminary experiments in guinea pigs, 200 mg. per

kg. of analogue given intravenously (see Goth, 1959) failed to inhibit increased permeability induced by histamine, globulin PF or compound 48/80 injected intracutaneously ½ to 2 hr. later. The same dose of 2-deoxy-(D)-glucose also failed to inhibit the increased permeability evoked by the injection of 18 µg. Cl. welchii α-toxin.

The effect of the analogue on the permeability response to U-V injury was tested in two ways. The first was that outlined for the testing of systemic antihistamines (see p.55, Fig. 7). Two groups of animals were injected with 2-deoxy-(D)-glucose (200 mg. per kg.), 15 min. prior to irradiation, 3 hr. after irradiation and again 21 hr. after irradiation. In the first group, the first and second injections were given intraperitoneally and the third intravenously. In the second group of animals, the first injection was intravenous and the second and third were intraperitoneal. A further control group of guinea pigs was injected with equivalent volumes of saline by the same routes as the second group above.

On each animal, 10 sites were irradiated 15 min. after the first injection of analogue. All animals were "blued" 22 hr. after irradiation (i.e. 1 hr. after the last injection of glucose analogue) and examined ½ hr. later. In each case, the mean dye equivalent of the lesions in the treated groups (125 and 72 µg. per ml. respectively) exceeded that in the controls (55 µg. per ml.).

The analogue was then assessed against the developing

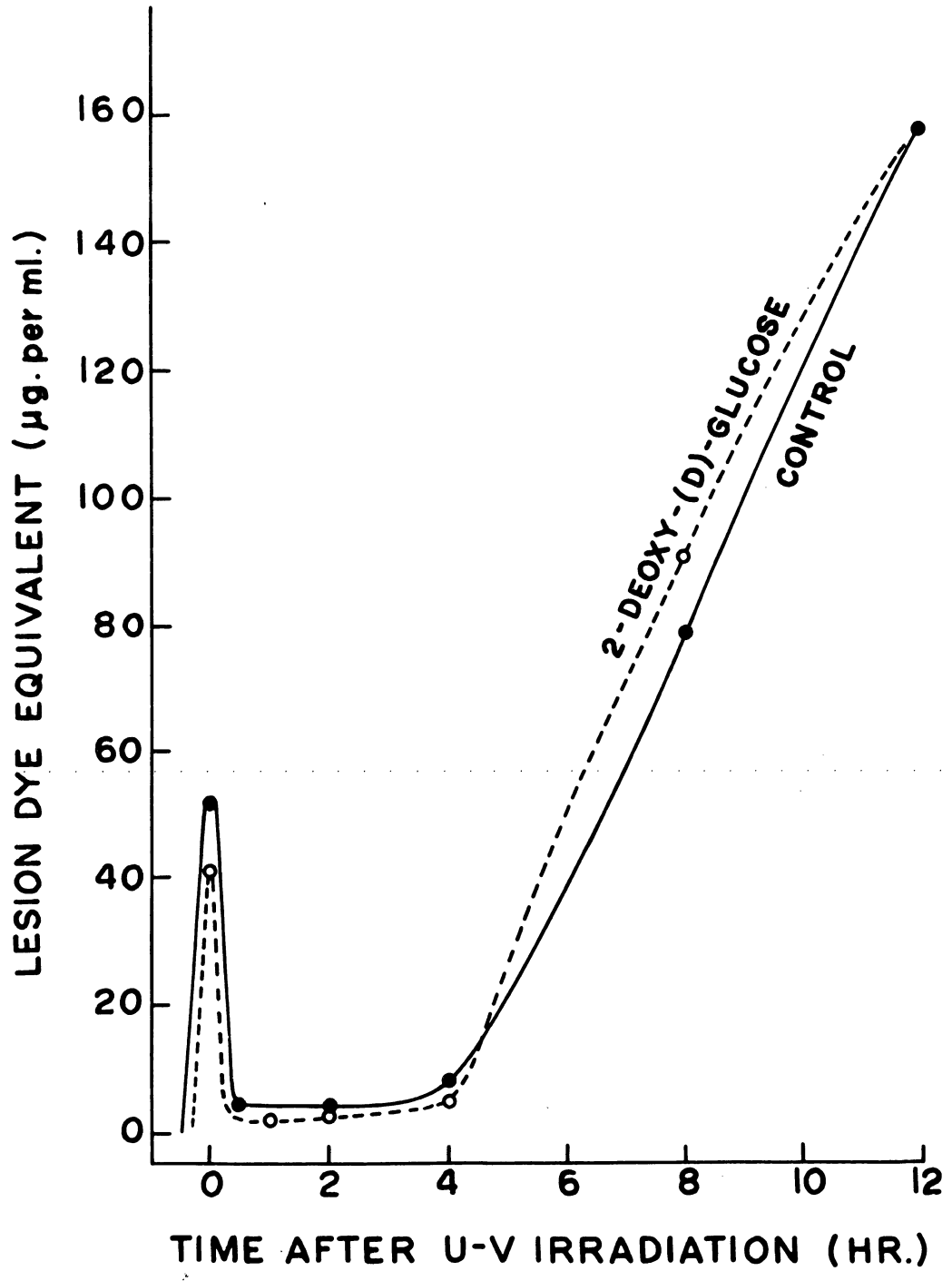


Fig. 13 The effect of a glucose analogue on the late permeability response to U-V injury

stage of the late permeability response in the manner outlined for the testing of Trasylol and the lecithinase antagonists (see p. 65).

Skin sites were irradiated for 20 sec. in two groups of guinea pigs, so that, at "blueing", the animals bore lesions 0, 1, 2, 4, 8 and 12 hr. old respectively. One group of animals was given intravenous 2-deoxy-(D)-glucose (200 mg. per kg.) immediately before the initial irradiation, and further intraperitoneal doses after 3, 7 and 11 hr.

The second group served as controls and was injected with equivalent volumes of saline at the same intervals. All animals were "blued" 12 hr. after irradiation and examined 30 min. later.

Response lines for the animals treated with 2-deoxy-(D)-glucose were almost identical with those for the control animals and indicated no inhibition of the developing permeability response (see Fig. 13).

The effect of α-amylase

The buccal application of α-amylase in man has been claimed to reduce post-traumatic oedema in orthopaedic injuries (Thompson, Glick and Silverstein, 1960).

In doses up to 10 mg. per kg. subcutaneously, α-amylase has no inhibitory effect on the PF effect of histamine, globulin PF, synthetic bradykinin or the histamine liberators, compound 48/80 and polymyxin B sulphate.

The effect of α-amylase on the late U-V response was tested as for systemic antihistamines and 2-deoxy-(D)-glucose

(see above). Doses of 1 mg. per kg. were given intraperitoneally 1 hr. before applying U-V radiation, 2½ hr. after exposure, and intravenously, 21 hr. after exposure. When compared with control animals injected with saline, no inhibition of the permeability response was detected in the treated animals.

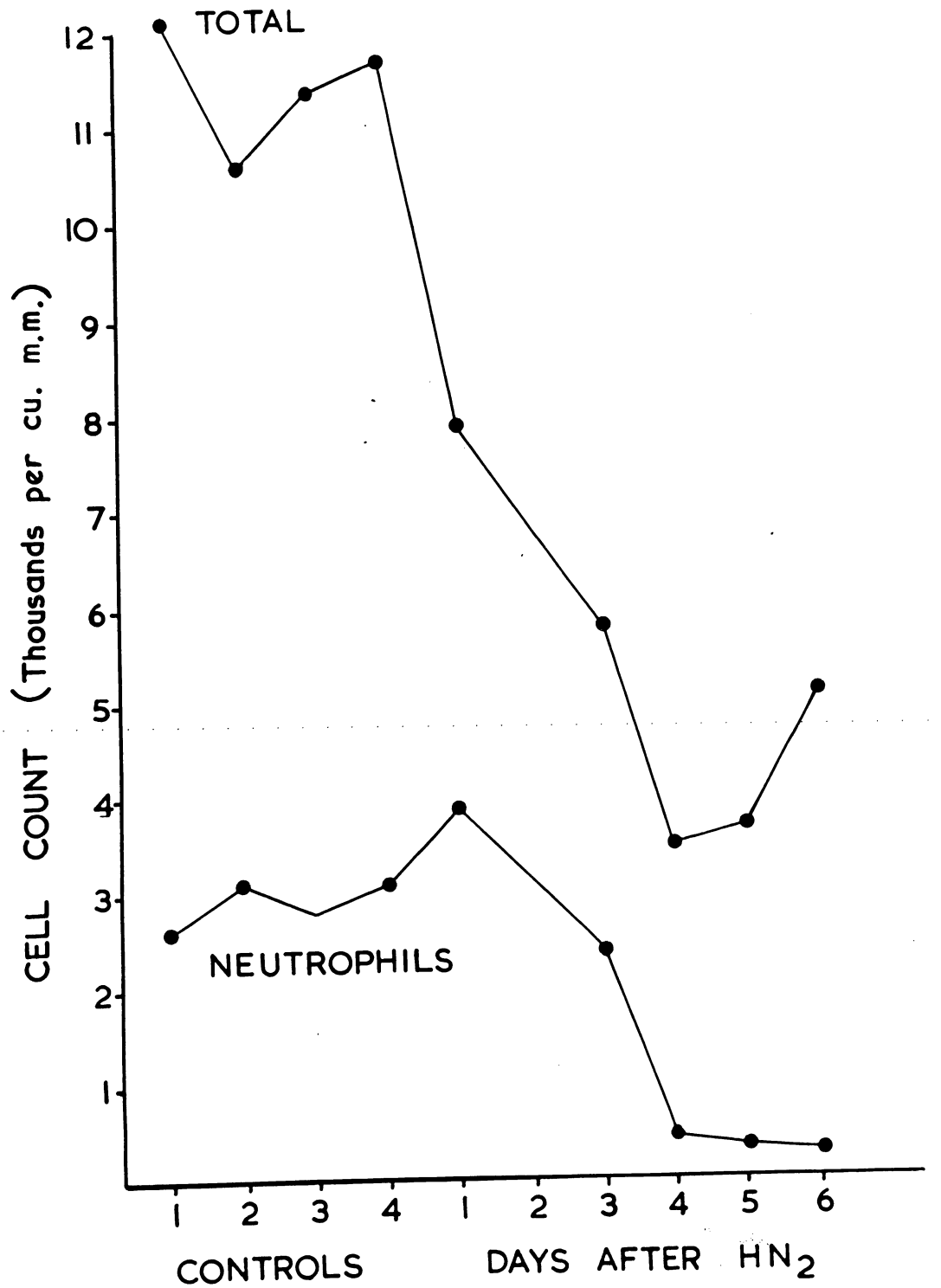


Fig. 14 Neutrophil counts in guinea pigs after the intravenous administration of nitrogen mustard

Chapter 11

THE EFFECT OF EXPERIMENTAL NEUTROPENIA ON THE LATE PERMEABILITY RESPONSE TO ULTRA-VIOLET INJURY IN GUINEA PIGS

The neutrophil response in irradiated skin sites develops and reaches its peak a few hours in advance of the late permeability response, suggesting that tissue leucocytosis might be related to, or even a necessary precursor of the late permeability response.

Male guinea pigs weighing 600 - 810 g. were anaesthetised with intraperitoneal pentobarbitone 40 mg. per kg. and subsequently injected aseptically with 2 mg. per kg. nitrogen mustard intravenously (Humphrey, 1955).

Daily total and differential white cell counts were performed for four days prior to the administration of nitrogen mustard and daily thereafter until the experiment was concluded. The absolute neutrophil counts calculated from the above results are recorded as mean values for all animals in Fig. 14.

For experimental purposes the animals were divided into two groups according to whether their neutrophil counts were lowest (a) 4 - 6 days, or (b) 5 - 7 days, after nitrogen mustard.

All animals appeared well until the fourth day, when their fur became ruffled. On the 5th day, many lay on their sides and appeared ill; one animal died on this day. All lost 20 - 70 g. weight during the 7 days of the test period.

In many animals, the wounds at the site of the intravenous injection of nitrogen mustard failed to heal normally

and exuded sero-purulent fluid by the 6th - 7th day.

Between the 4th and 6th days after the administration of nitrogen mustard, one group of 6 animals was tested by irradiating skin sites so that lesions ranging in age from 0 - 42 hr. were present at "blueing". Immediately after "blueing", the animals were injected intracutaneously with graded doses of histamine, globulin PF and compound 48/80, and the resultant dosage-response curves compared with pooled results of previous untreated controls.

The late permeability response in the treated animals was similar to that in control animals, though the early response was slightly diminished. Erythema also showed little difference in the two groups, though it tended to decrease more rapidly after 30 hr. in treated animals than in controls.

In animals tested between the 5th and 7th days after the administration of nitrogen mustard, both the early and late permeability responses were moderately depressed. Nevertheless, the depression appeared to be non-specific because, in the same animals, the PF responses to histamine, globulin PF and 48/80 were also decreased. In the two experiments, the histamine response was reduced 1- to 3-fold, that of globulin PF 2- to 5-fold, and that of 48/80, 10- to 25-fold.

The suppression of the U-V response must therefore be regarded as non-specific and probably related to the debili-

tated state of the animals.

Finally, although there was considerable reduction in the number of circulating neutrophils, the decrease was insufficient to eliminate tissue leucocytosis completely. Histological sections from lesions 12, 18 and 22 hr. old contained 0 - 5 neutrophils per HPF in the superficial part of the dermis.

Taken at face value, in the absence of <u>absolute</u> tissue neutropenia and in the presence of non-specific inhibition of permeability, the above results do not suggest any causal relation between tissue leucocytosis and increased vascular permeability.

Chapter 12

DISCUSSION

Diverse types of experimental injury to the skin of laboratory animals (see Chapter 3) result in a prolonged increase in vascular permeability lasting from 3 - 4 hr. in the case of mild thermal injury in guinea pigs (Wilhelm and Mason, 1960), to 23 days in the case of roentgen radiation injury in rabbits (Jolles et al., 1961).

In most types of injury the chief permeability response is preceded by an earlier, transient response which, in certain types of injury is mediated by histamine in the guinea pig (Sevitt, 1958; Wilhelm and Mason, 1958, 1960; Logan and Wilhelm, 1963) or 5-HT in the rat (Wilhelm and Mason, 1960; Logan and Wilhelm, 1965b).

The mediation of the principal phase of the permeability response to mild thermal injury in guinea pigs, rats and rabbits was extensively investigated by Wilhelm and Mason (1960). However, the identification of the relevant factors depended on the demonstration that the permeability response was suppressed by appropriate pharmacological antagonists. This requires in turn that the antagonists are present in effective concentrations for periods sufficiently long to influence the permeability response.

Ultra-violet injury has been investigated on similar lines for two main reasons - (1), the slower development and maturation

of the main permeability response may permit the attainment of effective concentrations of antagonists; and (2), the mediators of U-V injury may differ from those in thermal injury.

Nevertheless, the present investigation of the delayed phase of the permeability response in U-V injury in guinea pigs has still revealed no evidence that known permeability factors are the natural mediators of the response.

A significant difference between the early and late response in thermal injury in the rat is the fact that the permeability responses occur in different parts of the peripheral vascular bed (Cotran and Majno, 1964). The early transient response, which, in the rat, may be mediated by both histamine (Spector and Willoughby, 1958b; 1959b) and 5-HT (Wilhelm and Mason, 1960) occurs predominantly as the result of venular leakage. In this regard, it seems noteworthy that Majno and Palade (1961) reported that the injection of histamine (50 µg.) or 5-HT (5 µg.) in the cremaster muscle of rats induces the leakage of plasma between endothelial cells lining small venules 10 - 12 µ in diameter.

Rowley (1964) has demonstrated a similar leakage of carbon particles between the endothelial cells of venules in exposed sub-cutaneous tissue of the abdomen and also in the skin of the hind paw of the rat in response to injected histamine or 5-HT; and suggests that increased vascular permeability is simply due to increased venular hydrostatic pressure resulting from venous constriction induced by the injected agents.

Alksne (1959), on the other hand, using histamine applied topically to the skin of the mouse, reported trans-cellular, intravesicular transfer of colloid markers across the endothelial cytoplasm of capillaries rather than through intercellular spaces. The apparent discrepancy may be partly explained by the high concentration of histamine (4 per cent in distilled water) used by Alksne; and the results of Majno and Palade (1961) appear more relevant to this discussion.

The delayed permeability response, on the other hand, occurs predominantly in true capillaries (cf. Voisin and Toullet, 1959; Wells and Miles, 1963). Increased vascular permeability in these vessels develops after a latent interval and usually does not proceed to a stage of complete stasis and obstruction to blood flow (Cotran and Majno, 1964). In the present work, a delayed response, comparable to the above, occurred in superficial capillaries immediately subjacent to the epidermis as well as in the superficial dermis, after U-V irradiation of the skin of both guinea pigs and rats; and no stasis or crowding of vessels with erythrocytes was demonstrable in superficial vessels in histological sections except where frank necrosis occurred. Another point against stasis and in favour of increased blood flow in irradiated sites is the transient blue "flash" of colour that such lesions demonstrate within 5 sec. of the injection of Evans blue.

Biochemical mediation of permeability responses in tissue injury

Evidence has been presented in earlier chapters that strongly suggests that the early permeability response in tissue

injury is mediated by histamine in the guinea pig, and 5-HT in the rat. No evidence has been obtained, however, to implicate any of the known chemical factors as mediators of the late permeability response.

Interest in histamine as a mediator of the late response was re-awakened by Schayer (1960) who demonstrated increased formation of histamine from C^{14} labelled histidine in the skin of the mouse after various stimuli such as exposure to cold, the injection of histamine, serotonin or bacterial endotoxin. Maximal increase in histidine decarboxylase activity occurred in about 6 hr. and had returned to normal after 24 hr. Despite the above observations, no evidence was found in the guinea pig that histamine participates in the delayed permeability response to either mild thermal injury (Spector and Willoughby, 1958b, 1959b; Wilhelm and Mason, 1960) or ultra violet injury (see Chapter 7). 5-HT activity is also apparently absent in the late permeability response to U-V injury in the rat (see Chapter 7).

The relation of the histamine- or 5-HT-mediated early response to the late permeability response in both the above species also remains uncertain. Edery and Lewis (1962) observed that scalding the hind limb of dogs at 80°C for 15 sec., or the intra-arterial injection of histamine resulted in an increased flow of lymph containing an elevated level of kinin-forming enzyme. The increased concentration of enzyme, but not the increased flow of lymph, was suppressed by the prior administration of the anti-histamine, mepyramine.

Partial inhibition of the increased permeability induced by injected histamine after the administration of ε-ACA, an inhibitor of the fibrinolytic system, has been noted in the present work (see Chapter 8), and suggests a possible interrelation between the two systems. Any such relation, however, must have little importance because the early permeability response to U-V injury can be suppressed in both guinea pigs and rats without apparent effect on the maturation of the late response.

The activation of proteolytic enzymes in injury has been noted by various workers including Beloff and Peters (1944-45) and Ungar (1947). Hilton and Lewis (1957) subsequently suggested that bradykinin, a nonapeptide resulting from the action of kinin-forming enzymes on plasma proteins, might fill the role postulated for H-substance by the late Sir Thomas Lewis (1927) and Krogh (1929). G.P. Lewis (1963) further urged the claims of bradykinin and a related decapeptide, kallidin, and indicated the conditions in inflammation which favour their optimal activity. Miles and Wilhelm (1960) had previously pointed out that criticism of kinins on the score of short duration of permeability effects might be compensated by the large reservoir of kinin precursors in the plasma proteins.

Further evidence for the participation of proteases obtained by Hladovec, Mansfeld and Horakova (1958) who observed that oedema and exudation induced in the hind limb of the rat by the subaponeural injection of 10 per cent sterile kaolin suspension, were suppressed by the systemic administration of

trypsin inhibitors from soy bean, lima bean or potato, in doses of 50 mg. per kg. Ovomucoid trypsin inhibitor was ineffective. The vegetable inhibitors also suppress the permeability effects of guinea pig globulin PF activated by dilution in glass vessels (Miles and Wilhelm, 1955; Margolis, 1959).

Wilhelm and Mason (1960) claim that the suppression of oedema after kaolin injury noted by Hladovec, Mansfeld and Horakova was probably non-specific because they observed suppression of the permeability increasing effect of histamine by soya inhibitor in doses of 3.3 mg. per kg. or greater. In view of the observations of Edery and Lewis (1962) on the effect of mepyramine on the level of kinin-forming enzyme in lymph after thermal injury, as well as the present observations on the effect of ε-aminocaproic acid on the PF response to histamine, the conclusion of Wilhelm and Mason seems to need further investigation.

A further aspect of the use of antagonists can be illustrated by reference to protease inhibitors. These were used in minimally effective concentrations in an attempt to minimise non-specific effects. Though laudable in purpose, this approach has certain disadvantages. The maximal effect of trypsin inhibitors is obtained only when the PF and its inhibitor are mixed before injection. In skin sites injected with 10 µg. of trypsin inhibitors from soya or potato and immediately superinjected with globulin PF, considerably less inhibition occurs than when the PF and antagonist are mixed before injection. If the superinjection of globulin PF is delayed for 20 min. after the

injection of inhibitor, virtually no inhibition occurs.

Furthermore, doses of inhibitor at the lower limit of effectiveness and injected up to 15 min. before irradiation, are likely to be markedly diluted, especially during the early phase of increased permeability, thus further reducing their already limited effectiveness. In any case, it is hardly to be expected that such small doses of inhibitor given at infrequent intervals and with such a limited duration of activity, would exert a substantial effect on a time course of increased vascular permeability extending over 24 - 72 hr.

ε-ACA is an extremely soluble compound and is rapidly excreted both in the rabbit and man. The doses of ε-ACA used in this study theoretically were sufficient to secure optimally effective blood levels of the preparation, at least for short periods. However, without precise information on the actual blood levels of the inhibitor at varying intervals after administration, it is difficult to draw firm conclusions concerning the activity of plasmin during the principal phase of permeability.

The results as they stand, though necessarily qualified, suggest no participation in the delayed permeability response to U-V injury by short-acting PF's such as histamine, serotonin, bradykinin, or other proteolytic enzymes such as globulin PF, kallikrein or plasmin. Indeed, since such PF's induce only <u>venular</u> changes of short duration and never the <u>capillary</u> changes characteristic of the delayed permeability response, Wells and Miles (1963) suggest that a <u>prima facie</u> case exists against their

participation in the delayed permeability response to tissue injury.

The apparent similarity between the permeability responses in guinea pigs to injected Cl. welchii α-toxin (Elder and Miles, 1957) and mild thermal injury (Wilhelm and Mason, 1960) in the first place, and between the delayed permeability responses to injected Cl. oedematiens α-toxin (Elder and Miles, 1957) and U-V injury (Logan and Wilhelm, 1963) in the second place, suggested that lecithinases might mediate the late response. However, the results with lecithinase inhibitors appear clear cut. While permeability induced by Cl. welchii α-toxin is markedly inhibited by EDTA-Ca_2, the developing, delayed response to U-V injury is unaffected (see Fig. 10).

The experiments with the lecithin analogue yielded similar, though less convincing results. However, it is noteworthy that Rosenthal and Geyer (1962) found it necessary to mix the analogue and enzyme before the addition of substrate to achieve maximal inhibitory effect. The addition of analogue after the enzyme had reacted for a short time with substrate caused comparatively poor inhibition. In the same way it is possible that a lecithinase activated by U-V injury might unite with its endogeneous substrate before the poorly diffusible inhibitor, injected in suspension, had an opportunity to exert its effects. However, since repeated systemic doses of EDTA-Ca_2 had no effect on the U-V permeability response while having a substantial effect on injected Cl. welchii α-toxin (Fig. 10), lecithinase activity in the principal phase

of the U-V permeability response would appear to be unlikely.

Metabolic inhibitors such as sodium α-naphthylacetate (the sodium salt of an analogue of acetic acid), 2-Deoxy-(D)-glucose (a glucose analogue) and α-amylase were also ineffective.

Tissue leucocytosis and the delayed permeability response

Moses, Ebert, Graham and Brine (1964) have recently described the elaboration of a permeability-increasing and fever-inducing substance by granulocytes obtained from peritoneal exudates in rabbits. This substance was heat stable and, in the skin of "blued" rabbits, induced a delayed permeability response which became maximal in 1½ - 2 hr.

In the guinea pig experiments reported in Chapter 6, it was noted that a wave of tissue leucocytosis (the cells being predominantly neutrophils) preceded the delayed permeability response by a few hours (Fig. 4). On the other hand, no similar and consistent relationship between leucocytosis and increased permeability was demonstrated in either the rat or rabbit. Indeed, in the rabbit, the neutrophil response reached a peak after the maximal permeability effects were observed, and appeared to be related to the formation of slough rather than to the development and subsidence of the permeability response.

It might be argued that the occurrence of tissue necrosis and slough formation in the rat and the rabbit masked a response similar in these two species to that occurring in the guinea pig. However, the results of experimental neutropenia in the guinea pig did not support the concept of the delayed permeability

response being mediated by a PF elaborated by neutrophil leucocytes.

Conclusion

The results of the present work on vascular permeability induced by ultra-violet irradiation extend and support the results of Wilhelm and Mason (1960) on mild thermal injury. The earlier proposals concerning the biochemical substances that mediate the principal phase of the permeability response in various types of injury are clearly becoming increasingly discredited, and require re-evaluation and considerably more investigation.

A logical forward step would seem to be the elucidation of the events occurring at an ultrastructural level during the delayed permeability response in different types of injury; and a comparison of ultrastructural changes induced by various agents might well provide a clue to an as yet unidentified PF, or alternatively disclose a hitherto unsuspected mode of action of currently favoured PF's.

In the meantime, it is sobering to reflect that despite prodigious effort, in terms of factual information concerning the mechanism of increased vascular permeability in inflammation, we have advanced little since Cohnheim (1889) first postulated that the vascular events in the inflammatory reaction result from a "molecular change" that occurs in the walls of blood vessels, and further that "it is only and solely the vessel wall which is responsible for the entire series of events".

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