HISTAMINE METABOLISM IN DIABETES MELLITUS AND VASCULAR DISEASE

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DOCTOR OF PHILOSOPHY

by

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ABSTRACT

The studies reported in this thesis consist of investigations of histamine metabolism in (a) various tissues and plasma of diabetic rats, and (b) in the leucocytes, platelets and plasma of patients with diabetes mellitus (DM) and peripheral occlusive vascular disease (PVD). In addition, we have investigated the factors influencing histamine uptake by normal human platelets.

In DM rats histamine synthesis, as reflected by histidine decarboxylase activity, was found to be markedly elevated in the aortae and various other tissues; there was no concomitant alteration in histamine degradation. This was accompanied by a significant increase in plasma histamine concentrations. One of the likely consequences of elevated plasma and aortic histamine in DM rats would be an increase in vascular endothelial permeability. However, unlike previous reports, elevations in aortic permeability in our DM rats was not affected by the *in vivo* administration of either H_1 or H_2 receptor antagonists.

In DM and PVD patients, there was a marked increase in plasma histamine concentrations. In PVD platelets there was a concomitant increase in the histamine content of leucocytes and platelets, without any alteration in histamine synthesis or breakdown. It is therefore likely that increased intraplatelet and intraleucocyte histamine in PVD is due to increased uptake by these activated cells from a 'histamine rich' plasma. We also observed an accelerated platelet histamine uptake *in vitro* as a result of mild platelet activation.

Our studies indicate marked alterations in histamine metabolism in both experimental DM and in patients with DM and vascular disease. It is likely that an altered histamine status in the plasma and blood vessels contributes to increased vascular permeability in DM and vascular disease. Since increased vascular permeability is one of the key events in the pathogenesis of diabetic microvascular disease and atherosclerosis, it is possible that histamine plays an important role in the pathogenesis of these conditions.

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CHAPTER I

INTRODUCTION

Histamine belongs to a group of diverse substances called "autocoids", which are released from tissues during injury or inflammation. They differ widely in their structural and pharmacological activities. The group includes the biogenic amines, histamine and serotonin; small peptides, such as the kinins; and lipids, such as the prostaglandins. Their action can be attenuated, blocked or potentiated by a wide variety of agents, either by interacting at receptors or by blocking the enzymes that synthesize or metabolise them.

Histamine was the first of these substances to be discovered. Early research gave an indication of the possible involvement of histamine in inflammatory and anaphylactic reaction. However, the progress of knowledge in this area has been slow. The two most important reasons for this were the lack of availability of specific agonists and antagonists, and the lack of sensitive and specific methodology for the detection and measurement of histamine.

Realization of the physiological importance of histamine dates from 1910 when Sir Henry Dale (Dale & Laidlaw, 1910) demonstrated its powerful effects on smooth muscle and blood pressure. Dale was quick to realize that the substance in question was identical to β-imidazolylethylamine, which was chemically synthesised in 1907. The principal pharmacological activities of histamine were described in a series of elegant papers by Dale and Laidlaw from 1910 to 1919 when they, together with co-workers, outlined all the major actions of histamine except one: the stimulation of gastric secretion.

By 1919, Dale and Laidlaw (1919) had made two general conclusions about histamine. They noted that when applied locally it produced redness, swelling and oedema; and it did not escape their notice that these were the features of mild inflammation. The dilatation of the capillaries, pooling of blood in these vessels, and the exudation of plasma through capillary walls would result in the familiar sequence leading to weal formation in man. Their second general conclusion was that the administration of histamine

in large doses into the circulation reproduces symptoms similar to those of shock produced by trauma and anaphylactic reactions.

Additional functions have also been postulated for histamine. Schayer (1965) proposed that an adaptive mechanism exists whereby histamine synthesis in small blood vessels can be induced solely by tissue stress, which leads to vasodilatation, and that this newly formed histamine exerts its effects locally and is not stored. This so-called 'inducible' histamine is similar to the 'nascent' histamine described by other workers, who have shown that there is increased histamine synthesis in many tissues undergoing rapid growth or repair. It is also thought to be a minor mediator of exocrine secretions, particularly salivation.

It is now recognised that histamine is a normal constituent of blood and of most tissues, although its function in many tissues remains obscure. In the mammalian brain, for example, Schwartz *et al.* (1980) have shown that histamine acts as a central neurotransmitter. In most tissues, histamine is stored mainly in mast cells (Schayer, 1956; Riley & West, 1966); in blood, histamine is stored almost exclusively in leucocytes, with the basophil being by far the richest histamine-containing cell. Eosinophils, neutrophils, monocytes and lymphocytes are also known to contain histamine in appreciable amounts (Graham *et al.*, 1955). The human blood platelet was thought not to contain any histamine at all; but this is now known not to be the case (Brown *et al.*, 1980). Human platelets contain very small amounts of histamine, but with the huge number of circulating platelets they can collectively contain approximately 5% of total blood histamine. Human plasma contains minute amounts of histamine (Heavey *et al.*, 1984) unlike rat plasma, which can have up to 400 times more histamine than human plasma (Hollis *et al.*, 1985). Erythrocytes do not contain significant amounts of histamine.

The role of histamine in health and disease has intrigued investigators for more than half a century. Its role in gastric acid secretion and as a mediator of allergic reactions is well established, while its role in several other physiological functions and pathological conditions is suspected.

As mentioned earlier, histamine is known to be a normal constituent of most mammalian tissue and blood cells. It is thought to exist in at least three distinct pools (Schayer, 1965): (1) the tissue non-mast cell 'inducible'

(nascent, intrinsic) pool; (2) the tissue mast cell-bound histamine pool; and (3) the blood-borne histamine pool.

Although tissue histamine is stored predominantly in mast cells (Riley & West, 1966), a pool of non-mast cell histamine also exists in certain tissues like the stomach and blood vessels (Kahlson & Rosengren, 1968; Hollis & Rosen, 1972). The so-called non-inducible mast cell pool is not thought to play a major role in the physiology and pathology of histamine, apart from allergic reactions. The interaction of antigens with surface immunoglobulin E (IgE) or the action of agents on appropriate receptors yields an activated mast cell which responds with degranulation and subsequent histamine release (Read *et al.*, 1974).

On the other hand, the 'inducible' nascent non-mast cell histamine pool in tissues is variable in content and is dependent on the rate of histamine synthesis and breakdown. Alterations in this 'inducible' pool have been shown to be accompanied by changes in the tone and permeability of blood vessels (Schayer, 1966). The correlation between changes in HDC activity and circulatory changes has been taken by some authors as a basis for the hypothesis that induced histamine serves as a modulator of the functional state of the terminal blood vessels, induced histamine being formed at a rate required to maintain homeostasis (Schayer, 1962). This inducible form of HDC is believed to be located in vascular endothelial cells and is known to have a very short half-life (3-6 hours). This rapid turnover pool is also believed to play a key role in the intrinsic regulation of microcirculatory vessels (Schayer, 1962), in the slow phase of inflam-mation (Schayer, 1961) and in neurotransmission (Green *et al.*, 1978).

The third distinct pool of histamine is contained within blood cells, and is contained almost exclusively in leucocytes.

Mast cells, leucocytes and most tissues have the capacity to synthesise, take up and release histamine (Schayer, 1956; Holcslaw *et al.*, 1985; Lagunoff, 1972; Catini *et al.*, 1984; Day & Stockbridge, 1964; Stewart *et al.*, 1979; Green 1967).

A. *Histamine Metabolism*

(a) Biosynthesis

Histamine is derived solely from the decarboxylation of the essential amino acid L-histidine, although this is not the only route of histidine catabolism (Figure 1). Two enzymes capable of decarboxylating L-histidine have been found in mammalian tissues (Boeker & Snell, 1972). Histidine decarboxylase (HDC; EC 4.1.1.22) is specific for L-histidine. On the other hand, aromatic L-amino acid decarboxylase [dihydroxy-phenylalanine (DOPA) decarboxylase; EC 4.1.1.26] is a non-specific enzyme capable of decarboxylating a variety of natural aromatic amino acids, including DOPA and 5-hydroxytryptophan; it is widely distributed in animal tissues. Histidine has a greater affinity for HDC than for the non-specific enzyme, and evidence suggests that the decarboxylation of histidine is performed *in vivo* solely by the specific decarboxylase (Schwartz, 1975).

The two enzymes may be distinguished on the basis of their optimal pH (6-7 for the specific enzyme and 8-9.5 for the non-specific enzyme), by their reaction to benzene, which activates DOPA decarboxylase but not HDC, and by the action of inhibitors. The most potent inhibitor of HDC is 4-bromo-3-hydroxy-benzyloxyamine (NSD-1055; Brocresine). However, the mechanism of action of this compound appears to be by reaction with pyridoxal phosphate and it is therefore not specific, since both decarboxylases use this co-enzyme (Leinweber, 1968). 4-imidazolyl-3-amino-2-butanone (McN-A-1293) is less potent than NSD-1055 but is a specific competitive inhibitor for HDC (Taylor *et al.*, 1973). Alpha-hydrazinohistidine (αHH) and alpha-fluoromethyl-histidine (α-FMH) are more effective and irreversible inhibitors of the specific enzyme than the non-specific enzyme (Levine *et al.*, 1965a; Kollonitsch *et al.*, 1978). On the other hand, α-methyl-3,4-dihy-droxyl-L-phenylalanine (α-methyl DOPA-αMD) is a more potent and specific inhibitor of the non-specific decarboxylase enzyme (Mole & Shepherd, 1972).

HDC has been identified in a variety of tissues (Boeker & Snell, 1972), most notably in the stomach and in rapidly growing normal and abnormal tissue. An important characteristic of HDC is that it is inducible in certain conditions like trauma, cold and local inflammation (Maslinski, 1975a). Induction is at the local level of protein synthesis (Morris & Fillingame, 1974). There is also evidence that release of histamine from a tissue results

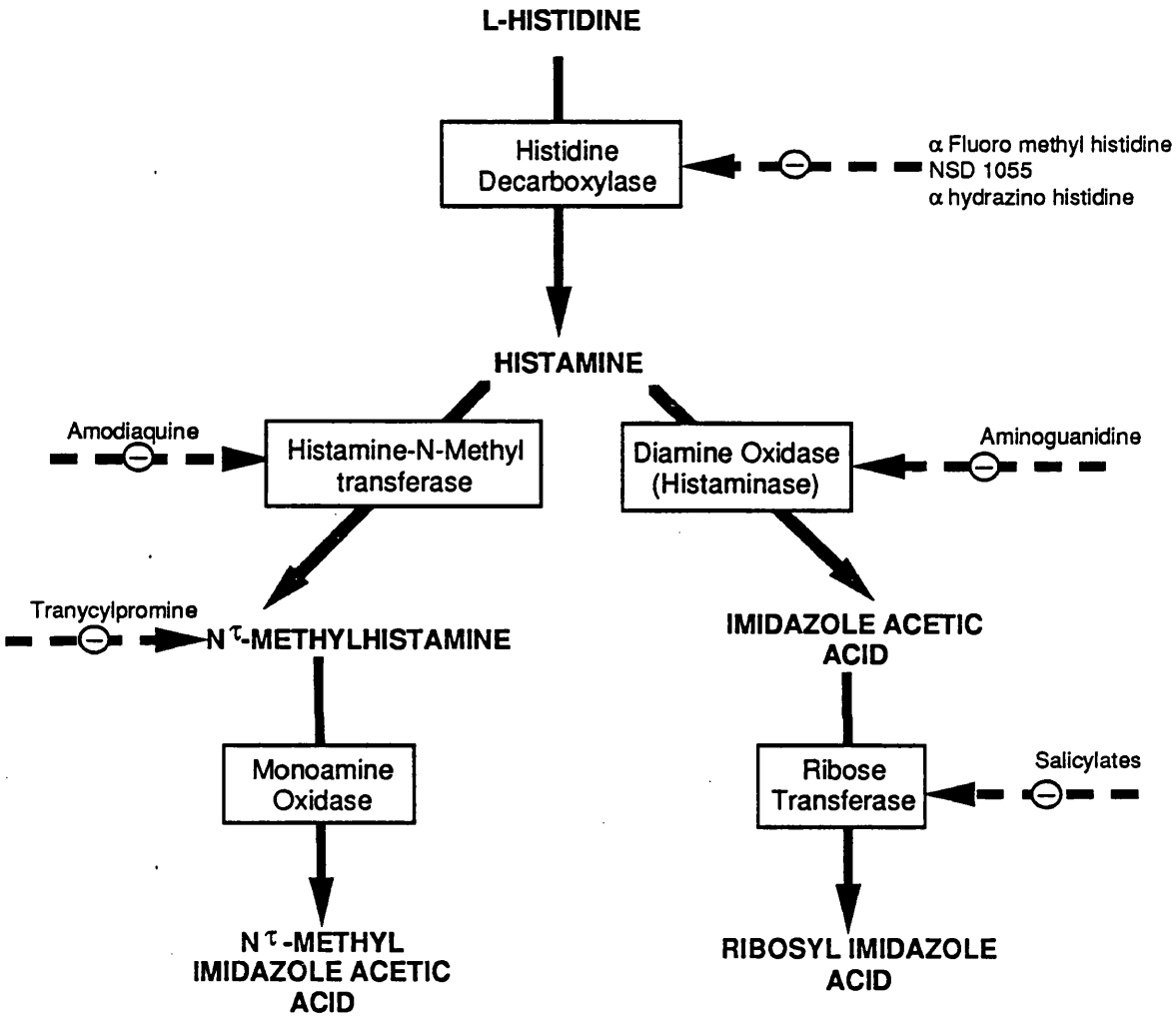

Figure 1

Major synthetic and catabolic pathways of histamine

in an increase in HDC activity (Schwartz *et al.*, 1972). Evidence has also been presented that HDC levels are controlled not only by the rate of synthesis of histamine, but also by its rate of degradation (Morris & Fillingame, 1974).

(b) Catabolism

Histamine is catabolised by two routes (Wetterquist, 1978; Maslinski, 1975). In one, histamine is methylated to form 1-methyl-4-[β-aminoethyl]-imidazole (methylhistamine) through transfer of a methyl group from S-adenosylmethionine (SAM) in the presence of the enzyme, histamine-N-methyltransferase (HMT). The specificity of HMT for methylating histamine is the basis for the widely used isotopic assay of histamine (Beavan & Horakova, 1978). Methylhistamine is subsequently deaminated by the Type B monoamine oxidase to form 1-methylimidazole-4-acetic acid (methylimidazole acetic acid). In the other route, histamine is deaminated by diamine oxidase (DAO; histaminase) to form imidazole acetic acid (Wetterquist, 1978), which is then conjugated and excreted as 1-ribosyl-imidazole-4-acetic acid (ribosyl imidazole acetic acid) (Figure 1). The conjugation with ribose is unique in that histamine is the only known compound to be metabolised by conjugation with this sugar. The purpose of this conjugation is unclear, since imidazole acetic acid is excreted as readily as the ribosyl derivative.

The relative importance of these metabolic pathways varies between species (Maslinski, 1975b). It has been shown that deamination of histamine and the conversion to ribosyl imidazole acetic acid is the major pathway in the rat and a minor one in man, cat and dog. In rabbits and guinea-pigs, both routes appear to be equally important (Kahlson & Rosengren, 1968).

Inhibitors of the various catabolic steps include the hydrazine derivative aminoguanidine, which is a potent and highly specific inhibitor of DAO (Severs *et al.*, 1970). HMT is inhibited by a variety of antimalarials, although relatively high doses of these compounds are needed (Beavan & Shaff, 1979).

The body appears to have a great capacity to destroy histamine. This ability of the body to degrade histamine rapidly appears to be necessary because of the potentially lethal quantities of histamine in some tissues, such as the stomach. The presence of two catabolic pathways also provides the body with a flexible system and a large capacity to degrade histamine.

(c) Histamine Receptors and their Antagonists

As identified on a physiological and pharmacological basis, there are three known receptors for histamine, classified as histamine H_1, H_2 and H_3. The most well-known antihistamines are H_1 receptor antagonists and have been in existence since before the second world war; they include early compounds such as mepyramine (pyrilamine) and highly potent drugs such as diphenhydramine and tripelennamine. The H_1 receptors are known to mediate the actions of histamine in anaphylaxis and allergy.

The other well-known class of histamine antagonists inhibit the actions of histamine at the H_2 receptor. H_2 antagonists like cimetidine and ranitidine are potent inhibitors of gastric acid secretion, and have been extremely successful in the clinical treatment of peptic ulcers.

The third, and most recently discovered, H_3 histamine receptor is thought to be involved in the feedback control of histamine synthesis and release in the brain (Arrang *et al.*, 1983, 1987). The H_3 receptor is not inhibited by either H_1 or H_2 antagonists.

Studies of DPPE, an antiproliferative agent, have shown that it inhibits histamine binding at a novel (non-H_1, non-H_2, non-H_3) site. These studies are leading to discussions of a possible fourth distinct histamine receptor (Brandes *et al.*, 1987, 1988).

Histamine receptors have been identified on virtually all blood leucocytes (Melmon *et al.*, 1972; Plaut & Lichtenstein, 1982; Dy *et al.*, 1981) as well as in most tissues, even though the function of these receptors in many tissues and cells remains unclear.

B. *Histamine-induced vascular permeability*

(a) Endothelial permeability

The increase in capillary permeability observed during the acute inflammatory process has been ascribed to the liberation of various chemical mediators, including histamine. The role of histamine in increasing capillary permeability was first described by Eppinger (1913) and was subsequently confirmed by Sollmann & Pilcher (1917) and Dale & Richards (1918). Histamine had been reported by Gaddum (1948) to produce leakage of circulating proteins and protein-bound dye into the tissues in many species, including man. Since then, several investigators have confirmed the role of histamine in causing vasodilatation and an increase in capillary permeability in association with acute inflammation (Wilhelm, 1962; Lichtenstein *et al.*, 1973). It has now been shown that histamine exerts its effects on capillary permeability via the histamine H_1 receptor, since the administration of H_1 agonists can enhance permeability, while H_1 antagonists reduce capillary permeability. H_2 agonists and antagonists have been shown not to affect capillary permeability (Bhargava *et al.*, 1977).

The endothelial cell is uniquely situated to play an active role in the induction of the inflammatory response, affording the venular endothelial cell a central role in the barrier function of the vessel wall. The concept of endothelium serving as an inert barrier between blood and the surrounding tissue is now known to be grossly inaccurate. According to that model, molecular exchange across the blood vessel wall was simply regulated by the interplay between hydrostatic and osmotic pressures, endothelium serving the sole purpose of providing a physical barrier. It is now known that the endothelium not only serves as a physical barrier but is also a widespread organ of multiple capabilities. It is actively involved in a number of processes, including maintenance of the vessel wall integrity, the blood clotting system, the clearance of circulating lipids, the renin-angiotensin system, the endocrine system and the immune response.

Arterial endothelium is also known to respond to histamine and other inflammatory mediators such as serotonin and bradykinin. It is generally agreed that increased arterial endothelial permeability to macromolecules represents one of the most important initial events in the pathogenesis of

atherosclerosis (Hüttner *et al.*, 1970; Schwartz *et al.*, 1978; Stefanovich & Gore, 1971; Veress *et al.*, 1970). These infiltrated macromolecules subsequently evoke a variety of reactive processes within the arterial wall. Endothelial cells are known to contain both histamine H_1 and H_2 receptors (Heltianu *et al.*, 1983; Rotrosen & Gallin, 1986).

(b) Other endothelial actions of histamine

Histamine has been shown to evoke a multitude of changes in the endothelium, including an increase in arterial endothelial permeability (Majno *et al.*, 1967; Killacky *et al.*, 1986; Rotrosen & Gallin, 1986), an increased widening of inter-endothelial gaps by endothelial contraction (Majno *et al.*, 1969), increased dissociation of actin cables (Welles *et al.*, 1985; Rotrosen & Gallin, 1986), an elevation in endothelial prostaglandin production (Baenziger *et al.*, 1981; Haddock *et al.*, 1987; Revtyak *et al.*, 1988), and an increase in endothelial pinocytosis (Orlidge, 1983). Such effects are likely to increase the transport of plasma macromolecules such as lipids into the arterial wall.

Other effects of histamine which would affect endothelial function and integrity include polymorphonuclear leucocyte migration into the subendothelial layer (Doukas *et al.*, 1987), an increase in cytosolic free ionised calcium concentration in endothelial cells (Exton, 1985; Rotrosen & Gallin, 1986); the induction of the synthesis of platelet activating factor (PAF) by endothelial cells (McIntyre *et al.*, 1985); an increase in the release of Von Willebrand factor by the endothelium (Hamilton & Sims, 1987); and the stimulation of the release of endothelial tissue plasminogen activator (tPA; Levin & Santell, 1988).

C. *The Pathogenesis of Atherosclerosis:*

Is there a Role for Histamine?

As mentioned earlier, it is now widely accepted that the initial event in atherogenesis is an increase in the permeability of atherogenic plasma macromolecules (Ross, 1986) into the endothelium of the intimal lining of arteries. It has therefore been suggested that an alteration in histamine metabolism within blood vessels may be an important factor responsible for increased endothelial permeability. Theodore Hollis and his colleagues have been actively pursuing this issue since the mid-1970s. They have proposed that initial increases in aortic endothelial permeability may be mediated, at least in part, through accelerated histamine synthesis *de novo* within the wall of the aorta mediated by the enzyme HDC.

Hollis and his co-workers reported that significant increases in aortic HDC activity occur in reponse to a variety of atherogenic stresses, including transient neurogenic hypertension (Bolitho & Hollis, 1973; 1975) and mechanical hypertension (Yarnal & Hollis, 1976; Hollis *et al.*, 1972); following the exposure of aortae and aortic endothelial cells to elevated shear stress (De Forrest & Hollis, 1978; Hollis & Ferrone, 1974; Skarlatos & Hollis, 1987); as a result of diet-induced hypercholesterolaemia (Hollis & Sloss, 1975; Markle & Hollis, 1975; Markle & Hollis, 1977; Hollis & Furniss, 1979; Owens & Hollis, 1979) and experimental diabetes (Hollis & Strickberger, 1985; Orlidge & Hollis, 1982; Gallik & Hollis, 1981). More importantly, it has been clearly shown that the increase in *de novo* aortic histamine synthesis correlates significantly with increased endothelial permeability and therefore the initial events leading to atherosclerosis (Hollis & Furniss, 1980; Hollis *et al.*, 1983; Carroll & Hollis, 1985).

(a) Histamine and Hypercholesterolaemia

It is now well established that hypercholesterolaemia is associated with a marked increase in vascular wall permeability to macromolecules (Stefanovich & Gore, 1971; Veress *et al.*, 1970; Virag *et al.*, 1969). Closely related to this are the studies of Besterman (1970) and Cornhill & Roach (1976), who have shown the existence of a significant correlation between

serum cholesterol concentration and the severity of atherosclerotic lesions in aortae and coronary arteries.

It is now well accepted that hypercholesterolaemia constitutes a major risk factor for accelerated atherosclerosis. It is therefore interesting to note that marked alterations in arterial histamine synthesis have been reported in experimental hypercholesterolaemia induced by an increase in the dietary intake of cholesterol. Hollis and his co-workers have carried out a host of studies using hypercholesterolaemic rabbits, and have shown that increased aortic endothelial histamine synthesis is clearly associated with a pre-atherosclerotic state (Markle & Hollis, 1977). They have shown that HDC activity of aortic endothelial cells is markedly elevated in rabbits fed for two weeks on a high cholesterol diet. When these same rabbits are continued on this atherogenic diet for a further two weeks, changes of early histologically identifiable atherosclerosis are observed; however, the changes in aortic histamine synthesis begin to return to normal. This suggests that one pre-atherosclerotic metabolic change is an increased capacity of endothelial cells to form histamine, and that it is during this stage that the leaky endothelium causes the process of atherosclerosis to begin. Interestingly, Hollis and Sloss (1975) have shown that aortic lipid deposition does not occur in the above rabbits until the fourth week of high cholesterol feeding, by which time histamine abnormalities have subsided.

Inhibition of aortic histamine synthesis in hypercholesterolaemic rabbits has clearly been shown to be associated with a diminished risk of atherosclerosis. Owen and Hollis (1979) have shown that administration of αHH, a specific inhibitor of HDC, to hypercholesterolaemic rabbits causes a 31% decrease in aortic histamine synthesis, and this is accompanied by a 51% reduction in aortic permeability, as well as a 63% reduction in lipid accumulation. This study showed that the simple partial inhibition of one aortic enzyme, namely HDC, in an atherogenic environment can reduce permeability by half, as well as reducing the severity of atherosclerosis by over 60%.

To date, very little work has been conducted to investigate alterations in histamine metabolism in human atherosclerotic vascular disease. In one study, Kalsner & Richards (1984) reported that coronary arteries obtained from patients with a history of coronary artery disease contained twice as much histamine as coronary arteries obtained from non-cardiac patients.

Furthermore, they showed that atherosclerotic regions of the coronary arteries contained more histamine than non-atherosclerotic segments.

It has also been shown that plasma histamine concentrations are significantly elevated in patients with coronary artery disease (Yoshimura *et al.*, 1984) when compared with healthy control subjects. They also showed that plasma histamine concentrations were significantly higher in patients with three vessel disease when compared to one vessel disease, indicating increased concentrations of plasma histamine with severity of atherosclerosis. It must, however, be stressed that this report measured histamine by a fluorescence method which is not satisfactory in estimating the low levels of histamine found in human plasma. In their study, Yoshimura and colleagues reported a.plasma histamine concentration of 57 ng/ml in their control subjects, which is over 100 times greater than that reported by many authors using more specific and sensitive techniques (Heavey *et al.*, 1984; McBride *et al.*, 1988; Dyer *et al.*, 1982).

(b) Histamine and Diabetes Mellitus

Numerous epidemiological studies have clearly established that both macroangiopathy and microangiopathy are major complications of diabetes mellitus. Macroangiopathy is manifested as atherosclerotic vascular disease, while microangiopathy presents clinically through its effects on renal glomeruli and retinal microcirculation (Steiner, 1981).

Diabetes mellitus is one of the most important risk factors for atherosclerosis (Brownlee & Cahill, 1975; Jarrett & Keen, 1975; Mitchell & Schwarz, 1963; Robertson & Strong, 1968; Gordon *et al.*, 1977). Diabetics show more advanced and more severe atherosclerosis at any given age than their non-diabetic counterparts. Vascular complications of atherosclerosis are the principle events responsible for increased mortality in diabetic patients (Stout, 1979).

The prevalence of diabetes mellitus in the general population and the predilection of diseased individuals towards the development of atherosclerotic vascular disease have fostered considerable interest concerning mechanisms which might account for the close association between the two disease processes. However, while it is clear that a strong

association between these two diseases exists, there is surprisingly little evidence clarifying the mechanisms involved. In part, this may be due to our lack of understanding of the factors which precipitate initial atherogenic events. Gaps in our knowledge may also result from the fact that little research has centered on examining alteration in arterial wall metabolism in diabetes - alterations which are common in both disease processes. Clarification of these alterations may help explain the prevalence of macrovascular sequelae in the diabetic population.

Wolinsky *et al.* (1978) reported that several aortic smooth muscle cell hydrolases were decreased following the induction of experimental diabetes. That acid cholesteryl esterase activity was significantly reduced was of particular interest, since acid cholesteryl esterase is the principle enzyme involved in catabolism and subsequent clearance of low density lipoproteins (Basu *et al.*, 1976). The results of their investigations thus suggest that experimental diabetes may lead to an impaired ability of the aorta to clear infiltrated lipid.

Intravascular lipid accumulation may also result from an impairment in the functional ability of the arterial endothelium to selectively regulate transmural macromolecule flux. Hollis and his co-workers have been examining the possibility that these alterations may stem from changes in histamine metabolism in the arterial wall. In several recent investigations, Hollis has examined aortic histamine synthesis under a number of conditions which predispose to atherogenesis. A consistent observation has been that aortic histamine formation increases regardless of the type of atherogenic stress to which the animal is subjected.

As mentioned earlier, Hollis and his group have also been examining aortic histamine metabolism following induction of experimental diabetes (Gallik & Hollis, 1981; Orlidge & Hollis 1982; Hollis *et al.*, 1983). These investigations have revealed a diabetes-induced elevation in aortic histamine synthesis coupled with a concomitant decrease in aortic histamine catabolism. They have observed increases in the intracellular histamine content of both aortic endothelial and smooth muscle cells in strepto-zotocin-induced diabetes, and have proposed that one change occurring in experimental diabetes is an expansion in the inducible, or nascent, hista-mine pool in the aortae of these rats. If this pool expansion can be preven-ted, then there is no concomitant change in aortic albumin

accumulation that normally occurs in this form of diabetes (Hollis *et al.*, 1983).

No reliable human data is available to date on histamine metabolism in diabetes mellitus. One article by Yoshida and co-workers (1982) estimated plasma histamine concentrations in diabetic patients. This article is seriously flawed, since all estimations of plasma histamine use a fluorimetric method and, as explained earlier, this method is totally inadequate in measuring the pg/ml quantities of histamine in human plasma. Interestingly, the histamine concentrations of the controls and various diabetic groups are all above 50 ng/ml, which is over 100 fold greater than accepted plasma histamine concentrations in man. Despite this gross inaccuracy in estimating plasma histamine, Yoshida reports increased plasma histamine in patients with both Type I and Type II diabetes mellitus.

(c) Development of Atherosclerotic Lesions

Atherosclerosis is characterised by a focal intimal thickening of medium- and large-sized arteries. Its clinical manifestations include cerebral and myocardial infarction and peripheral vascular disease. Epidemiological studies have identified several factors asssociated with an increased incidence of coronary heart disease and cardiovascular disease, such as hypercholesterolaemia, hypertension, smoking and diabetes. Some further information has been provided by recent angiographic studies which have demonstrated an association between high levels of low density lipoprotein cholesterol and coronary atherosclerosis (Holmes *et al* , 1981; Millner *et al.*, 1981; Hamsten *et al* , 1986). However, since the early stages of lesion formation are characterised by intimal cell proliferation as well as macromolecule infiltration, it is not possible to explain the aetiology of atherosclerosis simply in terms of cholesterol deposition in the arterial wall. On the basis of the finding that platelets contain a potent mitogen for smooth muscle cells, Ross & Glomset (1976) suggested that atherosclerotic lesions develop in response to endothelial denudation and subsequent platelet adherence and release of growth factors. Lately this hypothesis has been questioned, since it has been difficult to demonstrate a clear association between endothelial denudation and the development of atherosclerosis *in vivo* (Reidy, 1985).

An alternative concept has developed during recent years as it has become clear that mitogens can also be produced by cells present in the arterial wall (Seifert *et al.*, 1984; Nilsson *et al.*, 1985; Shimokado *et al.*, 1985; Di Corletto & Bowen-Pope, 1983). These findings raise the possibility that stimulation of smooth muscle cell replication may be initiated independently of platelet aggregation and release of platelet mitogens, and instead occur as a result of an endogenous process in the arterial wall.

Despite these alternative hypotheses, it is undisputed that platelets are involved in atherosclerosis, since the human atherosclerotic lesion contains substantial amounts of platelet specific material (Pearson *et al.*, 1979).

In experimental dietary hypercholesterolaemia, monocytes can be found adhering to the endothelial surface (Gerrity, 1981; Joris *et al.*, 1983; Faggiotto *et al.*, 1984; Fagiotto & Ross, 1984), and signs of increased endothelial turnover are noted (Florentin *et al.*, 1969). Within a month, areas with intimal oedema are observed. Infiltration of monocytes through the endothelium occurs; these cells then accumulate as resident macrophages in the intima. Accumulation of lipid-filled intimal macrophages (foam cells) protruding into the vessel lumen represent the earliest type of atherosclerotic lesion, the fatty streak. At a later stage, proliferating smooth muscle cells give rise to fibrous plaques at the same anatomical sites (Joris *et al.*, 1983; Fagiotto *et al.*, 1984; Faggiotto & Ross, 1984).

As briefly mentioned above, the two main blood-borne cell types involved in atherosclerosis are platelets and leucocytes. In the present study, both of these cells have been examined for alterations in histamine metabolism.

Platelets in atherosclerosis

Mammalian blood platelets (thrombocytes) are biconvex discs about 2-3 μm in diameter and are formed in the bone marrow by the division of megakaryocytes. Human platelets have a half-life of 8-12 days and circulate in blood (200-350 million platelets per milliliter of blood). They are removed from the circulation either by incorporation in haemostatic plugs or by the reticulo-endothelial system.

The platelet plasma membrane is rich in glycoproteins, and it serves many functions similar to those of the plasma membranes of other cells: for example, the platelet membrane is involved in the active transport of ions and metabolites. The platelet membrane is also involved in functions unique to the platelet, such as the shape change phenomenon. During shape change the platelet, in response to appropriate stimuli (e.g. collagen, adrenaline), changes shape from a disc to a sphere and proceeds to secrete intracellular contents. It eventually aggregates with other platelets.

Platelets, in common with certain granulocytic leucocytes, contain intracellular storage granules. The two main granular organelles in platelets are the dense and alpha granules. The dense granules contain biogenic amines such as serotonin and histamine, together with adenosine nucleotides (Da Prada *et al.*, 1981). The alpha granules contain mainly platelet-specific proteins such as β-thromboglobulin and platelet factor 4, as well as mitogenic proteins such as platelet-derived growth factor (PDGF - Da Prada *et al.*, 1981; Ross *et al.*, 1974).

The main function of the platelet is aggregation, and platelet aggregation is essentially a process of platelet-to-platelet adhesion in response to activating stimuli. Normal platelets in the circulation do not adhere readily to each other or to the vascular endothelium. When activated, however, platelets readily adhere to each other or to any exposed subendothelium (for example, when the vascular endothelium is damaged by atheroma, angioplasty or bypass surgery; Baumgartner & Hosang, 1988).

When platelets aggregate with each other or adhere to the endothelium, they secrete constituents which activate other platelets flowing past. When these events occur on the vascular endothelium they can lead to the formation of an aggregate which eventually leads to the development of a mural platelet thrombus (Ross, 1986).

Platelet adhesion and aggregation are initiated by specific stimuli interacting with specific receptors on the platelet surface membrane. Platelet aggregation can be induced by a variety of stimuli such as ADP, adrenaline, collagen and thrombin.

As mentioned earlier, when platelets are activated they release intraplatelet constituents. This release reaction can be either the selective release of

storage granules (e.g. serotonin and histamine release) or the selective release of constituents that are synthesised and secreted when required and are not stored, e.g. thromboxane A_2 (Gordan, 1981).

Platelets acquire biogenic amines such as serotonin from the plasma by active uptake via a specific carrier mechanism followed by subsequent storage in dense granules. Serotonin uptake by human platelets has been very well documented over the past twenty years (Tuomisto, 1983). In contrast, histamine uptake by human platelets remains relatively un-explored. Histamine uptake by pig platelets has been extensively examined previously, but only a very small number of studies have investigated histamine uptake by human platelets in any detail (Wood *et al.*, 1983, 1984). These previous studies using human platelets have assessed histamine uptake using inappropriate methods: for example, the studies used platelets prepared from bloood anticoagulated with EDTA. EDTA is a known platelet inhibitor and it disrupts the platelet membrane (Pidard *et al.*, 1986). These studies assessed uptake by using platelets that were not stirred. Platelets are known to be totally non-responsive to any stimulus in the absence of stirring (Born & Cross, 1963). Furthermore, they assessed histamine uptake using concentrations of labelled histamine which were more than 300-fold greater than normal plasma histamine concentrations found in man (Brown *et al.*, 1980; Morel & Delaage, 1988). In this study we have examined histamine uptake by human platelets using methods that overcome the above problems.

The role of platelets in thrombus formation in atherosclerosis has been briefly mentioned earlier. Platelets are capable not only of forming thrombi at sites of vascular endothelial damage but are also capable, when activated by adherence, of secreting various mitogenic factors such as PDGF. PDGF released from platelets is capable of inducing both smooth-muscle migration and proliferation; a key event in atherogenesis. It is also known that when activated, platelets can release biogenic amines such as histamine. Histamine, as mentioned earlier, is capable of inducing a multitude of effects on the endothelium.

Leucocytes in atherosclerosis

Circulating blood contains colourless cells called white blood cells or leucocytes, which possess nuclei. There are three main varieties of leucocytes - the granulocytes, the lymphocytes and the monocytes.

The monocyte, 16-22 μm in diameter, is formed in the bone marrow and is the largest leucocyte in blood; it is mainly involved in phagocytosis. Lymphocytes are formed predominantly in the lymphatic tissues, although some are formed in the bone marrow. Both lymphocytes and monocytes contain significant amounts of histamine (Graham *et al.*, 1955).

There are three types of granulocytic leucocyte - the neutrophil, the eosinophil and the basophil. These granulocytes are distributed between the bone marrow, the circulating blood and the tissues. The lifespan of the mature granulocyte in blood is no more than about 30 hours. One of the main functions of granulocytes is protection of the body from infection, and phagocytosis is the main way in which these leucocytes exert this function.

The neutrophils and eosinophils both contain significant amounts of histamine, while the basophilic leucocyte contains very large amount of histamine (Graham *et al.*, 1955). It is often thought that the basophil in the blood is the equivalent of the tissue-bound 'mast' cell, since both these cells are anatomically almost identical, and both contain vast stores of histamine in their intracellular granules.

In the intact circulation, 5%-10% of circulating leucocytes are found as a 'marginal pool' slowly rolling along the endothelium of small blood vessels. In inflammation, adherence of leucocyctes to endothelial cells increases greatly (O'Flahertyt *et al.*, 1978). This increased adherence of leucocytes, preceding emigration into the vessel wall, is considered to be the key event in cellular reactions to vascular damage and induces endothelial damage and increased endothelial permeability (O'Flaherty *et al.*, 1978; Harlan, 1985). Hence, increased adherence of leucocytes is believed to initiate endothelial damage in atherosclerosis (Schwartz *et al.*, 1986). The ubiquitous fatty streak is the earliest lesion of atherosclerosis and is a lipid-rich lesion consisting of both leucocytes and smooth muscle cells (McGill, 1968). The fatty streak is known to progress and form a

smooth muscle rich fibrous plaque. Leucocyctes are again present in and around these plaques (Ross,. 1986).

Any attempt at explaining the aetiology and development of atherosclerosis must take into account several different aspects of the disease:
- which factors are responsible for the increased endothelial permeability and the intimal infiltration of monocytes seen in the early stages of the disease?
- how is the transition of smooth muscle cells into 'synthetic' phenotypes initiated and which factors influence smooth muscle cell replication in the arterial intima?
- which factors regulate the accumulation and clearance of intra- and extracellular lipids in atherosclerotic lesions?

It is clear that atherosclerosis constitutes the interaction of the endothelium and smooth muscle cell layer, not only with each other but also with blood-borne cells such as platelets and leucocytes; all of these cells are dependent on several initiating factors. It is one of these initiating factors, histamine, that this work aims to investigate.

D. Aim of Study

The main aim of this study was to investigate alterations in histamine metabolism (a) in experimental non-ketotic diabetes mellitus in the rat and (b) in patients with diabetes mellitus and/or peripheral vascular disease.

Plan of Study

a. Animal Studies

(i) Histamine metabolism (histamine content, histamine synthesis and catabolism) in various tissues and plasma of control and diabetic rats.

(ii) Histamine content and synthesis in various tissues of control and 3-day starved rats.

(iii) Determination of the effect of H_1 and H_2 receptor antagonists on histamine metabolism in various tissues of control and diabetic rats.

(iv) Determination of the effect of H_1 and H_2 receptor antagonists on aortic permeability in control and diabetic rats.

b. Human volunteer and patient based studies

(i) Histamine metabolism in plasma, leucocytes and platelets of controls and patients with DM and/or PVD.

(ii) Determination of the effects of various activating stimuli on [^{14}C]histamine uptake by platelets obtained from normal volunteers.

CHAPTER II

MATERIALS and METHODS

1. MATERIALS

a. Chemicals

All the chemicals used were of analytical grade: sodium hydroxide, sodium dihydrogen phosphate, disodium hydrogen phosphate, pyridoxal-5-phosphate, sodium hydrogen sulphate, streptozotocin anomers, hog kidney diamine oxidase, histamine dihydrochloride, horseradish peroxidase type II, 3-(dimethylamino) benzoic acid (DMAB) and 3-methyl-2-benzothiazolinone hydrazone hydrochloride monohydrate (MBTH) were purchased from the Sigma Chemical Company (Poole, Dorset, UK). Disodium ethylenediamine tetraacetic acid (EDTA), trisodium citrate, acetyl salicyclic acid, sodium chloride, diethyl ether, hydrochloric acid, sulphuric acid, tris hydrochloride, bovine serum albumin, Folin-Ciocalteu's reagent, calcium chloride, magnesium sulphate, magnesium chloride, sodium bicarbonate, potassium chloride were purchased from BDH (Enfield, Middlesex, UK). Sagatal was purchased from May and Baker Limited (Dagenham, Essex, UK). Amberlite CG50 (100-200 and 200-400 wet mesh) was purchased from Fluka Chemicals (Basle, Switzerland).

b. Radionuclides

[methyl-^{3}H]-S-adenosylmethionine, [ring-2-^{14}C]histamine dihydrochloride, L-[2,-5-^{3}H]histidine were obtained from the Amersham Radiochemical Centre (Aylesbury, Bucks, UK). [^{125}I]bovine serum albumin (60,000 Mwt) was purchased from New England Nuclear (Herts, UK).

c. Commercially available monoclonal histamine radioimmunoassay was obtained from Immunotech SA (Marseille, France).

d. Scintillation fluids

Liquiscint was obtained from National Diagnostics (New Jersey, USA).

e. Test tubes and Dispensaries

10 ml conical polypropylene tubes, 10 ml sterile conical polystyrene tubes were obtained from Sterilin Limited (Middlesex, UK). Eppendorf 1.5 ml microcentrifuge tubes from Eppendorf (Alan Medical Limited, Sussex, UK). Micropipettes and automatic liquid dispensers from Gilson (Anachem Limited, Luton, Beds., UK); Bio-Rad Econo columns from Bio Rad Limited (Stevenage, Herts, UK); Multistix from the Ames Division of Miles Laboratories Limited (Stoke Poges, Bucks, UK).

f. Instruments

1. Centrifuges
   (a) IEC Centra 7R (refrigerated) and IEC Microcentaur microfuge from International Equipment Company (New Jersey, USA)
   (b) MSE Bench Centrifuge from MSE Instruments (Sussex, UK)
   (c) Sorrall ultracentrifuge from Sorrall Limited (Herts, UK)
2. Shaking temperature controlled water bath from Grant Instruments (Cambridge, UK)
3. Whirlimix vortex mixer and magnetic stirrer from Gallenkamp (Middlesex, UK)
4. pH meter from Corning Science Products (Beds, UK)
5. Precisa 80A balance from Precisa Limited (Switzerland)
6. Spectrophotometer SP1700 Ultraviolet from Pye Unichem (Cambridge, UK)
7. LKB Wallac 1280 Ultragamma gamma counter and LKB Wallac 1219 Rackbeta beta counter from LKB Wallac (Switzerland)
8. Vacuum temperature-controlled dessicator and MSE Soniprep 150 from MSE Limited (Sussex, UK)
9. Kinematic Polytron homogeniser from Kinematica Ltd (Basle, Switzerland)
10. Omniscribe chart recorders, Coulter ZM Counter, Chronolog aggregometers, siliconised aggregometer tubes, mini magnetic stir bars and Coulter Channelyser with X-Y recorder from Coulter Ltd (Beds., UK)
11. ICA 1 ionized calcium analyzer from Radiometer (Copenhagen, Denmark)
12. YSI 23 AM glucose analyzer from Yellow Springs Instruments Ltd (Michigan, USA)
13. SMAC Autoanalyzer from Technicon Instruments (Herts., UK)

2. ASSAYS

HISTAMINE ASSAY

A. Double isotope radioenzymatic assay

(a) The Principle

This assay is based on the method of Beavan and Horakova (1978), with modifications by Keeling (1984). The assay uses a double isotope method, where histamine is methylated by the enzyme histamine-N-methyl-transferase (HMT; EC 2.1.1.8). If the source of the methyl group is labelled [^{3}H], the enzyme product also becomes labelled, and the amount of label can be determined in a suitable counter.

To determine the efficiency of the enzyme conversion and of the isolation technique, a second isotope is employed. A known quantity of [^{14}C]-histamine can be added at the beginning of the reaction and the amount of labelled [^{14}C]methylhistamine recovered can be determined. The proportion of the [^{14}C]--histamine that has been converted gives an indication of the total histamine that has been methylated by the enzyme and the extraction efficiency.

(b) Preparation of histamine N-methyltransferase and storage of radioactive materials

Histamine N-methyltransferase (HMT) was prepared by the method of Shaff and Beavan (1979). The kidneys from seven male Sprague-Dawley rats were washed and homogenised in ice-cold 0.25 M sucrose (10 ml/g tissue) using a Polytron homogeniser (speed 3 for 1 min). The homogenate was centrifuged at 126,000 *g* for 60 min at 4 °C and the pellets discarded. That fraction of the supernatant precipitating between 45% and 70% saturation with ammonium sulphate (at 4 °C) was resuspended in 15 ml 10 mM sodium phosphate buffer, pH 7.4, and dialysed at 4 °C for 36 h against 3 changes of 2 l sodium phosphate buffer. After dialysis, the HMT preparation was frozen in 1 ml aliquots and stored at -40 °C. The protein content of the HMT preparations was estimated and was usually between

12-15 mg/ml. This was diluted 10-fold with 0.1 M sodium phosphate buffer (pH 7.4) immediately before use.

[Methyl-^{3}H]-S-adenosylmethionine was diluted with 0.5 mM sulphuric acid to 50 µCi/ml before being flash-frozen in liquid nitrogen in aliquots and stored at -40 °C. Before use, the aliquots were allowed to thaw at room temperature and then kept on ice.

[ring-2-^{14}C]histamine was diluted with 1 mM HCl to 0.05 µCi/ml before being flash-frozen in liquid nitrogen in aliquots and stored at -40 °C. Aliquots were allowed to thaw at room temperature before use and then kept on ice.

(c) The assay procedure

Incubations in the histamine assay were performed in a total volume of 500 µl in 10 ml conical polypropylene tubes and consisted of the following:

(1) sample material or standard made up to 380 µl with 0.1 M sodium phosphate buffer, pH 7.8;

(2) 10 µl [methyl-^{3}H]-S-adenosylmethionine ([^{3}H]-SAM 500 mCi/mmol : 0.5 µCi);

(3) 10 µl [ring-2-^{14}C]histamine dihydrochloride (56 mCi/mmol : 1000 dpm);

(4) 100 µl histamine-N-methyltransferase preparation.

Incubations were started by the addition of HMT preparation to the other components, and were continued for 90 min at 25 °C. The reactions were stopped with 200 µl 5 M NaOH. The radiolabelled methylhistamine was extracted into 5 ml chloroform. After vigorous shaking for 20 sec, the phases were separated by centrifugation at 1500 *g* at room temperature for 5 min and the aqueous layer was removed and discarded. Care was taken to remove as little of the chloroform layer as possible. The chloroform layer was washed with 1 ml 3.3 M sodium hydroxide, shaken and centrifuged as described above. The aqueous phase was again discarded. Of the remaining chloroform phase, 4 ml aliquots were transferred to scintillation vials and were then evaporated to dryness in a vacuum dessicator (connected to an electric pump). Liquid scintillation fluid (Liquiscint), in 10 ml

aliquots, was added to the vials, which were then counted for [^{3}H] and [^{14}C] radioactivity simultaneously, using an LKB Rackbeta scintillation counter.

To determine the amount of [^{3}H] labelled methylhistamine in the assay, a standard curve was obtained in the range 0-20 ng histamine free base (using histamine dihydrochloride in 1 mM HCl) by substituting aliquots of sample material with standard histamine solutions. In calculating the data, the [^{3}H]methylhistamine detected was corrected for recovery of [^{14}C]histamine. A typical standard curve is illustrated in Figure 2.

(d) Assay Characteristics

The lower limit of detectability in this double isotope radioenzymatic assay was 0.7 ng/ml histamine. The intraassay coefficient of variation (n=10) on both samples and standards was 8.2% and the interassay coefficient of variation was 10.5%. Intra-individual variation in the leucocyte content of histamine, assessed in 6 healthy volunteers, was 13.2%.

B. Plasma histamine determination by radioimmunoassay (RIA)

The RIA used in this study was purchased as a kit from Immunotech SA (Marseille, France). This new monoclonal antibody RIA for the measurement of histamine in biological fluids is the only assay currently available which can adequately measure histamine concentrations in human plasma without having to perform thin layer chromatography or high performance liquid chromatography.

(a) The Principle

The assay is based on the conversion of histamine in samples to an 'acylated' succinyl-glycinamide derivative (Morel & Delaage, 1988). The acylated histamine then mimics the immunogen used to generate the highly specific monoclonal antibody. The assay has been shown to exhibit a linear response from 0.1 to 5.0 ng/ml of histamine and the monoclonal antibody used is very specific for histamine, and with minimal modifications, the assay can accurately measure histamine in human plasma (McBride *et al.*, 1988). The histamine determination makes use of the

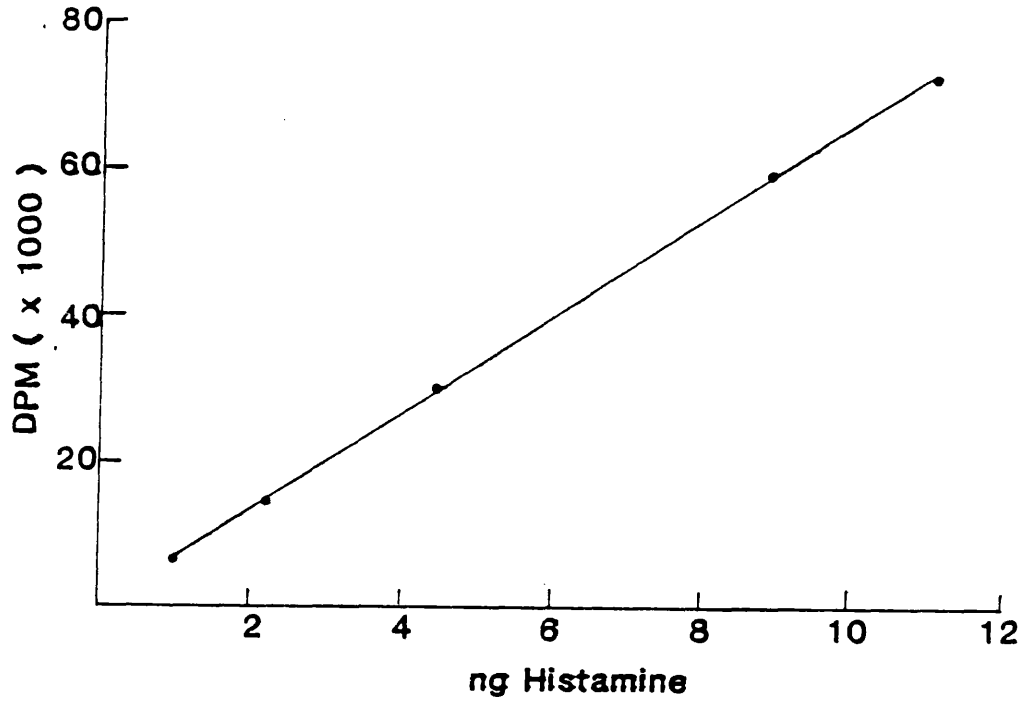

Figure 2. A typical standard curve for histamine when using the double isotope radioenzymatic method.

competition of 'acylated' histamine and ^{125}I-acylated histamine for their binding to mouse monoclonal antibody coated on to plastic tubes.

(b) Reagents

The manufacturer's kit provides the following reagents:

1. ^{125}I-radiolabelled histamine (1 vial: 55 ml). The vial contains 4 μCi ^{125}I-acylated histamine diluted in a buffer containing protein, sodium azide and a red dye. It was stored at 4 °C.

2. Acylation reagent (enough for 50 tubes). Each tube contains 1 mg lyophilised acylating reagent; stored at 4 °C.

3. Standards (7 vials: 1 ml) containing respectively 0, 0.5, 1.5, 5, 15, 50 and 150 nM histamine in buffer with 5 mM sodium azide as a preservative. These standards were kept frozen to increase shelf life.

4. Acylation buffer (1 vial: 5 ml borate buffer, pH 8.2).

5. 100 anti-histamine antibody coated tubes.

(c) Radioimmunoassay procedure

Acylation of samples and standards

(a) The acylating reagent (succinyl glycinamide N-hydroxysuccinimide ester) was collected at the bottom of the polypropylene conical tube by gentle tapping on the laboratory bench.

(b) The tubes were then acylated tube by tube:
- 100 μl standard solution or plasma was added to acylating powder
- this was immediately followed by the addition of 50 μl acylating buffer
- the conical tubes were recapped and the constituents mixed on a vortex mixer rapidly until complete solubilisation of the reagent (even traces remaining on the cap)
- the tubes were then incubated for 30 min at room temperature.

Competition radioimmunoassay

At room temperature:

- 50 µl acylated standard of acylated plasma was added to coated antibody tubes
- 500 µl iodinated tracer was added to this
- two regular tubes were kept for total and 0 standard radioactivity determination

At 4 °C:

- the antibody tubes were incubated for 18 h minimum at 2-6 °C
- the tubes were aspirated (at 4 °C). All the fluid was drawn up using a Pasteur pipette attached to a vaccum source
- all the emptied tubes were counted for gamma radioactivity in an LKB gamma counter for 1 min.

The standard curve and results

A standard curve was drawn on semi-logarithmic graph paper by plotting histamine concentration of standards on the horizontal axis, and average (of duplicates) bound/0 standard counts (B/Bo) values on the vertical axis.

The histamine concentration of plasma was determined by direct reading on the standard curve. A typical standard curve is illustrated in Figure 3.

(d) Assay characteristics

The lower limit of detectability in this modified RIA was 10 pg/ml histamine, similar to that of HPLC methodology. The intra-assay co-efficient of variation (n=6) on both plasma samples and standards was 7.3% and the inter-assay variation was 9.5%. Intra-individual variation of plasma histamine concentration, assessed in 6 healthy volunteers, was 11.9%.

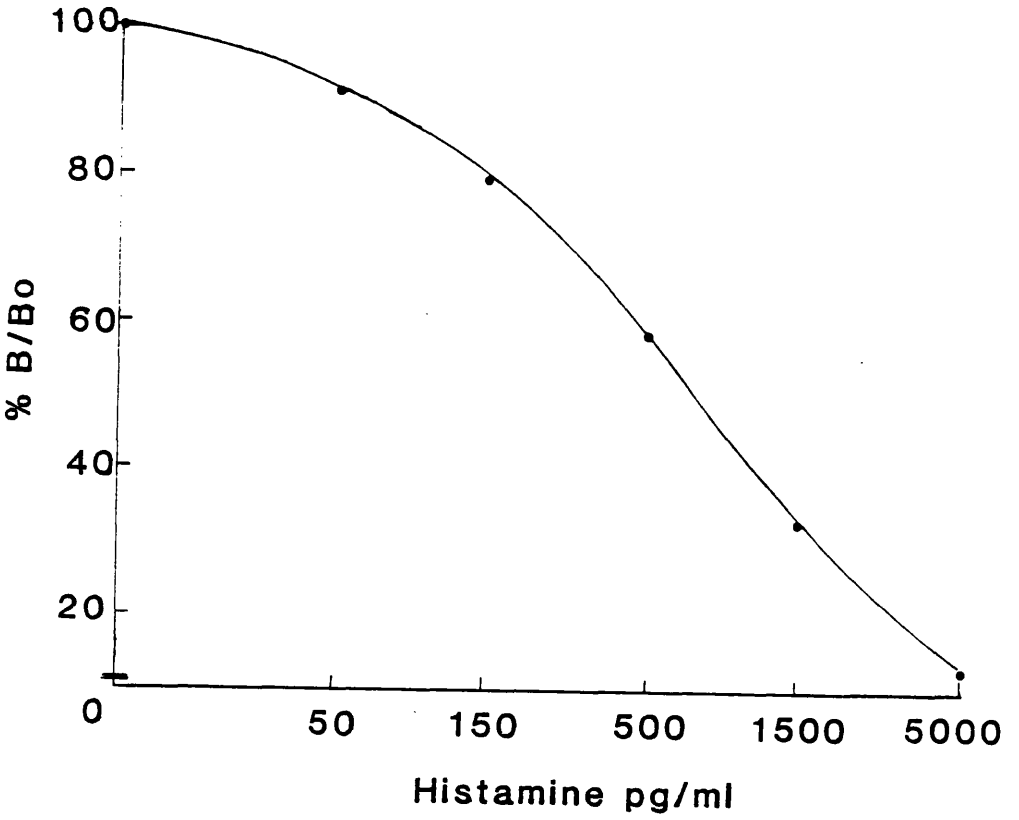

Figure 3. A typical standard curve for histamine when using the monoclonal antibody radioimmunoassay method.

HISTIDINE DECARBOXYLASE ASSAY

(a) The principle

The assay is based on the conversion of [^{3}H]labelled histidine to [^{3}H]-labelled histamine by HDC (EC 4.1.1.22). (Baudry *et al.*, 1973; Keeling & Smith, 1979). A known quantity of [^{3}H]-labelled histidine and necessary co-factors were incubated with the tissue homogenate containing the enzyme.

The converted [^{3}H]histamine was then isolated from unconverted [^{3}H]-histidine by three different isolations, involving chloroform extraction, ion-exchange chromatography and finally a scintillant extraction. The [^{3}H]-histamine was then counted by β-scintillation spectrometry.

(b) Purification of [^{3}H]-histidine

[ring 2,5-^{3}H]-histidine was stored in sealed vials at 4 °C. Sufficient radioactivity for one day's work was removed for purification, using a syringe. This was applied to a small Amberlite column (200-400 mesh; 50 µl bed volume) equilibrated in 0.1M Tris/Cl, pH 8.0. The column was washed with distilled water (twice the volume of applied radioactivity). The combined effluents contained the purified [^{3}H]-histidine (0.33 µCi/µl).

(c) Preparation and use of Amberlite

The ion-exchange resin used in the HDC assay was Amberlite CG 50 (H form; 100-200 mesh). A finer mesh Amberlite (200-400) was used to purify the [^{3}H]histidine. The Amberlite resin was equilibrated in bulk, using the appropriate buffer: 0.1 M Tris/Cl, pH 8.0. The drug resin was first suspended in five volumes of distilled water and allowed to settle. The cloudy supernatant containing fine particles was discarded. This was repeated twice before the resin was suspended in 1 M Tris base and poured into a large column. 0.1 M Tris/Cl buffer, pH 8.0, was then passed through the column (10 ml/min initially; slowing down to 2 ml/min after 4 h) until the pH of the material eluting from the column was 8.0.

The equilibrated resin was stored with an equal volume of equilibrating buffer such that 1 ml of the slurry produced 0.5 ml bed volume of resin.

For the separation procedure in the HDC assays, poly-propylene Econo-columns were used. A 1 ml aliquot of resin slurry was added to the column to give a 0.5 ml bed volume. The columns were held in a rack such that direct elution into a tray of scintillation vials was possible. The resin was added to the columns within 30 min of sample application and was discarded after one use.

The ion-exchange column used to purify [^{3}H]histidine was made by cutting the end off a 200 µl Gilson pipette tip. A disc cut from the filter of a Bio-Rad Econo-column was fixed to the end of the pipette tip with Superglue; this small column held the 50 µl of ion-exchange resin used to purify the [^{3}H]histidine. Slight positive pressure was sometimes required to force solutions through the mini-column; this was achieved using a 10 ml syringe.

(d) The assay procedure

Incubations (50 µl) were performed in capped 1.5 ml Eppendorf tubes. Sample material (0-30 µl) was made up to 40 µl with 0.1 M sodium phosphate buffer, pH 7.2. The reaction was then started by the addition of 10 µl freshly prepared cocktail containing the following:

2 µl 0.25 mM pyridoxal-5-phosphate
2 µl 2.5 mM histamine
3 µl purified L-[2,5-^{3}H]-histidine (40-60 Ci/nmol; ≅ 1µCi)
3 µl 0.1 M sodium phosphate buffer, pH 7.2

After a 120 min incubation at 37 °C the reactions were stopped by the addition of 250 µl of 0.3 M sodium acetate buffer, pH 4.5, containing 10 µg histamine, followed by 1 ml chloroform containing 0.1 M diethylhexyl-phosphoric acid (DEHPA). This effectively acts as the first isolation procedure, since it allows only [^{3}H]histamine (and [^{3}H]methylhistamine) to pass into the organic solvent phase but prevents movement of [^{3}H]histidine. After the addition of stopping solutions, the tubes were thoroughly mixed on a vortex mixer and centrifuged at 2600 *g* for 2 min in a micro-centrifuge. The aqueous phase was removed and discarded, and the remaining chloroform washed twice with 250 µl of 0.3 M sodium acetate buffer, pH 4.5. The [^{3}H]histamine was back extracted with 200 µl 1M HCl

and mixed with 5 ml 0.1 M Tris/Cl buffer, pH 8.0, which contained sufficient 5 M NaOH to give a final pH of 7.5-8.5 (approximately 40 µl). This mixture was loaded into an ion-exchange column containing Amberlite CG50 ion exchange resin (100-200 wet wash; 0.5 ml bed volume) equilibrated in 0.1 M Tris/Cl, pH 8.0. The columns were washed with 2x10 ml 0.1 M Tris/Cl and any bound labelled product eluted with 1.5 ml 1 M HCl into plastic scintillation vials containing 3.5 ml 0.3 M sodium acetate buffer and sufficient 5 M NaOH to give a final pH of 4.5-5.0 (approximately 130 µl). Toluene (10 ml), containing 0.4% (w/v) diphenyloxazole (PPO) and 0.1 M diethylhexylphosphoric acid (DEHPA), was added and the vial shaken vigorously for approximately 5 sec to ensure complete extraction into the scintillant. The radioactivity in the organic toluene phase could then be determined without separation of the aqueous layer. Only 0.09 ± 0.006% (SEM; n=5) of the radioactive substrate remaining in the aqueous phase could be detected by the scintillant.

Assay blanks were determined by replacing sample material with 0.1 M sodium phosphate buffer, pH 7.2, or by stopping an incubation containing homogenate at zero time. The amount of radioactivity observed in such blanks was 40-50 dpm (scintillant background 20-25 dpm).

(e) Calculation of HDC activities

The histidine concentrations used in this assay were in the range 0.3 µM-0.5 µM. This is more than 100 times lower than reported K_m values for the enzyme (Keeling & Smith, 1979; Palacios *et al.*, 1976) under the conditions used in this assay. The observed rate of reaction should therefore be proportional to the concentration of histidine used. The concentration of [^{3}H]-histidine used in each set of assays was determined by counting an aliquot of the assay cocktail prepared on that day. In order to standardise the results between sets of assays, the observed enzyme activity was divided by the concentration of histidine used (in µM) to give that activity which would have been observed had the histidine concentration been 1 µM.

(f) Assay Characteristics

The sensitivity of the assay, defined as that HDC activity (measured at 1 μM histidine) giving double the assay blank after a 120 min incubation, was 0.9 fmol/min/ml. The intraassay coefficient of variation (CV) was 6.2% and the interassay CV was 8.1%. Intra-individual leucocytic HDC variation was 14%, as assessed in six healthy volunteers.

HISTAMINASE (DIAMINE OXIDASE) ASSAY

(a) The Principle

This assay is based on the spectrophotometric method of Stoner (1985) and it involves the generation of hydrogen peroxide from histaminase (EC 1.4.3.6.) by a coupled enzymatic reaction. In the presence of hydrogen peroxide and peroxidase, the chromagen 3-methyl-2-benzothiazolone (MBTH) is oxidatively coupled to 3-(dimethyamine) benzoic acid (DMAB), forming a purple indamine dye, which has an absorption maximum at 595 nM. Therefore, using hog kidney diamine oxidase (histaminase) as a standard, unkown enzyme activities can be estimated.

(b) The assay procedure

Reagents

- Hog kidney diamine oxidase (DAO; EC1.4.3.6, 0.06 units/mg) was dissolved in sodium phosphate buffer (0.07 M, pH 6.7) at a concentration of 0.5 mg DAO/ml. The DAO solution was freshly prepared, kept at 4 °C and used only for each day's work. One unit of DAO will deaminate 1.0 μmole of putrescine/h at pH 7.2 at 37 °C.

- Horseradish peroxidase (EC 1.11.1.7, Type II, 150-200 purpurogallin units/mg) was dissolved in sodium phosphate buffer at a concentration of 0.34 mg/ml. The peroxidase solution was freshly prepared on the day of the assay. One unit will form 1 mg purpurogallin in 20 s from pyrogallol at pH 6 at 20 °C.

- MBTH was dissolved in distilled water at a concentration of 0.6 mmol/l. This solution was stable for 3 weeks at room temperature.

- DMAB was dissolved in sodium phosphate buffer at a concentration of 18 mmol/l. This solution was stable for 3 weeks at room temperature.

Spectrophotometric measurements

Absorbance measurements were obtained with a SP 1700 UV spectrophotometer at 25 °C using quartz cuvettes with a 1 cm path length.

Assay of DAO activity

Reaction mixtures consisted of the following in 2 ml:

- 17 µg horseradish peroxidase (50 µl)
- 20 µmol/l MBTH (100 µl)
- 1.0 mmol/l DMAB (170 µl)
- 0.7 mmol/l histamine (50 µl)
- Sodium phosphate buffer (1380 µl)

- DAO - standard or sample (250 µl)

Reagent blanks consisted of all the above chemicals in the reaction mixture except the substrate (DAO). The assay was initiated by the addition of DAO standard or unknown sample. Samples consisted of tissue homogenate supernatants (centrifuged at 10,000 *g*.) and rat plasma samples. Leucocyte samples were assayed as homogenates without centrifugation. Ten minutes after the addition of substrate, the absorption was read at 595 nm. A typical standard curve is illustrated by Figure 4.

Diamine oxidase activity was also assayed as a function of time. This mixture contained:

- 17 µg horseradish peroxidase
- 20 µmol/l MBTH
- 1.0 mmol/l DMAB
- 0.7 mmol/l histamine
- Sodium phosphate buffer

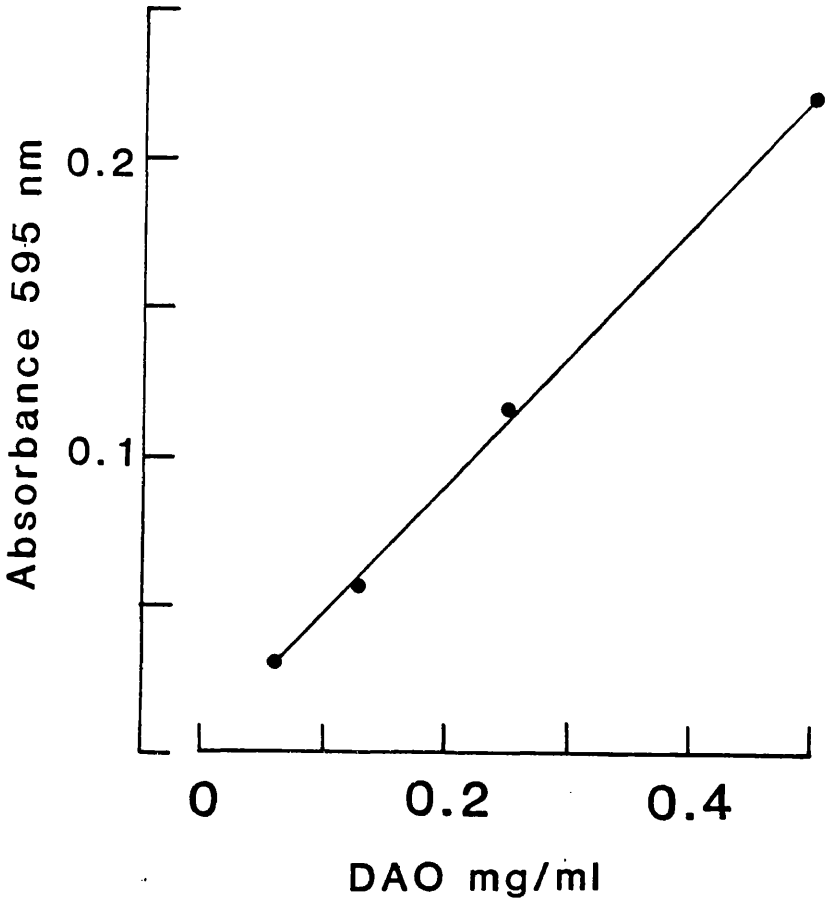

Figure 4. A typical standard curve for histaminase when using the spectrophotometric method.

The reaction was started by the addition of various amounts of DAO, bringing the total volume to 3 ml and read over a period of 10 min. See Figure 5.

(c) Assay characteristics

The sensitivity of this modified spectrophotometric assay was 0.9 pmol/h/ml homogenate or plasma. DAO has previously been assayed by other workers, either by biologic (Ahlmark, 1944) or colorimetric (Kapeller-Adler & McFarlane, 1963) procedures, and by the deamination of ^{14}C-putrescine according to the procedure developed by Okuyama & Kobayashi (1961). Both types of assays give comparable values and appear to be equally sensitive (Tryding & Willert, 1968). The colorimetric method of Stoner (1985), employed in this study, has several advantages over the putrescine assays. The methodology is relatively simple and the reagents required cost less collectively than ^{14}C-putrescine alone. Furthermore, this is a direct assay of histaminase activity, since histamine is used as a substrate rather than putrescine.

The intraassay CV for this assay was 9.1% (n = 10) and the interassay CV was 13% (n = 4).

Measurement of protein concentration (Lowry *et al.*, 1951)

A micro-adaptation of the method of Lowry was used to determine protein in the leucocyte samples and in the rat tissues.

(a) Reagents

I. Alkaline sodium carbonate solution:

Sodium carbonate -	200 mmol/l	(Na_2CO_3)
Sodium hydroxide -	100 mmol/l	(NaOH)

II. Copper sulphate - 40 mmol/l ($CuSO_4$)

III. Sodium potassium tartrate - 80 mmol/l

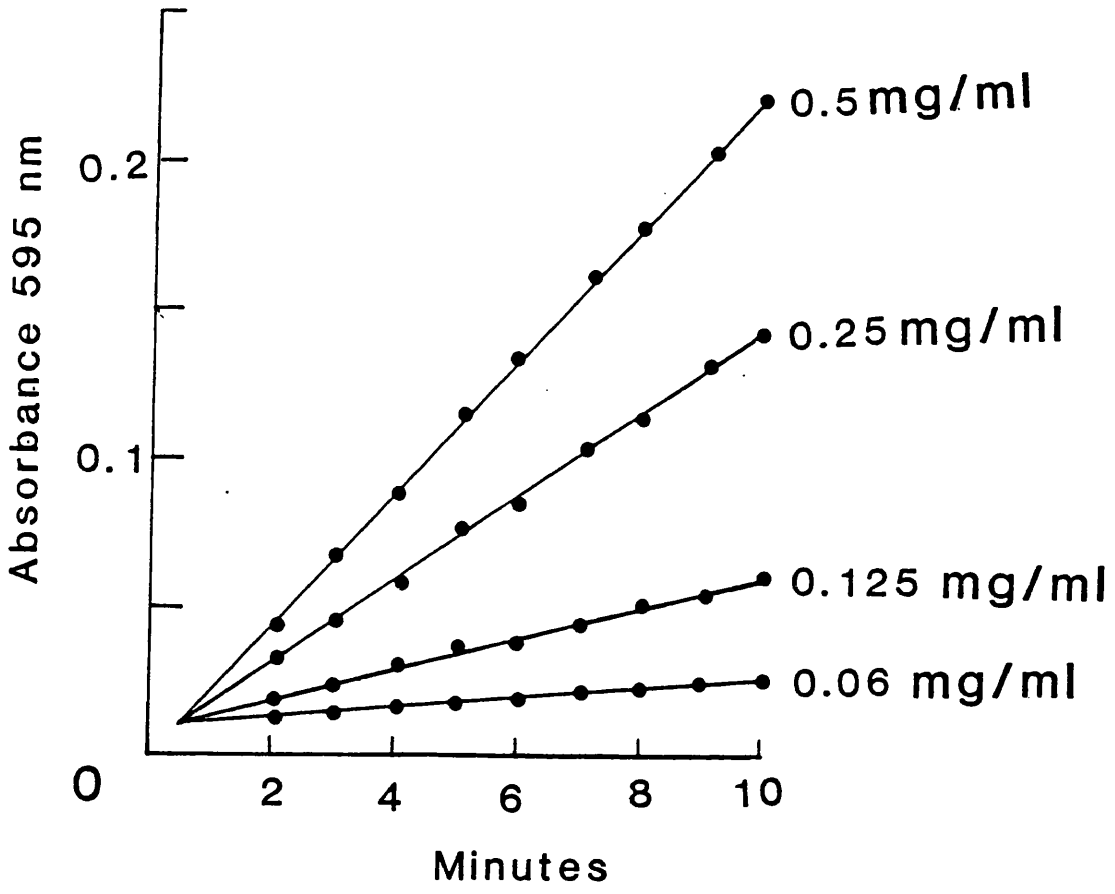

Figure 5. A typical curve of diamine oxidase activity as a function of time, using various concentrations of diamine oxidase.

IV. 'Alkaline solution' - prepared by adding 1 ml sodium potassium tartrate followed by 1 ml copper sulphate solution to 100 ml alkaline sodium carbonate solution. A fresh solution was prepared for each assay.

V. Folin-Ciocalteau reagent - commercial reagent was diluted 1:1 with distilled water prior to use.

(b) Standard Protein Solution

A standard commercial protein solution was diluted appropriately, or else a solution was prepared from bovine serum albumin. All working standards were diluted in solution I to give final concentrations of 0.05, 0.1, 0.15, 0.2 and 0.25 g/l.

(c) Assay Procedure

0.2 ml of digested leucocyte or tissue sample, or of protein standard, was taken into microcuvettes in duplicate, with 0.2 ml of Solution I as a blank. 1 ml of alkaline solution was added to each and left for 10 min. Folin-Ciocalteu reagent (0.1 ml) was then added to each cuvette and immediately mixed by inversion, with parafilm over the end of each cuvette. The spectrophotometer was zeroed by reading the blank against distilled water. The absorbance of the sample was read after 30 min at 700 nm. The protein content of each sample was calculated from the standard protein curve. A typical standard curve is illustrated in Figure 6.

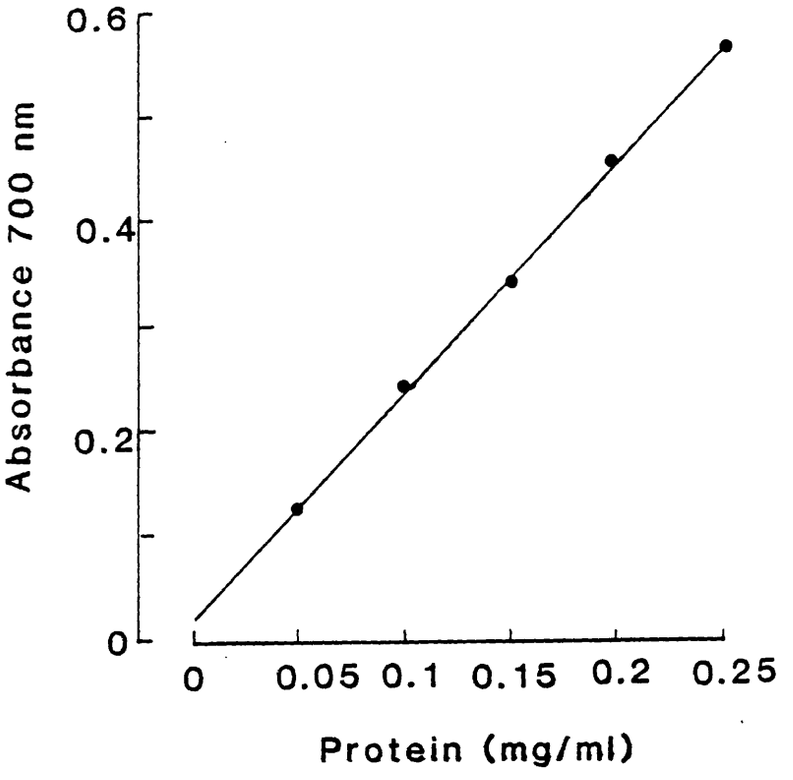

Figure 6. A typical standard curve for protein measurement using the Lowry method.

3. ANIMAL STUDIES

Experimental non-ketotic diabetes

The animals used in the diabetic studies were male albino Sprague Dawley rats of initial weight between 230 and 240 g. They were housed in groups in wire-bottomed cages with a dark/light cycle lasting 12 h each and provided food (Diet 41B) and water *ad libitum.*

Induction of Diabetes in rats

Animals were left in a warmed cage (heated with a 100 w light bulb) for 30 min to induce peripheral vasodilatation. After 30 min the animals were removed individually and introduced into a restraining cage. The animals were then anaesthetised with diethyl ether (soaked into cotton wool and held at the breathing aperture of the restraining cage). A tourniquet (rubber tubing) was then applied to the top of the rat's tail and one of the four lateral tail veins injected with 0.5 ml streptozotocin (65 mg/kg body weight in 0.01 M sodium citrate buffer, pH 4.5). Control animals were injected with 0.5 ml sodium citrate buffer only.

Three days after injection with streptozotocin, urinary glucose and ketones were measured using Multistix enzyme reagent strips. Of the streptozotocin-injected animals, only those that developed marked glycosuria were diagnosed as having diabetes. Animals injected with streptozotocin not exhibiting glycosuria were sacrificed and not included in any other group.

On the day of the experiment (30 days after the detection of glycosuria for the permeability and antagonist experiments; 60 days after the detection of glycosuria for all other experiments), the animals were injected with pentobarbitone (Sagatal; 90 mg/kg body weight) intraperitoneally. After the onset of anæsthesia, a cardiac puncture (one per animal) was performed to exsanguinate the animal. The blood was collected in fluoride/oxalate bottles for glucose measurement, and in lithium heparin (2 iu/ml) bottles for plasma biochemistry and histamine measurement. The abdomen was shaved and a 5 cm^2 sample of abdominal skin was removed, the underlying fat stripped, and skin biopsies were obtained using a 5 mm diameter

ophthalmic trephine. The entire length of the thoracic aorta was removed and cleared of its periadventitial fat. Relevant tissues were then removed and cleared of attached fat. All tissues were quickly washed in ice-cold 0.1 M sodium phosphate buffer (pH 7.4). The tissues were then divided into three pieces and immediately stored at -40 °C in 5 volumes of 0.1 M phosphate buffer.

Analysis of blood samples

Plasma concentrations of the following were determined using standard methodology for the SMAC AutoAnalzyer: sodium, chloride, total calcium, bicarbonate, potassium and urea. Blood glucose was determined using a YSI glucose analyzer. Whole bood calcium was determined using an ICA 1 ionized calcium analyzer. Three values were obtained using this instrument: blood pH, and actual and corrected (pH 7.4) ionised calcium concentration, the latter being a derived value.

On the day of the appropriate assay, the tissues were allowed to thaw at room temperature and then homogenised at 4 °C in 10 volumes of sodium phosphate buffer, pH 7.4, using a Kinematic Polytron homogeniser (3 x 10 s, speed 8). The homogenates were centrifuged at 1000 x *g* for 5 min and the supernatants were then assayed for either histamine, HDC or histaminase. Tissue histamine was assayed using the double isotope radioenzymatic method.

Three-day Starvation Model in Sprague Dawley rats

The animals used in the 3-day starvation study were all male albino Sprague-Dawley rats. They were housed in groups in wire-bottomed cages (to minimise coprophagia) with a dark/light cycle lasting 12 h each. The normal control fed rats were allowed access to water and food (Diet 41B) *ad libitum*. The starved rats were allowed free access to water and had their food removed for 3 days before the experiment.

On the day of the experiment, the animals were anaesthetised with pento-barbitone (Sagatal; 90 mg/kg body weight) injected intraperitoneally as previously described by Jeremy *et al.* (1985). Blood was collected by cardiac

puncture (once only from each animal) and placed in fluoride/oxalate bottles for glucose measurement and in lithium heparin (2 iu/ml) bottles for plasma biochemistry and histamine measurements.

The entire length of the thoracic aorta and both kidneys were removed as previously described and assayed for histamine and HDC.

Experiment to determine the effect of histamine H_1 and H_2 receptor antagonists on histamine metabolism and aortic permeability in control and diabetic rats

Diabetes was induced in 21 rats as previously described. A further 21 rats were injected with citrate buffer only. The animals were randomly selected from an initial group of 42 rats each weighing between 255-270 g.

Control rats

The 21 control rats were further subdivided into 3 groups of 7 rats each. Four weeks after the injection of saline (via tail vein, as control for DM induction), one group was intraperitoneally injected with saline only (daily for two weeks), while the other two groups were injected with mepyramine or cimetidine respectively.

Diabetic rats

Of the 21 rats injected with streptozotocin, only 18 developed glycosuria on the 3rd day. These 18 rats were subdivided into three groups of six rats each. Four weeks after the induction of diabetes, one group was injected with saline only (daily for two weeks) intraperitoneally, while the other two groups were injected with mepyramine or cimetidine respectively.

Mepyramine administration

Mepyramine was dissolved in isotonic saline and was administered intraperitoneally (0.5 ml) at a concentration of 1 mg/kg body weight daily

for two weeks. This concentration was known to cause total H_1 receptor blockade in rats (Al Haboubi & Zeitlin, 1979; Parsons, 1988).

Cimetidine administration

Cimetidine was dissolved in isotonic saline and was administered intraperitoneally (0.5 ml) at a concentration of 10 mg/kg body weight daily for two weeks. This concentration was known to cause total H_2 receptor blockade in rats (Parsons, 1988).

On the day of sacrifice (2 weeks after the administration of H_1 and H_2 antagonists; 6 weeks after induction of diabetes), the animals were anaesthetised with pentobarbitone as previously described, and the blood and tissues removed and processed as previously described.

A 1 cm segment of aorta from each rat was removed into Krebs Ringer bicarbonate buffer (37 °C, pH 7.4) for aortic permeability experiments.

Rat aortic permeability experiments

This novel method involved the incubation of opened 1 cm segments of rat aortae with 1 μCi ^{125}I-bovine serum albumin (2 mCi/mg) in 2 ml Krebs Ringer bicarbonate buffer (KRB; pH 7.4) at 37 °C for 2 h. At the end of the incubation period, the segments were removed and thoroughly washed with normal saline before digestion in 4% trichloroacetic acid (TCA) at 37 °C for 2 h. After digestion of aortae with TCA, the samples were homogenised in a Kinematic Polytron homogeniser (speed 9 for 20 sec x 3) and then centrifuged for 3 min at 10 000 x *g* at room temperature. Protein bound radioactivity in the pellet was determined in a LKB gamma counter. The results were corrected for dry weight of TCA precipitable tissue. Results were expressed as cpm.

4. HUMAN VOLUNTEER-BASED STUDIES

Histamine uptake by human platelets

Human platelets have previously been shown to take up histamine (Wood *et al.*, 1983, 1984). These studies were, however, limited by three factors. Firstly, the concentrations of histamine used during the incubation, *in vitro*, were almost 300-fold greater than those found in human plasma (Brown *et al.*, 1980; Morel & Delaage, 1988). Secondly, the platelets were prepared in ethylenediamine tetraacetic acid (EDTA) at concentrations which have been shown to inhibit platelet function (Pidard *et al.*, 1986). Thirdly, since the incubations were carried out in conventional water baths they were performed without significant stirring. It is well known that platelets do not aggregate efficiently *in vitro* unless they are stirred (with a stirring bar at 1000 rpm in conventional aggregometers; Born & Cross, 1963). Blood is also subject to turbulence and mixing *in vivo*. It is possible that functions of platelets other than aggregation may also be dependent on turbulence/stirring. Our novel method involves the investigation of histamine uptake by human platelets in experimental conditions which overcome the problems mentioned above.

500 µl of platelet rich plasma (PRP; prepared in trisodium citrate) was allowed to stand or be stirred in siliconised glass aggregometer cuvettes, and a 6 µl portion was taken for platelet counting in a Coulter counter ZM. The histamine uptake was then started by the addition of 25 µl prediluted [^{14}C]histamine label (final concentration in aggregometer cuvette, 2.5 nmol/l), followed immediately by up to 10 µl isotonic saline (for control samples) or desired concentration of test substance. During the incubation (up to 90 min) with saline or aggregating agent, the amount of aggregation occurring in the Chronolog dual channel aggregometer was constantly monitored on Omniscribe chart recorders. Aggregation was expressed as the percentage fall in optical density after addition of agonist. The difference in optical density between PRP and platelet poor plasma (PPP) was defined as 100%. After incubation, a second 6 µl aliquot was removed for platelet counts, and the remainder of the PRP was transferred to a 1.5 ml polypropylene Eppendorf tube containing a solution of aspirin in saline (final concentration of aspirin after adding the PRP: 100 mg/l). Preliminary experiments had shown that histamine uptake was approximately 15% greater if aspirin was added at this stage, presumably because the platelet

release reaction is inhibited by aspirin (Best *et al.*, 1981), and some degree of activation could occur during these stages. The PRP was then immediately centrifuged at 10000 x *g* for 1 min in an IEC-micro-centrifuge. The supernatant was removed and the pellet was resuspended and washed with isotonic saline. The pellet was then resuspended and ultrasonicated (to break up platelets) in 500 µl fresh saline using an MSE Soniprep 150 for 20 s at an amplitude of 18 microns. Liquiscint scintillant (5 ml) was added and the resulting suspension was counted for ^{14}C-radioactivity in a LKB Rackbeta counter.

Subjects

Subjects were healthy males and females who had not ingested any drugs for 2 weeks prior to the experiment. Their median age was 29 (range: 21-44) years. The number of subjects in each experiment is mentioned in the detailed description of experiments below.

Preparation of platelet rich plasma

Venous blood was collected in trisodium citrate (0.38% final concentration) and PRP was prepared by centrifugation at 150 x *g* in an MSE bench centrifuge at room temperature. Thereafter all PRP was kept at 37 °C, since it has been shown that cooling influences platelet function (Mikhailidis *et al.*, 1983). The resultant PRP was used in uptake and aggregation studies.

Preparation of [^{14}C]histamine label

[ring-2-^{14}C]histamine dihydrochloride (56 mCi/mmol; Amersham International, UK) was diluted in isotonic saline so that 25 µl gave a final concentration of 2.5 nmol/l [^{14}C]histamine in the aggregometer cuvette.

1. ***The determination of the effect of stirring on [^{14}C]histamine uptake in PRP***

PRP was incubated with [^{14}C]histamine label at 37 °C for up to 90 min in aggregometer cuvettes (a) with spinning, (b) without spinning. Incubations were stopped as described above.

2. ***The determination of the effect of conventional aggregating agents and various agonists, antagonists and inhibitors on [^{14}C]histamine uptake in PRP***

Effect of Collagen

PRP was incubated for 30 min in aggregometer cuvettes as described in (a) and (b) above. Incubations were started by the simultaneous addition of [^{14}C]histamine label and several concentrations of collagen (range: 0.01 - 0.1 mg/l) dissolved in isotonic saline.

Effect of adrenaline

PRP was incubated for 15 min with adrenaline dissolved in isotonic saline as desribed for collagen above, except that the same amount of adrenaline was added every 5 min because of the instability of adrenaline in plasma (Chattoraj *et al.*, 1986). Final concentrations of adrenaline ranged between 0.02 - 0.05 µmol/l.

Effect of adenosine diphosphate (ADP)

PRP was incubated for 15 min with ADP dissolved in isotonic saline as described for collagen. The same amount of ADP was added every 5 min in order to minimise the effects of intrinsic ADPase activity (Hutton *et al.*, 1984). Final concentrations of ADP ranged between 0.02 - 0.1 µmol/l.

Effect of iodoacetate, aspirin, imipramine, cimetidine and mepyramine

PRP was incubated for 30 and 60 min with various concentrations of the above substances dissolved in isotonic saline. All these substances, except aspirin, were added in 25 µl such that the 25 µl, when added to 450 µl of PRP, resulted in the required final concentration of substance. Only 10 µl of aspirin was added and at the end of the incubation period, to inhibit possible release of [^{14}C]histamine taken up.

Control experiments

I. Estimation of the amount of residual radioactivity in the platelet pellet after ultrasonication

PRP was prepared from 3 subjects and individually incubated for 30 min as described above in Experiment 1b. Each PRP was divided into two portions: one was ultrasonicated as described in 1b above, whereas the other was not. Platelet counts on both samples were then carried out using a Coulter Counter ZM (see above). The two samples were then centrifuged (10 000 x *g*, at room temperature, for 2 min) and the pellets washed as desribed above. The pellets from both samples were resuspended and ultra-sonicated for 20 s and the amount of ^{14}C radioactivity was determined as described above. In these experiments the supernatants were not discarded, as previously, but counted for radioactivity.

II. The effect of using EDTA as an anticoagulant on platelet aggregation, ionised calcium concentration and [^{14}C]histamine uptake

PRP was prepared using an anticoagulant solution made up as described by Wood *et al.* (1984), consisting of 27 mmol/l disodium EDTA, 120 mmol/l NaCl and 6 mmol/l glucose. Blood (10 parts) was mixed with this solution (1 part). The sample was then centrifuged at 140 x *g* for 15 min, as previously described. PRP was also prepared from blood obtained at the same venepuncture using trisodium citrate only as an anticoagulant, as described above. Platelet counts and platelet aggregation (for a 3-minute period) were carried out as described above. The uptake of [^{14}C]histamine into platelets of both samples was carried out as described in 1a and b above. Ionised calcium was measured in both samples using an ICA 1 ionised calcium analyzer (Radiometer, Copenhagen, Denmark).

III. The effect of platelet count in PRP on [^{14}C]histamine uptake

PRP and PPP were prepared as previously described. The PRP was diluted 1 : 1 and 1 : 4 with autologous PPP. Platelet counts were performed as

previously described. The uptake of [^{14}C]histamine into platelets was carried out as described in 1a and b above.

IV. The effect of stirring, non-stirring and various aggregating agents on the release of β-thromboglobulin (βTG) and thromboxane A_2 (TXA_2) from human platelets

PRP was prepared as previously described, and incubated with 2.5 nmol/l histamine (non-labelled) and various aggregating agents for 30 min as described above in experiments 1a and 1b. After the incubation period the PRP was centrifuged at 100 x *g* for 10 min for measurements of βTG in the PPP. For measurement of TXA_2, the PRP was added to 1 ml of absolute alcohol and centrifuged at 1500 x *g* for 15 min. The supernatant was stored frozen (-20 °C) until assay of TXA_2. The βTG and TXA_2 assays were carried out by Dr J.Y. Jeremy (Department of Chemical Pathology, RFHSM) and Dr R. Hutton (Haemophilia Centre, RFHSM), using standard purchased RIA kits.

V. The effect of temperature on [^{14}C]histamine uptake by platelets

All incubations for histamine uptake experiments were conducted at 37 °C. For this experiment, platelets were incubated at 37 °C, 27 °C, 17 °C and 4 °C. All these temperatures except 4 °C were achieved in conventional temperature-controlled water baths (hence with no stirring of platelets). To achieve 4 °C, the platelets were incubated with [^{14}C]histamine in an ice batch. The incubations were allowed to proceed for 30 min and 60 min.

5. PATIENT-BASED STUDIES

Four groups of patients and two groups of controls were studied.

Control Volunteers

Controls consisted of two groups of normal subjects. The first group comprised healthy young male and female volunteers (n=39) from the research staff of the Royal Free Hospital School of Medicine. None of these controls had DM or any history of cardiovascular disease. The median age of the younger controls was 36 years, with a range between 18 and 63 years. These volunteers had not ingested any drugs for at least two weeks prior to sampling of blood for the studies.

An older control group of normal subjects was obtained from a local geriatric day centre, and consisted of male and female volunteers (n=25). The median age of this older goup was 72 years, with a range between 65 and 80 years. None of these volunteers had any known history of cardio-vascular disease or DM.

Collectively the entire control population (n=64) had a median age of 49 years with a range of 18-80 years.

Patients

All the patients included in these studies were attending the diabetic or vascular clinics at The Royal Free Hospital.

Type I diabetes mellitus

This group consisted of both male (n=24) and female (n=20) insulin-dependent diabetics (IDDM) with no known complications (n=44). The criteria for inclusion in this group were: the onset of diabetes mellitus before the age of 25 years, a history of ketosis and treatment of hyper-glycaemia with exogenous insulin. The median age of this group was 40 years, with an age range of 19-72 years. The duration of diabetes mellitus ranged between 3 and 40 years. The median blood glucose was 11.1 mmol/l

(range: 3.6-29.1) and the median glycosylated haemoglobin (HbA_1) was 9.9% (range: 5.0-11.8). The patients were defined as smokers if they presently smoked more than 5 cigarettes per day. Patients were defined as hypertensive if they had a diastolic blood pressure >95 mmHg on two separate occasions. In the IDDM group, there were 12 smokers and 18 hypertensive patients.

Type II diabetes mellitus

This group consisted of male (n=25) and female (n=13) non-insulin-dependent diabetics (NIDDM) with no known complications (n=38). The criteria for inclusion in this group were: diagnosis of diabetes after the age of 35 years, and treatment with oral hypoglycaemics or diet only without the use of exogenous insulin. The median age of this group was 65 years, with an age range of 41 to 86 years. The duration of diabetes ranged between 1.0 and 27 years. The median blood glucose was 11.5 mmol/l (range: 5.0-13.7) and median HbA_1 10% (range 7.5-15.4). Seven NIDDM patients admitted to being smokers and 20 of the 38 patients in this group were hypertensive.

Peripheral vascular disease (PVD)

This group consisted of male (n=28) and female (n=12) patients with a confirmed diagnosis of PVD (n=40). The criteria for inclusion in this group were: (a) intermittent claudication for more than 6 months; (b) ankle/arm systolic blood pressure (SBP) ratio <0.85 in both arteries (dorsalis pedis and posterior tibial) of the worse leg; (c) pain-free walking time of less than 6 min on a treadmill at 1/10 incline at speeds between 2 and 5 km/h (individually adapted during test). Patients taking vasodilators for claudication were excluded from this study. The median age of this group was 73 years, with an age range of 50 to 85 years. Twenty-one of these patients were smokers. Of the remaining 19, 12 were previous smokers. Twenty-two of the patients in this group were hypertensive.

Peripheral vascular disease and diabetes mellitus (PVD-DM)

This group consisted of male (n=12) and female (n=5) patients with a diagnosis of PVD with concomitant diabetes mellitus (n=17) (both IDDM [n=4] and NIDDM [n=13]). The criteria for inclusion in this group were the same as for the PVD and diabetic groups. The median age of this group was 68 years (range: 56-88 years) and duration of DM was between 5 and 22 years. Seven of these patients were current smokers and 9 were hypertensive.

It should be noted that throughout the studies, some patients and volunteers allowed just enough blood for plasma histamine estimation *or* measurement of their leucocyte/platelet histamine concentration, and therefore the numbers of patients in each group do not necessarily tally in the various indices measured.

Drugs

Healthy subjects denied taking drugs for at least two weeks prior to sampling. Diabetic patients were on standard treatment regimens with insulin/oral hypoglycaemic agents and diet. Hypertensive PVD patients were on a combination of nifedipine and bendrofluazide.

Blood sample collection and processing

Blood was taken from the antecubital vein of patients and volunteers with minimal stasis, and nine parts of blood were added to one part of 3.8% w/v trisodium citrate, sodium EDTA (5 mmol/l final concentration) and lithium heparin (2 iu/ml) for plasma biochemistry measurements. Plasma for histamine measurements was prepared by centrifuging EDTA anticoagulated blood for 20 min at 1500 x g at 4 °C. The supernatant was frozen immediately and kept at -40 °C until assay. Leucocytes were separated from blood samples anticoagulated with lithium heparin using the Dextran sedimentation technique as previously described by Baron & Ahmed (1969).

Separation of leucocytes

The dextran sedimentation method was used, with some small modifications. Venous blood (10 ml) was collected in heparinised tubes (2 iu/ml final concentration). An equal amount of buffer (Solution I, pH 7.4), pre-warmed in a water bath at 37 °C, was added. Dextran (Solution III, pH adjusted to 7.4) was warmed and added to blood in a ratio of 1:4. The mixture was shaken very gently and the air bubbles (if any) at the top of the column were removed. The mixture was left to stand for 15 min. The red blood cells (RBC) were allowed to sediment to the bottom of the tube and the resulting supernatant, rich in leucocytes, was removed using a Pasteur pipette into sterile conical tubes. The supernatant was centrifuged at room temperature for 4 min at 250 x *g* and the supernatant discarded. The pellet at the bottom contained the leucocytes, with some contaminating RBC. The RBCs were lysed hypotonically by adding 3 ml distilled water and whirlimixing for 8 - 10 s. One ml Solution II was rapidly added and mixed to restore isotonicity. The mixture was centrifuged at 250 x *g* for 4 min. The layer of haemoglobin and RBC ghosts was blown away by gently using a Pasteur pipette along the side of the conical tube without disrupting the tightly packed leucocytes; the supernatant containing the haemoglobin and RBC ghosts was then discarded. The pellets containing the mixed population of leucocytes were resuspended in 1 ml 0.1 M sodium phosphate buffer (pH 7.4) for estimation of histamine, HDC and protein.

Prior to assay of histamine and HDC, the leucocyte suspension was ultrasonicated for 10 s x 3 at an amplitude of 20 microns, followed by repeated freezing and thawing.

Separation of platelets

For the separation of platelets, blood was anticoagulated with trisodium citrate. Blood (9 ml) was added to 1 ml 3.8% trisodium citrate in polyethylene tubes to give a final concentration of 0.38% trisodium citrate. Platelet rich plasma (PRP) was prepared as described by Mikhailidis *et al* (1984). Briefly, anticoagulated blood was centrifuged at 150 x *g* at room temperature for 15 min in an MSE bench centrifuge. The resultant PRP (supernatant) was transferred to Eppendorf tubes for the preparation of

platelet pellets. Aliquots of PRP (6 µl) were then removed for platelet counts in a Coulter counter ZM. Platelet pellets were prepared by centrifuging the PRP at 10 000 x *g* for 1 min in a microcentrifuge. The pellets were then stored at -40 °C until analysis.

Prior to histamine, HDC and histaminase assay, the platelet pellets were ultrasonicated in 500 µl of 0.1 M sodium phosphate buffer, pH 7.4, for 10 s (x 3) at an amplitude of 18 microns using an MSE Soniprep sonicator. In order to ensure that our sonication procedure fully disrupts platelets, we counted, sized and plotted platelet population before and after sonication. For this purpose, platelets were obtained from healthy volunteers and the above parameters were assessed using a Coulter ZM counter with a Channelyzer C-1000 and X-Y Recorder. Following sonication, the platelet count was reduced to <5% of the original and the mean platelet volume became unmeasurable.

Separation of plasma for histamine estimation

Blood collection for plasma histamine determination needs special care (Lorenz *et al.*, 1972) since the blood leucocytes are known to contain large quantitites of histamine per cell, while human plasma contains 1000 fold (50-100 pg/ml) less histamine per millilitre. Thus any degranulation of leucocytes, especially basophilic leucocytes, would grossly contaminate the plasma histamine estimation. Blood for plasma histamine was therefore collected from the antecubital fossa into plastic syringes. 10-20 ml syringes were used in order to reduce pressure on cells entering the narrow needle. Furthermore, histamine can be adsorbed onto glass surfaces, and hence only plastic syringes and tubes should be used. Thus, blood samples were collected through an indwelling venous cannula (21 gauge: Butterfly) into plastic tubes containing EDTA (5 mmol/l final concentration after addition of blood) for all studies or heparin for plasma biochemistry. Some earlier experiments used heparinised blood for plasma histamine estimation but EDTA was chosen later because it is known to inhibit leucocyte degranulation without affecting the assay of histamine (Heavey *et al.*, 1984), as well as inhibiting histaminase activity in plasma (Brown *et al.*, 1980).

The anticoagulated blood was centrifuged within 20 min at 1000 x *g* for 15 min at 4 °C. After centrifugation, the plasma was removed by plastic

pipettes without disturbing the packed cells and keeping well clear of the buffy white cell layer. The plasma was then stored at -70 °C until assay.

6. STATISTICAL METHODS

The data for [^{14}C]histamine uptake experiments and for histamine, HDC and histaminase from animals and patients were assumed to be non-parametrically distributed; the results are therefore expressed as medians, with corresponding ranges in parentheses.

The Mann-Whitney U-test (two-tailed) was used for comparing unpaired data and the Wilcoxon match-pairs signed rank test (two-tailed) was used for comparing paired data.

Correlation coefficients were calculated by Spearman's method for non-parametric data using a validated computer program in use in the Department of Chemical Pathology and Human Metabolism at The Royal Free Hospital.

CHAPTER III

RESULTS

1. CONTROL EXPERIMENTS

A considerable amount of work was carried out to determine the optimal incubation times and optional volume of homogenate of each tissue to be used in the histamine, HDC and histaminase assays. Tables 1 and 2 show the incubation time and volume of homogenate used for each of the blood cell and animal tissues used in this study for histamine and HDC assays. For the histaminase assay, 100 µl of homogenate were incubated for each tissue for 120 min.

The HDC assay employed in this study is a very sensitive assay of histamine formation from histidine. Since there are two enzymes capable of decarboxylating histidine, we used specific inhibitors of both enzymes to determine their relative contributions to total decarboxylase activity. Figures 7 and 8 show the inhibition of decarboxylase activity by αFMH (a specific inhibitor of HDC) and αMD (a specific inhibitor of DOPA decarboxylase) in various tissues obtained from control and diabetic rats. These data clearly show that in all the tissues examined the vast majority of decarboxylation is carried out by the specific enzyme HDC. In control animals, the inhibitor data for aorta, heart, whole brain and lungs and kidneys show that HDC accounts for over 80-85% of total decarboxylating activity in these tissues, with DOPA decarboxylase activity accounting for the rest. In diabetic animals, the data again show that most of the decarboxylation of histidine occurs via HDC (90-95%). However, the proportion of total decarboxylase activity accounted for by HDC is significantly greater in diabetic animals than that in controls ($P < 0.03$ for all tissues except brain). For example, in the aortae of control animals, 12% of total histamine synthesis was by DOPA decarboxylase with 88% accounted for by HDC. In the aortae of diabetic animals, however, HDC accounted for 94% of the total with only 6% accounted for by the non-specific enzyme. Therefore, DOPA decarboxylase activity was halved in aortae from diabetic animals despite a massive increase in total activity (113%), indicating a marked elevation in HDC activity in these animals. In other words, the actual amount of

Table 1

The volume of homogenate and incubation times utilised for the various tissues in the assay of histamine. Leucocytes and platelets were prepared from human blood; all others tissues were obtained from rats.

	Histamine assay (radioenzymatic)	
	Volume of homogenates (µl)	Incubation time (min)
Aorta	20	60
Heart	20	90
Kidneys	30	90
Lung	20	90
Whole brain	30	60
Stomach	10	60
Skin	20	60
Plasma	20	60
Leucocytes	20	60
Platelets	30	90

Table 2

The volume of homogenate and incubation times utilised for the various tissues in the HDC assay. Leucocytes and platelets were prepared from human blood; all other tissues were obtained from rats.

	HDC Assay	
	Volume of homogenates (µl)	Incubation time (min)
Aorta	20	60
Heart	30	60
Kidneys	30	60
Lung	20	60
Whole brain	20	60
Stomach	5	60
Skin	40	120
Leucocytes	10	60
Platelets	40	120

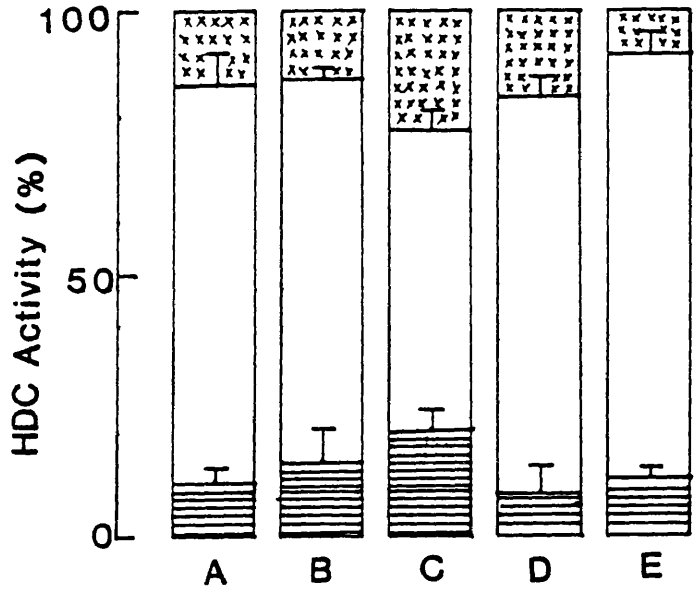

Figure 7. The effect of α-fluoro methylhistidine and α-methyl DOPA on HDC activity of aorta (A), heart (B), kidneys (C), whole brain (D) and lungs (E) obtained from control rats (n = 7).

- inhibition by α-fluoro methylhistidine

- inhibition by α-methyl DOPA

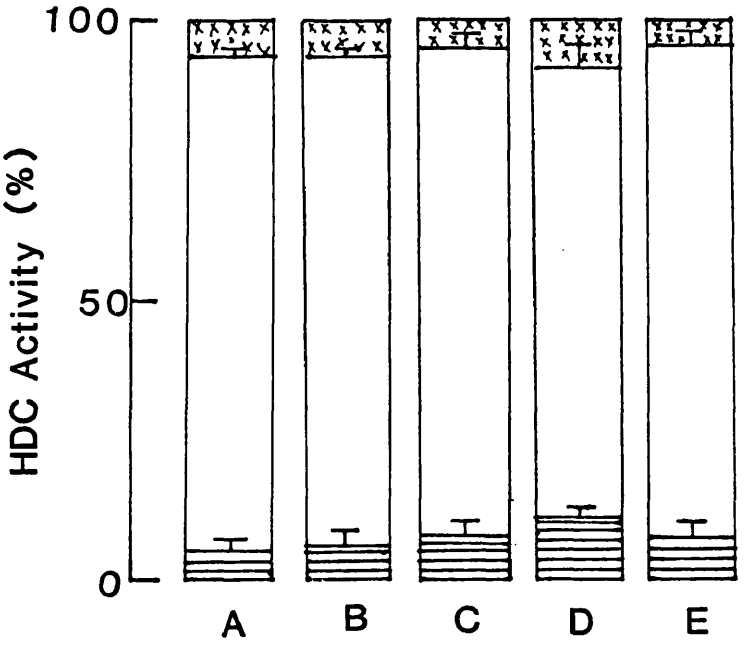

Figure 8. The effect of α-fluoro methylhistidine and α-methyl DOPA on HDC activity of aorta (A), heart (B), kidneys (C), whole brain (D) and lungs (E) obtained from diabetic rats (n = 7).

- inhibition by α-fluoro methylhistidine

- inhibition by α-methyl DOPA

histamine synthesised by DOPA decarboxylase in diabetic animals did not alter, while that by HDC increased markedly. Interestingly, the DOPA decarboxylase contribution to total activity in both control and diabetic rats accounted for the same amount of histamine synthesis [control aortae = 378 pmol/min/g protein (12% of total); diabetic aortae = 397 pmol/min/g protein (6% of total)] despite different percentage contributions.

In the other tissues, the relative proportions of DOPA decarboxylase were as follows; for controls: heart 13%, kidney 21%, brain 13% and lungs 11%; and for diabetic tissues: heart 7%, kidneys 7%, brain 10% and lungs 6%. In all these tissues the actual DOPA decarboxylase activity was not altered, despite differences in the percentage of total activity.

These preliminary control experiments served as an early indicator that in diabetic rats, HDC activity may be elevated in most of the tissues chosen for this study.

2. ANIMAL STUDIES

A. *The physical and biochemical characteristics of the control, 3-day starved and diabetic groups* (Tables 3 and 4)

The data presented in Tables 3 and 4 are the physical and biochemical characteristics of the two treatment groups and are data obtained from controls and 3-day starved diabetic animals.

The data show that streptozotocin-induced diabetes mellitus in the male Sprague-Dawley rat is characterised by severe hyperglycaemia and glycosuria. In this model there was no ketonuria. These observations are consistent with those reported previously for streptozotocin-induced DM (Junod *et al.*, 1969). The data in Table 4 also show significant changes in plasma urea, sodium and chloride concentrations in diabetic rats, with no significant changes in potassium or bicarbonate concentrations. There was no glycosuria or ketonuria in the starved animals.

The data presented in Table 3 are the pooled values of all the diabetic and starved animals used in this study, and clearly show that the diabetic animals have a severe reduction in weight gain - a characteristic of streptozotocin-induced diabetes in growing rats (Jeremy *et al.*, 1985). The 3-day

Table 3

Body weights, glycosuria and ketonuria in various groups. Data are expressed as median and (range); * denotes significant difference from control (P <0.01).

Group	n	Initial body weight (g)	Final body weight (g)	Urinary glucose	Urinary ketones
Control (DM)	28	234 (230-239)	509 (407-557)	negative	negative
Diabetic	26	235 (230-240)	299* (176-349)	positive	negative
Control (starved)	7	230 (223-241)	268 (240-277)	negative	negative
3-day starved	7	237 (221-251)	201* (190-217)	negative	negative

Table 4

The biochemical characteristics of the treatment groups.

Data are expressed as median and (range);

* denotes significant difference from control (P <0.01).

Group	n	Blood glucose (mmol/l)	Urea (mmol/l)	K^+ (mmol/l)	Na^+ (mmol/l)	HCO_3^- (mmol/l)	Cl^- (mmol/l)
Control (DM)	14	8.3 (6.4-10.3)	5.4 (5.1-5.9)	4.6 (3.8-7.7)	142 (141-144)	21.5 (18-25)	103 (100-105)
Diabetic	13	32.4* (26.5-42.0)	6.9* (6.6-11.1)	5.2 (4.2-6.5)	133* (126-140)	19.5 (14-21)	92.5* (87-96)
Control (starved)	7	8.9 (7.1-11.3)	–	–	–	23 (17-28)	–
3-day starved	7	7.2 (5.4-8.4)	–	–	–	20 (16-26)	–

starved animals exhibited severe weight loss when compared to corresponding controls.

B. ***The histamine concentration of plasma and the histamine content of various tissues obtained from control and diabetic rats***

Plasma (Figure 9)

There was a 164% increase (p <0.002) in plasma histamine concentrations in diabetic rats (median and [range]: 74 [55-96] ng/ml) compared to control rats (28 [14-40] ng/ml).

Aorta (Figure 9)

There was an 84% increase (p<0.001) in the histamine content of the aortae of diabetic rats 665 [610-715] ng/mg protein) compared to the control rats (360 [315-405] ng/mg protein).

Heart (Figure 10)

There was a 28% increase (P <0.01) in the histamine content of the hearts of diabetic rats (4.9 [4.0-5.5] ng/mg protein) compared to control rats (3.8 [3.2-4.3] ng/mg protein).

Lungs (Figure 10)

There was a 32% increase (P <0.02) in the histamine content of the lungs of diabetic rats (7.3 [4.7-10.1] ng/mg protein) compared to control rats (5.5 [3.5-7.2] ng/mg protein).

Kidneys (Figure 11)

There was an 89% increase (p <0.001) in the histamine content of the kidneys of diabetic rats (0.85 [0.70-0.99] ng/mg protein) compared to control rats (0.45 [0.35-0.55] ng/mg protein).

Whole Brain (Figure 11)

There was a 41% increase (P <0.03) in the histamine content of the brains of diabetic rats (0.78 [0.60-0.91] ng/mg protein) compared to control rats (0.55 [0.37-0.80] ng/mg protein).

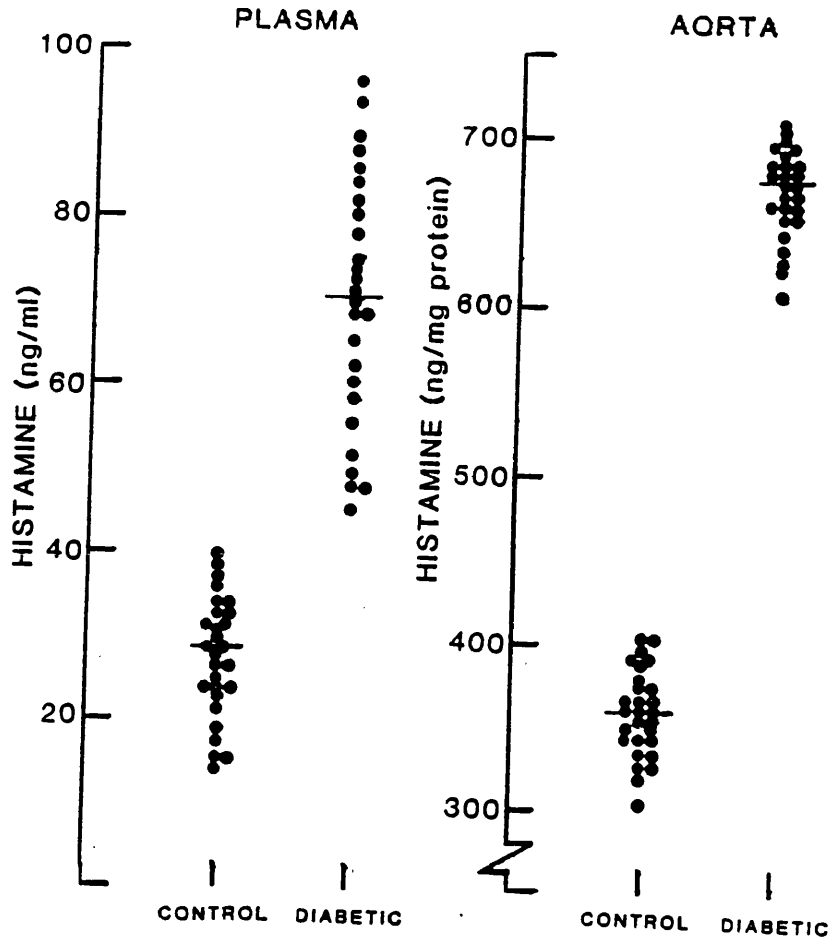

Figure 9. (a) The plasma histamine concentration of control (n = 28) and diabetic (n = 26) rats. Control *vs* diabetic, $p < 0.002$. (b) The aortic histamine content of control and diabetic rats. Control vs diabetic, $p < 0.001$.

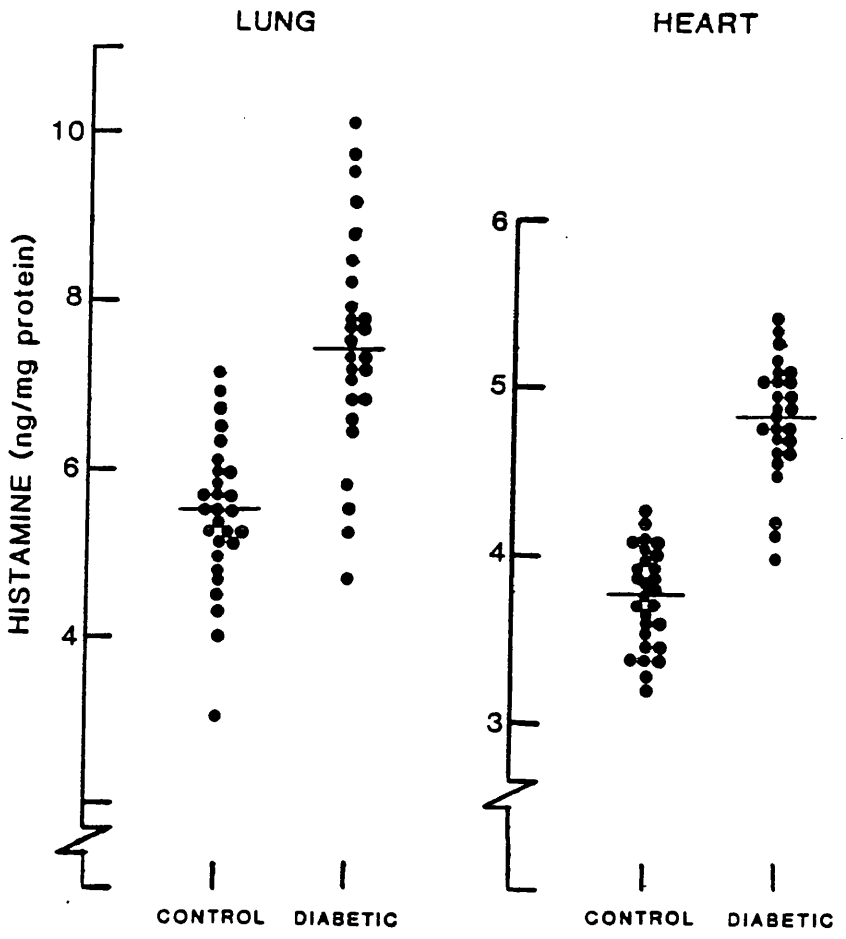

Figure 10. The histamine content of (a) lung and (b) heart of control and diabetic (n = 26) rats. (a) control *vs* diabetic, $p < 0.02$. (b) control *vs* diabetic, $p < 0.01$.

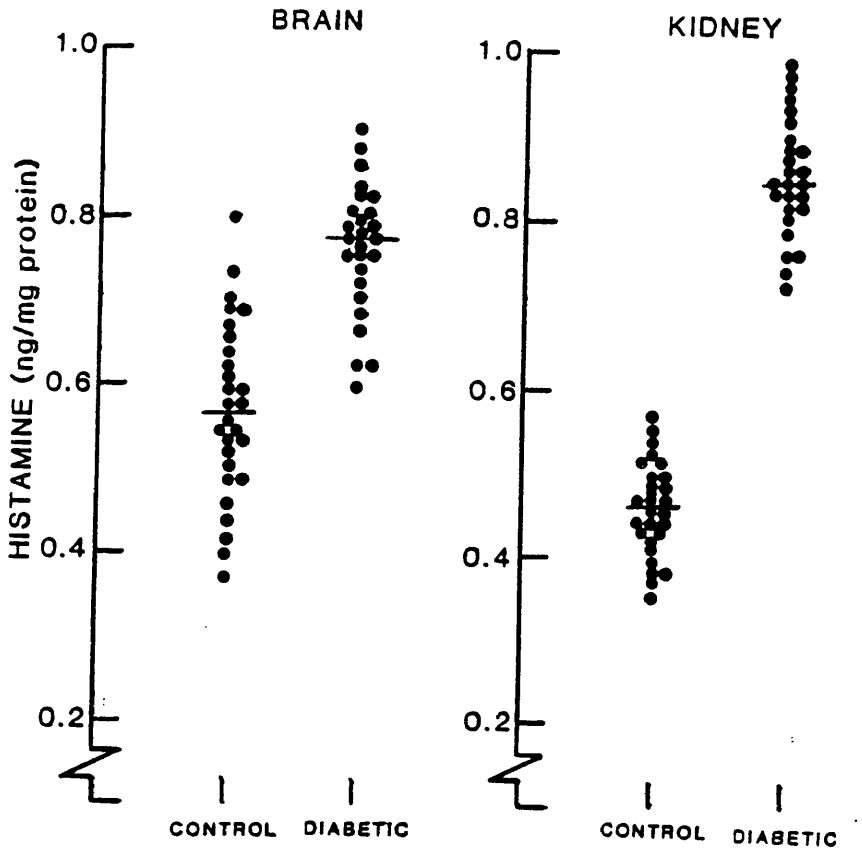

Figure 11. The histamine content of (a) brain and (b) kidney of control (n = 28) and diabetic (n = 26) rats. (a) control *vs* diabetic, $p < 0.03$. (b) control *vs* diabetic, $p < 0.001$

Stomach

There was a 30% decrease (not statistically significant) in the histamine content of the stomachs of diabetic rats (1400 [1100-2700] ng/mg protein; n=26) compared to control rats (2000 [1400-2900] ng/mg protein; n=28)

Skin

There was a 14% decrease (not statistically significant) in the skin of diabetic rats (1700 [1100-3300] ng/mg protein; n=26) compared to control rats (1960 [1300-4100] ng/mg protein; n=28).

C. ***The HDC activity of various tissues obtained from control and diabetic rats***

Aorta (Figure 12)

There was a 119% increase (p <0.002) in HDC activity of the aortae of diabetic rats (median and [range]: 7600 [6100-9800] pmol/min/g protein) compared to control rats (3500 [2600-6300] pmol/min/g protein).

Heart (Figure 12)

There was an 80% increase (p <0.005) in HDC activity of the hearts of diabetic rats (18.5 [15.7-22.7] pmol/min/g protein) compared to control rats (10.3 [8.0-11.9] pmol/min/g protein).

Kidneys (Figure 13)

There was a 79% increase (p <0.001) in HDC activity of the kidneys of diabetic rats (22.7 [15.8-26.4] pmol/min/g protein) compared to control rats (12.7 [11.8-14.3] pmol/min/g protein).

Lungs (Figure 13)

There was a 40% increase (p <0.02) in HDC activity of the lungs of diabetic rats (111 [93-131] pmol/min/g protein) compared to control rats (74 [49-103] pmol/min/g protein).

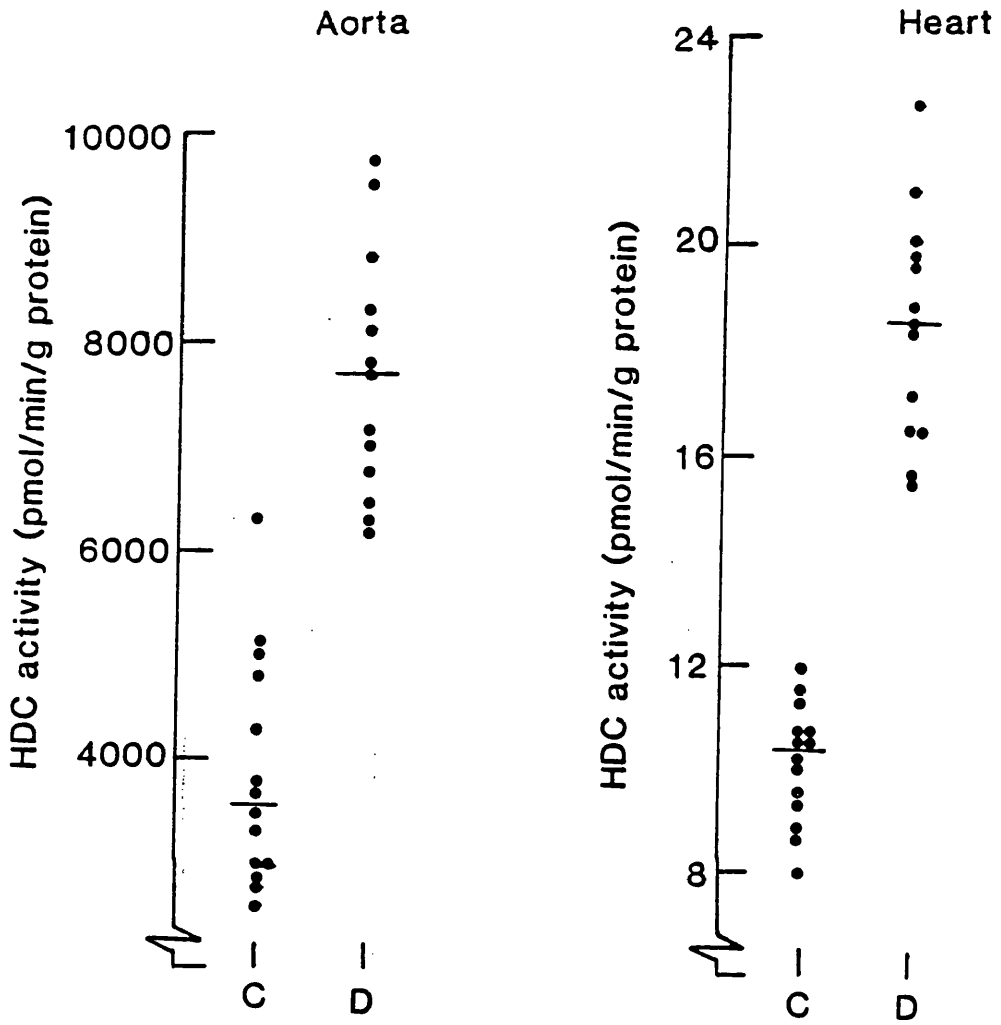

Figure 12. The HDC activity of (a) aorta and (b) heart of control (C; n = 14) and diabetic (D; n = 13) rats. (a) control *vs* diabetic, $p < 0.002$. (b) control *vs* diabetic, $p < 0.005$.

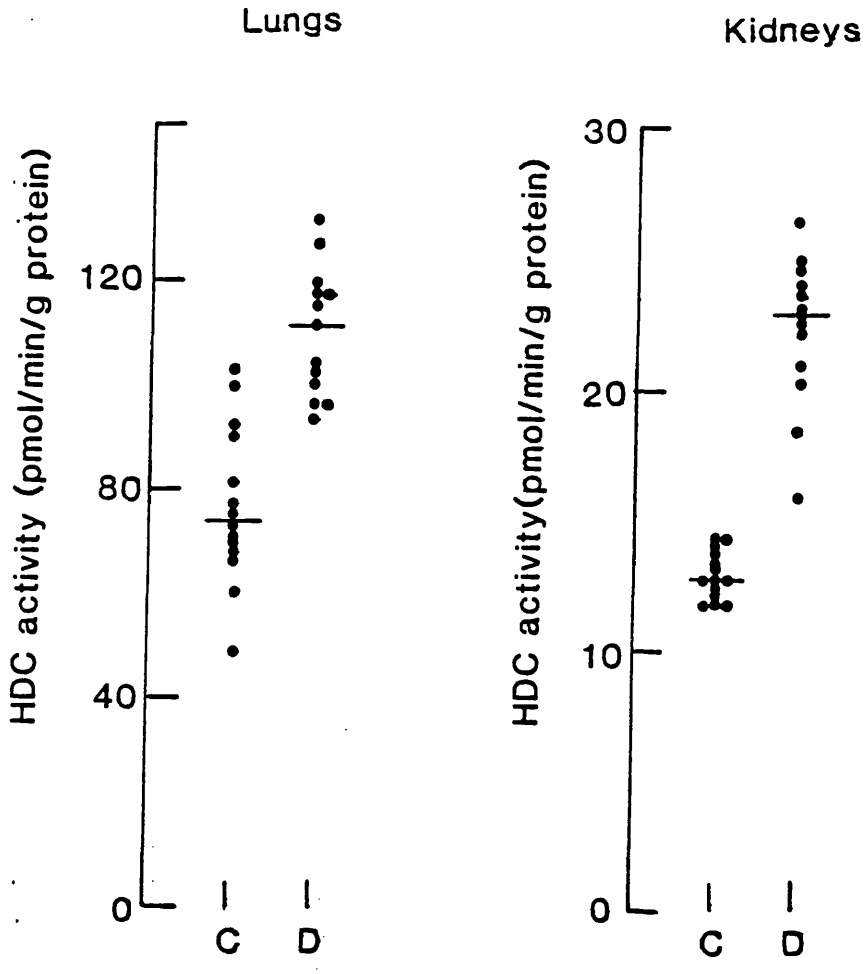

Figure 13. The HDC activity of (a) lungs and (b) kidneys of control (C; n = 14) and diabetic (D; n = 13) rats. (a) control *vs* diabetic, $p < 0.02$. (b) control *vs* diabetic, $p < 0.001$.

Whole Brain

There was a 3% increase (not statistically significant) in HDC activity of the whole brain of diabetic rats (610 [530-710] pmol/min/g protein) compared to control rats (590 [430-710] pmol/min/g protein).

Stomach

There was a 16% decrease (not statistically significant) in HDC activity of the stomachs of diabetic rats (8800 [7200-14 200] pmol/min/g protein) compared to control rats (10 500 [7900-17 200] pmol/min/g protein).

Skin

There was no detectable HDC activity in the skin samples of either control or diabetic rats despite repeated attempts to measure this using numerous rats.

D. *The histaminase activity of plasma and various tissues obtained from control and diabetic rats*

Histaminase activity was greatest in the kidneys, followed by the heart, the skin, the aorta, the stomach and the brain and was lowest in the plasma. Experimental diabetes did not alter histaminase activity in any of the tissues examined (Table 5). Histaminase activity of the lungs from both control and diabetic rats was not detectable using the assay methodology described.

E. *The effect of histamine antagonists on aortic permeability and histamine metabolism of various tissues in diabetic rats*

The data in Table 6 clearly show that diabetic rats suffer from statistically significant weight loss, while the control animals continue with expected and normal weight gain over the six-week period. There were no significant differences in weight gain in the control groups with either mepyramine or cimetidine administration. Similarly, there were no significant differences in weight loss between any of the three diabetic groups. Blood glucose concentrations were elevated, as expected, in the

Table 5

Histaminase activity of plasma and various tissues from control and diabetic rats, expressed as median and (range). Tissue histaminase is expressed as pmol/h/mg protein and plasma histaminase as pmol/h/ml. ND = non-detectable. There were no significant differences between control and diabetic rats in any of the tissues examined.

	Control (n=28)	Diabetic (n=26)
Plasma	3.0 (1.3-2.8)	2.3 (1.4-2.7)
Aorta	13.3 (9.7-15.1)	14.3 (10.6-17.1)
Heart	18.7 (17.3-24.1)	16.6 (15.9-19.3)
Kidneys	22.5 (18.0-24.7)	23.2 (17.6-24.0)
Whole brain	11.8 (10.6-20.0)	11.5 (9.3-15.8)
Lung	ND	ND
Stomach	12.5 (11.4-13.0)	12.9 (11.6-13.4)
Skin	17.3 (13.1-18.9)	18.6 (12.4-29.3)

Table 6

The initial, intermediate and final body weights of the various rat treatment groups. Data are expressed as median and (range).

H_1 = mepyramine; H_2 = cimetidine.

• denotes significant difference from initial body weight;

+ denotes significant difference from corresponding control.

	n	Initial body weight (g)	4 weeks post-DM (g)	7 weeks post DM (g)
Control	7	255-270	440• (425-459)	512• (481-523)
Control H_1	7	255-270	423• (419-471)	446• (435-501)
Control H_2	7	255-270	436• (418-448)	482• (428-530)
Diabetic	6	250-258	262+ (195-273)	236•+ (181-255)
Diabetic H_1	6	250-258	259+ (230-268)	215•+ (210-226)
Diabetic H_2	5	250-258	253+ (249-263)	236•+ (218-254)

diabetic rats but were not affected by mepyramine or cimetidine in the control or diabetic groups.

Four animals died in the three diabetic groups, resulting in only 6 in the diabetic and diabetic H_1 groups and only 5 in the diabetic H_2 group. Death is common in this model of experimental diabetes. It is usually the result of severe hyperglycaemia and has previously been described by other workers (Junod *et al.*, 1969).

In vitro aortic permeability

Aortic permeability to ^{125}I-albumin was 290% greater (p <0.01) in diabetic rats (220 cpm/mg tissue) when compared to controls (87 cpm/mg tissue). Permeability was not altered by mepyramine or cimetidine in either control or diabetic rats (Figure 14).

HDC activity

HDC activity was markedly (p <0.001) increased in the aortae of diabetic rats (median and [range]: 6800 [6100-8800] pmol/min/g protein) when compared to controls (3200 [2500-5300] pmol/min/g protein); but HDC activity was not altered by the administration of either mepyramine or cimetidine in control or diabetic rats (Figure 15).

The HDC activity of the heart, kidneys and lung was significantly (p <0.02) greater in the diabetic animals, as previously demonstrated. However, the administration of either H_1 or H_2 antagonists did not significantly alter histamine synthesis. The whole brain did not exhibit increased HDC activity in the diabetic animals, confirming previous observations (Tables 7 and 8).

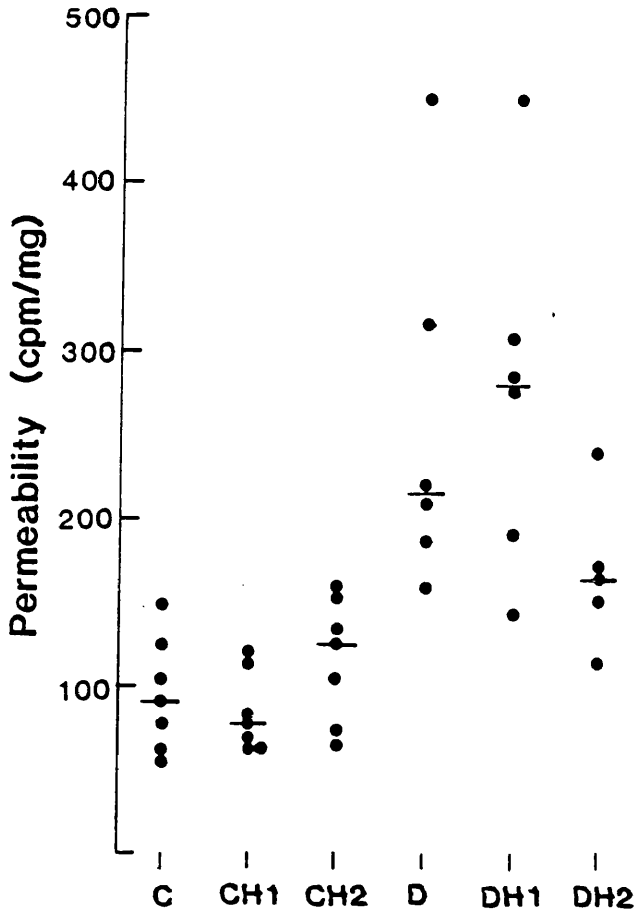

Figure 14. The effect of mepyramine (H_1) and cimetidine (H_2) on aortic permeability to ^{125}I-albumin in control (C) and diabetic (D) rats. C, CH_1 and CH_2 *vs* D, DH_1 and DH_2, p <0.01. There was no significant difference between C, CH_1 and CH_2 or between D, DH_1 and DH_2.

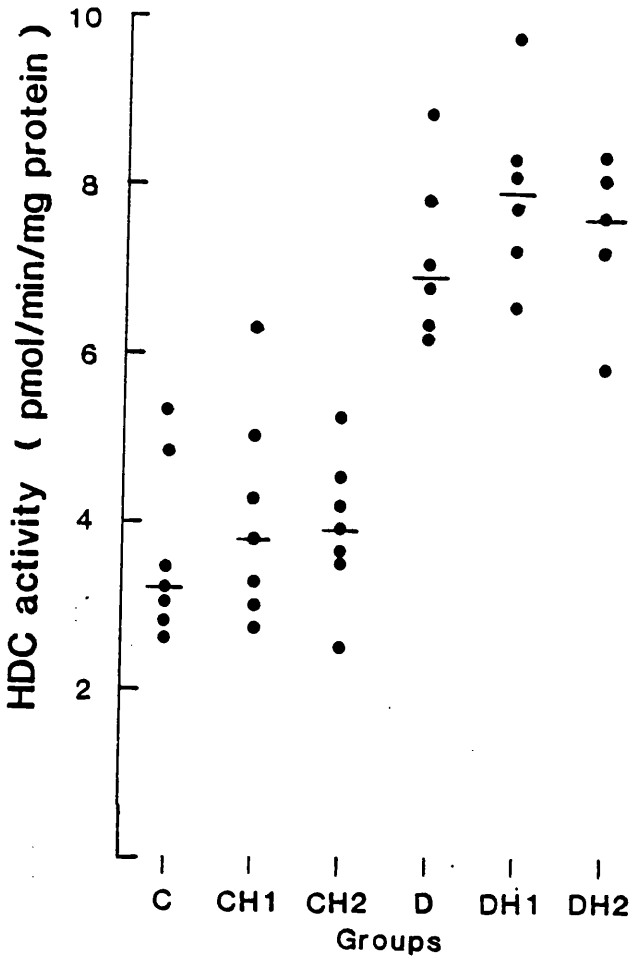

Figure 15. The effect of mepyramine (H_1) and cimetidine (H_2) on aortic HDC activity in control (C) and diabetic (D) rats. C, CH_1 and CH_2 *vs* D, DH_1 and DH_2, p <0.001.

Table 7

The HDC activity of various tissues obtained from control rats given saline, mepyramine or cimetidine. Results are expressed as median and (range) pmol/min/g protein. There were no significant differences between the various groups.

	Control (saline), n=7	Control (mepyramine), n=7	Control (cimetidine), n=7
Aorta	3200 (2500-5300)	3800 (2600-6200)	3900 (2400-5300)
Heart	9.7 (7.3-13.1)	11.4 (8.2-12.7)	10.9 (8.0-13.1)
Kidneys	13.0 (11.3-15.1)	11.6 (9.2-14.7)	12.1 (10.6-14.2)
Whole brain	500 (430-700)	560 (460-630)	540 (470-600)
Lung	84 (53-112)	89 (59-96)	78 (63-93)

Table 8

The HDC activity of various tissues obtained from diabetic rats given saline, mepyramine or cimetidine. Results are expressed as median and (range) pmol/min/g protein. There were no significant differences between the various groups.

	Diabetic (saline), n=6	Diabetic (mepyramine), n=6	Diabetic (cimetidine), n=5
Aorta	6800 (6100-8800)	7900 (6500-9700)	7600 (5800-8300)
Heart	17.3 (14.7-23.0)	18.9 (15.1-21.3)	18.4 (15.0-22.6)
Kidneys	23.1 (16.1-27.3)	22.7 (16.8-26.4)	29.1 (18.3-31.5)
Whole brain	620 (530-720)	580 (480-610)	610 (580-710)
Lung	124 (93-172)	138 (74-181)	114 (90-171)

Histamine Content

The histamine content of plasma, aorta, heart, kidneys, whole brain and lung was significantly (p <0.01) greater in the diabetic animals when compared to controls (Tables 9 and 10). The administration of either mepyramine or cimetidine did not alter histamine content in any tissue except the kidney, in which there was a significant reduction in the histamine content in diabetic animals treated with either mepyramine (p <0.01) or cimetidine (p<0.03). This observation is inconsistent with all the other data from our studies and is as yet unexplained, since neither HDC nor histaminase activity in the kidney were altered by either antagonist in control or diabetic animals.

Histaminase activity

Histaminase activity was not altered in any tissues of diabetic rats, and furthermore was not affected by either H_1 or H_2 antagonists in control or diabetic rats (Tables 11 and 12); nor was there any detectable histamine activity in the lung, confirming previous observations.

F. *The histamine content and HDC activity of plasma, aorta and kidneys obtained from 3-day starved rats*

The starved rats exhibited severe weight loss after three days of food restriction (Table 3). The control rats gained 38 g in three days, while the starved rats lost 36 g. The starved animals had normal blood glucose levels and did not exhibit ketonuria (Tables 3 and 4).

There were no significant differences in the histamine content of plasma, aorta or kidneys of diabetic animals when compared with control animals (Table 13). Furthermore, there were no alterations in the rate of histamine synthesis in the aorta or kidneys of 3-day starved rats (Table 14).

Table 9

The histamine content of various tissues obtained from control rats given saline, mepyramine or cimetidine. Results are expressed as median and (range) ng/mg protein for tissues and ng/ml for plasma.

There were no dignificant differences between the various groups.

	Control (saline), n=7	Control (mepyramine), n=7	Control (cimetidine), n=7
Plasma	28.7 (15.3-32.9)	29.5 (14.9-35.6)	24.8 (23.1-33.4)
Aorta	371 (320-410)	361 (330-371)	363 (324-412)
Heart	3.1 (2.8-4.1)	3.8 (2.8-4.7)	3.3 (3.0-4.3)
Kidneys	0.68 (0.53-0.81)	0.64 (0.40-0.90)	0.74 (0.58-0.85)
Whole brain	0.56 (0.37-0.72)	0.49 (0.36-0.70)	0.58 (0.42-0.69)
Lung	5.9 (4.9-7.4)	5.6 (3.1-7.1)	6.1 (4.3-7.2)

Table 10

The histamine content of various tissues obtained from diabetic rats given saline, mepyramine or cimetidine. Results are expressed as median and (range) ng/mg protein for tissues and ng/ml for plasma.

* denotes significant difference from diabetic saline group, $P < 0.03$;

** denotes significant difference from diabetic saline group, $P < 0.01$.

	Diabetic (saline), n=6	Diabetic (mepyramine), n=6	Diabetic (cimetidine), n=5
Plasma	59.3 (31.0-74.6)	74.3 (60.4-96.5)	70.0 (59.9-93.4)
Aorta	667 (623-711)	620 (588-721)	632 (521-677)
Heart	4.7 (4.2-5.6)	4.8 (3.9-6.0)	5.4 (4.2-6.2)
Kidneys	1.15 (1.10-1.20)	0.81** (0.61-0.86)	0.85* (0.50-1.10)
Whole brain	0.71 (0.37-0.80)	0.69 (0.51-0.79)	0.80 (0.57-0.91)
Lung	7.9 (5.9-8.4)	9.2 (5.3-11.1)	8.4 (7.6-9.4)

Table 11

The histaminase activity of various tissues obtained from control rats given saline, mepyramine or cimetidine. Results are expressed as median and (range) pmol/h/mg protein. There were no significant differences between any of the groups for any tissue. ND = not detectable.

	Control (saline), n=7	Control (mepyramine), n=7	Control (cimetidine), n=7
Aorta	12.2 (9.3-15.2)	15.2 (10.7-17.1)	13.5 (9.7-16.0)
Heart	15.3 (12.1-19.4)	17.7 (14.0-18.9)	17.6 (13.0-23.6)
Kidneys	21.5 (14.9-32.3)	29.1 (19.6-33.9)	22.7 (17.2-25.7)
Whole brain	11.6 (10.3-19.1)	11.8 (11.0-12.9)	12.3 (9.0-15.6)
Lung	ND	ND	ND

Table 12

The histaminase activity of various tissues obtained from diabetic rats given saline, mepyramine or cimetidine. Results are expressed as median and (range) pmol/h/mg protein. There were no significant differences between any of the groups for any tissue. ND = not detectable.

	Diabetic (saline), n=6	Diabetic (mepyramine), n=6	Diabetic (cimetidine), n=5
Aorta	14.3 (10.8-16.3)	13.8 (9.9-17.0)	15.1 (9.1-19.1)
Heart	16.3 (13.2-21.1)	17.2 (11.6-20.0)	15.8 (12.9-19.8)
Kidneys	24.4 (15.7-31.7)	20.1 (16.2-26.1)	27.6 (18.0-32.3)
Whole brain	12.1 (11.0-16.4)	11.7 (10.2-13.5)	10.9 (9.1-12.3)
Lung	ND	ND	ND

Table 13

The histamine content of plasma, aorta and kidneys obtained from control and 3-day starved rats. Results are expressed as median and (range) ng/mg protein for aorta and kidneys and ng/ml for plasma. There we no significant differences between the two groups and in any of the tissues.

	Control n = 7	3-day starved n = 7
Plasma	27.3 (18.8-32.4)	31.8 (22.4-33.7)
Aorta	387 (312-420)	360 (320-415)
Kidneys	0.42 (0.34-0.62)	0.46 (0.34-0.57)

Table 14

The HDC activity of aorta and kidneys obtained from control and 3-day starved rats. Results are expressed as median and (range) pmol/min/g protein. There were no significant differences between the two groups.

	Control n = 7	3-day starved n = 7
Aorta	3900 (2700-5300)	4100 (3000-6000)
Kidneys	13.8 (11.0-15.3)	12.1 (10.7-14.8)

3. HUMAN VOLUNTEER-BASED STUDIES

Histamine Uptake by human platelets

a. The effect of stirring on [^{14}C]histamine uptake by platelets in PRP (Figure 16)

There was linear uptake of [^{14}C]histamine for up to 90 min in the stirred (S) and unstirred (US) samples. The correlation coefficient (r) and the *p* values for uptake vs time (up to 60 min incubation) were r = 0.85 and $p < 0.001$ for the S samples (n=7) and r=0.96 and $p < 0.001$ for the US samples (n=7). The results for histamine uptake for S and US samples are shown in Figure 16 for incubations of up to 60 min. Samples from only 3 subjects were incubated for up to 90 min. Uptake in the S samples (206 fmol histamine/ 10^8 platelets/min) of all subjects was always greater than that in the US samples (127 fmol histamine/10^8 platelets/min). After incubation for 60 min, the platelet count dropped by approximately 7% of the baseline value in both the S and US samples.

b. The effect of conventional aggregating agents on [^{14}C]histamine uptake by platelets in PRP

(i) Effect of collagen (Figure 17)

Collagen, at a final concentration of 0.05 mg/l, significantly ($p<0.01$) enhanced [^{14}C]histamine uptake by platelets in PRP. The drop in platelet number during this experiment was similar to that in saline controls. Lower concentrations of collagen (0.01 and 0.03 mg/l) did not significantly enhance [^{14}C]histamine uptake. Higher concentrations of collagen (0.07 and 0.1 mg/l) did not enhance [^{14}C]histamine uptake, but did induce a concomitant fall in platelet count of >11%. In some subjects these concentrations (0.07 and 0.1 mg/l) induced significant platelet aggregation (>10% change in optical density). In the US samples, collagen did not significantly enhance [^{14}C]histamine uptake.

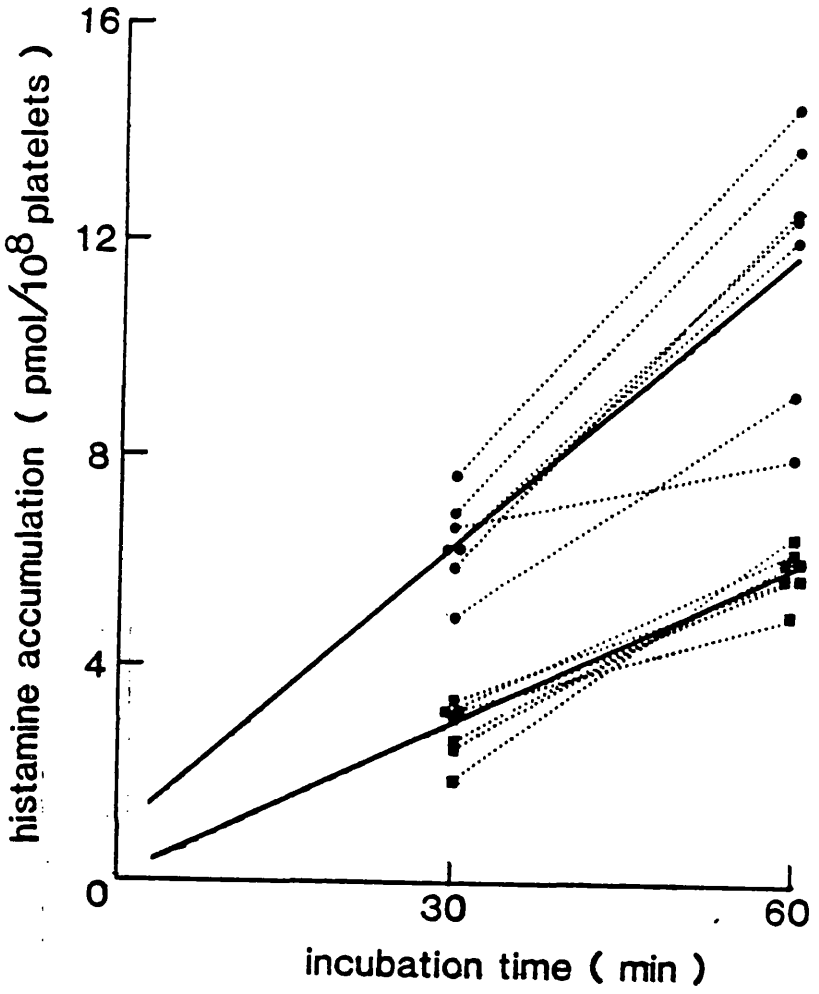

Figure 16. The effect of stirring on [^{14}C]histamine uptake by human platelets in PRP. Stirred *vs* unstirred, $p < 0.01$ at 30 and 60 min. The correlation coefficient (r) for the stirred samples was r = 0.85, $p < 0.001$ and for the untirred samples r = 0.96, $p < 0.001$.
■ - unstirred platelets; ● - stirred platelets

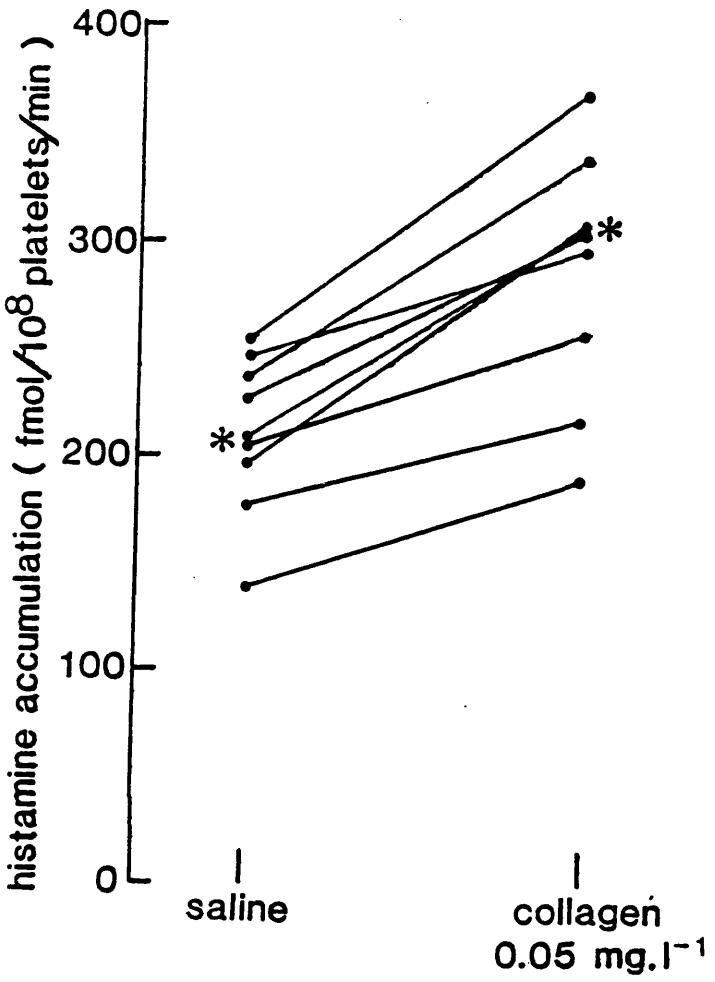

Figure 17. The effect of collagen on [^{14}C]histamine uptake by human platelets in PRP. * = median value. Saline *vs* collagen, $p < 0.01$.

(ii) Effect of adrenaline (Figure 18)

Adrenaline at a final concentration of 0.02 μmol/l significantly (p <0.01) enhanced [^{14}C]histamine uptake by platelets (with a 27% fall in platelet count). Higher concentrations of adrenaline (0.03 and 0.05 μmol/l) caused an increase in uptake, but they also caused a marked decrease in platelet numbers (>46%). In some subjects these higher concentrations caused aggregation as observed by changes in optical density (>10%). A decrease in platelet numbers occurred at lower adrenaline concentrations even in those samples in which no recordable aggregation was observed.

In the US samples, there was marginal enhancement of uptake following the addition of adrenaline.

(iii) Effect of ADP (Figure 19)

ADP at a final concentration of 0.03 μmol/l and 0.05 μmol/l margin-ally but significantly increased [^{14}C]histamine uptake (the fall in platelet count at the end of the incubation was similar to that in saline controls, i.e 5%). Higher concentrations (0.1 μmol/l) of ADP did not significantly enhance uptake, but did cause a concomitant drop in platelet numbers (>22%). Although most subjects showed appreciable enhancement of histamine uptake, the concentrations of ADP required to show this effect varied substantially between subjects. They were, however, all in the range of 0.02 -0.06 μmol/l ADP.

In the US samples there was no significant change in [^{14}C]histamine uptake following the addition of ADP.

c. Control Experiments

(i) Estimation of the amount of residual radioactivity in the platelet pellet after sonication.

This experiment confirmed that [^{14}C]histamine was located inside the platelets and that sonication released the ^{14}C radioactivity, leaving a very small residual amount (<2%) associated with the platelet fragment pellet. Platelet lysis was confirmed by the absence of platelet counts in the soni-cated PRP.

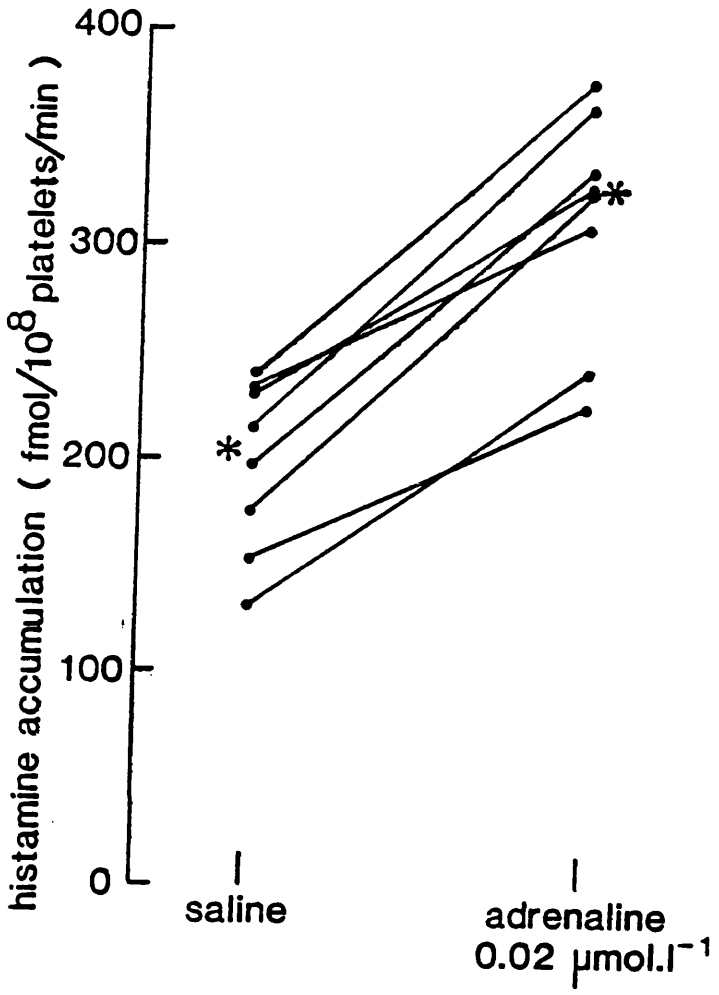

Figure 18. The effect of adrenaline on [^{14}C]histamine uptake by human platelets in PRP. ✻ = median value. Saline *vs* adrenaline, $p < 0.01$.

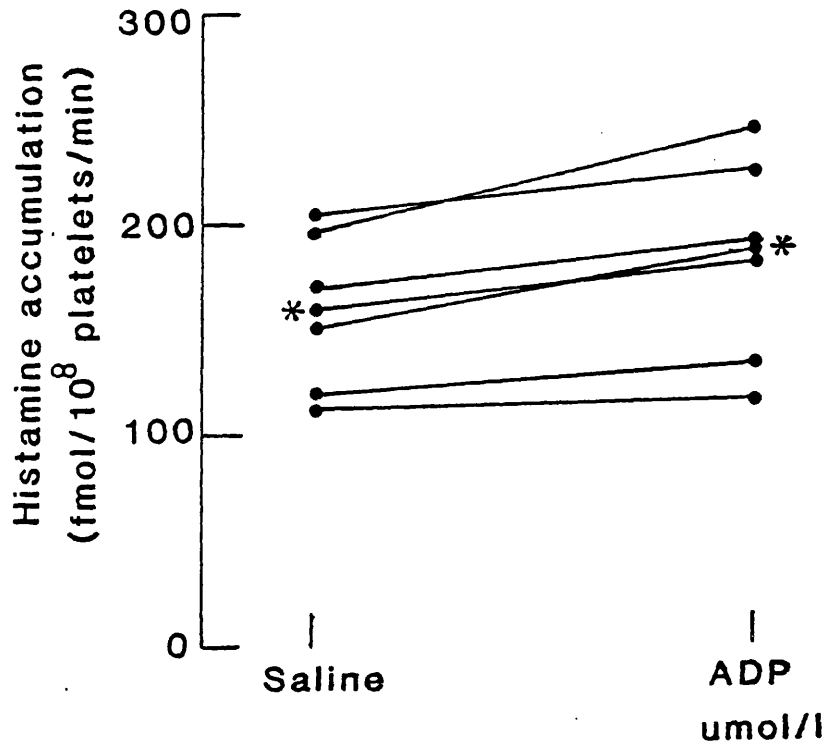

Figure 19. The effect of 0.05 µmol/l ADP on [^{14}C]histamine uptake by human platelets in PRP. * = median value. Saline *vs* ADP, $p < 0.01$.

(ii) The effect of using EDTA as an anticoagulant on platelet aggregation, ionised calcium concentration, and [^{14}C]histamine uptake

Platelet counts were significantly greater in samples prepared with EDTA as an anticoagulant than those prepared with citrate. PRP prepared with EDTA as an anticoagulant did not, however, show any aggregation even when high concentrations of agonists (collagen, adrenaline and ADP) were used (Table 15).

In the US samples, the [^{14}C]histamine uptake by platelets in the EDTA-PRP was approximately 20% (25 fmol histamine/10^8 platelets/min) of that in platelets prepared in citrate (121 fmol histamine/10^8 platelets/min; n=4). In the S samples, the [^{14}C]histamine uptake by platelets prepared in EDTA was approximately 50% (107 fmol histamine/10^8 platelets/min) of that in the platelets prepared in citrate (208 fmol histamine/10^8 platelets/min; n=4). The median concentration of ionised calcium in the EDTA-prepared PRPs was 0.11 (range: 0.09-0.12) mmol/l; and in the citrate-prepared PRPs, 0.09 (0.08-0.1) mmol/l.

(iii) The effect of platelet count in PRP on [^{14}C]histamine uptake

In the S samples there was no significant difference in the rate of [^{14}C]histamine uptake between undiluted PRP (215 fmol histamine/10^8 platelets/min), 1:1 diluted PRP (212 fmol histamine/10^8 platelets/min) and 1:4 diluted PRP (207 fmol histamine/10^8 platelets/min). In the US samples, there was again no significant difference in the rate of [^{14}C]histamine uptake between undiluted PRP (131 fmol histamine /10^8 platelets/min), 1:1 diluted PRP (130 fmol histamine/10^8 platelets/ min) and 1:4 diluted PRP (137 fmol histamine/10^8 platelets/min).

(iv) The effect of iodoacetate on [^{14}C]histamine uptake

30 min incubations (Table 16)

Iodoacetate, a known inhibitor of energy-requiring metabolic processes and in particular glycolysis (Clusin, 1983), at final concentrations of 10 (p <0.05), 50 and 100 mM significantly (p <0.03) inhibited [^{14}C]histamine uptake by platelets when incubated and stirred for 30 min at 37 °C. There was a statistically significant (p <0.05) reduction in uptake with 50 mmol/l iodoacetate when compared to 10 mmol/l iodoacetate. There was no reduction in [^{14}C]histamine uptake by iodoacetate at a final concentration of 1 mM.

Table 15

The inhibitory effects of EDTA on platelet aggregation (for 3 minutes). Results are expressed as median and (range) percentage aggregation; n = 6

	Aggregating agent dose		
	ADP (10 µmol/l)	Adrenaline (5 µmol/l)	Collagen 1 mg/l)
PRP prepared in citrate	66 (58-90)	61 (21-90)	71 (56-90)
PRP prepared in EDTA	0	0	0

Table 16

The inhibition of [^{14}C]histamine uptake by iodoacetate.

Results are expressed as median and (range); n = 5.

Basal uptake of histamine with only saline added was

214 fmol histamine/10^8 platelets/min for 30 minute incubations and

207 fmol histamine/10^8 platelets/min for 60 minute incubations.

* P <0.05, ** P <0.03 compared to corresponding saline only.

§ P <0.05, 50 mmol/l vs 10 mmol/l

	% inhibition of total [^{14}C]histamine uptake	
	30 min	60 min
Saline only	0 (0-0)	0 (0-0)
1 mmol/l iodoacetate	0 (0-0)	13 (9-16)*
10 mmol/l iodoacteate	30 (27-35)*	49 (43-58)**
50 mmol/l iodoacetate	48 (46-53)**§	67 (57-75)**§
100 mmol/l iodoacetate	54 (50-59)**	71 (65-79)**

Iodoacetate at 50 and 100 mM concentrations caused a 4% and 7% decrease respectively in platelet counts when compared to baseline. There were no changes in platelet counts with saline, 1 mM and 10 mM iodoacetate. In the unstirred samples, only 100 mM iodoacetate caused any inhibition of uptake. This inhibition was not statistically significant.

60 min incubation (Table 16)
Iodoacetate at final concentrations of 1 (p <0.05), 10, 50 and 100 mM significantly (p <0.03) inhibited [^{14}C]histamine uptake when incubated and stirred for 60 min at 37 °C. There was a statistically significant reduction in histamine uptake (p <0.05) at 50 mmol/l iodoacetate when compared to 10 mmol/l iodoacetate. After a 60 min incubation, the platelet count dropped by approximately 7% for saline only, 7% for 1 mM iodoacetate, 10% for 50 mM iodoacetate and 12% for 100 mM iodoacetate. In the unstirred samples, iodoacetate at 50 mM and 100 mM concentration caused small, statistically non-significant reductions in histamine uptake.

These results for both 30 min and 60 min incubations with iodoacetate show that approximately 30% of [^{14}C]histamine uptake was not inhibited at all.

(v) The effect of temperature on [^{14}C]histamine uptake by platelets

All incubations for platelet histamine uptake studies had been conducted at 37 °C. The lowering of temperature successively by 10 °C caused significant (p <0.01) reductions in [^{14}C]histamine uptake for both 30 and 60 min incubations (Table 17). A 10 °C decrease in incubation temperature to 27 °C caused 35% and 32% decreases in [^{14}C]histamine uptake for 30 min and 60 min incubations respectively. A further 10 °C decrease to 17 °C caused a 47% (30 min) and 48% (60 min) reduction in [^{14}C]histamine (p <0.01). Incubations carried out at 4 °C caused 71% (30 min) and 72% (60 min) decreases in [^{14}C]histamine uptake when compared to incubations carried out at 37 °C (p <0.01). Again, approximately 30% of uptake could not be inhibited, even at 4 °C. It must be stressed that these experiments on the effect of temperature were conducted in temperature-controlled water baths and were hence without stirring (only vigorous shaking). Since stirring itself has been shown by earlier experiments to increase uptake, it is possible that in these experiments we have only examined the passive, non-activated histamine uptake system.

Table 17

The effect of temperature on [^{14}C]histamine uptake by platelets. The results are expressed as median and (range), n=5. Basal uptake of histamine at 37 °C was 131 fmol histamine/10^8 platelets/min for 30 minute incubations (unstirred samples).

* P <0.01 when compared to 37 °C; § P <0.05 for 17 °C when compared to 27 °C; §§ P < 0.05 for 4 °C when compared to 27 °C.

	% inhibition of total uptake	
	30 min	60 min
37 °C	0 (0-0)	0 (0-0)
27 °C	35 (33-37)*	32 (26-36)*
17 °C	47 (43-57)*	48 (45-51)*§
4 °C	71 (60-90)*§§	72 (61-84)*§§

(vi) Estimation of β–thromboglobulin (β–TG) activity as an indicator of platelet activation in uptake studies

The results show that activation by aggregating agents was dependent on stirring of the platelets (Table 18). The addition of saline did not cause significant release of β-TG from the platelets in either S or US samples (30 min incubation); however, the addition of sub-aggregatory doses of agonists (without changes in optical density-aggregation) elicited marked release of β-TG in the stirred samples but not to any extent in the unstirred samples (30 min incubation).

(vii) Estimation of TXA_2 concentration as an indicator of platelet activation in uptake studies

The results clearly show that platelet activation can occur by the simple process of stirring. Furthermore, only marginal activation occurs in the US samples regardless of stimuli. The same concentration of aggregating agent which elicits marginal or no TXA_2 release in the US samples can cause large TXA_2 release over a 30 min period in the S samples (Table 19).

(viii) The effect of aspirin, imipramine, cimetidine and mepyramine on [^{14}C]histamine uptake by platelets

Aspirin: In four separate experiments, acetylsalicylic acid at a concentration of 100 mg/ml, when added at the end of a 30 min incubation, significantly [234 (201-247) fmol histamine/10^8 platelets/min, p <0.01) increased [^{14}C]histamine content of the platelets when compared to the addition of saline [174 (161-189) fmol histamine/10^8 platelets/min).

Imipramine: The antidepressant imipramine, a known inhibitor of serotonin (5HT) uptake (Tuomisto, 1973), did not signficantly alter [^{14}C]histamine uptake by platelets, in four separate experiments.

Cimetidine (Table 20): In four separate experiments, cimetidine (at final concentrations between 10 nmol/l and 100 μmol/l), a specific inhibitor of histamine at the H_2 receptor, did not alter [^{14}C]histamine uptake by platelets.

Mepyramine (Table 21): Mepyramine (at final concentrations between 1 nmol/l and 10 μmol/l), a specific antagonist of histamine H_1 receptors

Table 18

The activation of platelets as assessed by β-thromboglobulin release after activation with stirring and sub-aggregatory doses of various agonists (30 minutes). n = 1 volunteer.

		β-thromboglobulin pmol/ml unstirred	stirred
Saline only		63	72
Adrenaline	0.02 μmol/l	-	189
Adrenaline	0.03 μmol/l	-	271
Adrenaline	0.05 μmol/l	55	1087
Collagen	0.05 mg/l	76	134
ADP	0.5 μmol/l	129	72

Table 19

The activation of platelets as assessed by TXA_2 release after activation with stirring and sub-aggregatory doses of various agonists.

n = 1 volunteer

		ng/ml TXA_2	
		unstirred	stirred
Saline only		0.25	0.8
Adrenaline	0.05 µmol/l	0.25	2.5
Adrenaline	0.03 µmol/l	-	2.0
Adrenaline	0.02 µmol/l	-	2.4
Collagen	0.05 mg/l	0.8	2.0
ADP	0.05 µmol/l	0.8	2.0
ADP	0.03 µmol/l	-	1.0

Table 20

The effect of cimetidine (30 min incubation) on [^{14}C]histamine uptake by platelets. Results are expressed as median and (range), n=4. There were no significant differences between saline and any of the cimetidine concentrations

		[^{14}C]histamine uptake fmol histamine/10^8 platelets/min
Saline		210 (184-239)
Cimetidine	10 nmol/l	193 (171-222)
Cimetidine	100 nmol/l	207 (189-239)
Cimetidine	1 µmol/l	217 (183-251)
Cimetidine	10 µmol/l	213 (200-231)
Cimetidine	100 µmol/l	202 (180-215)

Table 21

The effect of mepyramine (30 min incubation) on [^{14}C]histamine uptake by platelets. Results are expressed as median and (range), n=4. There were no significant differences between saline and any of the mepyramine concentrations

		[^{14}C]histamine uptake fmol histamine/10^8 platelets/min
Saline		210 (184-239)
Mepyramine	1 nmol/l	212 (179-240)
Mepyramine	10 nmol/l	200 (181-219)
Mepyramine	100 nmol/l	217 (170-253)
Mepyramine	1 µmol/l	197 (168-218)
Mepyramine	10 µmol/l	207 (190-240)

and a known inhibitor of 5HT uptake by platelets (Bevan & Heptinstall, 1983) did not affect [^{14}C]histamine uptake by human platelets in four separate experiments.

4. PATIENT-BASED STUDIES

A. *The histamine content of plasma, leucocytes and platelets in normal controls and patients with IDDM, NIDDM, PVD and PVD-DM*

Plasma (Figure 20)

Histamine concentrations in plasma from patients with PVD (median and [range]: 520 [446-650] pg/ml; n=23), PVD-DM (400 [256-538] pg/ml; n=17), IDDM (466 [110-615] pg/ml; n=26) and NIDDM (430 [218-1600] pg/ml; n=29) were significantly (p <0.002) greater than those in age-matched control subjects (87 [35-247] pg/ml, n=38). Patients with PVD and concomitant DM had plasma histamine concentrations similar to those in patients with IDDM and NIDDM. There were no statistically significant differences in plasma histamine levels between IDDM and NIDDM patients. There was no age-related change in plasma histamine concentrations in the control population. The median plasma histamine concentrations were 81 (range: 55-200) pg/ml in the younger controls (n=22) and 75 (range: 35-247) pg/ml in the elderly controls (n=16). Most importantly, the median histamine concentration (75 pg/ml) in the elderly controls was significantly lower than that in patients with PVD (520 pg/ml) who were of comparable age. There were no significant differences in plasma histamine between males and females, smokers and non-smokers, hypertensive and normotensive patients, DM patients with complications and DM patients without complications.

There were no significant correlations between plasma histamine concentrations and duration of DM and/or PVD. Furthermore, no correlation between plasma histamine and the severity of DM (blood HbA_1 and blood glucose) or the severity of PVD (ankle/arm systolic blood pressure [SBP] ratio and claudication-provoking time) was observed; neither were there any correlations between plasma histamine and leucocyte or platelet histamine levels.

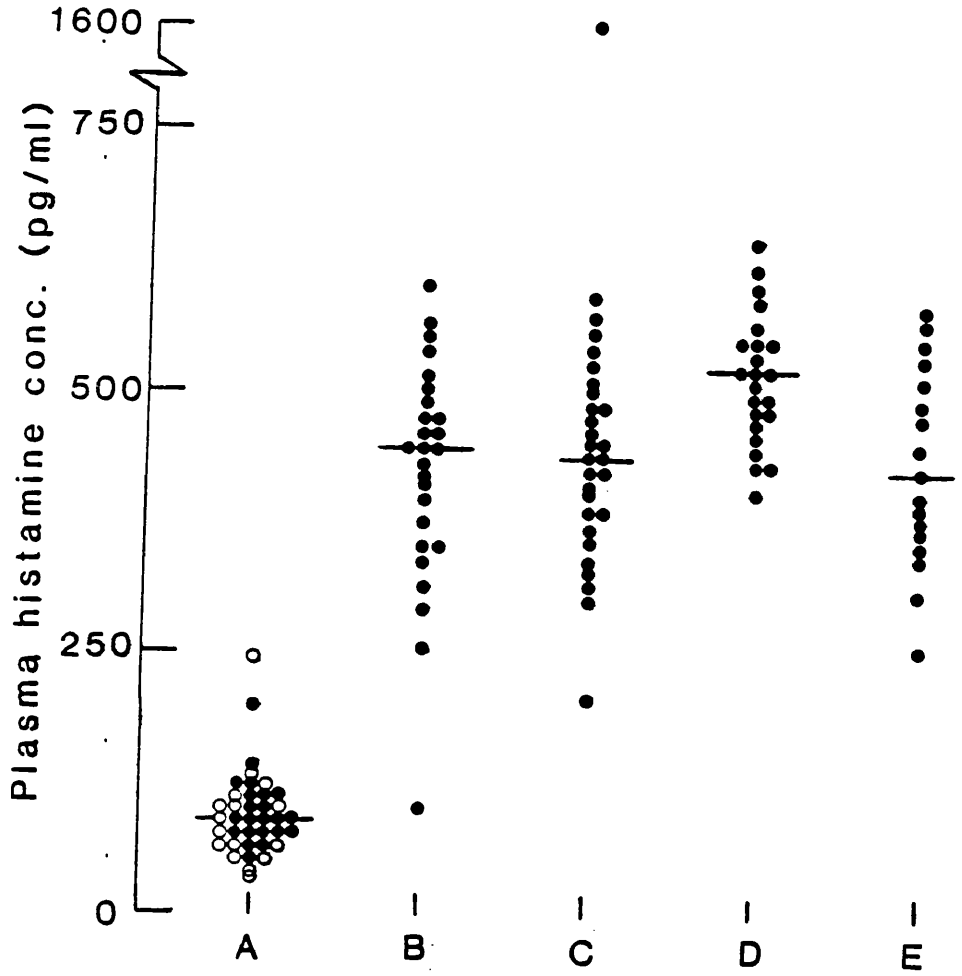

Figure 20. Plasma histamine concentrations. (A) controls, n=38; (•) young controls under the age of 65 years, n=22; (o) elderly controls over the age of 65 years, n=16. (B) Insulin-dependent diabetics, n = 29. (C) Non-insulin-dependent diabetics, n=23. (D) Patients with peripheral vascular disease, n=23. (E) Patients with peripheral vascular disease and concomitant diabetes mellitus (n=17). A *vs* B, A *vs* C, A *vs* D and A *vs* E, $p < 0.003$. All other permutations are non-significant.

Leucocytes (Figure 21)

The histamine content of leucocytes from patients with PVD (104 [73-139] ng/mg protein; n=33) was significantly (p <0.001) greater than that in controls (69 [20-100] ng/mg protein; n=55) and patients with IDDM (69 [25-106 ng/mg protein; n=39) or NIDDM (86 [31-200] ng/mg protein; n=38). Patients with PVD-DM (IDDM or NIDDM) had significantly (p <0.01) higher leucocyte histamine content (129 [74-152] ng/mg protein; n=16) than that in PVD patients. Leucocyte histamine content in NIDDM and IDDM patients was not significantly different from that in controls. Some NIDDM patients had leucocyte histamine contents comparable to the levels found in patients with PVD and PVD-DM. These outliers may have had as yet undiagnosed occult vascular disease.

There was no significant correlation between age and leucocyte histamine content in any of the patient groups or in the controls. Controls below the age of 65 had leucocyte histamine content (68 ng/mg protein; n=30) which was almost identical to controls over 65 years old (69 ng/mg protein; n=25). There was also no significant difference in leucocyte histamine content between males and females in both controls and patients. No apparent differences existed in leucocytic histamine in smokers and non-smokers and hypertensives and normotensives in any of the patient groups. Furthermore, no correlation was observed between leucocyte histamine and the severity of disease (DM - blood HbA_1, blood glucose; PVD - ankle/arm SBP ratio and claudication-provoking time). There were also no apparent correlations between leucocyte histamine content and the duration of disease (both DM and PVD). There was a highly significant correlation ($r = 0.69$; P <0.001) between leucocyte and platelet histamine contents of the patients and controls investigated (DM and PVD; Figure 22). Plasma histamine concentrations did not correlate with leucocyte histamine contents.

Platelets (Figure 23)

The histamine content of platelets was significantly (p <0.01) greater in PVD patients (11.9 [8.3-12.7] ng/10^8 platelets) than in controls (8.2 [7.1-10.1] ng/10^8 platelets) or in patients with IDDM (8.5 [7.1-10.2] ng/10^8 platelets) or NIDDM (8.5 [6.9-10.5] ng/10^8 platelets). The concomitant presence of

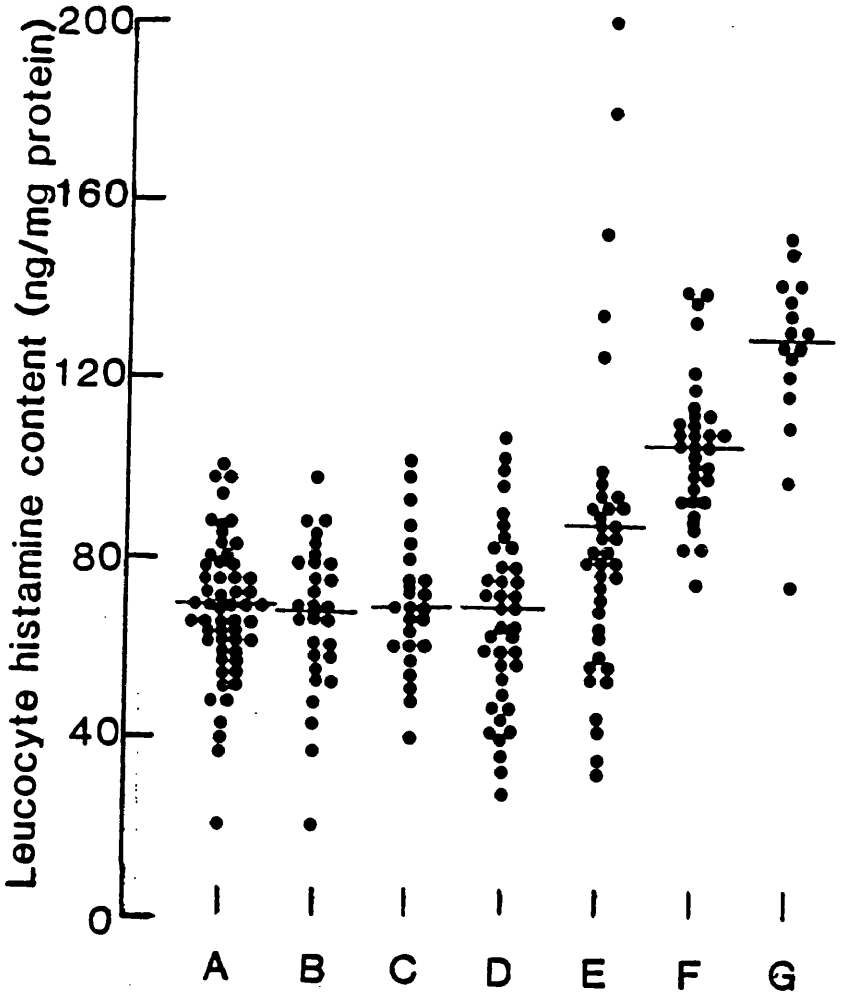

Figure 21. The histamine content of leucocytes of: (A) controls, n = 55; (B) controls under the age of 65 years, n = 30; (C) controls over the age of 65 years, n = 25; (D) insulin-dependent diabetics, n = 39; (E) non-insulin-dependent diabetics, n = 38; (F) patients with peripheral vascular disease, n = 33; (G) patients with peripheral vascular disease and concomitant diabetes mellitus (n = 16). A *vs* F and A *vs* G, $p < 0.01$. There were no significant differences between A - E in any permutation.

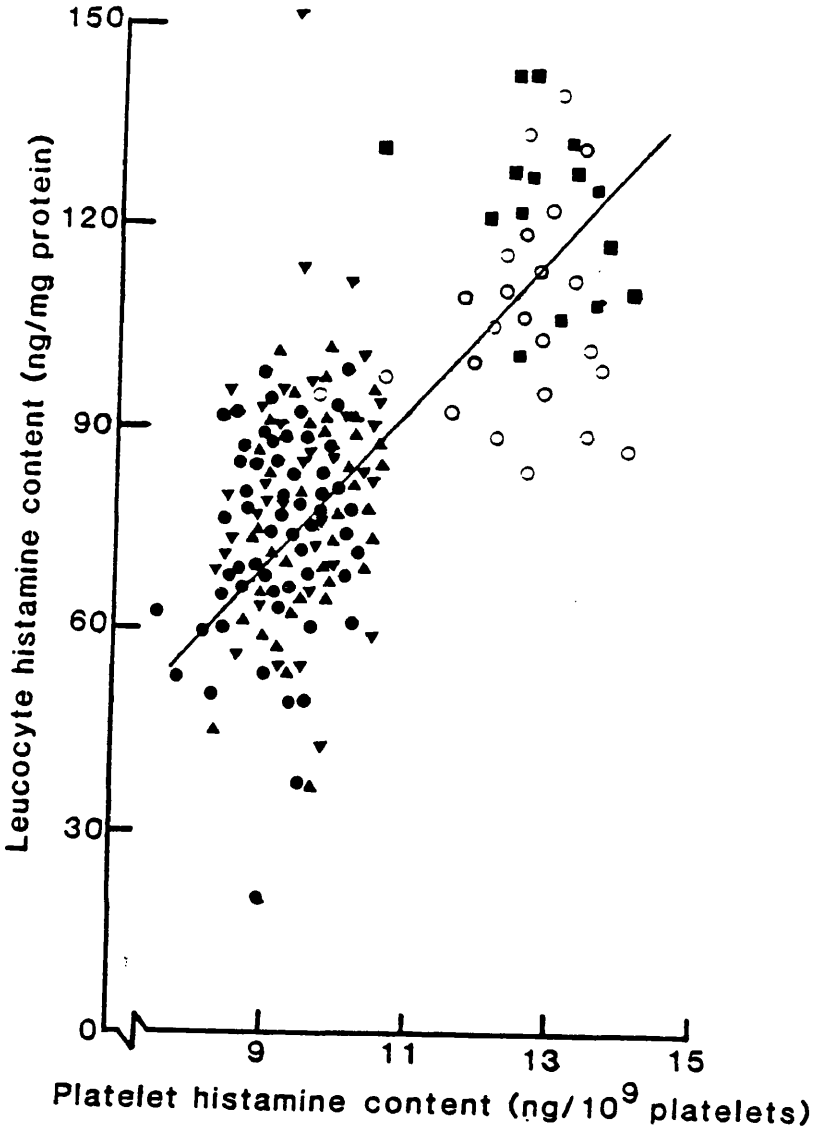

Figure 22. The correlation between leucocyte and platelet histamine content. Controls (•; n = 54), insulin-dependent diabetics (▲; n = 37), non-insulin-dependent diabetics (▼; n = 31), patients with peripheral vascular disease (○; n = 24), patients with peripheral vascular disease and concomitant diabetes mellitus (■; n = 15. r = 0.69; p <0.001.

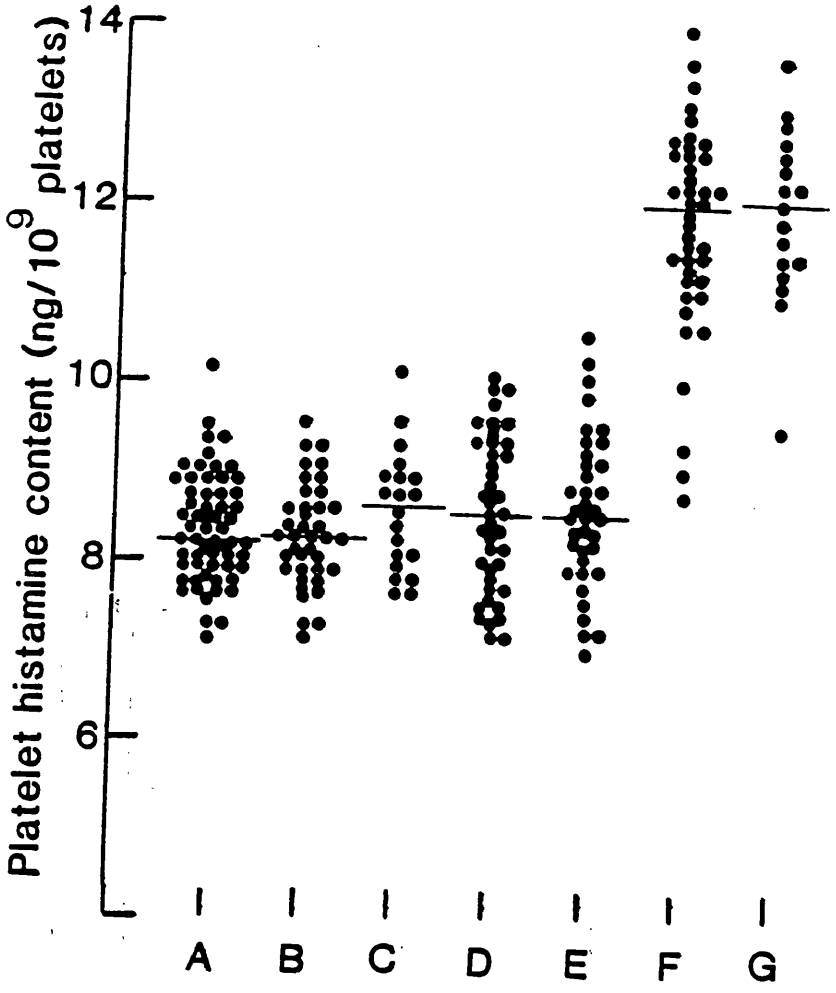

Figure 23. The histamine content of platelets of: (A) controls, n = 59; (B) controls under the age of 65 years, n = 39; (C) controls over the age of 65 years, n = 20, (D) insulin-dependent diabetics, n=44; (E) non-insulin-dependent diabetics, n = 38; (F) patients with peripheral vascular disease, n=40; (G) patients with peripheral vascular disease and concomitant diabetes mellitus, n = 17. A *vs* F and A *vs* G, $p < 0.001$. There were no significant differences between A - E in any permutation.

diabetes with PVD did not further affect the histamine content of platelets (12.1 [9.2-13.9] ng/10^8 platelets). The histamine content of platelets from IDDM and NIDDM patients did not differ from that in controls, and there was no difference between platelet histamine of NIDDM and IDDM patients.

There was no age-related difference in the histamine content of platelets from controls. Controls above the age of 65 years had a median platelet histamine content of 8.6 ng/10^8 platelets, and controls below the age of 65 years had a median platelet histamine content of 8.3 ng/10^8 platelets.

There were no significant correlations between platelet histamine content and severity of DM or PVD, or with the duration of DM and/or PVD. There was a highly significant correlation between platelet histamine content and leucocyte histamine content in the patient and control groups ($r = 0.69$; $p < 0.001$; Figure 22). There were also no significant differences between males and females, smokers and non-smokers and hypertensives versus normotensives.

B. *The HDC activity of leucocytes and platelets in controls and patients with IDDM, NIDDM, PVD and PVD-DM*

Plasma

HDC activity was not assessed in plasma

Leucocytes (Table 22)

There were no significant differences in leucocyte HDC activity between controls, IDDM, NIDDM PVD and PVD-DM patients. There was no correlation between leucocyte HDC activity and age, or the severity or duration of DM and PVD. Furthermore, no significant correlation between leucocyte HDC activity and leucocyte histamine content, plasma histamine and platelet histamine content was observed. There were no differences between males and females, smokers and non-smokers, hypertensives and normotensives in either patients or controls.

Table 22

The HDC activity of leucocytes in various patient groups. Results are expressed as median and (range). There were no significant differences between any of the groups.

Group	n	HDC activity (pmol/h/mg protein)	
Controls	47	11.7	(8.4-15.8)
IDDM	33	10.7	(8.3-17.3)
NIDDM	34	11.0	(8.9-18.4)
PVD	23	10.5	(7.1-17.5)
PVD-DM	17	11.0	(6.2-16.4)

Platelets

There was no detectable HDC activity in the platelets of the controls or any of the patient groups.

C. *The histaminase activity of leucocytes and platelets in controls and patients with IDDM, NIDDM, PVD and PVD-DM*

Plasma

Histaminase activity of plasma was not assessed

Leucocytes (Table 23)

There were no significant differences between the histaminase activity of leucocytes obtained from control subjects and from patients with IDDM, NIDDM, PVD and PVD-DM.

Furthermore, no correlation between histaminase activity and age, duration of disease or severity of disease was observed. There were also no significant correlations between leucocyte histaminase and leucocyte HDC, leucocyte histamine, plasma histamine or platelet histamine content.

Platelets

No detectable histaminase activity was observed in the platelets of the controls or of the various patient groups.

Table 23

The histaminase activity of leucocytes in various patient groups. Results are expressed as median and (range). There were no significant differences between any of the groups.

Group	n	Histaminase activity (pmol/h/mg protein)
Controls	14	8.7 (6.4-10.1)
IDDM	14	9.3 (7.3-11.0)
NIDDM	14	7.7 (6.0-8.4)
PVD	10	8.0 (5.8-9.3)
PVD-DM	10	7.8 (6.1-9.9)

CHAPTER IV

DISCUSSION

As discussed in Chapter I, histamine is a normal constituent of most mammalian tissues and blood. In the present study, we have examined alterations in histamine metabolism in (a) various tissues of rats with experimentally-induced non-ketotic diabetes mellitus and (b) the various components of human blood.

In our animal studies we used tissues that were readily accessible and which facilitated rapid removal from the animal post-mortem. The aorta from diabetic animals had previously been shown to exhibit marked alterations in histamine metabolism (Hollis & Strickberger, 1985; Hollis *et al.*, 1983, 1984) and was therefore an obvious choice for further examination. The other tissues investigated in our studies were the heart, the kidneys, the lungs and the brain. Plasma was obtained to assess the concentrations of histamine to which the vascular endothelium would be subjected.

In our human studies, on the other hand, the only readily available tissue was blood, and therefore the determination of alteration in histamine metabolism in the various components of human blood was the most practical choice. Blood leucocytes, platelets and plasma are known to contain histamine and are known to be involved in a variety of physiological processes too numerous to mention. Furthermore, platelets and leucocytes are both known to be intimately involved in the pathogenesis of atherosclerosis. In our experiments to isolate human leucocytes we used the dextran sedi-mentation method (Ahmed, 1970), which is rapid and simple and which yields a mixed population of polymorphs and mononuclear leucocytes in the same proportion as in peripheral blood. The method of isolation for human platelets from venous blood is relatively simple and rapid (15 minutes) and requires small quantities of blood which can yield 200-350 million platelets per ml of plasma (Born & Cross, 1963).

Our control experiments on the various rat tissues used in this study clearly demonstrate that the highest proportion of histamine synthesis in these tissues was by HDC and not by the non-specific DOPA decarboxylase

enzyme. This confirms previous reports which have shown that HDC is the sole enzyme responsible for *in vivo* histamine synthesis (Morris & Fillingame, 1974). These data were also the first indicator that diabetic tissues had elevated histamine synthesis primarily through the increased activity of the specific HDC enzyme, since the non-specific DOPA decarboxylase enzyme activity was similar in control and diabetic rats.

A. ANIMAL STUDIES

Our data demonstrate that the aorta, the kidneys, the lungs and the heart of diabetic rats have a significantly greater HDC activity than that in controls (Gill *et al.*, 1988a), without any concomitant decrease in histaminase activity (Gill *et al.*, 1990a). The increase in histamine synthesis in the aorta was the most impressive, as was the increase in aortic histamine content. The increase in histamine content was paralleled by an increase in HDC activity in all tissues except the brain, stomach and skin. It is therefore likely that the increase in histamine content in various tissues in diabetic animals is due to an increase in histamine synthesis, since there is no decrease in histamine breakdown. Previous work by Orlidge & Hollis (1982), however, has shown a significant increase in the histaminase activity of aortic endothelial and smooth muscle cells from diabetic rats. It is possible that these changes were masked in our study, since we assayed whole tissue histaminase rather than histaminase activity of specific vascular cells. Furthermore, Orlidge & Hollis (1982) have shown that the magnitude of increase in aortic HDC activity in diabetic rats is markedly greater than the magnitude of decrease in histaminase activity. Therefore, any changes in histamine synthesis would be more apparent than changes in histamine breakdown, especially when whole tissue extracts are assayed. The brain of the diabetic animals showed no increase in HDC activity but it did show a significant increase in histamine content; this may reflect the presence of residual histamine 'rich' plasma and, possibly, cerebrospinal fluid. Since the aortae from diabetic animals showed the greatest increase in HDC activity, the possibility that the presence of blood vessels largely contributes to the increase in HDC activity in other organs has to be considered. This is relevant to all tissues investigated except the brain, since the brain had high HDC activity in spite of the fact that it did not alter with diabetes. This fact raises the possibility that a circulating mediator

inducing changes in tissue histamine synthesis is not able to cross the blood brain barrier. The selectivity of the blood brain barrier in experimental diabetes is well documented (Lorenzi, 1986).

The skin and stomach are the two tissues highly subject to the effects of histamine, and neither showed any changes in histamine metabolism in diabetic rats. It is important to realise that the greatest amount of histamine in the stomach and skin of rats is stored in mast cells (Parratt & West, 1956; Foley & Glick, 1962), and it is possible that diabetes does not influence this so-called non-inducible pool. Any changes in the blood vessels of the stomach or skin of rats would be diluted by the vast amounts of histamine naturally present in non-vascular tissue. Tissue HDC activity in the brain, the lungs, the stomach and kidneys of control rats in our experiments was similar to that previously reported (Fisher *et al.*, 1981; Yamada *et al.*, 1980). However, HDC activity of the heart was different to that reported previously: whereas Yamada *et al.* (1980) could not detect HDC activity in the hearts of Sprague Dawley rats, we consistently observed a small HDC activity in rat hearts. This may be due to differences in assay method, since the methodology employed by us is much more sensitive than the o-phthal-aldehyde method employed by Yamada.

Our experiments on diabetic animals clearly indicated that there were no alterations in DOPA decarboxylase activity in any of the tissues examined. Since DOPA decarboxylase is the enzyme responsible for the biosynthesis of serotonin and catecholamines, any changes in serotonin and catecholamines in experimental diabetes would be independent of the serotonin and catecholamine synthesis. Recent work by Barradas *et al.* (1990) have indeed shown a marked decrease in the serotonin content of all those tissues in experimental diabetes in which we observed an increased histamine content. It is very probable, therefore, that these reduced tissue serotonin levels are the result of an increase in serotonin catabolism and/or an inability of tissues to store serotonin. These data on serotonin further support the concept that the increase in histamine content of the tissues of diabetic rats is due to an increase in histamine synthesis mediated by specific increases in HDC and is not the result of an indiscriminate elevation in non-specific decarboxylase activity.

Hollis and his colleagues have, for the past 15 years, been working on the premise that accelerated *de novo* histamine synthesis is at least one

important event occurring in experimental diabetes. Increased synthesis is causally related to certain increased vascular wall permeability characteristics in diabetes and in the pathogenesis of atherosclerosis. They have shown that aortic histamine metabolism and, in particular, increased histamine synthesis by HDC, is altered in a variety of atherogenic risk situations. It has been shown that shearing stresses like those found in atherosclerosis and hypertension create a frictional interaction with the aortic endothelium; and Fry (1968, 1969) has shown that these stresses can create a vascular injury, at least when these stress forces are elevated. Hollis and his co-workers have shown marked increases in endothelial HDC activity in aortae exposed to elevated shear stress (De Forrest & Hollis, 1978; Hollis & Ferrone, 1974; Skarlatos & Hollis, 1987). Rosen *et al.* (1974) have shown that endothelial histamine synthesis is extremely sensitive to shear stress exposure, and have suggested that the HDC system may represent an enzymatic coupler between haemodynamic stresses and subsequent permeability alterations, as in atherosclerosis. In another study it has been shown that cultured bovine aortic cells when exposed to oscillatory shear stress which closely mimics the disturbed pulsatile flow of blood in the diseased aorta, respond by rapidly synthesising histamine. They showed that these changes were more dependent on the shear stress exposure duration rather than on the stress intensity. This same study demonstrated that endothelial cells respond to shear stress by initially (6 h) taking up histamine from the media and subsequently (12-24 h) synthesising histamine intracellularly to such an extent that it results in leakage of histamine from the endothelial cells to the media (Skarlatos & Hollis, 1987). Owen & Hollis (1981) have also shown that there are marked elevations in histamine synthesis and albumin permeability in aortic locations exposed to locally disturbed blood flow as assessed by the uptake of Evans Blue dye.

In a separate batch of studies, Hollis and his team have looked at the effect of transient neurogenic and mechanically induced hypertension (Bolitho & Hollis, 1975; Hollis *et al*, 1972). They found that aortic HDC activity is markedly elevated in rats with both types of hypertension, indicating HDC induction in the blood vessels with increased vascular stress as a result of hypertension.

Hypercholesterolaemia is known to be a risk factor for atherosclerosis. As a result, Hollis and co-workers have examined aortic histamine metabolism

in dietary hypercholesterolaemia in rabbits (Hollis & Sloss, 1975; Markle & Hollis, 1975, 1977; Hollis & Furniss, 1980; Owens & Hollis, 1979). In two of these studies (Hollis & Sloss, 1975; Markle & Hollis, 1977) they examined aortic HDC activity in rabbits subjected to short-term, relatively low dosage cholesterol feeding with the specific intention of measuring aortic histamine synthesis in both the preatherosclerotic state and in the early stages of histologically discernible aortic lipid deposition. They found that aortic HDC activity was markedly elevated in the hypercholesterolaemic rabbits when they were in the preatherosclerotic state (no aortic lipid deposition), but that the elevation in HDC (albeit significantly higher) started to normalise towards control values when aortic lipid deposition started to occur. This indicated that, at least in mild dietary hypercholesterolaemia, elevation in aortic histamine synthesis was transient. In a subsequent study (Hollis & Furniss, 1980) it was shown that the increased aortic endothelial histamine synthesis was strongly correlated with increased aortic albumin permeability; again both these changes were transient in nature and consistent with the findings of Robertson and Khairallah (1973), who have also shown that aortic permeability changes during atherogenesis are in many cases transient.

If, as suggested, increased aortic histamine synthesis is one of the factors responsible for increased aortic macromolecular uptake, then the inhibition of HDC should reduce the incidence and severity of atherosclerosis in hypercholesterolaemic rabbits. This has been clearly demonstrated to be the case by Owens and Hollis (1979). They have shown that the simple partial inhibition of HDC by αHH, a specific inhibitor of this enzyme, not only reduces aortic histamine synthesis by over 30% but also causes a 51% reduction in aortic albumin accumulation and a 63% decrease in the severity of atherosclerosis when assessed histologically.

Diabetes mellitus is a known risk factor for atherosclerosis, and the majority of the animal studies in this work were conducted on rats with experimentally induced diabetes. Very little work has been conducted to examine specific metabolic changes occurring in the large arteries of diabetic animals or patients. Wolinsky *et al.* (1978), however, reported that several aortic smooth muscle hydrolases were decreased following streptozotocin-induced experimental diabetes. They showed that the largest increase occurred with acid cholesteryl esterase, the principle enzyme involved in LDL catabolism and clearance. This study suggests

that LDL clearance is impaired in experimental diabetes which, in conjunction with the elevated total blood lipid concentrations in experimental diabetes, would result in the deposition of LDL in the vessel wall. Factors such as histamine which are closely associated with vessel wall permeability to macromolecules such as LDL, could therefore be important in the pathogenesis of diabetic atherosclerosis.

The data presented in this study, along with the work of Hollis, strongly suggest that histamine metabolism is considerably altered in experimental diabetes. Others, as well as ourselves, have shown that in experimental diabetes an increase in histamine content occurs in various highly vascular tissues and in plasma, and this is associated with an increase in HDC activity of various tissues. Hollis, in a series of important studies, has pursued the issue of histamine metabolism in experimental diabetes. He has shown that in this condition, aortic endothelial and smooth muscle cells show increases of 150% and 165% respectively in their histamine content (Orlidge & Hollis, 1982). This increase in histamine content is also accompanied by a 260% increase in HDC activity in the endothelial cells and a striking 300% increase in the smooth muscle cells - increases that are of a similar order to, but higher than, those reported in the present study. While Orlidge and Hollis have used pure aortic endothelial and smooth muscle cells, we have used whole aorta preparations. If the increases in histamine synthesis and content are wholly restricted to these cells, it is not surprising that the magnitude of the increases in our study are roughly half those reported by Orlidge and Hollis.

Orlidge and Hollis (1982) also demonstrated a reduction in histaminase activity by half in aortic endothelial and smooth muscle cells. Significantly, insulin treatment for a one-week period following three weeks of uncontrolled diabetes resulted in the complete reversal of all the above parameters. They concluded that histamine synthesis and catabolism account for 90% of the intracellular histamine present, and that these alterations in histamine metabolism are completely reversed by insulin treatment. Hollis has suggested that insulin modulates histamine metabolism in some way and that the aorta is particularly insulin-sensitive, at least in experimental diabetes. Furthermore, unlike aortic histamine alterations in dietary hypercholesterolaemia, alterations of histamine metabolism in experimental diabetes occur in both aortic endothelial and smooth muscle cells and are of a considerably greater

magnitude than those in hypercholesterolaemia; these changes are exacerbated over time and are not transient. Hollis has also shown that in experimental diabetes these alterations in aortic histamine metabolism are accompanied by massive increases in aortic albumin transfer (300%) as well as a 10-fold increase in aortic albumin accumulation (Hollis & Strickberger, 1985). They also showed that the albumin space in the aortae of diabetic animals is significantly lower than that in normal animals (Hollis *et al.*,1984). In addition, Hollis and his group have shown that in the rat, increases in aortic HDC activity occur within two weeks of the induction of diabetes (Orlidge & Hollis, 1982; Gallik & Hollis, 1981), indicating an extremely rapid response to the diabetic environment.

The most significant finding of these studies was that αHH administration to diabetic animals not only prevents increases in aortic HDC activity but also totally prevents an increase in albumin permeability, despite the presence of severe hyperglycaemia and hypoinsulinaemia (Hollis *et al.*, 1983; Hollis & Strickberger, 1985; Carroll & Hollis, 1985). All these studies strongly support the premise that the elevated aortic histamine synthesis which occurs in experimental diabetes is an important mediator of aortic macromolecular uptake.

In the present study, we have demonstrated an increase of 119% in plasma histamine concentrations in streptozotocin-induced experimental diabetes (Gill *et al.*, 1988b). It is interesting that Hollis and his co-workers (Hollis *et al.*, 1985) demonstrated a 100% increase in plasma histamine concentrations in their diabetic rats. However, Hollis also showed in his study that the elevation in plasma histamine in diabetic rats is almost certainly derived from the inducible HDC-dependent blood vessel histamine pool. This was indicated by the fact that insulin treatment, which prevents both hyperglycaemia and increased aortic histamine synthesis (Orlidge & Hollis, 1982) produces a significant reduction in plasma histamine concentrations, and that αHH administration produces an even greater decrease in the plasma histamine content The changes in plasma histamine observed in this study and in those reported by Hollis qualitatively parallel those changes in vascular histamine reported by us in this study, and reported previously by Hollis (Orlidge & Hollis, 1982; Hollis *et al.*, 1983).

In rats, as in man, most of the blood histamine is found in leucocytes, with the platelets containing negligible amounts (Almeida *et al.*, 1980; Graham

et al., 1955). However, since the alterations in plasma histamine in our present study qualitatively parallel those changes we report in aortic histamine content (Gill *et al.*, 1988b), we believe that the contribution by leucocytes to plasma histamine is minimal at best, and merely reflects endothelial histamine release. These data are supported by our studies with diabetic patients in which there were no alterations in histamine synthesis of degradation in the leucocytes. These studies will be discussed in more detail later in this chapter.

In the present study, we have reported marked increases in the histamine content and HDC activity in various other tissues such as the kidney, heart and lung. Hollis has also since shown that renal histamine is increased in streptozotocin-induced diabetes in rats; however, he has not investigated alterations in HDC activity in the kidney, and has wondered about the origin of elevated renal histamine (Markle *et al.*, 1986). He postulated that it might be the result of residual histamine 'rich' plasma, since the plasma histamine of untreated diabetic rats is markedly increased. In our study, we have clearly demonstrated that increased renal histamine is paralleled by an increase in renal HDC activity (Gill *et al.*, 1990a). A similar parallelism between histamine content and HDC activity was observed in the case of aortae of diabetic animals (Gill *et al.*, 1990a). It is of interest that Markle *et al.* (1986) were able to totally inhibit all changes in renal histamine content by insulin therapy, αHH administration, and a combination of both.

Our studies on the kidney are of special interest, since nephropathy and related proteinuria are prevalent in diabetes mellitus. It is known that proteinuria in early diabetes results from a 'functional' microangiopathy of the glomerulus. This functional microangiopathy, as described by Parving *et al.* (1976), develops prior to the structural microangiopathy and manifests as increased transglomerular passage of plasma proteins; but the mechanism for this protein leakage is not known. Thus it is possible that in view of the increases of vascular tissue histamine and permeability noted in the diabetic rat particularly related to endothelial histamine metabolism, the functional glomerular microangiopathy described above by Parving may involve an increase in the renal 'inducible' vascular histamine pool.

Histamine is known to have a profound effect on cardiac function (Flacke *et al.*, 1967), although it elicits different cardiovascular responses in different species of animals (Beavan, 1978). The heart is very responsive to

histamine and therefore the increase in histamine synthesis in the hearts of diabetic rats, shown in this study, may lead to an alteration in many cardiac functions such as the sinus rate and contractility acutely, while promoting coronary atherosclerosis in the long term. The coronary arteries are the most atherosclerosis-prone vessels in the body and, in fact, coronary artery disease is the predominant manifestation of atherosclerosis. Therefore, any alteration in the permeability characteristics of coronary vessels as a result of histamine disturbances can have potentially disastrous consequences. Furthermore, histamine has been shown to induce coronary spasm in swine, leading to changes phenomenologically similar to those of an anginal attack in patients with coronary spasm (Egashira *et al.*, 1986). Since it has been shown that atherosclerotic coronary arteries contain significantly higher histamine contents than non-atherosclerotic arteries (Kalsner & Richards, 1984), it is possible that local release of histamine from the coronary vessels may induce a cononary spasm, culminating in angina.

The lung is another organ which responds to histamine and apart from its role in allergic reactions, histamine is suspected to be a mediator of hypoxic pulmonary vascoconstriction leading to pulmonary hypertension (Fishman, 1976). The lung was investigated primarily because it contains vast amounts of capillary vasculature. The lung also contains large amounts of histamine, predominantly contained in mast cells (Hoffman *et al.*, 1977). It is therefore not surprising that in diabetic rats the histamine content of the lung increases by only 32% and HDC activity increases only by 40%. If, as postulated, increases in histamine synthesis occur in the non-mast cell 'inducible' pool, then any increases in the pulmonary capillary endothelium would be diluted by the presence of vast amounts of mast cell histamine when the tissues are homogenised for assay.

Retinal histamine synthesis is also known to be elevated in experimental diabetes (Carroll & Hollis, 1987) and this in itself is very interesting since diabetes mellitus is known to cause abnormal blood retinal barrier permeability, which leads to diabetic retinopathy (Cunha Vaz *et al.*, 1975). Others have also observed changes in blood-ocular barrier permeability in diabetic humans and rats, noting that these changes occur before any ophthalmoscopic or angiographic signs of diabetic retinopathy develop (Waltman *et al.*, 1970; Ishibashi *et al.*, 1980). The causes of blood-ocular leakage in diabetes are not fully known; however, it is interesting that the

chronic intravenous infusion of histamine in non-diabetic rats to levels found in diabetic rats causes a marked increase in blood retinal albumin.

It has also been demonstrated that insulin therapy and αHH therapy reverse blood retinal permeability (Carrol & Hollis, 1987; Enea *et al.*, 1985). This is an interesting observation, since insulin therapy and αHH also reverse aortic albumin permeability. In recent studies (Hollis *et al.*, 1988; Dull *et al.*, 1986) it was demonstrated that the simultaneous administration of H_1 and H_2 antagonists to diabetic rats dramatically reduces retinal permeability, despite the presence of severe hyperglycaemia. The authors concluded that elevated blood retinal barrier permeability can be reversed by H_1 and H_2 receptor antagonists, and that the interaction of these antagonists with elevated endogenous histamine synthesis may mediate retinal barrier permeability. These facts suggest a pathogenic role for histamine in diabetic retinopathy.

In the present study, the administration of H_1 and H_2 receptor antagonists to diabetic rats did not alter the histamine content, histamine synthesis or histamine degradation in any of the tissues examined, including the aorta, and plasma. In this same study, the albumin accumulation of the aortae of diabetic animals was 290% greater than that of the aortae from control animals; again, the adminstration of H_1 and H_2 antagonists did not significantly alter aortic albumin accumulation in either control or diabetic rats. It is interesting that blood retinal permeability in diabetic rats can be reduced so dramatically by H_1 and/or H_2 antagonists, yet these same antagonists do not alter histamine metabolism or aortic permeability in the same animals. Insulin and αHH, on the other hand, affect both systems equally.

There are several reasons why H_1 and H_2 receptor antagonists did not affect histamine metabolism in our experiments. Firstly, there is no evidence to suggest that inhibition of the histamine H_1 and H_2 receptors which are known to exist on vascular endothelial cells (Heltianu *et al.*, 1982) should in any way prevent the induction of HDC in endothelial and smooth muscle cells. Furthermore, it is possible that the concentrations of both inhibitors were inadequate and did not achieve total blockade of endothelial H_1 and H_2 receptors. This is unlikely because the concentrations used for both antagonists have been used previously to achieve total body receptor blockade (Al Haboubi & Zeitlin, 1979; Parsons,

1988). Lastly, it is also possible that an H_3-like receptor (Arrang *et al.*, 1987) may be involved in the actions of histamine in this system.

Aortic albumin accumulation was more likely to be inhibited by histamine antagonists, since they are known to inhibit retinal permeability (Carroll & Hollis, 1987; Enea *et al.*, 1985; Hollis *et al;*, 1988). Again, this was not the case in our study. It was clear in our experiments that diabetic aortae were appproximately three times more permeable to albumin than control aortae; however, the administration of H_1 and H_2 antagonists did not significantly reduce albumin permeability in either controls or diabetics. There was a definite trend for diabetic animals treated with cimetidine to have lower permeability to albumin than untreated diabetic rats; however, probably because of a limited number of animals (n=5; two animals died during the experiment), the results did not achieve statistical significance. It must be stressed that our permeability studies were conducted *in vitro* and it is possible that our methodology was not sufficiently sensitive to detect any alterations; however, the fact that we demonstrated a 290% increase in albumin accumulation in diabetic rats when compared to controls points to a relatively simple but sensitive method. It must be stressed that Hollis's experiments to assess the effects of αHH and insulin on aortic permeability were conducted *in vivo*.

In our methodology we have assessed not only endothelial albumin accumulation but also total aortic accumulation of exogenous albumin, which is a combination of permeability by both the endothelial and adventitial route. It is known that albumin enters the media of the aortic wall by both routes *in vivo* (Bratzler *et al.*, 1977). Our methodology cannot therefore serve as a quantitative estimate of the permeability of any one route of albumin entry, nor was it intended to do so; it was designed to be a rapid and relatively simple method to assess the overall permeability of a vessel.

Experimental diabetes, in the rat, represents a severe catabolic state as evidenced by weight loss. The possibility thus exists that increased histamine synthesis in the various tissues is as a result of complications induced by this sverely catabolic state and may not be the results of diabetes. We therefore starved rats for three days, to induce a severe catabolic state in which animals is utilise body stores of fats and proteins in order to provide energy; a situation similar to that in diabetic rats, albeit at an accelerated rate. The data clearly show that in the 3-day starved rat, despite gross

weight loss, there were no alterations in histamine synthesis in the aorta or kidneys and there was no increase in the histamine content of plasma. It is highly probable that the alterations in histamine synthesis in various tissues are the result of diabetes mellitus and not of reduced growth rate. It may, however, be argued that semi-starvation for a protracted period (9 days) may more closely resemble the non-ketotic diabetes we investigated, since Hollis and his group (Orlidge & Hollis, 1982) have shown altered histamine metabolism within only 14 days.

B. HUMAN STUDIES

Our data show conclusively that the increase in plasma histamine concentrations previously observed in experimental diabetes by ourselves (Gill *et al.*, 1988b) as well as others (Hollis *et al.*, 1985) also occur in patients with DM and PVD.

The present study also demonstrates that the histamine content of leucocytes and platelets in patients with PVD is significantly greater than that in control subjects (Gill *et al.*, 1988c). The age of the patients or volunteers did not have any effect on the content of histamine in these cells or in plasma. Neither IDDM nor NIDDM were associated with an increase in histamine content in leucocytes or platelets, but some patients with NIDDM had leucocyte histamine contents which were comparable to those in patients with PVD. These patients with NIDDM were elderly and may therefore have had atherosclerosis at a subclinical level. There was no increase in the HDC activities of leucocytes from patients with PVD, DM or PVD-DM. Platelets exhibited no HDC activity, whether obtained from patients or controls. There was no change in the histaminase activity of leucocytes in any of the patient or control groups. Platelets did not exhibit any histaminase.

It has been postulated that the increase in plasma histamine concentrations observed in experimental diabetes may be the result of leakage of histamine from the markedly expanded histamine pool in the blood vessels of these animals (Owens & Hollis, 1979; Orlidge & Hollis, 1982; Gill *et al.*, 1988b). We have also demonstrated marked increases in plasma histamine in DM and PVD patients (Gill *et al.*, 1989). Yoshimura *et al.* (1984) have shown that plasma histamine concentrations are elevated in

patients with coronary artery disease (CAD) and that the more severe the arterial lesion, the higher the plasma histamine concentration. As mentioned earlier, it is of interest to note that a marked increase in histamine content has been observed in the coronary arteries of patients with CAD by Kalsner & Richards (1984). They showed that the concentration of histamine was nearly doubled in the arteries of patients with CAD and that the arteries from such patients were hyperreactive to histamine and responded by more marked contraction at lower concentrations of histamine. Furthermore, the atherosclerotic regions of the arteries had significantly higher histamine than the non-atherosclerotic regions of the same arteries. Kalsner & Richards postulated that the sudden release of histamine due to injury would induce a powerful contraction or spasm of a coronary vessel segment and precipitate a cardiac crisis such as angina or dysrhythmia. Furthermore, any locally released histamine would cause a substantial increase in coronary vessel permeability (Pilati & Maron, 1988), which can in turn accelerate the atherosclerotic process and possibly lead to myocardial oedema (Pilati & Maron, 1988).

Ginsburg *et al.* (1981) have demonstrated that an intracoronary infusion of histamine provokes coronary artery spasm, and they have implicated histamine in the pathogenesis of angina pectoris. Shimokawa *et al.* (1983) have also shown that angiographically visualised coronary artery spasm can be induced in the miniature swine with experimentally induced atherosclerosis, but not in control pigs, by the intravenous or intracoronary administration of histamine and that this action can be blocked by H_1 receptor antagonists.

Antihistamines have been shown to inhibit cholesterol-induced atherosclerosis in rabbits (Harman, 1962; Hollander *et al.*, 1974). These authors showed that histamine H_1 antagonists not only inhibit the increase of permeability to lipids into the aorta in this model of atherosclerosis, but also suppress the formation of the atherosclerotic plaque. Interestingly, H_1 receptors on the vascular endothelium (Simionescu *et al.*, 1982) are implicated in virtually every histamine action that can be determined to be atherogenic; this will become more apparent later in the discussion.

All the above studies strongly suggest an expansion in the vascular histamine pool of patients with atherosclerosis, similar to that observed in experimental diabetes and experimental atherosclerosis. It is therefore

possible that the markedly increased plasma histamine concentrations observed in patients with PVD in this study, and in patients with CAD in other studies, is the result of leakage of histamine from the vasculature which, in atherosclerosis, is now known to contain increased amounts of histamine. Despite the fact that there is no demonstration of increased blood vessel histamine in diabetic patients, it is likely that a similar increase to that demonstrated in experimental DM occurs in human DM and results in leakage of histamine into the blood.

Our data on the histamine content of leucocytes and platelets are qualitatively comparable and there was a highly significant correlation between platelet and leucocyte histamine content in this study. Leucocytes are known to contain HDC; this was demonstrated by us in this study, as well as by others (Gill *et al.*, 1989; Grzanna, 1984). Human leucocytes also contain histaminase but again, histaminase activity was not altered in the leucocytes of any of the patient or control groups. Human leucocytes also have an uptake system for histamine, with a vast capacity for increasing intracellular histamine content (Catini *et al.*, 1984). Catini and co-workers also demonstrated that neutrophils and eosinophils, but not basophils, increase their intracellular content by uptake of histamine. Therefore, it is possible that in our study the increase in leucocyte histamine in PVD occurs primarily in neutrophils and esoinophils and is the result of histamine uptake from a 'histamine rich' plasma, rather than the result of increased synthesis or decreased breakdown of histamine.

Atherogenesis involves interplay between a multitude of factors and it is becoming increasingly apparent that altered leucocyte adhesion and migration on the vascular endothelium are two of the most important of these factors. Leucocyte-endothelial interactions are intimately involved in the physiological traffic of leucocytes from the blood to the blood vessel wall and back. Altered interactions between these two cell types are known to occur in atherosclerosis. It has been shown that leucocyte accumulation in arterial wall lesions of hypercholesterolaemic animals occurs within a few weeks after the initiation of an atherogenic diet (Ross, 1981). Leucocytes can be observed to adhere to the endothelium and are found in the junctions between endothelial cells and subendothelial intima; accumulation of leucocytes occurs before any proliferation of smooth muscle cells. Electron microscopic studies of emigrating leucocytes have shown that they first adhere to the endothelium and then extend

pseudopods, which appear to probe for the path of least resistance at interendothelial junctions. These cells then insert a cytoplasmic probe into the junction,and crawl between the endothelial cells. After penetrating the intracellular junction, leucocytes migrate into the basal lamina (Marchesi & Florey, 1960; Shaw, 1980).

Numerous studies have shown that leucocytes are intimately involved in both the initial and late events of atherosclerosis. Early and advanced lesions of atherosclerosis both consist of smooth muscle cells, leucocytes and foam cells (fat-filled leucocytes). The above accumulation of leucocytes is considered by many authors to be a defensive response by the vessel wall which has not subsided but instead progressed to a pathologic response (see Ross, 1986, for a review of studies).

Platelets are also known to interact with the vascular endothelium and it is thought that some of these platelet interactions are involved in the initial development of atherosclerosis (Ross, 1986). We therefore investigated histamine metabolism in platelets and found that platelet histamine content is markedly elevated in PVD but not in DM. There was no detectable platelet histamine synthesis or degradation in either controls or patients. We therefore investigated histamine uptake by human platelets and have demonstrated that human platelets have a capacity for the uptake of histamine, and for the first time shown that this uptake can be enhanced by various activating stimuli such as simple stirring or by the addition of adrenaline, ADP and collagen, which are all known platelet aggregating agents (Gill *et al.*, 1987).

It is of interest that this stimulatory effect of adrenaline, ADP and collagen is exerted at concentrations smaller than those required to induce aggregation of platelets. Clearly, therefore, histamine uptake by platelets is a process which may well occur continuously while the platelet is in the circulation. Furthermore, histamine uptake may be stimulated by circulating adrenaline, the release of ADP from aggregating platelets, or at sites of vascular injury where exposure to collagen occurs. The concentrations of adrenaline used in our experiments were of a similar order to those observed in patients with cardiogenic shock (Laing *et al.*, 1983) (range: 2-63 nmol/l) or during hypoglycaemia (Horie *et al.*, 1984) (range: 3.4-4.5 nmol/l). It is noteworthy that at higher concentrations of adrenaline and collagen the histamine uptake did not increase; it actually fell. This is

probably due to the fact that at these agonist concentrations, the release of histamine from platelets exceeds the uptake. We have observed that at these concentrations, β-thromboglobulin and TXA_2 are released by platelets and that platelets counts in PRP fall. Both these indicators point to activation of platelets, with the formation of platelet clumps.

Our ability to demonstrate consistent histamine uptake by platelets at low concentrations added of [^{14}C]histamine (nanomolar vs micromolar quantities used previously; Wood *et al.*, 1984) and to stimulate histamine uptake by platelet agonists over shorter periods of incubation (15 or 30 min vs 90 min previously) is due to two main reasons. Firstly, our studies on the uptake of [^{14}C]histamine were carried out in PRP prepared from citrated blood and not in EDTA. This difference is relevant, since our results clearly demonstrate that EDTA inhibits platelet aggregation whilst citrate does not. EDTA-induced alteration/damage in platelet function and structure have also been described by others (Pidard *et al.*, 1986). It is noteworthy that the differences in platelet function in PRP coagulated with EDTA or citrate are not dependent upon ionised calcium concentrations in PRP, since this value was similar with both anticoagulants. Others have also reported that EDTA-induced loss of platelet aggregation could not be reversed by adding calcium (Zucker & Grant, 1978). The second reason why [^{14}C]histamine uptake was greater in our experiments was because platelets were constantly stirred. Stirring is necessary for eliciting aggregation of platelets, and we reasoned that other functions of platelets may be dependent on a similar 'trigger'. Indeed, our experiments show that [^{14}C]histamine uptake is considerably reduced in the absence of stirring. Furthermore, the agonists (ADP, collagen and adrenaline) did not increase [^{14}C]histamine uptake by platelet unless these cells were stirred. Our data indicate that under normal circumstances platelet histamine uptake is a relatively slow and passive process which, given the right stimulus, can be activated to increase histamine uptake. We reasoned that if uptake could be stimulated, it might also be possible to inhibit uptake by various inhibitory agents. It is known that active energy-requiring processes, unlike totally passive uptake processes, can be slowed down by decreasing the temperature or by inhibiting metabolic energy-producing enzymes.

Incubation of platelets at 4 °C markedly (>70%) reduced histamine uptake; however, this process was not totally abolished. Reduction of temperature by 10 °C to 27 °C and 17 °C also caused stepwise decreases in histamine

uptake of 35% and 47% respectively. There was also an inhibition of histamine uptake by iodoacetate, a known inhibitor of metabolic processes (Clusin, 1983). Increasing concentrations of iodoacetate caused a stepwise decrease in histamine uptake; however, approximately 30% could not be inhibited. These two experiments indicate that approximately 70% of total histamine uptake is sensitive to temperature and metabolic inhibition, and that approximately 30% of uptake cannot be affected and is probably totally passive and dependent on an osmotic gradient of histamine.

Imipramine is a known inhibitor of carrier-mediated serotonin uptake into human platelets (Tuomisto, 1973) but it did not inhibit the uptake of histamine. This indicates that these two bioamines are not taken up into platelets by the same carrier mechanism in spite of the fact that they are stored together in the same dense granules when inside the platelet. Aspirin caused a slight increase in histamine content of platelets; this was probably because aspirin is known to inhibit the release reaction of platelets (Best *et al.*, 1981) and histamine, once taken up, may therefore be prevented from being released during the preparation of platelet pellets. Mepyramine and cimetidine did not affect platelet histamine uptake and therefore the inhibition of H_1 and H_2 receptors on platelets did not affect the histamine uptake mechamism.

In conclusion, histamine is taken up by blood platelets and this uptake is stimulated by stirring, adrenaline, ADP and collagen. The method used in this study to obtain these data is simple, rapid and cheap, and provides consistent results.

Our data indicate that platelets obtained from normal subjects and various patient groups exhibited no histamine synthesis or degradation; therefore the increase in intraplatelet histamine content in PVD patients is probably the result of increased histamine uptake from a 'histamine rich' plasma. Alternatively, it is possible that immature platelets may have increased histamine synthesis or that megakaryocytes may have increased intracellular histamine content. It must be stressed, however, that megakaryocytes have not, as yet, been shown to contain HDC.

Interestingly, the platelets of patients with DM did not show any increases in intracellular histamine despite the fact that these platelets are also exposed to a 'histamine rich' plasma. Since we have demonstrated increased histamine uptake with platelet activation (Gill *et al.*, 1987), it is

possible that PVD platelets, which are known to be markedly hyper-aggregable (Mikhailidis *et al.*, 1985), take up histamine more avidly because they are activated and that DM platelets, which are relatively normal (Peacock *et al.*, 1986), do not. This is reflected in the increased platelet histamine content in PVD and PVD-DM, but not in DM alone (Gill *et al.*, 1988c).

As mentioned earlier, platelets are implicated in the pathogenesis of atherosclerotic lesions. Vascular endothelial cells are normally non-thrombogenic and produce anti-thrombotic substances like prostacyclin (PGI_2) and endothelium derived relaxing factor (EDRF). PGI_2 and EDRF have marked anti-aggregatory properties and inhibit platelet adherence (Ross, 1986). However, in certain conditions, such as hypercholesterol-aemia (Faggiotto *et al.*, 1984) or endothelial injury due to balloon angioplasty or cardiac bypass surgery (Brown *et al.*, 1982), the endothelium can become more 'sticky' to platelets. Furthermore, endothelial injury and contraction can lead to the exposure of sub-endothelial layers in the vessel wall which are highly thrombogenic in character and lead to the formation of platelet clumps on the exposed subendothelial collagen rich surface (Baumgartner & Haudenschild, 1972).

When leucocytes and platelets adhere, aggregate and infiltrate the vascular wall, they form platelet and/or leucocyte clumps which stimulate both cell types to release their intracellular contents (Baumgartner *et al.*, 1972). Both these cell types contain a host of mediators with various physiological functions. Platelets, when activated, secrete various factors such as PDGF, which is pro-aggregatory to platelets and is both mitogenic and chemotactic to leucocytes and can induce increases in smooth muscle migration and proliferation, an important initial event in atherosclerosis. Platelets also produce platelet factor 4 (PF_4), which is capable of inducing chemotaxis in monocytic leucocytes (Ross, 1986). Leucocytes, when activated by aggre-gation or adherence, also release a multitude of vasoactive factors such as fibroblast growth factor, interleukins, prostaglandins and leukotrienes (Ross, 1986).

Histamine is also released when leucocytes and/or platelets are activated by adherance and/or aggregation. In the present study we have shown elevation of histamine in the leucocytes and platelets of patients with PVD. The formation of a leucocyte/platelet clump on blood vessels in PVD

patients can therefore lead to the release of extra histamine, which then exerts a large variety of effects on the vessel wall and other attached leucocytes and platelets. For example, human platelets when stimulated with collagen have been shown to release an unidentified substance which causes a marked release of histamine from human leucocytes (Orchard *et al.*, 1986). PF_4, also a platelet specific substance, is known to cause the release of histamine from human basophilic leucocytes (Brindley *et al.*, 1983). The released histamine, whether from platelets, leucocytes or endothelial cells, would have a variety of effects on the blood vessel wall.

The most prominent effect of histamine is in provoking a large increase in endothelial permeability to plasma macromolecules (Carter *et all.*, 1974; Killackey *et al.*, 1986; Majno *et al.*, 1967; Rotrosen & Gallin, 1986). Histamine primarily increases endothelial permeability by inducing endothelial cell contraction via the H_1 receptor, which in turn leads to a widening of interendothelial gaps (Majno *et al.*, 1967, 1969; Horan *et al.*, 1986). These widened endothelial junctions allow for easier access of macromolecules into the blood vessel wall.

It is known that the arterial endothelium is a selective barrier and allows the passage of only certain macromolecules; this selectivity is thought to be the result of a dual pore permeability system. One pore system has a functional diameter of less than 90 Å and the other has large pores of some 200-1500 Å diameter (Bruns & Palade, 1968). The small pore system is believed to be the structural equivalent of the intercellular junction (Cotran & Karnovsky, 1968) and is therefore dependent on endothelial contraction as described above. The large pore system is dependent on vesicular transport or pinocytosis (Karnovsky, 1968); this is a process in which plasma molecules enter invagination on the plasma membrane of the luminal surface of endothelial cells. The invagination separates off into the endothelial cytoplasm to form pinocytic vesicles ranging in size from 400 to 1500 Å diameter, which move from the luminal to the abluminal or junctional surface of the endothelial cell. Histamine has been shown to markedly increase endothelial pinocytosis (Orlidge, 1983; Davies *et al.*, 1983).

It is clear, therefore, that histamine increases both the large and small pore permeability systems of the vascular endothelium Many macromolecules have been investigated in the above permeability systems, including

albumin, dextran, horseradish peroxidase, fibrinogen, Evans Blue and ferritin. However, none of these macromolecules are in themselves atherogenic. Cholesterol (in particular, low density lipoprotein; LDL) has conclusively been shown by numerous studies to be one of the most atherogenic of various macromolecules in plasma (Steinberg, 1983). Many of these studies have shown that endothelial influx of LDL is grossly elevated in atherosclerosis (Bremmelgaard *et al.*, 1986; Steneler & Hjelms, 1987). Histamine has been shown to markedly increase the passage of LDL through human aortic endothelial cells by inducing endothelial contraction (Langler *et al.*, 1989). LDL may enter the endothelium through the large pore pinocytic system, since its diameter is 220 Å diameter and this would allow it a ready entry through the endothelium. Any increase in local histamine concentration due to endothelial/leucocyte/platelet release would increase endothelial pinocytosis and endothelial contraction and thereby increase the influx of LDL into the blood vessel wall.

Interestingly, histamine-induced alterations in both endothelial contraction and endothelial pinocytosis are mediated via the histamine H_1 receptor (Killackey *et al.*, 1974; Rotrosen & Gallin, 1986; Orlidge, 1983; Davies *et al.*, 1983). Histamine action via the H_1 and H_2 receptor has also been shown to induce increased calcium binding to the endothelium (Barman *et al.*, 1987); these authors speculated that this altered binding of calcium is associated with increased endothelial permeability. Endothelial calcium influx (D'Amore & Shepro, 1977) and intracellular calcium concentrations are increased in the presence of histamine (Rotrosen & Gallin, 1986; Hamilton & Sims, 1987) and again the effect is inhibited by H_1 receptor antagonists.

The histamine-induced increase in cytosolic endothelial calcium has been demonstrated to be accompanied by a marked release of endothelial von Willebrand Factor (vWF; Hamilton & Sims, 1987). vWF is a potent platelet agonist and has been shown to enhance platelet adhesion to the endothelium and to mediate platelet aggregation (Weiss *et al.*, 1989). Endothelial platelet activating factor (PAF) synthesis is also enhanced by histamine (McIntyre *et al.*, 1985), which leads to increased platelet adhesion and aggregation on the endothelium. Alterations in endothelial calcium, vWF and PAF would all result not only in increased permeability but also in increased thrombogenicity of the endothelium; both of these processes are well known sequences in atherogenesis.

As stressed earlier, the process of leucocyte diapedesis, in which leucocytes breach the endothelial cell barrier and enter the vessel wall, is of prime importance in the development of atherosclerosis. It is therefore of interest that histamine also modifies the endothelium to enhance polymorphonuclear leucocyte diapedesis (Doukas *et al.*, 1987). Furthermore, histamine also decreases endothelial stress fibre formation by increasing dissociation of actin cables (Welles *et al.*, 1985; Rotrosen & Gallin, 1986). Therefore histamine-induced alteration in the endothelial cytoskeleton would allow not only increased leucocyte infiltration, as demonstrated above, but also increased permeability to macromolecules. The above histamine actions on the cytoskeleton are known to be mediated via the H_1 receptor.

Arteries in humans are known to secrete factors which are chemotactic for leucocytes (Mazzone *et al.*, 1983). It is possible that the histamine rich blood vessels found in DM and vascular disease release increased amounts of histamine locally, which in turn lead to an acceleration of the changes mentioned above. These may, in time, progress into an atherosclerotic lesion.

Histamine increases the synthesis and release of prostacyclin from human endothelial cells (Baenziger *et al.*, 1981; Haddock *et al.*, 1987; Schellenberg *et al.*, 1986), stimulates the synthesis and release of endothelial HETE products (Revtyak *et al.*, 1988) and increases inositol phosphate accumulation by the endothelium (Carson *et al.*, 1989). All these changes are also mediated by the H_1 receptor. Tissue plasminogen activator (tPA; Levan & Santell, 1988) and EDRF (Bregestoviski *et al.*, 1988) are also released by endothelial cells in response to histamine.

Histamine has many other effects on both the vascular endothelium and smooth muscle cells which are too numerous to mention; but it is clear from all the above studies that histamine in undoubtedly involved in altering blood vessel physiology in conditions predisposing to atherosclerosis. One could legitimately label histamine as an atherogenic substance when released in and around blood vessels.

C. GENERAL CONCLUSION

In conclusion, our data clearly demonstrate altered histamine metabolism in experimental diabetes and in patients with DM and PVD.

Our animal studies confirmed the previous observations of an increase in histamine content of plasma and aortae of diabetic rats. We extended these observations to show that this elevated histamine content is not merely confined to the aorta or plasma but is also apparent in other tissues (e.g. kidney; heart). Furthermore, we have provided evidence that this increase in histamine is the result of activation of histamine synthesis via the enzyme HDC, and is not due to increases in non-specific decarboxylase activity. A decrease in tissue histamine catabolism via the enzyme histaminase also appears to be unlikely in view of the findings presented in this thesis. The administration of histamine H_1 and H_2 receptor antagonists to control and diabetic rats did not alter any of the changes in histamine metabolism mentioned above. This is not surprising, since neither H_1 nor H_2 histamine antagonists are known to alter histamine synthesis or breakdown.

Aortic endothelial permeability is known to be increased by histamine and has been shown to be elevated in experimental diabetes. Using novel *in vitro* techniques, we have confirmed these previous observations by demonstrating a three-fold increase in aortic albumin permeability in diabetic rats. Interestingly, the administration of H_1 and H_2 antagonists did not alter aortic permeability in control or diabetic rats. These surprising results indicated that histamine-associated increases in vascular permeability in DM rats may not be mediated via the H_1 or H_2 receptors.

In our human-based studies we have, for the first time, demonstrated that the alterations in histamine metabolism documented in experimental diabetes are also apparent in patients with DM. We have extended these observations to show similar alterations in the histamine status of patients with PVD. As in experimental diabetes, there are marked increases in plasma histamine concentrations in patients with DM and PVD. Furthermore, the histamine content of platelets and leucocytes was elevated in samples obtained from PVD and PVD-DM patients but not from DM only patients. The increase in histamine content in leucocytes

and platelets of PVD patients was not accompanied by either increased histamine synthesis or decreased histamine degradation.

It is thus likely that the increase in platelet and leucocytic histamine content in PVD is due to an increased uptake of histamine from plasma. As mentioned earlier, both leucocytes and platelets are also capable of taking up histamine from the circulation. Using a novel technique, we have demonstrated that human platelets can take up histamine and have shown for the first time that platelet activation further stimulates increases in histamine uptake.

Patients with PVD have previously been shown to have markedly hyperactive platelets (Mikhaildis *et al.*, 1985), while patients with DM (with no complications) are known to have platelets that are less hyperactive (Peacock *et al.*, 1986). It is therefore possible that leucocytes and platelets in PVD have higher histamine contents because they are exposed to higher concentrations of histamine in plasma and because the hyperactive platelets of PVD patients have an increased uptake of this amine. This is reflected in the increased platelet histamine content in PVD and PVD-DM but not in DM alone. Since the magnitude of increase in the histamine content of PVD leucocytes is of a similar order to that of PVD platelets, it is probable that a similar 'histamine plasma concentration/cellular activation'-dependent process, as in PVD platelets, occurs in PVD leucocytes. Studies must be performed to investigate histamine uptake by leucocytes following activation and exposure to high concentrations of histamine (this is discussed under 'Suggestions for Future Studies').

Our data, coupled with previous observations, suggest that there is a marked expansion in the histamine pool of blood vessels and various tissues of diabetic animals, and that release or leakage of histamine from these 'histamine rich' tissues results in increased plasma histamine concentrations. Since atherosclerotic human blood vessels have previously been shown to have markedly higher histamine contents, it is possible that the elevated plasma histamine concentrations in patients with DM and PVD in our study are the result of a similar process.

Thus, the possible local release of histamine from blood vessels in DM and PVD shown in our study would, as discussed earlier, induce changes in arterial endothelial permeability; this would facilitate the influx of atherogenic blood-borne macromolcules such as LDL. This process would

therefore constitute one of the initial events of atherogenesis. In time, this event would accelerate as more endothelial histamine is released and results in the formation of fatty streaks. Fatty streak formation has been shown to lead to smooth muscle proliferation and the adherence and infiltration of platelets and leucocytes. Adherent activated platelets and leucocytes release various mediators, including more histamine. This process eventually culminates in the formation of an occluding atherosclerotic plaque (see Ross, 1986, for a review of the atherosclerotic process). A similar process could also occur in diabetic capillary endothelium and lead to the many complications of diabetic microangiopathy.

It is obvious that additional studies must be undertaken to understand the true significance of our data and to make definitive statements. Such studies were beyond the scope of this thesis, and have been discussed under 'Suggestions for Future Studies'. However our data, coupled with previous observations on the atherogenic effects of histamine, give strong support to the premise that histamine may be one of the factors involved in the pathogenesis of both micro- and macro-vascular disease.

Our work has provided some clarification of the histamine-related interactions between blood vessels and the cells circulating in the blood, in health and disease. It is hoped that these observations will point the way towards further investigations that will increase our understanding of the role played by histamine in the atherogenic process.

SUGGESTIONS FOR FUTURE STUDIES

1. *The effect of αFMH on capillary permeaibility in normal volunteers and DM/PVD patients*

In our studies we have clearly demonstrated marked alterations in histamine metabolism both in DM rats and in patients with DM and/or PVD. Previous inhibition of aortic or retinal HDC by specific inhibitors, such as αHH, not only prevents the increase in histamine synthesis in these tissues but more importantly normalises diabetes-associated increases in aortic and retinal permeability.

With the recent use of αFMH (a potent and specific inhibitor of HDC: Kollonitsch *et al.*, 1978) in man (Tung *et al.*, 1985), it may be possible to determine the effect of inhibiting histamine synthesis on *in vivo* capillary endothelial permeability in normal volunteers and/or DM/PVD patients.

Capillary permeability could be evaluated by a relatively novel, simple and accurate radioisotope technique (Valensi *et al.*, 1987). The assessment of capillary permeability using this method involves the injection of 99m technitium labelled albumin, which is probably the major disdvantage of our study. Alternatively, it may be possible to assess the effects of αFMH on microalbuminuria (a capillary permeability related phenomenon) in DM and PVD patients. Assessment of microalbuminuria is totally non-invasive and requires only a simple 24-hour urine collection.

2. *Further investigation of [^{14}C]histamine uptake by human platelets and leucocytes*

We have shown in the present work that platelets obtained from patients with PVD have significantly higher histamine contents than platelets obtained from normal volunteers or diabetic patients. This increase in intraplatelet histamine was present without any concomitant alteration in histamine synthesis or breakdown.

Since we have, in our present studies, also demonstrated accelerated histamine uptake by activated human platelets, we reasoned that the

increased histamine content of PVD platelets was probably the result of elevated uptake from a 'histamine rich' plasma. Furthermore, we suggested that DM platelets have relatively normal histamine contents (despite exposure to similar plasma histamine concentrations) because DM platelets are less hyperactive than PVD platelets and as a result take up plasma histamine less avidly.

There are two possible approaches which can be used to clarify the above situation:

(a) [^{14}C]histamine uptake by normal human platelet following in vivo *exposure to a diabetic environment*

It could be argued that DM platelets have normal histamine contents despite the presence of high plasma histamine because histamine uptake is inhibited in diabetic patients. It is possible that the diabetic platelet environment (hyperglycaemia, various pH's, high non-esterified fatty acids, ketone bodies, marked alterations in insulin concentrations) may be inhibitory as a result of one or a combination of the above diabetic blood components. It is there-fore possible to investigate [^{14}C]histamine uptake by normal platelets following exposure to a so-called diabetic environment (Jeremy *et al.*, 1983). One could evaluate both basal (i.e. stirred only) and activated (stirred + low dose agonist) histamine uptake.

(I) The major problem with the above study would be the reproduction of an accurate diabetic environment. The diabetic environment is constantly changing, and it is well-established that numerous blood components are either elevated or decreased in diabetics. Various plasma proteins and cell membrane protein are glycosylated to varying degrees. It would be virtually impossible to reproduce the *in vivo* diabetic environment.

(II) The duration of exposure to the diabetic environment poses additional problems, since *in vivo* the diabetic platelet can be exposed to its environment for 10-12 days. In comparison, in the above study exposure would be limited to approximately 30 minutes.

(b) [^{14}C]histamine uptake by platelets obtained from patients with DM and PVD

These experiments would probably yield more accurate data than the *in vitro* exposure experiments mentioned above. This study would involve obtaining platelets from PVD and DM patients and assessing [^{14}C]histamine uptake *in vitro*. Again, both activated and basal uptake could be determined.

The main problems with this study are as follows:

(I) Loss of hyperactive larger platelets and platelet aggregation in DM and PVD patients during the preparation of PRP. Since PVD platelets are known to be markedly hyperaggregable, it is highly likely that the more active PVD platelets would be selectively lost (this is evidenced by low platelet counts in PVD PRP). It is these lost activated platelets that probably take up histamine more avidly.

(II) Obtaining sufficient numbers of patients with DM and PVD who are not on drugs which inhibit platelet function, including non-steroidal anti-inflammatory drugs.

(III) Difficulty in in obtaining age-matched controls for the older PVD patients.

(c) [^{14}C]histamine uptake by leucocytes obtained from normal volunteers and PVD/DM patients

In our studies, we demonstrated that PVD and PVD-DM leucocytes had siginficantly higher histamine contents than control or DM only leucocytes. We postulated that this might be the result of increased uptake of 'activated' leucocytes from a 'histamine rich' plasma.

To further evaluate this hypothesis, it is necessary to investigate histamine uptake not only in normal volunteers (e.g. as in platelets; with and without stirring and the effect of sub-aggregatory doses of agonists) but also in patients with DM and PVD.

3. ***The effect of H_1 and H_2 receptor antagonists on* in vivo *aortic ^{125}I-albumin permeability in DM rats and hypercholesterolaemic rabbits***

It has been clearly established that histamine plays a major role in the increased aortic endothelial permeability commonly associated with experimental DM and experimental hypercholesterolaemia. In our studies, the administration of H_1 and H_2 receptor antagonists to DM rats for two weeks did not alter the DM-associated elevation in aortic albumin permeability. This is surprising, since histamine synthesis is clearly associated with increased endothelial permeability and one would expect the actions of histamine to be mediated via H_1 and/or H_2 receptors. However, our experiments were conducted *in vitro*, and it is possible that the aorta becomes non-responsive upon removal from the animal. Therefore the adminstration of H_1 and H_2 antagonists to DM and atherosclerotic animals, followed by an *in vivo* assessment of aortic permeability, may clarify the above situation.

4. ***The effect of near-perfect diabetic control with (a) continuous subcutaneous insulin infusion (CSII) on plasma/platelet/leucocyte histamine concentration content in poorly-controlled IDDM; and (b) strict dietary/drug control in NIDDM***

The induction of excellent control over a period of four to six weeks is likely to induce a reduction in plasma histamine concentration and this may well contribute to the reduction in microalbuminuria known to occur following the induction of such control in insulin-dependent diabetics.

REFERENCES

Ahlmark A (1944)
Histaminease and related amine oxidases
Acta Physiol Scand, **9** (Suppl 28), 1-8

Ahmed SA (1970)
Ionic and acid base composition of human leucocytes
PhD thesis, University of London

Al-Haboubi HA & Zeitlin IJ (1979)
The actions of cimetidine, mepyramine, indomethacin and aprotinin (Trasylol) on the inflammatory response in adjuvent rats
Proc Brit Pharm Soc, July, 446-447

Almeida AP, Flye W, Deveraux D, Horakova Z & Beavan MA (1980)
Distribution of histamine and histaminase (diamine oxidase) in blood of various species
Comp Biochem Physiol, **67c**, 187-190

Arrang JM, Garbarg M & Schwartz JC (1983)
Auto-inhibition of brain histamine release by a novel class (H3) of histamine receptor
Nature, **302**, 832-837

Arrang JM, Garbarg M, Lancelot JC, Leconte JM, Pollard H, Robba M, Schunack W & Schwartz JC (1987)
Highly potent and selective ligands for histamine H3-receptors
Nature, **327**, 117-123

Baenziger NL, Fogerty FJ, Mertz LF & Chernuta LF. (1981)
Regulation of histamine-mediated prostacyclin synthesis in cultured human vascular endothelial cells.
Cell, **24**, 915-923.

Barman SA, Olson MD & Saari JT (1987)
Histamine-induced alteration of calcium binding to microvascular endothelium as indicated by use of ionic lanthanum
Cardiovascular Research, **21**, 576-581

Barradas MA, Thompson CS, Gill DS & Dandona P (1990)
Serotonin content of tissues from rats with diabetes mellitus
Clin Sci, **78**, 27P

Basu SK, Goldstein JL, Anderson RGW & Brown MS. (1976)
Degradation of cationized low density lipoproteins and regulation of cholesterol metabolism in homozygous familial hypercholesterol-aemia fibroblasts
Proc Natl Acad Sci USA, **73**, 3178-3182

Baudry M, Matres MP & Schwartz JC (1973)
The subcellular localization of histidine decarboxylase in various regions of rat brain
J Neurochem, **21**,1301-1305

Baumgartner HR & Haudenschild C (1972)
Adhesion of platelets to sub-endothelium
Ann NY Acad Sci, **201**, 22-36

Baumgartner HR & Hosang M (1988)
Platelets, platelet-derived growth factor and arteriosclerosis
Experientia, **44**, 109-112

Baumgartner HR (1972)
Platelet interactions with vascular structures
Thromb Diath Haemorrh, **51**, 161-176

Beavan MA & Horakova Z (1978)
The enzymatic isotopic assay of histamine. In: *Handbook of Experimental Pharmacology*, Vol. XVIII (ed. 2), Ed. Rocha e Silva M, pp 1512-153
Berlin: Springer-Verlag

Beavan MA & Shaff R. (1979)
New inhibitors of histamine-N-methyltransferase.
Biochem Pharmacol, **28**, 183-188.

Beavan MA (1978)
Histamine: its role in physiological and pathological processes.
In: Dukor P *et al.*, *Monographs in Allergy*, Vol. 13
S. Karger, Basel

Best LC, Holland TK, Jones PBB & Russell RGG (1981)
The interrelationship between thromboxane biosynthesis, aggregation and 5-hydroxytryptamine secretion in human platelets *in vivo*
Thromb Haemostas, **43**, 38-40

Besterman EMM (1970)
Experimental coronary atherosclerosis in rabbits.
Atherosclerosis, **12**, 75-81.

Bevan J & Heptinstall S (1983)
Effects of ketanserin and mepyramine on platelet aggregation and on the uptake of 5-HT into platelets
Thromb Res, **30**, 415-423

Bhargava KP, Nath P & Palit G. (1977)
Nature of histamine receptors concerned in capillary permeability.
Br J Pharmacol, **59**, 349-351.

Boeker EA & Snell EE. (1972)
In PD Boyer (ed) *The Enzymes*, 3rd Edition, Vol. VI, p 217
New York: Academic Press.

Bolitho GA & Hollis TM (1973)
Rat aortic intima media histamine synthesis in experimental neural hypertension
Proc Pa Acad Sci, **47**, 141-147

Bolitho GA & Hollis TM (1975)
Aortic histamine synthesis in experimental neurogenic hypertension.
Proc Soc Exp Biol Med, **148**, 1189-1193.

Born GVR & Cross MJ (1963)
The aggregation of blood platelets
J Physiol, **168**, 178-195

Brandes LJ, Bogdanovic RP, Cawker MD & LaBella FS (1987)
Histamine and growth: interaction of antiestrogen binding site ligands with a novel histamine site that may be associated with calcium channels
Cancer Res, **47**, 4025-4031

Brandes LJ, Gerrard JM, Bogdanovic RP, Lint DN, Reid RE & LaBella FS (1988)
Correlation of the antiproliferative action of diphenylmethanine derivative antiestrogen binding site ligands with antagonists of histamine binding
Cancer Res, **48**, 3954-3958

Bratzler RL, Chisolm GM, Colton CK, Smith KA, Zilversmith DB & Lees RS (1972)
The distribution of labeled albumin across the rabbit thoracic aorta *in vivo*
Circ Res, **40**, 182-190

Bregestovski P, Bakhramar A, Danilov S, Moldobaeva A & Takeda K (1988)
Histamine-induced inward currents in cultured endothelial cells from human umbilical vein
Br J Pharmacol, **95**, 429-436

Bremmelgaard A, Stender S, Lorentzen J & Kjeldsen K (1987)
In vivo flux of plasma cholesterol into human abdominal aorta with advanced atherosclerosis
Arteriosclerosis, **6**, 442-452

Brindley LL, Sweet JM, Goetzi EJ (1983)
Stimulation of histamine release from human basophils by human platelet factor 4
J Clin Invest **72**, 1218-1223

Brown BG, Cukingnan R, Petersen RB, Pierce CD, Bolson EL & Dodge HT (1982)
Perianastomotic arteriosclerosis in grafted human coronary arteries: prevention with platelet-inhibiting therapy
Am J Cardiol, **49**, 968-971

Brown MJ, Ind PW & Jenner DA. (1980)
Platelet histamine.
N Engl J Med, **302**, 756

Brown MJ, Ind PW, Barnes PJ, Jenner DA & Dollery CT (1980)
A sensitive and specific radiometric method for thre measurement of plasma histamine in normal individuals
Analyt Biochem, **109**, 142-146

Brownlee M & Cahill GF (1979)
Diabetic control and vascular complications. In: Paolotti RW, Gotto AM (eds)
Atherosclerosis Reviews, Vol 4, pp 29-70.
New York: Raven Press.

Bruns RR & Palade GE (1968)
Studies on blood capillaries II Transport of ferritin molecules across the wall of muscle capillaries
J Cell Biol, **37**, 277-299

Carroll WC, Hollis TM & Gardner TW (1988)
Refined histamine synthesis is increased in experimental diabetes
Invest Ophthalmol Vis Sci, **29**, 1201-1204

Carroll WJ & Hollis TM (1985)
Aortic histamine synthesis and aortic albumin accumulation in diabetes: activity-uptake relationships.
Exp Mol Pathol, **42**, 344-352.

Carson MR, Shasby SS & Shasby DM (1989)
Histamine and inositol phosphate accumulation in endothelium: cAMP and a G protein
Am J Physiol, **257**, L259-L264

Carter RD, Joyner WL & Renkin EM (1974)
Effects of histamine and some other substances on molecular selectivity of the capillary wall to plasma protein and dextran
Microvascular Research, **7**, 31-48

Catini C, Gheri G, Grampoli M & Miliani A (1984)
Histamine uptake by leucocytes *in vitro*
Basic Appl Histochem, **28**, 329-336

Chattoraj SC & Watts NB (1986)
Endocrinology. In: *Textbook of Clinical Chemistry* , ed. Tietz NW, pp 1144-1145
WB Saunders: Philadelphia

Clusin WT (1983)
Mechanism by which metabolic inhibitors depolarize cultured cardiac cells
Proc Natl Acad Sci USA, **80**, 3865-3869

Cornhill JF & Roach MR (1976)
A quantitative study of the localization of atherosclerotic lesions in the rabbit aorta.
Atherosclerosis, **23**, 489-493.

Cotran RS & Karnorsky MJ (1968)
Ultrastructural studies on the permeability of the mesothelium to horseradish peroxidase
J Cell Biol, **37**, 123-137

Cunha Vaz JG, Faria de Abreu JR, Campos AJ & Figo GM (1975)
Early breakdown of the blood retinal barrier in diabetes
Br J Ophthalmol, **59**, 649-656

D'Amore P & Shepro D (1977)
Stimulation of growth and calcium influx in cultured bovine endothelial cells by platelets and vasoactive factors
J Cell Physiol, **92**, 177-184

Da Prada M, Richards JG & Kettler R (1981)
Amine storage organelles in platelets. In: *Platelets in Biology and Pathology II*, ed. JL Gordon
Elsevier/North Holland Press, Amsterdam, pp 105-145

Dale HH & Laidlaw PP. (1910)
The physiological action of β-imidazolylethylamine.
J Physiol (Lond), **41**, 318-344.

Dale HH & Laidlaw PP. (1910)
Histamine shock.
J Physiol (Lond), **42**, 355-390.

Dale HH & Richards AN.. (1918)
The vasodilator action of histamine and of some other substances.
J Physiol (Lond), **52**, 110-165.

Davies PF, Dewen GF, Bussolari SR, Gordon EJ & Gimbrone MA (1983)
Influence of hemodynamic forces on vascular endothelial function
J Clin Invest, **73**, 1121-1129

Day M & Stockbridge A. (1964)
The effects of drugs on the uptake of amines by mast cells.
Br J Pharmacol, **23**, 405-419.

De Forrest JM & Hollis TM. (1978)
Shear stress and aortic histamine synthesis.
Amer J Physiol, **234**, 701-707.

Di Corletto PE & Bowen-Pope DF. (1983)
Cultured endothelial cells produce a platelet-derived growth factor-like protein.
Proc Natl Acad Sci (USA), **80**, 1919-1923.

Doukas J, Shepro D & Hechtman HB. (1987)
Vasoactive amines directly modify endothelial cells to affect polymorphonuclear leucocyte diapedesis *in vitro*.
Blood, **69**, 1563-1569.

Dull RO, Vergis GJ & Hollis TM (1986)
Effect of chronic histamine infustion on the permeability of the blood retinal barrier
Fed Res, **45**, 462-471

Dy M, Lebel F & Kamaun P. (1981)
Histamine production during the anti-allograft response.
J Exp Med, **153**, 293-309.

Dyer J, Warren K, Merlin S, Metcalf DD & Kalner M (1982)
Measurement of plasma histamine: description of an improved method and normal values
J Allergy Clin Immunol, **70**(2), 82-87

Egashira K, Tomoike H, Yamamoto Y, Yamada A, Hayashi Y & Nakamura M (1986)
Histamine-induced coronary spasm in regions of intimal thickening in miniature pigs: role of serum cholesterol and spontaneous or induced intimal thickening
Circulation, **74**, 826-837

Enea NA, Kern JA, Hollis TM & Gardner TW (1985)
Altered histamine receptor activity may mediate altered blood-retinal barrier permeability in experimental diabetes
Diabetes (Suppl 1), **34**, 215A

Eppinger H. (1913)
Uber eine eigentumliche hautreaktion, hervorgernfen durch ergamin.
Wien, med, Wschr.,**63**, 141

Exton JH. (1985)
Role of calcium and phosphoinositols in the actions of certain hormones and neurotransmitters.
J Clin Invest, **75**, 1753-1757.

Faggiotto A & Ross R (1984)
Studies of hypercholesterolaemia in the non-human primate, Part 2.
(Fatty streak conversion to fibrous plaque).
Arteriosclerosis, **4**, 341-346

Faggiotto A, Ross R & Harker L. (1984)
Studies of hypercholesterolaemia in the non-human primate, Part 1. (Changes that lead to fatty streak formation).
Arteriosclerosis, **4**, 323-327

Fisher DZ, Meier E, Fitzpatrick D (1981)
Histamine, free histidine decarboxylase, histamine methyltransferase activity in developing rat tissues
Comp Biochem Physiol, **68**, 231-234

Fishman AP (1976)
Hypoxia on the pulmonary circulation
Circ Res, **38**, 222-231

Flacke W, Atanackovic D & Gillis RA (1967)
The action of histamine on the mammalian heart
J Pharmacol Exp Ther, **155**, 271-278

Florentin RA, Nam SC, Lee KT & Thomas WA. (1969)
Increased ^{3}H-thymidine incorporation into endothelial cells of swine fed cholesterol for 3 days.
Exp Mol Pathol, **10**, 250-253.

Foley WA & Glick D (1962)
Histamine, mast and parietal cells in stomach of rats and effects of cortisone treatment
Gastroenterology, **43**, 425-429

Fry DL (1968)
Acute vascular endothelial changes associated with increased blood velocity gradients
Circulation Res, **22**, 165-169

Fry DL (1969)
Certain histaological and chemical responses of the vascular interface to acutely induced mechanical stress in the aorta of the dog.
Circulation Res, **24**, 93-97

Fukami MH, Holmsen H & Ugurbil K (1984)
Histamine uptake in pig platelets and isolated dense granules
Biochem Pharmacol, **33**, 3869-3874

Gaddum JH. (1948)
Histamine.
Br Med J, **i**, 867-873.

Gallick SG & Hollis TM (1981)
Studies on aortic histamine synthesis in experimental diabetes.
Proc Soc Exp Biol Med, **166**, 496-500.

Gerrity RG. (1981)
The role of the monocyte in atherogenesis.
Amer J Path, **103**, 181-191.

Gill DS, Barradas MA, Fonseca VA & Dandona P (1989)
Plasma histamine concentrations are elevated in patients with diabetes mellitus and peripheral vascular disease
Metabolism, **38**, 243-247

Gill DS, Barradas MA, Fonseca VA, Gracey L & Dandona P (1988b)
Increased histamine content in leucocytes and platelets of patients with peripheral vascular disease
Am J Clin Path, **89**, 622-626

Gill DS, Barradas MA, Mikhailidis DP & Dandona P (1987)
Histamine uptake by human platelets
Clin Chim Acta, **168**, 177-185

Gill DS, Thompson CS & Dandona P (1988a)
Increased histamine in plasma and tissues in diabetic rats
Diabetes Res, **7**, 31-34

Gill DS, Thompson CS & Dandona P (1988c)
Tissue histamine synthesis and content in streptozotocin-induced diabetes mellitus in rats
Diabetologia, **31**, 494A

Gill DS, Thompson CS & Dandona P (1990a)
Histamine synthesis and catabolism in various tissues in diabetic rats
Metabolism, **39**, 815-818

Gill DS, Thompson CS, Barradas MA & Dandona P (1990b)
The effect of histamine antagonists on aortic permeability and histamine metabolism in streptozotocin-induced diabetes mellitus in the rat
Clin Sci, **78**, 39P

Ginsburg R, Bristow MR, Kantrowitz N, Baim DS & Harrison DC (1981)
Histamine provocation of clinical coronary artery spasm: implication concerning pathogenesis of variant angina pectoris
Am Heart J, **102**, 819-822

Gordon JL (1981)
Platelets in perspective. In: *Platelets in Biology and Pathology II*, ed. JL Gordon
Elsevier/North Holland Press, Amsterdam, pp 1-17

Gordon T, Castelli WP, Hjortland MC, Kannel WB & Dawber TR. (1977)
Predicting coronary heart disease in middle-aged and older persons. The Framingham Study.
JAMA, **238**, 497-499.

Graham HT, Lowry OH, Wheelwright FL, Lenz D & Parish H (1955)
Distribution of histamine among leucocytes and platelets.
Blood, **10**, 467-481.

Green JD, Johnson CL, Winstein H (1978)
Histamine as a neurotransmitter. In: Lipton M, Dimascio A, Killman K, eds. *Psychopharmacology - A generation of progress*
Raven Press, New York, pp 319-332

Green JP. (1967)
Uptake and binding of histamine.
Fed Proc, **26** 211-218.

Grzanna R (1984)
Histidine decarboxylase: isolation and molecular characteristics
Neurochem Res, **9**, 993-1009

Haddock RC, Mack P, Fogerty FJ & Baenziger NL. (1987)
Role of receptors in metabolic interaction of histamine with human vascular endothelial cells and skin fibroblasts.
J Biol Chem, **262**, 10220-10228.

Hamet P, Sugimoto H, Umeda F, Lecavalier L, Franks J, Orth DN & Chiassou JL(1985)
Abnormalities of platelet-derived growth factors in insulin-dependent diabetes
Metabolism, **34**, 25-31

Hamilton K & Sims PJ. (1987)
Changes in cytosolic Ca^{2+} associated with von Willebrand factor release in human endothelial cells exposed to histamine
J Clin Invest, **79**, 600-608.

Hamsten A, Walldius G, Szamesi A, Dahlèn G & de Faire U (1986)
Relationship of angiographically defined coronary artery disease to serum lipoproteins and apoproteins in young survivors of myocardial infarction.
Circulation, **73**, 1097-1100.

Harlan JM (1985)
Leucocyte-endothelial interactions
Blood, **65**, 513-525

Harman D (1962)
Atherosclerosis-inhibiting effect of an antihistamine drug, chlorpheniramine
Circ Res, **XI**, 277-282

Harman D (1962)
Atherosclerosis-inhibiting effect of an antihistaminic drug, chlorpeniramine
Circ Res, **11**, 277-282

Hearey DJ, Ind PW, Miyatake A, Brown MJ, Macdermott J & Dollery CT (1984)
Histamine released locally after intradermal antigen challenge in man
Br J Clin Pharmacol, **18**, 915-919

Heltianu C, Simionescu M & Simionescu N. (1982)
Histamine receptors of the microvascular endothelium revealed *in situ* with a histamine-ferritin conjugate: characteristic high-affinity binding sites in venules.
J Cell Biol., **93**, 357-364.

Hoffmann EA, Munroe ML & Tucker A (1977)
Histamine H_1 and H_2 receptors and their roles during alveolar hypoxia
Resp Physiol, **29**, 255-264

Holcslaw TL, Nichols G & Wilson C. (1985)
Studies on uptake and catabolism of vascular histamine in spontaneously hypertensive rats.
J Pharm Exp Ther, **233**, 352-360.

Hollander W, Kramsch DM, Franzblanc C, Paddock J & Colombo MA (1974)
Suppression of atheromatous fibrous plaque formation by antiproliferative and anti-inflammatory drugs
Circ Res, **34**, 131-137

Hollis TM & Ferrone RA (1974)
Effect of shearing stress on aortic histamine synthesis.
Exp Mol Pathol, **20**, 1-4.

Hollis TM & Furniss JV. (1980)
Relationship between aortic histamine formation and aortic albumin permeability in atherogenesis.
Proc Soc Exp Biol Med, **165**, 271-274.

Hollis TM & Rosen LA (1972)
Histidine decarboxylase activity of bovine aortic endothelium and intima-media
Proc Soc Exp Biol, **141**, 978-986

Hollis TM & Sloss RJ (1975)
Rabbit aortic histamine synthesis following short-term cholesterol feeding.
Atherosclerosis, **21**, 125-134.

Hollis TM & Strickberger SA (1985)
Inhibition of aortic histamine synthesis by α-hydrazinohistidine inhibits increased aortic albumin accumulation in experimental diabetes in the rat.
Diabetologia, **28**, 282-285.

Hollis TM, Enea JA, Enea NA & Cosgarea AJ. (1985)
Changes in plasma histamine concentration in the streptozotocin-diabetic rat.
Exp Mol Pathol, **43**, 90-96.

Hollis TM, Enea NA & Kern JA (1984)
Time-dependent changes in aortic albumin permeability characteristics in experimental diabetes
Exp Mol Pathol, **41**, 207-217

Hollis TM, Gallik SG, Orlidge A, Yost JC. (1983)
Aortic endothelial and smooth muscle histamine metabolism - relationship to aortic ^{125}I-histamine accumulation in experimental diabetes.
Arteriosclerosis, **3**, 599-606.

Hollis TM, Gardner TW, Vergis GJ, Kirbo BJ, Butler C, Dull RO, Campos MT & Enea NA (1988)
Antihistamines reverse blood-ocular barrier breakdown in experimental diabetes
J Diabetic Complications, **2(1)**, 47-49

Hollis TM, Yarnal JR, Ferrone RA & Anthony A (1972)
Changes in aortic histidine decarboxylase activity in hypertensive rats
Fed Proc, **131**, 292-294

Holmes DR, Elveback LP, Frye RL, Kottke BA & Ellefson RD (1981)
Association of risk factor variables and coronary artery disease documented with angiography.
Circulation, **63**, 293-297.

Horan KL, Adamski SW, Ayele W, Langone JJ & Grega GJ (1986)
Evidence that prolonged histamine suffusions produce transient increases in vascular permeability subsequent to the formation of venunal macromolecular leakage sites: proof of the Majno-Palade Hypothesis
Am J Pathol, **123**, 570-576

Hüttner J, More RM & Rona G. (1970)
Fine structural evidence of a specific mechanism for increased permeability in experimental hypertension.
Amer J Path, **61**, 395-400.

Hutton RA, Barradas MA, de Albarran R & Dandona P (1984)
Adenine nucleotide metabolism in diabetes
Diabetologia, **26**, 89-90

Ishibashi T, Tanaka K & Taniguchi Y (1980)
Disruption of blood-retinal barrier in experimental diabetic rats.
An electron microscopic study
Exp Eye Res, **30**, 401-410

Jarrett RJ & Keen H. (1975)
Diabetes and atherosclerosis. In: Keen H, Jarrett RJ (eds)
Complications of Diabetes, pp 179-205.
London: Edward Arnold.

Jeremy JY, Mikhailidis DP & Dandona P (1983)
Simulating the diabetic environment modifies *in vitro* prostacyclin synthesis
Diabetes, **32**, 217-221

Jeremy JY, Thompson CS, Mikhailidis DP & Dandona P (1985)
Experimental diabetes mellitus inhibits prostacyclin synthesis by rat penis: pathological implications
Diabetiologia, **28**, 365-368

Joris I, Zand T, Nunnari JJ, Krolikowski FJ & Majno G (1983)
Studies on the pathogenesis of atherosclerosis.
Amer J Path., **113**, 341-347.

Junod A, Lamber AE, Stauffacher W & Renold AE (1969)
Diabetogenic action of streptozotocin: relationship of dose to metabolic response.
J Clin Invest, **48**, 2129-2139

Kahlson G & Rosengren E (1968)
New approaches to the physiology of histamine
Physiol Rev, **48**, 155-196

Kalsner S & Richards R (1984)
Coronary arteries of cardiac patients are hyperreactive and contain stores of amines: a mechanism for coronary spasm
Science, **223**, 1435-1437

Kapeller-Adler R & McFarlane H (1963)
Purification and identification of hog-kidney histaminase
Biochim Biophys Acta, **67**, 543-550

Keeling DJ & Smith IR (1979)
Study of histidine decarboxylase from guinea-pig brain: a novel bioassay for histidine decarboxylase activity
Biochem Soc Trans, **7**, 672-673

Keeling DJ, Smith IR & Tipton KF (1984)
A coupled assay for histidine decarboxylase: *in vivo* turnover of this enzyme in mouse brain
Naunyn-Schmiedeberg's Arch Pharmacol **326**, 215-221

Killacky JJF, Johnston MG & Movat MG. (1986)
Increased permeability of microcarrier-cultured endothelial monolayers in response to histamine and thrombin.
Am J Pathol, **122**, 50-61.

Kollonitsch J, Patchett AA, Marburg S, Maycock AL, Perkins LM, (1978)
Doldouras GA, Duggan GE & Aster SD.
Selective inhibitors of biosynthesis of aminergic neurotransmitters.
Nature, **274**, 906-908

Langeler EG, Snetling-Hannga I & van Hinsberg VWM (1989)
Pasage of low density lipoprotein through monolayers of human arterial endothelial cells: effects of vasoactive substances in an *in vitro* model
Arteriosclerosis, **9**, 550-559

Leinweber FJ. (1968)
Mechanism of histamine decarboxylase inhibition by NSD-1055 and related hydroxylamines.
Mol Pharmacol, **4**, 337-348.

Levin EG & Santell L. (1988)
Stimulation and desensitization of tissue plasminogen activator release from human endothelial cells.
J Biol Chem, **263**, 9360-9365

Levine RJ, Sato TL & Sjoerdsma (1965)
Inhibition of histamine synthesis in the rat by α-hydrazino analog of histidine and 4-bromo-3-hydroxy-benzyl oxyamine.
Biochem Pharmacol, **14**, 139-149.

Lichtenstein LM, Plant M, Henney C & Gillespie J (1973)
The role of H_2 receptors as cells involved in hypersensitivity reactions. In: CJ Wood & MA Simkins (eds) *International Symposium on Histamine H_2-receptor Antagonists*, pp 187-207.
WGC, SK & F Labs Ltd.

Lorenz W, Reimann JH, Barth H, Kusche J, Meyer R, Doenicke A & Hutzel M (1972)
A sensitive and specific method for the determination of histamine in human whole blood and plasma
Hoppe-Seyler's Z Physiol Chem, **353**, 911-920

Lorenzi M, Healy DP, Hawkins R, Printz JM & Printz MP (1986)
Studies on the permeability of the blood-brain barrier in experimental diabetes
Diabetologia, **29**, 58-62

Lowry OH, Rosenberg NJ, Farr AL & Randall RJ (1951)
Protein measurement with the folin-phenol reagent
J Biol Chem, **193**, 265-275

Majno G, Gilmore V & Leventhal M. (1967)
On the mechanism of vascular leakage caused by histamine-type mediators. A microscopic study *in vitro*.
Circ Res, **21**, 833-847.

Majno G, Shea SM & Leventhal M. (1969)
Endothelial contraction induced by histamine-type mediators. An electron-microscopic study.
J Cell Biol, **42**, 647-672.

Marchesi V & Florey H (1960)
Electron micrographic observations on the emigration of leucocytes
Q J Exp Physiol, **45**, 343-349

Markle RA & Hollis TM (1975)
Rabbit aortic endothelial and medial histamine synthesis following short-term cholesterol feeding.
Exp Mol Pathol, **23**, 417-420.

Markle RA & Hollis TM (1977)
Variations in rabbit aortic endothelial and medial histamine synthesis in pre- and early atherosclerosis.
Proc Soc Exp Biol Med, **155**, 365-368.

Markle RA, Hollis TM & Cosgarea AJ (1986)
Renal histamine increases in streptozotocin-diabetic rats
Exp Mol Pathol, **44**, 21-28

Maslinski C. (1975a)
Histamine and its metabolism in mammals. Part I.
Agents and Actions, **5**, 89-107.

Maslinski C. (1975b)
Histamine and its metabolism in animals, Part II.
Agents and Actions, **5**, 183-225.

Mazzone T, Jensen M & Chait A (1983)
Human arterial wall cells secrete factors that are chemotactic for monocytes
Proc Natl Acad Sci USA, **80**, 5094-5097

McBride P, Bradley D & Kaliner M (1988)
Evaluation of a radioimmunoassay for histamine measurement in biologic fluid
J Allergy Clin Immunol, **82**, 638-646

McGill HS Jr (Ed.) (1968)
The Geographic Pathology of Atherosclerosis
Williams & Wilkins, Baltimore, pp 1-193

McIntyre TM, Zimmerman GA, Satoh K & Prescott SM. (1985)
Cultured endothelial cells synthesize both platelet activating factor and prostacyclin in reponse to histamine, bradykinin and adenosine triphosphate.
J Clin Invest, **76**, 271-280.

Melmon KL, Weinstein Y, Shearer GM, Bourne HR & Sela M (1972)
Immunological implication of extracellular receptors for histamine on human leucocytes and mouse spleen cells
Israel J Med Sci, **8**, 641-643

Mikhailidis DP, Barradas MA, Jeremy JY & Dandona P (1985)
Heparin-induced platelet aggregation in anorexia nervosa and peripheral vascular disease
Eur J Clin Invest, **15**, 313-319

Mikhailidis DP, Hutton RA, Jeremy JY & Dandona P (1983)
Cooling decreases the efficiency of prostaglandin inhibitors of platelet aggregation: effects of possible relevance in cold-induced pathology
Microcirculation, **2**, 413-423

Mikhailidis DP, Mikhailidis AM, Woolard ML & Dandona P (1982)
Protection of prostacyclin-like activity in human plasma: a non-enzymatic mechanism
Clin Sci, **62**, 177-181

Millner NE, Hammet F, Saltissi S, Rao S, van Zeller H, Coltart J & Lewis B (1981)
Relationship of angiographically defined coronary artery disease to plasma lipoprotein subfractions and apolipoproteins.
Br Med J, **282**, 1741-1745.

Mitchell J & Schwartz C (1965)
Arterial Disease, pp 375-390
Oxford: Blackwell

Mole KH & Shepherd DM (1972)
Inhibition of histidine decarboxylase *in vivo*
Arch Int Pharmacodyn, **31**, 109-147

Morel AM & Delaage MA (1988)
Immunoanalysis of histamine through a novel chemical derivatization
J Allergy Clin Immuno, **82**, 646-654

Morris DR & Fillingame RH. (1974)
Regulation of amino acid decarboxylation.
Ann Rev Biochem, **43**, 303-325.

Nilsson J, Sjölund M, Palmberg L, Thyberg J & Heldin CH (1985)
Arterial smooth muscle cells in primary culture produce a platelet-derived growth factor-like protein.
Proc Natl Acad Sci (USA), **82**, 4418.

O'Flaherty J, Craddock PR & Jacobs HS (1978)
Effect of intravascular complement activation on granulocyte adhesiveness and distribution
Blood, **51**, 731-739

Okuyama T & Kobayashi Y (1961)
The radioisotope assay of diamine oxidase
Arch Biochem Biophys, **95**, 242-250

Orchard MA, Kagy-Sabetka A, Proud D & Lichtenstein LM (1986)
Basophil histamine release induced by a substance from stimulated human platelets
J Immunol, **136**, 2240-2244

Orlidge A & Hollis TM (1982)
Aortic endothelial and smooth muscle histamine metabolism in experimental diabetes.
Arteriosclerosis, **2**, 142-150.

Orlidge A. (1983)
Bovine aortic endothelial cells - histamine metabolism and its relationship to fluid phase endocytosis: regulation by insulin, histamine receptors and alpha hydrazinohistidine.
PhD Thesis. The Pennsylvania State University Park, PA, USA.

Owen GK & Hollis TM (1979)
Relationship between inhibition of aortic histamine formation, aortic albumin permeability and atherosclerosis
Atherosclerosis, **34**, 365-373

Owens GK & Hollis TM (1981)
Local aortic histamine metabolism and albumin accumulation.
Difference between blue and white areas
Arteriosclerosis, **1**, 265-272

Palacious JM, Mengod G, Picatoste F, Grace M & Blanco I (1976)
Properties of rat brain histidine decarboxylase
J Neurochem, **27**, 1455-1460

Parratt JR & West GB (1956)
Tissue histamine and 5-hydroxytryptamine
J Physiol (Lond), **132**, 40-45

Parsons ME (1988)
Personal Communication
SK & F Research institute, Welwyn, Herts.

Parving HH, Noer I, Deckert T, Evrin PE, Nielsen SL, Lyngse J, Magersen CE, Rorth M, Svendsen PA, Trap-Jensen J & Lassen NA (1976)
The effects of metabolic regulation in microvascular permeability to small and large molecules in short-term juvenile diabetes
Diabetologia, **12**, 161-166

Peacock I, Hawkins M, Heptinstall S (1986)
Platelet behaviour in non-insulin dependent diabetes - influence of vascular complications, treatment and metabolic control
Thromb Haemostas, **55**, 361-365

Pearson TA, Dillman J, Solez K & Heptinstall RH (1979)
Monoclonal characteristics of organising arterial thrombi: significance in the origin and growth of human atherosclerotic plaques
Lancet, **I**, 7-11

Pidard D, Didry D, Kunicki TJ & Nurden AT (1986)
Temperature-dependent effects of EDTA on the membrane glycoprotein IIb-IIIa complex and platelet aggregation
Blood, **67**, 604-611

Pilati CF & Maron MB (1988)
Effect of pathological blood histamine levels on canine coronary vascular permeability
Am J Physiol, **254**, H912-918

Plaut M & Lichtenstein M. (1982)
Histamine and immune responses. In: Ganellin CR, Parsons ME (eds)
Pharmacology of Histamine Receptors, pp 392-435.
Boston: Wright, PSG.

Rapoport SI (1976)
Blood Brain Barrier in Physiology and Medicine
Raven Press, New York

Read GW, Ortner MJ, Hino R & Lenney JF (1974)
Studies on the mast cell receptor(s) responsible for histamine release
Clin Pharmacol, **15**, 217-220

Reidy MA (1985)
A reassessment of endothelial injury and arterial lesion formation.
Lab Invest, **53**, 513-520.

Revtyak GE, Hughes MJ, Johnson AR & Campbell WB (1988)
Histamine stimulation of prostaglandin and HETE synthesis in human endothelial cells.
Am J Physiol, **225**, C214-C225.

Riley JF & West GB (1966)
The occurrence of histamine in mast cells. *Handbook of Experimental Pathology, Vol XVIII. Histamine and Antihistamines, Part 1*, ed. Rocha e Silva M; pp 116-135
Springer Verlag: New York

Robertson AL & Khairallah PA (1973)
Arterial endothelial permeability and vascular disease: the 'trap door' effect
Exp Mol Pathol, **18**, 241-245

Robertson WB & Strong JP (1968)
Atherosclerosis in persons with hypertension and diabetes mellitus.
Lab Invest, **18**, 538-551.

Rosen LA, Hollis TM & Sharma MG (1974)
Aleration in bovine endothelial histidine decarboxylase activity following exposure to shearing stress
Exp Mol Pathol, **20**, 329-331

Ross R & Glomset JA (1976)
The pathogenesis of atherosclerosis.
N Engl J Med, **295**, 369-420.

Ross R (1986)
The pathogenesis of atherosclerosis
New Engl J Med, **314**, 488-500

Ross R, Glomset J, Kariya B & Harker L (1974)
A platelet derived serum factor that stimulates the proliferation of arterial smooth muscle cells *in vitro*
Proc Natl Acad Sci USA, **71**, 1207-1210

Ross R. (1986)
The pathogenesis of atherosclerosis - an update.
N Engl J Med, **314**, 488-501.

Rotrosen D & Gallin J. (1986)
Histamine type 1 receptor subtype occupancy increases endothelial cytosolic calcium, reduces F-actin and promotes albumin diffusion across cultured endothelial monolayers.
J Cell Biol, **103**, 2379-2387.

Schayer RW (1961)
Histamine and circulatory homeostasis
Fed Proc, **24**, 1295-1297

Schayer RW (1961)
Significance of induced synthesis of histamine in physiology and pathology
Chemotherapia, **3**, 128-136

Schayer RW (1962)
Evidence that induced histamine is a regulator of the microcirculatory system
Amer J Physiol, **202**, 65-72

Schayer RW (1965)
Histamine and circulating homeostasis.
Fed Proc **24**, 1295-1297.

Schayer RW (1966)
Enzymatic formation of histamine from histidine. In: *Handbook of Experimental Pharmacology*, ed. Eichler O & Farah A
Berlin: Springer-Verlag, vol 18, pp 688-725

Schayer RW. (1956)
Formation and binding of histamine by free mast cells of rat peritoneal fluid.
Am J Physiol, **186**, 199-202.

Schellenberg RR, Dugg MJ, Foster A & Paddon HB (1986)
Histamine releases PGI_2 from human pulmonary artery
Prostaglandins, **32**, 201-205

Schwartz CJ, Gerrity RG & Lewis LJ. (1978)
Arterial endothelial structure and function with particular reference to permeability. In: R Paolotti & AM Gotto (eds)*Atherosclerosis Reviews*, Vol 3, p 109.
New York: Raven Press.

Schwartz CJ, Valente AJ, Sprague GA, Kelly JL, Suenram CA,
Graves DT, Rozek MM, Edwards EH & Delgado R (1986)
Monocyte macrophage participation in atherogenesis: inflammatory components of pathogenesis
Sem Thromb Hemostas, **12**, 79-86

Schwartz JC, Lampart C & Rose C. (1972)
Histamine formation in rat brain *in vivo*: Effects of histidine loads.
J Neurochem, **19**, 801-810.

Schwartz JC, Pollard H & Quach TT. (1980)
Histamine is a neurotransmitter in mammalian brain:
Neurochemical evidence.
J Neurochem, **35**, 26-33.

Schwartz JC. (1975)
Histamine as a transmitter in brain
Life Sci, **17**, 503-517

Seifert RA, Schwartz SS & Bowen-Pope DF (1984)
Developmentally regulated production of platelet-derived growth factor-like molecules.
Nature, **311**, 669-672.

Severs WB, Gordon JW & Beavan MA. (1970)
Some observations on aminoguanidine pharmacology.
Pharmacology, **3**, 201-208.

Shaff RE & Beavan MA (1979)
Increased sensitivity of the enzymatic isotopic assay of histamine: measurement of histamine in plasma and serum.
Analyt Biochem, **94**, 425-430

Shaw JO (1980)
Leucocytes in chemotactic fragment-induced lung inflammation: Vascular emigration and alveolar surface migration
Am Pathol, **101**, 283-290

Shimokado K, Raims EW, Madtes DK, Barrett TB, Benditt EP & Ross R (1985)
A significant part of macrophage-derived growth factor consists of at least two forms of PDGF.
Cell, **43**, 277-280.

Shinokawa H, Tomoike H, Nabeyama S, Yamamoto H, Araki H & Nakamura M(1983)
Coronary artery spasm induced in atherosclerotic miniature swine
Science, **221**, 560-561

Simionescu N, Heltianu G, Antohe F & Simionescu M (1982)
Endothelial cell receptors for histamine
Ann NY Acad Sci, **401**, 132-149

Skarlatos SI & Hollis TM (1987)
Cultured bovine aortic endothelial cells show increased histamine metabolism when exposed to oscillatory shear stress.
Atherosclerosis, **64**, 55-61.

Sollmann T & Pilcher JD. (1917)
Endermic Reactions.
I J Pharmac , **9**, 309-340.

Stefanovich V & Gore I. (1971)
Cholesterol diet and permeability of rabbit aortas.
Exp Mol Pathol, **14**, 20-26.

Stefanovich V & Gore I. (1971)
Cholesterol diet and permeability of rabbit aortas.
Exp Mol Pathol, **14**, 20-25.

Steinberg D (1983)
Lipoprotein and atherosclerosis: a look back and a look ahead
Arteriosclerosis, **3**, 283-301

Steiner G (1981)
Diabetes and atherosclerosis - an overview.
Diabetes, **30** (Suppl 2), 1-7.

Stender S & Hjelms E (1987)
In vivo transfer of cholesterol from plasma to human aortic tissue
Scand J Clin Lab Invest, **47**, 21-29

Stewart J, Jones DG & Kay AB. (1979)
Metabolic studies on the uptake of [^{14}C]-histidine and [^{14}C]-histamine and histamine synthesis by guinea-pig basophils *in vitro*.
Immunology, **36**, 539-548.

Stoner P (1985)
An improved spectrophotometric assay for histamine and diamine oxidase (DAO) activity
Agents and Actions, **17**(1), 5-9

Stout RW. (1979)
Diabetes and atherosclerosis: the role of insulin.
Diabetologia, **16**, 141-150.

Taylor RJ, Leinweber FJ & Braun GA. (1973)
4-imidazolyl-3-amino-2-butanone (McN-A-1293), a new specific inhibitor of histidine decarboxylase.
Biochem Pharmacol, **22**, 2299-2310.

Tryding N & Willert B (1968)
Determination of plasma histaminase in clinical practice. A comparison between a biological method and a radiochemical micromethod
Scand J Clin Lab Invest, **22**, 29-32

Tung AS, Blake JT, Roman IJ, Vlasses PH, Ferguson K & Zweerink HJ (1985)
In vivo and *in vitro* inhibition of human histidine decarboxylase by (s)-α fluoromethyl-histidine
Biochem Pharmacol, **34**, 3509-3515

Tuomisto J (1973)
A new modification for studying 5-HT uptake by blood platelets: a re-evaluation of tricyclic antidepressants as uptake inhibitors
J Pharm Pharmac, **26**, 92-100

Valensi P, Attali JR, Behar A & Sebaoun J (1987)
Isotopic test of capillary permeability to albumin in diabetic patients. Effects of hypertension, microangiopathy and duration of diabetes.
Metabolism, **36**, 834-839

Veress B, Balint A, Kocze A, Nagy Z & Jellinek H (1970)
Increasing aortic permeability by atherogenic diet.
Atherosclerosis, **11**, 369-376.

Virag S, Denes R, Pozsonyi T (1969)
Uptake of [^{125}I]-β-lipoprotein by aortas and veins of normal and cholesterol fed rabbits.
Atherosclerosis, **11**, 361-370.

Waltman SR, Oestrich C, Krupin T, Hanish S, Ratzan S, Santiago J & Kilo C (1970)
Quantitative vitreous fluorophotometry. A sensitive technique for measuring early breakdown of the blood retinal barrier in young diabetic patients
Diabetes, **27**, 85-87

Weiss HJ, Hawiger J, Ruggeri ZM, Turritto VT & Thiagarajan P (1989)
Fibrinogen-independent platelet adhesion and thrombus formation on sub-endothelium mediated by glycoprotein IIb/IIIa complex at high shear rate
J Clin Invest, **83**, 288-297

Welles SL, Shepro D & Hechtman HB (1985)
Vasoactive amines modulate actin cables (stress fibres) and surface area in cultured bovine endothelial cells.
J Cell Physiol, **123**, 337-342.

Wetterquist H (1978)
Histamine metabolism and excretion. In Rocha E Silva (ed.): *Handbook of experimental Pharmacology*, Vol. 18, Part 2, pp 131-150.
Berlin: Springer Verlag.

Wilhelm DL. (1962)
The mediation of increased vascular permeability in inflammation.
Pharmac Rev, **14**, 251-280.

Wolinsky H, Goldfischer S, Capron L, Capron F, Coltoff-Schuller B (1978)
& Kosak L.
Hydrolase activities in the aorta. I. Effect of diabetes mellitus and insulin treatment.
Circ Res, **42**, 821-831.

Wood K, Harwood J & Coppen A (1983)
Platelet accumulation of histamine in depression
Lancet, **2**, 519-520

Wood K, Harwood J & Coppen A (1984)
Platelet accumulation of histamine in controls, depressed and lithium-treated patients
J Affective Disord, **7**, 149-158

Yamada M, Watanabe T & Harina S (1980)
The effect of protease inhibitors on histidine decarboxylase activities and assay of enzyme in various rat tissues
Biochim Biophys Acta, **615**, 458-464

Yarnal JR & Hollis TM (1976)
Rat aortic histamine synthesis during short-term hypertension.
Blood Vessels, **13**, 70-76.

Yoshida K, Hashimoto M & Nagase M (1982)
The clinical role of plasma DβH activity, histamine and serotonin in diabetes mellitus
The Autonomic Nervous System (Osaka), **19**, 200-208

Yoshimura F, Hakuta T, Koneko Y & Yoshida Z (1984)
Evalulation of plasma histamine in patients with coronary artery disease
Jap J Med, **23**(2), 127-134

Zucker MB & Grant RA (1978)
Non-reversible loss of platelet aggregability induced by calcium deprivation
Blood, **52**, 505-513

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Histamine uptake by human platelets

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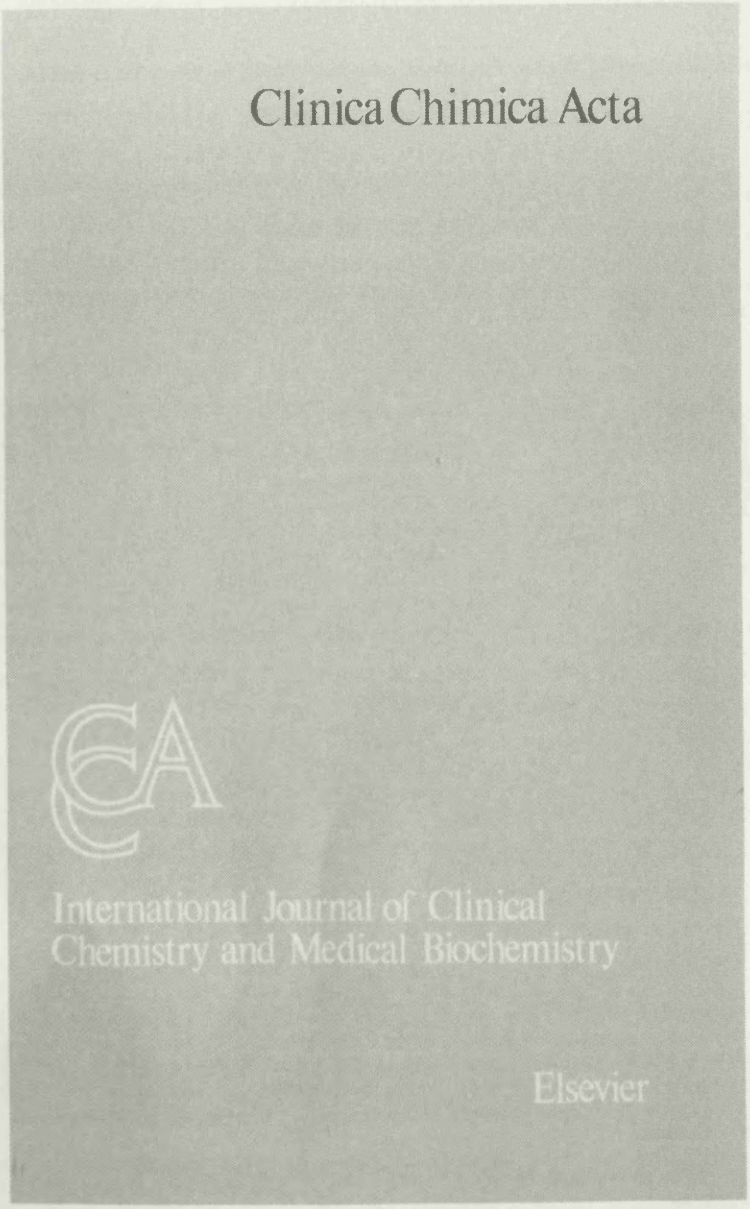

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Histamine uptake by human platelets

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Summary

We have investigated the uptake of histamine by human platelets. Incubations were carried out in platelet rich plasma prepared by using sodium citrate as an anticoagulant at histamine concentrations of 2.5 $nmol \cdot l^{-1}$, with and without stirring, in a platelet aggregometer cuvette at 37°C. Stirring increased platelet histamine uptake significantly. Conventional platelet aggregating agents (e.g. adrenaline) significantly increased platelet histamine uptake at sub-aggregatory concentrations. Histamine uptake by platelets may be a useful index of platelet behaviour when studying the effect of subaggregatory concentrations of platelet agonists in conditions where platelet aggregation is altered, e.g. peripheral vascular disease and diabetes mellitus.

Introduction

Human platelets have previously been shown to take up histamine [1,2]. Previous studies are, however, limited by 3 factors. Firstly, the concentrations of histamine used during the incubations, in vitro, were almost 300-fold greater than those found in human plasma [3]. Secondly, the platelets were prepared in ethylenediamine tetraacetic acid (EDTA), at concentrations which others, as well as ourselves, have shown to inhibit platelet function [4]. Thirdly, since the incubations were carried out in conventional water baths, they were performed without stirring. It is well known that platelets do not aggregate efficiently, in vitro, unless they are stirred (e.g. with a stirring bar at 1000 rpm in conventional aggregometers) [5]. Blood is also subjected to turbulence and mixing in vivo.

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The present study investigates histamine uptake by human platelets in experimental conditions which overcome the problems mentioned above.

Materials and methods

Subjects

Subjects were healthy males and females who had not ingested any drugs for 2 wk prior to the experiment. Their median age was 29 yr (range: 21–44 yr). The number of subjects in each experiment is mentioned in the detailed description of experiments below.

Preparation of platelet rich plasma

Venous blood was collected in trisodium citrate, and platelet rich plasma (PRP) was prepared by centrifugation at $140 \times g$ as previously described [6]. Thereafter all PRP was kept at 37 °C since we have shown that cooling influences platelet function [7].

Preparation of [^{14}C]histamine label

[ring-2-^{14}C]histamine dihydrochloride (56 mCi/mmol; Amersham International, UK) was diluted in isotonic sodium chloride (154 mmol/l) so that 25 μl gave a final concentration of 2.5 nmol $\cdot$ l^{-1} [^{14}C]histamine in the aggregometer cuvette.

Structure of individual experiments

1. The determination of the effect of stirring on [^{14}C]histamine uptake by human platelets in PRP

PRP was incubated at 37 °C for up to 90 min in siliconised glass aggregometer cuvettes (Coulter Electronics Ltd., Luton, UK): (a) in a shaking water bath; and (b) in chronolog aggregometers (Coulter Electronics Ltd., Luton, UK) with a teflon-coated stirring bar spinning at 1000 rpm [6].

2. The determination of the effect of conventional aggregating agents on [^{14}C]histamine uptake by human platelets in PRP

(a) Effect of collagen. PRP was incubated for 30 min in aggregometer cuvettes as described in 1a and b above. Incubations were started by the simultaneous addition of [^{14}C]histamine label and several concentrations of collagen (Horm-Chemie, Munich, FRG) dissolved in isotonic sodium chloride (154 mmol/l). The concentrations of collagen used are shown in Table I.

(b) Effect of adrenaline. PRP was incubated for 15 min with adrenaline (Sigma Chemical Co., Poole, Dorset, UK) dissolved in isotonic sodium chloride (154 mmol/l), as described above for collagen except that the same amount of adrenaline was added every 5 min because of adrenaline instability [8]. The concentrations of adrenaline used are shown in Table I.

TABLE I

The effect of stirring and aggregating agents on [^{14}C]histamine uptake by platelets

a. The effect of stirring

Stirred (S) $n = 7$ (a)	Unstirred (US) $n = 7$ (b)	p values
206 (132–240)	127 (112–134)	(a) vs (b) < 0.01

b. The effect of collagen

Saline only $n = 9$ (a)	Collagen 0.05 mg·l^{-1} $n = 9$ (b)	Collagen 0.1 mg·l^{-1} $n = 7$ (c)	p values
207 (112–255)	306 (187–366)	201 (149–296)	(a) vs (b) < 0.01 (a) vs (c) n.s.

c. The effect of adrenaline (Adr)

Saline only $n = 8$ (a)	Adr 0.02 μmol·l^{-1} $n = 8$ (b)	Adr 0.03 μmol·l^{-1} $n = 7$ (c)	Adr 0.05 μmol·l^{-1} $n = 7$ (d)	p values
205 (131–240)	327 (226–376)	296 (204–385)	278 (212–362)	(a) vs (b) < 0.01 (a) vs (c) < 0.01 (a) vs (d) < 0.01

d. The effect of ADP

Saline only $n = 7$ (a)	ADP 0.03 μmol·l^{-1} $n = 7$ (b)	ADP 0.05 μmol·l^{-1} $n = 7$ (c)	p values
200 (113–257)	225 (128–281)	235 (169–299)	(a) vs (b) < 0.01 (a) vs (c) < 0.01

These data take into account the fall in platelet numbers, since they are expressed as fmol histamine/10^8 platelets/min, median and (range). n, Number of subjects sampled; n.s., not significant. Statistical evaluation: paired Wilcoxon test (two-tailed).

(c) Effect of adenosine diphosphate (ADP). PRP was incubated for 15 min with ADP (Sigma Chemical Co., Poole, Dorset, UK) dissolved in isotonic sodium chloride (154 mmol/l), as described above for collagen. The same amount of ADP was added every 5 min in order to minimise the effects of intrinsic ADPase activity [9]. The concentrations of ADP used are shown in Table I.

Procedure for assessing platelet [^{14}C]histamine uptake

PRP (500 μl) was allowed to stand or stir in an aggregometer cuvette for 1 min and a 6 μl portion taken for platelet counting in a Coulter ZM (Coulter Electronics Ltd., Luton, UK). The histamine uptake was then started by the addition of 25 μl of prediluted [^{14}C]histamine label (final concentration in aggregometer cuvette: 2.5 nmol·l^{-1}), followed immediately by up to 10 μl of isotonic sodium chloride (154

mmol/l) (for control samples) or desired concentrations of the aggregating agent (for test samples). During the incubation with saline or aggregating agent the amount of aggregation occurring in the Chronolog dual channel aggregometers was constantly monitored on Omniscribe chart recorders as previously described [10]. After incubation, a second 6 μl portion was removed for platelet counts and the remainder of the PRP was transferred to a polypropylene Eppendorf tube containing an aspirin solution in saline (final concentration of aspirin after adding the PRP: 100 mg $\cdot$ l^{-1}). Preliminary experiments have shown that histamine uptake was approximately 15% greater if aspirin was added at this stage, presumably because the platelet release reaction is inhibited by aspirin [11] and some degree of activation could occur during these stages. The PRP was then immediately centrifuged at 10000 $\times$ g for 1 min in an Eppendorf microfuge (Eppendorf, Munich, FRG). The supernatant was removed and the pellet washed with isotonic sodium chloride (154 mmol/l). The pellet was then resuspended and ultrasonicated (to break up platelets) in fresh saline using an MSE Soniprep 150 (M.S.E., Crawley, Sussex, UK) for 20 s at an amplitude of 18 μm. Liquiscint (National Diagnostics, NJ, USA), 5 ml, was added and the resulting suspension was counted for ^{14}C-radioactivity in an LKB rackbeta counter (L.K.B. Wallac, Sweden).

Control experiments

I. Estimation of the amount of residual radioactivity in the platelet pellet after ultrasonication. PRP was prepared from 3 subjects and individually incubated for 30 min as described above in expt 1b. Each PRP was divided into two portions: one was ultrasonicated as described in 1b above, whereas the other was not. Platelet counts on both samples were then carried out using a Coulter ZM (see above). The two samples were then centrifuged (10000 $\times$ g, at room temperature, for 2 min) and the pellets washed as described above. The pellets from both samples were resuspended and ultrasonicated for 20 s and the amount of ^{14}C radioactivity was determined as described above.

II. The effect of using EDTA as an anticoagulant on platelet aggregation, ionised calcium concentration and [^{14}C]histamine uptake. PRP was prepared using an anticoagulant solution made up as described by Wood et al. [1], consisting of 27 mmol $\cdot$ l^{-1} disodium EDTA, 120 mmol $\cdot$ l^{-1} NaCl and 6 mmol $\cdot$ l^{-1} glucose. Blood (10 parts) was mixed with this solution (1 part). The sample was then centrifuged at 140 $\times$ g for 15 min as previously described [6]. PRP was also prepared from blood obtained at the same venepuncture using citrate only as anticoagulant, as described above. Platelet counts and platelet aggregation were carried out as described above. The uptake of [^{14}C]histamine into platelets of both samples was carried out as described in 1a and b above. Ionised calcium was measured in both samples using an ICA 1 ionised calcium analyzer (Radiometer, Copenhagen, Denmark).

III. The effect of platelet count in PRP on [^{14}C]histamine uptake. PRP and platelet poor plasma (PPP) were prepared as previously described [6]. The PRP was

diluted 1:1 and 1:4 with autologous PPP. Platelet counts were performed as previously described. The uptake of [^{14}C]histamine into platelets was carried out as described in 1a and b above.

Statistical analysis and presentation of results

Results are expressed as median and (range). p values were determined using the non-parametric paired Wilcoxon test (two-tailed). The correlation coefficient (r) and its p value were calculated using a verified computer programme.

Results

Experiment 1: determination of the effect of stirring on [^{14}C]histamine uptake by human platelets in PRP (Table Ia)

There was linear uptake of [^{14}C]histamine for up to 90 min in the stirred (S) and unstirred (US) PRP samples. The correlation coefficient (r) and the p values (for 60 min incubations) were: $r = 0.85$ and $p < 0.001$ for the S samples ($n = 7$) and $r = 0.96$ and $p < 0.001$ for the US samples ($n = 7$). The results for histamine uptake for S and US samples are shown in Table Ia for incubations of up to 60 min. Samples from only 3 subjects were incubated for up to 90 min. Uptake in the S samples of all subjects was always greater than that in the US samples. After incubation for 60 min the platelet count dropped by approximately 7% of the baseline value in both the S and US samples.

Experiment 2: the determination of the effect of conventional aggregating agents on [^{14}C]histamine uptake by platelets in PRP

(a) Effect of collagen (Table Ib). Collagen at a final concentration of 0.05 $mg \cdot l^{-1}$ significantly ($p < 0.01$) enhanced [^{14}C]histamine uptake by platelets in PRP. The drop in platelet number was similar to that of saline controls. Lower concentrations of collagen (0.01 and 0.03 $mg \cdot l^{-1}$) did not significantly enhance [^{14}C]histamine uptake. Higher concentrations of collagen (0.07 and 0.1 $mg \cdot l^{-1}$) did not enhance [^{14}C]histamine uptake, but they induced a concomitant fall in platelet count of $> 11\%$. In some subjects these concentrations (0.07 and 0.1 $mg \cdot l^{-1}$) elicited significant changes ($> 10\%$ aggregation) in optical density. In the US samples collagen did not significantly enhance [^{14}C]histamine uptake.

(b) Effect of adrenaline (Table Ic). Adrenaline at a final concentration of 0.02 $\mu mol \cdot l^{-1}$ significantly ($p < 0.01$) enhanced [^{14}C]histamine uptake by platelets (with a 27% fall in platelet count). Higher concentrations of adrenaline (0.03 and 0.05 $\mu mol \cdot l^{-1}$) caused an increase in uptake, but they also caused a marked decrease in platelet numbers ($> 46\%$). In some subjects these higher concentrations caused aggregation as observed by changes in optical density ($> 10\%$). A decrease in platelet numbers occurred at lower adrenaline concentrations even in those samples in which no recordable aggregation was observed.

In the US samples, there was marginal enhancement of uptake following the addition of adrenaline.

(c) Effect of ADP (Table Id). ADP at a final concentration of 0.03 $\mu mol \cdot l^{-1}$ and 0.05 $\mu mol \cdot l^{-1}$ marginally but significantly increased [^{14}C]histamine uptake (the fall in platelet count at the end of the incubation was similar to that in saline controls, i.e. 5%). Higher concentrations (0.1 $\mu mol \cdot l^{-1}$) of ADP did not significantly enhance uptake, but did cause a concomitant drop in platelet numbers (> 22%). Although most subjects showed appreciable enhancement of histamine uptake, the concentrations of ADP required to show this effect varied substantially between subjects. They were, however, all in the range of 0.02–0.06 $\mu mol \cdot l^{-1}$ ADP.

In the US samples there was no significant change in [^{14}C]histamine uptake following the addition of ADP.

Control experiments

I. Estimation of the amount of residual radioactivity in the platelet pellet after sonication. This experiment confirmed that [^{14}C]histamine was located inside the platelets and that sonication releases the ^{14}C radioactivity, leaving a very small residual amount (< 2.0%) associated with the platelet fragment pellet. Platelet lysis was confirmed by the absence of platelet counts.

II. The effect of using EDTA as an anticoagulant on platelet aggregation, ionised calcium concentration, and [^{14}C]histamine uptake. Platelet counts were significantly greater in samples prepared with EDTA as an anticoagulant than those prepared with citrate. PRP prepared with EDTA did not, however, show any aggregation even when high concentrations of agonists (collagen, adrenaline and ADP) were used (Table II).

In the US samples, the [^{14}C]histamine uptake by platelets in the EDTA-PRP was approximately 20% of that in the platelets prepared in citrate ($n = 4$). In the S samples, the [^{14}C]histamine uptake by the platelets prepared in EDTA was approximately 50% of that in the platelets prepared in citrate ($n = 4$). The median concentration of ionised calcium in the EDTA-prepared PRPs was 0.11 (range: 0.09–0.12) mmol/l and in the citrate-prepared PRPs, 0.09 (0.08–0.1) mmol/l.

TABLE II

The inhibitory effect of EDTA on platelet aggregation

	Aggregating agent dose		
	ADP ($10 \mu mol \cdot l^{-1}$) $n = 6$	Adrenaline ($5 \mu mol \cdot l^{-1}$) $n = 6$	Collagen ($1 mg \cdot l^{-1}$) $n = 6$
PRP prepared in citrate	66 (58–90)	61 (21–90)	71 (56–90)
PRP prepared in EDTA	0	0	0

Figures are median and (range) percentage aggregation.

III. The effect of platelet count in PRP on [^{14}C]histamine uptake. In the S samples there was no significant difference in the rate of [^{14}C]histamine uptake between undiluted PRP (215 fmol histamine/10^8 platelets/min), 1 : 1 diluted PRP (212 fmol histamine/10^8 platelets/min) and 1 : 4 diluted PRP (207 fmol histamine/10^8 platelets/min). In the US samples, there was again no significant difference in the rate of [^{14}C]histamine uptake between undiluted PRP (131 fmol histamine/10^8 platelets/min), 1 : 1 diluted PRP (130 fmol histamine/10^8 platelets/min) and 1 : 4 diluted PRP (137 fmol histamine/10^8 platelets/min).

Discussion

Our experiments demonstrate that platelets take up histamine and that this process is stimulated by adrenaline, ADP and collagen, which are known platelet aggregating agents. It is of great interest that this stimulatory effect of adrenaline, ADP and collagen is exerted at concentrations smaller than those required to induce aggregation of platelets. Clearly, therefore, histamine uptake by platelets is a process which may well occur continuously while the platelet is in circulation. Furthermore, histamine uptake may be stimulated by circulating adrenaline, the release of ADP from aggregating platelets, or at sites of vascular injury where exposure to collagen occurs. The concentrations of adrenaline used in our experiments were of a similar order to those observed in patients with cardiogenic shock [12] (range: 2–63 $nmol \cdot l^{-1}$) or during hypoglycaemia [13] (range: 3.4–4.5 $nmol \cdot l^{-1}$). It is noteworthy that at higher concentrations of adrenaline and collagen, the histamine uptake did not increase, it actually fell. This is probably due to the fact that at these agonist concentrations, the release of histamine from platelets exceeds the uptake. We have observed that at these concentrations, β-thromboglobulin is released by platelets and that platelet counts in PRP fall. Both these indicators point to activation of platelets, with the formation of platelet clumps.

Our ability to demonstrate consistent histamine uptake by platelets at low concentrations of [^{14}C]histamine label (nanomolar vs micromolar quantities used previously) [1], and to stimulate histamine uptake by platelet agonists over shorter periods of incubation (15 or 30 min vs 90 min previously) is due to two main reasons. Firstly, our studies on the uptake of [^{14}C]histamine were carried out in PRP prepared from citrated blood and not in EDTA. This modification is relevant since our results clearly demonstrate that EDTA inhibits platelet aggregation whilst citrate does not. EDTA-induced alteration/damage in platelet function and structure have also been described by others [4]. It is noteworthy that the differences in platelet function in PRP anticoagulated with EDTA or citrate are not dependent upon ionised calcium concentrations since this value was similar with both anticoagulants. Others have also reported that EDTA-induced loss of platelet aggregation could not be reversed by adding calcium [14]. The second reason why [^{14}C]histamine uptake was greater in our experiments was because platelets were constantly stirred. Stirring is necessary for eliciting aggregation of platelets, and we reasoned that other functions of platelets may be dependent on a similar 'trigger'.

Indeed, our experiments show that [^{14}C]histamine uptake is considerably reduced in the absence of stirring.

Having demonstrated enhanced uptake of histamine by activated platelets, it would be of interest to use this methodology to investigate various pathological conditions associated with platelet hyperaggregability (e.g. peripheral vascular disease [15], diabetes mellitus [16], anorexia nervosa [17] and cystic fibrosis [18]).

In conclusion, histamine is taken up by blood platelets and its uptake is stimulated by stirring, adrenaline, ADP and collagen. The method used in this study to obtain these data is simple, rapid and cheap, and provides consistent results.

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References

1 Wood K, Harwood J, Coppen A. Platelet accumulation of histamine in controls, depressed and lithium-treated patients. J Affective Disord 1984;7:149–158.
2 Wood K, Harwood J, Coppen A. Platelet accumulation of histamine in depression. Lancet 1983;2:519–520.
3 Brown MJ, Ind PW, Barnes PJ, Jenner DA, Dollery CT. A sensitive and specific radiometric method for the measurement of plasma histamine in normal individuals. Analyt Biochem 1980;109:142–146.
4 Pidard D, Didry D, Kunicki TJ, Nurden AT. Temperature-dependent effects of EDTA on the membrane glycoprotein IIb-IIIa complex and platelet aggregability. Blood 1986;67:604–611.
5 Born GVR, Cross MJ. The aggregation of blood platelets. J. Physiol 1963;168:178–195.
6 Mikhailidis DP, Mikhailidis AM, Woollard ML, Dandona P. Protection of prostacyclin-like activity in human plasma — a non-enzymatic mechanism. Clin Sci 1982;62:177–181.
7 Mikhailidis DP, Hutton RA, Jeremy JY, Dandona P. Cooling decreases the efficiency of prostaglandin inhibitors of platelet aggregation: effects of possible relevance in cold-induced pathology. Microcirculation 1983;2:413–423.
8 Chattoraj SC, Watts NB. Endocrinology. In: Tietz NW, ed. Textbook of clinical chemistry. Philadelphia: W.B. Saunders, 1986;1144–1145.
9 Hutton RA, Barradas MA, de Albarran R, Dandona, P. Adenine nucleotide metabolism in diabetes. Diabetologia 1984;26:89–90.
10 Mikhailidis DP, Barradas, MA, Maris A, Jeremy JY, Dandona P. Fibrinogen mediated activation of platelet aggregation and thromboxane A_2 release; pathological implications in vascular disease. J Clin Pathol 1985;38:1166–1171.
11 Best LC, Holland TK, Jones PBB, Russell RGG. The interrelationship between thromboxane biosynthesis, aggregation and 5-hydroxytryptamine secretion in human platelets in vivo. Thromb Haemostas 1981;43;38–40.
12 Laing GS, Kumar PS, Frayn KN, Little RA. Cardiac arrest and plasma catecholamines. J Roy Soc Med 1983;76:1080–1081.
13 Horie H, Matsuyama T, Namba M, Itoh H, Nonaka K, Tarui, S, Yamatodani A, Wada H. Response of catecholamines and other counterregulatory hormones to insulin-induced hypoglycaemia in totally pancreatectomized patients. J Clin Endocrinol Metab 1984;59:1193–1196.
14 Zucker MB, Grant RA. Non-reversible loss of platelet aggregability induced by calcium deprivation. Blood 1978;52:505–513.
15 Mikhailidis DP, Barradas MA, Jeremy JY, Gracey L, Wakeling A, Dandona P. Heparin induced platelet aggregation in anorexia nervosa and in severe peripheral vascular disease. Eur J Clin Invest 1985;15:313–319.

16 Banga JD, Sixma JJ. Clinics in haematology, diabetes mellitus, vascular disease and thrombosis. vol. 15. In: Chesterman CN, ed. Philadelphia; W.B. Saunders, 1986:465–492.
17 Mikhailidis DP, Barradas MA, De Souza V, Wakeling A, Dandona, P. Adrenaline-induced hyperaggregability of platelets and enhanced thromboxane release in anorexia nervosa. Prostagl Leukotr Med 1986;24:27–34.
18 Stead RJ, Barradas MA, Mikhailidis DP, Jeremy JY, Hodson ME, Batten JC, Dandona P. Platelet hyperaggregability in cystic fibrosis. Prostagl Leukotr Med 1987;26:91–103.

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3 Winder AF. Factors influencing the variable expression of xanthelasmata and corneal arcus in familial hypercholesterolaemia. In: Bergsma D, Berman ER, eds. Genetic disease of the eye. New York: A.R. Liss, 1982;449–462.

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Histamine Synthesis and Catabolism in Various Tissues in Diabetic Rats

D.S. Gill, C.S. Thompson, and P. Dandona

In view of the observations that (1) plasma histamine concentrations are significantly higher in diabetic patients and diabetic rats than those in controls, and (2) tissue concentrations of histamine are elevated in rats with experimental diabetes, we have investigated histamine synthesis, as reflected by histidine decarboxylase (HDC) activity, and histamine catabolism, as reflected by histaminase activity, in various tissues of the diabetic rat. Rats with streptozotocin-induced diabetes mellitus (DM) showed an increase in histamine synthesis in various tissues; this was most marked in the aorta and to a lesser, but significant, extent in the kidneys, lungs, and heart, but not in the brain, stomach, or skin. Tissue content of histamine was significantly increased in all tissues except the stomach and skin. We conclude that tissue histamine synthesis is significantly increased in diabetic animals and that this increase is most marked in the aorta. The elevation in HDC activity in these tissues probably accounts for the increase in tissue and plasma concentrations of histamine in diabetic animals, since there is no change in histamine catabolism. This increase in histamine synthesis and release may contribute to the pathogenesis of endothelial damage in diabetic microangiopathy and macroangiopathy.

IT IS WELL ESTABLISHED that diabetes mellitus (DM) is an important risk factor for atherosclerotic vascular disease.[1,2] Diabetics show more widespread and more severe atherosclerosis than nondiabetics of similar age, and vascular complications are the main cause of increased mortality among diabetic patients.[3]

Recent work has demonstrated that in experimental DM in the rat, plasma histamine concentrations are markedly elevated and that these changes can be reduced by the administration of α-hydrazinohistidine (αHH; a specific inhibitor of HDC, the enzyme responsible for the biosynthesis of histamine).[4] Others, as well as ourselves, have shown that in experimental DM, an increase in histamine concentration occurs in various tissues, including kidneys,[5] aorta, lungs, and heart.[6] It is believed that an inducible histamine pool is expanded in experimental diabetes.

Changes in histamine metabolism also occur in vascular disease in humans. We have recently shown that there is a marked increase in the histamine content of leucocytes and platelets in peripheral vascular disease (PVD),[7] and that plasma histamine concentrations are elevated in DM and PVD.[8] Other investigators have demonstrated marked increases in the histamine content of arteries of patients with atherosclerosis.[9]

It is thought that increased arterial macromolecular uptake is one of the factors responsible for increased susceptibility to atherosclerosis in diabetics.[10] Inhibition of aortic macromolecule uptake should therefore reduce the incidence and severity of atherosclerosis: this has been shown by different groups in both rat and rabbit. αHH treatment of rabbits with cholesterol-induced atherosclerosis caused a 51% decrease in aortic albumin accumulation and a 63% decrease in severity of atherosclerosis.[11] More recently, Hollis et al have clearly shown that αHH administration to diabetic rats prevents the increase in histamine content and histamine synthesis in aortic endothelial and smooth muscle cells.[12,13] Furthermore, they have demonstrated that administration of αHH also reduces the aortic albumin accumulation that occurs in experimental diabetes.[14]

The present study was undertaken to determine whether the increased histamine content in the various tissues of animals with experimental DM is due to decreased histamine breakdown and/or increased histamine synthesis. Therefore, we measured histaminase and HDC activity in various tissues of diabetic and control animals.

MATERIALS AND METHODS

Materials

Streptozotocin, hog kidney diamine oxidase, horse radish peroxidase type II, 3-(dimethylamino) benzoic acid (DMAB), 3-methyl-2-benzothiazolinone hydrazone hydrochloride monohydrate (MBTH), diethylhexylphosphoric acid, Amberlite CG50, and S-adenosylmethionine chloride were purchased from the Sigma Chemical Company, Poole, Dorset, UK. Multistix were purchased from the Ames Division of Miles Laboratories, Stoke Poges, Berks, UK. Sagatal was purchased from May and Baker, Dagenham, Essex, UK. The YSI 23AM glucose analyser was purchased from Yellow Springs Instruments, Yellow Springs, OH. S-[^{3}H]-adenosylmethionine (500 mCi/mmol [^{3}H]SAM), [ring-2-^{14}C] histamine dihydrochloride (50 mCi/mmol) and L-[2,-5-^{3}H]histidine (50 Ci/mmol) were obtained from the Radiochemical Centre, Amersham, Bucks, UK. Rat Diet 41B was (standard rat chow in the UK) obtained from Grain Harvesters Ltd, Kent, UK.

Methods

Male Sprague Dawley rats (n = 13) weighing between 230 and 240 g were rendered diabetic with a single injection of 0.5 mL streptozotocin (65 mg/kg body weight) in sodium citrate buffer (0.01 mol/L, pH 4.5) into the tail vein. Control animals (n = 14) received a single dose of sodium citrate buffer only. All animals were given food (Diet 41B) and water ad libitum. Three days after injection of streptozotocin, urinary glucose and ketones were measured using enzyme reagent strips (Multistix). All 13 animals developed glycosuria and were diagnosed as having DM. Their blood glucose concentrations were measured at the end of the experiment, 60 days after the detection of glycosuria, and were consistently found to be elevated.

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Tissue Preparations

At the end of the experiment (60 days), the animals were anesthetised with pentobarbitone (Sagatal; 90 mg/kg body weight) and exsanguinated from the heart. The blood was collected in fluoride/oxalate for glucose measurement.

The abdomen was shaved and a 5-cm^2 sample of abdominal skin was cut away, from which the underlying fat was removed. Skin biopsies were obtained using a 5-mm ophthalmic trephine. The heart, whole brain, both kidneys, whole stomach, and lungs were removed. The entire length of the thoracic aorta was also removed and cleared of its periadventitial fat. All tissues were cleaned of fat and capsules and were then quickly washed in ice-cold sodium phosphate buffer (pH 7.4) and immediately stored at −40°C in sodium phosphate buffer (pH 7.4). On the day of assay, all tissues were homogenized at 4°C using a Kinematic Polytron homogenizer.

Assay of Histamine Content

Histamine content in the homogenates was determined by the double isotope radioenzymatic assay of Beavan and Horakova,[15] with modifications of Keeling et al.[16] The method involves the incubation of homogenates with the methyl donor [^{3}H]SAM and histamine methyltransferase (HMT, EC 2.1.1.8). Trace amounts of [^{14}C]histamine are added to correct for extraction and conversion efficiency. Histamine standards were run simultaneously with each assay. Rat kidney HMT was prepared by the method of Shaff and Beavan.[17]

After incubation at 37°C, the assay resulted in the formation of [^{3}H]methylhistamine, which was separated from [^{3}H]SAM by chloroform extraction from an alkaline aqueous phase followed by ion-exchange chromatography using Amberlite CG50 resin (100 to 200 mesh).

Assay of HDC Activity

HDC activity was determined using the coupled HDC assay as described by Keeling et al.[16] The assay involved the incubation of L-[2,5-^{3}H]-histidine, S-adenosylmethionine, pyridoxal-5-phosphate, and HMT preparation with homogenates. The incubation resulted in the formation of [^{3}H]methylhistamine, which was separated by chloroform extraction from an alkaline aqueous phase followed by ion-exchange chromatography using Amberlite CG50 resin (100 to 200 mesh) and finally by liquid ion-exchange chromatography using diethylhexylphosphoric acid.

Protein contents were determined by means of the method described by Lowry,[18] using bovine serum albumin as the standard.

Assay of Histaminase Activity

Histaminase (diamine oxidase, EC 1.4.3.6) activity was assayed by the improved spectrophotometric method of Stoner.[19] The method involves the generation of hydrogen peroxide from histaminase by a coupled enzymatic reaction. In the presence of hydrogen peroxide and peroxidase, the chromagen MBTH is oxidatively coupled to DMAB, forming a purple indamine dye, which has an absorption maximum at 595 nm. Therefore, using hog kidney diamine oxidase as a standard, unknown enzyme activities can be estimated.

Statistical Analysis

Since the data obtained from the above experiments were nonparametrically distributed, they were compared using the Mann-Whitney U test for unpaired data.

RESULTS

Characteristics of the control and diabetic animals are given in Table 1, which shows final body weight, blood glucose, urinary glucose, and urinary ketones. These data show that the diabetic group exhibit marked hyperglycemia and glycosuria with no ketonuria.[20] There was also a severe reduction in weight gain characteristic of streptozotocin-induced diabetes in growing rats.

Table 1. Characteristics of Control and Diabetic Groups

Group	(n)	Blood Glucose (mmol/L)	Final Body Weight (g)	Urinary Glucose	Urinary Ketones
Control	(14)	8.6 (6.9-10.8)	513 (413-543)	−	−
Diabetic	(13)	33.6 (26.1-43.7)	302 (176-349)	+	−

HDC Activity

HDC activity was greatest in the stomach, followed by the aorta, the brain, the lung, the kidneys, and the heart. There was no measurable HDC in the skin, in spite of the very high histamine content in this tissue. Repeated assays of HDC in skin homogenates confirmed the absence of HDC activity.

HDC activity in the aorta, kidney, lung, and heart of diabetic animals was significantly greater than that in controls (Table 2). The percentage increases in HDC activity were as follows: aorta, 119; kidneys, 79; heart, 80; and lungs, 40. There was no significant difference in HDC activity of the brain or stomach when diabetic and control animals were compared.

Histamine Content

The histamine content in the aorta, kidneys, heart, lungs, and whole brain in diabetic animals was significantly greater than that in controls (Table 3). The percentage increases in histamine content were aorta, 78; kidneys, 74; heart, 36; lungs, 42; and whole brain, 32. There were no significant differences in the histamine content of stomach and skin in control and diabetic animals.

Table 2. HDC Activities of Various Tissues From Control and Diabetic Rats

	Control (pmol/min/g protein)	Diabetic (pmol/min/g protein)	Percent Increase	
Aorta	3,500 (2,600-6,300)	7,600 (6,100-11,000)	119	$P < .002$
Heart	10.3 (8.0-11.9)	18.5 (15.7-22.7)	80	$P < .005$
Kidneys	12.7 (11.8-14.3)	22.7 (15.8-26.4)	79	$P < .001$
Lung	79 (60-103)	111 (93-181)	40	$P < .002$
Whole brain	590 (430-710)	610 (530-710)	3	NS
Stomach	10,500 (7,900-17,200)	8,800 (7,200-14,200)	0	NS
Skin	ND	ND	—	—

NOTE. Values are medians (and range).
Abbreviation: ND, not detectable.

Table 3. Histamine Content of Various Tissues From Control and Diabetic Rats

	Control (ng/mg protein)	Diabetic (ng/mg protein)	Percent Increase	
Aorta	361 (311-402)	642 (607-696)	78	$P < .001$
Heart	3.6 (3.2-4.3)	4.9 (4.2-5.4)	36	$P < .005$
Kidneys	0.51 (0.39-0.58)	0.89 (0.70-0.99)	74	$P < .001$
Lung	5.7 (4.3-6.8)	8.1 (6.4-9.7)	42	$P < .01$
Whole brain	0.52 (0.37-0.71)	0.69 (0.59-0.83)	32	$P < .02$
Stomach	2,000 (1,400-2,900)	1,400 (1,100-2,700)	0	NS
Skin	1,960 (1,300-4,100)	1,700 (1,100-3,300)	0	NS

NOTE. Values are medians (and range).

Histaminase Activity

Histaminase activity was greatest in the kidneys, followed by the heart, the skin, the aorta, the stomach, the brain, and the lungs. Experimental diabetes did not alter histaminase activity in any of the tissues examined (Table 4).

DISCUSSION

Our data demonstrate that the aorta, the kidneys, the lungs, and the heart of diabetic rats have a significantly greater HDC activity than that in controls, without any concomitant decrease in histaminase activity. The increase in histamine synthesis in the aorta was the most impressive, as was the increase in aortic histamine content. The increase in histamine content in all tissues except the brain, stomach, and skin was paralleled by an increase in HDC activity. Therefore, it is likely that the increase in histamine content in various tissues in diabetic animals is due to an increase in histamine synthesis, since there is no increase in histamine breakdown. However, previous work by Orlidge and Hollis[21] showed a significant increase in the histaminase activity of aortic endothelial and smooth muscle cells from diabetic rats. It is possible that these changes were masked in our study, since we assayed whole tissue histaminase rather than histaminase activity of specific vascular cells. Furthermore, Orlidge and Hollis[21] have shown that the magnitude of increase in aortic HDC activity in diabetic rats is markedly greater than the magnitude of decrease in histaminase activity. Therefore, any changes in histamine synthesis would be more apparent than changes in histamine breakdown, especially when whole tissue extracts are assayed. The brain of the diabetic animals showed no increase in HDC activity, but it did show a significant increase in histamine content; this may reflect the presence of residual histamine-"rich" plasma and, possibly, cerebrospinal fluid. Since the aorta from diabetic animals showed the greatest increase in HDC activity, the possibility that the increase in HDC activity in other organs is largely contributed to by the presence of blood vessels has to be considered. This is relevant to all tissues investigated except the brain, since the brain had high HDC activity in spite of the fact that it did not alter with diabetes. This fact raises the possibility that a circulating mediator inducing changes in tissue histamine synthesis is not able to cross the blood brain barrier.

Table 4. Histaminase Activity of Various Tissues From Control and Diabetic Rats

	Control (pmol/h/mg protein)	Diabetic (pmol/h/mg protein)
Aorta	13.3 (9.7-15.1)	14.3 (10.6-17.1)
Heart	18.7 (17.3-24.1)	16.6 (15.9-19.3)
Kidneys	22.5 (18.0-24.7)	23.2 (17.6-24.0)
Whole brain	11.8 (10.6-20.0)	11.5 (9.3-15.8)
Lung	ND	ND
Stomach	12.5 (11.4-13.0)	12.9 (11.6-13.4)
Skin	17.3 (13.1-18.9)	18.6 (12.4-29.3)

NOTE. Values are medians (and range). There were no significant differences between control and diabetic rats in any of the tissues examined.

The skin and stomach are the two tissues highly subject to the effects of histamine, and neither showed any changes in histamine metabolism in diabetic rats. It is important to realize that the vast majority of histamine in the stomach and skin of rats is stored in mast cells,[22,23] and it is possible that diabetes does not influence this so-called noninducible pool. Any changes in the blood vessels of the stomach or skin of rats would be diluted by the vast amounts of histamine naturally present in nonvascular tissue. Tissue HDC activity in the brain, the lungs, the stomach, and kidneys of control rats in our experiments were similar to those previously reported.[24,25] However, HDC activity of the heart was different to that reported previously. Yamada et al[25] could detect no HDC activity in the hearts of Sprague-Dawley rats; we consistently observed a small HDC activity in rat hearts. This may be due to differences in assay methodology, since the assay we used is much more sensitive than the o-phtalaldehyde method employed by Yamada.

These increases in HDC activity observed in experimental DM by ourselves and in experimental atherosclerosis by others may also occur in diabetic and atherosclerotic patients, since it has been shown that arteries from patients with atherosclerosis have a higher histamine content than those from normal subjects.[9] Furthermore, we have shown a marked increase in plasma histamine concentrations in patients with DM with a further increase in PVD and there is a concomitant increase in histamine content in the platelets and leucocytes of PVD patients.[7] Interestingly, there were no significant differences between normal and diabetic leukocyte and platelet histamine contents. This may reflect good diabetic control, since it has been shown that insulin therapy to diabetic rats reverses the changes that occur in histamine content in the endothelial and smooth muscle cells in the aorta.[21] This study clearly demonstrates an increase in HDC

activity not only in the aorta and kidneys as previously reported, but also in other organs of diabetic rats. Furthermore, we did not observe a decrease in histaminase activity in the aorta or any other organ in these animals unlike one previous report.[21]

Whatever the mechanism underlying the increase in histamine concentration in plasma or the various tissues in diabetic animals, it is possible that histamine contributes to an increase in the permeability of the endothelium of capillaries and of the tunica intima of larger blood vessels.[26,27] Increased histamine concentrations may also contribute to endothelial contraction and a widening of interendothelial gaps,[28] dissociation of actin cables,[29] an increase in PGI_2 production,[30] increases in endothelial pinocytosis,[31] and an increase in polymorphonuclear leukocyte migration across the endothelium.[32] These changes would promote (1) an increase in the influx of atherogenic macromolecules into the intima of blood vessels and therefore contribute to the pathogenesis of diabetic macroangiopathy; and (2) microangiopathy through increased capillary permeability.

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REFERENCES

1. Jarrett RJ, Keen H, Chakrabarti R: Diabetes, hyperglycaemia and arterial disease, in Complications of Diabetes (ed 2). London, England, Arnold, 1982, pp 179-203

2. Stout RW: Diabetes and atherosclerosis—the role of insulin. Diabetologia 16:141-150, 1979

3. Garcia MJ, McNamara PM, Gordon T, et al: Morbidity and mortality in diabetics in the Framingham population. Sixteen year follow-up study. Diabetes 23:105-111, 1979

4. Hollis TM, Kern JA, Enea NA, et al: Changes in plasma histamine concentration in the streptozotocin-diabetic rat. Exp Mol Pathol 43:90-96, 1985

5. Markle RA, Hollis TM, Cosgarea AJ: Renal histamine increase in streptozotocin-diabetic rat. Exp Mol Pathol 44:21-28, 1986

6. Gill DS, Thompson CS, Dandona P: Increased histamine in plasma and tissues of diabetic rats. Diabetes Res 7:31-34, 1988

7. Gill DS, Barradas MA, Fonseca VA, et al: Increased histamine content in leucocytes and platelets of patients with peripheral vascular disease. Am J Clin Pathol 89:622-626, 1988

8. Gill DS, Barradas MA, Fonseca VA, et al: Plasma histamine concentrations are elevated in patients with diabetes mellitus and peripheral vascular disease. Metabolism 38:243-247, 1989

9. Kalsner S, Richards R: Coronary arteries of cardiac patients are hyperreactive and contain stores of amines: A mechanism for coronary spasm. Science 223:1435-1437, 1984

10. Faggiotto A, Ross R, Harker L: Studies of hypercholesterolemia in the nonhuman primate, part 1. Changes that lead to fatty streak formation. Arteriosclerosis 4:323-340, 1984

11. Owens GK, Hollis TM: Relationship between inhibition of aortic histamine formation, aortic albumin permeability and atherosclerosis. Atherosclerosis 34:365-373, 1979

12. Gallick SG, Orlidge A, Yost JC, et al: Aortic endothelial and smooth muscle histamine pools in streptozotocin-induced diabetes. Fed Proc 40:328, 1981

13. Hollis TM, Gallik SG, Orlidge A, et al: Aortic endothelial and smooth muscle histamine metabolism. Relationship to aortic ^{125}I-albumin accumulation in experimental diabetes. Arteriosclerosis 3:599-606, 1983

14. Hollis TM, Strickberger SA: Inhibition of aortic histamine synthesis by α-hydrazinohistidine inhibits increased aortic albumin accumulation in experimental diabetes in the rat. Diabetologia 28:282-285, 1985

15. Beavan MA, Horakova Z: The enzymatic isotopic assay of histamine, in Rocha é Silva M (ed): Handbook of Experimental Pharmacology, vol XVIII (ed 2). Berlin, FRG, Springer-Verlag, 1978, pp 151-173

16. Keeling DJ, Smith IR, Tipton KF: A coupled assay for histidine decarboxylase: In vivo turnover of this enzyme in mouse brain. Naunyn Schmiedebergs Arch Pharmacol 326:215-221, 1984

17. Shaff RE, Beavan MA: Increased sensitivity of the enzymatic isotopic assay of histamine: Measurement of histamine in plasma and serum. Anal Biochem 94:425-430, 1979

18. Lowry OH, Roseborough NJ, Farr AL, et al: Protein measurement with the folin phenol reagent. J Biol Chem 193:265-275, 1954

19. Stoner P: An improved spectrophotometric assay for histamine and diamine (DAO) activity. Agents Actions 17:5-9, 1985

20. Jeremy JY, Thompson CS, Mikhailidis DP, et al: Experimental diabetes mellitus inhibits prostacyclin synthesis by rat penis: Pathological implications. Diabetologia 28:365-368, 1985

21. Orlidge A, Hollis TM: Aortic endothelial and smooth muscle histamine in experimental diabetes. Arteriosclerosis 2:142-150, 1982

22. Parratt JR, West GB: Tissue histamine and 5-hydroxytryptamine. J Physiol (Lond) 132:40-45, 1956

23. Foley WA, Glick D: Histamine, mast and parietal cells in stomach of rats and effects of cortisone treatment. Gastroenterology 43:425-429, 1962

24. Fisher DZ, Meier E, Fitzpatrick D: Histamine, free histidine decarboxylase, histamine methyltransferase activities in developing rat tissues. Comp Biochem Physiol 68:231-234, 1981

25. Yamada M, Watanabe T, Harino S, et al: The effect of protcese inhibitors on histidine decarboxylase activities and assay of enzyme in various rat tissues. Biochim Biophys Acta 615:458-464, 1980

26. Majno G, Gilmore V, Leventhal M: On the mechanism of vascular leakage caused by histamine-type mediators. A microscopic study in vitro. Circ Res 21:833-847, 1967

27. Killacky JJF, Johnston MG, Morat MG: Increased permeability of microcarrier-cultured endothelial monolayers in response to histamine and thrombin. Am J Pathol 122:50-61, 1986

28. Majno G, Shea SM, Leventhal M: Endothelial contraction induced by histamine-type mediators. An electron microscopic study. J Cell Biol 42:647-672, 1969

29. Welles SL, Shepro D, Hechtman HB: Vasoactive amines modulate actin cable (stress fibres) and surface area in cultured bovine endothelium. J Cell Physiol 123:337-342, 1985

30. Baenziger NL, Fogerty FJ, Mertz LF, et al: Regulation of histamine-mediated prostacyclin synthesis in cultured human vascular endothelial cells. Cell 24:915-923, 1981

31. Orlidge A: Bovine aortic endothelial cells- Histamine metabolism and its relationship to fluid phase endocytosis: Regulation by insulin, histamine receptors and alpha-hydrazinohistidine. PhD thesis, The Pennsylvania State University, Park, PA, 1983

32. Doukas J, Shepro D, Hechtman HB: Vasoctive amines directly modify endothelial cells to affect polymorphonuclear leucocyte diapedesis in vitro. Blood 69:1563-1569, 1987

Plasma Histamine Concentrations are Elevated in Patients With Diabetes Mellitus and Peripheral Vascular Disease

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Previous work has shown that plasma and tissue concentrations of histamine are elevated in rats with experimental diabetes mellitus and that leucocytes and platelets from patients with peripheral vascular disease have a higher histamine content than those from controls. In the present study, we have measured: (a) plasma histamine concentrations; (b) leucocyte and platelet histidine decarboxylase (the enzyme responsible for the biosynthesis of histamine) in patients with diabetes mellitus (Types I and II) and peripheral vascular disease; and (c) platelet and leucocyte histamine content. Plasma histamine concentration was significantly higher in patients with diabetes and peripheral vascular disease respectively than that in age-matched controls. Leucocyte histidine decarboxylase activity in diabetic and peripheral vascular disease patients was similar to that in controls, while platelets had no histidine decarboxylase activity. The leucocyte and platelet content of histamine were greater in patients with peripheral vascular disease than those in controls, but they were not altered in diabetic patients. There was no correlation between plasma histamine concentration, leucocyte and platelet histamine content, and histidine decarboxylase activity. We conclude that plasma histamine is elevated in diabetics and in patients with peripheral vascular disease and that platelet and leucocyte histamine content is increased in the latter. This increase in platelet and leucocyte histamine content is not due to an increase in histidine decarboxylase activity of these cells. The increase in plasma and cellular histamine content may contribute to the pathogenesis of increased endothelial permeability in diabetes and to the pathogenesis of intimal damage in atherosclerosis.

IT IS WIDELY recognized that diabetes mellitus (DM) and hyperlipidemia are important independent risk factors for atherosclerotic vascular disease and for the significantly higher mortality and morbidity rate of these patients.[1,2] Both DM[3] and hypercholesterolemia[4] have been shown to induce an increased permeability of the aorta to plasma macromolecules in both human and experimental animals.

An increase in endothelial permeability in DM has been shown to be associated with a significant increase in the histamine content of the aorta,[5] the plasma,[6] the kidneys,[7] the lung and the heart.[8] Furthermore, the administration of α-hydrazinohistidine (αHH, a specific inhibitor of histidine decarboxylase [HDC], the enzyme responsible for the biosynthesis of histamine) to animals with experimentally induced DM and diet-induced hypercholesterolemia has been shown to prevent the increase in both aortic[4,9] and plasma histamine content[9] as well as aortic vascular permeability to albumin.[5] Such changes in histamine metabolism may also occur in diabetic and atherosclerotic patients. Thus, it has been shown that coronary arteries from patients with ischemic heart disease contain significantly higher concentrations of histamine than those from control patients with no history of cardiac disease.[10] We have recently shown that the histamine content of leucocytes and platelets from patients with peripheral vascular disease (PVD) is significantly higher than that in control subjects.[11]

It is, therefore, possible that in DM and hypercholesterolemia, histamine contributes to the pathogenesis of atherosclerosis by increasing vascular endothelial permeability to large plasma molecules and cellular components of blood. In vitro experiments using human endothelium have also shown that histamine increases vascular permeability via a direct effect on H_1 receptors.[12]

Since the histamine content of leucocytes and platelets in PVD is significantly increased, and since a substantial increase in plasma histamine concentration has been shown to occur in experimental diabetes, we embarked on an investigation of the histamine content and HDC activity in leucocytes and platelets, and plasma histamine concentrations in patients with DM and PVD.

MATERIALS AND METHODS

Materials

Plasma histamine radioimmunoassay (RIA) kits were purchased from Immunotech SA, Marseille, France. S-[^{3}H]-adenosylmethionine (500 mCi/mmol), [ring-2-^{14}C] histamine dihydrochloride (50 mCi/mmol) and L-[2,5-^{3}H] histidine (50 Ci/mmol) were obtained from the Radiochemical Centre (Amersham, Bucks, UK). S-adenosylmethionine was obtained from the Sigma Chemical Company (Poole, Dorset, UK).

Patients and Controls

Four groups of patients and two groups of controls were studied. Controls consisted of two groups of normal subjects. The first group comprised 17 healthy young volunteers (12 men, five women). None of these controls had DM or any history of cardiovascular disease. The median age of the younger controls was 27 years (range 19 to 45 years). None of these volunteers had ingested any drugs for at least 2 weeks before the study.

An older control group was also obtained from a local geriatric day center for the study and consisted of ten volunteers (six men, four women). The median age of this group was 69 years (range 59 to 80 years). None of these volunteers had any known history of cardiovascular disease or DM. Collectively, the entire control population had a median age of 39 years (range 19 to 80 years).

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All patients included in this study were attending the Diabetic or the Vascular clinics at The Royal Free Hospital.

Type I diabetes mellitus. This group consisted of 14 patients (eight men, six women) with insulin-dependent diabetes mellitus (IDDM) with no known complications. The median age of this group was 37 years (range 19 to 63 years). The duration of diabetes ranged between 3 and 40 years.

Type II diabetes mellitus. This group consisted of 14 patients (seven men, seven women) with non–insulin-dependent diabetes mellitus (NIDDM) with no known complications. The median age of this group was 65 years (range 41 to 83 years). The duration of diabetes ranged between 1.5 and 27 years.

Peripheral vascular disease. This group consisted of 13 patients (eight men, five women) with a diagnosis of PVD. The median age of this group was 73 years (range 56 to 85 years). The criteria for inclusion in this group were intermittent claudication for more than 6 months and ankle/arm systolic blood pressure (SBP) ratio <0.85.

Peripheral vascular disease–diabetes mellitus (PVD-DM). This group consisted of 11 patients (eight men, three women) with a diagnosis of PVD with concomitant DM (two IDDM, nine NIDDM). The median age of this group was 68 years (range 56 to 88 years); the duration of diabetes was between 5 and 22 years.

Some patients and controls allowed us just enough blood for plasma histamine measurements. Measurements of their leucocyte/platelet histamine concentrations could not, therefore, be carried out.

Collection and Preparation of Samples

Venous blood was drawn into plastic syringes using an indwelling venous cannula into tubes containing heparin as anticoagulant (2 IU/mL). Plasma was obtained by centrifuging at 1000 g for 15 minutes at 4°C. The plasma was removed well clear of the buffy layer and stored at −70°C until assay. Venous blood was collected for platelets in trisodium citrate, and platelet rich plasma (PRP) was prepared by differential centrifugation at 140 g at room temperature, as previously described.[13] Platelet counts were performed in a Coulter ZM (Coulter Electronics, Luton, Beds, UK). The PRP was centrifuged at 1000 g for 10 minutes at room temperature, and the resulting platelet pellet was washed with 0.9% wt/vol saline and finally resuspended in 0.1 mol/L sodium phosphate buffer, pH 7.4, and frozen at −70°C after ultrasonication for 3 × 10 seconds at an amplitude of 18 μm (Soniprep 150; MSE, Crawley, Sussex, UK). Leucocytes were isolated using the method of Baron and Ahmed[14] whereby leucocytes are separated using density gradient centrifugation followed by hypotonic lysis to remove contaminating erythrocytes. The resulting leucocyte pellets were resuspended in 0.1 mol/L sodium phosphate buffer, pH 7.4, ultrasonicated for 3 × 10 seconds at an amplitude of 20 μm, and stored at −70°C in separate aliquots until assay.

Assay of Histamine Content

Plasma. Plasma histamine concentrations were determined using a sensitive and specific monoclonal antibody RIA for histamine. The assay involves the acylation of histamine by a novel acylating reagent provided by the manufacturers of the kit. The histamine determination makes use of the competition of acylated histamine and ^{125}I acylated histamine for their binding to the mouse monoclonal antibody fixed on tubes. The lower limit of detectability in this modified RIA was 10 pg/mL histamine. The standard curve allowed the measurement of concentrations of histamine between 10 pg/mL and 15 ng/mL. Histamine concentrations of 0.2 ng/mL can consistently be measured by this RIA. Intraassay variation was 7.3% and interassay variation was 9.5% (both on plasma samples and the standards). Intra-individual variation assessed on six healthy volunteers was less than 12%.

Leucocytes and platelets. Histamine contents were determined by the double isotope radioenzymatic method of Beavan and Horakova[15] with the modifications of Keeling et al.[16] This sensitive method involves the incubation of sonicated cell preparation with the methyl donor S-[^{3}H] adenosylmethionine ([^{3}H]-SAM) and rat kidney histamine-N-methyltransferase (HMT; EC 2.1.1.8). Trace amounts of [^{14}C]-histamine were added to correct for extraction and conversion efficiency. Histamine standards were run simultaneously with each assay. Rat kidney HMT was prepared by the method of Shaff and Beavan.[17]

After incubation at 37°C, the reaction between [^{3}H]-SAM, HMT and histamine resulted in the formation of [^{3}H]-methylhistamine, which was then separated from [^{3}H]-SAM by extraction into chloroform from an alkaline aqueous solution. Further separation from [^{3}H]-SAM was achieved by ion-exchange chromatography using Amberlite CG50 ion-exchange resin (Sigma Chemical, Pode, Dorset, UK) as described by Keeling et al.[16] The amount of [^{3}H]-methylhistamine radioactivity present was then determined by liquid scintillation counting in a Rackbeta Scintillation Counter (LKB, Wallac, Sweden). Interassay and intraassay variation were 12% and 5%, respectively.

Assay of HDC

HDC activity was determined using the coupled HDC assay as described by Keeling et al.[16] The assay involves the incubation of L-[2,5-^{3}H] histidine, S-adenosylmethionine, pyridoxal-5-phosphate and HMT preparation with sample material. This incubation resulted in the formation of [^{3}H]-methylhistamine, which was then separated from the [^{3}H] SAM by extraction into chloroform from an

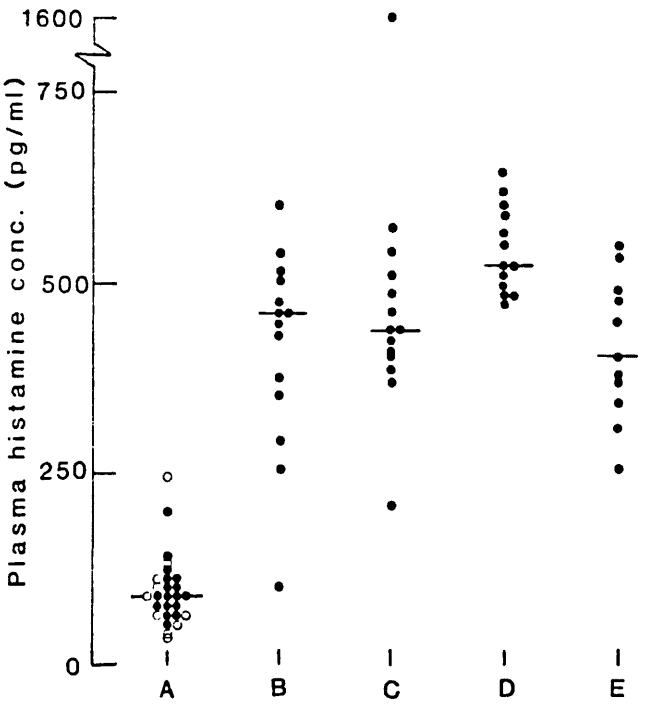

Fig 1. Plasma histamine concentrations. (A) controls: (●) young contols, (○) elderly controls. (B) Insulin-dependent diabetics. (C) Non-insulin-dependent diabetics. (D) Patients with peripheral vascular disease. (E) Patients with peripheral vascular disease and concomitant diabetes mellitus. A *v* B, A *v* C, A *v* D and A *v* E, $P < .002$. All other permutations are nonsignificant.

Table 1. The HDC Activity of Leucocytes in Various Patient Groups

Group	n	HDC Activity (pmol/mg/protein/h)
Controls	11	12.2 (8.4 to 15.8)
IDDM	10	10.1 (8.3 to 17.1)
NIDDM	14	11.2 (10.4 to 18.4)
PVD	8	10.6 (7.1 to 14.6)
PVD-DM	7	11.0 (6.2 to 16.4)

Results are expressed as median and (range). There were no significant differences between any of the groups.

alkaline aqueous phase, followed by ion-exchange chromatography using Amberlite CG50 resin and finally by liquid ion-exchange chromatography using diethylhexyl-phosphoric acid. The intraassay variation was 3% and interassay variation was 13%.

Protein contents were determined by the method of Lowry et al,[18] using bovine serum albumin (BSA) as the standard.

Statistical Analysis

Comparisons among data from various groups were made using nonparametric analyses (Mann-Whitney test, two-tailed).

RESULTS

Histamine concentrations in plasma from patients with PVD (median and [range]: 520 [446 to 650] pg/mL), PVD-DM (400 [256 to 538 pg/mL]) and IDDM (466 [110 to 615] pg/mL) and NIDDM (430 [218 to 1600] pg/mL) were significantly greater ($P < .002$) than those in age-matched control subjects (87 [35 to 247] pg/mL). Patients with PVD and concomitant DM had plasma histamine concentrations similar to those in patients with IDDM and NIDDM. There were no significant differences in plasma histamine levels between IDDM and NIDDM patients, and PVD and PVD-DM patients (Fig 1). There was no age-related change in plasma histamine concentration in the control population. The median plasma histamine concentrations were 87 (range: 55 to 200) pg/mL in the younger controls and 75 (range: 35 to 247) pg/mL in the elderly controls. Most importantly, the median histamine concentration (75 pg/mL) in elderly controls was significantly lower than that in patients with PVD (520 pg/mL) who were of comparable age.

There were no significant differences in leucocyte HDC activity between controls, IDDM, NIDDM, PVD and PVD-DM patients in any combination (see Table 1). There was no detectable HDC activity in the platelets of any of the groups.

The leucocyte and platelet contents of histamine were similar to those previously reported by us.[11] The histamine content of leucocytes from patients with PVD (103 ng/mg protein) was significantly greater than that in controls (72 ng/mg protein), IDDM patients (69 ng/mg protein) and NIDDM patients (87 ng/mg protein) ($P < .01$). Patients with PVD (103 ng/mg protein) and PVD-DM (111 ng/mg protein) had similar leucocyte histamine contents. The leucocytic histamine content in IDDM and NIDDM patients was not significantly different from that in controls (see Table 2).

The histamine content of platelets was significantly greater in PVD patients (11.9 ng/10^9 platelets) than in controls (8.4 ng/10^9 platelets) or in patients with IDDM (8.6 ng/10^9 platelets) or NIDDM (9.2 ng/10^9 platelets) ($P < .02$). There were no significant differences in platelet histamine contents between controls, IDDMs and NIDDMs (see Table 2).

There was no significant correlation between plasma histamine, leucocyte histamine, platelet histamine and leucocyte HDC activity. Furthermore, no correlation between age, duration of DM and/or PVD or severity of PVD (ankle/arm SBP ratio, claudication provoking time) was observed.

DISCUSSION

Our results show conclusively that the increase in plasma histamine concentrations previously observed in experimental diabetes also occurs in patients with DM and PVD. It has been postulated that the increase in plasma histamine observed in experimental DM may be the result of leakage from the markedly increased histamine pool in blood vessels of animals with experimental DM and atherosclerosis.[9] It is, therefore, of interest to note that a significant increase in histamine content has also been observed in the arteries of patients with ischemic heart disease.[10] It is possible that leakage of histamine from a similar expanded vascular pool may contribute to the higher plasma histamine concentrations found in PVD and DM. Since platelets exhibited no HDC activity, whether obtained from patients or controls, the increase in platelet histamine content in diabetics and PVD patients was probably the result of increased uptake of histamine from the plasma.

There was no increase in HDC activities of leucocytes from patients with PVD, DM and PVD-DM. It is thus likely

Table 2. The Histamine Content of Leucocytes and Platelets in Various Patient Groups

Group	n	Leucocyte Histamine Content (ng/mg Protein)	Platelet Histamine Content (ng/10^9 Platelets)
Controls	11	72 (30 to 112)	8.4 (7.2 to 10.1)
IDDM	10	69 (25 to 98)	8.6 (7.9 to 9.7)
NIDDM	14	87 (31 to 108)	9.2 (6.9 to 10.4)
PVD	8	103 (76 to 121)	11.9 (8.3 to 12.6)
PVD-DM	7	111 (73 to 135)	11.2 (9.2 to 12.7)

Results are expressed as median and (range).

Leucocytes: PVD or PVD-DM *v* control, $P < .01$; IDDM or NIDDM *v* control, not significant. Platelets: PVD or PVD-DM *v* control, $P < .02$; IDDM or NIDDM *v* control, not significant.

that the increase in leucocytic histamine content is not the result of increased histamine synthesis and is due either to increased uptake of histamine from the plasma or decreased catabolism or histamine in these cells. A decrease in histaminase activity has been observed previously in the aortic smooth muscle and endothelial cells of diabetic rats.[19] Leucocytes and platelets are also both capable of taking up histamine from the circulation. Human leucocytes, for example, have an uptake system with a vast capacity for increasing intracellular histamine content.[20] We have demonstrated previously that human platelets can take up histamine, and that platelet activation further stimulates increases in histamine uptake.[21]

Plasma concentrations of histamine in humans have been measured previously by sensitive enzymatic methods[22] as well as by RIA.[23] These assay methods demonstrated plasma histamine concentrations of 0.32 ng/mL and 0.19 ng/mL respectively, values which are very similar to those reported by us in both younger and older controls. In the rat, which has been the model for previous studies of this nature, plasma histamine concentrations are nearly 100-fold greater than those found in humans.[6,8]

Patients with PVD have been shown to have markedly hyperactive platelets,[24] while patients with DM (with no complications) are known to have platelets that are less hyperactive.[25] It is, therefore, possible that leucocytes and platelets in PVD have higher histamine contents because they are exposed to higher concentrations of histamine in plasma and because the hyperactive platelets of PVD patients have an increased uptake of this amine. This is reflected in the increased platelet histamine content in PVD and PVD-DM, but not in DM alone.[11]

The implications of increased plasma and blood cell histamine in the pathogenesis of vascular disease are clear. The formation of platelet clumps[26] and/or infiltration and collection of leucocytes[27,28] at sites of vascular injury may result in the release of histamine locally, which can then directly increase the vascular permeability of the endothelium to macromolecules in plasma.[29]

In conclusion, PVD and DM are both associated with a significant increase in plasma histamine concentrations. This increase is likely to be the result of leakage from the markedly increased histamine pool in the blood vessels of these patients. Furthermore, the increase in leucocyte and platelet histamine content previously observed in PVD and DM is not due to increased cellular histamine synthesis and is likely to be the result of histamine uptake from the plasma.

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REFERENCES

1. Jarrett J, Keen H, Chakrabarti R: Diabetes, hyperglycaemia and arterial disease, in Keen H, Jarrett J (eds): Complications of Diabetes. London, Arnold, 1982, pp 179-203

2. Stout RW: Diabetes and atherosclerosis—the role of insulin. Diabetologia 16:1343-1358, 1979

3. Hollis TM, Enea NA, Kern JA: Time-dependent changes in aortic permeability characteristics in experimental diabetes. Exp Mol Pathol 41:207-217, 1984

4. Owens GK, Hollis TM: Relationship between inhibition of aortic histamine formation, aortic albumin permeability and atherosclerosis. Atherosclerosis 34:365-373, 1979

5. Hollis TM, Gallik SG, Orlidge A, et al: Aortic endothelial and smooth muscle histamine metabolism. Relationship to aortic ^{125}I-albumin accumulation in experimental diabetes. Arteriosclerosis 3:599-606, 1983

6. Hollis TM, Kern JA, Enea NA, et al: Changes in plasma histamine concentrations in the streptozotocin-diabetic rat. Exp Mol Pathol 43:90-96, 1985

7. Markle RA, Hollis TM, Cosgarea AJ: Renal histamine increases in the streptozotocin-diabetic rat. Exp Mol Pathol 44:21-28, 1986

8. Gill DS, Thompson CS, Dandona P: Increased histamine in plasma and tissues in diabetic rats. Diabetes Res 7:31-34, 1988

9. Hollis TM, Strickberger SA: Inhibition of aortic histamine synthesis by α-hydrazinohistidine inhibits increased aortic albumin accumulation in experimental diabetes in the rat. Diabetologia 28:282-285, 1985

10. Kalsner S, Richards R: Coronary arteries of cardiac patients are hyperreactive and contain stores of amines: A mechanism for coronary spasm. Science 223:1435-1437, 1984

11. Gill DS, Barradas MA, Fonseca VA, et al: Increased histamine content in leucocytes and platelets of patients with peripheral vascular disease. Am J Clin Pathol 89:622-626, 1988

12. Killachey JJF, Johnston MG, Movat HZ: Increased permeability of microcarrier-cultured monolayer in response to histamine and thrombin. Am J Pathol 122:50-61, 1986

13. Mikhailidis DP, Mikhailidis AM, Woollard ML, et al: Protection of prostacyclin-like activity in human plasma—a non-enzymatic mechanism. Clin Sci 62:177-181, 1982

14. Baron DN, Ahmed SA: Intracellular concentrations of water and of the principal electrolytes determined by analysis of isolated human leucocytes. Clin Sci 37:205-219, 1969

15. Beavan MA, Horakova Z: The enzymatic isotopic assay of histamine, in Rocha e Silva M (ed): Handbook of Experimental Pharmacology, vol 18 (ed 2). Berlin, Springer-Verlag, 1978, p 151-173

16. Keeling DJ, Smith IR, Tipton KF: A coupled assay of histidine decarboxylase: *In vivo* turnover of this enzyme in mouse brain. Naunyn Schmiedebergs Arch Pharmacol 326:215-221, 1984

17. Shaff RE, Beavan MA: Increased sensitivity of the enzymatic isotopic assay of histamine: Measurements of histamine in plasma and serum. Anal Biochem 94:425-430, 1979

18. Lowry OH, Roseborough NT, Farr AL, et al: Protein measurement with the folin phenol reagent. J Biol Chem 193:265-275, 1954

19. Orlidge A, Hollis TM: Aortic endothelial and smooth muscle histamine metabolism in experimental diabetes. Arteriosclerosis 2:142-150, 1982

20. Catini C, Gheri G, Giampaoli M, et al: Histamine uptake by leucocytes in vitro. Basic Appl Histochem 28:329-336, 1984

21. Gill DS, Barradas MA, Mikhailidis DM, et al: Histamine uptake by human platelets. Clin Chim Acta 168:177-185, 1987

22. Dyer J, Warren K, Merlin S, et al: Measurement of plasma histamine: Description of an improved method and normal values. J Allergy Clin Immunol 70:82-87, 1982

23. Stockenhuber F, Sunder-Plassmann G, Balcke P: Increased plasma histamine levels in chronic renal failure. N Engl J Med 317:386, 1987

24. Mikhailidis DP, Barradas MA, Jeremy JY, et al: Heparin-induced platelet aggregation in anorexia nervosa and peripheral vascular disease. Eur J Clin Invest 15:313-319, 1985

25. Peacock I, Hawkins M, Heptinstall S: Platelet behavior in non–insulin-dependent diabetes—influence of vascular complications, treatment and metabolic control. Thromb Haemost 55:361-365, 1986

26. Baumgartner HR, Haudenschild C: Adhesion of platelets to subendothelium. Ann NY Acad Sci 201:22-36, 1972

27. Joris I, Zand T, Nunnari JJ, et al: Studies on the pathogenesis of atherosclerosis: Adhesion and emigration of mononuclear cells in the aorta of hypercholesterolaemic rats. Am J Pathol 113:341-358, 1983

28. Doukas J, Shepro D, Hechtman HB: Vasoactive amines directly modify endothelial cells to affect polymorphonuclear leukocyte diapedesis *in vitro*. Blood 69:1563-1569, 1987

29. Majno G, Gilmore V, Leventhal M: On the mechanism of vascular leakage caused by histamine-type mediators. Cir Res 21:833-847, 1967

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INCREASED HISTAMINE IN PLASMA AND TISSUES IN DIABETIC RATS

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SUMMARY In view of the previous observation that the histamine content of the aorta in rats with streptozotocin-induced diabetes mellitus is increased significantly over that in controls, we have investigated the effect of experimental diabetes on the histamine content of the plasma, the aorta, the kidney, the heart, the lung and the brain. Our data show that the histamine content of the plasma, the aorta, the heart, the kidney, the lung and the brain is significantly greater in diabetic rats than that found in controls. This increase in plasma and tissue histamine content may contribute to the increase in capillary and endothelial permeability known to occur in diabetes, and may have a role to play in the pathogenesis of diabetic micro- and macroangiopathy.

Key words: Histamine, diabetes, rats

INTRODUCTION

DIABETES mellitus (DM) is associated with two types of vascular disease: (a) macrovascular disease (1), which involves large and medium sized arteries and is indistinguishable from atherosclerosis except that it occurs much earlier in diabetics than in normal subjects; and (b) microvascular disease (2), which is specific to DM and is characterised by an increase in capillary permeability.

It is believed that the atherogenic effect of DM is at least partly due to an increase in the permeability of endothelium lining the arteries (3). If, indeed, one of the basic defects in the pathogenesis of diabetic macro- and microangiopathy is increased vascular permeability, the identification of the factor(s) responsible is important. Although no specific permeability-enhancing factors have hitherto been identified, the pioneering work of Hollis and colleagues has demonstrated that plasma concentration and aortic content of histamine in rats with experimental diabetes are significantly elevated (4, 5). Furthermore, the permeability of aortic endothelium, as assessed by the leakage of ^{125}I labelled albumin into subendothelial layers, is increased in diabetic rats (6). Diabetic rats treated with α-hydrazino-histidine (αHH), an inhibitor of histidine decarboxylase (the enzyme responsible for the biosynthesis of histamine) do not show either an increase in plasma (4) or aortic (5) histamine content, or an increase in endothelial permeability (6).

Our present studies are directed towards determining whether the DM-induced increase in histamine content observed in the aorta also occurs in other tissues like the heart, the lungs, the kidneys and the brain.

MATERIALS AND METHODS

13 male Sprague-Dawley rats weighing between 230–240 g were injected intravenously (through the tail vein) with 0·5 ml streptozotocin (65 mg/kg body weight) in sodium citrate buffer (0·01 M, pH 4·5). Control animals (n = 14) received an injection of the vehicle (sodium citrate buffer) only. All animals were allowed free access to food (Diet 41B, Grain Harvesters Limited, Kent, UK) and water. Three days after injection of streptozotocin, urinary glucose and ketones were monitored using enzyme reagent strips (Multistix). Persistent glycosuria was used as an indicator of diabetes; the latter was confirmed by measurement of blood glucose concentration at the end of the experiment 62 days after the detection of glycosuria.

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Plasma and Tissue Histamine Determination

At the end of the experiment, the animals were anaesthetised with pentobarbitone (Sagatal; 90 mg/kg body weight), and exsanguinated from the heart. The blood was collected in heparin and centrifuged; plasma was separated, and frozen at −40°C. The heart, brain, kidneys and lungs were removed. The entire length of the thoracic aorta was also removed, and cleared of its periadventitial fat. All tissues were then quickly washed in ice-cold sodium phosphate buffer (pH 7·8). These tissues were immediately stored at −40°C in 9 volumes of sodium phosphate buffer (pH 7·8). The following day, all tissues were homogenised using an Ultraturax homogeniser (Janke and Kunkel, FRG).

Blood glucose concentrations were measured using a YSI Model 23AM glucose analyser (Yellow Springs Instruments, Yellow Springs, USA).

Plasma urea, potassium, sodium, bicarbonate and chloride were determined using the SMAC autoanalyzer (Technicon Instruments Co. Ltd., Basingstoke, Hants, UK).

Histamine content in the supernatants was determined by the double isotope radioenzymatic method of Beavan and Horakova (7) with modification of Keeling *et al.* (8). This sensitive method involves the incubation of tissue homogenates or plasma with the methyl donor S-[^{3}H] adenosylmethianine (500 mCi/mmol, [^{3}H]-SAM) and rat kidney histamine N-methyltransferase (HMT, EC 2.1.1.8). Trace amounts of [^{14}C]-histamine (50 mCi/mmol) were added to correct for extraction and conversion efficiency. Histamine standards were run simultaneously with each assay. Rat kidney HMT was prepared by the method of Shaff and Beavan (9). The kidneys from 7 male Sprague-Dawley rats were washed and homogenised in ice-cold 0·25 M sucrose. The homogenate was centrifuged at 126,000 × g for 60 min at 4°C, and the pellets discarded. That fraction of the supernatant precipitating between 45 and 70% saturation with ammonium sulphate was resuspended and dialysed at 4°C for 36 h against 3 changes of 2 l of 10 mM sodium phosphate buffer, pH 7·4. After dialysis the HMT prepared was immediately frozen in liquid nitrogen and stored at −40°C.

After incubation at 37°C the reaction between [^{3}H]-SAM, HMT and histamine resulted in the formation of [^{3}H]-methylhistamine, which was then separated from the [^{3}H]-SAM by extraction into chloroform from an alkaline aqueous solution. Further separation from [^{3}H]-SAM was achieved by ion-exchange chromatography using Amberlite CG-50 ion-exchange resin. The amount of [^{3}H]-histamine radioactivity present was then determined by liquid scintillation counting in a Rackbeta Scintillation counter (LKB Wallac, Sweden). Protein contents were determined by using the Lowry method (10) using bovine serum albumin as the standard.

Statistical analysis Data was analysed using the Mann-Whitney test (two-tailed).

Materials

Streptozotocin and bovine serum albumin were purchased from Sigma Chemical Company, Poole, Dorset, UK. Multistix were purchased from the Ames Division of Miles Laboratories, Stoke Poges, Berks., UK. Sagatal was purchased from May and Baker, Dagenham, Essex, UK. S-[^{3}H]-adenosyl methionine and [ring-2-^{14}C] histamine dihydrochloride were purchased from the Radiochemical Centre, Amersham International, Slough, Bucks, UK.

Table 1(a) Characteristics of treatment groups

Group	*n*	*Final body wt. (g)*	*Urinary glucose*	*Urinary ketones*
Control	14	503 (407–557)	negative	negative
Diabetic	13	295* (188–324)	positive	negative

Results

Final body weight and the presence of urinary glucose and ketones are given in Table 1(a). Blood glucose concentrations and plasma urea and electrolyte measurements are given in Table 1(b). These data show that streptozotocin treatment led to a severe reduction in weight gain as well as to marked hyperglycaemia and glycosuria. There was no ketonuria. These observations are consistent with those previously reported for streptozotocin-induced DM (11). The data also show significant changes in plasma urea, sodium and chloride concentrations in diabetic rats with no significant changes in potassium and bicarbonate concentrations.

Histamine content in plasma, kidney, aorta, lungs, heart, and whole brain in diabetic animals was significantly greater than that in controls (Figures 1, 2, 3); however, the magnitude of this increase was different in various tissues. There was a 149% increase in plasma histamine concentration in diabetic rats compared with control rats. This increase in histamine content was also seen in the aorta (93%), kidney (81%), lungs (35%), whole brain (35%) and heart (26%). The histamine content of the aorta was the highest amongst the tissues investigated, whilst that in the brain and kidney was the lowest.

Discussion

Our data have confirmed the observations of Hollis and colleagues that the plasma concentration and the tissue content of histamine in the aorta and the kidney of the diabetic rat are significantly increased. Our observations,

Table 1(b) Biochemical characteristics of treatment groups

Group	*Blood glucose (mmol/l)*	*Urea (mmol/l)*	*K^+ (mmol/l)*	*Na^+ (mmol/l)*	*HCO_3^- (mmol/l)*	*Cl^- (mmol/l)*
Control	8·3 (6·4–10·3)	5·4 (5·1–5·9)	4·6 (3·8–7·7)	142 (141–144)	21·5 (18–25)	103 (100–105)
Diabetic	32·4* (26·5–42·0)	6·9* (6·6–11·1)	5·2 (4·2–6·5)	133* (126–140)	19·5 (14–21)	92·5* (87–96)

* Denotes significant difference from control ($p < 0·01$ Mann Whitney test—two-tailed).

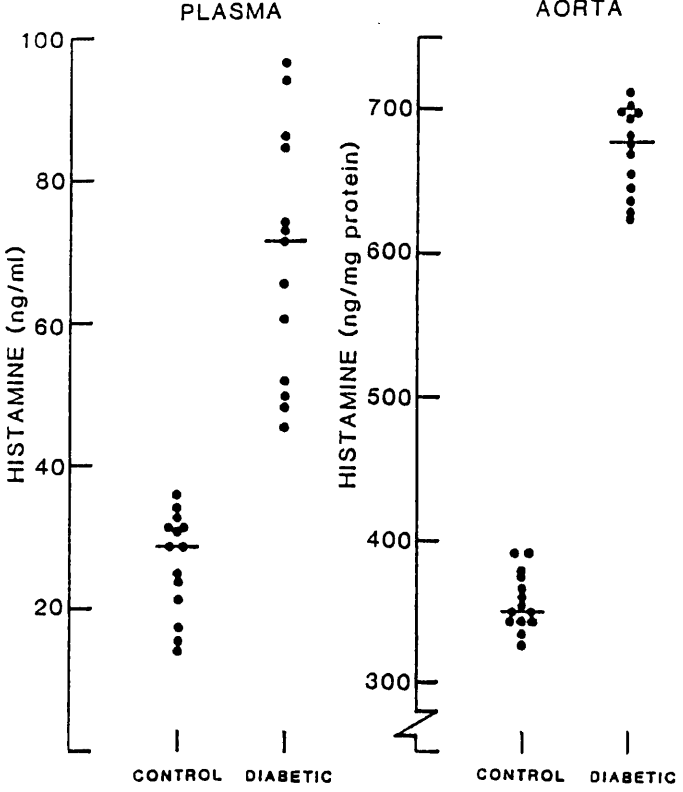

Figure 1. (a) The plasma histamine concentration (ng/ml) of control (n = 14) and streptozotocin diabetic (n = 13) rats. $p < 0.01$ difference from controls. (b) The aortic histamine content (ng/mg protein) of control (n = 14) and streptozotocin diabetic (n = 13) rats. $p < 0.001$ difference from controls.

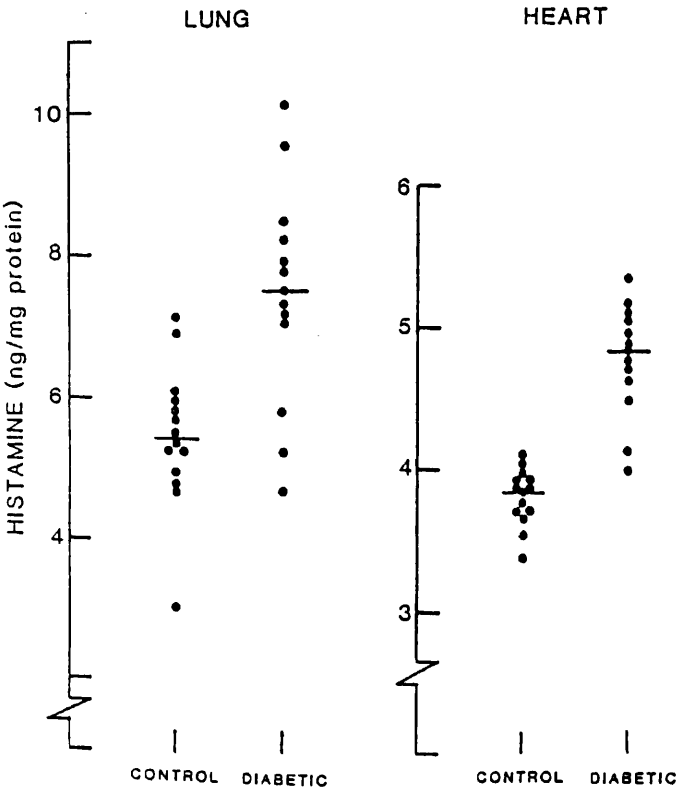

Figure 2. The histamine content (ng/mg protein) of (a) lung and (b) heart of control (n = 14) and streptozotocin diabetic (n = 13) rats. (a) $p < 0.01$ difference from control; (b) $p < 0.001$ difference from control.

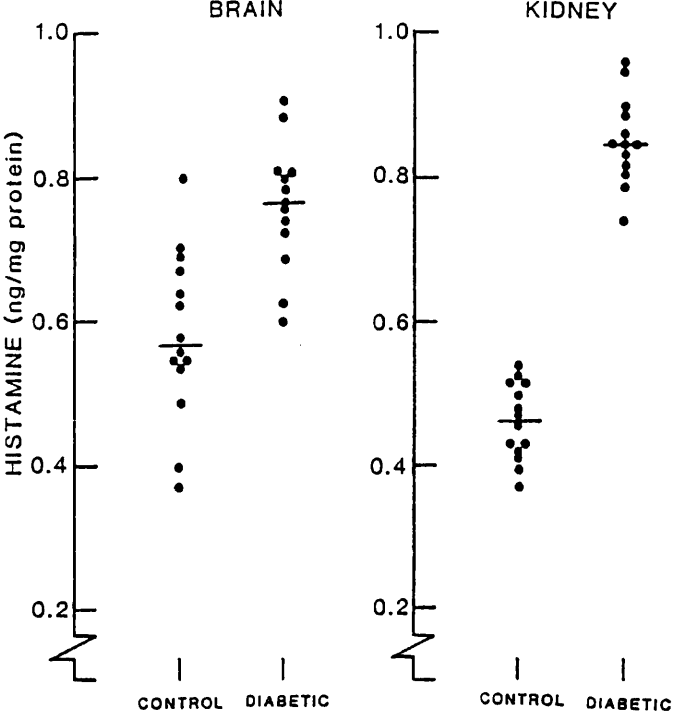

Figure 3. The histamine content (ng/mg protein) of (a) brain and (b) kidney of control (n = 14) and streptozotocin diabetic (n = 13) rats. (a) $p < 0.02$ difference from control; (b) $p < 0.001$ difference from control.

however, extend the previous ones to demonstrate that the histamine content in the lungs, heart and brain are also significantly altered. The increase in plasma and tissue histamine in the diabetic rat may be due either to increased synthesis, decreased catabolism, or both. All tissues investigated by us are known to have histidine decarboxylase, the enzyme responsible for the biosynthesis of histamine. It is therefore possible that the increase of tissue histamine in diabetic rats may be due either to an activation of this enzyme or to an increase in the synthesis of this enzyme. Orlidge and Hollis have shown that histidine decarboxylase action is increased, and that of histaminase is decreased, in aortae of diabetic rats (12).

The fact that the histamine content of all tissues examined was significantly increased suggests that DM has a generalised effect on inducing an increase in histamine. This has important implications in terms of capillary permeability, and may result in the leakage of macromolecules from plasma into tissue fluid. It is therefore of interest that the inhibition of histidine decarboxylase in diabetic rats has been shown to inhibit the increase in aortic histamine content as well as reducing the influx of macromolecules from the plasma into the aorta. We are currently investigating whether the inhibition of this enzyme results in a diminution of histamine content and macromolecule leakage into other organs like the brain, the heart and the lungs.

The increase in plasma histamine concentration is

probably the result of "leakage" of histamine from various organs. Whether the leucocyte population, known to have histidine decarboxylase (13), is also a significant contributor to the increase in plasma pool of histamine in diabetic rats is not clear. Our preliminary observations on human non-insulin-dependent diabetics (NIDD) indicate a significant increase in the content of histamine in leucocytes (14). We have hitherto not studied the histamine content of tissues obtained from diabetic patients.

Experimental diabetes is known to reduce prostacyclin (PGI_2) production in certain tissues like the aorta (15–17), the penis (15) and the brain (18). In contrast, it may enhance PGI_2 production by the urinary bladder (19). The reduction of PGI_2 production in vascular tissues, the aorta in particular, may result in the formation of platelet thrombi on the endothelial surface which may contribute to the pathogenesis of atherosclerosis. Whether histamine has an effect on rat aortic PGI_2 synthesis, and whether diminished PGI_2 synthesis in the aorta has an enhancing effect on aortic histamine synthesis is not known. These issues are the subject of investigations in our laboratory. It is noteworthy, however, that histamine stimulates PGI_2 production by human endothelial cells, but not that by human smooth muscle (20).

In conclusion, the induction of DM in rats results in a significant increase in plasma histamine concentration and tissue histamine content in the aorta, the heart, the lungs, the kidney and the brain. This increase may play a role in the pathogenesis of macro- and microvascular disease in DM. Further investigations into histamine synthesis and its catabolism are necessary to evaluate its specific role in these processes, and the possible use of inhibitors of its synthesis and/or action in the prevention/treatment of diabetic vascular disease.

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REFERENCES

1. Jarrett, R. J., Keen, H. and Chakrabarti, R. (1982). Diabetes, hyperglycaemia and arterial disease. In *Complications of Diabetes* (edited by H. Keen and R. J. Jarrett), pp. 179–203. Edward Arnold, London.
2. Spiro, R. G. (1971). Glycoproteins and diabetic microangiopathy. In *Joslin's Diabetes Mellitus* (edited by A. Marble, P. White, R. F. Bradley and L. P. Krall), pp. 146–156. Lea and Febiger, Philadelphia.
3. Bradley, R. F. and Ramos, E. (1971). The eyes and diabetes. In *Joslin's Diabetes Mellitus* (edited by A. Marble, P. White, R. F. Bradley and L. P. Krall), pp. 478–525. Lea and Febiger, Philadelphia.
4. Hollis, T. M., Kern, J. A., Enea, N. A. and Cosgarea, A. J. (1985). Changes in plasma histamine concentrations in the streptozotocin-diabetic rat. *Exp. Mol. Pathol.*, **43**, 90–96.
5. Gallik, S. G., Orlidge, A., Yost, J. and Hollis, T. M. (1981). Aortic endothelial and smooth muscle histamine pools in streptozotocin diabetes. *Fed. Proc.*, **40**, 328 (Abstract).
6. Hollis, T. M., Gallik, S. G., Orlidge, A. and Yost, J. C. (1983). Aortic endothelial and smooth muscle histamine metabolism. *Arteriosclerosis*, **3**, 599–606.
7. Beavan, M. A. and Horakova, Z. (1978). *Handbook of Experimental Pharmacology, Vol. XVIII* (second edition), (edited by M. Rocha e Silva), p. 151. Springer-Verlag, Berlin.
8. Keeling, D. J., Smith, I. R. and Tipton, K. F. (1984). A coupled assay for histidine decarboxylase: *in vivo* turnover of this enzyme in mouse brain. *Naunyn Schmiedeberg's Arch. Pharmac.*, **326**, 215–221.
9. Shaff, R. E. and Beavan, M. A. (1979). Increased sensitivity of the enzymatic isotopic assay of histamine: measurement of histamine in plasma and serum. *Anal. Biochem.*, **94**, 425–430.
10. Lowry, O. H., Roseborough, N. J., Farr, A. L. and Randall, R. J. (1954). Protein measurement with the folin phenol reagent. *J. Biol. Chem.*, **193**, 265–275.
11. Junod, A., Lambert, A. E., Stauffacher, W. and Renold, A. E. (1969) Diabetogenic action of streptozotocin: relationship of dose to metabolic response. *J. Clin. Invest.*, **48**, 2129–2139.
12. Orlidge, A. and Hollis, T. M. (1982). Aortic endothelial and smooth muscle histamine metabolism in experimental diabetes. *Arteriosclerosis*, **2**, 142–150.
13. Kraus, S., Gilbert, H. S. and Wasserman, L. R. (1968). Leucocyte histidine decarboxylase: properties and activity in myeloproliferative disorders. *Blood*, **31**, 300–306.
14. Gill, D. S., Menon, R. K. and Dandona, P. (1986). Histamine content of leucocytes and platelets in Type II (non-insulin dependent) diabetes mellitus. *Diabetologia*, **29**, 541.
15. Jeremy, J. Y., Thompson, C. S., Mikhailidis, D. P. and Dandona, P. (1985). Experimental diabetes mellitus inhibits prostaglandin synthesis by the rat penis: pathological implications. *Diabetologia*, **28**, 365–368.
16. Jeremy, J. Y., Mikhailidis, D. P. and Dandona, P. (1983). Simulating the diabetic environment alters aortic PGI_2 synthesis *in vitro*. *Diabetes*, **32**, 217–221.
17. Jeremy, J. Y., Thompson, C. S., Mikhailidis, D. P. and Dandona, P. (1987). Diabetes mellitus and fasting cause opposite effects on agonist stimulated PGI_2 synthesis by rat aorta. *Metabolism*, **36**, 616–620.
18. Mikhailidis, D. P., Jeremy, J. Y., Thompson, C. S. and Dandona, P. (1986). Changes in prostanoid synthesis by the brain of the diabetic rat. *Diabetologia*, **29**, 541.
19. Jeremy, J. Y., Mikhailidis, D. P. and Dandona, P. (1986). Effect of streptozotocin-induced diabetes on PGI_2 synthesis by rat bladder. *J. Urol.*, **135**, 1290–1292.
20. Baenziger, N. L., Fogerty, F. J., Mertz, L. F. and Chernuta, L. F. (1981). Regulation of histamine mediated prostacyclin synthesis in cultured human vascular endothelial cells. *Cell*, **24**, 915–923.

Increased Histamine Content in Leukocytes and Platelets of Patients with Peripheral Vascular Disease

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Since histamine has recently been shown to play an important role in the pathogenesis of atherosclerosis in experimental nonketotic diabetes, and since leukocytes and platelets contain most of the histamine in blood, we have determined the levels of histamine in these cells from patients with peripheral vascular disease (PVD), insulin-dependent diabetes mellitus (IDDM) and non-insulin-dependent diabetes mellitus (NIDDM). The leukocyte and platelet histamine concentration in PVDs was significantly greater than that in controls, IDDMs and NIDDMs. Histamine content of leukocytes and platelets from IDDMs and NIDDMs did not differ from that in control subjects. The higher histamine content of leukocytes and platelets in PVD may lead to a greater release of this amine at sites of vascular endothelial damage. Increased histamine release may increase endothelial permeability and contribute to further vascular injury as observed in experimental models of diabetes and hypercholesterolemia. (Key words: Vascular disease; Diabetes mellitus; Histamine; Leukocytes; Platelets) Am J Clin Pathol 1988;89:622–626

DIABETES MELLITUS and hypercholesterolemia are two major risk factors in the pathogenesis of atherosclerosis.[10,25] Both have been shown to induce an increase in the permeability of the aortic endothelium to macromolecules in experimental animals.[9,24] This increase in permeability of the endothelium has been shown to be associated with a significant increase in the histamine content of the aorta,[20] the plasma[8] and other tissues.[17] Furthermore, the administration of an inhibitor of histidine decarboxylase, α-hydrazinohistidine, to animals with streptozotocin-induced diabetes and diet-induced hypercholesterolemia has been shown to prevent both the increase in aortic histamine content and the increase in aortic vascular permeability to plasma macromolecules.[9,21] It is therefore possible that histamine may contribute to the pathogenesis of atherosclerosis by increasing vascular endothelial permeability to large molecules, including cholesterol and growth factors, and cellular components of blood like the mononuclear cells, which have recently been implicated in the pathogenesis of the initial atherosclerotic lesion.[4,11]

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Since the cellular components of blood, the leukocytes—mononuclear cells in particular—and platelets have been shown to be involved in the pathogenesis of atherosclerosis[22] in the human; and since they too contain histamine, we undertook a study to determine whether the histamine content of leukocytes and platelets is increased in clinical diabetes mellitus and clinical vascular disease. It is relevant to mention that patients with severe peripheral vascular disease have previously been demonstrated to have markedly hyperaggregable platelets,[18] and that high leukocyte counts are known to be associated with increased cardiovascular morbidity and mortality.[26]

Materials and Methods

Materials

Bovine serum albumin was purchased from the Sigma Chemical Company, Poole, Dorset, U.K. S-[^{3}H]-adenosylmethionine and [ring-2-^{14}C] histamine dihydrochloride were purchased from the Radiochemical Centre, Amersham International, Slough, Bucks, U.K.

Patients and Controls

Controls. This group consisted of 15 healthy volunteers (11 male, 4 female) from the research laboratories of the Medical School of The Royal Free Hospital, and 11 informed volunteers (5 male, 6 female) attending various clinics in the hospital. None of the controls had either diabetes mellitus or cardiovascular disease. In order to assess the effect of age on platelet and leukocyte histamine content, we divided the controls into those older than and those younger than age 65 years. The median age of the younger controls was 36 years (range, 25–63 years). The median age of the older controls was 74 years (range, 66–78 years). Collectively, the entire control population had a median age of 44 years (range, 25–78 years).

Group 1. This group consisted of 30 patients (16 male, 14 female) with Type I (insulin-dependent) diabetes mellitus (IDDM). The median age of the group was 46 years (range, 22–72 years). All patients in this group were diagnosed diabetics attending the diabetic clinic at The Royal Free Hospital.

Group 2. This group consisted of 26 patients (17 male, 9 female) with Type II (non-insulin-dependent) diabetes mellitus (NIDDM). The median age of this group was 66 years (range, 47–86 years). All patients in this group were diagnosed diabetics attending the diabetic clinic at The Royal Free Hospital.

Group 3. This group consisted of 23 patients (15 male, 8 female) with a diagnosis of peripheral vascular disease (PVD). The median age of this group was 70 years (range, 50–79 years). The criteria for inclusion in this group were (1) intermittent claudication for more than 6 months with ankle/arm systolic blood pressure (SBP) ratio < 0.85 in both arteries (dorsalis pedis and posterior tibial) of the more symptomatic leg; and (2) pain-free walking time of less than three minutes on a treadmill at 1/10 incline at a speed of 5 km/h. Patients taking vasodilators for claudication were excluded from the study.

Group 4. This group consisted of 12 patients (11 male, 1 female) with a diagnosis of PVD with concomitant diabetes mellitus (3 with IDDM, 9 with NIDDM). The median age of this group was 71 years (range 50–83 years).

Some patients and controls volunteered only enough blood for leukocytes or platelets; hence, the discrepancy between platelet and leukocyte numbers.

Drugs

The majority of control subjects had not ingested any drugs for at least two weeks prior to volunteering. Five elderly controls were on treatment for osteoporosis (calcium supplements). Diabetic patients were on standard treatment regimens with insulin/oral hypoglycemic agents. Patients with hypertension were on treatment with thiazide diuretics and/or nifedipine.

Collection and Preparation of Samples

Venous blood was collected for platelets in trisodium citrate, and platelet-rich plasma (PRP) was prepared by differential centrifugation at 140 *g* (at room temperature), as previously described.[19] Platelet counts were performed in a Coulter ZM® (Coulter Electronic Ltd., Luton, Beds., U.K.). The PRP was centrifuged at 1,000 *g* for ten minutes (room temperature). The platelet pellet was resuspended in an appropriate volume of 0.9% w/v saline and frozen at −20 °C after ultrasonication for 3 × 10 seconds at an amplitude of 18 μm (Soniprep 150, MSE Ltd., Crawley, Sussex, U.K.).

Leukocytes were isolated using the method of Baron and Ahmed[1]: 10 ml of heparinized venous blood was mixed with 4 mL of dextran (6%) in a standard buffer. After sedimentation, a supernatant containing an approximately equal number of leukocytes and erythrocytes was centrifuged and the cell pellet collected. The remaining erythrocytes were removed by a 12 s hypotonic lysis phase, and pure leukocytes collected after a second centrifugation. The leukocytes were then resuspended in 0.1 mol/L sodium phosphate buffer, pH 7.4, ultrasonicated for 3 × 10 seconds at an amplitude of 20 μm, and stored at −20 °C until assay.

Assay of Histamine Content

Histamine content was determined by the double isotope radioenzymatic method of Beavan and Horakova[3] with modification of Keeling and associates.[12] This sensitive method involves the incubation of sonicated cell preparation with the methyl donor S-[^{3}H]-adenosylmethianone ([^{3}H]-SAM) (500 mCi/mmol) and rat kidney histamine N-methyltransferase (HMT) (EC 2.1.1.8). Trace amounts of [^{14}C]-histamine (50 mCi/mmol) were added to correct for extraction and conversion efficiency. Histamine standards were run simultaneously with each assay. Rat kidney HMT was prepared by the method of Shaff and Beavan.[23] The kidneys from 7 male Sprague-Dawley rats were washed and homogenized in ice-cold 0.25 mol/L sucrose. The homogenate was centrifuged at 126,000 *g* for 60 minutes at 4 °C, and the pellets discarded. That fraction of the supernatant precipitating between 45 and 70% saturation with ammonium sulphate was resuspended and dialyzed at 4 °C for 36 hours against three changes of 2 L of 10 mmol/L sodium phosphate buffer, pH 7.4. After dialysis the HMT prepared was immediately frozen in liquid nitrogen and stored at −40 °C.

After incubation at 37 °C, the reaction between [^{3}H]-SAM, HMT, and histamine resulted in the formation of [^{3}H]-methylhistamine, which was then separated from the [^{3}H]-SAM by extraction into chloroform from an alkaline aqueous solution. Further separation from [^{3}H]-SAM was achieved by ion-exchange chromatography using Amberlite CG-50 ion-exchange resin. The amount of [^{3}H]-methylhistamine radioactivity present was then determined by liquid scintillation counting in a Rackbeta Scintillation® counter (LKB Wallac, Sweden). Protein contents were determined by using the Lowry method[15] using bovine serum albumin as the standard. The histamine content of platelets is expressed in ng/10^9 platelets, while that of leukocytes is expressed as ng/mg protein. We established a correlation between leukocyte counts and protein content in order to compare relative contents per leukocyte with that of platelets. This be-

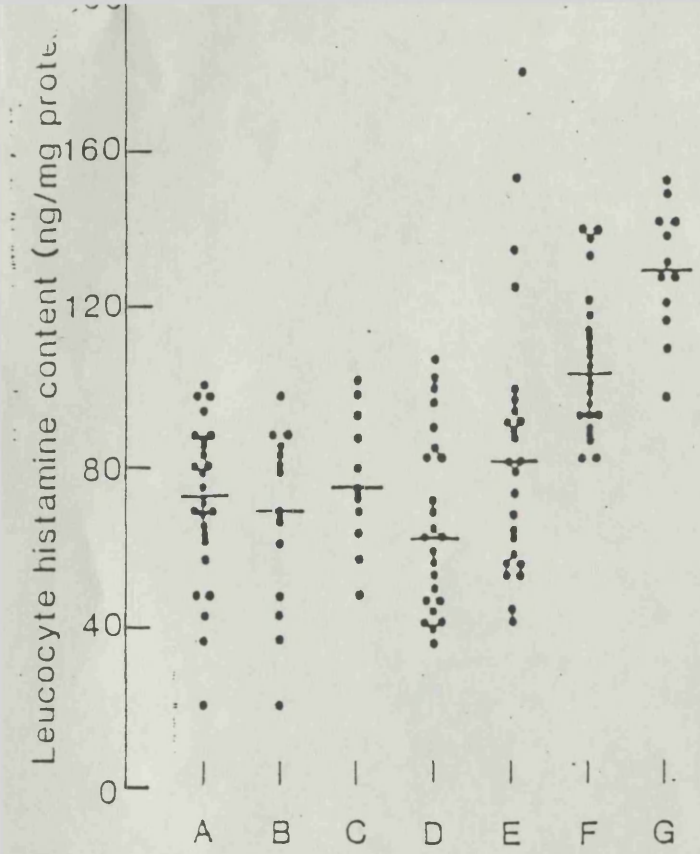

FIG. 1. The histamine content of leukocytes of controls (A), controls younger than age 65 years (B), controls older than age 65 years (C), insulin-dependent diabetics (D), non-insulin-dependent diabetics (E), patients with peripheral vascular disease (F), and peripheral vascular disease patients with concomitant diabetes (G). A *versus* F and A *versus* G, $P < 0.001$; F *versus* G, $P < 0.01$. There were no significant differences between A–E in any comparative permutation.

came necessary because leukocytes tended to clump in the Coulter ZM® used for platelet and leukocyte counts; we therefore avoided the use of this machine for leukocyte counts.

Statistical Analysis

Comparisons between data from various groups were made using the Mann-Whitney test (two-tailed). Correlations were established using linear regression analysis.

Results

Histamine content of leukocytes was much greater than that of platelets. This difference would to some extent be compensated by the greater total number of platelets in blood.

The histamine content of leukocytes from patients with PVD was significantly greater than that in controls, IDDMs and NIDDMs (Fig. 1). Patients with PVD and diabetes mellitus (IDDM or NIDDM) had higher leukocyte histamine content in NIDDM and IDDM patients was not significantly different from that in controls. Some NIDDM patients had leukocyte histamine content comparable to the levels found in patients with PVD and diabetes; these NIDDM patients tended to be elderly and may have occult vascular disease. Leukocyte histamine contents in NIDDM patients older than 65 years were not greater than those in younger NIDDM patients. Leukocytic histamine content in controls older than age 65 years did not differ from that in controls younger than age 65 years.

The histamine content of platelets was significantly greater in patients with PVD than that in controls, IDDMs or NIDDMs (Fig. 2). The concomitant presence of diabetes with PVD did not further affect the histamine content. Histamine content of platelets from IDDMs and NIDDMs did not differ from that in controls, and the histamine content of platelets from control subjects above the age of 65 years was also not signifi-

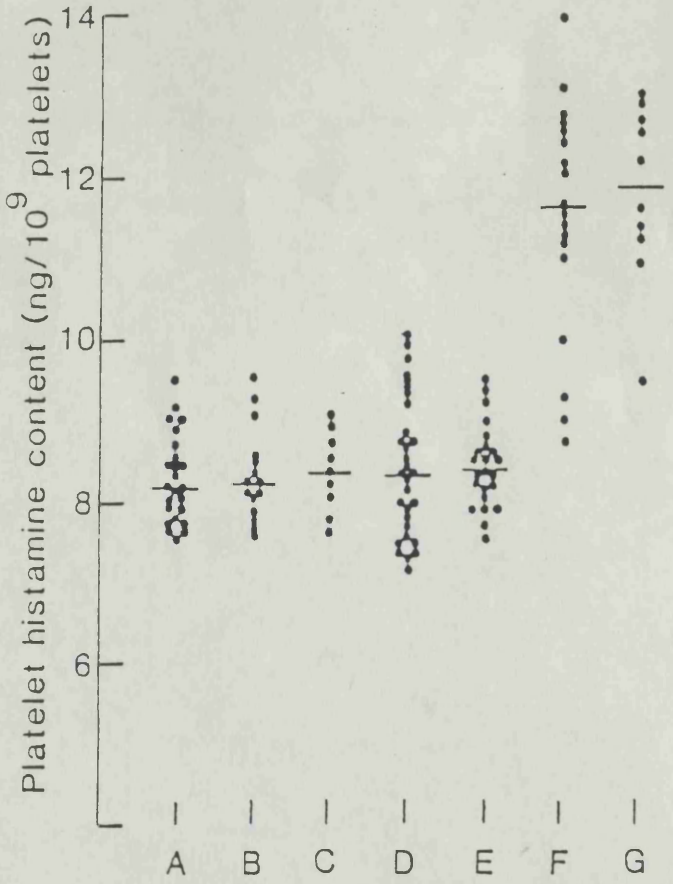

FIG. 2. The histamine content of platelets of controls (A), controls younger than age 65 years (B), controls older than age 65 years (C), insulin-dependent diabetics (D), non-insulin-dependent diabetics (E), patients with peripheral vascular disease (F), and peripheral vascular disease patients with concomitant diabetes (G). A *versus* F and A *versus* G, $P < 0.001$. There were no significant differences between A–E in any comparative permutation.

cantly different from that in controls below the age of 65 years.

There was a highly significant correlation ($r = 0.59$; $P < 0.0001$) between leukocyte and platelet histamine contents of the patients investigated (Fig. 3). Neither platelet nor leukocytic histamine was related to the duration of PVD or the severity of PVD as reflected in ankle–arm systolic pressure ratio, the claudication-provoking time and the maximum distance walked on the treadmill.

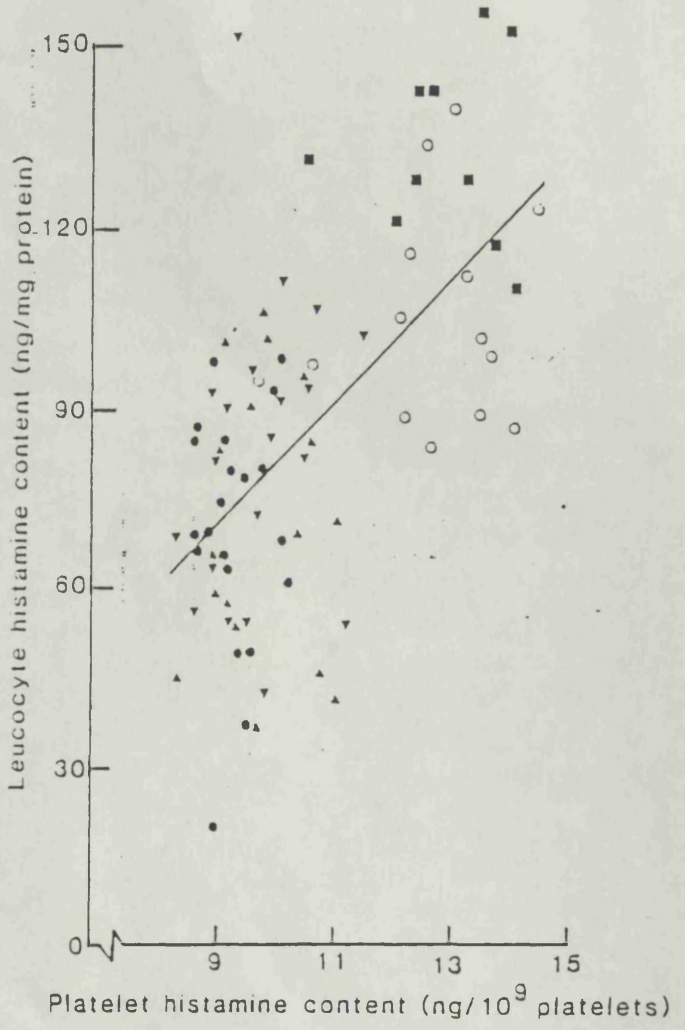

FIG. 3. The correlation between leukocyte and platelet histamine content. Controls (solid circles), insulin-dependent diabetics (solid upper-pointing triangles), non-insulin-dependent diabetics (solid downward-pointing triangles), patients with peripheral vascular disease (open circles), patients with peripheral vascular disease and diabetes (solid boxes). $r = 0.59$; $P < 0.0001$.

Discussion

Our results show clearly that the histamine content of leukocytes and platelets in patients with PVD is significantly greater than that in control subjects. Age did not have any effect on the content of histamine in these cells. Neither IDDM nor NIDDM were associated with an increase in histamine content in these cells, but some patients with NIDDM had leukocyte histamine contents which were comparable to those in patients with PVD. These patients with NIDDM were elderly, and may therefore have atherosclerosis at a subclinical level.

Data on the histamine content of leukocytes and platelets are qualitatively comparable and there was a highly significant correlation between platelets and leukocyte histamine content. Leukocytes have histidine decarboxylase,[7] the enzyme which is responsible for the synthesis of histamine, whereas information on the presence of this enzyme in human platelets is not available. Leukocytic histamine can therefore be produced by the leukocytes themselves while that in platelets may well represent the histamine taken up from plasma. Platelets have a system for histamine uptake which can be stimulated by platelet activation. Conventional aggregating agents like adrenaline, collagen and adenosine diphosphate at subaggregatory doses have recently been shown by us to stimulate the uptake of histamine by platelets.[5] It is possible that the activated, hyperaggregable platelets found in patients with PVD[18] take up histamine more avidly and therefore their histamine content increases.

The mechanism underlying the increased histamine content of leukocytes in PVD is not clear. Whether it reflects increased synthesis, decreased catabolism or even increased uptake has yet to be determined. The other issue which requires further elucidation is whether all leukocytes or only those abundant in histamine (basophils and eosinophils) are involved in these increases in histamine.

The potential implications of increased histamine in leukocytes and platelets regarding the pathogenesis of atherosclerosis are obvious. The formation of a mural thrombus with platelets would cause the release of histamine locally,[2] as would a collection of leukocytes at an injured site. The increased release of histamine at these sites would result in increased endothelial permeability and permit the entry of macromolecules into the intimal layer of the arteries.[16]

Histamine is thought to exist in at least three distinct pools[14]: (1) the tissue non-mast cell 'inducible' histamine pool; (2) the tissue mast cell-bound histamine pool; and (3) the blood-borne histamine pool. The 'inducible' non-mast cell histamine pool in tissues is variable in its content and is dependent upon the rate of histamine synthesis and catabolism.[13] It is postulated that this inducible pool is expanded in experimental diabetes.[9] This was confirmed by the previous observation of Hollis and co-workers[8] and our own more recent observations showing an increase in the histamine content and synthesis in tissues of rats with experimental diabetes.[6] However, there was no increase in the histamine content of platelets and leukocytes of diabetic pa-

ients, indicating no induction of histidine decarboxylase in the blood-borne pool in these patients.

The absence of a correlation between the histamine content of leukocytes and platelets with the clinical severity or duration of vascular disease suggests that there are other factors responsible for causing vascular damage, and that histamine may be only one of them.

In conclusion, PVD due to atherosclerosis is associated with a significant increase in the histamine content of leukocytes and platelets. The association of diabetes with PVD causes a further increase in the histamine content of leukocytes. It is possible that increased histamine content of platelets and leukocytes may play a role in the pathogenesis of endothelial vascular injury in atherosclerotic patients.

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References

1. Baron DN, Ahmed SA: Intracellular concentrations of water and of the principal electrolytes determined by analysis of isolated human leucocytes. Clin Sci 1969;37:205–219.
2. Baumgartner HR, Haudenschild C: Adhesion of platelets to subendothelium. Ann NY Acad Sci 1972;201:22–36.
3. Beavan MA, Horakova Z: Handbook of experimental pharmacology, vol. XVIII, second edition. Edited by M Rocha e Silva. Berlin, Springer-Verlag, 1978, p 151.
4. Gerrity RG: The role of the monocyte in atherogenesis. Am J Pathol 1981;103:181–190.
5. Gill DS, Barradas MA, Mikhailidis DP, Dandona P: Histamine uptake by human platelets. Clin Chim Acta 1987;168:177–185.
6. Gill DS, Thompson CS, Dandona P: Increased histamine in plasma and tissues in diabetic rats. Diabetes Res 1988 (in press).
7. Grzanna R: Histidine decarboxylase: Isolation and molecular characteristics. Neurochem Res 1984;9:993–1009.
8. Hollis TM, Kern JA, Enea NA, Cosgeara AJ: Changes in plasma histamine concentration in the streptozotocin-diabetic rat. Exp Mol Pathol 1985;43:90–96.
9. Hollis TM, Strickberger SA: Inhibition of aortic histamine synthesis by α-hydrazinohistidine inhibits increased aortic albumin accumulation in experimental diabetes in the rat. Diabetologia 1985;28:282–285.
10. Jarrett RJ, Keen H, Chakrabarti R: Diabetes, hyperglycaemia and arterial disease. Complications of diabetes. Edited by H Keen, RJ Jarrett. London, Edward Arnold, 1982, pp 179–203.
11. Jons I, Zand T, Nunnari JJ, Krolikowski JF, Maino G: Studies on the pathogenesis of atherosclerosis. Am J Pathol 1983;113:341–358.
12. Keeling DJ, Smith IR, Tipton KF: A Coupled assay for histidine decarboxylase: in vivo turnover of this enzyme in mouse brain. Naunyn-Shmiedeberg's Arch Pharmac 1984;326:215–221.
13. Levine RJ, Noll WW: Histidine decarboxylase and its inhibition. Ann NY Acad Sci, 1969;166:246–256.
14. Levine RJ, Sato TL, Stoerdsma A: Inhibition of histamine synthesis in the rat by α-hydrazino analog of histidine and 4-bromo-3-hydroxy benzyloxyamine. Biochem Pharmacol 1965;14:139–149.
15. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ: Protein measurement with the folin phenol reagent. J Biol Chem 1954;193:265–275.
16. Maino G, Gilmore V, Leventhal M: On the mechanism of vascular leakage caused by histamine-type mediators. Circulation Research 1967;21:833–847.
17. Markle RA, Hollis TM, Cosgarea AJ: Renal histamine increases in the streptozotocin-diabetic rat. Exp Mol Pathol 1986;44:21–28.
18. Mikhailidis DP, Barradas MA, Jeremy JY, Gracey L, Wakeling A, Dandona P: Heparin-induced platelet aggregation in anorexia nervosa and peripheral vascular disease. Europ J Clin Invest 1985;15:313–319.
19. Mikhailidis DP, Mikhailidis AM, Woollard MH, Dandona P: Protection of prostacyclin-like activity in human plasma: A non-enzymatic mechanism. Clin Sci 1982;62:177–181.
20. Orlidge A, Hollis TM: Aortic endothelial and smooth muscle histamine metabolism in experimental diabetes. Arteriosclerosis 1982;2:142–150.
21. Owen GK, Hollis TM: Relationship between inhibition of aortic histamine formation, aortic albumin permeability and atherosclerosis. Atherosclerosis 1979;34:365–373.
22. Ross R: The pathogenesis of atherosclerosis: An update. N Engl J Med 1986;314:488–500.
23. Shaff RE, Beavan MA: Increased sensitivity of the enzymatic isotopic assay of histamine: measurement of histamine in plasma and serum. Anal Biochem 1979;94:425–430.
24. Stefanovitch V, Gore I: Cholesterol diet and permeability of rabbit aortas. Exp Mol Pathol 1971;14:20–27.
25. Stout RW: Diabetes and atherosclerosis: The role of insulin. Diabetologia 1979;16:141–150.
26. Zalokas JB, Richard JL, Claude JR: Leucocyte count, smoking and myocardial infarction. N Engl J Med 1981;304:465–468.