THE MEDIATION OF INCREASED VASCULAR PERMEABILITY IN INFLAMMATION

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I. INTRODUCTION

The vascular and tissue reactions that characterize the early stages of inflammation are remarkably similar in different kinds of injury. Among these reactions, those of the blood vessels are outstanding; they include vasodilatation, increased capillary permeability, and the migration of leucocytes into the injured tissues.

The factors controlling the permeability changes in injury have not been fully established. Under physiological conditions, there is free movement of fluids and electrolytes across the wall of the terminal vascular bed, but that of plasma proteins is restricted, with only 5 to 10% escaping into the extravascular tissues, —*i.e.*, the capillary wall is freely permeable to water and solutions of most salts, but not to proteins. Fluid equilibrium is maintained by a balance between effective hydrostatic pressure in the capillary bed (84), which tends to drive fluid out of the vascular system, and the restraining effect of the equal and opposite osmotic pressure of the plasma proteins (160). However, it is noteworthy that Starling (160) considered the capillary endothelium itself to act merely as an inert filtering membrane.

In injury, the considerable increase of vascular permeability to fluid, electrolyte, and protein is not accompanied by an appropriate rise in hydrostatic pressure in the blood vessels (83). Accordingly, it seems probable that the endothelial barrier itself is affected.

The exact nature of the change is not understood. It does not appear to be due to physical effects like stretching, that might occur in vasodilatation (81), because increased permeability can be induced in constricted vessels (83), or to the direct effects of injury. Some form of biochemical mediation seems indicated,

and since various endogenous substances that increase vascular permeability are recoverable from both normal and inflamed tissues, there is good reason to assume that the permeability changes and other vascular phenomena of inflammation are mediated by endogenous pharmacological substances, released or activated by injury.

Since the ability to induce an increase in vascular permeability is exhibited by numerous endogenous preparations, those with high permeability-increasing potency are more likely to be the natural mediators, particularly if they are common to various mammalian species. This notion is attractive, on the grounds both of biological economy and of universality of mediation. However, proof that a substance is a natural permeability factor clearly requires considerably more evidence. This paper deals with substances that have been put forward as possible mediators of permeability responses in inflammation, and their shortcomings in this respect when put to the test in experimental injury.

Throughout the remainder of this paper, a substance considered to be a permeability factor is generally symbolized by the designation *PF*, and the permeability-increasing activity of such a substance is described as *PF activity.*

II. DEMONSTRATION OF INCREASED VASCULAR PERMEABILITY

At sites of increased vascular permeability, fluid, electrolytes, and proteins all escape in greatly increased amounts through the vascular endothelium. The exudation of any of the three groups of substances could justifiably be used as an index of the increased permeability. However, most investigations have been done in the depilated skin of laboratory animals, in which the exudation of a vital dye circulating in the blood is used as an index of increased permeability (see review by Spector, 154). Vital dyes like pontamine sky blue 6BX, Evans blue, or trypan blue, injected intravenously, become bound to plasma proteins (135) and in particular to albumins, and hence their accumulation in treated skin sites indicates exudation of plasma proteins.

Unfortunately, the evaluation of the results in such tests often lacks precision. For example, both the diameter and the colour-intensity of the blue lesions may be taken into account in recording the results on an arbitrary scale: ±, +, +±, ++, up to ++++ (126, 151, 177). Not only is this a crude assessment, particularly if the scale is not related to established concentrations of exuded dye, but it also limits effective comparison of results from different laboratories.

1. Estimation of PF potency of preparations injected intracutaneously. The investigation of test preparations by intracutaneous injection was put on a much more precise basis by Miles and Miles (114). They investigated the PF effects of histamine, of the histamine liberator, compound 48/80, and of leukotaxine in the depilated skin of the dorsal trunk of guinea pigs with circulating pontamine blue. In batches of 3 or 4 animals, the mean lesion-diameters induced by graded doses of these drugs in a constant injection-volume are proportional to the log doses injected, for lesion-diameters of 3.5 to 9.0 mm. The establishment of such dosage-response lines permits a fairly precise comparison of the potency of various test preparations, or the evaluation of the effects of antagonists on a

particular PF. Furthermore, the results are closely reproducible in different batches of animals.

Wilhelm *et al.* (181) used the same technique in rats and rabbits, and, as in guinea pigs, obtained consistent results provided the animals were not anaesthetized. Since the slope of the dosage-response lines for most PF's is similar for all three species, valid comparisons of potency can be made between one or more species (150). For this purpose, an effective blueing dose (EBD) of a PF has been defined as the amount of PF in a standard injection volume of 0.1 ml that in a particular species induces a mature skin lesion 6 mm in diameter (181) (see Table 1).

The shortcoming of assessing the PF potency of a preparation by measuring the diameter of the lesion induced is the lack of reference to concentration of dye in the lesion. For example, the lesions induced in rats by 5-hydroxytryptamine (5-HT, serotonin) are considerably darker in colour than lesions of similar size elicited by histamine. This deficiency in evaluating PF potency is not easily overcome because of the difficulty in extracting exuded dye from skin. Extraction is comparatively simple to perform with muscle (29, 140), which is readily macerated and has less avidity than skin for the dye. However, the development of this technique for skin work would permit much more precise evaluation of the amount of dye exuding at test sites.

TABLE 1

PF potency (EBD/mg) of various permeability factors: amines and their liberators (Group I), polypeptides (Group II), and proteases (Group III) in the guinea pig, rat, and rabbit*

Permeability Factor		EBD*/mg when Tested in		
Group	Substance	Guinea pig	Rat	Rabbit
I	Histamine†	32,200	1,400	37,000
	5-Hydroxytryptamine†	60	16,200	<20
	Compound 48/80†	3,500	6,800	30
	Polymyxin B‡	1,120	14,900	<20
II	Bradykinin (pure)§	2,700,000	8,100	(not tested)
	Bradykinin (crude)‡	170	50	300
	Leukotaxine‡	65	(not tested)	(not tested)
	"Leucotaxine"‡	320	120	80
III	Guinea pig globulin PF‡	38,700	1,100	130
	Rat globulin PF‡	1,400	620	400
	Rabbit globulin PF‡	930	120	22,000
	Kallikrein‡	240	70	1,130
	Trypsin¶	2,560	900	600

* EBD = Effective Blueing Dose (see p. 253 for definition).

† Data from Sparrow and Wilhelm (150).

‡ Data from Miles and Wilhelm (117).

§ Data from Logan and Wilhelm (91) with a preparation supplied by Sandoz Ltd.

¶ Data from Wilhelm (176).

2. Estimation of PF changes at sites of injury. Since experimental injury is generally induced by a standard stimulus, the lesions elicited should have a constant diameter; hence, of necessity, the estimation of PF changes at sites of injury is based on differences in colour-intensity (152, 178). Visual estimations are subject to daily variation in accuracy, and hence the development of a satisfactory technique of dye-extraction from skin is even more urgently required. Until this is available, even a rough assessment of the amount of local dye by comparison with standard lesions (178) seems preferable to the usual evaluation in terms of an arbitrary scale.

3. Other methods of estimating permeability changes. Another technique commonly used is the injection of test preparations into the paws of anaesthetized rats, or injury of the paws with appropriate stimuli. The resulting oedema is assessed by measurement of increased paw size or water content, by weight or by eye (154). If dye is injected intravenously before testing, the oedematous area becomes dye-stained. Despite the relative popularity of the rat's paw as a test preparation, the author agrees with Spector (154) that the technique gives less precise results than tests in the skin of the trunk. Furthermore, in such tests, the rats are usually anaesthetized, and anaesthesia depresses the permeability response of peripheral blood vessels (114, 176).

Spector (154) has recently reviewed the various methods of assessing permeability responses, and further information is available in his paper. His discussion of the relative merits of oedema and exudation of dye as indices of increased permeability is particularly noteworthy, because the use of different indices may well explain the varying reports concerning similar preparations tested in the same species. There is urgent need in this field for studies of the simultaneous exudation of water, electrolyte, and protein (preferably using preparations labelled with radioactive isotopes).

III. ENDOGENOUS PERMEABILITY FACTORS PROPOSED AS NATURAL MEDIATORS IN INJURY

Preparations with PF activity have been obtained from both normal and inflamed tissues by a variety of methods, including the elution of macerated tissues, perfusion of injured tissues, and disruption of cells.

For our purposes, only preparations that have been reasonably characterized need be considered; they fall into the following categories: 1) pharmacologically active amines—*e.g.*, histamine and 5-hydroxytryptamine (5-HT), and their liberators; 2) polypeptides—*e.g.*, leukotaxine, bradykinin, and kallidin; 3) proteases—*e.g.*, plasmin, kallikrein, and globulin permeability factor. Categories 2) and 3) overlap in that, even if proteases are subsequently identified as prime mediators, polypeptides as products of proteolysis may well be the final active substances. This is illustrated in the above list by the protease, kallikrein, and the corresponding polypeptide, kallidin. However, it is convenient to discuss them in the above descending order, which also coincides roughly with their chronological order of identification.

A. Histamine and 5-hydroxytryptamine

1. Histamine and its liberators. Eppinger (43) appears to have first described the PF activity of histamine; subsequently, Sollmann and Pilcher (149) and Dale and Richards (33) drew attention to its high PF potency. However, interest in its possible mediation of permeability changes in injury was stimulated largely by Sir Thomas Lewis's (89) observation that the triple response, induced by pricking histamine into human skin, was also evoked by thermal, chemical, mechanical, and electrical injury. Antihistamines were not available to Lewis; but despite their failure, when discovered, to suppress the permeability responses in injury, the histamine theory has not substantially decreased in popularity. When leukotaxine (108) appeared as a claimant to this role, histamine maintained its popularity because leukotaxine owed at least part of its PF effects to its being a histamine-liberator (38).

The histamine theory received fresh impetus when Feldberg and his colleagues (48, 49, 130) described the release of histamine from perfused skin by a variety of organic compounds, in particular, compound 48/80 (13, 130); and when Riley and West (137) reported the occurrence of histamine in tissue mast cells.

Whether or not histamine is a prime mediator of the permeability responses in injury (111), the normal tissue content of histamine appears inadequate *per se* to initiate and maintain the corresponding relatively prolonged events (178). This shortcoming, however, may be more apparent than real, in view of Schayer's (142, 143, 144, 145) observations that histidine decarboxylase is an adaptive enzyme, of which the activity in animal tissues increases in response to various stimuli such as heat, cold, the injection of *E. coli* endotoxin, delayed allergy, and treatment with histamine liberators or pertussis vaccine. Not only is the enzyme activated by stimuli that induce permeability changes, but its concentration at sites of local injury rises and falls in parallel with the time-course of the permeability events in, *e.g.*, bacterial infection of skin (24). Since histamine can induce many of the microcirculatory events elicited in injury, Schayer (143) proposed that the coincident fluctuation of decarboxylase activity with that of permeability response is too strong to be meaningless.

This general argument, however, is precisely that which has so long bedevilled this whole field of work. Proof of mediation is required, rather than circumstantial evidence supporting a possible role as mediator. But taken at face value, Schayer's observations (142, 143, 144) at least indicate how sustained local supplies of histamine might be brought about. Whether its function is that of a PF, or to partake in the metabolism of growth and repair (62, 75), or of some other type (98), remains to be determined.

Despite all the controversy provoked by the histamine theory, the drug remains one of the most potent PF's in man (117), the guinea pig, and the rabbit (Table 1). Its lesser activity in the rat, in turn, has aroused interest in another amine, 5-HT, which has high PF potency in this species (139, 150). The histamine-liberators, compound 48/80 and polymyxin B (25), probably owe their high activity in the rat to an associated release of 5-HT (17, 127).

The scope and activity of *histamine liberators*, particularly those which are simple chemical compounds, have been reviewed by Paton (131, 132). Various simple bases can form complexes with tissue acids; with nucleic acid, they do so proportionately to their ability to liberate histamine. Accordingly, liberators may mobilize histamine from a bound to diffusible form by acting as competitive bases.

Högberg and Uvnäs (68) presented evidence that compound 48/80 activates a lytic enzyme attached to the membrane of the mast cell. The enzyme, like lecithinase A, appears to depend on sulphydryl groups for its activity (69).

Although the naturally occurring and synthetic liberators serve as models for endogenous liberators in inflammation, their use as such is restricted in value since they exhibit wide species differences in PF potency (Table 1), marked variation in liberation of histamine from various tissues (47, 123), and considerable insusceptibility of their PF effects to antihistamine drugs (105). Accordingly, negative results must be interpreted with caution (117).

Permeability responses induced by injury have been investigated in rats depleted of histamine (157, 158) by intraperitoneal injection of compound 48/80 (50) or of the antibiotic, polymyxin B (25, 127). Both drugs fail to deplete the entire histamine content of tissues (50, 127), release 5-HT as well as histamine (17, 18), and induce non-specific suppression of the effects of both histamine-liberators and non-liberators (181).

2. Antagonists of histamine and its release. Of the three main types of antagonism to the effects of histamine release—physiological antagonism to histamine, competitive antagonism to histamine, and suppression of its release—only the first two are substantially effective; but, in general, all available preparations have restricted efficacy (131).

a. Antihistamines strongly suppress the PF effects of histamine injected in "blued" animals, but have low or negligible activity against those of histamine liberators (105, 117). Furthermore, their use in studying permeability changes is complicated by their non-specific side-effects (*e.g.*, anticholinergic and local anaesthetic).

Only two antihistamines, mepyramine maleate (22) and triprolidine hydrochloride (61), have effects sufficiently specific to justify their use in investigating permeability responses in injury. Both drugs have consistently high potency in guinea pigs and rats (105, 117). Although they have less potency in rabbits, they also appear to be the best compounds available for work in this species (105).

b. Endogenous histamine antagonists. Although heparin suppresses only moderately the PF effects of histamine or its liberators, its occurrence (73) with histamine (137) in mast cells means that it may be a natural histamine antagonist (99).

Preparations of leucocytes and, in particular, *eosinophils*, induce potent and prolonged suppression of histamine effects *in vivo* (4, 5, 46, 79, 167, 168).

The *serum proteins* of various species antagonize the effects of histamine *in vitro*. Two independent reports in 1953 described suppression of the contraction

of guinea pig ileum stimulated by histamine *in vitro*. In the first report (82) a histamine antagonist was noted in human serum, and in the second report (97) in guinea pig serum. Inhibitory activity of human serum (76) is associated with preparations containing γ-globulins, and of guinea pig serum with α_2-globulins. Parrot and Laborde (128) subsequently reported that sera from the guinea pig, rat, mouse, dog, and cat are all effective, but not those from the rabbit, sheep, cattle, horse, pig, or eel. The suppressive effects of serum proteins, and their possible relation to the antagonist in leucocytes seem to merit further investigation.

3. 5-Hydroxytryptamine (5-HT) and its liberators. The high PF potency of 5-HT in the rat (139) is particularly noteworthy because of the relatively low activity of histamine in this species. However, as Table 1 records, 5-HT is relatively ineffective in guinea pigs, and has no true PF effects in rabbits (150).

Since 5-HT, unlike histamine, does not occur mainly in mast cells (127), at least in skin, it is not surprising that substances like compound 48/80 and polymyxin B, which release both amines, differ quantitatively in their effects. Compound 48/80 more effectively releases histamine than 5-HT, whereas the reverse is true for polymyxin B. Reserpine, in large repeated doses, will deplete skin and other tissues of 80 to 95% of its 5-HT content, without substantially affecting that of histamine (127). However, depletion experiments with liberators of 5-HT probably are restricted in value for reasons similar to those given above for histamine-liberators.

4. Antagonists of 5-HT. A number of substances, including lysergic acid derivatives, antagonize the effects of 5-HT on preparations of smooth muscle *in vitro* (56). Lysergic acid diethylamide (LSD) has high suppressive activity *in vitro*, but its use *in vivo* is restricted by the drug's high toxicity. However, a bromine derivative (2-bromo-LSD, BOL 148) is relatively non-toxic, substantially specific in action, and strongly suppresses the PF effects of 5-HT in rats (178).

B. Polypeptides

Since the end of the last century (106, 107), many investigators have proposed that tissue breakdown products, particularly polypeptides, are the prime mediators of permeability responses in injury. However, the actual demonstration that endogenous peptides increase permeability had to await Menkin's (108) studies in 1938.

1. Leukotaxine. Because inflammatory exudates induce increased vascular permeability in the skin of "blued" rabbits, Menkin (108, 109) fractionated exudates induced by turpentine in the pleural cavity of dogs. Fractionation by a pyridine-acetone technique yielded a preparation of polypeptide, which Menkin named "leukotaxine." He proposed that in inflammation, leukotaxine is liberated from injured cells, is the primary mediator of increased vascular permeability, and induces diapedesis of neutrophil leucocytes.

As a PF, however, leukotaxine appears representative of various polypeptides. Thus, Duthie and Chain (35) obtained PF preparations by digesting albumin, globulin, and fibrin with pepsin, trypsin or papain; the resultant preparations had higher potency when digestion was partial rather than prolonged. Cullumbine

and Rydon (32) fractionated leukotaxine-like substances from suppurative exudates, blister fluid, pulmonary oedema fluid, peptic digests of fibrin, and succus entericus.

The growing misgivings concerning leukotaxine reached a climax when Spector (151) reported that tryptic and peptic digests of human fibrin mimic the permeability and leucocytic effects of leukotaxine, and also evoke swelling of capillary endothelial cells. Polypeptides with 8- to 14-amino-acid chains exhibited all three effects; those with 5-amino-acid chains showed only the first two. Spector's work further supported the growing feeling that leukotaxine was not a unique substance. In fact, even a crystalline peptide like pancreatic trypsin inhibitor could induce the effects of peptides with 8- to 14-amino-acid chains (151).

Menkin's studies have also been criticized because he failed to remove turpentine by-products from his preparations of leukotaxine (63), and because his extraction technique was essentially a removal of protein with acetone, the pyridine acting as a buffer to prevent adsorption of peptides on the precipitated protein (154). Nevertheless, Menkin's investigations started a new era in this field by demonstrating that various break-down products may mediate permeability responses in injury.

2. Bradykinin and kallidin. Of the various polypeptides isolated from tissues or prepared by proteolysis, the best characterized is bradykinin. This was first described by Rocha e Silva, Beraldo and Rosenfeld (138), who digested dog plasma with trypsin or certain snake venoms to prepare a polypeptide that stimulated smooth muscle and induced vasodilatation. The preparation, however, had only low PF activity (117).

Even before Menkin's work on leukotaxine, Frey and Kraut (54) observed a vasodepressor substance in urine. Since pancreatic juice and extracts of pancreas induced hypotension in dogs, they proposed that a substance "kallikrein" was released by the pancreas into the blood stream, where it formed an inactive complex. The complex could be reactivated under particular conditions, such as change in pH.

In 1937, Werle, Götze and Keppler (172) demonstrated that in blood treated with kallikrein, there developed a factor stimulating smooth muscle. However, the polypeptide nature of the active substance was not established until Rocha e Silva's (138) work on bradykinin. Then it became apparent that kallikrein had features common with those of proteolytic enzymes producing bradykinin from plasma proteins. Finally, in 1950, Werle and his colleagues (171, 173) established that treatment of plasma proteins with kallikrein yields a polypeptide, "kallidin," identical with, or closely related to bradykinin. Subsequent work has established that similar and pharmacologically active polypeptides (133) are produced under various circumstances *in vivo*, but until their identity as bradykinin or kallidin is established, the whole group is usually referred to as "plasma kinins." Polypeptides with similar activity not derived from plasma proteins are simply designated "kinins" (70) with a prefix denoting their origin, *e.g.*, wasp kinin (88).

a. Isolation and synthesis of bradykinin. Recent outstanding developments in the study of polypeptides have been the isolation of natural bradykinin by Elliott,

Lewis and Horton (41), and the synthesis of a nonapeptide (21, 125) identified as bradykinin (77, 78, 87), the structure of which agrees with that Elliott, Lewis and Horton (42) proposed for the natural product. Its structure is: Arg·Pro·Pro·Gly·Phe·Ser·Pro·Phe·Arg (87).

Bradykinin stimulates contraction of smooth muscle, induces vasodilatation and hypotension, increases capillary permeability (see Table 1), induces leucocytic diapedesis, and stimulates sensory pain fibres. Furthermore, its activity is of such high order that it ranks among the most active of biological substances (39, 40).

b. Bradykinin as a PF. Until bradykinin was isolated and structurally defined, the crude preparations available had comparatively low PF potency (Table 1). Nevertheless, their potential availability from the large protein reservoir in the body and their rapid release *in vivo*—*e.g.*, in a few seconds after stimulation of the chorda tympani (65, 66)—ensured continued interest in polypeptides as natural PF's. Any reservations based on the low PF potency of the former crude preparations available have now been swept aside by the demonstration that pure, synthetic bradykinin has outstanding PF activity (see Table 1). The recorded results confirm the earlier observations of Elliott, Horton and Lewis (39) and of Konzett and Boissonnas (77) concerning the high PF potency of bradykinin. Furthermore, since kallidin is pharmacologically similar to bradykinin, it also may exhibit high PF potency when highly refined preparations become available.

C. Proteases

Proteases, particularly plasma proteases, were considered possible mediators of permeability responses during the early investigations of the anaphylatoxin theory (52, 72; see also 14). Nevertheless, the current interest in proteases, and hence polypeptides and plasma kinins, as mediators in injury stems largely from Menkin's (108) discovery of leukotaxine, and the report of Beloff and Peters (16) on the influence of thermal burns on a skin proteinase.

The protease systems investigated include plasmin/plasmin inhibitor, and kallikrein/kallikrein inhibitor. The serum globulin PF, although not an established protease, is also considered in this section.

1. Plasmin. The blood of man and animals contains the inactive precursor, plasminogen (28, 92), of a fibrinolytic enzyme, plasmin. Plasminogen is activable by various kinases—*e.g.*, by the bacterial kinases, streptokinase and staphylokinase (57, 122), by cytofibrinokinase which is liberated in cell injury (9), by serofibrinokinase, occurring in serum as an inactive precursor, by antigen-antibody reactions and certain anaphylactoid stimuli (166), and by urokinase, occurring in urine (10).

The literature concerning the nature of this enzyme and its possible role in injury has been reviewed by Macfarlane and Biggs (95) and by Astrup (8). Like trypsin, it attacks synthetic substrates such as *p*-toluene sulphonyl L-arginine methyl ester (TAMe) and L-lysine ethyl ester (LEe) (165). However, it is now clear that guinea pig plasminogen is a mixture of at least two pro-enzymes, plasminogen C and plasminogen P (11). Each is the precursor of an esterase

which hydrolyzes TAMe. The former also attacks L-lysine methyl ester and is suppressed by soya bean trypsin inhibitor; the latter is insusceptible to soya inhibitor.

Mammalian tissues also contain at least two enzymes which form plasma kinins (88). The first, present in the skin of rats and cats, acts slowly and is antagonized by antiplasmin and soya bean trypsin inhibitor; the second, found in the secretions of the salivary, lachrymal, and pancreatic glands, as well as in urine, acts quickly and is insusceptible to antiplasmin or soya inhibitor. Furthermore, Astrup (8) reported that tissue extracts contain at least two plasmin activators: a local factor in the insoluble tissue proteins, and a soluble substance, which is rapidly formed from an inactive precursor in blood and distributed in the circulation.

Thus, the enzyme "plasmin" includes at least two esterases which are readily activated by endogenous and exogenous biological preparations, as well as by the treatment of serum with various substances such as chloroform (164) and peptone (166). Activation of plasmin, or other enzymes in blood which form plasma kinins, can also be induced by contact with glass (100, 101) or simply by dilution of plasma or serum in physiological solutions (96). Contact of cell-free plasma with glass activates unstable substances which accelerate blood coagulation (100) as well as a pain-producing substance (6). The pain-producing substance is a polypeptide similar to bradykinin, and is formed in two stages (101). In the first stage, a precursor (component A) in plasma is converted into an active substance designated as *contact factor*; in the second, contact factor interacts with another substance (component B) to form plasma kinin.

Activation by dilution (141) is discussed in the next section dealing with kallikrein.

Plasmin itself has little PF activity. About 20% of the PF activity in human plasma may be due to its plasmin content (121). In the guinea pig, rat and rabbit, plasmin has only slight activity (117).

2. Kallikrein. Frey and Kraut (54) considered that urinary kallikrein arose in the pancreas, circulated in the blood as an inactive precursor (kallikreinogen), and was finally excreted by the kidney (55, 80). It soon became apparent, however, that the enzyme arose in tissues other than pancreas (80, 170), and has since been identified in salivary gland and saliva (174), sweat (51), tears (86), and cerebrospinal fluid (27).

Although kallidin and bradykinin are similar in pharmacological activity, their elaboration by kallikrein and plasmin shows marked differences, particularly in the time-course of formation. Incubation of plasma globulins with salivary kallikrein results in maximal formation of plasma kinin in 3 to 5 minutes, with plasmin in 20 to 30 minutes. The difference does not appear to be associated with substances influencing kinin formation or destruction, but to represent a real difference in the ability of the respective proteases to hydrolyze globulins (87).

Schachter (141) described kinin release following dilution of serum or plasma from the ox, guinea pig, rat, dog, cat, and man, and postulated that the activated

protease might be kallikrein. His demonstration that a serum protease is activated by dilution is also noteworthy in view of the similar activation of globulin PF by the dilution of serum (97, 115). However, Schachter's proposal that kinin formation in diluted serum is due to activated kallikrein was disputed by Lewis (87), who observed that bovine plasma antagonized smooth muscle contraction to such an extent that kinin formation was not detectable after incubation with plasma protein. Nevertheless, kinins are formed when the plasminogen in bovine plasma is activated by such specific plasminogen activators as streptokinase and urokinase. There is still other evidence (87), from tests of heat stability and of trypsin treatment of serum, both for and against Schachter's proposal that dilution activates kallikrein. The matter requires further clarification.

Limited investigation (117) has revealed that kallikrein, like plasmin, has low PF potency. However, since kallidin has other pharmacological properties in common with bradykinin, kallidin, like bradykinin, may also have high PF activity.

In summary, plasma kinins such as bradykinin and kallidin are formed by corresponding proteases, plasmin and kallikrein, which are activated by various tissue and exogenous factors, as well as by contact with glass or by diluting plasma in saline. The protein substrate may well vary among species, but in some, at least, it appears to be an α_2-globulin. Plasmin and kallikrein themselves have little PF potency, but bradykinin is one of the most active PF's identified.

Some of the above features of plasmin and kallikrein are common to a third PF in blood—the globulin PF. However, although this PF appears to be a protease, its high PF potency does not appear to be due to the activation of kinins.

3. Globulin permeability factor (globulin PF). Globulin PF is present in inactive form (pro-PF) in the serum or plasma of the guinea pig (12, 97, 115, 179, 180), rat (152, 153, 181), rabbit (129, 181), and man (37, 121, 161). With a natural inhibitor (IPF) of the PF, it constitutes a pro-PF/IPF system that almost certainly is a feature of plasma, tissue fluid, and lymph (116) of all mammalian species (112, 162).

Each pro-PF in the above four species is readily and progressively activated when serum is diluted in physiological saline in glass containers, or fractionated in aqueous ethyl ether systems, except that rabbit PF is not activable by dilution (181). The IPF probably is present as such in serum, and slowly but progressively antagonizes the activated PF (115, 153, 179, 180, 181).

Activation of, *e.g.*, guinea pig pro-PF, probably is affected by contact with the surface of the glass container, rather than by dilution itself (100). When serum is collected and diluted in silicone-treated apparatus, activation of the PF is slow and feeble, but becomes rapid and strong when finely divided glass is added to the preparations (101). Similar activation is induced by other particles, such as cellulose, starch, or agar (112).

In the guinea pig, rat, rabbit, and man, the PF's are α- or β-globulins, according to the test species (117). Those from the guinea pig and rabbit, particularly when tested in the same species, have high PF activity, which is comparable with that of histamine. However, whereas guinea pig PF induces maximal lesions in

approximately 15 minutes, that from the rabbit takes 4 hours. Rat PF has relatively low potency. Although its potency is comparable with that of histamine in this species, it is much less than that of 5-HT in the rat, or of histamine in the guinea pig or rabbit (181). Serum fractions containing human PF have been investigated only in guinea pigs. In this species, they have low potency (121). The IPF can be isolated from fractions containing α-globulins in the guinea pig (179), albumins in the rabbit (129, 181), or both proteins in the rat (152, 181); although present, it has not been characterized in fractions of human serum (121).

There is good, but not conclusive, evidence that globulin PF is a protease and may hydrolyze amino acid esters *in vitro* (15, 118), and strong evidence that it does not owe its PF activity to being a histamine liberator (181). However, its relation, particularly to kallikrein, needs further clarification.

a. Evidence for enzymic nature of globulin PF. The evidence that the globulin PF is a protease is based on its high susceptibility to established protease inhibitors (15, 118), such as the trypsin inhibitors in soya bean (SBTI), lima bean (LBTI), and potato (PTI), and to the esterase inhibitor, di-*iso*propyl phosphorofluoridate (DFP; see ref. 1). Globulin PF has no caseinolytic activity, and no substrate for it has been identified. Moreover, the susceptibility of globulin PF to the above inhibitors has been demonstrated only when test preparations of PF were mixed with antagonist before injection into blued animals.

Probably the strongest evidence that the globulin PF is a protease is its progressive inhibition when PF and antagonist (*e.g.*, PTI or DFP) are held for increasing intervals before testing. This result seems to exclude antagonism of skin proteases (102, 103, 104) after injection of inhibitor (118).

Both *p*-toluene sulphonyl L-arginine methyl ester (TAMe) and benzoyl L-arginine methyl ester prevent inactivation of guinea pig PF by DFP. The former ester similarly protects rat PF. Accordingly, if the PF's are enzymes, they appear to attack ester linkages (15).

Evidence from another endogenous substance in guinea pigs indicates that esterases may have high PF activity. The secretion of the coagulating gland of the male guinea pig contains a PF with extremely high activity in both guinea pigs and rabbits (53). The PF can be isolated electrophoretically in a glycoprotein-containing fraction which has only feeble caseinolytic activity, but strong TAM esterase activity which is insusceptible to soya inhibitor. Heat stability tests suggest that the PF and esterase are identical.

b. Distinction of globulin PF from kallikrein and plasmin. Although the high activity of globulin PF suggests it is an enzyme, the evidence loses weight in view of the high PF potency of kinins like bradykinin. Furthermore, the failure to demonstrate a substrate for globulin PF means that the evidence for its enzymic nature rests on its susceptibility to trypsin and esterase inhibitors *in vitro* (118). However, kallikrein also is strongly susceptible to the same inhibitors and, like globulin PF, it is insusceptible to ovomucoid trypsin inhibitor (119).

Another similarity between the three preparations is the activation by dilution of plasmin (96) and allegedly of kallikrein (141) on the one hand, and of globulin

PF (115) on the other. For human plasmin and globulin PF, the effects of dilution seem to be different (37, 161); for guinea pig plasmin and globulin PF, tests of heat stability indicate that the two preparations are distinct (179). Furthermore, Lewis (85) has demonstrated that guinea pig plasmin, unlike globulin PF, attacks globulin preparations to form plasma kinin.

The distinction of globulin PF from plasmin seems established, but not that from kallikrein (see also Miles, 113).

c. Evidence against globulin PF being a histamine liberator. Much of the early interest in the possible role of globulin PF as a natural mediator of permeability changes in injury arose from its insusceptibility to antihistamines. Preparations of the PF from the guinea pig, rat, rabbit, and man are, at the most, only slightly susceptible to antihistamines (117), and fail to release histamine either *in vivo* or *in vitro* from preparations that readily yield histamine when treated with established liberators such as compound 48/80 (117, 181).

IV. CRITERIA FOR IDENTIFYING PERMEABILITY FACTORS AS POSSIBLE MEDIATORS IN INJURY

Since the ability to induce permeability effects is common to a wide range of substances, the presence of PF activity in an inflammatory exudate clearly may have no relation to that substance's actual function.

The criteria for identifying the natural mediators have been discussed in some detail by Miles (112) (see also Miles and Wilhelm, 117). They fall into two categories: those supporting the plausibility of the proposed PF as a natural mediator, and those actually proving its mediation.

The first category of criteria includes the distribution of a PF, both between different tissues in the same animal and between various species; its availability, activability, and potency; the presence of natural antagonists; and its ability to induce permeability effects similar in pattern and duration to those in injury.

1. Distribution. Histamine (45, 132) and 5-HT (31) occur in the tissues of a wide range of animals. Plasmin (8, 95) and globulin PF (117) probably are features of all mammalian plasma and hence are widely distributed in tissue fluid. Kallikrein also is present in blood and has been demonstrated in a variety of tissues, particularly glands (88). Plasma kinins are potentially available wherever proteins, especially globulins, occur.

2. Availability and activability. The histamine content of various tissues in the same or different species fluctuates between less than 1 μg/g in skeletal muscle of the horse and more than 100 μg/g in skin of the cat (45). However, the content of liberable histamine must be considered relative to other factors, such as its potency in the particular species and the amount of precursor available.

In general, the tissue content of each PF, or its precursor, is adequate to justify its consideration as a natural mediator. Furthermore, species differences in tissue content of, *e.g.*, histamine, have no particular relevance unless we seek a universal mediator.

On the score of activability, all the endogenous PF's qualify for consideration.

Histamine and 5-HT are readily released from mast cells, which in turn tend to be paravascular in distribution. Although practically any tissue manipulation releases histamine, it is noteworthy that the synthetic histamine liberators, in general, are strongly basic or highly surface-active substances (131). On the other hand, although mast cells are frequently degranulated in inflammatory tissue, this is not necessarily the case (148).

Plasmin probably is more readily activated than kallikrein, but itself may take part in activating kallikreinogen (86). Although the rate of formation of the corresponding plasma kinins appears to vary, it is in no way restricted. Globulin PF, at least *in vitro*, is readily activated by contact with glass (100, 101), and perhaps by dilution (97, 179).

3. Potency. That a PF with restricted activity in one or more species is unlikely to be a natural PF in injury is based on the notion that the natural mediator is common to all mammals. However, there is no reason why a PF with low potency in certain species should not be a natural, and even universal, mediator, provided that in such species it is available in correspondingly larger amounts.

Table 1 summarizes the PF potencies of various endogenous PF's in the skin of laboratory animals. Of the amines, either histamine or 5-HT has high potency in guinea pigs, rats, or rabbits. On the other hand, the established proteases, plasmin and kallikrein, have low PF potency, and if they play a natural role in injury, appear to owe their permeability effects to the production of plasma kinins such as bradykinin. The globulin PF from the guinea pig and rabbit has high potency in the homologous animal, but that from the rat is relatively inactive. Nevertheless, whatever the significance of potency variation according to species of test animal, the results recorded in Table 1 emphasize the importance of testing preparations in the *homologous* species.

4. Presence of natural antagonists. The case for the role of an endogenous PF is not compromised by the absence of natural antagonists. However, the effective concentration of a PF may be decreased by diffusion (20) as well as antagonism, although diffusion is likely to be more important for substances with low tissue affinity, *e.g.*, histamine (114), than for those with high affinity, *e.g.*, globulin PF (179). In any case, the short-term PF effects of released histamine are explicable in terms of endogenous antagonists like histaminase, diamine oxidase, acetylating and other enzymes (163), and plasma globulins (97, 128), and those of 5-HT by monoamine oxidase (MAO) (20).

Inactivation of plasma kinins may possibly be due to hydrolysis by peptidases. Various inhibitors of plasmin and kallikrein are present in both blood and tissue extracts (87).

Although the protease inhibitors in plasma require further characterization, they appear to overlap in nature with IPF, the natural plasma inhibitor of globulin PF (179).

5. Induction of permeability effects. Under this criterion, it appears necessary to establish not only that a substance can increase permeability, but also that its permeability effect is similar in pattern and duration to that in injury.

All PF's, by definition, increase vascular permeability. However, nearly all

have short-term effects that are in marked contrast to the relatively prolonged events in injury. This matter is more conveniently discussed in the next section, in association with the pattern of permeability response in injury.

V. TIME-COURSE OF PERMEABILITY CHANGES INDUCED BY INJURY AND BY ENDOGENOUS PERMEABILITY FACTORS

The PF changes in experimental injury vary in both pattern and duration, according to the type of injury and species of test animal. In general, however, they are characterized by a delayed response lasting several hours or days. In marked contrast, nearly all endogenous PF's elicit a response that appears rapidly and then declines quickly and disappears in 10 to 15 minutes.

It follows that the causal correlation of the presence of PF with observed permeability events requires the demonstration that the PF occurs in the injured tissue in amounts appropriate to the appearance and decline of the response. This in turn requires the establishment of a precise time-course of permeability events as a base-line for comparison. Otherwise, the role of the natural PF's with short-term effects cannot be reliably assessed. This argument seems valid whatever the methods used to identify the natural mediators in injury, whether by isolating the PF from inflamed tissue, or by demonstrating suppression of the permeability response in animals depleted of the PF or treated with specific antagonists.

Furthermore, the identification of the natural mediators in injury is more likely to be successful if the pharmacological picture is not complicated by non-specific breakdown products. Accordingly, the stimulus preferably should be the minimal that is effective. In general, the rapidity with which a permeability response appears in injury is directly proportional to the intensity of the stimulus, and hence minimal stimuli offer the further advantage of spreading the responses over a relatively long period, thereby facilitating the identification of different mediators of consecutive phases of a particular response.

1. The time course of permeability changes in mild injury. Figure 1 illustrates standard time-courses of the permeability changes induced in the skin of the guinea pig by a typical permeability factor (histamine) and by various kinds of injury.

The results for an endogenous permeability factor, histamine (curve A), bacterial infection (C), and *Cl. oedematiens* toxin (F) record the diameter of lesions at treated skin sites, and those for injury by xylol (B), heat (D), and ultraviolet irradiation (E), the amount of exuded dye (178). The time-courses were evaluated in guinea pigs given intravenous dye at the appropriate time intervals following injury (114), except that the events in the first few minutes were assessed in previously blued animals. The time-scale increases 10-fold from the top to the middle scale, and again from the middle to the bottom.

a. Bacterial infection. It is convenient to begin with the events in bacterial infection. Curve C records the biphasic response induced by each of 7 pathogens injected in guinea pig skin (24). An early phase begins immediately after injection

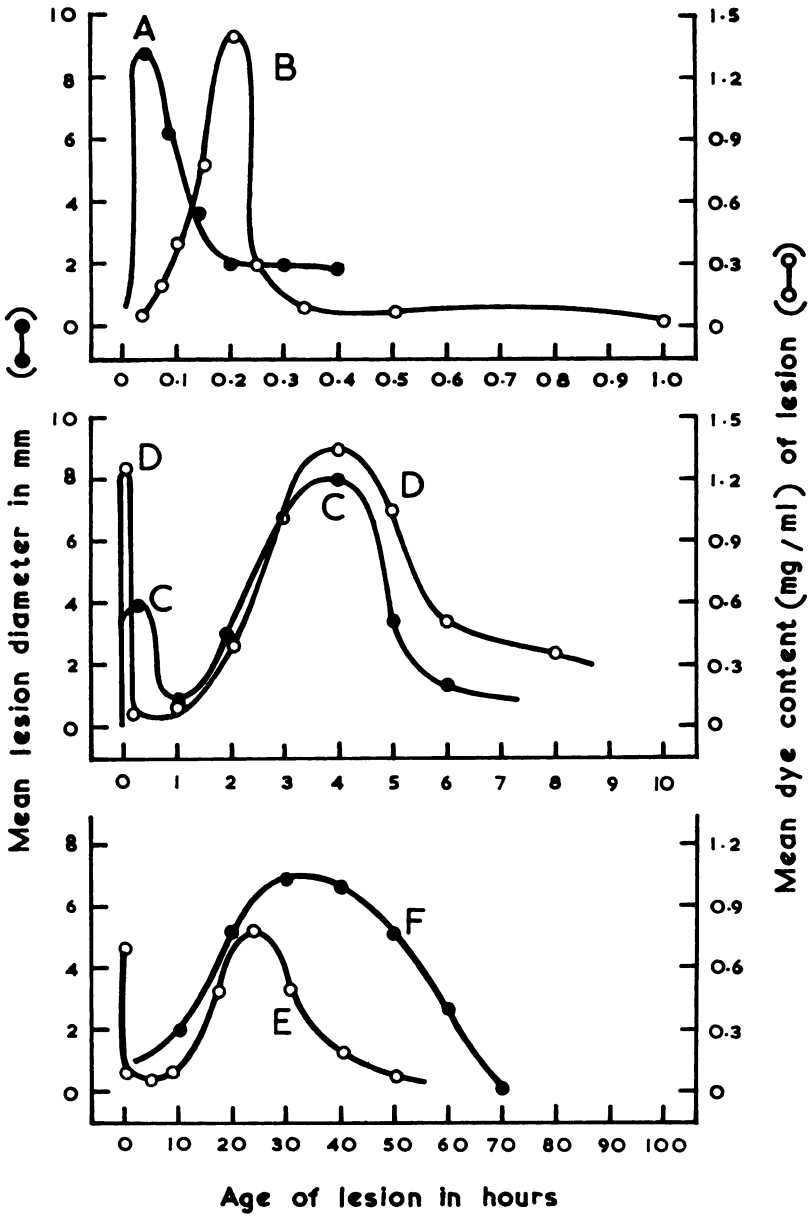

FIG. 1. The time-course of increased vascular permeability induced by various types of injury in the skin of the guinea pig. A = histamine (adapted from data by Miles and Miles, ref. 114); B = chemical injury (xylol; ref. 117, 176); C = acute bacterial infection (24); D = thermal injury, 54° for 20 sec (178); E = ultraviolet injury (91); F = *Cl. oedematiens* α-toxin (36).

and lasts 30 to 60 minutes, and a later phase appears during the second hour, becoming maximal in the third and fourth hours.

This diphasic pattern of response is elicited by *Staphylococcus aureus*, *Streptococcus pyogenes*, *Clostridium welchii*, *Listeria monocytogenes*, *Corynebacterium diphtheriae*, *Corynebacterium ovis*, or *Pseudomonas pyocyanea*. The immediate response may, in part, be due to adsorbed culture medium because it is decreased, but not eliminated, by repeatedly washing the bacteria. The delayed events, however, are characteristic of the response, and occur even in infections not evoking the immediate response—*viz.*, *Proteus vulgaris* or *Escherichia coli* (24).

The diphasic response is also induced by staphylococcal infection in the skin of the rabbit (134) and the rat (119). In the rabbit, both responses have a time-course like that in the guinea pig (Fig. 1, curve C), but in the rat, the delayed response appears more slowly, becoming maximal in 24 hours (*cf.* curve E).

b. Injection of Clostridial toxins. Elder and Miles (36) observed that sub-necrotizing doses of *Cl. welchii* toxins elicit a response similar in pattern and time-course to that induced by the corresponding bacteria (Fig. 1, curve C).

Clostridium septicum toxins also evoke a diphasic response, but the second phase becomes maximal in 45 minutes and is maintained for some 6 hours. The toxins of *Cl. oedematiens*, on the other hand, induce only the delayed response, which in this case is unusually prolonged. Beginning in some 6 hours, it becomes maximal in 24 hours and is sustained for a further 24 to 48 hours (curve F).

For each toxin, the main PF is the α-toxin, which, in the case of *Cl. welchii* is a lecithinase (94). *Clostridium septicum* toxin is enzymic in character, but apparently does not attack lecithin, cephalin, or the cerebrosides (93). Whether it attacks other lipids or proteins has not been determined.

Clostridium oedematiens toxins contain at least two lecithinases, which are specifically neutralized by anti-γ- and anti-β-sera, respectively, and are immunologically distinct from *Cl. welchii* lecithinase (93). The α-toxin has not been characterized.

c. Thermal injury. As early as 1873, Cohnheim (30) noted that a permeability response was evoked rapidly by burns and scalds, and considered it might be due to the direct effects of heat on the vascular endothelium. Subsequent workers postulated that the response was induced by breakdown products, but little progress was made until Lewis and Love (90) observed that mild heat, like histamine, induced the "triple response" in human skin.

In 1958, Sevitt (146) made a major contribution by demonstrating that heating skin sites in guinea pigs at 60°C for 5 to 60 seconds evoked both *early* and *delayed* phases of increased capillary permeability, and that heating for 5 seconds elicited an immediate mild response followed by a second stronger response in 30 to 60 minutes. These results have been confirmed in the guinea pig (177, 178), and similar responses to heating at 54 to 58°C have been demonstrated in the rat (156, 158, 178) and rabbit (178). A similar pattern of response can also be elicited in rabbit ear chambers (2).

As curve D illustrates, the time-course of the permeability events induced by mild thermal injury (54° for 20 seconds) in guinea pigs is remarkably like that in

bacterial infection (curve C). Although thermal injury at 56° and 58° for the same interval progressively decreases the immediate response, it both hastens the maturation and increases the intensity of the delayed response (178). The same effect is obtained in bacterial infection (24) or with necrotizing doses of clostridial toxins (36).

In rats and rabbits (178), the time-course of the two responses—immediate and delayed—to heating at 54° is basically similar to that in the guinea pig. In the rat, the immediate exudation of blue dye is slight or absent although, as in the guinea pig, substantial local oedema appears.

d. Ultrasonic injury. In unpublished work, Burke (23) observed that ultrasonic injury in guinea pig skin elicited a diphasic permeability response similar to that in thermal injury. The induction of an immediate response was comparatively easy, but the stimulus eliciting the delayed response was restricted in scope. Using an ultrasonic generator with a plate voltage of 1200 V and a pulse duration of 0.5 second, Burke elicited diphasic responses in focal lesions 3 mm in diameter, with the focal centre 1 mm below the stratum corneum. In such lesions, subsequent microscopic examination of paraffin sections revealed no necrosis.

e. Ultraviolet injury. Curve E in Figure 1 illustrates the diphasic response induced by ultraviolet injury in guinea pigs (91). In both pattern and delayed appearance, the response in guinea pigs is representative also of that in rats and rabbits (176).

f. Traumatic and chemical injury. In guinea pig skin, mechanical pinching (176), or the application of xylol, benzene, chloroform, or barium sulphide (117) induces a monophasic response that is maximal in 10 to 15 minutes and then steadily declines during the subsequent 30 to 40 minutes. However, normal low permeability is not restored even in 60 minutes (Fig. 1, curve B).

Treatment of guinea pigs by systemic administration of antihistamine before injury slightly, but consistently, depresses the permeability events in the first 10 to 12 minutes, but the response in 15 minutes has similar intensity to that in untreated controls [see Miles and Wilhelm (117), Fig. 6]. The results suggest that a minor histamine effect precedes the full response, which in turn is comparable to the accelerated delayed response in *moderate* thermal injury [see Wilhelm and Mason (178), Fig. 4], and that the response in traumatic and chemical injury differs from that in thermal injury or infection in time-course rather than in pattern—a conclusion that is supported by the results of Spector and Willoughby (152, 153, 155, 157) for turpentine pleurisy in rats. They observed that histamine and 5-HT in early exudates were replaced in older ones by globulin PF (or some unidentified mediator).

g. Summary. In summary, a *delayed* and prolonged permeability response is a characteristic feature of various types of experimental injury. Although an *immediate* and transient response is the rule in guinea pigs and rabbits, it may be feeble or absent in rats.

2. The time-course of permeability changes induced by endogenous permeability factors. A feature of the permeability response elicited by nearly all endogenous

PF's is their short duration. This is illustrated in Figure 1 by curve A, which records the typical response induced by histamine in guinea pigs, and exemplifies that induced by histamine, 5-HT, bradykinin, kallikrein, and globulin PF in guinea pigs, rats and rabbits (64, 114, 117, 136). The response is maximal in 3 minutes, and normal low permeability is restored in 10 to 15 minutes, or at the most, in 20 minutes.

Among endogenous PF's, the only exception to this rule is rabbit globulin PF which, in small doses, induces a prolonged effect in *rabbit* skin (117, 181), similar to that in bacterial infection and thermal injury (Fig. 1, curves C and D) in the guinea pig. Furthermore, the long duration of the response is peculiar both to the PF and to the species, because rabbit PF has the usual short-term effects (*cf.* curve A) in the guinea pig and rat, whereas rat and guinea pig PF have a short time-course in the rabbit.

The immediate response in injury clearly has a time-course like that of the endogenous PF's, and, in fact, probably is usually mediated by histamine or 5-HT (see below). That the delayed response is mediated by similar PF's with short-term effects would appear to require that 1) the local supply of PF is adequate to initiate and maintain the response, 2) the release of PF is reasonably continuous and prolonged, and 3) the blood vessels in all or part of the lesion can respond adequately to repeated stimulation over a relatively long period.

Even if all the histamine content of skin were released at temperatures of 50 to 60°C, used to induce experimental thermal injury, it would be inadequate to maintain the prolonged response induced (178). However, this objection loses force in view of the demonstration by Schayer and his colleagues (142, 143, 144, 145) that histidine decarboxylase is an adaptive enzyme—a result that is supported by the earlier findings of Dekanski (34) and Geiringer and Hardwick (59). On the other hand, it gains support from the failure to demonstrate an increased content of histamine in heated skin sites during the maturation of the delayed response (178).

The second of the three requirements seems the least difficult to attain. In general, the endogenous PF's are activated and released *in vitro* (111, 112, 117) with comparative ease; hence, if available in sufficient amount, they could well be released *in vivo* during periods sufficiently long to account for the delayed response.

Concerning the third requirement, that blood vessels be able to respond adequately to repeated and prolonged stimulation, there is good evidence indicating that the response of blood vessels declines when they are repeatedly stimulated by PF's. Following the injection of a PF such as histamine into the skin of animals, the blood vessels in the site become substantially refractory or immune to further injections of histamine or other PF's (114, 179). Refractoriness appears in one-half hour, and persists for some 4 hours. Furthermore, the effect is not specific in that a skin site injected with, *e.g.*, histamine, becomes refractory to the later effects of leukotaxine (114) or globulin PF (179). Whatever its cause—tachyphylaxis, increased back pressure of tissue fluid consequent to exudation, or some other factor—the phenomenon of refractoriness seems a serious obstacle

to accepting that the prolonged response in injury is mediated by continuous release of PF's with short-term effects.

The over-all picture lends itself to another explanation. The permeability events represented by the very tip of the peak of the prolonged response in curve C of Figure 1 (bacterial infection) reflect the events at the periphery of the lesion where the permeability response lasts only 20 to 30 minutes (117). Accordingly, the events represented by the tip of the peak might be mediated by a PF exemplified by curve A. This explanation, however, implies that in the prolonged response induced by, *e.g.*, the lecithinase in *Cl. welchii* α-toxin, the actual mediator is a transiently acting PF liberated by the lecithinase—an implication for which there is no evidence.

Until the prolonged effects of rabbit globulin PF were reported, only endogenous PF's with short-term effects could be put forward to explain the relatively prolonged and delayed response in injury. Now that substances—both endogenous (rabbit PF) and exogenous (clostridial toxins)—are known to elicit prolonged permeability effects, there seems good reason to doubt whether most of the established PF's with their transient effects are the natural mediators of the delayed response.

VI. METHODS OF IDENTIFYING NATURAL MEDIATORS IN INJURY

Methods of establishing that a PF is a natural mediator of increased permeability in injury fall into three categories: 1) isolation of the PF; 2) effects of depletion of a particular PF; 3) suppression of the PF response by specific antagonists. Of these, the first clearly is of prime importance, not only in actually identifying the mediator, but also in providing clues for the second and third categories of investigations.

1. Isolation of mediators. Since the permeability response provoked by injury may be diphasic, it seems likely that each phase is mediated by a different PF. Furthermore, since the delayed phase is relatively prolonged, it may well be induced by multiple stimuli. It follows that the mere recovery of a single PF from injured tissue at some stage during the inflammatory reaction is inadequate to explain *all* the permeability events up to that time. Rather, its appearance in active form in the injured tissue must be precisely correlated with the rise and fall of the permeability response.

Thus, Menkin (109) isolated leukotaxine in 24-hour-old exudates induced by turpentine in the pleural cavity of dogs. The doses of turpentine used almost certainly provoked a prolonged response. Accordingly, although the presence of leukotaxine may be relevant to the permeability events at 24 hours, it need have no relation to the earlier events.

The demonstration by Spector (152) of rat PF and IPF in 6- to 12-hour-old exudates in turpentine pleurisy is evidence that the exudate contains proteins similar in nature and stability to those in plasma, but no more. The same remarks apply to demonstrations of increased amounts of globulin PF in perfusates of guinea pig skin injured *in vivo* by mild thermal stimuli (178).

On the other hand, the demonstration by Spector and Willoughby (155) of

5-HT and histamine in 30-minute exudates offers much more convincing evidence of their role in the early stages of the response. Nevertheless, histamine and, to a lesser extent, 5-HT are readily liberated by various tissue manipulations, and hence their early presence may well be incidental to injury. Even the interaction of washed tissue debris and serum from the guinea pig results in histamine liberation (120). In fact, the problem seems difficult to resolve unless the mediators are extracellular, and hence isolable without disrupting the tissues. Even so, possible isolation might still be difficult if the factors are unduly labile, or quickly destroyed or inhibited by other enzymes or antagonists. A successful outcome seems most likely to be obtained by suppressing the natural response by appropriate inhibitors.

2. Depletion. Rat tissues are readily depleted of histamine by systemic treatment with compound 48/80 (50) or polymyxin B (127), and depleted of 5-HT by treatment with reserpine (44, 127). Although depletion is incomplete, the subsequent response to liberators of histamine or 5-HT is decreased. Corresponding suppression of permeability responses in injury (155) provides evidence that they are mediated by histamine and 5-HT. Thus, in turpentine injury of the pleural cavity in depleted rats, Spector and Willoughby (155) observed decreased exudation in the first half-hour, whereas at 4 hours exudation in depleted animals was similar to that in untreated controls. Nevertheless, such evidence remains suspect because the process of depletion has non-specific effects (181).

Furthermore, results in depleted animals are unconvincing unless depletion is complete. Since histidine decarboxylase may be an adaptive enzyme (143), complete depletion of histamine may not be practicable. For proteases and polypeptides, the large plasma reservoir of pro-factor or potential plasma kinins makes adequate depletion impossible.

Depletion experiments, carefully selected and adequately controlled, may provide supplementary evidence concerning PF's in inflammation, but the results must be assessed with caution.

3. Inhibition. In general, antagonists of PF's are not sufficiently specific in action to permit confident interpretation of their effects. For example, the antihistamines have anticholinergic and anaesthetic effects; likewise, although soya bean inhibitor may satisfactorily antagonize a protease like plasmin, it also suppresses kallikrein, trypsin, and globulin PF. Nevertheless, it is reasonable to expect that increasingly specific inhibitors will be developed as more is learned of the enzymes concerned.

The limiting factor in the use of inhibitors may well be similar to that in depletion; but, whereas depletion is a repetitive and slow process, satisfactory tissue concentrations of inhibitor can be obtained in minutes or hours.

VII. SUSCEPTIBILITY OF PERMEABILITY RESPONSES IN INJURY TO VARIOUS INHIBITORS

Three groups of inhibitors have been tested for their effects on the two permeability responses in injury: 1) antihistamines and 5-HT antagonists, 2) inhibitors

of proteases and esterases, and 3) non-specific antagonists like sodium salicylate and cortisone.

1. Susceptibility of the immediate response. Attention has previously been drawn to the similarity of the time-course of the immediate response to that of nearly all endogenous PF's. Judged by the effects of inhibitors, the response is probably mediated by the amines, histamine or 5-HT.

In staphylococcal infection in rabbits' skin, the immediate response is suppressed by the antihistamine, mepyramine maleate, 10 mg/kg subcutaneously (134). Smaller doses of mepyramine probably would be equally effective, because in guinea pigs the immediate response to both thermal (177, 178) and ultraviolet (91) injury is almost completely suppressed by only 0.1 to 1.0 mg antagonist/kg intravenously.

In rats, the immediate response (indicated by exuded dye) in thermal injury (54°C for 20 seconds) is minimal or absent (178), but this is not surprising in view of the low PF potency of histamine in this species (150). In another report (158), however, stronger heating (55° for 27 seconds) in rats induced substantial blueing in 30 minutes, which in turn was moderately suppressed by antihistamine. However, both groups of workers found that the early oedema induced by thermal injury was susceptible to antihistamine.

Ultraviolet irradiation in the rat, as in the guinea pig (Fig. 1) and rabbit, elicits a strong immediate response. That in the rat is highly susceptible to a 5-HT antagonist, and in the guinea pig or rabbit to antihistamines (91, 176).

In turpentine injury of the pleural cavity, the early permeability response is substantially suppressed by the antihistamine, diphenhydramine (157).

Xylol, benzene, chloroform or barium sulphide applied to guinea pig skin each elicits a monophasic response (Fig. 1, curve B) that during the first 10 minutes is slightly but consistently suppressed by triprolidine or mepyramine (117).

Disagreement with the conclusion that the immediate response is mediated by histamine (146) or 5-HT is the exception to the rule, and probably is explained by differences in technique (178). However, there is unanimity that the immediate response is insusceptible to inhibitors of proteases and esterases.

2. Suppression of the delayed response. Although the mediation of the immediate response seems established, that of the delayed response—the major permeability event in injury—is uncertain.

Despite the strong effects of *antihistamines* on the earlier response, they in no way influence the delayed events in bacterial infection (134) or in thermal (146, 147, 156, 158, 169, 175, 177, 178), ultraviolet (176), or chemical injury (117, 155, 157). The antihistamines are equally ineffective when given locally or systemically, either before or during the maturation of the response. Moreover, giving the drugs prior to inducing injury suppresses the immediate response without affecting the delayed, so that the immediate permeability response does not appear a necessary precursor for the later events (175). Rather, the appearance of the former may simply reflect the ease with which histamine is liberated by almost any type of injury. Nevertheless, Schayer's (143) demonstration of a rise and fall in histidine decarboxylase activity, corresponding in time-course to the

delayed permeability response, means that the non-participation of histamine as a mediator is not finally settled.

Inconsistency of the results obtained by different workers with *protease inhibitors* restricts the value of the corresponding reports. The oedema induced in rats' paws by injecting kaolin has been reported to be suppressed by the trypsin inhibitors from soya bean, potato, pancreas, or egg white (67). The dosage of each antagonist was large (50 mg/kg, intraperitoneally) and repeated; on the other hand, more than twice this dosage intravenously does not affect the volume of pleural exudate in rats (157).

Other results with these inhibitors have been obtained in tests using exuded dye as the index of increased permeability; however, the validity of their comparison with those measuring oedema has not been established (60, 158). Nevertheless, if we take results in *blued* rats at face value, intravenous doses of soya inhibitor (3 mg/kg) do not affect the delayed response in thermal injury (178). The results from tests with higher doses are unreliable, because the suppression of permeability responses becomes non-specific.

Even the negative results in thermal injury with small, intravenous doses of soya inhibitor are of doubtful value, because systemic soya inhibitor only slightly suppresses globulin PF, despite the PF's high susceptibility to treatment *in vitro* with the inhibitor (118). The relative inefficiency of systemic soya inhibitor, in turn, may be due to several factors, such as the presence in blood of various proteases that quickly deplete the plasma concentration of inhibitor, the adsorption of inhibitor to plasma proteins, and its large molecular size and consequent lack of mobility. Study of the role of proteases and esterases in injury at present is seriously handicapped by the lack of inhibitor preparations which are both specific in action and readily distributed in the tissues.

The esterase inhibitor, di-*iso*propyl phosphorofluoridate (DFP) is an organophosphorus compound that has a low molecular weight relative to that of soya inhibitor. However, the organophosphorus compounds are among the most toxic known (1), and Wilhelm and Mason (177, 178) considered that the toxicity of DFP and the consequent non-specific depression of permeability responses precluded the drug's use by systemic administration. Injected locally in small doses into injured skin sites, DFP still induced local effects, such as muscular fibrillation, but did not affect the delayed permeability response in thermal injury in the guinea pig, rat, or rabbit.

On the other hand, Spector and Willoughby (156, 158, 159) observed that intraperitoneal DFP decreased both oedema and the exudation of dye in the late phase of increased permeability induced by thermal injury in rats. Despite the drug's suppression of the response to various unrelated PF's, Spector and Willoughby (158) considered its suppressive effects in thermal injury might be in part general, and presumably non-specific, but in part also due to "inhibition of the activation of globulins or other permeability-increasing substances."

The difference of opinion concerning the suitability of DFP for systemic administration is possibly in part due to variations in technique, such as making tests in anaesthetized or non-anaesthetized animals, and using different prepara-

tions of DFP; but, until the general toxicity of DFP can be accurately assessed, claims that its effects are even partly specific are difficult to evaluate.

In a subsequent paper, Spector and Willoughby (159) studied the effects of large doses of various substances (quinine, quinidine, chloroquine, caffeine, and salicylate), as well as DFP—compounds that inhibit the carboxylic esterase and esterase-endopeptidase group of enzymes. Again, both the late permeability response in thermal injury and that to histamine, substance P, 5-HT, and compound 48/80 were suppressed. Although these results support the earlier conclusion (158) that the late permeability response may be mediated by esterase-like substances, they are open to the same criticism as those above concerning DFP.

3. Miscellaneous antagonists. Sodium salicylate suppresses the permeability effects of various preparations, including both histamine and globulin PF (121). The suppression is peculiar in that it involves the colour-intensity of exuded dye, and not the size of the lesions.

Since the permeability response in, *e.g.*, thermal injury, is usually evaluated by changes in colour-intensity of exuded dye, it is not surprising that the drug strongly suppresses both the early and late phases of the permeability response (158).

Cortisone suppresses both the permeability effects and leucocytic diapedesis in inflammation elicited in rabbits (3, 110), but not the permeability response to injected PF preparations in guinea pigs (180).

Sodium α-naphthyl acetate, in large subcutaneous doses, has been reported (71) to suppress oedema in formalin injury of rats' paws. Comparatively small doses, however, do not suppress exudation of dye in thermal injury (178), but dosage was restricted to obviate non-specific effects.

EDTA-Na_2. Decreased concentrations of calcium ions in or near capillary endothelial cells have long been suspected to increase vascular permeability (7, 26, 153). The metal chelating agent, disodium ethylenediaminetetraacetate (EDTA-Na_2), which chelates divalent calcium ions, strongly suppresses the delayed permeability effects in guinea pigs induced by *Cl. welchii* toxin (19, 124), but has no effect on the delayed response in thermal injury (178).

4. Resolution of the "histamine controversy." Previous reference has been made to Lewis's (89) evidence that histamine mediates oedema formation in injury—evidence that has been a large factor in the retention of the histamine theory of inflammation, despite its subsequent lack of experimental support, particularly from tests with antihistamines. However, the apparent discrepancy seems explicable in terms of varying techniques. As described earlier in this paper, tests with antihistamines in guinea pigs indicate that the minor immediate permeability response in thermal injury (54° for 5 to 20 seconds) is mediated by histamine, whereas the delayed response is insusceptible to antihistamine. The above order of heating "dissects" the elicited permeability response into two distinct phases, immediate and delayed. Moderately higher orders of heating (56 to 58° for 20 seconds) somewhat suppress the immediate response, and both hasten the onset and increase the intensity of the delayed response (178). Thus,

moderate heating results in an over-all permeability effect that is largely an accelerated delayed response, and consequently insusceptible to antihistamine. This evidence from tests in guinea pigs seems relevant to those in man and the rabbit, because in all three species histamine has high PF potency (117). Accordingly, the results in guinea pigs seem to explain why mild heating (43 to 52° for 5 to 180 seconds) in man induces only a histamine response (90), whereas stronger heating—60° for 15 seconds in man (147), or 96° for 10 seconds in the rabbit (169)—elicits a substantial permeability response which is insusceptible to antihistamine. Nevertheless, the issue concerning the mediator of the major and delayed permeability response remains unsettled, and there is no consistent and convincing evidence that amines, polypeptides, or proteases are the key substances inducing the response.

VIII. CONCLUSIONS

If the experimental evidence is a reliable guide to the vascular permeability response in inflammation, two sets of facts emerge. Firstly, the response is complex and consists of at least two phases. The early one is transient and appears irrelevant to the later, which constitutes the essential part of the response. Secondly, histamine and 5-HT are the only natural mediators that have been firmly identified, but their role is confined to the early and minor events.

This information has been established largely by forsaking the gross procedures formerly used to induce experimental inflammation. If further progress is to be achieved, it is more likely to come by studying the reactions elicited by mild stimuli in animals that are not assaulted by overwhelming doses of drugs.

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