SMALL-FIBRE STUDIES IN DIABETIC NEUROPATHY

David Michael Levy

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to the memory of
Samuel Levy
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Abstract

Distal small fibre studies in diabetes have been hindered by methodological problems. Psychophysical thermal thresholds have high variability and may not measure pure small fibre function, and most methods for measuring sympathetic function are complex and not quantitative. Three simple techniques for quantitating sympathetically-mediated sweating have been developed: computerised counts of pilocarpine-activated sweatspots, dynamic sweat responses to acetylcholine, and measurement of the sympathetic skin response, in order (a) to establish the frequency of sweating dysfunction; (b) to address the question of the length dependence of diabetic neuropathy; and (c) to compare sweat function with other peripheral nerve measurements. 14–20% of randomly-selected diabetic patients had diminished sweat-gland activation after cholinergic stimulation and abnormal sympathetic skin responses, many of whom had other evidence of neuropathy. Other neurological tests were as frequently abnormal, and had similar intraindividual coefficients of variation, though thermal thresholds were more frequently abnormal (30%). Approximately 5% had increased sweatspot density and acetylcholine-evoked sweat secretion, suggesting denervation supersensitivity. Some of these patients also had autonomic and somatic nerve dysfunction. Dynamic infrared pupillometry confirmed that proximal small-fibre function is less frequently abnormal than distal small-fibre measurements.

The function and structure of neuropeptide-immunoreactive cutaneous sensory and autonomic nerves were investigated in two studies. Neuropathic patients showed supersensitivity to intradermal methacholine, and these responses were decreased by the addition of substance P (SP) and vasoactive intestinal polypeptide (VIP). The effect was more marked with VIP in non-neuropathic, compared with normal and neuropathic subjects. This result may be due to upgrading of sweat gland VIP receptors. Quantitative immunohistochemistry of lower limb skin biopsies confirmed depletion of epidermal nerves immunoreactive for PGP 9.5 and the neuropeptide calcitonin gene-related peptide (CGRP) in neuropathic patients. Non-statistically significant depletion was found in specimens from non-neuropathic patients. Some diabetic subjects had higher dermal and sweat gland immunoreactivity than expected. Nerve proliferation may therefore occur in diabetes, and may contribute to the structural basis for the enhanced functional sweat gland responses.
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**Ethics Committee approval**

All studies were approved by the Ethics Committees of the Central Middlesex Hospital and Parkside Health Authority. Informed consent was given by all subjects for all the studies.
Some of the work contained in this Thesis has been presented in the following publications:


*In press:*


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1 Small fibre abnormalities in diabetes

1.1 What constitutes a "small fibre"?

Research on the properties of unmyelinated nerve fibres has progressed much more slowly than that on myelinated fibres, largely on account of their small size, and it was not until 1895 that Tuckett gave the first histological description of unmyelinated fibres (Thomas and Ochoa 1984). Small, lightly-myelinated fibres (Aδ fibres) and unmyelinated fibres are now known to be the most numerous fibres in a mixed nerve, while large myelinated fibres, including α-motor axons and primary sensory afferent fibres, comprise only about 30% (Ochoa and Mair 1969; Jacobs and Love 1985). Unmyelinated fibres form two functionally distinct groups; afferent sensory (C) fibres derived from dorsal root ganglia, the greater proportion, and efferent postganglionic parasympathetic and sympathetic autonomous fibres supplying cutaneous appendages. Although there is a continuum of diameters, fibre analysis of mixed nerves confirms the presence of the two major populations of nerves, reflected in their conduction velocities. The largest motor and sensory fibres conduct most rapidly (between 10 and 60 m/s). Nerve conduction distribution methods can estimate conduction velocities down to approximately 10 m/s (Dorfman et al. 1983), but below this range indirect methods are usually used. For example, using a method for measuring response times to thermal stimuli, Fowler et al. (1988) estimated that afferent fibres conveying cool sensation (largely Aδ fibres) conduct at approximately 4.5 m/s and those conveying warm sensation at about 2.1 m/s; other indirect methods in postganglionic autonomous fibres indicate lower velocities, in the range 1–1.5 m/s (Shahani et al. 1984). Such values are consistent with those derived from microneurographic measurements (Wallin 1988; see section 1.4.2).

The justification for the use of the global term "small fibres" in diabetes to include both afferent (sensory) and efferent (autonomic) nerve fibres has rested on two presuppositions; first, diabetic neuropathy is a length-dependent "dying back" neuropathy, and it is therefore justified to consider both populations of distal unmyelinated nerve fibres together; and second, because of the structure of unmyelinated fibres, they are in some way especially vulnerable to the pathophysiological processes leading to diabetic neuropathy. This view is reflected in some of the classifications of the diabetic neuropathies (for example, those of Thomas and Brown (1987) and of Brown and Asbury (1984)) which are in part at least based on neuropathological distinctions, and include a category ("pseudosyringomyelic" form) where
small fibres are selectively or predominantly affected – in contrast to the more usual topographical groupings (for example, that of Dyck et al. (1987a)). While occasional clinical cases of "pseudosyringomyelic" diabetic neuropathy have been described (Said et al. 1983), instrumental studies show that a truly selective small-fibre form of neuropathy is unusual (see section 1.5), in comparison with, for example, amyloid neuropathy, where the burden of neuropathological damage occurs in unmyelinated fibres, and myelinated fibres are largely preserved (McLeod and Pollard 1984). Nevertheless, small unmyelinated fibres form a well defined group in terms of their origin, electrophysiological properties and functional roles, and these commonalities are widely taken as justification for grouping them together as "small fibres" (Thomas and Ochoa 1984).

1.2 Historical aspects of small-fibre disturbances in diabetic neuropathy

John Rollo, at the end of the 18th century, was probably the first to describe symptoms of small-fibre dysfunction in diabetes when he noted the occurrence of pain, particularly in the legs (Rollo 1798). The first modern description has been attributed to Marchal de Calvi (1864) who suggested that the sciatic pains and areas of peripheral anaesthesia were the result, rather than the cause of, diabetes. Claude Bernard described autonomic symptoms in diabetes, including disturbances of sweating, temperature control of the peripheries and postural hypotension resulting from "piqûre" of the fourth ventricle of the brain (Bernard 1877). Pavy's celebrated account of diabetic peripheral neuritis included descriptions of lancinating pains and their frequent nocturnal exacerbation, and abnormalities of sweating, including one case where sweating was normal on one side of the body, and absent on the other (Pavy 1885). This observation was also later made by Pryce (1893). In 1887, Pavy gave the following vivid description of neuropathic symptoms, presumably referring to type 2 (non-insulin-dependent) diabetic patients, who at the time would have at least survived to develop diabetic neuropathy:

... the number of cases I find to be affected ... is striking. The remark does not apply to young subjects, but to persons beyond the middle period of life. I used to come across persons who complained of pain in the legs, put down as cases of gout or rheumatism; and I took it as simply coincidental to the diabetes, without anything important in it.

... there are usually associated some anaesthesia, and hyperaesthesia, and various forms of paraesthesia. Persons feel as though they were walking on pebbles. The flesh is tender, so that when the leg is grasped it gives pain. This comes oftener in the legs than in the upper extremity; sometimes, however, it affects the upper also. Then there is an aching of the bones. The patient complains of it especially at night, in bed. The condition appears to be due to peripheral neuritis. (Pavy 1887)
Two important papers in the 1930s and 40s established the autonomic nervous system as a source of profound symptoms in diabetic patients. Jordan (1936) described neuritic manifestations of diabetes in 226 patients seen at the Joslin Clinic over a three-year period. He drew attention to the characteristics of pain in diabetic neuropathy, and described definite pupillary abnormalities in approximately 9%, including five with true Argyll-Robertson pupils after exclusion of neurosyphilis, eight with a sluggish response to light, irregular pupils in seven and unequal pupils in five. Later, Rundles reviewed 125 sequential cases of diabetic neuropathy seen in a university hospital between 1936 and 1945 (Rundles 1945). He pointed to the diagnostic confusion with autonomic features of neurosyphilis, particularly Argyll-Robertson-like pupils, and abnormal postural regulation of blood pressure. "Shooting pains" in deep and superficial tissues occurred in more than 25% and were characteristically worse in the cold and at night. About one-quarter of his group of patients had abnormal pupillary signs, including miosis, a sluggish light response, irregularity and inequality of pupillary size, and frank Argyll-Robertson pupils in two subjects. Bowel disturbances, thought to be due to autonomic neuropathy, were very common; 42% of his patients had constipation, frequently refractory. Twenty-one percent had diarrhoea of varying severity; eight percent had almost continuous diarrhoea. Twenty-eight per cent of his male patients had complete impotence and 10% some degree of urinary incontinence.

Evidence of involvement of the autonomic fibres of peripheral nerves had been accumulating during the 1940s (see Guttmann 1940). Rundles described the sweating deficiencies, loss of vasomotor and pilomotor control, dependent oedema and trophic skin changes due to diabetic autonomic neuropathy. Thirteen (10%) of his patients noted decreased or absent sweating in the legs and feet, usually in areas of sensory abnormality; this was paradoxically associated with drenching night sweats in six who were confirmed to be free of infection, including tuberculosis. Anhidrosis of the lower limbs was confirmed in eleven patients with a starch-iodine test (List and Peet 1938a).

Rundles (1945) established the association between diabetic neuropathy, symptomatic orthostatic hypotension and orthostatic tachycardia, though it had been previously described in other neurological conditions. Three of his patients also had substantial but asymptomatic postural hypotension, and he suggested that the essential lesion causing both postural hypotension and Argyll-Robertson pupils lay in the hypothalamus. By measuring skin temperature he found that reflex vasoconstriction and vasodilatation were impaired in neuropathic subjects.
Martin (1953a) described 150 cases at King’s College Hospital with definite symptoms and signs of diabetic neuropathy. Pupillary abnormalities similar to those reported by Rundles occurred in 9% of his patients, though true Argyll-Robertson pupils were present in only two. Like Rundles, Martin attributed peripheral oedema in the absence of other causes to peripheral autonomic dysfunction. Absent sweating in the feet and atrophy of the skin were common. He found no cases of orthostatic hypotension, but there were three instances of “simple” tachycardia, also attributed to autonomic dysfunction. He obtained similar results to Rundles when vasomotor and sudomotor function was measured.

Goodman (1966) defined a symptom complex he named “diabetic anhidrosis”, consisting of the paradoxical association noted by Rundles, that is, excessive perspiration in the upper half of the body with anhidrosis below the waist. This had been noticed previously in non-diabetic subjects undergoing sympathectomy (List and Peet 1938b). Their teleological explanation for this finding was that under conditions of heat stress, adequate thermoregulation could be achieved only if there was a compensatory hyperhidrosis of the normally-innervated parts of the body. Earlier neurologists had described an area of hyperhidrosis below a spinal transection; since this did not occur in diabetic neuropathy, Goodman concluded that the lesion lay in the peripheral nerves. While this accounts for the lower limb anhidrosis, it less easily accounts for the occurrence of night sweats. There have been no experimental studies of this phenomenon.

1.3 Pathology of unmyelinated nerves in diabetes

1.3.1 Morphometric analyses of nerves

Morphological changes in unmyelinated fibres are difficult to assess. Although they are 3–5 times more numerous than myelinated fibres, they are small (0.1–3.0 μm diameter), and electron microscopy is required. The complex changes seen with normal aging have only recently been fully reported (Kanda et al. 1991) and confirm that while total unmyelinated fibre density in sural nerve does not change, more subtle measurements show significant changes. For example, the number of Schwann cell subunits with and without axons and the mean number of Schwann cell profiles per axon increased with age, while the percentage of subunits containing unmyelinated axons and the mean number of axons in single axon-containing Schwann cell subunits decreased.

There have been several studies in diabetic patients. Martin (1953b) examined qualitatively ten peripheral nerves from patients with symptomatic neuropathy. There was a greater
reduction in the number of unmyelinated fibres, compared with the myelinated ones, demonstrated by closely-packed clumps of “naked” axis cylinders, and he suggested that axonal degeneration occurred before reduction in myelin sheaths. Isolated quantitative analyses of small fibre populations in rare neuropathies had been undertaken before the 1970s, but Behse et al. (1975) were the first to investigate unmyelinated fibres in more common peripheral neuropathies. Three of their 45 subjects were known to have diabetic neuropathy, though the changes were similar in all the neuropathies studied. Simple counts of unmyelinated nerve fibres were unchanged in patients compared with controls, because the range of normal values was so wide, and because regenerating fibres frequently replaced lost fibres. The only quantitative abnormality was an increase in the number of empty Schwann cell sub-units. The authors speculated that this was due either to survival of the Schwann cells when their contained axons had degenerated and disappeared, or to Schwann cell proliferation. This was the only feature statistically correlated with a decrease in the number of myelinated fibres; however, in one-third to one-half of the nerves studied there was evidence of regeneration of unmyelinated nerve fibres, demonstrated by an increase in the number of small unmyelinated nerves, usually found in clusters of three or more.

Low et al. (1975) examined the sural nerves of two diabetic patients with an acute onset of severe autonomic dysfunction. The main finding was of active axonal degeneration, affecting predominantly unmyelinated and small myelinated fibres. Brown et al. (1976) studied small fibres in patients with painful diabetic neuropathy and found a reduction in density of small myelinated fibres and an excess of regenerating unmyelinated fibres.

Said et al. (1983) examined sural nerve biopsies from five patients with advanced neuropathic symptoms compatible with widespread small-fibre abnormalities ("pseudosyringomycelic" form). Myelinated fibres showed segmental demyelination and/or remyelination in 16–46% of fibres and axonal degeneration in 4–24%. All specimens showed a decrease in myelinated fibre density compared with a control specimen, but there was also severe depletion of unmyelinated axons, in contrast to Brown’s findings. They concluded that in the "small fibre" variant of diabetic neuropathy unmyelinated and small myelinated fibres are involved before large myelinated fibres.

Shahani et al. (1984) undertook a clinico-pathological study of sural nerve morphometry in various peripheral neuropathies, including diabetes. Three patients who had an absent sympathetic skin response (see section 2.5.4 and Chapter 5) all had decreased unmyelinated fibre density in the sural nerve, while of five patients with a preserved response three had
normal numbers of unmyelinated fibres and two had decreased numbers, though they also had complex and rare neuropathies in association with diabetic neuropathy.

Until recently, there have been no systematic clinico-pathological studies of unmyelinated fibre populations in diabetes; Dyck’s “index of pathology” (Dyck et al. 1985), is closely associated with clinical measurements of small-fibre function, but it is an agglomerate score of teased fibre analysis and morphometry which therefore quantifies predominantly myelinated fibre pathology. Longitudinal studies of changes in myelinated fibre morphology after treatment with the aldose reductase inhibitor Sorbinil have been reported (Sima et al. 1988), but so far there have been no reports of any effects on unmyelinated fibres. Recently, Llewelyn et al. (1991) reported morphometry of myelinated and unmyelinated fibres in patients with either painless or painful neuropathy. Median unmyelinated fibre size and total unmyelinated fibre density were reduced in all patients. Vibration perception thresholds were, as expected, correlated with total number of myelinated fibres. Thermal thresholds correlated with median unmyelinated axon diameter, but not with total unmyelinated fibre numbers, again probably as a result of the prominent regenerative activity found in all specimens, regardless of the presence of pain. The authors concluded that the presence of regenerating fibres does not reliably identify patients with painful symptoms. A recent study (Veves et al. 1992), while confirming decreased in unmyelinated fibre diameter and increased Schwann cell densities, found no association between morphometric measurements and quantitative sensory testing.

There is one report of quantitative unmyelinated fibre morphometry in the vagus nerve of two patients with intractable vomiting as a result of diabetic gastroparesis (Guy et al. 1984). The normal infradiaphragmatic portion of the vagus consists almost solely of unmyelinated fibres (Keen 1966); both specimens contained only about 25% of the control number of unmyelinated fibres. The remaining fibres were of small diameter, and as in the study of Behse et al. (1975) numerous Schwann cell processes not associated with axons were seen. Schwann cell basal laminae were considerably thickened. Endoneurial capillaries were also abnormal, with thickened, reduplicated basal laminae. In summary, the findings in diabetic nerve comprise a generalised decrease in unmyelinated (and myelinated) fibre density, together with a variety of changes seen in normal aging nerve, but to a more marked degree.

Sima and co-workers have reported progressive structural abnormalities in the vagus nerve of the spontaneously diabetic BB rat, suggesting a primary axonopathy of parasympathetic nerves (Yagihashi and Sima 1986; MacEwen and Sima 1987). These are the first studies indicating that structural abnormalities in unmyelinated fibres precede evidence of functional
changes, assessed by decrease in RR interval variation with respiration. Ultrastructural
analysis of the thoracic sympathetic ganglia showed progressive changes in postganglionic
(unmyelinated) fibres, but not the preganglionic fibres (Yagihashi and Sima 1985). Paro et
al. (1989) studied the extrinsic nerves of the bladder in the BB rat, and found that although
there was evidence of marked axonal atrophy in efferent preganglionic fibres, this occurred
after axonal degeneration of afferent sensory myelinated fibres, suggesting that in this
experimental model, large-fibre changes preceded small-fibre abnormalities.

1.3.2 Neuropathology of small fibres in target organs

There have been several studies of unmyelinated fibres in autonomically innervated structures,
using both light and electron microscopy. Smith (1974) found pronounced lymphocytic
infiltration of the ganglia of the oesophageal myenteric plexus in diabetic patients, a process
noted also to occur in autonomic ganglia (Duchen et al. 1980). She attempted to distinguish
parasympathetic and sympathetic fibres with silver staining techniques, and thought that the
former were more frequently affected than the latter, both in the myenteric plexus and in the
extrinsic vagal trunks, though changes in the finer sympathetic fibres may have been more
difficult to detect. Apart from the lymphocytic infiltration, the neurons themselves appeared
morphologically normal. Johnson (1981) described perineurial cell basement membrane
thickening in skin biopsy specimens.

Faerman and his colleagues reported pathological findings in a variety of autonomically-
innervated organs in human diabetes. Skin biopsies were taken from the thigh and forearm,
stained with silver and examined under light microscopy (Faerman et al. 1982). Nine of
eleven subjects had hypo- or anhidrosis, assessed by the starch-iodine test (see section
2.5.1.2). Lower limb biopsies in all patients showed several qualitative abnormalities of the
sympathetic fibres, including hyperargentophilia, beaded or vacuolated thickenings,
argentophilic spherical swellings and fragmentation of fibres. Changes were most marked near
the sweat glands, and were indistinguishable from specimens taken from two non-diabetic
patients who had undergone sympathectomy for peripheral vascular disease. Upper limb
biopsies were all normal. Silver staining, however, cannot distinguish unmyelinated
autonomic from somatic sensory fibres, and the distinction was made on the location of fibres
in the skin. Similar findings were reported in the bladder in nine patients (Faerman et al.
1973) and confirmed by Mastri (1980). All Faerman's patients had clinical evidence of
peripheral neuropathy and absent acetylcholinesterase staining in muscle and nerve. Similar
neuropathological findings occurred also in the myocardium (Faerman et al. 1977), suggesting that abnormal autonomic innervation might explain the occurrence of painless myocardial infarction in diabetes, and in the corpora cavernosa of impotent diabetic patients (Faerman et al. 1974). Lourie and King (1966) examined skin biopsies taken from patients with localised hyperpathia caused by a variety of peripheral nerve lesions. The findings were similar in all cases, consisting of scarring and proliferation of Schwann cells, and loss of myelinated fibres, leaving a preponderance of unmyelinated fibres.

Immunological abnormalities may contribute to pathological changes in small fibres. Round cell infiltration of target organs (Smith 1974; Mastri 1980) suggested involvement of T lymphocytes in autonomic neuropathy. Appenzeller et al. (1965) induced autonomic neuropathy (assessed by absence of reflex vasodilatation to heating), in rabbits immunised with an antigen extracted from autonomic ganglia, but not in those immunized with antigens from other nervous tissue. Half of the animals showed mild perivascular infiltration with chronic inflammatory cells in nervous tissue, suggesting a delayed hypersensitivity reaction. More recently, clinical studies have suggested an association between HLA antigen status and iritis, an unusual and severe manifestation of autonomic neuropathy (Guy et al. 1984b; Gilbey et al. 1986; Gilbey et al. 1988), and Barzilay et al. (1992) found that the presence of the HLA-DR3/4 haplotype conferred an increased risk of cardiovascular autonomic neuropathy, compared with other HLA-DR haplotypes. Sundkvist et al. (1991) found a high prevalence of autonomic nerve antibodies in patients with abnormal cardiovascular autonomic function.

1.3.3 Histochemical studies

Histochemical techniques, developed to reflect the functional as well as the structural state of neurons, have been increasingly used over the past decade. They were originally used to localise "conventional" neurotransmitters, acetylcholine (or acetylcholinesterase) and catecholamines. More recently, peripheral nerves have been found to immunostain for a prodigious variety of peptide substances, themselves postulated to act as neurotransmitters or, more likely, neuromodulators (Bloom and Polak 1983; Kupferman 1991). Substance P (SP) and calcitonin gene-related peptide (CGRP) are the most important peptides found in "sensory" nerves, and are thought to mediate neurogenic inflammation and the microvascular leakage elicited by axon reflexes (Foreman 1987; Fuller et al. 1987), as well as playing a role in nociception. In addition to a transient axon-reflex vascular response, CGRP elicits a long-lasting non-neurologically mediated vasodilatation (Brain et al. 1985; Brain et al. 1986).
There is growing evidence that SP and CGRP may also have trophic functions in tissue maintenance and renewal during normal function (Kruger et al. 1989). Neuropeptides associated with peripheral autonomic innervation include vasoactive intestinal polypeptide (VIP), which is found predominantly in nerves supplying sweat and salivary glands (see section 2.2.4), but also in the vascular innervation, particularly that of the brain (Larsson et al. 1976); and neuropeptide Y, a potent vasoconstrictor, which is also found in perivascular nerves. Co-localisation of neurotransmitters, both conventional and neuropeptide, is now thought to be the rule rather than the exception (Krug 1987), though the functional significance of co-localised transmitters is often obscure (Kupferman 1991). Many other neuropeptides have been localised in immunohistochemical studies of the dermal innervation. Somatostatin, galanin and atrial natriuretic peptide have been found in presumed autonomic nerves (Tainio et al. 1987; Johansson et al. 1985; Johansson et al. 1988; Hökfelt et al. 1977), and other tachykinins, for example neurokinin A, in sensory nerves (Björklund et al. 1986).

Much of the experimental work on neuropeptides in small fibres has been carried out in the gut, which is richly supplied with both conventional and neuropeptide transmitters. After longstanding (5–7 months) streptozotocin diabetes in the rat, Schmidt et al. (1981) found degenerative changes in terminal autonomic axons of the colon and in the extrinsic nerves of the mesentery, but not in controls or animals with islet-cell transplantation. There was a reduction both in the number and intensity of staining of adrenergic and cholinergic terminals. Belai et al. (1985) investigated changes in gut neurotransmitters of the streptozotocin rat after a much shorter duration of diabetes, eight weeks. There was increased intensity and density of VIP-immunoreactive (IR) nerve fibres and cell bodies in the myenteric plexus and circular smooth muscle of the ileum and proximal colon, and a significant increase in total VIP content. Ultrastructurally, the varicosities of VIPergic nerves were markedly enlarged. In contrast, there were no changes in the number or intensity of SP-IR fibres. In the same animal model the same workers found a general reduction in CGRP-IR, intrinsic nerves being affected more than extrinsic nerves (Belai and Burnstock 1987).

Functional differences were studied in 8-week streptozotocin rat ileum (Belai et al. 1987). After electrical stimulation of the gut, there was no reduction in release of acetylcholine, serotonin or substance P. There was not the expected increased release of CGRP or of VIP; the former was explained by the decrease in immunoreactive CGRP, while the latter may have been due to impaired axonal VIP transport, or diminished release at the nerve terminals. In this animal model, therefore, changes are time-dependent and suggest disruption of the
normal co-ordination of gut neuropeptide activity in diabetes (Belai et al. 1988). The factors determining these differential changes with time are not known. There is recent evidence that neuropeptide-IR may not be accompanied by changes in the general innervation. Webster et al. (1991) found that while there was decreased neuropeptide-IR in the perivascular sympathetic neurons of the streptozotocin-diabetic rat, the overall innervation, revealed by immunostaining for the general neuronal marker protein gene product (PGP) 9.5 (Thompson et al. 1983), was unchanged.

There have been no histochemical studies of neurotransmitters in the human diabetic gut, and the functional relevance of the experimental findings to human diabetic gastroenteropathy is not known. However, there are data suggesting that similar processes may occur in the iris of streptozotocin-diabetic rats, where there was an increase in VIP-immunoreactive fibres, but not of neuropeptide Y-, SP- or catecholamine-containing nerves (Crowe and Burnstock 1988). Karanth et al. (1990) found that epidermal and dermal CGRP, and VIP around sweat glands and blood vessels were markedly increased in the lip and footpad after twelve weeks of streptozotocin diabetes in rats, and in contrast to the findings of Webster et al. (1991) these changes were accompanied by increases in the general neuronal marker PGP 9.5.

The reasons for finding increased immunoreactivity in some animal models are not known, but the target-organ derived nerve growth factor (NGF; Levi-Montalcini and Angeletti 1968) may be involved in the nerve regeneration. Ribiero-da-Silva et al. (1991) found increased NGF receptor-like-IR (NGFr-LI) in larger nerves innervating rat skin after sensory denervation, and increased NGFr-LI in degenerating nerves around blood vessels and smooth muscle fibres after sympathetic denervation. Confirmation of the association between increased neuropeptide-IR and NGF will require simultaneous studies of NGF, NGFr-LI, neuropeptides and neuropeptide receptors.

A recent human study in the uncommon painful cutaneous condition of notalgia paraestheticca showed an increase in the number of intraepidermal PGP 9.5-IR fibres, without a concomitant increase in neuropeptide immunoreactivity (Springall et al. 1991), suggesting that proliferation of immunohistochemically undifferentiated terminal small fibres may be responsible in part for the generation of pain.

Cholinergic, adrenergic and VIPergic nerves are all now thought to play an important role in penile erection (Polak et al. 1981; Gu et al. 1983), and VIP-IR is reduced in rats with streptozotocin-induced diabetes (Crowe et al. 1983). Lincoln et al. (1987) performed immunocytochemical and biochemical investigations on cavernous tissue from a group of
patients with diabetic impotence and another group with non-neurological causes of impotence. VIP- and acetylcholinesterase-IR nerves were markedly decreased in the diabetic compared with the non-diabetic group and the noradrenaline content of the corpus cavernosum was significantly decreased in the diabetic subjects; one diabetic patient with no clinical evidence of neuropathy had markedly depleted immunoreactivity.

It is thought that immunohistochemical studies give a qualitative indication of the functional state of nerve terminals, but interpretation is difficult as there may be several processes occurring simultaneously, some leading to increased, others to decreased, immunoreactivity. Levy et al. (1989a) studied neuropeptide distribution in diabetic skin, and found that there was a general, and often profound, decrease in the number and intensity of staining of autonomic (VIP-, NPY-IR) and sensory (CGRP-, SP-IR) fibres, together with a decrease in the total number of nerve fibres staining for the general neuronal marker PGP 9.5. Lindberger et al. (1989) counted epidermal CGRP- and SP-IR fibres, and found that all diabetic subjects, whether neuropathic (defined using Dyck’s neuropathy symptom and disability scores) or not, had decreased numbers of immunoreactive fibres.

1.4 Assessment of small fibre function

1.4.1 Clinical features

Unmyelinated fibres mediate many of the symptoms of diabetic neuropathy. Abnormalities in afferent unmyelinated fibres cause pain, while failure of efferent sympathetic outflow gives rise to orthostatic hypotension and deficient sweating in the peripheries; together they contribute to Charcot neuroarthropathy (Edmonds et al. 1982). There is also a poorly understood connection between pain and the sympathetic nervous system; several causalgic syndromes can be relieved, at least temporarily, by sympathetic blockade (Stanton-Hicks 1989), and painless myocardial infarction in diabetic patients is thought to be due to afferent sympathetic abnormalities (see section 1.3.2). Efferent parasympathetic dysfunction results in gastroparesis and impotence. Finally, an intriguing qualitative sign of defective sympathetic innervation, lack of skin wrinkling on prolonged immersion of the hands in water, has been described (Braham et al. 1979; Clark et al. 1984; Alvarez et al. 1980). Its physiological basis has not been determined.

Few studies have been undertaken to assess symptoms specifically resulting from small-fibre dysfunction. The prevalence of the commonest, impotence, may be as high as 50% of middle-aged diabetic patients (Boulton et al. 1985), compared with less than 5% of age-matched non-
diabetics. When caused by autonomic nerve damage, as opposed to vascular abnormalities, impotence is thought to be the earliest symptom of autonomic neuropathy, and may be an isolated finding (Ewing et al. 1980b). In contrast, gastroparesis and orthostatic hypotension are relatively uncommon, are associated with other neuropathic abnormalities, and, in some series, carry a poor prognosis (Ewing et al. 1980b). Pain syndromes are common in diabetes. A cross-sectional study of a large unselected clinic population of diabetic patients gave a prevalence of typical lower-limb neuropathic pain of 5% (Levy and Abraham, unpublished observations). Reliable subjective assessments of pain can be made using visual analogue scales (Scott and Huskisson 1976), and similar scales have been used for other symptoms. Clinical symptom scores have also been developed, the most notable being the neurological symptom score of Dyck et al. (1987a), though it emphasises symptoms thought to be due to large-fibre, rather than small-fibre, dysfunction.

1.4.2 Microneurography

Much of our current knowledge of efferent sympathetic postganglionic function is derived from microneurography, a technique pioneered by Hagbarth and colleagues in the late 1960s, in which a tungsten microelectrode is inserted distally into a peripheral nerve fascicle (Hagbarth and Vallbo 1968). Impulses can be recorded from the two major components of peripheral sympathetic innervation, supplying skeletal muscle and skin. The former regulates muscle blood flow, and can be identified by bursts of activity (muscle sympathetic activity, MSA) coupled to the cardiac rhythm. Skin sympathetic activity (SSA) consists of sudomotor and vasoconstrictor efferents (Bini et al. 1980). Depending on the recording site and the height of the subject, the onset latency from the start of the burst is 0.5–1.0 s. Reflex latencies can therefore be used as an indirect measurement of peripheral sympathetic conduction velocity (Fagius and Wallin 1980); using these methods, sudomotor fibres have been found to conduct at 1.0–1.4 m/s, vasoconstrictor fibres at about 0.7 m/s (Wallin 1988). There may also be minor pilomotor and vasodilator components. MSA and SSA can be individually recorded from nerves supplying the extremities, suggesting that the two fibre populations are intermingled. At rest, and at normal room temperature, SSA consists of spontaneous, irregular activity, often occurring synchronously with the respiratory rhythm, though synchronicity with the cardiac rhythm has also been described (Bini et al. 1981). Skin blood flow and skin resistance measurements confirm that some bursts of SSA cause simultaneous vasoconstriction and sweating, while others are purely vasoconstrictor. The general independence of the two supplies is confirmed by the finding of increased
vasoconstrictor impulses during cooling, and increased sudomotor impulses during warming. Sudomotor activity, like MSA, may be time-locked to the cardiac cycle, but the significance of this finding is not known (Wallin 1988).

Arousal stimuli, including auditory (Nilsson 1982) and tactile stimulation, regularly evoke a single burst of SSA, as do deep breaths — the neurophysiological correlate of the skin sympathetic response (see section 2.5.4 and Chapter 5). During insulin-induced hypoglycaemia, inhibition of vasoconstrictor SSA usually, but not always, results in increased skin blood flow (Berne and Fagius 1981); MSA, also vasoconstrictor, increases, resulting in decreased muscle blood flow (Fagius et al. 1986); and, most prominent of all, the discharge in sudomotor nerves increases dramatically. Emotional stimuli, such as pressurised mental arithmetic or stressed conversation, usually lead to a longer-lasting increase in SSA which occasionally persists long after the end of the stimulus. In view of these rapidly changing and adapting sympathetic responses, the concepts of basal “sympathetic tone” and of the sympathetic nervous system as a slow-reacting “vegetative” assemblage should now be reconsidered.

1.4.3 Microneurographic studies in peripheral neuropathies

In up to 64% of diabetic patients with clinical evidence of peripheral neuropathy, adequate microneurographic recordings cannot be obtained, whereas recording failures were found in only 19% of subjects with non-diabetic neuropathy (Fagius 1982). Subjects in whom there was a failed recording were more likely to have a low peroneal or median motor conduction velocity, but judging from the graphical plot Fagius provided, there were at least 6 subjects with normal reflex latencies who had subnormal motor conduction velocities (<c.40 m/s). This study also found that sympathetic reflex latency measurements in diabetic subjects were either normal or absent, and never prolonged. The author suggested two explanations for this finding: first, reduced conduction velocity is usually associated with demyelination, a process which clearly does not occur in unmyelinated C-fibres; and second, it was postulated that the disease process caused such rapid functional derangement and conduction failure in individual fibres that a recording would be unlikely to detect any transient phase of reduced conduction. While microneurography is the only method available for the direct measurement of nerve activity in small fibres, it is a specialised and invasive technique, inappropriate for large scale studies, and currently not easily quantifiable.
1.4.4 Thermal and heat pain sensation

Clinical and experimental studies have established that cool sensation is conveyed in lightly myelinated (Aδ) fibres, while warmth (and heat pain) is conveyed in slower-conducting unmyelinated (C) fibres (Konietzny and Hensel 1975; Hallin et al. 1982; Fowler et al. 1988; Yarnitsky and Ochoa 1990), though the receptors involved have not been identified with certainty. Hensel et al. (1974) found that cold spots in the face of the cat were supplied by thinly myelinated fibres that branched close to, and ended in, the basal layer of the epidermis. The nature of mammalian warm receptors, thought to be present in much lower densities than cool receptors, has not been determined (Iggo and Andres 1982), but are probably situated deep in the epidermis or dermis (Light and Perl 1984). Polymodal nociceptors (possibly in part composed of “free” nerve endings) are involved in nociception and respond to heat pain.

Microneurography (see section 1.4.3) is impractical in the clinical situation, and most clinical studies of peripheral small-fibre function have measured detection thresholds of thermal sensation, usually using devices based on psychophysical principles. Rapid and precise control of the temperature of stimulating thermodes can now be achieved with the use of Peltier-controlled elements, first introduced into thermal sensation research by Kenshalo and Scott (1963). They are made of a metal bilayer whose temperature can be alternately raised or lowered by reversing the applied current. Dissipation of excess heat is achieved either by air-cooling through fins attached to the thermode, or by water circulating through the thermode.

Though convenient for routine clinical use, psychophysical methods introduce several poorly quantifiable factors: temporal and spatial summation of thermal stimulation; conduction is measured in a long multineuronal pathway from the peripheral receptor to the cortical levels involved in decision-making, and not only the specific peripheral fibre components; and finally, there are the factors involved in attention and decision-making, broadly termed “central processing”. These may be particularly important in diabetic patients, where cortical function may be affected by changes in blood glucose levels that do not cause overt hypoglycaemic symptoms (Frier 1986), and in whom there is evidence from several studies of central neuropathy (Donald et al. 1981; Cracco et al. 1984; Fedele et al. 1986; Goldsher et al. 1986). There is recent evidence that psychomotor slowing is itself correlated with the degree of peripheral neuropathy (Ryan et al. 1992).

Some factors affecting “central processing” can be controlled by simple measures, such as ensuring patient comfort and normoglycaemia, minimising extraneous sensory input, and limiting the duration of the test so as not to exceed the subject’s attention span. Some authors
have claimed that a “forced-choice, two-alternative” technique, in which the subject is compelled to decide between the presence or absence of a stimulus, or whether one stimulus is warmer (or cooler) than another, is preferable to a “method of limits”, where the subject decides when a continuously-changing stimulus is perceived (Dyck et al. 1978; Jamal et al. 1985a). It is argued that since a forced-choice method incurs less response bias than the method of limits, the results should be more precise and reproducible, but a study by Levy et al. (1989) which compared the two techniques in diabetic neuropathy suggested that the variability of the two methods and their sensitivity in detecting abnormality was comparable in a large group of diabetic patients tested under clinical, rather than laboratory conditions.

Since “central processing” factors cannot be strictly controlled (Fagius and Wahren 1981), much effort has been devoted to controlling peripheral stimulus-related factors. The test site should be constant, as skin thickness and presumably also receptor density varies between sites. The basal thermode temperature should be in the zone of thermal neutrality (30–32°C) (Kenshalo 1977); temperatures above and below this range strongly influence thresholds. Spatial summation of thermal sensation is complex, but Kenshalo (1970) confirmed earlier reports (Lele et al. 1954) that for testing the limbs, a stimulating area of more than 8 cm² yielded consistent thresholds. Where thermal receptor density is thought to be higher, for example on the face, smaller thermodes can be used (Fowler et al. 1987). Thermode pressure has been assumed rather than proven to be important in thermal threshold measurements (Bertelsmann et al. 1985). Jamal et al. (1985a, 1985b) controlled this factor by using the weight of the thermode itself as a constant applied pressure. Where alternating thermodes at different temperatures need to be applied to the same site (Arrezzo et al. 1986; Bertelsmann et al. 1985), this factor is less easy to control. However, under physiological conditions, mechanoreceptors are not activated by thermal stimuli (Konietzny and Hensel 1975), and although in theory increased numbers of thermoreceptors could be stimulated when high thermode pressures were used, the importance of controlling this factor in clinical methods is not known.

Kenshalo (1977) found that the rate of change of thermode temperature was also important. Temporal adaptation to thermal stimuli is complex (Kenshalo and Scott 1966), but in clinical testing thresholds remain constant if the rate of temperature change of the thermode is greater than approximately 1°C/sec. However, when a method of limits is used in clinical testing, thermal thresholds increase as the rate of temperature change increases, as a result of the increased patient response time. This factor accounts for the frequently-noted differences between thermal thresholds measured clinically with the two methods (Kennedy et al. 1989;
Levy et al. 1989b), those using the method of limits being consistently higher. When large numbers of patients are studied, the results obtained with the two methods are statistically weakly correlated (Levy et al. 1989b).

Most methods can be used or modified to measure separate warm and cool thresholds, but their significance is disputed. In the original description of the Marstock method (method of limits) Fruhstorfer et al. (1976) measured a combined threshold by allowing the thermode temperature to oscillate above and below the reference temperature, the subject alternately indicating warm and cool thresholds. Because of differences in methodology and patient selection, claims that measurement of one or other threshold is more sensitive (and that one is an earlier indicator of nerve dysfunction than the other) should be interpreted with caution. Sosenko et al. (1988) adapted the Sensortek thermal tester, a forced-choice, two-alternative method (Arrezzo et al. 1986) to measure separate warm and cool thresholds, and suggested that diabetic subjects have greater warm insensitivity than cool. In our own study (Levy et al. 1989), warm and cool thresholds measured with the Marstock method were closely correlated, and we found, like Jamal et al. (1985b), that the prevalence of abnormal warm and cool thresholds was similar. In smaller groups of patients, Claus et al. (1987) and Hilz et al. (1988) concluded that abnormal cool perception may precede abnormal warm perception. Dyck et al. (1987b) used only cooling thresholds in a well-validated diagnostic neuropathy screen. Standardisation of thermal testing methods and long-term follow-up studies are required before the important question of preferential impairment of cool or warm sensation can be answered.

1.4.5 Pain thresholds

Pain sensation, mediated through C-fibres, is commonly abnormal in diabetes (Morley et al. 1984; Anon 1985; Navarro et al. 1989). Kennedy et al. (1984b) found that the clinical extent of pain deficit in the leg correlated well with abnormal sweating in the feet in diabetic patients. Quantitation of pain thresholds is difficult; clinical algometers, usually measuring pinch-pressure pain, have been described, but reproducibility of the methods and their application in diabetic patients has not been established. Polymodal nociceptors are activated by heat pain, and by increasing the rate of temperature rise of the Marstock thermode, heat pain thresholds can be measured using the method of limits. However, to avoid thermal damage to the skin, conservative automatic cut-out temperatures (around 50°C) have usually been employed. Heat pain thresholds are therefore frequently not detected in neuropathic
subjects (Levy et al. 1989b), and in this study the prevalence of abnormal heat pain thresholds was much lower than that of warm or cool thresholds.

Jamal et al. (1989) modified the Glasgow thermal testing system (Jamal et al. 1985a) to measure late cortical responses to warm, non-painful stimuli. Carbon dioxide laser stimuli have been used to elicit pain, and to record further "ultralate" cortical evoked potentials (Bremm and Treede 1987). Such measurements may prove to be more sensitive and objective than psychophysical methods, as they permit the delivery of known amounts of energy to the skin, and the measurement of cortical potentials obviates the need for decision-making, but they have not, as yet, been used in diabetes.

Measurement of cold pain thresholds, potentially less hazardous than heat pain, have not been of value in diabetic neuropathy (Levy et al. 1989b). Using the Marstock method, we found that many diabetic and normal subjects did not feel pain even at thermode temperatures of 5°C, though Yarnistky and Ochoa (1990) were readily able to measure cold-induced pain sensation in 19 normal subjects (mean threshold 13°C).

Spontaneous pain in the feet in diabetic subjects, which is of a dysaesthetic quality that suggests peripheral small fibre dysfunction, is common, but its cause remains unknown, and there are no reliable objective methods for its assessment other than through the use of patient- or doctor-administered subjective pain scores, often based on visual analogue scales. Morley et al. (1984) found that raised glucose levels depressed pain perception thresholds in normal subjects, and that glucose itself may modulate endogenous opiate receptors (Morley et al. 1981), but this does not localise the origin of the pain in these cases. However, recent evidence (Ross and Varipapa 1989; Tandam et al. 1992) that pain in diabetic neuropathy can be relieved by topical capsaicin, which depletes peripheral sensory C fibre terminals of substance P and calcitonin gene-related peptide, suggests a peripheral site, either pain receptors themselves or their afferent unmyelinated axons.

1.4.6 C nociceptor function

Polymodal nociceptors mediate the effects of direct physical damage, chemical insults and heat pain through axon reflex mechanisms (Foreman 1987), local neuronal circuits composed of afferent and efferent branches of the same C fibre (Clander and Folkow 1953) (see section 2.1.2.3). The effector response usually measured is vasodilatation, as it can be quantitated by measuring the area of flare or its intensity, usually with laser doppler flowmetry. Several recent studies have reported decreased flare responses in diabetic patients
(Aronin et al. 1987; Westerman et al. 1988; Parkhouse and Le Quesne 1988b; Bennaroch and Low 1991). However, flare induced by polymodal nociceptor stimulation is not purely neural; certain agents, for example acetylcholine, act through both neural and endothelium-dependent mechanisms (Saenz de Tejada et al. 1989). Moreover, there is evidence that part of the final vasodilator response is produced by substances such as histamine released from mast cells (Foreman 1987; Church et al. 1989).

Parkhouse and Le Quesne (1988a, 1988b) found that impairment of neurogenic inflammation in diabetic subjects with neuropathic foot lesions paralleled clinical diminution of pain sensation. Neurogenic stimulation of sweat glands has been investigated by Low et al. (1983, 1986) (see section 2.5.2.2), who found a high concordance between absent axon-reflex sweating and cardiac vagal abnormalities. Axon reflex sweating appears to be less easy to elicit than vasodilatation, but may be a more appropriate model for examining the role of peripheral small fibre dysfunction in diabetes.

1.4.7 Studies of the peripheral microvasculature

Sympathetic denervation in neuropathic diabetic patients, causing increased arteriovenous shunting, may result in high capillary blood flow (Edmonds et al. 1982; Flynn et al. 1988). However, there are other factors in diabetes that may affect microvascular responses: these include the mechanical effects of basement membrane thickening, rheological abnormalities, and increased capillary permeability. It is difficult to assess the relative importance of neurological, microvascular and rheological factors in the control of local blood flow in diabetes; all may be involved in causing the reduced hyperaemia that follows skin trauma in diabetic patients (Rayman et al. 1986; Walmsley et al. 1989).

1.4.8 Integrated peripheral vascular responses

The sympathetic vasoconstrictor response to a deep breath is impaired in peripheral neuropathies of various aetiologies, including diabetes (Aminoff 1980). Among the cardiac autonomic tests, the diving reflex, consisting of bradycardia and peripheral vasoconstriction, can be elicited by applying a cold stimulus to the face. The place of this response in the assessment of the sympathetic-vascular smooth muscle pathway has recently been re-evaluated. It has been found to be a repeatable test, which is well-tolerated when carried out under controlled conditions (Heath and Downey 1990). Another recent study (Gilmore et al.
investigated the relationship between vasoconstrictor responses to a deep breath and body cooling, and cardiovascular vagal tests; while there was good concordance between these responses (measured with venous occlusion plethysmography) and the cardiovascular tests, there was a small number of patients with electrophysiological evidence of neuropathy who had normal cardiovascular tests but abnormal vasoconstrictor function, suggesting to the authors that there might be increased diagnostic yield from the use of both tests.

1.4.9 Assessment of proximal small fibre function

1.4.9.1 Cardiac autonomic function

Sharpey-Schafer and Taylor (1960) were the first to quantify abnormal vagal function in diabetic neuropathy, but it was not until the 1970s that measurements of these responses, which are based on changes in heart rate during and after various simple standardised manoeuvres, became practical for routine diagnostic use (Ewing et al. 1973; Ewing 1978). Since then, the methods have been refined and adapted for simple computers. Three tests, heart rate variation with deep breathing (respiratory RR variation), and the heart rate responses to the Valsalva manoeuvre (Valsalva ratio), and to change in posture from lying to standing (30:15 ratio; Bellavere and Ewing 1982) have become established in the evaluation of diabetic neuropathy (Ewing 1990; Ryder and Hardisty 1990). They are among the most sensitive and reproducible of autonomic tests (Schumer et al. 1988). Pharmacological blocking studies have demonstrated that they are mediated predominantly through the vagus (parasympathetic), but power spectral analysis of RR interval variation has identified low frequency components which are abolished by sympathetic blockade (Weise et al. 1988), and which correlate poorly with the results of the three standard tests (Bellavere et al. 1992). The Valsalva manoeuvre tests the efferent sympathetic system, which mediates the reflex tachycardia and the peripheral vasoconstriction which occurs during the manoeuvre. The results of the three tests are strongly intercorrelated and significantly correlate with the results of many other tests of small- and large-fibre function (Pfeifer et al. 1985).

Most studies in normal subjects show marked age-related deterioration in these tests (O’Brien et al. 1985), but this is not a consistent finding in studies of the Valsalva manoeuvre (see Chapter 1). Most authors recommend the use of age-related normal values (Smith 1982), although after logarithmic transformation the lower limits remain approximately linear with age. Subjects are therefore usually concordant for abnormality whether age-related or non-age-related values are used, and the latter values are considered adequate for screening
purposes (Ewing and Clarke 1986b). It is generally agreed that a diagnosis of cardiovascular vagal autonomic neuropathy requires clearly abnormal values in two or more of the tests; several authors have used scoring systems based on Ewing's non-age-related normal, "borderline" and abnormal values. Various measures of respiratory RR variation have been advocated, but the difference between minimum and maximum heart rate while breathing at six breaths per minute, a rate which maximises the RR variation, is regarded as a sensitive measure (Ewing et al. 1981). Using a vectorial analysis (circular mean resultant) Pfeifer has largely eliminated the influence of basal heart rate and ectopic beats on respiratory RR variation (Pfeifer and Peterson 1987). With this technique, a mean coefficient of variation of 24% for respiratory RR variation and 18% for the Valsalva ratio has been reported in a multicentre clinical trial (Schumer et al. 1988). Pfeifer regards RR variation as a more sensitive indicator of parasympathetic damage than the Valsalva ratio, though the latter may be of value in monitoring advanced autonomic neuropathy, by which stage respiratory RR variation has become undetectable (Rothschild et al. 1987).

To what extent do these tests represent true "small fibre" function? Vagal nerve fibres are predominantly unmyelinated (Keen 1966) and therefore the responses are mediated through small fibre pathways. The pathways, however, are polysynaptic and the pathological processes affecting synapses may not be the same as those affecting the unmyelinated axons themselves. The end-organ response (heart rate) may itself be affected by several non-neurological factors. Pfeifer's group has shown, for example, that respiratory RR variation is depressed up to six months after myocardial infarction (Rothschild et al. 1988), while other measures of parasympathetic function (pupillary latency time and dark-adapted pupil size) remain unchanged, suggesting that myocardial as well as neuronal dysfunction may contribute to abnormal cardiovascular reflexes. There is also evidence that autonomic dysfunction and subclinical abnormalities of cardiac function are associated even in the absence of ischaemic heart disease (Zola et al. 1986). In summary, cardiovascular vagal tests are precise and reproducible indicators of proximal ("central") autonomic dysfunction in diabetes, but the complexity of the pathways suggests that caution should be exercised in interpreting them as pure indicators of small-fibre function.

1.4.9.2 Blood pressure changes

Changes in blood pressure are less sensitive indicators of autonomic dysfunction than tests measuring heart rate changes (Ewing and Clarke 1986a, 1986b), but are the most convenient
measures of sympathetic function. Abnormal blood pressure regulation is caused by efferent baroreflex dysfunction, resulting in failure of splanchnic and subcutaneous vasoconstriction through sympathetic denervation of resistance vessels (Hilsted et al. 1981). Some of the lack of sensitivity may be due to the relatively crude methods for routine measurement of blood pressure, which are unable to follow rapid changes during and after a manoeuvre. The usual definition of significant postural hypotension, that is, a systolic fall of >30 mmHg (Ewing and Clarke 1986b), is somewhat arbitrary, and others have proposed a value of >20 mmHg (Weiling et al. 1983). In addition, maximal postural drop may not occur until several minutes after standing, and the time at which the standing blood pressure is taken should be defined. Blood pressure and heart rate responses to standardised isometric exercise (for example, sustained handgrip) have also frequently been used to assess sympathetic function. In this test, a diastolic blood pressure rise of ≤10 mmHg is regarded as an abnormal response (Ewing and Clarke 1986b).

1.4.9.3 Quantitation of pupillary responses

Normal pupillary responses depend on intact innervation from both sympathetic and parasympathetic systems. The two commonest clinical abnormalities in diabetes are miosis and reduced reflexes to light (Smith and Smith 1983a). The small pupil, dilating poorly in darkness, is thought to be due to sympathetic dysfunction, rather than increased stiffness of the iris musculature, the presence of retinopathy, or the presence of the small pupil itself restricting light-induced constriction (Hreidarsson 1982; Smith and Smith 1983b). Pfeifer et al. (1984) found that dark-adapted pupil size after autonomic blockade was reduced in newly-diagnosed type 1 (insulin-dependent) patients, confirming that retinopathy or other long-term tissue changes did not contribute to the small pupil size.

Infrared videopupillography is required to measure dynamic pupillary changes to a light stimulus. Using this technique, Smith and Smith (1983b) confirmed the reduced light reflex in diabetes, and suggested that small diabetic pupils had the same aetiology as those of normal old age, that is reduction in “sympathetic tone”. Although the validity of this term is now under question (see section 1.4.2), there is some pharmacological evidence for age-related pupillary changes in normal old age and diabetes, as both groups show sympathetic pupillary supersensitivity to phenylephrine. Sigsbee et al. (1974) demonstrated pupillary parasympathetic denervation supersensitivity to methacholine in diabetic neuropathy, and Hayashi and Ishikawa (1979) sympathetic supersensitivity to adrenaline. These responses
would be expected in accordance with Cannon’s law of denervation supersensitivity (see section 2.3.1).

There is conflicting evidence over whether pupillary latency time is abnormal in diabetes; Hreidarsson and Gundersen (1985) found no change, but other studies have detected prolonged latencies (Smith et al. 1979; Lanting et al. 1988). Pfeifer et al. (1984) found prolonged latency times only in newly-diagnosed Type 1 but not Type 2 patients, but Hreidarsson (1981) found that the only abnormality in newly-diagnosed diabetes, under conditions of poor control, was a prolongation of the redilatation time. A reduction in pupillary unrest (hippus; Hreidarsson and Gundersen 1988), and prolonged or absent pupil cycling (Martyn and Ewing 1986; Clark 1988) have also been described in diabetes. The pupillometric method and variations in population selection may be important factors, but changes in the pupillary light reflex in diabetes appear to be very subtle; even with the most sophisticated equipment, and measuring many variables, Hreidarsson and Gundersen (1985) were able to detect a slight diminution only in reflex amplitude in their large group of Type 1 (insulin-dependent) patients, compared with normal subjects, and after allowing for the decrease in pupillary diameter in diabetes, most dynamic measurements were found to be normal (see Chapter 8).

Reports of the associations between pupillary and peripheral nerve function have yielded variable results. Lanting et al. (1988) found a significant association with thermal discrimination thresholds, but not with vibration thresholds or motor nerve conduction, while an inverse association between dark-adapted pupil area and vibration threshold was found by Hreidarsson (1982). Pfeifer et al. (1984) found more widespread associations between pupillary measurements and peripheral sensory and motor nerve function. Using a simple static method for measuring dark-adapted pupil size as a ratio of the iris diameter, Smith and Smith (1986) found that 80% of diabetic patients with one or more abnormal vagal cardiovascular autonomic test had small pupils.

1.5 Relative importance of small- and large-fibre abnormalities in diabetes

There has been continuing controversy over the importance of small- and large-fibre abnormalities in diabetic neuropathy. Cross-sectional studies are difficult to interpret on account of the differing sensitivities of tests designed to test the two major fibre groups, and there are no reports of longitudinal studies of small- and large-fibre tests. In a late edition of his popular study of diabetes Lawrence (1965) stated that peripheral small-fibre abnormalities
preceded other neuropathic damage. He did not quote supporting evidence for this view, though as discussed above, many positive symptoms of diabetic neuropathy (pain, postural hypotension, impotence, anhidrosis) are caused by small-fibre abnormalities.

There have been several cross-sectional studies comparing function of myelinated and lightly- or un-myelinated nerve fibres in diabetes. Guy et al. (1985) found that there was a small number of symptomatic diabetic patients who had abnormal small-fibre function (thermal sensation) with normal (large-fibre function) vibration sensation, but no patients with abnormal vibration sensation and normal thermal sensation in the foot. It was concluded that small fibres are more susceptible to damage, and that impairment of thermal sensitivity is the rule in diabetic neuropathy. Heimans et al. (1986) stressed the increased prevalence of abnormal thermal thresholds compared with vibration thresholds in patients with painful neuropathy. Ziegler et al. (1988) reached a similar conclusion in a study of age-matched patients with and without painful diabetic neuropathy, and suggested that selective lower limb small-fibre involvement was 2–4 times more frequent than selective large-fibre involvement.

A study using different devices for thermal and vibration testing (Levy et al. 1987a) in unselected patients confirmed the infrequent finding of abnormal vibration sensation together with normal thermal sensation; 35% of patients had abnormal thermal sensation and 18% abnormal vibration sensation. However, an even higher proportion of patients (54%) had an undetectable medial plantar sensory action potential, the most distal sensory electrophysiological measurement currently available, suggesting that with sufficiently sensitive methods, large-fibre dysfunction could be detected as frequently as small-fibre abnormalities. Contrary results were obtained by Dyck et al. (1985), who administered multimodal neurological tests to 180 diabetic subjects; 69% had abnormal nerve conduction in two or more nerves, vibration detection thresholds were abnormal in 44%, and cooling thresholds in 35%. The high proportion of patients with abnormal large-fibre function (vibration thresholds and electrophysiology) suggests that patient selection and the use of different testing methods are important confounding factors.

The pathogenesis of diabetic neuropathy is multifactorial and may vary with the stage and type of diabetes (Sima et al. 1988), and also in relation to the fibre type. In animal studies, for example, acute osmotic effects cause a decrease in nerve conduction velocity through axonal shrinkage (Dyck et al. 1981), but it is not known whether this is more pronounced in myelinated than unmyelinated fibres. Parallel morphometric studies of both fibre types are required to settle this question definitively, though Dyck et al. (1986) suggested from studies of sural nerve biopsies that predominant small- and large-fibre involvement represent
extremes of a continuum of neural damage. The results of the clinical studies mentioned above support this view.

1.6 Length-dependence of small-fibre abnormalities in diabetes

The length-dependence of myelinated fibre function in diabetic neuropathy has been documented in several studies, and this proximal-distal gradient is consistent with a random-distribution pattern of lesions in myelinated fibres (Waxman et al. 1976). Until recently, few studies had investigated this question in relation to unmyelinated fibre function. Ewing et al. (1981a) found that cardiac parasympathetic tests became abnormal before sympathetic tests, and suggested that this might be due to the greater length of cardiac parasympathetic fibres compared with the sympathetic. In the eye the sympathetic nerves take a longer course than the parasympathetic, and might be expected to be more prone to damage. Hreidarsson and Gundersen (1985) supported this view with their finding that prolonged pupillary redilatation (sympathetic) coexisted with a normally constricting pupil (parasympathetic), within the reduced dynamic range of the small diabetic pupil. Low et al. (1983) found that sweating abnormalities were much less common in the upper limb than in the lower limb in neuropathic patients and Kennedy et al. (1989) found similar, though less pronounced differences in pilocarpine-stimulated sweatspot activation between the arm and leg. In the study of Ryder et al. (1988) the number of acetylcholine-activated sweatspots in the foot was correlated with dark-adapted pupil size, and the sweat test was more commonly abnormal than the pupillary test. While there is therefore accumulating clinical evidence that deranged distal unmyelinated fibre function is commoner than proximal, this question can be resolved only by using tests of similar sensitivity. Clinical abnormalities of distal sweating function may precede other symptoms of autonomic dysfunction (Shahani 1985), but symptoms of small-fibre dysfunction are likely to be unreliable, since most autonomic deficits are clinically inapparent, even when advanced (Bilous 1990).
2 Eccrine sweat glands and their abnormalities in diabetes

2.1 Neural pathways involved in eccrine sweating

2.1.1 Sudomotor innervation of the general body surface

Sudomotor and vasomotor pathways originate in the hypothalamus, pons and medulla, though other less important centres of origin have been found in the rat brain (Nathan and Smith 1987). In the rat, cat and monkey, direct connections between the hypothalamus and the sympathomotor area of the thoracic cord have been traced along the ventrolateral surface of the medulla (Saper et al. 1976). Early workers (Foerster 1939; Foerster et al. 1939; Laruelle 1937 [quoted in Nathan and Smith 1987]) localised descending vasomotor and sudomotor tracts predominantly to the intermediolateral and intermediomedial gray columns of the spinal cord. Using postmortem material after anterolateral cordotomies Nathan and Smith (1987) found that sympathetic fibres lie in the medial part of the equatorial plane of the cord, extending from the base of the posterior horn and the lateral horn across the medial half of the white matter. The tracts are small, and although there is some overlap between sudomotor and vasomotor pathways, the sudomotor fibres are generally situated more ventrally. The fibres maintain this position within the cord as far as spinal segment L2 and are bilateral, so that extensive cord lesions are required to abolish sudomotor responses completely.

2.1.2 Sudomotor innervation of the head and neck: gustatory sweating

From clinico-pathological evidence, List and Peet (1938a) suggested that descending sudomotor pathways to the face originate in the dorsolateral "retro-olivary" area of the medulla. Nathan and Smith (1986) extended this area of origin. Efferent fibres run ipsilaterally and unilaterally. In the cervical cord sudomotor fibres lie in the region of the posterior angle of the anterior horn, from where they pass to supply the thoracic intermediolateral column.

There have been no quantitative studies of sudomotor function in the head and neck in diabetes. However, an interesting syndrome of excessive episodic sweating, known as gustatory sweating, was first described in the 1930s in non-diabetic individuals (Haxton 1948), and further characterised by Aagenaes (1962) [quoted in Ewing and Clarke (1987)], Watkins (1973), Bronshagg (1978) and Stuart (1978). It invariably occurs in patients with advanced neuropathy and microangiopathy. The symptoms often have an abrupt onset, and thereafter are permanent. Within a few minutes of eating food that would normally elicit
salivation, profuse symmetrical sweating breaks out on the forehead and spreads to involve the face, neck and upper anterior chest. In some it is provoked by tepid drinks or mild cheese. Its neuropathological basis is unknown; Watkins (1973) suggested that it results from aberrant nerve regeneration in the territory of the superior cervical ganglion. It can be suppressed by high doses of anticholinergic drugs.

2.1.3 Pre- and post-ganglionic pathways (Brodal 1981)

The cells of the intermediolateral cell columns from spinal segments T1 to L2 give rise to the sympathetic outflow which is carried in the ventral spinal roots. Fibres enter the sympathetic trunk via white rami communicantes and terminate in one or more sympathetic ganglia. Most postganglionic fibres re-enter the spinal nerve to supply small blood vessels, arrector pili muscles and sweat glands; a smaller proportion terminate in large vessels. Because fibres from more than one cord segment end in each ganglion, the sympathetic fibres retain a segmental distribution similar to, but less clear-cut than, their corresponding spinal accessory roots (Hyndman and Wolkin 1941a).

2.1.4 Local neuronal pathways: axon reflexes

The concept of a local, non-spinal, neuronal reflex involved in cutaneous responses originated with the work of Langley (1900) and was later extended by Sir Thomas Lewis (1927). These early studies showed that an intact sensory nerve supply was required for vasodilatation, sweating, and piloerection, and that the responses were abolished after section of the sensory nerve peripheral to the cell body in the dorsal root ganglion, after allowing time for degeneration of the peripheral component of the nerve. The concept of the axon reflex was necessary in order to explain the sensory and effector components of the responses being mediated through the same nerve; Celander and Folkow (1953) proposed a simple arrangement whereby the primary afferent neuron is assumed to have an input branch which is activated through polymodal nociceptors, and an effector branch, where neurotransmitters are released to generate the skin response through a target cell. A similar arrangement of neurons can be used to explain neurogenic inflammation (Foreman 1987), the pilomotor (Lewis and Marvin 1927) and sudomotor responses to faradic stimulation of the skin (Bickford 1938; Wilkins et al. 1938), and the pilomotor and sudomotor responses to intradermal injections of drugs with a nicotine-like action (Coon and Rothman 1951). The actual neuronal arrangement of the neurons involved in axon reflexes has not been demonstrated, and its characteristics have
largely been deduced from, and therefore defined in terms of, their physiological and pharmacological properties (see section 2.4). However, Coon and Rothman (1941) attempted to differentiate pharmacologically between two possible structural arrangements, namely an arborised network and a continuous network, and concluded that an arborisation better fitted the experimental data.

2.2 Morphology, neuroanatomy and histochemistry of eccrine sweat glands

2.2.1 Morphology of sweat glands

The eccrine sweat gland consists of a secretory coil (acinus) and a sweat duct. The secretory coil is situated in the middle or lower layer of the dermis (Montagna 1962) and is tightly and irregularly coiled. It leads to a straight segment extending to the epidermis. The epidermal portion is also coiled. Three cell types have been identified in the secretory coil: secretory cells (clear), dark cells (mucoid) and myoepithelial cells (Sato 1977). The clear cells are thought to secrete water and electrolytes, though Na⁺-K⁺-ATPase, indicating sodium pump activity, is located on the basolateral membrane, and not at the luminal surface (Quinton and Tormey 1976; Saga and Sato 1988). The secretory function of clear cells is also supported by the observation that they contain large numbers of mitochondria (Sato 1977). The cuboidal or pyramidal dark cells line the luminal aspect of the secretory tubule and may secrete mucosubstances into the sweat (Montagna 1962). The myoepithelial cells are unique, single spindle-shaped cells which lie on the basement membrane. They are filled with contractile microfilaments, and were previously thought to be responsible for expulsion of pre-formed sweat towards the skin surface. It is currently thought that the principal sweat gland neurotransmitters, acetylcholine and vasoactive intestinal polypeptide, exert their effects on the secretory elements via the myoepithelial cells (Collins 1988); they contract in response to cholinergic but not adrenergic stimulation. Sato et al. (1986) suggested an alternative mechanical function for the myoepithelial cells, that is, to provide support for the secretory coil wall against the increase in luminal hydrostatic pressure. The architecture of the openings of the sweat ducts at the skin surface is complex and varies between skin regions. On the palms and soles the ducts join the epidermis at the apices of the rete ridges, and thereafter become coiled. The secretory elements of sweat glands are therefore situated at different depths below the skin surface, depending on the local epidermal thickness.
2.2.2 Blood supply of sweat glands

Most sweat glands are supplied by a single arteriole, though larger glands may share an arteriolar supply from several vessels (Montagna 1962). The glomerular portion of the gland has a rich blood supply, from which arise parallel arterioles, cross-connected by capillary shunts, which accompany the straight portion of the ducts. These branch around the intra-epidermal coiled portion of the duct, and connect with the sub-epidermal arcades of blood vessels. The innervation of the sweat gland vasculature has not been studied.

2.2.3 Neuroanatomy of sweat glands (Brodal 1981)

Post-ganglionic sudomotor nerves branch to supply the secretory coil and the coiled portion of the duct. Several glands are probably innervated by the same sudomotor fibre and, conversely, the same glands receive terminals from several nerves. The anatomy of autonomic nerve-endings and their relationships with effector organs has been difficult to elucidate on account of the minute size of the nerve fibres. Early studies, using silver-staining, suggested the presence of a network of terminal fibres (Brodal 1981), the “ground plexus” of Hillarp (1959), consisting of extremely thin fibres about 200 Å in diameter. These early studies hinted that the terminal fibres of the syncytium were continuous with the cytoplasm of the effector cells. Most recent studies have not seen such close connections, though Tainio et al. (1986) visualised a sparse network of tyrosine hydroxylase-immunoreactive (IR) fibres applied closely to the sweat gland epithelium. At higher magnification, the sudomotor nerves are seen to contain a string of varicosities along their length, similar to the terminal boutons and boutons en passage in the central nervous system (Collins 1988). These represent presynaptic vesicles containing neurotransmitter substances in secretory granules. Other portions of the sweat gland may also have a nerve supply; Lundberg et al. (1979) observed a dense network of both thin and varicose vasoactive intestinal polypeptide (VIP)-IR fibres around the acini and ducts of the cat sweat gland.

2.2.4 Immunohistochemistry of eccrine sweat glands

Dale and Feldberg (1934) characterised the cholinergic sympathetic transmission at the sweat gland, and until recently, acetylcholine was the only neurotransmitter to have been localised in sudomotor nerves. Lundberg et al. (1979) found VIP-like IR co-localised with acetylcholine, and suggested that co-secretion of these two substances might account for the
simultaneous vasodilatation and sweat secretion that occurs when secretomotor nerves are stimulated. Further evidence for the role of VIP in sweat secretion was produced when VIP-IR was found to be deficient in the sweat glands of children with cystic fibrosis, a disease still diagnosed on the basis of abnormal sweat composition (Heinz-Erian et al. 1985). The same group has now demonstrated low and high affinity groups of VIP receptors on human sweat gland epithelium (Heinz-Erian et al. 1986). Eedy et al. (1990) confirmed the presence of binding sites for both VIP and the related substance peptide histidine methionine (PHM) on sweat gland secretory cells, and suggested that these peptides were colocalised in the same neurons. Using reverse-phase HPLC, this same group identified both peptides in concentrated human sweat.

Other neuropeptides have been found in nerves near sweat glands, but in much lower concentrations than VIP, and their functional role in sweating is even less clear. Neuropeptide Y (NPY) is a neuropeptide that is commonly colocalised with biogenic amines. It is found in autonomic, predominantly sympathetic, structures (Inagaki and Kito 1986; Schalling et al. 1991), and might be expected to be a significant peptide in sudomotor nerves. Weak NPY-IR has indeed been found around normal sweat glands in biopsies taken without the use of adrenaline-containing local anaesthetic (Karanth et al. 1989), though Tainio et al. (1986) were unable to detect NPY-IR. Evidence for the presence of the “sensory” neuropeptides substance P (SP) and calcitonin gene-related peptide (CGRP) in sudomotor nerves is also conflicting (see section 1.3.3). Landis and Fredieu (1986) found CGRP-IR and VIP-IR co-localised in nerves to rat sweat glands, and Gibbins et al. (1987) found abundant CGRP-IR in nerves around sweat glands, and weaker somatostatin-IR in the same neurons, in human skin. SP-IR has been found in many secretory structures, including salivary glands (Hökfelt et al. 1977a). Recent studies have not detected SP-IR nerves innervating human sweat glands (Gibbins et al. 1987; Tainio et al. 1987), though dense SP-IR has been detected in fibres innervating sweat glands in the cat, where it coexists with VIP-IR and CGRP-IR (Lindh et al. 1988). These variable findings suggest that there is considerable inter-species variation in neuropeptide distribution in sweat glands. Tainio et al. (1987) also detected atrial natriuretic peptide-IR and galanin-IR in human sweat gland nerves.

Wada (1950) and Haimovici (1950), and later Foster et al. (1970) described sweating induced by local intradermal injection of catecholamines, though catecholamines were not localised histochemically in sudomotor nerves until several years later (Uno 1977). Their functional role in sweating in adult animals is still not known, but they may remain as ontological markers of their embryonic adrenergic origin. Landis and Keefe (1983) showed that although
rat sudomotor nerves lose endogenous catecholamines during development, after maturation into cholinergic nerves they still retain the capacity to take up and store exogenous catecholamines. This capability may be induced by the sweat gland itself; using an in vivo transplantation technique Schotzinger and Landis (1988) showed that the sweat gland itself can induce normally adrenergic neurons to express cholinergic transmission. The importance of various neurotransmitters in sweating may therefore vary with time and with the amount of residual amount of neuropeptides remaining during development of the sweat gland (Landis 1988).

2.3 Sweat gland denervation and re-innervation

2.3.1 Effects of acute and chronic denervation of sweat glands: Cannon's law of denervation and denervation supersensitivity

Surgical section of a peripheral nerve markedly reduces or abolishes the response of sweat glands to pilocarpine, providing sufficient time has elapsed for nerve degeneration to occur (Guttmann 1940; List and Peet 1938a). Randall and Kimura (1955) confirmed this finding three months after thoracic ganglionectomy for hypertension. In short-term studies Kennedy and Sakuta (1984d) found that after sciatic and saphenous nerve section in the mouse, there were no detectable pilocarpine-activatable foot-pad sweat glands after 96 hours, and Simeone et al. (1951) demonstrated an absent galvanic skin response six days after denervation of the cat hind paw. Early clinical reports in man (List and Peet 1938b) suggested that when sufficient precautions are taken to ensure complete postganglionic denervation there is minimal sweat gland responsiveness to pilocarpine. Hyndman and Wolkin (1941b) found that after preganglionic denervation pilocarpine-induced sweating was normal, but after postganglionic denervation there was sweat gland unresponsiveness after a brief period of increased reactivity. There is therefore experimental and human evidence that sweat glands form an exception to Cannon's law of denervation which stated: "When in a series of efferent neurons a unit is destroyed, an increase in the irritability to chemical agents develops in the isolated structure or structures, the effects being maximal in the part directly denervated" (Cannon 1939). The biological basis of this law has been analysed in detail in skeletal muscle, in which denervation results in a complex series of biochemical changes, culminating in de novo synthesis of muscarinic acetylcholine receptors, which spread uniformly over the muscle cell surface, rather than being concentrated at the sites of the neuromuscular junctions (Thesleff and Sellin 1980). The situation in sympathetically-innervated tissues is different; supersensitivity occurs immediately after denervation, compared with hours or days after
skeletal muscle denervation. Here the mechanism is thought to be due to loss of uptake of transmitter by the presynaptic neurons, increasing the amount of transmitter or agonist which reaches the postsynaptic receptors with a resulting increase in responsiveness of the tissues. The reasons for sweat glands appearing to be an exception to this rule are unknown. It has been suggested that unreactivity may be due to sweat gland atrophy; histological studies, however, show no significant changes in sweat gland morphology (Löfgren 1950), and it is unlikely that morphological abnormalities would occur so soon after denervation, though Silver et al. (1964b) demonstrated histochemical absence of acetylcholinesterase in sudomotor nerves after denervation resulting from peripheral nerve injuries. It is also unlikely that changes in vascular reactivity could account for such profound changes, particularly as increased, rather than decreased, skin blood flow occurs after denervation. Receptor changes are probably responsible, but the nature of such changes is unknown. Sweat glands are the best-known example, but there are other exceptions to Cannon's Law, for example parasympathetic denervation of the submaxillary gland (Pierce and Gregerson 1948), and the secretory response to urecholine of the vagotomised stomach (Antia and Ivy 1949).

2.3.2 Experimental studies of sweat gland re-innervation

Kennedy and Sakuta (1984d) showed experimentally that sweat gland reinnervation after denervation occurs over a wide area. Their experiments were based on the finding that pilocarpine-induced sweating in the innervated region is completely inhibited up to seven days after nerve section (see section 2.3.1), but returns with reinnervation. In the mouse, only small numbers of glands are innervated by the saphenous, compared with the sciatic nerve, but all saphenous-innervated glands are also innervated by the sciatic. After sciatic nerve section, the area of pilocarpine-stimulated sweat glands increased three or four-fold; the same response was also elicited by saphenous nerve stimulation, suggesting that the newly activated sweat glands had been collaterally reinnervated from the saphenous nerve. This pattern of widespread reinnervation extending beyond the original dermatomal boundaries resembles that of nociceptive fibres (Devor et al. 1979) and mechanoreceptors (Brown et al. 1981) rather than that of α-motorneurons, which occurs over a more limited area. Kennedy et al. (1988) found that complete reinnervation of sweat glands occurred whether the nerve was transected or frozen, but that after freezing there was more rapid re-innervation, probably due to preservation of axis cylinders. During reinnervation, sweat glands were more responsive to pilocarpine than to heat; the authors suggest that this may be due to the newly regenerated axons having a high threshold, but may also be due to changes in sweat gland receptors.
Sweat gland reinnervation in clinical neuropathies is likely to be similar to the effects of experimental freezing, rather than nerve transection.

2.4 Pharmacology of eccrine sweating

2.4.1 Response of sweat glands to cholinomimetic agents

2.4.1.1 Direct sweat gland stimulation by muscarinic agents

The similarity in action between acetylcholine and muscarine on autonomic effector organs, smooth muscle and glands gave rise to the term “muscarinic” effects, to distinguish them from the “nicotinic” effects of acetylcholine on ganglion cells and striated muscle. Pilocarpine is the best-known substance acting directly on sweat gland epithelium through muscarinic acetylcholine receptors. List (1936) [quoted in Randall and Kimura (1955)] suggested that pilocarpine acted on sudomotor nerve terminals; however, List and Peet (1938a) found that six weeks after cervicodorsal ganglionectomy, sweat responses, albeit reduced, were still present, suggesting that pilocarpine acted directly on sweat glands, rather than on nerve terminals, which were assumed to have degenerated by that time. Pilocarpine resists degradation by acetylcholinesterase, and has a prolonged and intense action which is confined strictly to the area of application (Kennedy et al. 1984b). It acts on muscarinic M2-receptors (Barnes 1992), which presumably exist on sweat gland epithelium, but have not yet been demonstrated. Methacholine (acetyl-β-methylcholine), an acetylcholine analogue which is relatively resistance to acetylcholinesterase, has a shorter-acting muscarinic effect than pilocarpine.

2.4.1.2 Axon-reflex sweating mediated by nicotinic agents

Coon and Rothman (1941) and later Collins and Weiner (1961) described the transience and extent of the sweat response to intradermal nicotine. Sweat glands up to 5 cm from the forearm injection site were activated in a longitudinal ellipse, beginning 10–15 s after injection, reaching a maximum at 15–45 s and fading after 1–3 min. The intensity and extent of the response was related to local sweat gland density, and enhanced in a warm, and inhibited in a cool, environment. The response was abolished by prior treatment with local anaesthetic. Nicotine injected into an atropinised area abolished sweating in the area of the wheal, and diminished the response in the periphery, suggesting that sweating was completely inhibited at the neuroglandular junction and partly inhibited by receptor blockade. Both
hexamethonium and curare blocked the receptor side of the axon reflex arc, confirmed by the preservation of thermal sweating in areas treated with hexamethonium. These pharmacological methods are unable to determine whether the neuroglandular junction or "free" nerve endings serve as the receptors. Nerve fibres themselves are not the receptor sites, as they are not excited by nicotine (Brink et al. 1946 [quoted in Collins and Weiner 1961]). The receptor sites act pharmacologically like autonomic ganglia, in being stimulated by low concentrations of nicotine-like drugs and paralysed by high concentrations (Coon and Rothman 1941; Collins and Weiner 1961), though there is no histological evidence for the presence of peripheral ganglia in the sudomotor pathway.

2.4.1.3 Sweat responses to acetylcholine

There is conflicting evidence on the sudorific actions of acetylcholine. Coon and Rothman (1941) found that high concentrations (1:1,000 to 1:10,000) of intradermal acetylcholine gave a muscarinic sweating response extending only a few millimetres from the point of injection, corresponding to the region of vascular diffusion of the chemical, reaching a maximum at 5–10 min. In lower concentrations (1:10,000 to 1:80,000), characteristic axon-reflex sweating was seen, comprising a transient area of sweating (maximum at 1–2 min) about 5 cm in diameter. Chalmers and Keele (1952), however, were able only to detect muscarinic sweating in response to a wide range of acetylcholine concentrations (1:100 to 1:10,000,000). Wada et al. (1952) distinguished between the nicotinic and muscarinic actions of acetylcholine by applying an elastic band around the limb being tested sufficiently tightly to prevent vascular diffusion (i.e. the muscarinic effect) of the agent injected to one side of the band, while allowing the nicotinic action to be detected on the other side. In contrast to Chalmers and Keele (1952) they were able to detect axon reflex sweating over a wide range of acetylcholine concentrations (1:100 to 1:1,000,000). Low et al. (1983), using a compartmentalised sudorometer to exclude direct muscarinic sweat responses (see section 2.4.1.4), was able to detect nicotinic sweat responses after iontophoresis of a 10% acetylcholine solution.

Gordon and Maibach (1966) used a modified Bullard sudorometer (see section 2.5.2.2) to quantify sweat output after 1:100-1:10,000 intradermal acetylcholine; the responses, which peaked at 2–3 min, and fell to near-basal levels after 15 min, suggest that the muscarinic, rather than the nicotinic, actions of acetylcholine were being detected. These results suggest that over a wide range of concentrations, acetylcholine stimulates both nicotinic and
muscarinic forms of sweating; exclusion of the more intensive muscarinic response is required in order to detect the weaker nicotinic response.

2.4.1.4 Denervation supersensitivity to cholinergic agents

In diabetes, the small fibres of a mixed nerve trunk will at any one time be at different stages of dysfunction, degeneration, and possibly regeneration, and the comparison with surgical nerve transection is likely to be inaccurate. Accordingly, there have been several incidental descriptions of sweat gland hyper-responsiveness in chronic diabetic neuropathy. Low et al. (1983) used, in effect, an elastic band technique (see section 2.4.1.3) to study purely nicotinic sweat gland responses and found several patients with "partial denervation" who showed a short sweat response latency and a subsequent prolonged high sweat output. Ahmed and Le Quesne (1986) used Low's method in diabetic patients with diabetic foot lesions and also found exaggerated responses in 3 of 37 patients. These studies suggest that sweat gland supersensitivity to the nicotinic action of acetylcholine is a common finding in diabetic neuropathy. Muscarinic cholinergic supersensitivity has been described in the neuropathic pupil (see section 1.4.9.3), but not in the sweat gland. Non-diabetic patients with pure autonomic failure do not demonstrate sweat gland supersensitivity to methacholine (Baser et al. 1991), but this study used a relatively insensitive method to measure total sweat output.

2.4.2 Catecholamine-induced sweating

Although there is doubt about the physiological relevance of catecholamine-induced sweating, several studies have shown that sweat glands respond to intradermal noradrenaline and adrenaline (see section 2.2.4). Alpha-receptor blockade abolishes catecholamine sweating, suggesting the presence of specific adrenergic receptors on sweat glands; this has been confirmed by the elegant in vitro work of Sato (1977). Catecholamine-induced sweat responses are much smaller than those induced by cholinergic agents, though they have a similar duration of action. Foster et al. (1970) found that the threshold concentration of intradermal catecholamines was greater than that of acetylcholine, though Chalmers and Keele (1951) found no difference between adrenaline and acetylcholine thresholds. Intradermal adrenaline does not, as previously suggested (Kuno 1965), potentiate the cholinergic sweat response (Foster et al. 1970).
2.4.3 The role of neuropeptides in eccrine sweating

While several neuropeptides have been demonstrated in nerves around sweat glands (see section 2.2.4) current evidence is that only VIP is functionally involved in the modulation of sweating, and its role in vivo is uncertain. Sato and Sato (1987) studied isolated (ie. denervated and devascularised) simian sweat glands in vitro, and showed that VIP given alone causes sweat production that is as profuse as that elicited by cholinergic agents, but of a shorter duration of action, possibly as a result of desensitization of receptors. VIP-induced sweating is mediated through cAMP. This sudorific effect is additive to that of methacholine and of isoproteenol-stimulated cAMP accumulation (Sato et al. 1989).

The effect of neuropeptides on sweating in vivo has been poorly explored. Unpublished findings reported by Heinz-Erian et al. (1986) suggested that iontophoresed VIP caused sweating in human subjects, but details of the methods used to iontophoresese VIP, a polypeptide, have not been forthcoming. Yamashita et al. (1987) examined the effects of injected VIP on human forearm sweating. In a “cool” environment (ambient temperature 23°C) VIP alone did not increase skin water loss above basal rates; however, when given together with methacholine, it modestly increased peak sweating rate compared with methacholine alone. The effect of VIP was greatest (approximately 2-fold) at a concentration of \(10^{-5}\) g/ml. In a second experiment, subjects were exposed to a high environmental temperature (35°C) to induce passive vasodilatation, but also given a vasoconstrictor forearm nerve block, which inhibited sweating. VIP facilitation of sweating was reduced compared to the first experiment. The authors concluded that VIP may be involved in securing an ample oxygen supply to sweat glands, particularly when vasoconstrictor tone is relatively high. The effects on sweating of neuropeptides other than VIP have not been investigated.

2.5 Development of methods for studying sudomotor function

Many qualitative and quantitative methods have been devised to test the integrity of different portions of the sudomotor pathway (see sections 2.1.1–2.1.4). Thermoregulatory sweat tests (see section 2.5.5) test the whole pathway from hypothalamus to sweat gland; they can delineate large anhidrotic areas resulting from lesions of brain, spinal cord and peripheral nerves (List and Peet 1938a,b,c). By their nature, they are poorly reproducible, and they have only recently been quantified (Fealey et al. 1989). They are less appropriate in diffuse peripheral nerve diseases, where clinical sweating abnormalities are uncommon, usually not focal, and can not be used reliably to detect enhanced sweat responses. The sympathetic skin
response (see section 2.5.4) can be used to measure conduction in a long pathway which shares a common postganglionic portion with that of the thermoregulatory sweat pathway. Most methods, however, test the postganglionic pathway, and more specifically, the terminal ramifications of sudomotor nerves. These tests are of two main varieties: those that count activated sweat gland density, and those that quantitate sweat output, both methods measuring sweating function over relatively small skin areas.

2.5.1 Quantitation of activated sweat glands

2.5.1.1 Impression methods

The phenomenon of the unresponsiveness of completely denervated sweat glands to locally applied cholinergic agents (see section 2.3.1) has been used in several studies to quantify sympathetic denervation in peripheral neuropathies. Only direct histological examination can reveal the total number of sweat glands present in the skin, in contrast to the number functionally active (Szabó 1962). Several techniques have been developed to count sweat spots in small skin areas. Thomson and Sutarman (1953) were the first to use an impression method, in which a rapidly-drying plastic embedding medium was spread over the sweating skin and the resulting sweat spot impressions in the solidified medium quantified by microscopy or projection of an enlarged photograph of the plastic film. Sarkany and Gaylarde (1968) used this method in combination with silver chloride precipitation. Modifications of the method have been widely used in pharmacological research into the peripheral effects of anticholinergic agents (Clublecy et al. 1978). Using this technique one study estimated the proportion of poorly-functioning sweat glands after pilocarpine or thermal stimulation to be approximately 10%, by repeatedly counting activated sweat glands over precisely defined skin areas (Willis et al. 1972). Kennedy et al. (1984) were the first to measure sweat spot densities in diabetes, using an impression method. They also measured sweat spot diameters in order to estimate approximate sweat volumes, and recently employed computerised image-analysis to increase the precision of counting (Kennedy and Navarro 1989b).

2.5.1.2 Colorimetric methods

Several methods have been developed which use a colour reaction between an indicator substance and either the water or one of the chemical constituents of sweat, to demonstrate areas of active sweating. Early work used dyes (such as the starch-iodine method of Minor
(Sato et al. 1988)) or quinizarine (Guttmann 1941) to register gross areas affected either by peripheral denervation or defective central thermoregulatory control. Modifications of these techniques have allowed quantitation of sweat gland density and size. The most widely used is again the starch-iodine method (Randall 1946; Dole and Thaysen 1953) in which the colour of the mixture changes to blue on contact with the water of sweat. Iodine can either be incorporated into starch-containing paper by sublimation, or painted directly on the skin as a mixture with oil and starch. A variety of other compounds has been used, for example ferric chloride-tannic acid (Silverman and Powell 1944). The use of bromophenol blue was first described in 1950 by Boymond, and later by Manuila [cited in Herrmann et al. (1951)] and Le Quesne et al. (1989). Herrmann et al. (1951) also carried out a gravimetric study of various indicator methods, and concluded that bromophenol blue was the most sensitive when used in indicator papers. More complex chemical reactions have been used, for example ninhydrin (Pontén 1960), which reacts with amino acids present in sweat.

2.5.1.3 Choice and administration of cholinergic agents for sweatspot activation studies

Collins and Weiner (1961), using a starch-iodine imprint method, were able to detect the transient sweat response elicited by nicotine, starting about 15 s after injection. However, quantitation of these transient responses is probably best recorded with a continuous monitoring system (see section 2.5.2.2). The intensity and extent of the sweat response to nicotine is very variable, and we have not been able to detect nicotine-induced sweating on the forearm using bromophenol blue papers (Levy and Berg, unpublished observations, 1989). Sweatspot activation studies have, therefore, usually measured the more intense and long-lasting muscarinic sweating produced by acetylcholine, its cholinesterase-stable analogue methacholine (acetyl-β-methylicholine) or pilocarpine.

Whether injection or iontophoresis is a more appropriate method for introducing cholinergic agents has also been a matter of debate (Levy and Abraham 1988). Using intradermal injection the precise dose of drug administered is known (though the concentration at the sweat gland epithelium or nerve endings is not), but it is difficult to give injections consistently at the same skin depth, the area of the injection bleb itself cannot be used for sweatspot counting (Ryder et al. 1988), and there are potential hazards associated with injections into neuropathic feet.
All cholinergic agents have effects on skin vasculature (Parkhouse and Le Quesne 1988a, 1988b), which are difficult to quantify. Injections of muscarinic agents rely for their sudorific effect on vascular spread, and are affected by those factors, for example, skin temperature, which themselves influence skin blood flow. Iontophoresis with a constant current generator allows standardisation of the vascular effects as well as the dose of sudorific agent, and can be used to stimulate sweat glands uniformly over relatively large areas; if sudomotor denervation is patchy, the increased measurement area may be important in improving precision. Iontophoresis, however, cannot be used to introduce non-polar or weakly-polar compounds, or polypeptides (see section 2.4.3), and, as with injections, it is not possible to know the drug concentration at the sweat gland, as the dynamics of drug transfer through the skin are known only for a few substances. However, the maximum amount of drug that is deposited on the skin surface can be calculated from Faraday’s law (Webster 1983).

2.5.2 Quantitative assessment of sweat output

2.5.2.1 Gravimetric methods

The earliest methods for quantifying fluid loss from the skin measured changes in total body weight. With developments in weighing technology, methods were improved to enable water loss from small surface areas to be measured, usually by absorption onto filter papers and subsequent weighing. At their most sophisticated, gravimetric methods are able to detect water loss from non-sweating skin (transepidermal water loss (TEWL), see below, section 2.5.2.2). Pinson (1942) measured TEWL by this method to be approximately 0.12 mg·cm⁻²·hr⁻¹. Similar results were obtained for water diffusion through dead skin and toe nails (Burch and Winsor 1946).

2.5.2.2 Hygrometric methods: measurement of transepidermal water loss

Gravimetric methods were unable to monitor rapidly changing water loss, and were supplanted by electrical hygrometry during the 1960s. Nakayama and Takagi (1959), using a hygroscopic plant pith as a sensor, were the first to make continuous recordings of sweating, and estimated “moderate” thermal sweating over the chest at about 2 mg·cm²·hr⁻¹. Bullard (1964) used a hygrometer with an improved, lithium chloride sensor and most studies since then have used a modification of his “ventilated-capsule” method, in which a carrier gas of known humidity and flow rate is passed into a capsule enclosing a known skin area.
Having evaporated the sweat, the effluent air of increased humidity is conducted out of the capsule to the hygrometer, where the humidity is measured as relative humidity (RH) or, in some instruments, dewpoint temperature (dp). Using a high gas flow (up to 18l/min) Bullard's instrument was just sensitive enough to detect insensible water losses.

Several studies have attempted to quantify TEWL (Rothman 1954), and to determine how much of this water vapour is the result of passive diffusion through the skin interstitium and how much the product of active, but subclinical, sweat gland activity. Using a non-ventilated capsule method, Rosenberg's (1962) measurement of TEWL, approximately 0.2 mg·cm⁻²·hr⁻¹, was similar to the value measured using earlier gravimetric methods. TEWL was similar in normal subjects, subjects whose sweat glands had been inactivated by iontophoresis of scopolamine, and in one subject with congenitally absent sweat glands. He concluded that there was no evidence of subthreshold eccrine sweating contributing to TEWL.

Baker and Kligman (1967) concluded that the variation in TEWL found in previous studies (range 0.2–6.0 mg·cm⁻²·hr⁻¹) was due to a combination of experimental error and undetected sweat gland activity. Grice et al. (1971) found that TEWL after inactivation of sweat glands increased exponentially with surface temperature, confirming that TEWL is a passive diffusional process limited by the barrier function of the epidermis. Regional variations in TEWL (forehead and palm have higher values than the general body surface) are related to the thickness of the stratum corneum (Baker and Kligman 1967), and physical or chemical disruption of the epidermis by progressive stripping increases TEWL by up to a factor of 30.

There have been few studies of TEWL without inactivation of sweat glands. Since the skin diffusion layer is disrupted by any device which is placed on the skin surface (see Chapter 2), TEWL measurements will vary for each apparatus, and because of the contribution of sweat gland activity to TEWL, will vary from one skin region to another, depending on regional sweat gland densities as well as epidermal thickness. Using similar devices, the results are fairly consistent for a given skin site; for example, an early study by van Gasselt and Vierhout (1963) using an electrolytic water analyser measured TEWL at 0.5–0.9 mg·cm⁻²·hr⁻¹, and the baseline values in the paper of Yamashita et al. (1987), using a modern hygrometer, were approximately 0.9 mg·cm⁻²·hr⁻¹. These higher values for basal evaporative loss obtained without inactivating sweat glands confirm that it represents a mixture of diffusional loss and variable sweat gland activity. Basal evaporative skin water loss has not been studied in peripheral neuropathies.
2.5.3 Factors affecting sweat gland activation

The physiology of sweating is complex, and the factors influencing it are incompletely understood. The following summarises some of the factors that may influence clinical sweat measurements. Most of the comparative studies discussed have used measurements of activated sweat-gland densities after pharmacological or thermal stimulation.

2.5.3.1 Measurement technique

There are no comparative studies of sweat gland activation measured by different techniques. However, starch-iodine techniques typically produce lower counts than impression methods. For example, impression studies in the thigh have yielded densities between 52 (Knip 1969) and 86 glands/cm² (Thomson 1954), while starch-iodine counts range from 45 (Bar-Or et al. 1968) to 81 glands/cm² (Randall and McClure 1949). Kenney and Fowler (1988) attributed this discrepancy to air bubbles trapped beneath the impression medium, but a more likely explanation is that small sweat spots register in an impression medium but not through the starch-iodine layer, and therefore do not become visible. Apart from the study of Herrmann et al. (1951) previously mentioned (see section 2.5.1.2) there are no comparative data on indicator paper methods.

2.5.3.2 Site

There is a large literature on this subject. The results are confounded by considerations of gender and race (see below), and by the disparity between the density of glands found on histological examination and the functionally active density. For example, histological densities as high as 120/cm² are reported for the thigh (Szabó 1962), while functionally active counts range from 45-86/cm² (Kenney and Fowler 1988).

Thomson (1954) found the lowest densities of thermally-activated sweat gland on the abdomen, thigh, chest and leg (88/cm²), and the highest on the hand and forehead (238/cm²). Proximal areas of limbs have fewer glands than distal parts (Clubley et al. 1978), and there are higher densities on the arms than the legs (Bar-Or et al. 1968). There are considerable inter-individual differences in activated sweat gland densities at the same site (Thomson 1954). Less attention has been paid to sub-regional differences. These may be significant; for example, Kennedy et al. (1984) found the density of pilocarpine-activated glands of the
1st-2nd interspace of the dorsum of the foot to be approximately four times that of the skin over the dorsolateral aspect of the foot, over extensor digitorum brevis (see Chapter 3).

2.5.3.3 Physical characteristics

The total number of sweat glands on the body surface does not change after about two years of age (Kuno 1956), and some of the inter-individual differences in sweat gland density may be due to differences in adult physical characteristics. Thomson (1954) found a significant negative correlation between average sweat gland density in eleven skin areas and calculated total skin surface area, and Bar-Or et al. (1968) found a close inverse relation between heat-activated gland density and skinfold thickness. Higher densities were found in lean compared with obese subjects. Using an in vitro preparation, Sato and Sato (1983) found increased methacholine sensitivity and glandular hypertrophy in subjects who exercised compared with sedentary subjects, though Kenney and Fowler (1988) suggested that the age-related decreased in methacholine responsiveness of sweat glands was unrelated to body composition, aerobic fitness level or degree of thermal acclimatisation.

2.5.3.4 Gender

Gender comparisons are complicated by differences in threshold to cholinergic agents between males and females reported by some authors (Janowitz and Grossman 1950; Kral 1969 [quoted in Dobson and Sato 1972]), but not by others (MacMillan and Spalding 1969), and the effect of physical characteristics (see above). Kahn and Rothman (1942) found that there was a larger qualitative sweat response to intradermal acetylcholine and pilocarpine in men, and other than Chalmers and Keele (1952), who found no gender difference, most authors have reported increased sweat gland responsiveness in males. In an enormous series of more than 4,000 normal subjects, Schwachman and Mahmoodian (1967) found that quantitative pilocarpine-induced sweat output was consistently lower in adult females than males. Gibson and Shelley (1948) obtained a similar result for sweat gland activation after intradermal acetylcholine.
2.5.3.5 Age

Most studies have failed to find significant changes in heat- or pharmacologically-stimulated sweat gland density with age, once adulthood has been reached (Schwachman and Mahmoodian 1967; Ryder et al. 1988). However, the functional power of each sweat gland, and therefore total sweat output, appears to decrease in old age (Silver et al. 1964; Kenney and Fowler 1988).

2.5.3.6 Temperature

Local sweating rate is under the control of a central thermoregulator (Gisolfi et al. 1988) which is activated by changes in internal and mean skin temperature, acting in an additive fashion. The effect of local skin temperature is thought to be multiplicative, rather than additive (Sato and Sato 1983). Quantitative studies estimate the $Q_{10}$ for skin temperature to be approximately 2.5 (Ogawa and Asayama 1986), while a rise in core temperature of about 1°C causes a linear 2-3 fold rise in forearm sweat rate (Breben and Kerslake 1961). The influence of pharmacological stimulation has been less thoroughly investigated. Collins and Weiner (1961) found that the latency of sweating both to nicotinic and muscarinic agents was increased in a cool environment, suggesting that local temperature affects both nerve endings and muscarinic sweat gland receptors. In the presence of subthreshold concentrations of pilocarpine, sweating occurred after local warming that by itself gave rise to no sweat activity (MacIntyre et al. 1968). Kuno (1956) and Dobson and Sato (1972) found that sweat-gland responsiveness, as expected, increased during the summer months. There is also a less well-studied diurnal variation in sweating threshold (Crockford et al. 1970).

2.5.3.7 Ethnic origin

Gibson and Shelley (1948) recorded activated sweatspot densities in response to intradermal acetylcholine and pilocarpine in 4 groups of male and female, black and white Americans. They concluded that the sweat glands of black subjects “appeared to be slightly more responsive” than those of white subjects. Re-analysis of their data (Wilcoxon signed ranks test) shows that although there are pronounced differences between males and females when white and black subjects were considered together, there were no significant differences in sweat gland activation between the two ethnic groups. Thomson (1954) found no differences in heat-activated sweat gland densities between two groups of young Africans and Europeans,
and concluded that very large numbers of subjects would have to be studied in order to demonstrate a statistically significant difference between them.

2.5.3.8 Thermogenic vs. pharmacological stimulation of sweating

Thermal sweating is more intense than pharmacologically-stimulated sweating, Sato and Dobson (1970) finding a 6-fold difference, and Kenney and Fowler (1988) a doubling of sweat rate. Sato et al. (1970) found that activated sweat gland density was similar after pilocarpine and thermal stimulation, suggesting that the increased sweating after thermal stimulation is due to increased sweat output per gland. In vitro studies have shown that sufficient ATP is generated after methacholine stimulation to account for the sweat rates encountered in thermal sweating (Sato and Dobson 1970), and these authors suggested that pharmacological sweating is simply “unphysiological”. There are also chemical differences between thermal and pharmacological sweat; pilocarpine-induced sweat has a higher potassium concentration than thermal sweat, and its sodium content may also be higher, though this latter finding has been attributed to experimental artefact (Sato et al. 1970). Studies on forehead sweating suggest that the distribution of sweating areas is similar after exercise and systemic pilocarpine (Antonacci et al. 1988).

2.5.3.9 Hormonal factors

Some of the gender differences in sweating have been attributed to hormonal changes during the menstrual cycle (Dobson and Sato 1972). A recent study (Hassan et al. 1990) found the high progesterone and oestriol levels during the luteal phase of the menstrual cycle to be associated with impaired postural vasoconstrictor responses in the feet, but there have been no similar studies of sweat responses. Clinical abnormalities of sweating are common in thyrotoxicosis, and even of diagnostic and therapeutic significance in acromegaly, but there have been no systematic studies of sweating in these conditions. There is, however, recent evidence that growth hormone deficient adults have reduced pilocarpine-induced sweating capacity compared with age-matched normals (Pedersen et al. 1989). The mechanism by which growth hormone modulates sweat function is not known, but sweating was shown to be impaired in a patient with Laron dwarfism, emphasising the importance of IGF-1 in mediating the tissue effects of growth hormone (Main et al. 1990). Growth hormone receptor-like immunoreactivity has been detected in sweat gland epithelium in human skin biopsies (Lobie et al. 1990).
2.5.3.10 Sweat gland “training” and “fatigue”

Sweat gland responsiveness is influenced by “conditioning”, the difference in the response of sweat glands to the same stimulus under different conditions. The major conditioning factors are skin temperature and season, but there is also the important, but less well-understood phenomenon of acclimatisation. Repeated daily exposure to heat causes a progressive increase in sweat rate (Collins et al. 1965; Dobson and Sato 1972), and repeated intracutaneous injection of cholinergic agents causes increased sweat gland responsiveness (“training”) if the drug is given in small quantities, or, paradoxically, decreased responsiveness (“fatigue”) when high-dose injections are given. The mechanisms involved in these phenomena are not known. Sweat-gland “training” may result from an increase in sweat output per gland, rather than increased sweat gland recruitment (Dobson and Sato 1972), and Collins et al. (1959) proposed that “fatigue” may be due to neuroglandular block resulting from desensitisation of sweat gland receptor sites.

2.5.3.11 Laterality

Some authors have found right-left differences in sweat gland function, and Kral et al. (1969), quoted in Dobson and Sato (1972), found increased sweating in nearly all normal subjects on the non-dominant side; sweat production was also increased in right-handed, compared with left-handed people, but since sweat gland densities were not measured it is not known whether this finding is due to differences in sweat-producing capacity or in the number of actively-secreting glands. Clubley et al. (1978) found non-significant left-right differences in activated sweat gland densities, and concluded that any such differences were small relative to other sources of variation.

2.5.4 Sympathetic skin responses

The change in the resistance of the skin (the galvanic skin response) after sympathetic stimulation is a venerable observation, attributed to the French clinician Féré (1888) [quoted in Wang 1957], though it had been observed in the cat foot-pad 10 years earlier by Hermann and Luchsinger [quoted in Wang 1957]. The corresponding change in skin potential was first measured by Tarchanoff in 1890. There has been confusion over the terminology of skin bioelectric recordings, as they can be measured in different ways; external electrical versus no external electrical source, constant voltage versus constant current, and using different
methods of stimulation. Low (1983) has summarised the complex nomenclature; the trend in neurological work is to use the term sympathetic skin response (SSR), regardless of the mode of stimulation or recording.

There is conclusive physiological and clinical evidence that the response is caused by depolarisation-repolarisation changes in sweat glands (Nilsson 1982). People with congenitally absent sweat glands do not generate an SSR, and animals with sweat glands only on their paws have absent responses elsewhere on their body (Wang 1957). The response is inhibited by locally iontophoresed atropine (Lader and Montagu 1962) and the magnitude of the response varies with the regional density of sweat glands.

While the response can be readily elicited, its precise neurological pathway is not known. The final common pathway is the preganglionic neuron with its cell body in the intermediolateral column of the thoracolumbar cord, and the postganglionic fibres supplying the sweat glands (see section 2.1.3). The efferent pathway in experimental animals arises from the posterior hypothalamus, passing to the spinal cord via the ventrolateral portions of the brainstem (Davison and Koss 1975). It is therefore a complex multisynaptic pathway.

2.5.4.1 Components of the sympathetic skin response

The characteristic response consists of a fast negative component, followed by a slow positive wave, usually of greater amplitude. In the cat paw, Shaver et al. (1962) showed that the first component originated in the sweat glands, while the second component disappears when the epidermis is removed. Skin drilling reduces the amplitude of the response, confirming that an intact epidermis is required for its full expression. The onset latency of the first negative response, 1–3 s in the upper limb, 2–4 s in the lower limb, represents the time taken for conduction in the afferent and efferent portions of the sympathetic pathway (Low 1983). Much of the latency is thought to be due to delay at the neuroglandular junction, as conduction in the pre- and postganglionic pathways is thought to occupy only approximately 0.2 s (Low 1983), though this is a low estimate given the data discussed in sections 1.1 and 1.4.2, which estimates postganglionic conduction velocity to be approximately 1m/s. The amplitude of the first negative wave represents the amount of sweat secreted under the active portion of the electrode, but as yet no functional significance can be attached to measurements of the amplitude of the second, positive component.
2.5.4.2 Factors influencing the sympathetic skin response

There are several factors which affect the response and may increase its variability. In common with other sympathetic responses, it habituates rapidly, and stimuli must therefore be delivered infrequently. Because of the long duration of the response, conventional averaging techniques cannot usually be used. Filter settings can appreciably affect the time course and magnitude of the response, as can the electrolyte composition of the recording electrodes (Fowles and Schneider 1978). The waveform is affected by local temperature, a diphasic wave at 20°C becoming monophasic at 40°C, and the latency of the response decreases with increasing skin temperature (Yokota et al. 1959). Low (1983) stated that thyroid hormone caused a depression in electrodermal resistance and potential, but there have been no systematic studies of thyroid hormone status.

Knezevic and Bajada (1985) found no association between age or limb length and the latency or amplitude of the response, in contrast to Shahani et al. (1984), who found that latency increased with age and height in normal subjects. Gender effects have not been studied, but Christie and Venables (1972) found a systematic right-left difference in palmar skin potentials, with significantly higher amplitudes in the dominant limb. Other, less well-defined factors may affect the response, including degree of stress and mental set of the patient.

2.5.4.3 Studies of the sympathetic skin response in peripheral neuropathies

Ten of 33 neuropathic patients studied by Shahani et al. (1984) were diabetic. He elicited SSR using electrical stimuli and a deep inspiration, and found that the latter stimulus gave a consistently larger response. This study attempted to correlate quantitative sural nerve pathology with the presence or absence of the SSR. All 3 nerves taken from patients with an absent SSR had low unmyelinated axon densities, while only 2 of 5 nerves from patients with an intact response had abnormal small fibre numbers. There was little association between presence or absence of the response and symptoms of dysautonomia, which are usually parasympathetically mediated. He concluded that the sympathetic skin response was a poor indicator of symptomatic autonomic neuropathy, but that it was a helpful index of unmyelinated fibre dysfunction in peripheral neuropathies, particularly if used in combination with measurement of RR interval variation to deep breathing (Shahani et al. 1990). Knezevic and Bajada (1985) recorded electrically stimulated SSR, and found that the response amplitude was significantly reduced in diabetic patients, though there was no difference in the mean onset latency of the response.
Three studies have recently examined the relationship between SSR and other measures of peripheral nerve function. Maselli et al. (1989) compared Low’s axon reflex sweat test (see section 2.5.2.2 and Chapter 2) with SSR in a group of diabetic patients, most of whom had autonomic symptoms. They found a high concordance between SSR amplitude and the sweat test, though quantitative comparisons in the arm were made difficult by the presence of unusually large and prolonged sweat responses (“hung-up” response). There was also a high concordance with respiratory RR variation, but 20% had an absent sweat response with normal RR variation. They concluded that the two tests are complementary in the assessment of peripheral sympathetic function in diabetes.

Niakan and Harati (1989) investigated the relationship between SSR amplitude, electrophysiology and the Valsalva ratio. All patients had abnormal electrophysiology and 83% an absent SSR. Increasing abnormality in SSR (normal → absent in feet → absent in hands and feet) was reflected in decreasing nerve conduction velocities and Valsalva ratio, and in an increasing proportion of patients with orthostatic hypotension.

Wieman et al. (1989) compared peripheral sensory nerve function and SSR in diabetic patients. Clinical scores of sensory loss to pain, light touch and temperature, and respiratory RR variation, but not vibration score, were significantly associated with SSR amplitude.

2.5.4.4 Sympathetic conduction velocity calculated from measurements of latency of sympathetic skin response

Several studies have estimated conduction velocity in the sympathetic pathway by recording SSR simultaneously at two sites. Knezevic and Bajada (1985) estimated “central” sympathetic conduction velocity by recording the SSR at the palm and the sole. The mean velocity in 5 normal subjects was 1.28 m/s. This procedure is open to question because of the uncertainty of the position of the spinal sympathetic outflow to the upper and lower limbs. Three studies have estimated peripheral autonomic conduction velocity by the well-validated procedure of recording SSR at two sites in the same limb, and calculating conduction velocity from the differences in onset latency. Shahani et al. (1984) estimated mean arm conduction velocity to be 1.57 m/s and leg velocity 1.02 m/s. Leg conduction was estimated by Day et al. (1986) to be 1.49 m/s, and by Elie and Guiheneuc (1990) 1.40 m/s. These estimates are in line with direct microneurographic measurements (see section 1.4.2) (Fagius and Wallin 1980; Wallin 1988).
2.5.5 Thermoregulatory sweat tests

SSR tests integrated sudomotor function in a long pathway. Thermoregulatory sweating is an even more complex function that depends on intact temperature-sensitive afferent pathways from the skin and in the spinal cord as well as the pre- and postganglionic pathways. Rundles' early study (1945) found that thermoregulatory sweating was frequently abnormal in his severely affected patients, and Bárány and Cooper (1956) demonstrated varying degrees of sweating loss in the extremities. The test is primarily a qualitative or descriptive one: sweating after exposure to heat, hot drinks and aspirin is monitored over the whole body surface using an indicator such as quinizarin which changes colour on exposure to the water of sweat. Recently, Fealey et al. (1989) described this test in a group of relatively elderly symptomatic diabetic patients. Using planimetry they were able to measure semi-quantitatively the percentage of the body surface that was anhidrotic. This result correlated well with an index of clinical autonomic neuropathy. Anhidrosis was most common distally, and more marked in the feet than in the hands, but they also found cases of global anhidrosis, and of focal anhidrosis in patients with thoracolumbar radiculopathies. All patients with abnormal thermoregulatory sweating also had reduced axon reflex sweating in the feet (Low et al. 1983) and this was taken as an indication that the two tests were of similar sensitivity in detecting distal sudomotor neuropathy. There may be a role for thermoregulatory sweat tests in the clinical demonstration of unusual neuropathic syndromes, though they remain cumbersome and impractical for general use, and difficult to quantify for research purposes.
1.1 Introduction

Many methods have been described for qualitative and quantitative assessment of neurological function in diabetes (Consensus Proceedings 1992); those used in the comparative studies which follow are described in this chapter. They are predominantly quantitative tests of small-fibre function, but certain large-fibre tests, electrophysiology and vibration perception thresholds, have been included, for purposes of comparison with the small-fibre tests, and because they have themselves been used to define minimal diagnostic criteria for diabetic neuropathy (Dyck et al. 1985). Emphasis is placed on the methods and the rationale behind the test protocols, and on derivation of reference ranges for normal data. Confidence intervals were calculated using standard methods (Gardner and Altman 1989).

1.2 Assessment of large-fibre function

1.2.1 Vibration perception thresholds

Psychophysical vibration perception thresholds (VPT) were measured at the right medial malleolus and tip of the great toe with a Biothesiometer (Ohio Biomedical Instruments, Ohio, USA; Bloom et al. 1984) which had been calibrated by an accelerometer (courtesy of ICI Pharmaceuticals, Alderley Edge, UK; Goldberg and Lindblom 1979). The subject was supine in a warm room, and acquainted with the sensation by setting the applied voltage to >40 V (maximum obtainable 50 V) and applying the biothesiometer head to the medial malleolus. If vibration was not felt, progressively more proximal sites were used. Three measurements were then taken at each test site. At the medial malleolus the foot was everted so that the biothesiometer head could rest perpendicularly, using the weight of the instrument as a standard applied pressure. Voltage was slowly increased until the patient signalled that vibration was felt. Measurements were recorded to the nearest volt. The instrument was removed from the skin between each test and the voltage decreased to below threshold level.

For testing the great toe, the biothesiometer probe was applied perpendicularly to the great toe pulp, avoiding contact with the toe nail. The mean of three readings was taken as the vibration perception threshold. Median intra-subject coefficients of variation (CV%) for the measurements, in 6 normal subjects (mean age 48 y, range 21–63 y) studied twice, up to 12 months apart, were 25.3% (range 0–61.0%) for the medial malleolus, and 36.7% (range 9.9–48.6%) for the great toe.

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1.2.2 Normal values

Thresholds at the medial malleolus were measured in 75 normal subjects, mean age 42 y (range 19–70) and at the great toe in 71. Logarithmic transformation of the thresholds resulted in a normal distribution of data. 95% confidence limits were calculated from the t-distribution and linear regression coefficients, and back transformed, using standard methods (Gardner and Altman 1989). 95% confidence intervals for the normal range are shown in Figure 1.

![Graphs showing vibration perception thresholds at the medial malleolus and great toe.](image)

Figure 1: Vibration perception thresholds in normal subjects measured at the medial malleolus (left panel) and the pulp of the great toe (right panel). Curves of the 95% confidence limits for are shown. Vibration perception thresholds at the great toe were slightly lower than those at the medial malleolus.

1.3 Electrophysiology

Electrophysiological measurements were made on the dominant side using a Nicolet Viking electromyograph (Nicolet Biomedical Instruments, Madison, Wisconsin, USA). Surface electrodes, made from strips of silver 2 cm × 4 mm covered with gauze and soaked in 0.9% NaCl, were used, with a separation of 5 cm for motor recordings and 4 cm for sensory recordings. Supramaximal stimuli were delivered using a constant current generator, at 1 Hz (motor) or 1.5 Hz (sensory), and with a stimulus duration of 0.2 ms. Muscle action potential amplitudes were measured from baseline to peak, and sensory action potentials from peak to peak. Thirty-two responses were averaged for the sensory recordings. Skin temperature was
at least 30°C; if necessary, the limb was warmed by convection. Measurements were made from the following nerves:

(a) **Common peroneal nerve.** Motor conduction velocity (CPMCV, m/s); compound distal motor action potential amplitude (CPDAMP, mV); minimum f wave latency (ms) (32 stimuli)

(b) **Sural nerve.** Sensory action potential amplitude (SSAP, µV) and conduction velocity (SSCV, m/s)

(c) **Medial plantar nerve (Guiloff and Sherratt 1977; Abraham and Abraham 1987).** The sensory action potential amplitude (MPSAP, µV) was recorded using surface electrodes (compare Levy et al. 1987) stimulating with ring electrodes at the great toe. Up to 64 responses were averaged if after 32 no potential was visible. With older equipment, MPSAP responses of < 0.6µV could not reliably be distinguished from the baseline, and were regarded as absent. With the improved resolution of the Viking electromyograph, potentials as low as 0.2 µV could be resolved.

Recordings were not made from the tibial nerve on account of the depth of the nerve at the popliteal fossa and the frequent need to deliver painful supramaximal stimuli.

### 1.3.1 Normal values

Common peroneal nerve studies were performed in 43 normal subjects, and sural nerve and medial plantar nerve studies in 41 (mean age 35y (SD 12), range 17-68). Most electrophysiological measurements show age-related deterioration; f wave latencies are strongly associated with height (Lachman et al. 1980). Results were approximately normally distributed. For the minimum f wave latencies, height-related values were calculated. MPSAP but not SSAP showed an age-related decrease in this population; the mean SSAP amplitude was 21.3 µV (SD 8.2, range 8.1-36.7), yielding a lower limit of normal of 4.9 µV. Mean MPSAP was 2.2 µV (SD 1.3, range 0.6-5.5); the lower 95% confidence limit for MPSAP included 0 µV at all ages over 50. Normal ranges for common peroneal conduction velocity and distal compound motor action potential amplitude are shown in **Figure 2**.
1.4 Assessment of small fibre function

1.4.1 Psychophysical measurements of thermal thresholds

Thermal thresholds at the right foot were measured with an apparatus modified from the "Marstock" device described by Fruhstorfer et al. (1976) (Thermotest, Somedic AB, Stockholm, Sweden). Thermal stimuli were delivered through a water-cooled metal thermode, 25 mm × 50 mm, maintained at a basal temperature of 32°C, i.e. within the range of thermal neutrality (see Introduction, section 1.4.4). The thermode was constructed on the Peltier principle, so that the metal bi-layer of which it was constructed could alternately be warmed and cooled by reversal of the current passing through it.

The temperature of the thermode could be raised and lowered at a pre-determined rate. In the apparatus used here, the rate of change of thermode temperature could be varied as a percentage of the maximum (100%). From the data of Claus et al. (1987), at the setting used in this study (25%), the rate of temperature change for both warm and cool thresholds was calculated to be approximately 1.4°C/s. For heat pain threshold testing, the thermode current was raised to 60% (approximately 5.0°C/s). Cold pain thresholds were not measured (see Introduction, section 1.4.5), as most normal and diabetic subjects were unable to detect a distinctly painful sensation at the minimum achievable thermode temperature (approximately 10°C) (Levy et al. 1989).
Patients were seated comfortably with the foot flat on the floor. Skin temperature was measured and maintained at 32°C with local convective warming if necessary. The tests were carried out at the eminence of the extensor digitorum brevis muscle, close to the site used for the sweat tests (Chapters 3 and 4), and for measurement of the common peroneal compound action potential amplitude and the sympathetic skin response (Chapter 5). The thermode was applied in a longitudinal direction, using a light flexible Velcro-fastened strap. Uniform contact between the skin and thermode was ensured by inspection.

Subject and operator each held a control switch. After explanation of the test procedure, the operator switch was pressed out of sight of the subject; when the stimulus was felt, the subject pressed the control switch, returning the thermode to the basal temperature. Stimuli were delivered at irregular intervals so that the subject did not become accustomed to a particular rhythm of change. Warm, cool and heat pain thresholds were measured sequentially. The output was recorded on a calibrated chart recorder. In preliminary studies on 6 normal subjects, the intra-individual coefficient of variation for warm thresholds was found to be minimum when the values of the first five stimuli were ignored and the threshold calculated from the subsequent 10 values (Levy et al. 1989). Fifteen stimuli were therefore used for warm and cool threshold testing, and the last ten used for calculation of the mean threshold. Heat pain thresholds were measured in the same way, but using only five stimuli. An automatic cut-out temperature of 50°C was set, in order to avoid thermal injury in insensitive patients. Thresholds were calculated as limens, that is, the mean difference between the basal and threshold temperatures, rather than as absolute temperature thresholds.

1.4.2 Normal values

In 78 normal subjects (mean age 50.9 y, range 18–67), there was a small but statistically significant deterioration in all thresholds with age (warm: r = 0.48, p < 0.001; cool: r = 0.23, p = 0.05; heat pain: r = 0.34, p = 0.002) (Levy et al. 1989b; Abraham and Levy 1990). Upper 95% confidence limits for the limens were: warm: 8.6°C, cool: 4.7°C, heat pain: 16.5°C.

The reproducibility of the method was tested in 5 normal subjects, by testing on 3 occasions over a period of six weeks. Median coefficient of variation (CV%) was 14% for warm threshold and 42% for cool threshold (Levy et al. 1987).
1.5 Cardiac autonomic function tests

1.5.1 Tests of RR interval variation measurement

Three cardiovascular autonomic function tests, primarily measuring vagal function (see Introduction, section 1.4.9.1), were performed using a computerised method, modified by ICI (Alderley Edge, UK) for use in clinical trials. Three standard disposable ECG electrodes (3M, Minnesota, USA) were attached to the chest wall, and a trace with a positive R wave deflection $>10$ mm established. The ECG output was connected to a BBC microcomputer, which measured and plotted instantaneous RR intervals.

1.5.1.1 RR interval variation to deep breathing (respiratory RR variation)

Patients were tested while sitting. With the aid of a visual prompt on the computer screen, deep breaths, 5 s inspiration and 5 s expiration, were taken for 1 min. Results for the first and last breath were discarded, and three of the remaining 4 were chosen for calculation of the mean RR interval variation, expressed in beats per minute (Ewing 1990). If the variations were below the pre-set detection limit, there was a facility for decreasing hysteresis, and therefore increasing detection sensitivity.

1.5.1.2 RR interval variation during the Valsalva manoeuvre (Valsalva ratio)

The changes in heart rate during and after the Valsalva manoeuvre (forced expiration with closed glottis) are complex, and test the cardiac innervation, the baroreceptor reflex, and the efferent limb of the sympathetic nervous system. The unitless ratio (Valsalva ratio) between the shortest RR interval during the manoeuvre (a reflex tachycardia resulting from the impeded venous return to the heart) and the longest RR interval after it (a compensatory bradycardia resulting from an increased cardiac output being pumped into a vasoconstricted peripheral vasculature) has become the commonly accepted measurement (Ewing and Clarke 1986b). The test was conducted with the subject sitting upright, using an aneroid sphygmomanometer connected to a plastic mouthpiece. The subject was instructed to exhale to maintain a pressure of 40 mmHg for 15 s. The ratio was computed automatically after clearance of ectopic beats. The test was performed three times, and the mean ratio calculated. Patients with past or current proliferative retinopathy, cerebrovascular disease or uncontrolled hypertension were not tested.
1.5.1.3 30:15 ratio (lying: standing ratio)

The unitless ratio between the longest and the shortest RR interval after standing (occurring respectively at or around the 30th and 15th beat after standing) was used as the third measure of vagal function. Subjects lay supine for 2 min before the test, and were instructed to stand up in their own time. Measurement of RR intervals was begun as the subject began to stand up. The ratio was automatically computed after clearance of ectopic beats.

1.5.1.4 Normal values: age-related changes

Age-related changes in these tests have been described in several reports (see Introduction, section 1.4.9.1). Respiratory RR interval variation and 30:15 ratio both show marked deterioration in normal subjects with age, but the evidence for age-related changes in the Valsalva ratio is less consistent (O’Brien et al. 1985; Ewing et al. 1985; Kennedy et al. 1989a). Using the method described, we established normal ranges for the 3 vagal autonomic reflexes (RR variation in 91 subjects, mean age 40.7 y (SD 14.1); 30:15 ratio in 87, mean age 40.8 y (SD 14.3) and Valsalva ratio in 85, mean age 40.9 y (SD 14.2)). Data were non-normally distributed. Logarithmic transformation resulted in satisfactory normalisation for respiratory RR variation, but an improved fit for the 30:15 ratio, particularly for lower values, was achieved by using log (ratio−0.7) a method first described by Box and Cox (O’Brien et al. 1985; Ziegler et al. 1992). 95% confidence intervals for the normal data were calculated using the method of Gardner and Altman (1989), and back-transformed. Plots of the normal values for respiratory RR variation and 30:15 ratio are presented in Figures 3 and 4. There was no significant change in the Valsalva ratio with age (Figure 5) and a non-age-related lower limit of 1.13 has been used.
Figure 3: The relationship between respiratory RR variation and age in 91 normal subjects. Upper and lower 99%, 95% and 90% confidence intervals are shown.

1.5.2 Sympathetic function: postural change in blood pressure

This is the most convenient test of sympathetic dysfunction, though criteria for abnormality and the optimum time after standing for measuring the blood pressure are controversial (Wieling 1988; see Introduction, section 1.4.9.2). In the current studies the subject lay supine for 5 min. Blood pressure was taken with a Hawksley random zero sphygmomanometer to the nearest 2 mmHg, using Phase V (disappearance of Korotkoff sounds) for the diastolic pressure. Blood pressure was taken again 1 min after standing. The criteria of abnormality quoted by Wieling et al. (1983) and by Dambrink and Wieling (1987) have been used; that is, a systolic fall of 20 mmHg or greater and/or a diastolic fall of 5 mmHg or greater, 1 min after standing.
**Figure 4:** Age-related changes in 30:15 ratio in normal subjects (n=87). Optimum normalisation of the data was achieved by using log (30:15 ratio - 0.7). Lines representing the 95% confidence interval are shown.

**Figure 5:** Valsalva ratio and age in normal subjects (n=85). There was no significant change with age (r=0.07, p=0.53), and a lower limit of normal of 1.13 has been used for all ages. The 95% confidence interval is shown.
1.6 Symptom assessment

The value of assessments of symptomatic peripheral and autonomic neuropathy is confounded by the lack of agreement on a standard format. Dyck has devised a widely-used neuropathy symptom score, which correlates well with histological abnormalities of myelinated fibres in sural nerve biopsies (Dyck et al. 1985). In the current studies there was a need for a symptom questionnaire that more closely reflected abnormalities in small fibre function. A simple set of questions was therefore devised, emphasising somatic sensory and autonomic symptoms (Figure 6). The questionnaire was explained to and then completed by the patient, a nurse helping if there were difficulties with comprehension or interpretation.

Symptomatic peripheral neuropathy was considered to be present if patients registered any of the listed symptoms bilaterally in the upper or lower legs, with or without symptoms in the upper limbs. Upper limb sensory symptoms on their own were not considered to be due to diabetic polyneuropathy, nor were symptoms in axial portions of the body. Because of the more diffuse nature of autonomic symptoms, any autonomic symptom (including impotence alone) was considered indicative of autonomic neuropathy. No attempt at grading symptoms was made.
**Figure 6: Symptom questionnaire administered to patients**

**SYMPTOM ASSESSMENT (this information remains strictly confidential)**

**Name .....................**

<table>
<thead>
<tr>
<th align="left">Please indicate with a tick if you have had any of the symptoms listed below over the past 6 months</th>
<th>Top Feet</th>
<th>Sole Feet</th>
<th>Shin</th>
<th>Calf</th>
<th>Thigh</th>
<th>Hand</th>
<th>Arm</th>
<th>Shoulder</th>
</tr>
</thead>
<tbody>
<tr>
<td align="left">Pain</td>
<td>....</td>
<td>....</td>
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<td>....</td>
<td>....</td>
<td>....</td>
<td>....</td>
<td>....</td>
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<tr>
<td align="left">Numbness</td>
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<tr>
<td align="left">Burning</td>
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<tr>
<td align="left">Tingling/pins and needles</td>
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<tr>
<td align="left">Stabbing</td>
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<tr>
<td align="left">Cramps</td>
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<td>....</td>
<td>....</td>
<td>....</td>
<td>....</td>
<td>....</td>
</tr>
<tr>
<td align="left">Discomfort when clothes/bedclothes touch your skin</td>
<td>....</td>
<td>....</td>
<td>....</td>
<td>....</td>
<td>....</td>
<td>....</td>
<td>....</td>
<td>....</td>
</tr>
<tr>
<td align="left">None of these</td>
<td>....</td>
<td>....</td>
<td>....</td>
<td>....</td>
<td>....</td>
<td>....</td>
<td>....</td>
<td>....</td>
</tr>
</tbody>
</table>

Please indicate with a tick if you have had any of these symptoms over the past 6 months:

- Feeling of weakness or faintness when you stand up ....
- Nausea, vomiting or a bloated feeling after meals ....
- Constipation ....
- Diarrhoea that comes on for no apparent reason ....
- Difficulties in controlling your urine ....
- Sweating abnormalities, for example loss of sweating in the feet or severe sweating of the face after eating food ....
- Unawareness of going hypo (if you are taking insulin) ....
- None of these ....

**MALE PATIENTS ONLY:**

- Difficulties with obtaining or maintaining an erection ....
- Loss of morning erections ....
- Difficulties with ejaculation ....
- None of these ....

**Of ALL these symptoms, which is the ONE that causes you the most trouble at the moment?**

.................................................................
1.7 Miscellaneous measurements: height, weight, haematology and biochemistry

Height was measured without shoes to the nearest millimetre, using a standard pediatric stadiometer. Weight was measured to the nearest 0.1 kg without shoes and outer clothing, using digital scales, zeroed before each reading.

Before sweat testing, capillary blood glucose was measured in all diabetic patients (Glucometer II/Glucostix, Ames, Stoke Poges, UK). At the end of the test session, venous blood was drawn for the following tests:

- Full blood count
- Potassium, creatinine and urea
- Liver function tests: ALT, AST, albumin, γGTP, alkaline phosphatase
- Total T₄, TSH
- Glycated haemoglobin (affinity chromatographic method)
- Random plasma glucose (Yellow Springs)

One male type 1 (insulin-dependent) diabetic patient was found to be subclinically hypothyroid after initial neurological tests. These results were discarded, thyroxine replacement given, and the patient recalled for tests 3 months after he had become biochemically euthyroid.
Chapter 2  Development of a direct-reading sudorometer for the study of sweat responses in diabetic neuropathy

2.1 Introduction

Humidity is difficult to measure precisely, largely because air-water mixtures exhibit non-ideal behaviour, particularly at low water vapour pressures. Individual national humidity standards exist, but most hygrometers are not calibrated against them. Two methods are currently used in physiological research. The Evaporimeter (Nilsson 1977) is a commercially-available static humidity sensor, while the remainder are modifications of the ventilated-capsule method described by Bullard (1962) (see Introduction, section 2.5.2.2). The aims of the current study were to examine critically the potential performance of the Evaporimeter, and develop a direct-reading ventilated-capsule method based on up-to-date hygrometric principles.

2.2 Relative humidity vs. dewpoint determinations: analysis of the performance of the Evaporimeter

Most hygrometers measure relative humidity (RH), defined as the mixing ratio of moist air at a given pressure and temperature to the saturation mixing ratio at the same pressure and temperature (where mixing ratio is the ratio of the mass of water vapour to the mass of dry air with which the water vapour is associated). The resulting value is a unitless ratio, usually expressed as a percentage (RH%). RH is related to atmospheric pressure and ambient temperature, and for comparative purposes RH measurements must therefore be made under standard environmental conditions. The Evaporimeter was designed to give a direct estimate of surface evaporation rates by taking two RH readings at fixed distances above the evaporating surface, and calculating water loss from the difference between them (Nilsson 1977). It is convenient and simple to use, consisting of a hand-held probe connected to a portable display unit. However, an analysis of its performance, using data from Wheldon and Monteith (1980) suggests that the performance of its RH sensors may not be suitable for precise measurements of water vapour loss.
The flux-diffusion equation for water vapour at a surface is given by the equation

\[ E = (D/s) \delta x \]

where \( E \) = evaporation rate

\( D = \) the molecular diffusion coefficient of water vapour in air (at 25°C \( D = 2.49 \times 10^3 \) m²/s and increases by about 0.7% per °C)

\( s = \) separation of the two sensors

and \( \delta x = \) difference in water-vapour concentration.

\( D/s = 1/r_s \)

where \( r_s = \) diffusion resistance of the cell

From the data of Wheldon and Monteith (1980) the diffusion resistance can be calculated as 0.82 Ks/m.

Hence

\[ \delta x = E \times S/D = E \times r_s \]

For a typical moderate transepidermal water evaporation rate of 5 g·m⁻²·hr⁻¹

\[ \delta x = 1.275 \text{ g/m}^3 \]

For ambient air at 50% RH and 25°C, the moisture loading is 11.5 g/m³. With a diffusion gradient of 1.275 g/m³, the moisture loading immediately above the skin surface is 12.775 g/m³. The sensors in the Evaporimeter are 4 mm apart, while the cell itself is 15 mm long; the moisture loading differential between the sensors is therefore

\[ 4/15 \times 1.275 = 0.34 \text{ g/m}^3 \]

Under these ambient conditions a change in moisture loading of \( \pm 1.275 \text{ g/m}^3 \) corresponds to an increase in RH of 5.8%. Thus at the skin surface the RH should be 55.8%. This corresponds to a dewpoint change from 14.1°C to 15.55°C. Because of the proximity of the sensors, the actual change between them is only 4/15 of this i.e. 1.55% RH or a dewpoint change of 0.38°C.

In the absence of other errors the RH accuracy of each sensor would need to be \( \pm 0.075\% \) in order to yield an accuracy of \( \pm 10\% \). This is beyond the precision even of national humidity standards. The precision of currently-available high precision RH meters is approximately \( \pm 5\% \) under ideal conditions (MCM Ltd, personal communication 1989). Wheldon and Monteith (1980) estimate that the Evaporimeter underestimates "insensible perspiration" (\( \approx 2 \text{ mg·cm}^2·\text{hr}^{-1} \)) by about 10%, but high sweating rates (\( \approx 80 \text{ mg·cm}^2·\text{hr}^{-1} \)) by as much as 50%.
2.3 Estimation of magnitude of temperature dependence of RH

Assume, for example, that the ambient temperature changed by 0.5°C, from 25°C to 25.5°C; then for the same degree of moisture loading the RH would change by 1.67% ie. from 50.0% to 48.33%. This is more than the RH differential between the sensors. Therefore a temperature variation of as little as ±0.125°C could result in the RH difference being eliminated or doubled. In practice the temperature differential may be considerably higher (for example a skin surface temperature of 32°C and an ambient temperature of 21°C ie. a temperature gradient of 11°C). Under these conditions the temperature of each sensor would need to be kept to ±0.0125°C in order to allow a precision of ±10-20%. The constraints of both the variation in ambient temperature and the precision of two sensors situated 4 mm apart can only be met under strict experimental conditions. In clinical use these potential errors will be compounded by:

(a) Localised convection currents in the cell, producing temperature and moisture disturbances

(b) Variations in the air flow across the open end of the cell, causing disturbances in the diffusion pattern

(c) Variations in ambient RH and temperature

These large temperature effects can therefore markedly affect sweat measurements. Low et al. (1983) used an RH meter in their important work on axon reflex mechanisms in diabetic neuropathy, but overcame the dependence of RH on temperature by incorporating a heating block so that effluent air from the sweat capsule was kept at a constant 40°C.

2.4 Hygrometry by dewpoint measurement

Because of these limitations of RH measurements a more precise measurement was required, one that preferably could be converted directly to masses of water evaporated. This requirement is fulfilled by the use of dewpoint (dp) measurements. Dewpoint (defined by Resolution 166, International Meteorological Organization, Conference of Directors, Washington, 1947) is the temperature to which air must be cooled in order that it is saturated with respect to water at an initial pressure p and mixing ratio r (see section 2.2).

The potential advantage of dp over RH, that it can be more accurately converted to vapour pressure, thence to mass of water evaporated, has been confounded by the imperfect design of the experimental apparatus used to measure vapour pressure in the laboratory, particularly
at low water vapour pressures (ie. low dewpoints). Various determinations have yielded results differing from each other by as much as 70% at -67°C (Wexler 1977). These difficulties can be overcome by using thermodynamically derived formulae which take into account the non-ideal behaviour of water vapour in air.

2.5 Calculation of saturated vapour pressure of water at the dewpoint temperature

I am grateful to MG O'Donnell, formerly of the Heat Transfer and Properties of Fluids Division of the National Engineering Laboratory, Glasgow, for providing the equations for calculation of vapour pressure, based on data from Wexler et al. (n.d.).

The vapour pressure over liquid water is given by:

\[ \ln p_w = \sum_{i=-1}^{3} g_i T^i + g_4 \ln T, \text{ Pa} \]

where

\[ g_1 = -0.58002206 \times 10^4 \]
\[ g_2 = 0.13914993 \times 10^1 \]
\[ g_3 = 0.48640239 \times 10^1 \]
\[ g_4 = 0.41764768 \times 10^4 \]
\[ g_5 = -0.14452093 \times 10^7 \]
\[ g_6 = 0.65459673 \times 10^1 \]

The vapour pressure of ice is given by:

\[ \ln p_i = \sum_{i=0}^{5} m_i T^{i-1} + m_6 \ln T, \text{ Pa} \]

where

\[ m_0 = -0.56745359 \times 10^4 \]
\[ m_1 = 0.63925247 \times 10^1 \]
\[ m_2 = -0.96778430 \times 10^2 \]
\[ m_3 = 0.62215701 \times 10^6 \]
\[ m_4 = 0.20747825 \times 10^8 \]
\[ m_5 = -0.94840240 \times 10^{-12} \]
\[ m_6 = 0.41635019 \times 10^1 \] (all temperatures in K)
2.6 Evaluation of enhancement factor

In order to calculate evaporated volumes of water the vapour pressure requires conversion to a humidity ratio ie. weight of water vapour per weight of dry air. This requires evaluating a so-called “enhancement factor” which takes account of the non-ideal behaviour of the air-water vapour mixture. It is normally a function of many parameters. Over a physiological temperature and humidity range the enhancement factor is relatively constant, but may be calculated very accurately from the dp temperature. The formula (courtesy of Dr O’Donnell) is given by:

\[ F = \left( \frac{A}{T} \right) + B + (C \times T) + (D \times T^2) \]

where
- \( F \) = the enhancement factor,
- \( T \) = is the dew-point temperature in Kelvin,

and the coefficients are:
- \( A = 38.48149 \)
- \( B = 0.6205401 \)
- \( C = 0.001281876 \)
- \( D = -0.000001442007 \)

Once the enhancement factor has been calculated, the humidity ratio can be calculated from the following equation:

\[ W = \left( \frac{MW_w}{MW_a} \right) \times F \times P_w \]

\[ P_{atm} - (F \times P_w) \]

where
- \( W \) = the humidity ratio in (kg water vapour) per (kg of dry air)
- \( MW_w \) = the molecular weight of water (18.01528)
- \( MW_a \) = the molecular weight of air (28.9645)
- \( P_{atm} \) = atmospheric pressure (101,325 Pa)
- \( F \) = the enhancement factor, and
- \( P_w \) = the water vapour pressure in Pascals

From the humidity ratio the water loss rate (mg·cm⁻²·hr⁻¹) in our own experimental arrangement can be derived from the following formula:
\[
TEWL = \left( \frac{P_{atm} \times 293}{W \times 1.2 \times 101325 \times T \times (flowrate \times 60)} \right) / 1.716
\]

where

- \( TEWL \) = transepidermal water loss rate
- \( P_{atm} \) = the atmospheric pressure
- 293 = standard temperature (K)
- \( W \) = the humidity ratio
- 1.2 = the density of dry air at standard temperature and pressure (kg/m\(^2\))
- \( T \) = air temperature (K)
- flow rate = air flow (cm\(^3\)/min)
- 1.716 = internal surface area of sweat cell (cm\(^2\))

The maximum error of water content derived in this way from dp measurement is less than 0.2%, with an average error of approximately 0.05% (MG O'Donnell, personal communication, 1989).

### 2.7 Sensor requirements

From the discussion above it is apparent that there are certain requirements for a sensor required to measure physiological humidity accurately. (1) It should measure dp, or a quantity strictly related to dp, rather than RH; (2) it should provide a signal output which is proportional to dp, and therefore which can be related to water content; (3) it should have a rapid response time so as reliably to detect transient peaks of humidity; (4) it should be stable over long periods; (5) its calibration should be directly relatable to nationally agreed humidity standards; and (6) it should provide accurate readings over the humidity range likely to be encountered in clinical situations. The problem of choosing a suitable sensor has been made more difficult by the unreasonable claims of some authors and manufacturers for the accuracy of their equipment; the manufacturers of the hygrometer used in the study of Scott et al. (1982), for example, suggested that the sensor had an accuracy of ±1 ppm. At 0°C, for example, this represents a change of approximately 0.003°C dp. It is generally accepted that the maximum achievable precision attainable at present is 0.5–2°C dp. On the basis of these factors, a silicon chip-based system (MCM Dewluxe DL 20, Moisture Control and Measurement, Wetherby, Yorkshire, UK) was adapted for the current study (Figure 1).
Figure 1: Schematic diagram of the sudorometer. See text for details.
2.7.1 Response time of sensor

The response time of the sensor depends predominantly on the material of the sensor and its construction. The commonly-used thin-film aluminium oxide sensors, for example, have a typical dry-to-wet response time of 10–15 min and a wet-to-dry response time of 1–4 h. They may therefore fail to detect transient peaks of humidity. In addition, previous studies using these sensors, which suggested that TEWL could be measured accurately with a 2 min recording period, are likely also to have underestimated basal skin water loss (eg. Bettley and Grice 1965). Thick-film aluminium oxide sensors have even slower responses, typically 30–60 min, but unlike other sensors have a faster wet-to-dry response than dry-to-wet. Neither of these sensors is therefore likely to be of value in physiological measurements. The silicon-chip sensor has a much shorter response time. For example, for a humidity step change between +5 and −20°C dp, the 63% response time was found to be 7 s for rising humidity and 9 s for falling. The settling times to within ±1°C were 25 s and 54 s respectively. In laboratory conditions dead time for both rising and falling humidity was <2 s.

In order to estimate the response time under clinical conditions, a simple experiment was conducted with the apparatus set up as follows. Output from the hygrometer was recorded with a chart recorder running at 20 cm/s. The apparatus was dried down to approximately −40°C dp. The sweat capsule was placed on a plastic sheet, which itself was placed over a saline-soaked swab. Quickly removing the plastic sheet allowed a metal probe inside the sweat cell to complete a simple circuit with the saline. At an air flow rate of 300 ml/min the time between circuit completion and the beginning of the upstroke of the hygrometer recording ie. the response time, was found to be 1.04 s (mean of 6 measurements).

2.7.2 Long term stability of sensor

Aluminium oxide sensors are subject to appreciable drift over long periods. The silicon-chip sensor is notably stable: a recent independent test over 1000 h operation yielded an output drift of 0 to −2.1°C dp (Bruce Wallis, MCM Ltd, personal communication 1989).

2.7.3 Precision

Many instruments read accurately at the wetter end of the range; in drier conditions, aluminium oxide sensors are generally unable to read to within ±7°C dp of the generated humidity after 45 min exposure. Under similar conditions errors in the silicon-chip sensor
range from +0.1 to -0.3°C dp. This currently represents the best precision available in commercially available hygrometers.

2.7.4 Range

Previous clinical studies were hampered by the limited range of the hygrometers available. Bullard's pioneering work, for example, required one wide range (5–40% RH) and 6 narrow range sensing elements to cover adequately a physiological range of humidities. In ventilated-capsule methods, adjustment of the air-flow rate can be used to bring readings within the most sensitive range of the sensor. Most modern hygrometers operate over a wide humidity range; the one used in the current studies had a working range of -50°C – +10°C dp, equivalent to approximately 50–12,000 ppm water vapour (w/v). At a gas flow of 300 ml/min this is equivalent to evaporation rates of 0.1–60 mg·cm⁻²·hr⁻¹, a range that includes the lowest values of basal water loss and moderate pharmacologically stimulated sweating.

2.7.5 Calibration

The Evaporimeter can be calibrated by using saturated salt solutions known to develop certain vapour pressures (Nilsson 1977). This is a prolonged procedure. Apparatus which measures dp in gas flowing over the sensor is easier to calibrate since industry standard calibrators generate moisture of known dp. However, neither of these methods is convenient for a clinical sudorometer. The silicon-chip hygrometer, being stable over long periods, is less likely to lose its calibration. Since it measures water content direct, its calibration can be simply checked by evaporating known quantities of water and integrating the resulting masses of water. Sterile water was dispensed by a micropipette (1–10 µl, SGE, Victoria, Australia) on to a specially-constructed stainless steel recessed plate into which the sweat capsule was fitted; in order to increase the rate of evaporation, both the plate and the water were heated to 32°C. The remainder of the apparatus was set up as for the clinical experiments. Air was passed at 300 ml/min and evaporation continued until baseline was reached. 1, 3, 5, 7 and 9 µl samples were tested on each of 5 occasions over a period of 5 months. The results are shown in Figure 2.
**Figure 2:** Sudorometer calibration. The volumes of water (µl) dispensed from the micropipette are shown on the x axis, the retrieved amounts, derived from integration of the evaporation curve, on the y axis. Values are shown as the mean of 5 calibrations carried out over a period of 5 months; error bars represent the standard deviation. The line of identity, representing 100% retrieval, is also plotted. Dispersed volumes of less than about 5 µl are slightly under-retrieved (mean 89%-96%), while there is apparent over-retrieval at higher volumes (mean 102%-108%). This is probably due to the response time of the sensor, which may under-read the narrow, transient peaks caused by evaporation of small volumes.

2.8 Alteration of skin micro-environment by the presence of the detecting probe

The presence of any detecting probe inevitably affects the boundary layer of moisture surrounding the skin surface. The depth of the layer depends on the site, air speed, and the magnitude of both free and forced convection. For example, the boundary layer of a nude person standing in a light draught is about 7 mm, but this depth is reduced to 1 mm at a windspeed of 5 m/s (Scott et al. 1982). Boundary layer resistance is increased by the presence of the probe cylinder of the Evaporimeter and by the presence of the two humidity detectors. Increased diffusion from the skin further increases the resistance, and this accounts for the increasing disparity between true and recorded evaporation rates measured with the Evaporimeter. The disturbance of the skin micro-environment is likely to be even greater with a ventilated capsule, but will be constant for a given air flow. The presence of dry air passing over the skin may change the hydration of the upper layers of the stratum corneum, affect the diffusion of water from the skin, and may itself cause local stimulation of sweat gland
activity. The effect is likely to increase with increasing gas flow, and to be compounded by turbulent non-laminar gas flow within the capsule. Earlier workers with ventilated-capsules either did not address this question (e.g. Grice et al. 1971) or considered the effect to be negligible (Van Gasselt and Vierhout 1963). An estimate of this effect in the present experimental arrangement was obtained by applying the sweat capsule to the volar surface of the forearm (surface temperature 33.3°C, air temperature 23.5°C) recording an initial baseline value for 2 min and recording for a further 40 min. The humidity level gradually fell, reaching a plateau at approximately 0.01 mg·cm⁻²·hr⁻¹ below the baseline. The integrated volume below baseline over 20 min was (-)0.104 mg, over 40 min (-)0.288 mg. Over the relatively short recording periods used in the clinical studies, it is therefore a small effect, and is unlikely to be an appreciable effect when measuring active sweating.

2.9 Adaptation of the silicon-chip hygrometer for sweat measurement

The apparatus is shown in schematic form in Figure 1. A brief description of each element, with critical reference to the factors affecting sweat measurement, follows:

(1) Gas supply

In different studies the favoured gas has been either air or nitrogen. They are both inert and unlikely to affect the sweating skin surface other than through the drying effect discussed above. Neither RH nor dp measurements are affected by the nature of the carrier gas; however, for direct reading of evaporated volumes, it is necessary to know the behaviour of water vapour in the carrier gas at low partial pressures. The equations for water in air, as discussed above, are accurately known. Where prior calibration of known evaporated volumes in arbitrary units is used to interpolate experimental results, as in the method of Low et al. 1983), the nature of the carrier gas is unimportant.

(2) Two-stage gas regulator

(3) Needle valve for fine regulation of gas flow

(4) Tubing

Metal tubing is the ideal material, as it is impermeable to water, but its rigidity makes it impractical for use in physiological studies. Most conventional plastic tubing is permeable to water vapour. A practical compromise is polytetrafluoroethylene (PTFE, Teflon) tubing
whose water permeability can be neglected for practical purposes. All joints in the apparatus
are either metal or PTFE.

(5) Gas dryer

We measured the water content of bottled air to be approximately 80 ppm w/v (≈ -40°C dp).
For accurate detection of low transpidermal water loss rates, levels of <2 ppm were
achieved using a molecular sieve gas dryer. This was recharged periodically by passing air
through it at 4–6 l/min for 4–6 h while accumulated moisture was evaporated by a built-in
heating circuit.

(6) Flow cell

The flow cell was made of Teflon (MCM Ltd), with wall thickness approximately 1.5 mm
and internal diameter 11 mm (skin surface area 1.7 cm²). Inflow and outflow steel pipes
(outside diameter 3/16") were fixed to the top of the cell with epoxy glue. The cell was
attached to the skin surface with a flexible tourniquet; leaks at this site could readily be
detected by observing variations in air flow at the flow meter. Inevitably, the pressure of the
cell results in indentation of the skin. Low et al. (1983) used this necessity to exclude the
direct muscarinic effect of acetylcholine on sweat glands, while allowing nicotinic responses
to be measured (see Chapter 3). Distortion of the skin surface by sweat capsules and its
possible effects on sweat rates have not been investigated.

(7) Flow meter

The integration of evaporated volumes is dependent on the accuracy of the measurement of
air flow through the sweat cell, and it is likely that this accounts for the largest potential error
in the method. From the humidity ratio equation (section 2.6), water loss rate is directly
proportional to the flow rate; at an indicated air flow rate of 300 ml/min an error of
10 ml/min (the maximum likely magnitude of misjudgment of the float position on the scale
of the rotameter (Type 1100 AA 300, Tube size E3-300, KDG Instruments, Burgess Hill,
Sussex, UK)) would result in a 3% error in water loss rate. A higher flow rate would yield
an underestimate. The flowmeter was calibrated by hydraulic pressure test at 1 bar, 15°C and
760 mm Hg absolute pressure. At an indicated flow of 300 ml/min, the actual flow, 303
ml/min, represents an error of 0.99%. The maximum total error resulting from the flow
meter is therefore approximately 4%.
In order to avoid dry air passing through the flowmeter and spuriously increasing humidity before reaching the hygrometer, the meter was placed distal to the hygrometer. Its integral needle valve was fully opened so that pressure in the tubing remained atmospheric.

(8) Flow rate

Previous studies have used widely different flow rates according to the experimental arrangements. In early work high flow rates were used, where because of the low sensitivity of hygrometers, large surface areas needed to be studied. Recent studies using sensitive hygrometry and smaller skin surface areas have used flow rates between 200 and 500 ml/min. In view of the dehydrating effect of prolonged exposure to dry air (section 2.13) the gas flow rate should be minimised, while ensuring that sweat evaporation is complete. Low et al. (1983) achieved this by heating the nitrogen carrier gas to 100°F (38°C). The possible effect on sweating resulting from the use of warmed gas was not investigated. In order to avoid this potential effect we used air at ambient temperature; calculations show that a flow rate of 300 ml/min is sufficient to evaporate sweat at rates of up to 50 mg·cm⁻²·hr⁻¹. This value is rarely exceeded in studies of pharmacologically stimulated sweating; for studies of thermal sweating (up to 200 mg·cm⁻²·hr⁻¹) the flow rate would need to be increased.

(9) Analysis of hygrometer output

By direct measurement we confirmed that the current output (0–20 mA) of the hygrometer was proportional to the logarithm of the dewpoint temperature. The output was directed through an interface box (containing a fixed resistance of 10Ω) to a BBC Master Computer (512K RAM). Software was written in BBC Basic, incorporating the vapour pressure equations above, to convert dp to weight of water evaporated in real time. Sampling was usually every second, but for rapid responses occurring over a few seconds, the program could sample up to 5 times a second. Sampling was displayed simultaneously on screen as parts per million (ppm), instantaneous evaporation rate (mg·cm⁻²·hr⁻¹) and dewpoint (dp), and plotted also in real time, on a logarithmic scale. The baseline evaporative water loss, from which the stimulated sweat output was integrated, was initially plotted over 2 min. An average value for the 120 points was calculated by the program, and plotted as a straight line. Curves of stimulated sweat output were integrated from this baseline to yield total weight (mg) of water evaporated. Particular sections of interest under the curve could be integrated as required. Records were stored as datafiles on disc. At the end of each sweat test, a minute-by-minute integration of sweat output was printed out, together with the peak sweat rate achieved, and the baseline water loss. At a sampling rate of 1 every second, recordings could
be continued for up to 45 min. Options in the initial input screen included air temperature, atmospheric pressure, air flow rate, baseline sampling parameters and duration of experimental recording (autoscaling).
3.1 Introduction

The accurate measurement of evaporative water loss from human skin is important in several fields, and many different methods have been devised since the first estimation was made by Sanctorius in 1614, using simple accurate weighing (Kuno 1956). With advances in the technology of moisture-sensitive substances during the 1940s and 1950s several devices were introduced which permitted increasingly precise estimates of skin water loss. The introduction of the ventilated capsule (Bullard 1964) and its subsequent modifications have allowed more accurate determinations in clinical studies. Over the past decade neurologists interested in diabetic peripheral neuropathy have developed more sophisticated techniques for the pharmacological stimulation of sweating, and for measuring stimulated sweat output (Kennedy et al. 1984a, 1984b; Low et al. 1983). No studies to date have used techniques sensitive enough to measure basal evaporative skin water loss; this measurement is of interest because of the contribution of basal sweat gland activity to transepidermal water loss (TEWL, see Introduction, section 2.5.2.2). The aim of the current study was therefore to use the technique developed in Chapter 2 for the accurate quantitation of sweat output to study dynamic acetylcholine-induced sweat responses in diabetic and non-diabetic subjects, and to compare the results with those of other neurological tests, particularly those reflecting small-fibre function.

3.2 Subjects and methods

3.2.1 Subjects

Forty-six normal subjects and 86 randomly selected diabetic patients were studied; clinical details of both groups are given in Table 1 (see Appendix A for details of selection criteria). Their mean ages were not significantly different.
Table 1: Clinical characteristics of normal and diabetic subjects. Mean (SD, range) [number of subjects]

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>Diabetic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>46</td>
<td>86</td>
</tr>
<tr>
<td>Gender (M:F)</td>
<td>23:23</td>
<td>53:33</td>
</tr>
<tr>
<td>Age, y</td>
<td>44.9 (12.1, 19-67)</td>
<td>42.7 (14.9, 17-66)</td>
</tr>
<tr>
<td>Duration diabetes, y</td>
<td>10.8 (9.0, 0-34)</td>
<td></td>
</tr>
<tr>
<td>Symptomatic peripheral neuropathy</td>
<td>31</td>
<td>24</td>
</tr>
<tr>
<td>Symptomatic autonomic neuropathy</td>
<td>11.4 (3.0, 6.5-20.1) [64]</td>
<td></td>
</tr>
<tr>
<td>Glycated haemoglobin, %(^1)</td>
<td>11.4 (3.0, 6.5-20.1) [64]</td>
<td></td>
</tr>
<tr>
<td>(^1) Reference range &lt;8.0%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

3.2.2 Sweat testing method

3.2.2.1 General

A detailed critique of the principles involved in the development of the sweat testing method was given in Chapter 2 (a schematic diagram is shown on page 81); it is a development of the ventilated-capsule method originally described by Bullard (1962).

In all cases the skin over the right extensor digitorum brevis was tested without prior preparation; footwear and stockings were removed at least 10 min before testing. Ambient and skin temperatures were recorded, and ambient relative humidity (RH) measured using a hair hygrometer (Fischer, DDR). Atmospheric pressure (mm Hg) was measured on the day of the test with a standard laboratory barometer. Skin temperature was maintained above 30°C and ambient temperature at 22-25°C; if necessary the foot was warmed with a convection heater. Some of the tests were performed during a particularly warm summer, and the test room was air-conditioned to a temperature of 24°C and RH of 40%, having allowed the subjects to acclimatise for at least 30 min before testing. Subjects were tested in a quiet room in a reclining chair, and were asked to discontinue using all skin preparations at least 2 weeks before the test.

Basal skin water loss (transepidermal water loss, TEWL) was measured by placing the sweat capsule (surface area 1.7 cm\(^2\)) over the test site. Evaporation of accumulated water on the skin usually gave a transient initial peak of humidity. Recordings were continued at a sampling rate of 1/second until the measurement was stable for 2 min. Mean TEWL was
calculated as the mean of the 120 values, and this rate was plotted as a straight line by the 
computer to form the reference baseline for subsequent integration of total sweat volumes 
after stimulation.

3.2.2.2 Induction of sweating

Sweating was stimulated with a battery-powered, rechargeable constant-current generator 
(Wescor sweat inducer 3600, Chemlab Scientific Instruments, Romford) developing 1.5 mA, 
in general paediatric use as a sweat-inducer for cystic fibrosis testing. A constant-current 
device was important as skin resistance tends to decrease as iontophoresis proceeds (Webster 
1983). The apparatus had an internal timer which terminated iontophoresis after 5 min, a 
system for gradually increasing and decreasing the current delivery during the first and last 
30 s of iontophoresis, in order to minimise discomfort to the subject, and a safety cut-out 
device which sounded an alarm and stopped iontophoresis if the skin resistance exceeded pre-
set limits. We confirmed that the timing and current generation were accurate, and established 
the cut-out resistance limit, not quoted by the manufacturer, to be approximately 70 kΩ. Many 
normal Afro-Caribbean and some Asian patients have static skin resistance levels in the feet 
exceeding this value (RM Abraham, personal communication, 1988). When iontophoresis was 
successful, most normal and diabetic subjects felt the direct current as a slight tingling or 
burning sensation, very occasionally as mild stinging pain.

The standard metal and lint electrodes in general use for cystic fibrosis testing were 
unsatisfactory for three reasons: (1) over time they develop an irregular surface resulting in 
uneven current delivery, with iontophoresis not occurring over certain areas of the skin; (2) 
the pads are insufficiently flexible to cover irregular surfaces such as the dorsum of the foot; 
and (3) lint pads tend to leak fluid under pressure, and cause unacceptable degrees of skin 
hydration (Kennedy et al. 1983). In order to overcome these difficulties, we subsequently 
adopted the method of Webster (1983), incorporating 1% acetylcholine bromide (Sigma 
catalogue No. A6500) into a 2.5 cm diameter flexible disc made of 30% standard grade phar-
maceutical gelatine (Rothery’s granulated gelatine).

The literature on the heat stability of acetylcholine salts is limited, but there is some data on 
acetylcholine bromide, which retains over 90% of its activity in a 10% aqueous solution for 
more than 3 months, and is heat-stable up to 50°C (Pharmaceutical Society of Great Britain, 
personal communication 1988). Preliminary studies using the agar gel found satisfactory for 
sweatspot testing (Chapter 4) confirmed that:
(a) Adequate mixing of acetylcholine bromide solution and 4% agar gel could not be achieved at temperatures lower than 50°C, by which time the gel had already begun to set. Pilocarpine salts, on the other hand, are heat-stable to much higher temperatures.

(b) Iontophoresis through blank discs of 4% agar left a residue of water on the skin surface which was detected by the hygrometer at 500-1000 ppm, and which was not completely evaporated by 10 min.

We therefore experimented with other gels that were still fluid at 35-45°C, while at a higher concentration than 4% in order to avoid the problem of fluid exudation and skin hydration. The optimum readily available gel was found to be pharmaceutical grade gelatine, which has a standardised viscosity when made up in solution (Bloom grade 250). A 30% gelatine disc was found to cause minimal water exudation (or stimulation of sweat output) after iontophoresis at 1.5 mA for 5 min. Integrated water output after iontophoresis through blank discs in 2 normal subjects was found to be approximately 0.1 mg over a 10 min recording period.

After recording baseline TEWL, iontophoresis was carried out with a blank gelatine disc as the cathode placed proximal to the lateral malleolus, and the acetylcholine-gel anode disc at the test site. After iontophoresis, the acetylcholine-gel disc was replaced by the capsule, and recording started immediately. Recordings were initially continued for 20 min. Minute-by-minute analysis of the preliminary group of subjects showed that there were no significant differences in sweat volumes between diabetic (n=28) and normal (n=15) subjects beyond 12 min (Levy et al. 1991); moreover, 74% of responses in normal subjects had reached within 1 SD of the mean TEWL value by 12 min. Analyses are therefore presented for the 12 min recordings. Three measurements were made:

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) Basal transepidermal water loss (TEWL), mg·cm⁻²·hr⁻¹</td>
<td></td>
</tr>
<tr>
<td>(b) Total integrated sweat volume to 12 min above basal TEWL (TVol12), mg</td>
<td></td>
</tr>
<tr>
<td>(c) Peak sweating rate, mg·cm⁻²·hr⁻¹</td>
<td></td>
</tr>
</tbody>
</table>
3.2.3 Reproducibility

The reproducibility of the method was assessed in 8 normal and 8 diabetic subjects, randomly selected, tested on a separate occasion 2-12 weeks after the first test.

3.3 Pharmacological studies

In order to characterise the pharmacology of the response to acetylcholine, two adjacent areas of the forearm of a normal male subject (age 34) were pre-treated with intradermal injections (in 0.2 ml total volume) of the ganglion-blocking agent hexamethonium bromide (200 μg), and atropine (0.2 μg) (see Collins and Weiner 1961). Ten minutes later, acetylcholine was iontophoresed over the same area and the sweat responses measured.

3.4 Neurological tests

As described in Chapter 1, patients underwent neurological testing of small- and large-fibre tests. The numbers (%) who had each additional test are given below:

<table>
<thead>
<tr>
<th>Test</th>
<th>Count</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Electrophysiology</td>
<td>70</td>
<td>(75%)</td>
</tr>
<tr>
<td>Vibration perception thresholds</td>
<td>69</td>
<td>(74%)</td>
</tr>
<tr>
<td>Marstock thermal thresholds</td>
<td>81</td>
<td>(87%)</td>
</tr>
<tr>
<td>Autonomic function tests:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Respiratory RR variation</td>
<td>86</td>
<td>(92%)</td>
</tr>
<tr>
<td>30:15 ratio</td>
<td>85</td>
<td>(91%)</td>
</tr>
<tr>
<td>Valsalva ratio</td>
<td>71</td>
<td>(76%)</td>
</tr>
<tr>
<td>Postural blood pressure</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(random zero sphygmomanometer)</td>
<td>74</td>
<td>(80%)</td>
</tr>
</tbody>
</table>

3.5 Statistical methods

Probability plots showed that TEWL, TVol12 and peak sweating rate were normalised by logarithmic transformation. Parametric statistics (Student’s t-test and Pearson correlation coefficients) were therefore performed on log-transformed data. Linear least squares regression analysis was used for more detailed analysis of individual associations. 95% confidence intervals for normal values were established using logarithmically transformed data and back-converted (Gardner and Altman 1989). Group comparisons between small numbers of subjects were performed with the non-parametric Mann-Whitney U-test.
3.6 Results

3.6.1 Normal subjects

The normal response consisted of a rapid rise in humidity after the end of iontophoresis to a peak usually between 2-3 min. Thereafter the humidity declined at a variable rate until the recording was terminated at 12 min (Figure 1). Mean TEWL was 1.215 mg·cm⁻²·hr⁻¹; there was no significant change with age (r=0.02, p=0.88). The logarithmically transformed upper 95% confidence limit was 1.979 mg·cm⁻²·hr⁻¹, lower limit 0.717 mg·cm⁻²·hr⁻¹ (Figure 2). Mean TVol12 was 2.398 mg, with no significant change with age (r=0.04, p=0.78); the upper 95% confidence limit was 6.061 mg, the lower limit 0.696 mg (Figure 3). Mean peak sweating rate was 13.035 mg·cm⁻²·hr⁻¹. Like the other measurements, this did not change significantly with age (r=0.26, p=0.09), but the plot of the 95% confidence intervals shows that after logarithmic transformation, the upper limit of normal decreased markedly from 32 mg·cm⁻²·hr⁻¹ at age 20 to 18 mg·cm⁻²·hr⁻¹ at age 70 (Figure 4). These age-related values have been used in the further analysis. There were no gender differences in sweat measurements.
Figure 2: Effects of hexamethonium (200 μg) (curve 2) and atropine (0.2 μg) (curve 3) on acetylcholine-induced sweating (curve 1). Total sweat output (integrated areas under curve) was reduced from 7.35 mg to 3.90 mg (53%) after hexamethonium and to 0.35 mg (5%) after atropine. A similar minimal response (0.50 mg) was obtained after injection of hexamethonium alone (curve 4).

3.6.1.1 Pharmacological studies

Pre-treatment of the iontophoretic area with intradermal hexamethonium reduced the acetylcholine-induced sweat response by approximately 50%; hexamethonium alone gave no significant sweating, and pre-treatment with atropine almost completely abolished the sweat response.

3.6.2 Diabetic patients

Mean TEWL was 1.304 mg·cm⁻²·hr⁻¹, TVol12 2.251 mg and peak sweating rate 13.694 mg·cm⁻²·hr⁻¹. None of these values was significantly different from those in normal subjects. Twelve subjects (14.0%) had abnormal TEWL, 8 high and 4 low (Figure 2). TVol12 was abnormal in 5 subjects (5.8%), 2 with high sweat volumes, 3 with low (Figure 3). Twelve subjects (14.0%) had abnormal peak sweating rates, 9 high, 3 low (Figure 4). TEWL decreased significantly with age (r=-0.27, p=0.01) (Figure 2) but neither TVol12 nor peak rate showed a significant change with age. TEWL was associated with peak sweat rate (r=0.32, p=0.003) but not with TVol12 (r=0.07), and peak sweat rate was closely
correlated with TVol12 (r=0.87, p<0.001). There were no associations with known duration of diabetes, though weight (but not height or body mass index) was significantly correlated with TVol12 (r=0.24, p=0.03) and peak sweat rate (r=-0.26, p=0.02).
Figure 2: Relationship between TEWL rate and age. Upper and lower 95% confidence intervals for the normal range are shown, together with the plotted values for the diabetic patients. There is no significant change of TEWL with age in normal subjects, but a weak age-related change in diabetic patients ($r=-0.27$, $p=0.01$).

Figure 3: Relationship between sweat volume at 12 min (TVol12) and age. There is no significant change with age in either normal or diabetic subjects.
Figure 4: Peak sweating rate after acetylcholine iontophoresis and its relation with age. In common with other measurements requiring logarithmic transformation to achieve normality, the upper 95% confidence interval for the normal population changes significantly with age, while the lower interval shows little change. In this instance, both limits may have physiological relevance.

Figure 5: The weak, though statistically significant, correlation between evoked sweat output and glycated haemoglobin. The relationship ($r = -0.32, p = 0.009$) is further weakened ($r = -0.27, p = 0.03$) by removal of the outlying patient with very poor diabetic control ($HbA_1c = 20\%$). The regression line for the whole group is shown.
3.6.2.1 Diabetic control

Glycated haemoglobin level was negatively correlated with TVol12 (r=-0.32, p=0.009) though the correlation was weakened (r=-0.27, p=0.03) by removal of one subject with a high glycated haemoglobin level (20%) (Figure 5). There was a weakly significant association with peak sweat rate (r=0.24, p=0.05). When one very high random blood glucose measurement (30 mmol/l) had been excluded there was a significant association only with TEWL (r=0.26, p=0.04).

![Figure 6](image)

**Figure 6:** Transepidermal water loss rate (TEWL) and foot temperature in normal subjects. There is a significant increase in TEWL with increasing foot temperature; from the fitted linear regression line, TEWL doubles with an increase of 10°C in foot temperature (r=0.41, p=0.004). This association was not statistically significant in diabetic patients.

3.6.2.2 Effect of environmental factors

Skin surface temperature is known to be an important determinant of TEWL (Bettley and Grice 1965); in this study, there was a limited range of foot temperatures (30-35°C) but there was still a significant correlation with TEWL in normal subjects (r=0.41, p=0.004) (Figure 6) in normal subjects, but not in diabetic patients (r=0.13, p=0.24). Using the linear regression equation, in normal subjects TEWL increased by 0.11 mg·cm⁻²·hr⁻¹ per °C rise in foot temperature, an approximate doubling of TEWL with a 10°C rise. In normal subjects TVol12 showed a decrease with increasing atmospheric pressure (r=-0.28, p=0.06), and a
significant increase with RH \((r=0.38, \ p=0.01)\), but these relationships were not found in diabetic patients. Peak sweating rate was not influenced by environmental factors. In normal subjects foot temperature closely followed ambient temperature \((r=0.42, \ p=0.004)\), but not in diabetic patients \((r=0.09, \ p=0.38)\). In contrast with TEWL, there were no significant correlations between TVol12 and peak sweating rate with surface temperature either in normal or diabetic subjects.

### 3.6.2.3 Association with other neurological measurements (Table 2)

There were significant correlations with systolic and diastolic blood pressure fall, though not with absolute blood pressure measurements. The scattergrams of systolic blood pressure fall with TEWL \((r=0.30, \ p=0.01)\) and peak sweat rate \((r=0.32, \ p=0.007)\), Figures 7 and 8, show that as postural drop increases, sweat function decreases, though the relationships are not linear. Most of the cardiac vagal autonomic function tests, but none of the thermal or vibratory thresholds were significantly associated with all three measurements. Among the electrophysiological measurements, only the f wave latency showed significant associations, with TVol12 and peak sweat rate \((r=-0.30, \ p=0.02; \ r=-0.49, \ p=0.007)\).

**Figure 7:** Postural systolic blood pressure change and TEWL in diabetic patients. Positive values represent a fall, negative values a rise, in blood pressure. Increasing blood pressure fall is associated with decreased transepidermal water output. Quadratic smoothing is shown; the linear regression analysis yields \(r=-0.40, \ p<0.001\). With exclusion of the outlying patient, \(r=-0.39, \ p=0.001\).
Figure 8: Postural systolic blood pressure change and peak sweating rate in diabetic patients. Quadratic smoothing is shown, resulting in a U-shaped distribution, suggesting that patients with either low or high peak sweat rates have disordered sympathetic function, resulting in greater postural blood pressure fall.

Table 2: Correlations between the three primary measurements and autonomic function tests. Numbers of patients are in parentheses; r values are for Pearson correlation coefficients

<table>
<thead>
<tr>
<th></th>
<th>TEWL</th>
<th>TVol12</th>
<th>Peak sweat rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Systolic BP fall</td>
<td>-0.40 (73)</td>
<td>-0.30 (70)</td>
<td>-0.35 (71)</td>
</tr>
<tr>
<td>p &lt; 0.001</td>
<td>p = 0.01</td>
<td>p = 0.004</td>
<td>p = 0.039</td>
</tr>
<tr>
<td>Diastolic BP fall</td>
<td>-0.11 (73)</td>
<td>-0.34 (70)</td>
<td>-0.35 (71)</td>
</tr>
<tr>
<td>p = 0.35</td>
<td>p = 0.004</td>
<td>p = 0.01</td>
<td></td>
</tr>
<tr>
<td>Respiratory RR variation</td>
<td>0.28 (86)</td>
<td>0.21 (83)</td>
<td>0.40 (84)</td>
</tr>
<tr>
<td>p = 0.02</td>
<td>p = 0.07</td>
<td>p = 0.01</td>
<td></td>
</tr>
<tr>
<td>Valsalva ratio</td>
<td>0.34 (71)</td>
<td>0.21 (69)</td>
<td>0.29 (70)</td>
</tr>
<tr>
<td>p = 0.009</td>
<td>p = 0.1</td>
<td>p = 0.02</td>
<td></td>
</tr>
<tr>
<td>30:15 ratio</td>
<td>0.22 (85)</td>
<td>0.22 (82)</td>
<td>0.27 (83)</td>
</tr>
<tr>
<td>p = 0.1</td>
<td>p = 0.06</td>
<td>p = 0.02</td>
<td></td>
</tr>
</tbody>
</table>

3.6.2.4 Relationship between symptomatic autonomic and peripheral neuropathy, sweat measurements and other neurological tests

[For the working definitions of symptomatic autonomic and peripheral neuropathy, based on a symptom questionnaire, see Chapter 1].

Thirty one (36%) of this group of diabetic patients were considered to have symptomatic peripheral neuropathy, and 24 (28%) to have symptomatic autonomic neuropathy; 19 (23%)
had both. It can be seen from Table 3 that both groups were significantly older than asymptomatic patients. Although most measurements show broad concordance in their degree of abnormality compared with asymptomatic patients, TVol12 and peak sweat rates tended to be more abnormal in patients with symptomatic peripheral, rather than autonomic neuropathy. Three of the 8 patients with a postural systolic blood pressure drop of >20mm Hg had low peak sweat rates. Of the remaining 5, all but 1 had symptomatic autonomic neuropathy but had no abnormalities in any of the three sweat measurements.

Table 3: Comparison of neurological measurements in patients with symptomatic autonomic (A) and peripheral (P) neuropathy. Probability values are given for Student’s t-test, standard deviations in parentheses.

<table>
<thead>
<tr>
<th></th>
<th>Symptomatic</th>
<th>Asymptomatic</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>A</td>
<td>49.2 (14.0)</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>51.2 (13.1)</td>
<td>31</td>
</tr>
<tr>
<td>Duration diabetes, y</td>
<td>A</td>
<td>13.0 (11.3)</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>12.1 (9.7)</td>
<td>31</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>A</td>
<td>25.7 (4.4)</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>25.9 (4.6)</td>
<td>26</td>
</tr>
<tr>
<td>TEWL, mg·cm⁻²·hr⁻¹</td>
<td>A</td>
<td>1.14 (0.31)</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>1.15 (0.32)</td>
<td>30</td>
</tr>
<tr>
<td>TVol12, mg</td>
<td>A</td>
<td>2.31 (1.69)</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>1.85 (1.31)</td>
<td>31</td>
</tr>
<tr>
<td>Peak sweating rate, mg·cm⁻²·hr⁻¹</td>
<td>A</td>
<td>12.85 (8.09)</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>10.53 (5.77)</td>
<td>31</td>
</tr>
<tr>
<td>Marstock warm threshold, °C</td>
<td>A</td>
<td>10.0 (5.6)</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>8.8 (6.3)</td>
<td>29</td>
</tr>
<tr>
<td>Marstock cool threshold, °C</td>
<td>A</td>
<td>4.4 (5.9)</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>4.7 (5.5)</td>
<td>29</td>
</tr>
<tr>
<td>Marstock heat pain threshold, °C</td>
<td>A</td>
<td>13.7 (8.4)</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>11.9 (10.3)</td>
<td>27</td>
</tr>
<tr>
<td>Systolic BP fall, mmHg</td>
<td>A</td>
<td>9 (15)</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>10 (13)</td>
<td>25</td>
</tr>
<tr>
<td>Diastolic BP fall, mmHg</td>
<td>A</td>
<td>3 (12)</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>5 (12)</td>
<td>25</td>
</tr>
</tbody>
</table>

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3.6.2.5 Characteristics of patients with abnormal TEWL and peak sweating rates

Patients with abnormally low or high TEWL and peak sweat rates were evenly distributed throughout the age range studied (Figures 2 and 4). Of the 9 patients with high peak sweat rates, 4 had symptomatic peripheral neuropathy and 5 had symptomatic autonomic neuropathy; 4 had both. Three patients had low peak sweat rates. All had both symptomatic autonomic and peripheral neuropathy and 2 had had past episodes of neuropathic foot ulceration. A comparison of neurological measurements in the two groups is shown in Table 4. Patients with high peak sweat rates were non-significantly older, and the median known duration of diabetes was similar. They had a significantly higher body mass index. Thermal thresholds (apart from heat pain thresholds) were similar. Large-fibre tests (biothesiometry and electrophysiology) showed no differences between the two groups. Among tests of autonomic function, the 30:15 ratio was significantly higher, and postural blood pressure fall was lower. In a comparison with patients with peak sweating rates within the normal range, those with high sweat rates had significantly greater height (p=0.007), weight (p<0.001) and BMI (p=0.03), while apart from the strongly height-related shortest f latency (significantly prolonged: median 58.3 ms vs. 51.0 ms, p=0.04), there were no significant differences in neurological function.

Table 4: Comparison of patients with age-related high and low peak sweat rates after acetylcholine iontophoresis. Median values (range) [number of subjects]; p values refer to Mann-Whitney U test

<table>
<thead>
<tr>
<th></th>
<th>High (n = 9)</th>
<th>Low (n = 3)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>58 (29-66)</td>
<td>36 (21-60)</td>
<td>0.27</td>
</tr>
<tr>
<td>Duration diabetes, y</td>
<td>8 (2-34)</td>
<td>9 (2-32)</td>
<td>0.93</td>
</tr>
<tr>
<td>Type 1: type 2</td>
<td>4:5</td>
<td>3:0</td>
<td></td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>27.0 (23.7-35.2)</td>
<td>22.7 (21.6-23.7)</td>
<td>0.04</td>
</tr>
<tr>
<td>TEWL, mg.cm⁻².hr⁻¹</td>
<td>1.14 (1.03-2.61)</td>
<td>0.87 (0.64-1.19)</td>
<td>0.15</td>
</tr>
<tr>
<td>TVol12, mg</td>
<td>5.37 (3.38-6.12)</td>
<td>0.31 (0.17-1.20)</td>
<td>0.01</td>
</tr>
<tr>
<td>Respiratory RR variation, bpm</td>
<td>12 (2-27)</td>
<td>6 (5-8)</td>
<td>0.17</td>
</tr>
<tr>
<td>30:15 ratio</td>
<td>1.04 (0.99-1.64)</td>
<td>1.00 (1.00-1.02)</td>
<td>0.05</td>
</tr>
<tr>
<td>Valsalva ratio</td>
<td>1.51 (1.07-2.13)[7]</td>
<td>1.16 (1.11-1.21)[2]</td>
<td>0.24</td>
</tr>
<tr>
<td>Marstock warm threshold, °C</td>
<td>11.6 (5.1-16.2)</td>
<td>14.7 (13.5-15.8)</td>
<td>0.16</td>
</tr>
<tr>
<td>Marstock cool threshold, °C</td>
<td>4.2 (1.9-8.3)</td>
<td>10.7 (6.0-15.3)</td>
<td>0.19</td>
</tr>
<tr>
<td>Marstock heat pain threshold, °C</td>
<td>15.5 (14.5-16.8)[7]</td>
<td>17.8 (17.7-17.9)[2]</td>
<td>0.04</td>
</tr>
<tr>
<td>Systolic BP fall, mmHg</td>
<td>4 (-2-16)</td>
<td>22 (13-48)</td>
<td>0.04</td>
</tr>
<tr>
<td>Diastolic BP fall, mmHg</td>
<td>0 (-6-12)</td>
<td>6 (0-32)</td>
<td>0.09</td>
</tr>
</tbody>
</table>

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Eight patients had high TEWL, and 4 low TEWL. Only one of those with high TEWL had symptomatic peripheral neuropathy. Of those with low measurements, three had at least one age-related abnormal cardiovascular autonomic test, and all had symptomatic autonomic neuropathy. There were no significant differences in any of the neurological measurements between those with normal and those with abnormal TEWL, though random blood glucose was significantly higher in patients with high TEWL compared with those with normal TEWL; median (range) 12.9 mmol/l (6.8-16.8) vs. 7.6 (5.8-12.2), p=0.03).

3.6.3 Reproducibility

Repeat testing in 8 normal and 8 diabetic subjects gave the following coefficients of variation (CV%) in normal subjects: median CV% of TEWL was 7.9% (range 1.6-31.5), TVol12 24.0% (range 1.4-58.1) and of peak sweating rate 11.8% (range 2.2-141.4). In the diabetic patients median CV% of TEWL was 12.3% (range 3.6-32.0), TVol12 22.0% (range 5.0-42.0) and of peak sweating rate 16.7% (range 3.4-37.0).

3.7 Discussion

This study, using dewpoint measurements, has demonstrated that quantitative sweat responses can be measured with high precision and acceptable reproducibility. At this concentration of acetylcholine, the induced sweat response is quantitatively approximately 50% nicotinic in type, as hexamethonium pre-treatment reduced the acetylcholine sweat response by one-half. The precision of the sweat measuring method also allows basal sweat output (0.5–2.0 mg·cm⁻²·hr⁻¹), a measurement not previously evaluated in diabetic neuropathy, to be accurately quantified. After acetylcholine iontophoresis, we have defined a small group of unequivocally neuropathic subjects with sudomotor failure, and a larger group with high peak sweat output, probably due to denervation supersensitivity, of whom 50% had evidence of autonomic neuropathy. These patients were also significantly taller and heavier, suggesting that supersensitivity in this clinical model is length-dependent. Of the three sweat measurements, integrated sweat response up to 12 min after iontophoresis yielded the lowest rate of abnormality, whereas basal and peak sweating rates were abnormal in 13–14%. Sweat measurements were significantly associated with cardiac autonomic function tests and postural blood pressure changes, but not sensory thresholds or electrophysiology. The strong correlation between TEWL and postural systolic blood pressure fall suggests that this measurement is potentially useful in the assessment of sympathetic nerve function.
The only previous study to have examined dynamic sudomotor function in diabetes has been that of Low et al. (1983); his method differs from the current study in several respects. In both, the ventilated capsule has been modified to measure small quantities of sweat evaporated from the skin by a stream of dry gas. Low measured sweat with a relative humidity (RH) hygrometer. He overcame one of the problems of RH measurement, its temperature dependence, by heating the carrier gas (nitrogen) to approximately 38°C to ensure rapid evaporation of sweat, a temperature which may be high enough to stimulate thermal sweating (see Chapter 2). Since there is no simple relationship between RH and vapour pressure, the area under an RH curve cannot be integrated to yield directly the mass of water evaporated, and Low used a calibration curve to convert arbitrary measurement units into water mass. Use of dewpoint hygrometry permits direct quantitation of sweat volumes by the use of the dewpoint equations discussed in Chapter 2. The temperature of the carrier gas does not affect dewpoint measurements, and air at ambient temperature can be used.

The reproducibility of the current method is similar to that of other neurological measurements used in diabetic neuropathy. Low et al. (1983) did not quote reproducibility, but plotted a scattergram of duplicate measurements in 11 diabetic subjects. The regression line plotted suggests that there was a systematic decrease in sweat output between the two tests, compatible with a shift in sensor characteristics. However, recalculation of his data gives a median CV of approximately 14% (approximate range 0-30%). The CV in three subjects tested on 3-7 occasions was 10-50%.

A major difference between the studies relates to the nature of the sweat response. Low used a compartmentalised sweat capsule consisting of a central well, through which the gas was passed, and from which the sweat responses were measured, separated circumferentially from an outer iontophoresis well. By ensuring that the capsule was tightly attached to the skin, diffusion of the acetylcholine towards the centre well was prevented, and the direct muscarinic sweat responses thereby eliminated (see Introduction, section 2.4.1.3). Saline iontophoresis gave no sweat response, and infiltration of local anaesthetic in the space between the stimulating and recording wells abolished the acetylcholine response, confirming that sweating was mediated via unmyelinated fibres. The acronym “Q-SART” (quantitative sudomotor axon reflex test) was coined for this procedure. It precludes detection of muscarinic sweat gland supersensitivity; however, both his original study and that of Ahmed and Le Quesne (1986), using the same apparatus, confirmed that the method sometimes detects nicotinic sweat gland supersensitivity.
Low did not regard high sweat output, even that resulting from nicotinic cholinergic stimulation, as an adequate criterion of abnormality. Our own results confirm that 50% of patients with muscarinic sweat gland supersensitivity have documented evidence of neuropathy in other tests, and that increased peak sweating rate should, within the bounds of the reproducibility of the method, be regarded as evidence of sweat gland denervation supersensitivity and be also considered an abnormal finding. However, isolated high sweat responses must be interpreted with caution; psychological and thermal stimuli may cause spuriously high sweat output, and there were two young subjects with high peak sweat outputs in whom there was no evidence either of symptomatic neuropathy or of neurological abnormalities on objective testing.

Using simultaneous stimulation and recording Low was also able to measure the response latency. He regarded an abnormal latency as a less significant finding than an abnormal sweat volume, but he presented no evidence for this view. Even allowing for a delay in detecting moisture at the hygrometer the median latency of the Q-SART response in normal subjects (1.7–1.8 min) is longer than expected for an axon reflex; when cholinomimetic agents are given by intradermal injection, a sweat response occurs within a few seconds (Collins and Weiner 1961; Chapter 6). The time taken for conduction of impulses in the axon reflex pathway, even in a reticular arrangement of slowly conducting nerve fibres, and the time taken for neuroglandular transmission (see Chapter 5), are together unlikely to account for more than a few seconds. It seems likely that much of the observed latency is accounted for by the time taken for the iontophoresed acetylcholine to pass through the high resistance layer of the epidermis to the receptor points, though there is evidence that iontophoresed chemicals act on sweat glands via facilitated transport through the low-resistance channels of the sweat gland ducts themselves (Webster 1983). The latency found by Low et al. (1983) is consistent with either subepidermal or dermal nerves acting as receptors, or, as suggested by Low, the sweat gland epithelium itself. Further studies are required to assess the value of latency measurements as an indicator of nerve dysfunction.

Pharmacological characterisation of the response used in our study suggests that in the concentration used, acetylcholine was acting partly as a muscarinic, and partly as a nicotinic agent, since the sweat response was reduced by about 50% when the test area was infiltrated by hexamethonium. Low proposed that the nicotinic axon-reflex sweat response fails earlier than sweat responses elicited by muscarinic agents; in addition, he found that there was a small number of patients who had abnormal Q-SART responses in the presence of normal respiratory RR variation and Valsalva ratio (though there was also a similar number with nor-
nal Q-SART and abnormal vagal tests). He has therefore proposed the Q-SART as a more sensitive indicator of neural damage than measurement of the direct (muscarinic) sweat response. Since sweating thresholds to acetylcholine show high inter-subject variability (Chalmers and Keele 1952; MacMillan and Spalding 1969), care must be taken to deliver an adequate stimulus in order to avoid false negative responses in subjects with high sweat thresholds. Low et al. (1983) quoted two unpublished findings supporting the adequacy of the stimulus he used. First, he found that the Q-SART response was quantitatively larger than that evoked by intradermal acetylcholine and second, absent Q-SART responses were confirmed by the absence of response to intradermal acetylcholine. These findings are contrary to other reports in the literature. The direct sweat response is generally more intense than axon-reflex sweating (Randall and Kimura 1955). Since the response to intradermal acetylcholine is considered to be predominantly muscarinic (see Introduction, section 2.4.1.3) the converse would be expected; that is, the Q-SART response should be smaller than the response to intradermal acetylcholine. Since the effective stimulating dose when given by iontophoresis is not known, it is in any case difficult to compare the two responses. In addition, in the presence of denervation supersensitivity, intradermal acetylcholine would be expected to evoke a large response while the axon reflex response is absent. We have confirmed that intradermal methacholine, a stable acetylcholine analogue, does not give reduced responses in either non-neuropathic or severely neuropathic subjects, and indeed the response in definite small-fibre neuropathy is invariably increased (see Chapter 6). The question of false-negative results as a result of inadequate acetylcholine stimulation in Low's model therefore requires further consideration.

We have ensured an adequate stimulus by using sufficient time-current over a large area for electrophoresis so that a measurable sweat response is elicited in all subjects. This may, however, be achieved at the expense of some sensitivity. Our method also overcomes the problem of the density of receptors for axon-reflex sweating. Using point electrical stimulation to elicit axon-reflex sweating Bickford (1938) found a high variability of responses in the forearm and Chalmers and Keele (1952) and Collins and Weiner (1961) noted the high variability of axon reflex sweat responses to intradermal nicotine, suggesting that receptor points have both a low density and a variable threshold. Our own quantitative observations have confirmed these findings for intradermal nicotine (Berg et al. 1989, unpublished observations) and direct electrical point stimulation (Levy and Reid, 1989, unpublished observations) in pilot studies for the current work. Both these features are likely to be changed in neuropathic subjects. Low's stimulating well has an area of only approximately
7 mm². Until the receptor density is known, it would seem prudent to use a large, standardised stimulus.

Our study found a low prevalence of subnormal sweat responses, but when they occurred, they were often found in patients who had overt symptomatic neuropathy. They may also have evidence of structural degeneration of sweat glands, such as we found in skin biopsies of severely neuropathic diabetic patients (Levy et al. 1989). Other studies have found that structural changes in sweat glands require up to 2 years of complete denervation to occur (Silver et al. 1963), suggesting severe neuronal depletion. TEWL in this group was lower than in asymptomatic diabetics, and it is likely that this measurement, rather than the stimulated measurements, reflects the common observation of “dry skin” in neuropathic diabetic subjects. This group may be at high risk from heat stroke, because of their inability of their sweat glands to respond to heat stress. Collins and Weiner (1961), discussing axon-reflex sweating, considered that it played little or no role in thermoregulation. The Q-SART, therefore, would probably be of less value than the current test in identifying patients at risk from defective thermal sweating.

Several observations on environmental measurements may be of physiological significance in diabetes. There is a complex relationship between core and skin temperatures and sweating (Brener and Kerslake 1961; Ogawa and Asayama 1986). Core temperature was not measured in our study, but there is no indication that diabetic patients have abnormal body temperature. TEWL increased with increasing ambient temperature in normals but not in diabetic patients. This has not been noted previously, and may be another factor in the poor performance of diabetic patients under conditions of heat stress. Conversely, we have confirmed cross-sectionally that TEWL decreases with increasing ambient RH in normal subjects (Goodman and Wolf 1969; Grice et al. 1972). Goodman and Wolf (1969) analysed this relationship in detail. They concluded that the passive element of TEWL (diffusion through the stratum corneum) was relatively unaffected by ambient vapour pressure, and that most of the change in TEWL with increasing humidity was due to evaporation from a small number of sweat glands. The lack of change of TEWL with increasing RH in diabetic subjects therefore suggests dysfunction of sweat glands rather than of the skin barrier, and may also result in high skin water loss under conditions of high environmental humidity. This conclusion is supported by the experimental arrangement used here, where the air passing over the skin was of a constant and for practical purposes, negligible humidity, compared with the earlier studies, where conditioned air of different humidity was used. It appears that the eccrine glands of the body surface respond coherently to ambient humidity, though the mechanism
of this response is unknown. Our other finding, of an impaired increase in TEWL in a warm environment, suggests some degree of sympathetic dysfunction. High TEWL was found in several patients, but they were as a group non-neuropathic, both symptomatically and on the basis of neurological tests, and since this measurement is particularly strongly influenced by environmental and emotional factors (Nilsson 1978), it cannot be regarded as a neurologically significant finding, unlike elevated peak sweating rates.

The total volume of sweat produced yielded a lower rate of abnormality than either the peak sweat rate or the basal skin water loss, in direct contrast with the findings of Low et al (1983) who used evoked sweat volume as a primary neuropathic index. While the peak sweating rate is well correlated with the total sweat volume, in older normal subjects it was common to find that the sweat rates declined very slowly, rather than showing the rapid decline found in most normal subjects. This may represent a normal ageing process, resulting in minor unmyelinated nerve damage and consequent mild denervation supersensitivity, such as has been found in the pupil (see Introduction, section 1.4.9.3). This may be one reason why total sweat volume yielded a wide normal range and a consequent low rate of abnormality in diabetic patients. Another may be the high intensity of the stimulus, which may swamp the responses of those glands with borderline impaired innervation. A graded stimulus using various levels of current intensity might be able to identify more readily a population of glands with incipient abnormalities of threshold.

The number of abnormal responses should be interpreted in the light of the patient population studied. Low et al. (1986) tested a large group of patients, all with symptoms, signs, and abnormal nerve conduction studies. It was therefore a highly selected group, considerably older than those studied here (mean age 52.5y, maximum age 78y vs. mean age 42.7y, maximum age 66y); 55% had two abnormal cardiac autonomic tests (Valsalva ratio and respiratory RR variation), implying appreciable autonomic neuropathy. The patients in the current study were randomly selected from our clinic population, and the prevalence of neuropathy was lower. For example, just over one quarter had symptomatic autonomic neuropathy, and approximately one third symptomatic peripheral neuropathy. Using similar criteria to Low’s, 17 (22.6%) had abnormal age-related respiratory RR variation, 7 (10.9%) an abnormal Valsalva ratio, and only 5 patients (7.8%) had abnormalities in both tests. While the sensitivities of the individual tests will vary from centre to centre, it is clear that the current study has tested a less neuropathic group of patients.

The associations with other tests of neurological function showed some selectivity for autonomic measurements. The lack of association with tests of somatic nerve function (thermal
and vibratory thresholds and electrophysiology) is notable. In view of the known variability of postural blood pressure fall, the close association between the sweat measurements and systolic blood pressure fall is striking. These associations included both non-stimulated (TEWL) and stimulated (TVol12 and peak rate) sweat measurements; basal and stimulated sympathetic nerve activity appear both to be reflected in these blood pressure measurements. Microneurography (see Introduction, section 1.4.2.1) would be required to confirm this view.

The concordance of abnormal TEWL and peak sweat rate measurements was low. Eight of the 12 patients with abnormal (high or low) peak sweat rates, but only 4 of 12 with abnormal TEWL had symptomatic autonomic neuropathy, and only 2 had abnormalities in both measurements. There were no significant differences in any of the neurological measurements between those with normal and those with abnormal TEWL, though random glucose was significantly higher in patients with high TEWL compared with those with normal TEWL; furthermore, there was a significant positive association between TEWL and random blood glucose though not between TEWL and glycated haemoglobin. Several studies have demonstrated the association between glucose loading and enhanced sympathetic nerve activity; Berne et al. (1989) presented microneurographic evidence that muscle sympathetic activity, but not skin sympathetic activity, increases with oral glucose administration in normal people, and that this increase is correlated with an increase in plasma noradrenaline. The studies are of different design, and further experiments will be required to establish the effect of active glucose ingestion in diabetic subjects. If, as suggested, TEWL reflects skin sympathetic activity, there seems to be a paradoxical increase in basal sweating rates with glucose levels in diabetic subjects not observed in normal volunteers. Further studies are needed to explore the interrelationships between glucose levels, modulation of the sympathetic nervous system and sweating activity.

Low has not compared the Q-SART response with other measurements of small-fibre function, and its specificity as a measure of sympathetic function cannot be assessed. It has been shown to have a high concordance with the sympathetic skin response (Maselli et al. 1989), in that it is absent in a high proportion of subjects who have an absent skin response, but, as discussed in Chapter 5, the sympathetic skin response is an aggregate response in a long multineuronal pathway. Its absence cannot therefore be confidently ascribed to a lesion in the terminal portion of the pathway.

In conclusion, basal and acetylcholine-stimulated sweat responses are closely associated with other tests of autonomic function, especially those mediated through the sympathetic nervous
system. Elevated peak sweating rates have been demonstrated, suggesting denervation supersensitivity in some patients, but most of these patients do not have abnormal sweat volumes. These sweat responses are not associated with other conventional measurements of neurological function, in particular thermal threshold measurements, suggesting that peripheral sympathetic denervation proceeds independently of neurological dysfunction elsewhere in diabetes. The high interinividual variation of the measurements precludes their use as a diagnostic test of diabetic neuropathy. Basal transepidermal water loss rates can be accurately measured with the sudorometer described here, and under conditions of strict control of skin temperature may be a non-invasive quantitative index of skin sympathetic activity.
Chapter 4

Changes in cholinergic sweat gland activation in diabetic neuropathy identified by computerised sweatspot analysis

4.1 Introduction

Sweat secretion in response to local chemical stimulation is known to be dependent on an intact postganglionic sympathetic supply to the sweat glands (Kennedy et al. 1984a, 1984b) and early studies confirmed that sweat glands were unresponsive to cholinergic agents immediately after acute denervation (List and Peet 1938a; Simeone et al. 1951). In this respect they appeared to be an exception to Cannon's law, which linked supersensitivity of denervated autonomic structures to application of their endogenous neurotransmitter (Cannon 1939; see Introduction, section 2.3.1). However, the results of these acute studies may not accurately reflect the situation in chronic neuropathies, where the dual processes of denervation and reinnervation probably occur concomitantly (Kennedy and Sakuta 1984; Rogers and Levy 1988), and more recent studies have identified supersensitivity, both in the sweat glands (Low et al. 1983; Low et al. 1986; Chapter 3), and in the pupil (Sigsbee et al. 1974; Hayashi and Ishikawa 1979; Smith and Smith 1983).

Several studies have used manual methods to count activated sweat spots in the feet (Kennedy et al. 1984a, 1984b; Ryder et al. 1988), though recently Kennedy has used image-analysis for sweatspot counting in diabetic patients (Kennedy and Navarro 1989b). Both groups reported a decrease in the density of activatable sweat glands in diabetes which paralleled the deficit in other small nerve fibre measurements. In a distal part of the foot, Kennedy et al. (1984a, 1984b) found that up to 57% of diabetic patients had decreased pilocarpine-activated sweat glands, while Ryder et al. (1988) concluded that the number of acetylcholine-activated sweat glands was a more sensitive indicator of autonomic nerve dysfunction than either cardiovascular vagal function or dark-adapted pupil size. Ryder et al. (1988) studied impotent patients, while Navarro and Kennedy (1989) reported their findings in a large group of patients, most of whom had significant neuropathy, and many of whom were enrolled in the Minnesota pancreatic transplantation program.

The aim of the current study was to increase the precision of sweatspot measurements by developing a computerised method for measuring the density of pilocarpine-activated sweat glands in a group of randomly-selected diabetic patients. We have therefore used an image-analysis system to automate counting. These measurements have been compared with small- and large-fibre neurological tests in order to further characterise patients with abnormal sweat gland activation.
4.2 Subjects and methods

4.2.1 Subjects

(For details of selection of normal and diabetic subjects, see Appendix A). Fifty-three normal and 80 diabetic subjects were studied. Their clinical characteristics are given in Table 1. Subjects were asked not to use any skin preparation on the feet for 2 weeks before the test.

Table 1: Clinical characteristics of the normal and diabetic subjects. Data are presented as mean (SD, range) [number of patients]

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<tr>
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<th>Diabetic</th>
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<tr>
<td>Number</td>
<td>53</td>
<td>80</td>
</tr>
<tr>
<td>Age, y</td>
<td>45.6 (12.0, 19–79)</td>
<td>43.6 (14.9, 17–66)</td>
</tr>
<tr>
<td>Gender (M:F)</td>
<td>26:27</td>
<td>53:27</td>
</tr>
<tr>
<td>Type 1: Type 2</td>
<td>–</td>
<td>45:35</td>
</tr>
<tr>
<td>Duration diabetes, y</td>
<td>–</td>
<td>11.8 (9.2, 1–34)</td>
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<tr>
<td>Symptomatic peripheral neuropathy (%)</td>
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<td>30 (38%)</td>
</tr>
<tr>
<td>Age, y</td>
<td>–</td>
<td>52.1 (11.7, 23–66)</td>
</tr>
<tr>
<td>Duration diabetes, y</td>
<td>–</td>
<td>12.5 (9.6, 1–30)</td>
</tr>
<tr>
<td>Symptomatic autonomic neuropathy (%)</td>
<td>–</td>
<td>25 (32%)</td>
</tr>
<tr>
<td>Age, y</td>
<td>–</td>
<td>50.4 (12.7, 21–66)</td>
</tr>
<tr>
<td>Duration diabetes, y</td>
<td>–</td>
<td>13.2 (10.5, 2–34)</td>
</tr>
<tr>
<td>Glycated haemoglobin, %¹</td>
<td>–</td>
<td>11.2 (2.8, 6.5–16.5)</td>
</tr>
<tr>
<td>Random blood glucose, mmol/l</td>
<td>–</td>
<td>9.6 (4.3–19.0) [60]</td>
</tr>
</tbody>
</table>

¹ Reference range <8.0%

4.2.2 Methods: general aspects

Tests were performed in a warm room, 21–26°C, with the subject semi-recumbent. The foot was left uncovered for at least 10 min before testing started. Foot temperature was maintained at 30–34°C. If necessary, feet were warmed with cellular blankets and convective heating. The minimum temperature was not reached in 3 diabetic subjects (mean temperature 29.2°C) and in 1 normal subject. These data were not discarded as we were unable to find a relationship between between foot temperature and sweat responses in either diabetic or normal subjects (see Results, 4.4.1.3, 4.4.2.2).
4.2.3 Indicator method

Indicator papers were prepared according to the method of Sakurai and Montagna (1964). The principle of the test is based on the colour change of bromophenol blue in aqueous alkaline media from light tan to dark blue. Bromophenol blue (BDH Catalogue No. 20015, pH reference range 2.8–4.6) was dissolved in acetone to produce a 4% solution (weight/volume). Circles of Whatman filter paper (Type 1, diameter 4.25 cm; Whatman, Maidstone, UK) were dipped in the solution, rapidly air-dried, cut into semi-circles and stored in an airtight container until use.

4.2.4 Sweat induction

Sweating was stimulated using the iontophoresis equipment described in Chapter 2, section 3.2.2.2, delivering 1.5 mA. Pilocarpine was incorporated into 2.5 cm diameter agar gel discs for use in the recessed iontophoresis electrodes. Batches of discs were made up by melting 90 ml aliquots of 4% agar (Oxoid nutrient broth No.2). Anhydrous pilocarpine nitrate (Sigma Catalogue No. P6503) was stored in a desiccator at −20°C and weighed into 1 g aliquots. Pilocarpine is heat-stable up to 90°C (Pharmaceutical Society of Great Britain, personal communication, 1988) and was dissolved in 10 ml warm deionized water before mixing thoroughly with the agar. The resulting 100 ml of 1% solution was poured into 50 ml graduated petri dishes, cooled, and stored at 4°C. Discs for the indifferent electrode (cathode) contained agar only. The petri dishes were brought to room temperature about one hour before use, and discs cut with a stainless steel punch.

4.2.5 Test procedure

Iontophoresis was carried out with the anode over the eminence of the left extensor digitorum brevis and the cathode placed just proximal and medial to the ipsilateral lateral malleolus. Both electrodes were attached with Velcro straps, and inspected to ensure that they were in contact with skin over their whole area. The anodal disc was marked at four points around the edge with an indelible pen. After the 5 min iontophoresis period the electrodes were removed and a stop-clock started. In preliminary tests, very few sweat spots were activated immediately after iontophoresis. The first imprint was therefore taken at 4 min, and at 4 min intervals to 20 min, by which time there was no significant increase in sweatspot activation (see Results, section 4.4.1). In order to record sweatspots from the same anatomical area,
the middle of the edge of the semicircular indicator paper was aligned with one of the marks around the anode. The paper was applied to the skin with a 2.5 cm² block of foam rubber applied perpendicularly to the test site. Visible sweatspot patterns were consistently produced with 1 min application.

4.2.6 Image storage and analysis

Because of absorption of atmospheric moisture onto the paper, the sweatspot pattern progressively faded after about 48 h. The images were therefore photographed with colour film (Polaroid type 669) immediately after the test, using a Polaroid camera, at ×3 enlargement. In order to obtain consistent colour density, prints were allowed to develop for 5 min.

Previous studies of sweat gland distribution used manual counting methods. In the current study there were up to 600 sweatspot imprints at 20 min in normal subjects. Manual counting would have been subject to error, and the photographs were therefore processed by image-analysis (Quantimet 970, Cambridge Instruments [now Leica UK], Cambridge, UK).

The system used a video camera linked to a computer; an individual interactive software program was written for the analysis. The print, trimmed of white edging, was placed on an illuminated scanning table, the illumination level being automatically adjusted by the image-analyser to optimise contrast. Scaling and contrast sensitivity was established before each measuring session. The image was initially scanned 4 times, in order to minimise background artefact, and presented to the operator on a video display. Each sweat spot in the image was outlined and its maximum diameter measured. The area of interest was outlined by optical mouse, and the derived measurements based on the area of interest produced in hard copy. Edges of the indicator paper and large artefacts (eg. occasional photographic reflections), were excluded from the area analysed by use of the mouse device.

4.2.7 Measurements

The three primary measurements used were:

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>Total number of sweatspots in the area of interest</td>
</tr>
<tr>
<td>2</td>
<td>Area scanned (cm²)</td>
</tr>
<tr>
<td>3</td>
<td>Maximum diameter of each sweatspot (mm)</td>
</tr>
</tbody>
</table>

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Five derived measurements were produced:

1. Sweatspot density (no./cm²)
2. Mean maximum diameter of sweat spots (mm) and standard deviation
3. Estimated total volume of sweat (μl). Each sweat droplet was assumed to be hemispherical with maximum diameter as measured. Summation of all the individual sweat droplet volumes gave an approximate total volume of sweat secreted over each 4 min period, not taking into account the evaporation rate from the sweat spots between each sweat print.
4. Ratio of the area occupied by all sweat spots as a percentage of the total area scanned
5. Histogram of sweatspot size distribution, bandwidth 0.05 mm

For examples of the computerised presentation of these results, see Figures 2 and 3. In the analysis, sweatspot density and the mean sweatspot diameter have been used as the independent measures of sweating activity.

4.2.8 Other neurological measurements

Patients underwent other neurological tests, as described in Chapter 1. The number (percentage) who had each test was as follows:

<table>
<thead>
<tr>
<th>Test</th>
<th>Number (Percentage)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Electrophysiology</td>
<td>62 (79%)</td>
</tr>
<tr>
<td>Vibration perception thresholds</td>
<td>64 (82%)</td>
</tr>
<tr>
<td>Marstock thermal thresholds</td>
<td>74 (95%)</td>
</tr>
<tr>
<td>Cardiovascular autonomic function tests:</td>
<td></td>
</tr>
<tr>
<td>Respiratory RR variation</td>
<td>67 (85%)</td>
</tr>
<tr>
<td>Valsalva ratio</td>
<td>64 (82%)</td>
</tr>
<tr>
<td>30:15 ratio</td>
<td>76 (97%)</td>
</tr>
<tr>
<td>Postural blood pressure</td>
<td>67 (86%)</td>
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</tbody>
</table>

4.2.9 Reproducibility

To assess the variability of the computerised measurement method, randomly-selected sweatspot prints of 7 normal and 7 diabetic subjects were analysed on a different day. Intra-patient variability was assessed by repeat tests on the same foot in 6 normal and 8 diabetic patients, randomly-chosen, 2–24 weeks apart.
4.3 Statistical analysis

Probability plots confirmed that all data were normally distributed. Upper and lower 95% confidence limits were therefore derived from values of the mean and standard deviation. Comparisons between groups were made using Student’s t-test and correlations using Pearson correlation coefficients, with Bonferroni corrected probability values for multiple comparisons. Linear least squares regression analysis was used for more detailed analysis of individual associations. Non-parametric statistics were used for sub-group analysis of small numbers of subjects and for analysis of the coefficients of variation.

4.4 Results

4.4.1 Normal subjects

Iontophoresis was successful in all but one subject. The mean number of activated sweat glands was 65/cm² (SD 28) at 4 min and 72/cm² (SD 31) at 8 min, a significant change (p < 0.001). There was a further statistically significant rise (p < 0.001) at 12 min to 76/cm² (SD 30). Thereafter the number stayed constant at 76/cm² (SD 28) up to 20 min (Figure 1). Mean sweatspot size showed a slight early increase (0.295 mm (SD 0.054) at 4 min, 0.303 mm (SD 0.055) at 8 min, p = 0.07), but thereafter remained constant up to 20 min. The calculated total sweat volume also increased significantly between 4 min (1.71 µl (SD 1.54)), and 8 min (2.23 µl (SD 2.37), p = 0.02), but not thereafter. Therefore the early increase in sweat production after pilocarpine iontophoresis in normal subjects is predominantly due to sweat gland recruitment and not to the quantity of sweat secreted by each gland. None of the remaining derived parameters changed significantly between 8 and 20 min. In all subsequent analyses, the mean values of the measurements made at 8, 12, 16 and 20 min have been used, except for sweatspot density, for which the mean of the 12, 16 and 20 min values were used. Figure 2 shows a series of Polaroid photographs for a normal male subject, age 34.

In normal subjects, sweatspots were uniform in size and evenly distributed. In some subjects with high sweat gland densities, sweatspots in some areas appeared brownish-yellow rather than deep blue. Herrmann et al. (1951) found that this occurred in acidic conditions. While the sweatspot positions were obvious, there was insufficient contrast with the background to be reliably detected by the image analyser, and affected areas were excluded.

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4.4.1.1 Associations between sweatspot measurements

Mean sweatspot density and size were closely correlated ($r=0.60$, $p<0.001$), (Figure 3)) as were calculated sweat volume and both sweatspot size ($r=0.88$, $p<0.001$) and density ($r=0.75$, $p<0.001$).

4.4.1.2 Gender

Mean sweatspot density was non-significantly lower in females than males (male: 83/cm² (SD 26), n=26, female: 69/cm² (SD 30), n=27, $p=0.09$); the mean value for males and females grouped together has therefore been used (76/cm² (SD 28)), yielding an upper 95% confidence limit (mean + 2SD) of 132/cm² and a lower limit of 20/cm². However, the mean sweatspot size was significantly lower in females (male: 0.330 mm (SD 0.049), female: 0.272 mm (SD 0.035), $p<0.001$). Values derived from the sweatspot size were therefore also significantly lower eg. sweat volume, male: 1.41 µl (SD 0.51), female: 0.67 µl (SD 0.56), $p<0.001$; ratio of area occupied, male: 5.64% (SD 2.57), female: 2.74% (SD 1.98), $p<0.001$.

Figure 1: Sweatspot activation in normal (filled bars) and diabetic subjects (hatched bars). Sweatspot density increased significantly in normal subjects to 8 min, but to 12 min in diabetic patients.
Figure 2: (Previous page). Sweatspots in normal male subject age 34. The three sweatspot prints (left) were taken at 12, 16 and 20 min after the end of iontophoresis, and are shown at actual size (3x enlarged Polaroid print). There has been some loss of contrast of the image (originally blue sweatspots on pale yellow ground) in monochrome reproduction. The corresponding computerised measurements are shown on the right, together with the size-frequency distribution histograms. The mean sweatspot density shows little variation at 124-126/cm².
Figure 3: Correlation between mean maximum sweatspot diameter and sweatspot density in normal subjects (upper panel) and in diabetic patients (lower panel). Normal: \( r = 0.60, p < 0.001 \); diabetic: \( r = 0.41, p < 0.001 \).
DISTRIBUTION OF FOSSIL COUNT vs LENGTH

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Interpreted Count = 2
Overlooked Count = 2

LENGTH (mm)

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SWEET VOLUME / SQCM

2.28% based on dist. data

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DISTRIBUTION OF FOSSIL COUNT vs LENGTH

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SWEET VOLUME / SQCM

2.77% based on dist. data

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SWEET VOLUME / SQCM

2.65% based on dist. data

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122
Figure 4: (Previous page). Representative sweatspot prints (all taken at 16 min) in three diabetic patients, with their associated computer output. Top: Male type 1 (insulin-dependent) patient, age 43 (mean sweatspot density 73/cm²). Middle: Asymptomatic female type 2 patient, age 54 (mean density 17/cm²). Bottom: Female type 1 patient, age 44 with symptomatic autonomic neuropathy and a previous episode of painful neuropathy, showing increased sweatspot density (132/cm²). In all cases, sweatspot activation is subjectively more patchy than in the normal subject (Figure 2).
4.4.1.3 Age and skin surface temperature

No measurements changed significantly with age (sweatspot density: \( r=-0.10, p=0.57 \); sweatspot size: \( r=0.01, p=0.97 \); sweat volume: \( r=0.11, p=0.46 \)). Age-correction for these variables is therefore not required. Skin surface temperature and sweatspot measurements were not significantly correlated.

4.4.1.4 Ethnic origin

All but three normal subjects were European; insufficient data were therefore available to compare ethnic groups.

4.4.2 Diabetic patients

Iontophoresis was unsuccessful in 6 Afro-Caribbean and 3 Asian subjects. Three Afro-Caribbeans habitually used oil on the feet, and neither stopping the use of these preparations 2 weeks before the test nor gentle skin abrasion at the site of the indifferent electrode resulted in skin resistance levels below 100 k\( \Omega \). The 3 Asian subjects did not use skin preparations, but clinically the skin on their feet felt dry and hyperkeratotic.

Two subjects, both with severe symptomatic neuropathy, had no sweatspots detectable throughout the test; both were young and Caucasian (one male, age 23, one female age 30). In the remaining patients, the mean number of sweatspots at 4 min was 56/cm\(^2\) (SD 38). As in normal subjects there was a statistically significant rise at 8 min (65/cm\(^2\) (SD 42), \( p<0.001 \)) but there was a continuing slight rise at each 4 min interval to reach 74/cm\(^2\) (SD 43) at 20 min. None of these changes achieved statistically significant levels (Figure 1). Mean sweatspot density was slightly lower in diabetic patients at all times, but none of these differences was statistically significant, nor were the averaged values from 12–20 min.

Mean sweatspot size did not significantly increase during the test (4 min: 0.302 mm (SD 0.054); 20 min: 0.309 mm (SD 0.056), \( p=0.4 \)), and the calculated total sweat volume showed only a borderline significant increase between 4 min and 8 min (4 min: 1.69 \( \mu l \) (SD 1.74); 8 min: 2.07 \( \mu l \) (SD 2.56), \( p=0.07 \)), and between 8 min and 12 min (12 min: 2.39 \( \mu l \) (SD 3.15), \( p=0.05 \)). There were no significant differences in mean sweatspot size or mean sweat volume between normal and diabetic subjects. All measurements in diabetic subjects had a greater SD than in normal subjects. This quantitatively reflects the impression of variable size and distribution of sweat glands in diabetic subjects. Mean gland density and
sweatspot size were closely correlated \( r=0.41, p<0.001 \), but this relationship was not as close as in normal subjects (Figure 3).

### 4.4.2.1 Gender

The findings were similar to those in normal subjects. Mean sweatspot density was similar in males and females (male: 68/cm² (SD 44); female: 69/cm² (SD 41) \( p=0.9 \)), but since the mean sweatspot size in males was greater than that in females (male: 0.326 mm (SD 0.055); female: 0.274 mm (SD 0.044)), the total calculated volumes were again also greater (male: 1.37 µl (SD 1.35); female: 0.70 µl (SD 0.99), \( p<0.001 \)).

### 4.4.2.2 Age and skin surface temperature

Mean sweatspot density showed a significant negative correlation with age \( r=-0.38, p<0.001 \) (Figure 5). When taken as a group, there was no decrease in sweatspot size with age \( r=0.18, p=0.12 \), but in the more numerous male patients, this association was weakly significant (male: \( r=-0.33, (n=52), p=0.02 \); female: \( r=0.17, (n=26), p=0.41 \)). Derived sweat volume decreased slightly with age \( r=-0.26, p=0.02 \), but no measurements were significantly related to foot temperature, ambient temperature or humidity.

![Sweatspot density and age in diabetic patients. The 95% confidence intervals for the normal range are shown as dotted horizontal lines. In comparison with the normal subjects, there was a slight, though statistically significant, change in mean sweatspot density with age \( r=-0.38, p<0.001 \).](image.png)
4.4.2.3 Ethnic origin

Fifty-three patients were of European origin, 18 Asians and 9 Afro-Caribbean. Mean sweatspot density was significantly lower in both Asians (44/cm² (SD 31), p < 0.001) and Afro-Caribbeans (44/cm² (SD 26), p = 0.01) than in Europeans. Mean sweatspot size in Asian patients was smaller than in the other two groups (Asian: 0.29 mm (SD 0.05); European: 0.31 mm (SD 0.06); Afro-Caribbean: 0.31 mm (SD 0.05). There were no significant differences in sweatspot diameter between the three groups.
Figure 6: Sweatspot density distribution in normal and diabetic subjects, bandwidth 1 standard deviation (28 sweatspots/cm²).

4.4.2.4 Prevalence of abnormalities

Figure 6 shows the distribution of sweatspot densities in normal and diabetic subjects. The distribution in non-diabetic subjects was normal, in contrast to the diabetic patients, where there was a relatively constant proportion with densities up to 2 standard deviations (SD) above the mean normal value (76/cm²). This resulted in an excess of patients with low sweatspot counts (<20/cm²), a decrease in the proportion with densities up to 1 SD above the normal range, and an excess in the range 104–>160/cm².

Mean sweatspot density was the most frequent abnormality among the sweatspot measurements. Fourteen (17.5%) had a mean sweatspot density less than 20/cm², and 7 (8.8%) a density greater than 132/cm², yielding an overall abnormality rate (including the two subjects with no activatable sweatspots) of 28.8%. Five patients with low sweatspot density were either Asian or Afro-Caribbean. For mean sweatspot size and calculated sweat volume, separate normal ranges for males and females have been considered. Mean sweatspot size was above the normal ranges in 3 subjects (2 male, 1 female), of whom two also had increased sweatspot density and derived sweat volume, and below normal in 8 (all males), in 4 of whom it was an isolated abnormality. The range of normal values for calculated total sweat volume includes zero sweat secretion and therefore only 3 subjects (2 male, 1 female) 1 with values above the normal ranges can be considered abnormal on this measurement; 2 of them also had high sweatspot density and size.
4.4.2.5 Abnormalities in other neurological measurements

Among the electrophysiological measurements, common peroneal motor conduction velocity was low in 33% of those tested, common peroneal motor action potential amplitude in 7.5% and sural sensory action potential amplitude in 18.2%; 12.9% had prolonged shortest flat latency. Respiratory RR variation was low in 20.5%, the Valsalva ratio in 9.3% and the lying:standing (30:15) ratio in 13.0%, while 10.7% had a postural systolic blood pressure fall of >20 mmHg. In the psychophysical sensory threshold tests, 36.5% had an elevated Marstock warm threshold and 35.1% an elevated cool threshold, while vibration perception thresholds at the medial malleolus and great toe pulp were elevated in 20.3% and 16.9% respectively.

4.4.3 Reproducibility

4.4.3.1 Measurement variability

Median coefficient of variation (CV%) for repeated sweatspot densities in normal subjects was 14.1% (range 4.6–20.5), and in diabetic subjects 9.5% (1.4–20.0). Median CV% for mean sweatspot diameter in normal subjects was 4.0% (0.6–6.0), and in diabetic subjects 2.6% (0.7–4.0).

4.4.3.2 Intra-patient variability

Median CV% for mean sweatspot diameter in normal subjects tested in duplicate 2-24 weeks apart was 5.5% (range 2.9–12.8) and for mean sweatspot density 4.8% (range 2.3–25.4). Corresponding results for diabetic patients were: mean sweatspot diameter, 9.1% (0.5–20.3), mean sweatspot density 9.4% (4.3–49.4).

4.4.4 Associations with other measurements

4.4.4.1 Physical and biochemical variables

In normal subjects mean sweatspot size was strongly related to both height and weight (height: r=0.65, p<0.001; weight: r=0.57, p=0.005). There were no significant correlations with mean sweatspot density, nor between sweatspot size or density and body mass index (BMI, wt/ht2). Similar results were found in diabetic subjects (height and sweatspot size: r=0.40, p=0.001; weight and sweatspot size: r=0.30, p=0.01; height and
sweatspot density: $r=0.08$, $p=0.52$). Measurements did not change with known duration of diabetes, even considering type 1 (insulin-dependent) patients alone, nor with random plasma glucose or glycated haemoglobin at the time of the test.

### 4.4.4.2 Cardiac autonomic function

Mean sweatspot density was weakly but significantly associated with respiratory RR variation ($r=0.24$, $p=0.05$) (**Figure 6**) and Valsalva ratio ($r=0.28$, $p=0.03$), but not with 30:15 ratio ($r=0.09$) or with systolic or diastolic postural blood pressure changes. Mean sweatspot size was not significantly associated with any of the cardiovascular tests.

![Figure 6: Relationship between mean sweatspot density and respiratory RR variation in diabetic patients ($r=0.24$, $p=0.05$).](image)

### 4.4.4.3 Sensory thresholds

There was a striking lack of association between mean sweatspot density and size, and thermal and pain thresholds (eg. warm threshold: $r=-0.07$, $p=0.4$ (**Figure 7**)). Vibration perception thresholds were strongly negatively associated with mean sweatspot density (medial malleolus: $r=-0.44$, $p=0.001$ (**Figure 8**); great toe: $r=-0.49$, $p=0.001$).
4.4.4.4 Electrophysiology

Motor nerve conduction measurements were not significantly correlated with sweatspot density, but sural nerve conduction velocity and sensory action potential amplitude were more strongly correlated, though not statistically significantly (both $r=0.35$, $p=0.08$); statistical significance would probably be achieved with larger patient numbers.

Figure 7: Scattergram of relationship between Marstock warm thermal thresholds and mean sweatspot density in diabetic patients; there is no statistically significant correlation ($r=-0.07$, $p=0.4$).

Figure 8: Vibration perception threshold (VPT) at the medial malleolus and mean sweatspot density in diabetic patients ($r=-0.44$, $p=0.001$). VPT is shown on a logarithmic scale. This contrasts with the lack of statistical association between sweatspot density and thermal thresholds at the feet (see Figure 7 above).
4.4.5 Analysis of sweatspot size distribution histograms

Computerised data of the size distribution of the sweatspots were obtained in 53 normals and 77 diabetic patients. Histograms of sweatspots in 0.05 mm bandwidths were plotted for each sweat print (Figures 2 and 4). Using averaged values between 12 and 20 minutes, over which period sweatspot size did not change, and the total area scanned, the size distribution data was calculated as the number of sweatspots per cm$^2$ in each bandwidth. The mean total number of sweatspots used in each histogram was 1305 (range 240–2638) in normal subjects and 1274 (range 105–1274) in diabetic patients, and the mean areas scanned 4.3 cm$^2$ (range 2.6–5.5) for normal subjects and 4.2 cm$^2$ (range 2.2–4.9) for diabetic patients. The results for all normal and diabetic subjects are shown in Figure 9(a). In both groups modal diameter was 0.20–0.25 mm. The minimum diameter registered was 0.10 mm, but this limitation was due to sweatspot size rather than the resolution of the image-analysis system. The distribution was skewed, with a long tail towards the larger diameters. There were no sweatspots in any subjects more than 0.80 mm in diameter. The shape of the distribution was similar to that of Kennedy et al. (1984b), using a manual measuring method, though the modal diameter in his study was 0.10–0.12 mm, and because of artifact due to minute drops of sweat being retained within the dermal ridges, drops of less than 0.04 mm diameter were not included. Allowing for the approximately 2-fold difference in modal size between Kennedy's imprint method and ours, the arbitrary lower cut-off limit employed by Kennedy et al. (1984b) was similar to the limit of resolution of our own method. There were no significant differences between normal and diabetic subjects at any diameter, though there was a slight excess of smaller sweatspots (<0.35 mm) in normal subjects, and a slight excess of larger spots (>0.40 mm) in diabetic subjects.

Patients with symptomatic autonomic neuropathy showed no qualitative differences in the shape of the distribution histograms. In order to compare the distributions in normals and patients with low and high sweatspot activation, mean sweatspot counts were ranked and the highest and lowest 10% of subjects selected (ie. 8 diabetic patients and 5 normal subjects). Data for the normal subjects are shown in Figure 9(b). The modal diameter for the whole group was similar to those with high sweatspot activation. For the subjects with low sweatspot activation, the modal diameter was 0.15–0.20 mm. In diabetic patients, the same results were observed, Figure 9(c), with a greater spread of modal values consistent with the wider range of sweatspot activation in diabetic than in normal subjects. In patients with high densities, there were higher numbers of large sweatspots (>0.35 mm) than in normal subjects with high densities.
Figure 9: Sweatspot size distribution histograms. Top: combined results for all normal and diabetic subjects. Middle: normal subjects with high and low sweatspot densities. Bottom: diabetic subjects with high and low sweatspot densities. See text for details of methods of analysis.
4.4.6 Characterisation of the groups of diabetic patients with abnormal sweatspot densities

Clinical characteristics of the seven patients with increased, and of the 16 patients with decreased or absent sweatspot activation are given in Table 2. Those with increased activation were significantly younger, but had a similar duration of diabetes, compared with those who had decreased sweatspot activation. All but one were type 1 (insulin-dependent) patients. Three had symptomatic peripheral and autonomic neuropathy; one was a 21 year old female who had had an episode of painful peripheral neuropathy within the past year. Three had at least one abnormal (age-related) cardiovascular autonomic function test. Two had no abnormalities in peripheral neurological function. Median glycated haemoglobin level was similar in the two groups, but those with high sweatspot counts had significantly higher median blood glucose levels at the time of the test. Eight of the 14 patients with low sweatspot densities had symptomatic autonomic neuropathy.

Table 2: Comparison of patients with increased (n = 7) and decreased (n = 16) sweatspot activation. Results are presented as median (range); p values refer to Mann-Whitney U test statistics.

<table>
<thead>
<tr>
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<th>Increased (n = 7)</th>
<th>Decreased (n = 16)</th>
<th>p</th>
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<td>Age, y</td>
<td>31 (18-59)</td>
<td>50 (21-50)</td>
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<td>Duration diabetes, y</td>
<td>8 (3-30)</td>
<td>4.5 (1-32)</td>
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<td>Type 1: Type 2</td>
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<tr>
<td>Respiratory RR</td>
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<td>variation, bpm</td>
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<tr>
<td>Valsalva ratio</td>
<td>1.88 (1.61-2.24)</td>
<td>1.36 (1.09-1.63)</td>
<td>0.006</td>
</tr>
<tr>
<td>30:15 ratio</td>
<td>1.10 (0.96-1.37)</td>
<td>1.06 (0.98-1.44)</td>
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<td>Systolic BP fall, mmHg</td>
<td>0 (-10-12)</td>
<td>4 (-15-22)</td>
<td>0.07</td>
</tr>
<tr>
<td>Diastolic BP fall, mmHg</td>
<td>6 (-16-18)</td>
<td>4 (-15-22)</td>
<td>0.90</td>
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<td>Marstock warm threshold, °C</td>
<td>6.4 (4.3-15.5)</td>
<td>6.7 (4.5-15.8)</td>
<td>0.64</td>
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<tr>
<td>Marstock cool threshold, °C</td>
<td>4.1 (1.1-12.0)</td>
<td>6.0 (1.4-15.3)</td>
<td>0.76</td>
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<td>Random blood glucose, mmol/l</td>
<td>17.9 (12.2-19.0)</td>
<td>6.8 (4.8-15.4)</td>
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<td>Glycated haemoglobin, %</td>
<td>13.1 (8.6-15.1)</td>
<td>12.2 (7.3-16.5)</td>
<td>0.92</td>
</tr>
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</table>

1 Reference range <8.0%
4.5 Discussion

This study has simplified and automated the process of counting and measuring pharmacologically-activated sweat glands in the periphery of normal and diabetic subjects, a process previously carried out manually (Kennedy et al. 1984b; Ryder et al. 1988). More recently, Kennedy's group has reported results of sweatspot counting using a computerised image analysis system similar to that described here (Kennedy et al. 1989b). They have compared their previous manual method with the computerised system, and found that the increased precision resulted in a greater diagnostic yield. Ryder et al. (1988), using intradermal acetylcholine and a starch-iodine indicator system, was concerned that unevenness of sweatspot activation, particularly in neuropathic patients, would reduce the precision of his method, and rather than counting absolute numbers of sweatspots, used a method where scores were allocated according to the number of sweatspots in each division of a photographic print of the sweating area. Therefore, although he studied the same dorso-lateral area of the foot, the two studies are not directly comparable.

Repeatability measurements, hitherto not performed, suggest that the coefficient of variation of our method is lower than that of psychophysical sensory threshold measurements (Fagius and Wahren 1981; Abraham et al. 1988), and similar to that reported for cardiovascular autonomic tests (Schumer et al. 1988). The photographic method has allowed us to study the measurement variability, which was similar to that of the test-retest variability, though this could probably be improved by the use of a dedicated image-analyser. In this study, the repeat measurements were made using a system which had been used for unrelated studies in the period between the two analyses. The video camera therefore had to be set up on two separate occasions.

In our group of randomly-selected diabetic patients 20%, predominantly Type 2 (non-insulin-dependent) patients with widespread evidence of diabetic neuropathy, had low sweatspot densities, while a smaller group had high densities, presumably as a result of denervation supersensitivity. These patients were young (median age 31 y) and predominantly Type 1 (insulin-dependent). The results of conventional small-fibre tests in this group of patients were similar to those of the whole group of diabetic patients.

Previous studies (Kennedy et al. 1984b; Navarro et al. 1989; Ryder et al. 1988) have identified patients only with decreased sweat gland activation, up to 56% of the large population studied by Kennedy et al. (1989). However, Ionescu-Tirgoviste, using the starch-iodine method of Ryder et al. (1988) has demonstrated increased sweatspot densities in a
similar proportion of patients as in the current study (Ionescu-Tîrgoviște and Pitie, personal communication, 1992). The reasons for the lack of detection of the phenomenon of increased sweat gland activation are not clear. Ryder et al. (1988) investigated a small group of patients selected on the basis of impotence. Clinically, this group is unlikely to be comparable with the one studied here, but assuming a similar prevalence of supranormal responses, the sample size may have been inadequate to contain such a patient. This explanation is less likely in the more than 300 patients reported by Kennedy's group (Navarro et al. 1989). Approximately 50% of this large group of Type 1 (insulin-dependent) patients were taking part in the Minnesota pancreatic-renal transplantation program, and some may have had coexistent uraemic neuropathy (Kennedy et al. 1990). It is, nevertheless, surprising that in a group of type 1 (insulin-dependent) patients with established neuropathy, a similar phenomenon has not been previously described. The explanation may lie in two technical factors; we have stimulated a comparatively large area of the foot (4 cm²), and repeated the measurement over an identical area on three occasions after maximum sweat gland activation was achieved at 12 min. Kennedy stimulated a distal 1 cm² area of the foot with a mean normal sweat gland density of approximately 300/cm², and made only one imprint.

The reasons for increased sweatspot activation are not known. It appears to occur predominantly in insulin-dependent diabetes of varying duration. Some individual patients had evidence of abnormal nerve function, but not to the extent of the larger group with decreased sweatspot activation. The mechanisms involved in this presumed cholinergic denervation supersensitivity are not known, nor are the reasons why other patients with similar clinical characteristics do not exhibit the same phenomenon.

The histological density of sweat glands in this region of the foot has not been studied. However, separate studies on the skin of the thigh have found histological densities up to 120/cm² (Szabó 1962); methacholine-activated sweatspot counts are much lower, ranging from 45–86/cm² (Kenney and Fowler 1988), though a larger number is activated by heat stress (Dobson and Sato 1972). Therefore the number of potentially activatable glands is much greater than the number stimulated by pharmacological or physiological stimuli. Individual sweat glands are known to have highly variable stimulation thresholds to pharmacological stimuli (Sato and Sato 1983). In the presence of denervation supersensitivity, otherwise inactive glands with a high stimulation threshold would be recruited by a given cholinergic stimulus. The careful standardisation of the quantity of applied pilocarpine in this study, together with the two factors previously discussed, may explain why increased sweatspot densities have been detected in this study. Until repeated measurements over time have been
made, it is not possible to say how long this supersensitive state persists, nor whether it precedes or follows subnormal sweat gland activation. The known duration of diabetes in the group of patients with increased sweat gland activation was greater, though not significantly so, than those with decreased activation, and they were also younger. There may be parallels in this process with the evidence for regeneration of myelinated nerve fibres under the influence of the aldose reductase inhibitor Sorbinil, which appeared to have a greater effect in Type 1 than in Type 2 patients (Sima et al. 1988).

Since pilocarpine is a muscarinic agent acting directly on sweat gland epithelium, and not through neuronal pathways (Randall and Kimura 1955; see Introduction, section 2.4.1.1), it is likely that increased responses in this study reflect upgrading of cholinergic sweat gland receptors, though there is no direct evidence for this. Histological studies will be required to determine whether these changes are associated with changes in sweat gland innervation (see Chapter 7). However, Low et al. (1983, 1988) have also observed increased sweat output after iontophoresis with acetylcholine (see Chapter 3), using a method which stimulates only nicotinic acetylcholine receptors, and therefore changes in nerves as well as in the target organ would be expected. It is of interest that one of the patients with increased sweatspot density had recently had an episode of typical painful peripheral neuropathy associated with poor diabetic control; though skin biopsy data were not available for this patient (see Chapter 7), patients with the painful peripheral neuropathy known as notalgia paraesthetica have recently been shown to have increased unmyelinated nerve fibres in skin biopsy specimens (Springall et al. 1991). There may therefore be an association between increased pharmacological responsiveness of sweat glands and nerve proliferation; further studies are needed to elucidate this association. The group of supersensitive patients in the current study is heterogeneous, unlike the clearly neuropathic patients with decreased sweatspot activation. In the two patients with no evidence of neurophysiological abnormalities, there may be physiological reasons for increased responses, for example sweat gland training through environmental exposure (Collins et al. 1965) or physical training (Sato and Sato 1983; see Introduction, section 2.5.3.10); one of our patients had a strenuous manual job.

We chose to introduce the cholinergic agent by iontophoresis and not by intradermal injection (compare Ryder et al. 1988). If a compound is injected intradermally, it is not known (a) how the sweat response is affected by the local trauma of the injection, (b) to what extent its effect is dependent on vascular diffusion of the agent over the area to be studied, and (c) whether the depth of injection affects the sweat response. Pilocarpine and other cholinergic agents are known to have appreciable vasodilator effects, mediated through endothelium-dependent
mechanisms (Saenz de Tejada et al. 1989) and observed in this study as an area of long-lasting erythema (3–12 hours) limited to the area of iontophoresis. Iontophoresis itself also causes vasodilatation, as erythema, often of a similar degree to that noted under the active electrode, was noted at the indifferent electrode, which contained no pilocarpine. However, no sweatspots were detected when the skin area under the cathode was tested; the effect of vasodilatation caused by direct electrical current on sweat gland activation is therefore below the threshold of detection of this method, though it is possible that basal transepidermal water loss, independent of sweat gland activity, is enhanced by these stimuli (see Chapter 3). The advantages of iontophoresis over injection are therefore (a) the potential for uniform stimulation of a large area of skin, where the vascular effects of the iontophoretic current are constant, though not eliminated, and (b) iontophoresis is a harmless procedure with no potential for damage to the diabetic foot. There are, however, certain disadvantages. The epidermis is a high-resistance structure, approximately 1 mm thick; the secretory coils of sweat glands are at a depth of 2–3 mm. The mobility of pilocarpinium ions in the solution between the electrode and the skin surface is approximately 0.2 mm in 5 min (Webster 1983). The mechanisms that enhance this low mobility, and allow pharmacological concentrations of cholinergic agents to reach the sweat glands, are not known with certainty, but may themselves be affected by diabetes. It is thought that electrical transport through the epidermis is predominantly through the low-resistance pathways of the sweat gland ducts. The current density is lower below the epidermis, where ionic mobility is decreased; in addition there are the effects of competition with other physiological cations, and of dermal blood flow. There are therefore several factors which could increase the inter-individual variability of cutaneous transport of pilocarpine.

As with the study of acetylcholine-induced dynamic sweat responses (Chapter 3), there appears to be an ethnic effect; Afro-Caribbean and Asian subjects had lower sweatspot activation than European subjects, and although sweatspot size was smaller in Asian subjects, this was not statistically significant compared with the other two groups. We did not study an ethnically-matched normal population, and are unable to state whether this a phenomenon restricted to diabetic patients. If, as seems likely, there is an increased epidermal barrier to iontophoretic mobility in Afro-Caribbean and Asian patients, then this would be a disadvantage of the method, which may be shared by all methods which measure sweat gland responsiveness.

Only Kennedy and his colleagues have previously investigated the quantitative relationship between pilocarpine sweatspot activation and other neurological measurements in diabetes.
Navarro et al. (1989), in contrast with the current study, did not find any changes in sweatspot density with age or gender. Our finding of a significant decrease in sweatspot density with age in diabetic subjects, and in female, compared with male subjects, is in keeping with most neurological measurements in diabetes, both large- and small-fibre. Kenney and Fowler (1988) found that sweat gland activation in the thigh did not decrease with age in normal males, but the output per sweat gland diminished. Sweat gland size (related to sweat volume) was not significantly reduced in diabetic compared with normal subjects, but sweatspot diameter in males was significantly greater than in females. Decreased sweat output in male diabetic patients is therefore due to a combination of decreased numbers of activatable sweat glands and a decrease in output per gland. The situation in female patients may be complicated by hormonal influences (see Introduction, section 2.5.3.9).

Navarro et al. (1989) used a categorical score for thermal sensitivity, measured with the same apparatus as in the current study, and found that there was a close relationship between sweatspot density and impaired temperature sensation at the foot. In our correlation analysis, we were unable to find such a relationship with warm, cool, or heat pain thresholds. However, there was a negative correlation between sweatspot density and vibration perception thresholds. The discrepancy between the results for the sensory thresholds cannot readily be explained, particularly in view of the evidence that vibration sensation is transmitted through myelinated fibre pathways. Neither physical characteristics nor age are likely to be relevant. Weight, height, and body mass index, though strongly associated with vibration perception threshold (Gadia et al. 1987), are not associated with sweatspot density (though they are with mean sweatspot diameter). Age might also be considered to be common to all these measurements, but there is a comparably strong relationship between thermal and vibratory thresholds and age. We have found a weak correlation between respiratory RR interval variation and Valsalva ratio and sweatspot activation. Age again might be considered a related common factor, but we have been unable to find a deterioration in the Valsalva ratio with age, either in normal or diabetic subjects (see Chapter 1). Kennedy et al. (1989) presented a scattergram of the relation between respiratory RR variation and sweatspot density. Though the relationship is statistically significant ($r=0.47$, $p<0.0005$), it appears to be weak, and is influenced by the high proportion of subjects (84%) with abnormalities in both tests. In our randomly-selected, though smaller, population, the relation is statistically evident but also very weak. There were no associations with postural blood pressure changes. Overall, the degree of concordance with autonomic function tests was much lower than that found with other routinely used neurological measurements (San Antonio Consensus, 1988).
In this study, the prevalence of abnormal sweatspot densities was similar to that of cardiovascular autonomic tests, a finding in accordance with that of Kennedy et al. (1989) and Ryder et al. (1988). The relatively low prevalence of abnormality in sweat gland activation using directly-acting muscarinic agents in comparison with their axon-reflex sweat test has been noted by Low and his colleagues (Low et al. 1983), and it has been pointed out that the processes of sweat gland denervation and reinnervation, which probably occur at the same time, may result in apparently normal sweatspot densities, while active nerve degeneration and regeneration are occurring. This view cannot be refuted without a direct comparison between Low’s Q-SART test and the test described here, but the broad similarity between the rates of abnormality in pilocarpine sweatspot activation and other neurological tests suggests that the sensitivity of the method is comparable with that of other conventional tests.

The statistical weakness of the correlations between sweatspot activation and neurophysiological measurements suggests that the peripheral denervation of sweat glands differs from the deficits in the long neural pathways generally tested by conventional neurophysiological methods. The current test therefore cannot be used as a substitute for standard tests, and should be regarded as complementary to standard neurophysiology by reflecting events at the most distal autonomic nerve terminals.

In summary, this study has demonstrated that a computerised sweatspot counting method can identify a group of patients, probably exhibiting denervation supersensitivity, who have increased sweatspot densities after standardised pilocarpine iontophoresis, in addition to a larger group of unequivocally neuropathic patients with decreased activation. The significance of this new finding is not known, but it may represent a group of patients undergoing changes associated with active reinnervation, who should be followed up with sequential tests. The test is the simplest method currently available for the quantitative evaluation of peripheral sweat gland activity, being rapid, simple, and objective. Its reproducibility is similar to that of pupillometry and vagal cardiovascular autonomic tests. The test may be of value in monitoring the response of therapeutic agents, such as ACTH fragments and gangliosides, which experimentally have been found to increase peripheral nerve sprouting, and whose effects may not be adequately reflected in conventional neurophysiological measurements.
Chapter 5  Quantitative measures of skin sympathetic response in diabetes: relation to sudomotor and neurological function

5.1 Introduction

Recently there has been a resurgence of interest in the noninvasive measurement of sympathetic skin activity and a re-evaluation of its place in the study of peripheral neuropathies. There is conclusive evidence that the sympathetic skin response (SSR) is caused by changes in eccrine sweat glands (see Introduction, section 2.5.4). Several studies have investigated sympathetic skin response in diabetic subjects (Shahani et al. 1984; Knezevic and Bajada 1985; Maselli et al. 1989; Niakan and Harati 1989; Watahiki et al. 1989).

The aim of the current study was to standardise stimulus and recording parameters in order to increase the detection rate of the response, and to investigate the relationship between SSR and the sweat measurements described in previous chapters.

5.2 Subjects and Methods

5.2.1 Subjects

SSR was measured in 46 normal and 69 age-matched, randomly-selected diabetic subjects (see Appendix A). Their clinical characteristics are given in Table 1.

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>Diabetic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>46</td>
<td>68</td>
</tr>
<tr>
<td>Age, y</td>
<td>44.6 (12.7, 21-79)</td>
<td>41.0 (15.2, 17-66)</td>
</tr>
<tr>
<td>Duration diabetes, y</td>
<td></td>
<td>12.4 (9.3, 1-34)</td>
</tr>
<tr>
<td>Gender (M:F)</td>
<td>19:27</td>
<td>41:27</td>
</tr>
<tr>
<td>Type 1: Type 2</td>
<td></td>
<td>42:26</td>
</tr>
<tr>
<td>Treatment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Insulin</td>
<td>49</td>
<td></td>
</tr>
<tr>
<td>Oral hypoglycaemics</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>Diet alone</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Glycated haemoglobin, %¹</td>
<td>11.3 (2.8, 6.5-16.5)[52]</td>
<td></td>
</tr>
<tr>
<td>Plasma glucose, mmol/l</td>
<td>10.0 (4.4, 3.9-19.0)[52]</td>
<td></td>
</tr>
</tbody>
</table>

¹ Reference range <8.0%
5.2.2 Measurement of SSR

Tests were performed on the right leg, in a warm room at 21-24°C, with the patient recumbent. Foot temperature was maintained at 32-34°C, with local warming using blankets and convective heating if necessary. To ensure that no patient was hypoglycaemic, capillary blood glucose was measured before the test (Glucometer II/Glucostix, Ames, Stoke Poges, UK), and venous blood was taken for glucose and glycated haemoglobin at the end of the test. SSR was measured using a method modified from that of Shahani et al. (1984). All electrodes, apart from the earth, which was a plate EMG electrode attached to the lateral side of the leg, were standard Ag-AgCl disposable ECG monitoring electrodes (3M Corporation, Minnesota, USA), and recordings were made on a Nicolet Viking electromyograph (Nicolet Medical Instruments, Coventry, UK). In order to increase the size of the response, we used a deep inspiration as the stimulus, rather than electrical stimulation (Shahani et al. 1984). The stimulus was demonstrated to the subject, while emphasising that an adequate stimulus consisted of a sudden, rather than a prolonged, inspiration, and the subject was allowed to practice this unfamiliar manoeuvre. Intercostal EMG was recorded using two electrodes attached to the right lower chest wall (over 7th-9th intercostal spaces). SSR was recorded with one electrode on the mid-tarsal portion of the dorsum of the right foot and on the sole of the foot immediately opposite the dorsal electrode. Recordings were made simultaneously on two channels, one recording intercostal muscle activity, the other the SSR. Electromyograph settings were:

<table>
<thead>
<tr>
<th>Sweep speed:</th>
<th>500 ms/cm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity:</td>
<td>500 μV/cm (100–200 μV/cm if the responses were of small amplitude)</td>
</tr>
<tr>
<td>Band pass:</td>
<td>0.5 Hz–100 Hz</td>
</tr>
</tbody>
</table>

The filter settings (0.5 Hz and 100 Hz) were chosen as the lowest frequencies available on the Nicolet Viking, though it is likely that the recording would be further improved if an even lower filter frequency were available.

Twelve responses were recorded. In order to allow for the habituation of the response, stimuli were separated by at least 1 min. Many subjects had spontaneous skin responses, and if possible 1 min was allowed between the end of a spontaneous response and the next stimulus, though this was not always possible in subjects with frequent spontaneous activity. An ECG recording was frequently seen on the intercostal tracing (Figure 1), but the two could readily be distinguished. Tracings were stored on disc for later analysis. Digital averaging was not
available at the slow sweep speeds used, and each tracing was analysed individually using electronic cursor placements. In most instances (85%) the response showed an initial negative (upward) deflection followed by a delayed positive (downward) deflection, usually of greater amplitude. Absolute amplitudes of the first and second wave were recorded; in 15% of cases, the response was "inverted" (positive followed by a negative wave). There were no instances where the response orientation changed during the course of a test. In 8 patients, because of software failure, the responses could not be stored and measurements had to be made at the time of the test; because of time considerations, only onset latency and mean peak amplitudes (see below) were recorded.

The following measurements were made from the stored tracings, and the mean value of the 12 responses calculated:

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Onset latency (s)</td>
<td>Time between onset of intercostal activity and first (usually negative)</td>
</tr>
<tr>
<td>Amplitude (µV)</td>
<td>Baseline-peak measurement of the first and second deflections. In some cases</td>
</tr>
<tr>
<td></td>
<td>third (negative) and fourth (positive) deflections were seen</td>
</tr>
<tr>
<td>Peak latency (s)</td>
<td>Time between onset of first deflection and the first and second peaks</td>
</tr>
</tbody>
</table>

Figure 2 shows the conventions used for measuring latencies and amplitudes on a typical normal tracing.

5.2.3 Other neurological tests

Other neurological tests (see Chapter 1) were carried out in diabetic subjects. The number (%) undergoing each test was as follows:

<table>
<thead>
<tr>
<th>Test</th>
<th>Number (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Marstock thermal thresholds</td>
<td>60 (88%)</td>
</tr>
<tr>
<td>Vibration perception thresholds</td>
<td>58 (85%)</td>
</tr>
<tr>
<td>Autonomic function tests:</td>
<td></td>
</tr>
<tr>
<td>Respiratory RR variation</td>
<td>60 (88%)</td>
</tr>
<tr>
<td>30:15 ratio</td>
<td>61 (90%)</td>
</tr>
<tr>
<td>Valsalva ratio</td>
<td>51 (60%)</td>
</tr>
<tr>
<td>Postural blood pressure change</td>
<td>59 (87%)</td>
</tr>
<tr>
<td>Pilocarpine-activated sweatspot density</td>
<td>53 (77%)</td>
</tr>
<tr>
<td>Dynamic acetylcholine-induced sweat output</td>
<td>60 (87%)</td>
</tr>
<tr>
<td>Electrophysiology (common peroneal and sural nerves)</td>
<td>39 (57%)</td>
</tr>
</tbody>
</table>
Figure 1: Examples of SSR tracings in two diabetic patients. Four responses in each patient have been captured in these screen dumps, the upper of each pair of tracings (numbered 1, 3, 5 and 7) representing the SSR, the lower tracings (numbered 2, 4, 6 and 8) showing the intercostal surface EMG. The inspiratory gasp can be seen in the lower tracings, its onset marked with vertical electronic cursors labelled b. The miniature ECG tracing can be clearly seen superimposed on the intercostal recordings. The calibrations are as described in Methods, section 5.2.2. On the horizontal (time) axis, each division represents 500 ms. In the upper panel, the sensitivity has been set at 500 μV/cm, in the lower recording, which had smaller amplitudes, the sensitivity has been increased to 200 μV/cm.

Upper panel: asymptomatic male Type 1 (insulin-dependent) patient, age 24, duration of diabetes 10 years. Mean onset latency 1775 ms, mean peak 1 amplitude 670 μV.
Lower panel: female Type 2 (non-insulin-dependent) patient, known duration of diabetes 13 years. She had been converted from oral hypoglycaemic agents to insulin 6 months before her tests, on account of an episode of typical neuropathic pain, which was remitting at the time of the SSR measurement. She had no autonomic symptoms. Mean onset latency 1493 ms, mean peak 1 amplitude 133 μV.
5.2.4 Effect of foot temperature on latency and amplitude measurements

The effect of local foot temperature on the response was investigated in 4 normal male subjects (age 30-47 y). The foot was cooled using either cold air or ice-packs, and rewarmed by convection to approximately 35°C over a period of 30 min. Single responses were measured during rewarthing at 1 min intervals.

5.2.5 Reproducibility

Repeat tests were performed in randomly-selected subjects (5 normal, 5 diabetic), 2-12 weeks after the first test.

5.2.6 Simultaneous recording of SSR and quantitative sweat output

A comparison of the SSR recording and measurement of the sweat output using the sudorometer described in Chapters 2 and 3 was carried out in the upper limb of a normal male subject (age 30) by measuring the SSR across the palm and dorsum of the hand and simultaneously recording the sweat output by positioning the sudorometer capsule on the palm of the hand adjacent to the SSR electrode.
5.3 Statistical analysis

Probability plots were used to test normality of distribution. Mean onset latency was normally distributed without transformation, but the mean amplitude of the first (negative) and second (positive) peak and mean latencies of the two peaks were log transformed to achieve normality. 95% confidence intervals for the reference ranges were calculated using standard methods. Student’s t-test was used for group comparisons, and Pearson correlation coefficients, with Bonferroni corrections for multiple comparisons. Least squares regression analysis was used to evaluate particular associations. The non-parametric Mann-Whitney U-test was used for sub-group comparisons.

5.4 Results

5.4.1 Normal subjects

Responses were present in all subjects. The mean onset latency was 1564 ms (SD 319), the mean amplitude of peak 1 350 μV (range 87-772) and mean peak 2 amplitude 411 μV (range 26-2707) (Figures 3 and 4). Mean peak 1 latency was 960 ms (SD 269), and of peak 2, 2091 ms (SD 647). The upper limit of normal (mean + 2SD) for onset latency was 2202 ms, for peak 1 latency 1498 ms and for peak 2 latency 3385 ms. Third deflections (usually negative) occurred in less than half (21) of the normal subjects, fourth deflections (usually positive) in only 4. The significance of the late responses is unknown, and in view of their inconsistent presence no further analysis is presented. Using log transformed values for amplitude of peak 1 yielded a lower limit of normal (mean - 2SD) of 92 μV. There were no significant differences in responses between male and female subjects (latency: p=0.41, amplitude: p=0.95).
Figure 3: "Dit" plots of the distribution of SSR latencies in normal subjects (upper panel) and diabetic patients (lower panel). Latencies were significantly longer in diabetic than normal subjects (p < 0.001).

Figure 4: "Dit" plots of the distribution of SSR amplitudes (peak 1) in normal subjects (upper panel) and diabetic patients (lower panel). Amplitudes were significantly lower in diabetic patients (p = 0.006).

5.4.2 Associations with age and physical characteristics

There were no significant associations between the response variables and age, height, weight or body mass index; in particular there was no association between mean onset latency and age (r = 0.17, p = 0.26) or height (r = 0.14, p = 0.52), and there was no significant change in mean amplitude of peak 1 with age (r = 0.10, p = 0.52). Age- or height-corrections of these measurements are therefore not required.
5.4.3 Effect of foot temperature on latency and amplitude measurements

The results of the warming experiment in 4 normal subjects are shown in Figures 5(a) and (b). The latency plots show marked variation between the subjects over the wide temperature range studied. Two subjects showed a decrease in latency over the range 32-34°C, while the remaining two showed a slight increase. The mean change, calculated as a percentage of the latency at 34°C, was −2.5% per °C. Amplitudes showed a consistent increase with temperature in all subjects, averaging +8.5% per °C, over the same temperature range.

![Graph showing effect of foot temperature on latency](image)

**Figure 5(a):** Effect of change in local foot temperature on SSR latency in 4 normal subjects. The curves of the individual subjects, represented by different symbols (●, ■, ★, ■) were plotted using distance least-weighted squares (Systat). Vertical dotted lines represent the temperature range (32-34°C) used in the clinical study. Y-axis values were calculated as a percentage of the actual measurements obtained at 34°C, and not from the interpolated values.
Figure 5(b): Effect of change in local foot temperature on SSR amplitude in 4 normal subjects. The curves of the individual subjects, represented by different symbols (●, ■, □, △) were plotted using multiple least-squares fit (Systat). Vertical dotted lines represent the temperature range (32-34°C) used in the clinical study. Y-axis values were calculated as a percentage of the actual measurements obtained at 34°C, and not from the interpolated values.

5.4.4 SSR and sudorometer sweat measurements

Figure 6 shows three tracings depicting the onset of the deep breath, the resulting sympathetic skin activity and the simultaneous recording of the evoked sweat output using the sudorometer. The latency of the SSR in the upper limb was 1.7 s, and the latency of the direct sweat response 5.2 s. Allowing for the 1 s response time of the sudorometer (see Chapter 2), the remaining 2.5 s represents the time from depolarisation of the sweat glands to the emergence of the sweat at the skin surface; integration of the area under the curve yields a sweat volume of approximately 200 ng water vapour.

5.4.5 Diabetic patients

Three subjects had no recordable negative peaks; one of them had recordable positive peaks. When present, mean onset latency was significantly longer than in the normal group (1836 ms (SD 269), p < 0.001) (Figure 3). Mean amplitudes of the two peaks were both significantly lower than normal subjects (peak 1: 254 μV (range 42-671) vs. 350 μV (range 87-772), p = 0.006 (Figure 4); peak 2: 273 μV (range 27-943) vs. 411 μV (range 26-2707), p = 0.04).
The latency of peak 1, but not peak 2, was increased (1225 ms (SD 611) vs. 960 ms (SD 269) p=0.01).

Ten patients had a prolonged mean latency, and after including the 3 patients with an absent response, this yields an abnormality rate for latency of 19%. One of these patients, a very tall (189 cm) 66-year old type 2 (non-insulin-dependent) patient, had a dramatically prolonged onset latency (3265 ms). Figure 7 shows that this patient appreciably affects the relationship between onset latency and age, and the result has been excluded from further analysis.

Fourteen patients had a low (<92 μV) or absent peak 1 amplitude, an overall abnormality rate for this measurement of 25%. Excluding those with absent responses, only 5 patients shared an abnormal onset latency and amplitude. Eleven patients had a prolonged latency of peak 1, yielding a similar abnormality rate (21%). The peak amplitudes were negatively correlated with onset latency (peak 1: r=0.25, p=0.05; peak 2: r=0.28, p=0.03).

Fourteen patients had low amplitudes of peak 1 (<92 μV); the overall abnormality rate for this measurement was 25%.
Diaphragm EMG

Deep breath

Sympathetic skin activity

Sympathetic response

Sweat production

Sweat response 2.39 mg/cm²/h

Basal TEWL 2.35 mg/cm²/h

Seconds

Figure 6: SSR and the direct sweat response measured simultaneously at the palm of the hand. A deep breath (top trace) is followed 1.7 s later by the sympathetic skin response (middle trace), itself followed 3.5 s later by the direct sweat response detected by the sudorometer (see Chapters 2 and 3).
Figure 7: Relationship between SSR latency and age in diabetic patients. Latency shows a weak trend towards prolongation with increasing age. However, one outlying patient (latency 3200 ms) appreciably affects the statistical relationship: after exclusion of this patient, a weakly significant relationship \( r=0.26, p=0.03 \) becomes non-significant \( r=0.19, p=0.13 \).

5.4.5.1 Associations between SSR measurements

Onset latency was significantly associated with the latency of peak 1 \( r=-0.36, p=0.006 \), but not with the latency of peak 2 \( r=-0.10, p=0.17 \). As mean onset latency increased, the amplitudes of peaks 1 \( r=-0.25, p=0.05 \) and peak 2 \( r=-0.28, p=0.03 \) decreased.

5.4.5.2 Associations with age, duration of diabetes, physical characteristics, and metabolic measurements

As in normal subjects, there was no change in onset latency with age \( r=0.19, p=0.13 \), after excluding the patient discussed above. The latency of peak 1 increased significantly with age \( r=0.30, p=0.02 \). Amplitudes of peaks 1 and 2 decreased with age (peak 1: \( r=-0.47, p<0.001 \); peak 2: \( r=-0.23, p=0.06 \)).
**Figure 8:** The relationship between known duration of diabetes and SSR amplitude (peak 1) in Type 1 (insulin-dependent) diabetic patients. There is a weakly significant decline in SSR amplitude with known duration of diabetes ($r = -0.42$, $p < 0.001$). Of the sweat measurements discussed in this and previous chapters, this is the only one to show such a change with age.

In the whole group none of the measurements were related to known duration of diabetes (eg. mean latency: $r = -0.01$, $p = 0.95$), but mean onset latency increased with diabetes duration in Type 1 (insulin-dependent) subjects ($r = 0.29$, $p = 0.07$), and the mean amplitude of peak 1 significantly decreased ($r = -0.42$, $p < 0.001$; **Figure 8**).

Excluding the tall elderly neuropathic patient, there was no significant association between mean onset latency and height ($r = 0.14$, $p = 0.29$) (**Figure 9**). Latency significantly increased with weight ($r = 0.33$, $p = 0.009$), but not with body mass index ($r = 0.22$, $p = 0.09$). Blood glucose and glycated haemoglobin were not significantly associated with any of the SSR measurements.

**5.4.5.3 Associations with sudomotor function (see Chapters 3 and 4)**

Onset latency was not significantly associated with either pilocarpine-activated sweatspot density or the dynamic acetylcholine sweat response (mean sweatspot density (12-20 min): $r = -0.07$, $p = 0.63$; mean sweatspot diameter: $r = -0.01$, $p = 0.92$; acetylcholine sweat output (to 12 min): $r = 0.02$, $p = 0.85$). However, the peak amplitudes were significantly associated
with mean sweatspot count (peak 1: \( r = 0.32, p = 0.02 \) (Figure 10); peak 2: \( r = 0.27, p = 0.03 \)) but there were no significant associations with peak latencies (peak 1: \( r = -0.20, p = 0.20 \); peak 2: \( r = 0.20, p = 0.18 \)).

**Figure 9:** SSR latency and height in diabetic subjects. The outlying patient (shown also in Figure 6) alters the statistical relationship; removal of this result changes the regression from \( r = 0.24, p = 0.06 \) to \( r = 0.14, p = 0.29 \). The regression line is shown for the whole group.

**Figure 10:** SSR amplitude in diabetic patients reflects the density of pilocarpine-activated sweatspots (see Chapter 4); \( r = 0.32, p = 0.05 \).
5.4.5.4 Measurements reflecting small-fibre function

Warm thresholds were weakly associated with onset latency ($r=0.23$, $p=0.07$), but rather more strongly associated with amplitude of peaks 1 and 2 (both $r=-0.27$, $p=0.02$). Cool and heat pain thresholds showed no significant correlations. Respiratory RR variation was associated with onset latency ($r=-0.26$, $p=0.05$; Figure 11), peak 1 amplitude ($r=0.51$, $p < 0.001$) and peak 2 amplitude ($r=0.35$, $p=0.008$). There were no significant associations with the Valsalva ratio, 30:15 ratio or with postural change in blood pressure.

5.4.5.5 Large-fibre tests

Vibration perception thresholds showed a weak correlation with mean onset latency ($r=0.24$, $p=0.09$) but a much stronger relationship with peak 1 amplitudes (Figure 12). There were weak correlations with electrophysiological measurements in the smaller number of patients studied with these tests (eg. common peroneal motor conduction velocity and SSR latency: $r=-0.44$, $p=0.01$; shortest f latency and SSR latency: $r=0.40$, $p=0.05$; sural sensory action potential amplitude (SSAP) and SSR latency: $r=-0.37$, $p=0.06$; SSAP and SSR amplitude: $r=0.41$, $p=0.03$).

![Graph showing Respiratory RR variation and SSR latency in diabetic patients; $r=-0.26$, $p=0.05$.](image)
Figure 12: Vibration perception threshold at the medial malleolus and the amplitude of SSR peak 1 in diabetic patients. VPT is plotted on a logarithmic scale. There is a strong negative correlation between this large-fibre neurophysiological modality and the small-fibre-mediated SSR amplitude ($r = -0.51$, $p < 0.001$).

5.4.6 Reproducibility

5.4.6.1 Within-test variability

For the 12 responses in each test, the mean coefficients of variation (CV%) (range) for the 46 normal subjects were: onset latency: 17.3% (7.4–41.2%), peak 1 amplitude: 33.6% (16.1–79.9%). Variability was similar in diabetic patients; mean CV for onset latency: 16.9% (7.4–45.3%), peak 1 amplitude: 35.8% (16.8–96.7%).

Since the SSR is known to habituate with frequent repetition, the change between the first and the twelfth responses was analysed (paired t-test). There were no statistically significant changes in latency or amplitude in normal or diabetic subjects (Figure 13), though there was a tendency towards a longer onset latency in diabetic subjects (response 1: 1839 ms (SD 540), response 12: 1917 ms (SD 526), $p = 0.07$). No more than 12 responses should therefore be recorded in a single test session.
Figure 13: Changes in SSR latency (upper panel) and amplitude (lower panel) throughout the test in normal (○) and diabetic (▲) subjects. Error bars = SEM. There are no significant changes in amplitude between stimuli 1 and 12 (normal: p=0.25; diabetic: p=0.59); latency shows a slight but not statistically significant (p=0.07) lengthening in diabetic but not normal subjects.

5.4.6.2 Between-test variability

The results of repeat tests in 5 normal and 5 diabetic subjects over 2-12 weeks are shown in Table 2. The median CV of onset latency was similar to that of other autonomic function tests, and that of peak amplitudes comparable with conventional sensory and motor nerve action potential amplitudes.
Table 2: Coefficients of variation (CV%) of latency and peak amplitude measurements in normal (n = 5) and diabetic subjects (n = 5) tested on two occasions

<table>
<thead>
<tr>
<th></th>
<th>Median</th>
<th>Range</th>
<th>Interquartile range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal subjects</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Onset latency</td>
<td>15.7</td>
<td>1.9–21.8</td>
<td>6.9–20.6</td>
</tr>
<tr>
<td>Peak 1 amplitude</td>
<td>30.2</td>
<td>13.4–44.2</td>
<td>18.4–40.6</td>
</tr>
<tr>
<td>Peak 2 amplitude</td>
<td>17.2</td>
<td>9.2–28.2</td>
<td>10.8–25.2</td>
</tr>
<tr>
<td>Diabetic subjects</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Onset latency</td>
<td>8.7</td>
<td>4.3–13.7</td>
<td>5.4–9.9</td>
</tr>
<tr>
<td>Peak 1 amplitude</td>
<td>13.4</td>
<td>2.5–68.5</td>
<td>13.1–64.6</td>
</tr>
<tr>
<td>Peak 2 amplitude</td>
<td>16.3</td>
<td>4.5–103.0</td>
<td>11.2–83.1</td>
</tr>
</tbody>
</table>

5.4.7 Characteristics of patients with abnormal responses

The 13 patients with prolonged or absent latencies were significantly older than those with normal latencies. There were no significant differences in physical characteristics (Table 3). There were no significant differences in any of the small-fibre measurements apart from Marstock warm thresholds, which were significantly elevated (p=0.006). There was a reduction in activated sweat gland density and acetylcholine-induced sweating, though not to a statistically significant degree.

A similar comparison has been made for the 14 patients with low peak 1 amplitude (Table 4). There was a similar discrepancy in age, but those with abnormal amplitudes had a significantly shorter duration of diabetes. Diastolic blood pressure fall was significantly greater, and as expected, the density of pilocarpine-activated sweat glands significantly lower.
Table 3: Comparison of diabetic patients with absent or prolonged (> 2202 ms) mean onset latency (n = 13) vs. those with normal latencies (n = 55). Data are presented as median (range) [number of subjects]; statistical significances refer to the Mann-Whitney U-test

<table>
<thead>
<tr>
<th></th>
<th>Abnormal latency</th>
<th>Normal latency</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>54 (23-66) [13]</td>
<td>36 (17-66) [55]</td>
<td>0.02</td>
</tr>
<tr>
<td>Duration diabetes, y</td>
<td>5.5 (1-30) [13]</td>
<td>12.5 (1-34) [55]</td>
<td>0.33</td>
</tr>
<tr>
<td>Type 1: Type 2</td>
<td>6:7</td>
<td>40:15</td>
<td></td>
</tr>
<tr>
<td>Height, cm</td>
<td>166 (155-188) [13]</td>
<td>168 (151-191) [53]</td>
<td>0.88</td>
</tr>
<tr>
<td>Respiratory RR variation, bpm</td>
<td>8 (2-37) [12]</td>
<td>21 (3-45) [48]</td>
<td>0.02</td>
</tr>
<tr>
<td>Valsalva ratio</td>
<td>1.52 (1.07-2.24) [10]</td>
<td>1.69 (1.09-2.95) [41]</td>
<td>0.17</td>
</tr>
<tr>
<td>30:15 ratio</td>
<td>1.07 (0.98-1.37) [12]</td>
<td>1.10 (0.96-1.64) [49]</td>
<td>0.09</td>
</tr>
<tr>
<td>Systolic BP fall, mmHg</td>
<td>6.5 (-9-22) [10]</td>
<td>2.0 (-16-24) [48]</td>
<td>0.13</td>
</tr>
<tr>
<td>Diastolic BP fall, mmHg</td>
<td>-1.5 (-15-6) [10]</td>
<td>0 (-25-22) [48]</td>
<td>0.73</td>
</tr>
<tr>
<td>Marstock warm threshold, ºC</td>
<td>12.2 (4.9-16.2) [12]</td>
<td>6.4 (2.4-16.2) [50]</td>
<td>0.006</td>
</tr>
<tr>
<td>Marstock cool threshold, ºC</td>
<td>5.6 (1.4-12.0) [12]</td>
<td>2.5 (1.1-12.7) [49]</td>
<td>0.20</td>
</tr>
<tr>
<td>Marstock heat pain threshold, ºC</td>
<td>15.7 (14.2-17.7) [10]</td>
<td>15.8 (9.8-18.5) [49]</td>
<td>0.69</td>
</tr>
<tr>
<td>Vibration threshold, great toe, V</td>
<td>19.5 (10.5-45) [9]</td>
<td>10.0 (3.5-34.3) [40]</td>
<td>0.005</td>
</tr>
<tr>
<td>Vibration threshold, malleolus, V</td>
<td>18.4 (10.0-32.7) [9]</td>
<td>11.3 (3.8-48.0) [49]</td>
<td>0.06</td>
</tr>
<tr>
<td>CPMCV, m/s</td>
<td>43.8 (26.0-49.6) [10]</td>
<td>46.0 (31.0-52.0) [28]</td>
<td>0.19</td>
</tr>
<tr>
<td>CPDMAP, mV</td>
<td>4.6 (0.2-6.8) [10]</td>
<td>46.0 (31.0-52.0) [28]</td>
<td>0.01</td>
</tr>
<tr>
<td>SSAP, µV</td>
<td>3.9 (1.5-21.8) [8]</td>
<td>12.9 (5.7-28.2) [24]</td>
<td>0.01</td>
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Sweat measurements

<table>
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<tr>
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<th>Abnormal latency</th>
<th>Normal latency</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>TEWL, mg·cm⁻²·hr⁻¹</td>
<td>1.20 (0.93-4.00) [10]</td>
<td>1.42 (0.93-1.60) [50]</td>
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<td>Tvol12, mg</td>
<td>1.51 (0.17-4.16) [12]</td>
<td>2.20 (0.68-6.12) [49]</td>
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<tr>
<td>Mean sweatspots, no/cm²</td>
<td>43 (7-136) [11]</td>
<td>85 (7-161) [42]</td>
<td>0.13</td>
</tr>
<tr>
<td>Mean sweatspot diameter, mm</td>
<td>0.29 (0.22-0.36) [11]</td>
<td>0.32 (0.21-0.46) [42]</td>
<td>0.17</td>
</tr>
</tbody>
</table>

CPMCV, common peroneal motor conduction velocity; CPDMAP, common peroneal compound motor action potential amplitude; SSAP, sural sensory action potential amplitude; TEWL, transepidermal water loss rate; Tvol12, sweat volume to 12 min after acetylcholine iontophoresis (see Chapter 3).
Table 4: Comparison of diabetic patients with absent or low (<92 μV) mean amplitude peak 1 (n = 14), and those with normal amplitudes (n = 54). Data are presented as median [range] [number of subjects]; statistical significances refer to Mann-Whitney U-tests

<table>
<thead>
<tr>
<th></th>
<th>Abnormal amplitude</th>
<th>Normal amplitude</th>
<th>p</th>
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<tbody>
<tr>
<td>Age, y</td>
<td>47 [23-66] [14]</td>
<td>36 [17-66] [54]</td>
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<tr>
<td>Duration diabetes, y</td>
<td>6 [1-30] [14]</td>
<td>13 [1-34] [54]</td>
<td>0.02</td>
</tr>
<tr>
<td>Height, cm</td>
<td>167 [156-188] [13]</td>
<td>168 [151-191] [51]</td>
<td>0.56</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>24.4 [19.3-34.3] [50]</td>
<td>22.8 [17.7-35.2] [50]</td>
<td>0.06</td>
</tr>
<tr>
<td>Respiratory RR variation, bpm</td>
<td>11 [2-26] [13]</td>
<td>22 [3-45] [47]</td>
<td>0.02</td>
</tr>
<tr>
<td>Valsalva ratio</td>
<td>1.54 [1.07-2.14] [8]</td>
<td>1.68 [1.09-2.95] [43]</td>
<td>0.23</td>
</tr>
<tr>
<td>30:15 ratio</td>
<td>1.13 [0.98-1.37] [13]</td>
<td>1.13 [0.96-1.64] [48]</td>
<td>0.01</td>
</tr>
<tr>
<td>Systolic BP fall, mmHg</td>
<td>4.0 (-12-22) [11]</td>
<td>3.5 (-16-24) [46]</td>
<td>0.98</td>
</tr>
<tr>
<td>Diastolic BP fall, mmHg</td>
<td>-6 (-17-8) [11]</td>
<td>0.5 (-25-22) [46]</td>
<td>0.03</td>
</tr>
<tr>
<td>Marstock warm</td>
<td>10.2 [4.3-16.2] [12]</td>
<td>6.3 [2.4-16.2] [48]</td>
<td>0.03</td>
</tr>
<tr>
<td>threshold, °C</td>
<td>6.0 [1.4-12.0] [13]</td>
<td>2.3 [1.1-12.7] [45]</td>
<td>0.02</td>
</tr>
<tr>
<td>Marstock cool</td>
<td>16.8 [14.2-17.7] [11]</td>
<td>15.8 [9.8-18.5] [45]</td>
<td>0.71</td>
</tr>
<tr>
<td>threshold, °C</td>
<td>18.0 [9.7-45.0] [11]</td>
<td>9.5 [3.5-34.0] [37]</td>
<td>0.004</td>
</tr>
<tr>
<td>Vibration threshold,</td>
<td>18.0 [9.7-45.0] [11]</td>
<td>9.5 [3.5-34.0] [37]</td>
<td>0.004</td>
</tr>
<tr>
<td>great toe, V</td>
<td>16.0 [8.7-34.5] [12]</td>
<td>11.2 [3.8-48.0] [45]</td>
<td>0.01</td>
</tr>
<tr>
<td>Vibration threshold,</td>
<td>4.35 [26.0-44.6] [10]</td>
<td>46.0 [31.0-52.0] [29]</td>
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</tr>
<tr>
<td>malleolus, V</td>
<td>4.6 [0.2-9.7] [10]</td>
<td>5.6 [0.9-12.6] [29]</td>
<td>0.37</td>
</tr>
<tr>
<td>CPMCV, m/s</td>
<td>5.4 [1.5-16.3] [7]</td>
<td>11.9 [2.4-28.2] [25]</td>
<td>0.07</td>
</tr>
</tbody>
</table>

Sweat measurements

<p>| | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>TEWL, mg·cm⁻²·hr⁻¹</td>
<td>1.51 [0.93-2.24] [12]</td>
<td>1.39 [0.69-2.75] [49]</td>
<td>0.43</td>
</tr>
<tr>
<td>TVol12, mg</td>
<td>1.51 [0.17-4.16] [12]</td>
<td>2.07 [0.88-6.12] [49]</td>
<td>0.36</td>
</tr>
<tr>
<td>Mean sweatspots, no./cm²</td>
<td>26 [7-121] [11]</td>
<td>84 [8-161] [42]</td>
<td>0.02</td>
</tr>
<tr>
<td>Mean sweatspot diameter, mm</td>
<td>0.29 [0.22-0.34] [11]</td>
<td>0.32 [0.21-0.46] [42]</td>
<td>0.17</td>
</tr>
</tbody>
</table>

CPMCV, common peroneal motor conduction velocity; CPDMAP, common peroneal compound motor action potential amplitude; SSAP, sural sensory action potential amplitude; TEWL, transepidermal water loss rate; TVol12, sweat volume to 12 min after acetylcholine iontophoresis (see Chapter 3).

5.4.8 Patients with symptomatic autonomic neuropathy

Nineteen patients, 14 males, 5 females, had symptomatic autonomic neuropathy (see Chapter 1). They were significantly older than asymptomatic patients (median 51 y vs. 33 y, p=0.001). Where recordable, onset latency was significantly longer in symptomatic than non-symptomatic patients (mean 1976 ms (range 1069-3265) vs. 1755 ms (1266-2488), p=0.03),
and the amplitude of peak 1 was lower (142 μV (64-524) vs. 240 μV (42-671), p=0.04). The onset latencies of both peaks were not significantly different. Most other neurological measurements, being more or less age-related in diabetic subjects were significantly impaired in symptomatic subjects. Other sweat measurements, which are less affected by age, were not different in symptomatic patients; for example pilocarpine-induced sweat gland activation was similar in the two groups (symptomatic: mean 69 glands/cm² (range 7-131); asymptomatic: 76 glands/cm² (7-161), p=0.6); and dynamic acetylcholine-induced sweat output was also similar (symptomatic: 2.07 μl (0.17-6.1); asymptomatic: 2.02 μl (0.68-6.12), p=0.9).

5.5 Discussion

This study has shown that, using appropriate techniques, sympathetic skin responses can be measured quantitatively in almost all (96%) of a group of randomly selected diabetic patients; this represents a higher detection rate than distal sensory electrophysiological measurements, for example the medial plantar sensory action potential amplitude, which may be absent in up to one-half of a randomly-selected group of diabetic patients (Levy et al. 1987). The ability to quantify responses, rather than recording them as either "present" or "absent", and to define reference ranges, significantly enhances the use of this technique as a measurement of nerve function. The logarithmic distribution of SSR amplitudes in normal subjects means that after appropriate transformation, the lower limit of the reference range does not include an absent response. When present, latency measurements were longer, and response amplitudes smaller in diabetic subjects compared with age-matched normal subjects. Test-retest reproducibility was similar to that of other neurological measurements in diabetic neuropathy, though appreciably higher for SSR amplitude than for the latency. A recent microneurographic study found that although mean integrated neural activity was smaller with auditory tone bursts than nerve trunk stimulation, the amplitudes were relatively constant, the most important factor being that the stimuli should be delivered randomly in time (Satchell and Sears 1987).

The main limitation of amplitude measurements is their temperature dependence. The increase in amplitude with temperature over the range 32-34°C in 4 normal male subjects was 8% per °C, compared with, for example, approximately 2%/°C for motor nerve conduction velocities (≈ 1 m s⁻¹·°C⁻¹) (Halar et al. 1980), though the changes are not linear when measured over a wide temperature range (Todnem et al. 1989). A more apposite comparison would be sensory nerve action potential amplitudes, where, however, there is a decrease with increasing
local temperature. In the finger (median sensory action potential amplitude), Lang and Puusa (1981) found that amplitudes changed by approximately 13%/°C, comparable in magnitude, though opposite in direction, to the results found here. Diagnostic use of the SSR amplitude, like sensory action potential amplitudes, therefore requires comparably strict control of surface temperature.

The increased detection rate of SSR in this study compared with other reports may be due to the population studied, in particular its relatively young mean age and short duration of diabetes; in most previous reports, SSR has been studied in patients with symptomatic neuropathy, instrumental evidence of peripheral neuropathy, or both. Consequently the prevalence of undetectable responses has been reported as 40–83%. Niakan and Harati (1988) detected the response in the upper limb in all but 9% of diabetic patients, though the leg, where peripheral neuropathy is more severe, was not studied. However, in addition to patient selection, three technical factors may contribute to the increased detection rate. First, we used the lowest available cutoff frequency for the high pass filter; the higher settings used in previous studies would tend to decrease the response amplitude, making the take-off point less prominent, or even indistinguishable from background noise. Second, we used a deep inspiration rather than electrical nerve trunk stimulation as the stimulus, which Shahani et al. (1984) found yielded larger amplitude responses than direct electrical stimulation of nerve trunks. Third, the use of proprietary disposable electrodes appears to give larger and more consistent responses than conventional EMG metal plate electrodes with electrode paste. Fowles and Schneider (1978) recorded skin potentials using different electrolyte media, and concluded that media that caused greater degrees of skin hydration (including a standard electrode paste) reduced the magnitude of the recorded responses. The manufacturers (3M) of the electrodes used in this study would not disclose the composition of the electrolyte medium but it is likely that their consistent composition, diameter and thickness, together with their ability to maintain close contact with irregular skin surfaces, contributed to the increased sensitivity.

Fowles and Schneider (1978) also noted that repeated responses caused a progressive and consistent decrease in potentials which was greater for hydrating media; we have not demonstrated this effect in normal subjects with 12 responses over a test period of about 30 min, though there was a non-statistically significant tendency to increased latency in diabetic patients, and studies of longer duration (3–4 hours) have confirmed that there is a substantial reduction of about 50% in SSR amplitudes when recordings are made from electrodes continuously applied to the same test site on the foot. This finding has been
confirmed by Elie and Guiheneuc (1990), who found a 67% decrease in amplitude and a 14% increase in latency at the foot after 1 hour.

The variability and tendency to habituation of the SSR, as with other sympathetic responses, are important considerations if it is proposed as a routine clinical measurement. While frequently noted, these factors have not previously been quantified. The median intra-patient coefficient of variation for the onset latency using 12 stimuli was 17%, and higher (36%) for the amplitude measurements; we did not find a significant change in either SSR latency or amplitude over the course of a test session lasting approximately 30 min.

Our results for both latency and amplitude measurements differ from those of previous studies. Shahani et al. (1984) reported a mean latency of 1880 ms in the foot, Knezevic and Bajada (1985) 2070 ms, and Elie and Guiheneuc (1990) 2050 ms, all appreciably longer than the result obtained here, 1564 ms. This may be due to the longer afferent pathway when nerve trunk stimulation, rather than a deep breath, is used, though the technical factors referred to above, particularly the band pass, may also contribute to the discrepancy in latency measurements.

The differences in the amplitude measurements in different studies may likewise be due to various technical differences. The mean amplitude in the foot in the study of Shahani et al. (1984) (822 μV) was higher than ours, as was that found by Elie and Guiheneuc (1990) (1400 μV); Kenezvic and Bajada's (1985) was somewhat lower, 101 μV. Our result, with a mean value of 350 μV, was similar to that found by Maselli et al. (1989), but this was probably a chance finding, as the methods were different in the two studies. The sympathetic skin response probably arises from the difference in potentials generated by two populations of sweat glands of different densities (those on the plantar surface being approximately 4 times more numerous than those on the dorsum). Therefore, electrode placement, the effects of different electrolyte media, and the regional variations in sweat gland density over both surfaces of the foot, probably contribute to the marked differences between response amplitudes measured in different studies.

The final confounding factor contributing to the variability of SSR amplitudes is foot temperature, though the current study used the same temperature range (32–34°C) as the studies of both Shahani et al. (1984) and Niakan and Harati (1989). Increases in local foot temperature in normal subjects resulted in a consistent non-linear increase in amplitude over a wide range of temperatures (15–35°C). Over the range of temperatures used in the clinical study (32–34°C) the mean change was 8.5% of the amplitude at 34°C. This increase is
presumably due to increased neurotransmitter release at the neuroglandular junction. The changes in latency were less consistent and may reflect subtle effects of profound cooling of the foot on afferent central pathways which are represented in the latency measurement. This phenomenon deserves further investigation, but our results confirm that the changes in SSR latency with temperature are less marked than those of amplitude.

We have not found a significant association between height and onset latency of the SSR in normal or diabetic subjects, confirming the finding of Knezevic and Bajada (1985). Elie and Guiheneuc (1990) found a consistent correlation between height and latency both of the upper- and lower-limb SSR latency. Shahani et al. (1984) also reported that latency “correlated well” with the subject’s height, and presented a scattergram of onset latency in the arm and leg vs. height. The regression equation was not stated. Using least-squares regression to recalculate the lower-limb data from the published plot confirms that the relationship achieves statistical significance \( r=0.43, n=27, p=0.02 \), but it is a weak one, and after excluding one subject of short stature with a particularly short response latency, the relationship loses its statistical significance \( r=0.25, n=26, p=0.21 \). This weak relationship is confirmed by the current study, where one tall patient significantly affected the statistical association. The association with height is therefore less clear-cut than with other measures of nerve conduction velocity (eg. motor nerve conduction, f latency), or even with vibration perception thresholds (Gadia et al. 1987), probably again reflecting the complexity of the neurological pathway, particularly the ill-defined preganglionic portion, and the slowness of postganglionic conduction. Use of the current method to measure latencies would therefore not require height correction. After excluding the same tall, neuropathic, elderly diabetic patient we were also unable to find an association between age and onset latency, though the latencies of the two peaks remained weakly associated with age, as did the peak amplitudes; this latter relationship can be explained on the basis of the decreased number of pilocarpine-activatable sweat glands with age in diabetic subjects, as established in the study described in Chapter 4, and the consequent association of peak amplitude measurements and sweatspot densities. Thomas and Korr (1957) investigated this relationship in detail, by observing directly the number of sweat glands activated by thermal stress, and correlating this with the electrical skin resistance; in individual normal subjects the correlation was usually very strong. We have not been able to undertake such a detailed analysis, as the sweat gland counts and sympathetic skin response were measured on different occasions; in addition, the SSR measures spontaneous sweat production, in contrast with the pilocarpine-stimulated sweatspot counts. Nevertheless, the persistence of the association despite these confounding factors suggests that the recruitment
of sweat glands under active pilocarpine stimulation reflects also the basal state of innervation of the sweat glands.

Of the 13 patients with an absent or prolonged onset latency, all but three had abnormal Marstock warm thresholds, though 5 had normal respiratory RR interval variation. However 7 had three abnormal cardiovascular vagal autonomic tests, though of these only 1 had symptomatic autonomic neuropathy. The rate of abnormality in the whole group (onset latency 19.6%, peak amplitude 20.5%) was lower than that for thermal threshold testing (Marstock warm threshold 35%). There may be several factors responsible for this difference: technical factors, described previously, may be responsible for the high inter-patient variability of the response, leading to definition of a wide reference range. Second, registration of the SSR requires a coordinated sweat gland response, and the efferent pathway to a large group of glands probably has to be substantially damaged in order to delay the onset of the response. An abnormal psychophysical threshold, on the other hand, may occur when there are minimal abnormalities of temporal or spatial summation of impulses.

Shahani et al. (1984) suggested that the SSR reflects abnormal small-fibre function in predominantly axonal neuropathies, rather than clinically disordered autonomic function. This is to be expected, since most symptoms of autonomic dysfunction in diabetes are due to abnormalities of proximal parasympathetic, rather than of distal sympathetic function, symptoms of which (i.e. postural hypotension, gross abnormalities of sweating) are late features of diabetic neuropathy. In fact, the association between SSR, thermal and pain sensation and the other sweat tests (apart from the association between peak 1 amplitude and pilocarpine-activatable sweatspot density) was poor, reflecting the fact that local sweat tests assess function of postganglionic sympathetic nerve terminals and sweat gland receptors, and it is unlikely that their function changes pari passu with that of the whole sympathetic pathway. In the largely parasympathetically-mediated cardiac autonomic function tests only the most reproducible of the tests, respiratory RR variation, just reached statistically significant levels of association. The lack of association with postural blood pressure changes is unexpected, and at variance with the report of Watahiki et al. (1989), probably as a result of the variability and lack of standardisation of measurements. The strong correlation between SSR amplitude (though not latency) and vibration perception thresholds, noted also with pilocarpine-stimulated sweatspot density (see Chapter 4), is not readily explicable.

Despite the low abnormality rate in this study, and despite therefore a probable low sensitivity, the test appears to be specific, in that compared with thermal threshold testing and cardiac vagal autonomic tests, it rarely fails to detect an abnormality. Most patients with
abnormal SSR latency have abnormalities in thermal sensitivity or RR interval variation, or both. The concordance between sweatspot activation and abnormal SSR latency is poor; only 6 subjects with prolonged latency also had low sweatspot counts. Part of the discrepancy may be explained by the presence of abnormally large numbers of activatable sweat glands in some cases (see Chapter 4). Four such patients were identified in this study; two had evidence of diabetic neuropathy (either abnormal respiratory RR variation or Marstock warm threshold) and two had normal autonomic and thermal tests, but all four had very poor diabetic control. There were no supranormal peak amplitudes, suggesting that local changes causing increased sweat gland activation to pharmacological agents do not act through the same mechanisms as generalised sympathetic stimulation.

Maselli et al. (1989) found that there was high concordance between an absent SSR and sweating after axon reflex stimulation by acetylcholine (quantitative sudomotor axon reflex test, Q-SART; Low et al. 1983), but this finding does not support their conclusion that the combined use of these two tests may help uncover early distal sympathetic failure. Both tests would be expected to be abnormal in patients selected on the basis of abnormal peripheral nerve function, and although the Q-SART may be a sensitive indicator of sympathetic dysfunction, it has not been studied in randomly-selected diabetic subjects, nor compared with other standard neurophysiological measurements currently in use. Our study confirms the views of both Shahani et al. (1984) and Fagius and Wallin (1980) that abnormal sympathetic skin activity is a manifestation of relatively advanced neuropathy. The ability to quantify SSR latency allows qualification of Fagius and Wallin's view that sympathetic failure is an "all-or-none" phenomenon, on the basis that slowed sympathetic conduction in individual microneurographic recordings did not occur. They suggested that conduction was maintained at relatively normal levels until, finally, conduction block supervened and the function of that fibre failed. Our finding of a group of patients with prolonged SSR latencies is presumptive evidence of slowed sympathetic conduction in an integrated pathway, even though there may be "all-or-none" failure in individual sympathetic fibres.

In summary, this study has confirmed that if attention is paid to methodological detail, SSR is quantifiable in the majority of diabetic patients. Latency measurement is an objective and reproducible measure of conduction in a long multi-neural pathway, and may therefore be complementary to semi-quantitative psychophysical measurements. In itself, measurement of SSR amplitude appears to be no more sensitive than other measurements as a diagnostic tool in diabetic neuropathy, but it has acceptable reproducibility and is a measurement that can be easily measured in the EMG laboratory; we have confirmed that it is a valid measure of sweat
gland activation. However, like other neurophysiological measurements it varies with surface temperature, and other, more direct measures of peripheral sympathetic innervation, such as pilocarpine-activated sweatspot density, may be preferable for diagnostic purposes.
6.1 Introduction

Previous chapters have described some of the abnormalities of sweating in diabetic neuropathy. Microvascular responses are also known to be abnormal in diabetic skin (see Introduction, sections 1.4.7, 1.4.8). The mechanisms involved are complex and may involve both classical and peptide neurotransmitters.

Immunohistochemical studies have shown that vasoactive intestinal polypeptide (VIP) is the most abundant neuropeptide in sudomotor nerves, where it is co-localised with acetylcholine (Lundberg et al. 1979), and VIP receptors have been found on human sweat gland acini and ducts, suggesting a regulatory role in eccrine sweating (Heinz-Erian et al. 1986; Eedy et al. 1990). Yamashita et al. (1987) have shown an increase in methacholine-induced sweating in humans due to VIP. Other neuropeptides are found in small quantities in dermal nerves adjacent to sweat glands (see Introduction, section 2.2.4), and some, for example substance P (SP), are abundant in sudomotor nerves in non-human species (Lindh et al. 1988), but no data exist on their effects on sweating in humans. The content of substance SP is reduced in peripheral nerves in streptozotocin-diabetic rats (Willars et al. 1989) and we have shown that in patients with diabetic neuropathy there is decreased VIP- and SP-immunoreactivity in cutaneous and sudomotor nerves (Levy et al. 1989). Neurogenic flare responses to acetylcholine, SP, histamine, and capsaicin are also diminished in diabetic neuropathy (Aronin et al. 1987; Parkhouse and Le Quesne 1988).

In order to investigate further the effects of neuropeptides on these cutaneous responses, we have studied sudomotor responses to methacholine (acetyl-β-methylcholine), a long-acting muscarinic acetylcholine analogue (Collins et al. 1959; Gordon and Maibach 1966), and also the effects of SP and VIP on methacholine-induced sweat responses; in addition, we have studied the flare responses to the two neuropeptides, in normal subjects and in two age-matched groups of young diabetic patients with and without peripheral neuropathy, defined by two tests of small-fibre function, cardiac vagal autonomic tests and thermal thresholds (see Chapter 1).
6.2 Subjects and methods

6.2.1 Subjects

The subjects were all male Caucasians. Normal subjects were age-matched healthy hospital personnel, with no history or clinical evidence of neurological disease. Clinical and neurological data are shown in Tables 1 and 2. Subjects taking drugs with autonomic effects, or an alcohol intake of more than 14 units a week were excluded. Thirteen of the 14 diabetic subjects were type 1 (insulin-dependent). Patients were considered “neuropathic” if they had an abnormal Somedic Marstock warm threshold at the dorsum of the foot (> 8.6°C) together with at least one abnormal age-related cardiac vagal autonomic test (see Chapter 1). These measurements were normal in the “non-neuropathic” diabetic group; only one of this group had background retinopathy and none had microalbuminuria (early morning albumin/creatinine ratio > 2.0 mg/mmol; Gatling et al. 1988). The neuropathic diabetic subjects had a higher glycated haemoglobin than the non-neuropathic subjects (p = 0.03), implying poorer diabetic control, and a higher prevalence of sensory and autonomic symptoms. Although the neuropathic group was defined on the basis of small-fibre tests, electrophysiological measurements were also significantly different between the two diabetic groups, while, apart from the sural sensory action potential amplitudes, there were no significant differences between the non-neuropathic patients and the normal subjects (Table 2).

Table 1: Demographic and clinical data. Median values are given [interquartile range] (number of subjects)

<table>
<thead>
<tr>
<th></th>
<th>Normals (n = 6)</th>
<th>Non-neuropathic (n = 8)</th>
<th>Neuropathic (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>32 [26-42]</td>
<td>36.5 [28.5-43]</td>
<td>37.5 [25-45]</td>
</tr>
<tr>
<td>Duration of diabetes, y</td>
<td>–</td>
<td>14.5 [8.5-21.5]</td>
<td>20 [15-32]</td>
</tr>
<tr>
<td>HbA1c, %1</td>
<td>–</td>
<td>10.2 [8.0-11.2]</td>
<td>13.3 [11.6-15.8]*</td>
</tr>
<tr>
<td>Retinopathy2</td>
<td>–</td>
<td>1/8</td>
<td>6/6</td>
</tr>
<tr>
<td>Microalbuminuria3</td>
<td>–</td>
<td>0/8</td>
<td>2/6</td>
</tr>
<tr>
<td>Sensory symptoms</td>
<td>0/6</td>
<td>1/8</td>
<td>5/6</td>
</tr>
<tr>
<td>Autonomic symptoms</td>
<td>0/6</td>
<td>1/8</td>
<td>5/6</td>
</tr>
</tbody>
</table>

* p = 0.03 compared with non-neuropathic diabetic patients
1 Reference range < 8.0%  
2 Background retinopathy (> 5 microaneurysms) or previous laser therapy  
3 Overnight albumin/creatinine ratio > 2.0 mg/mmol
Table 2: Neurological measurements. Median values are given [interquartile range] (number of subjects)

<table>
<thead>
<tr>
<th></th>
<th>Normals (n = 6)</th>
<th>Non-neuropathic (n = 8)</th>
<th>Neuropathic (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Respiratory RR variation, bpm</td>
<td>30 [23-34] (5)</td>
<td>24.5 [22.5-26.0] (8)</td>
<td>7 **$§$ [6-9] (6)</td>
</tr>
<tr>
<td>Sowmedic warm threshold, °C</td>
<td>6.0 [3.7-8.7] (4)</td>
<td>4.9 [3.8-5.9] (8)</td>
<td>12.0 **$§$ [10.2-13.5] (6)</td>
</tr>
<tr>
<td>Sowmedic cool threshold, °C</td>
<td>1.8 [1.3-3.8] (4)</td>
<td>2.3 [1.9-4.2] (8)</td>
<td>9.0 $§$ [7.1-14.2] (6)</td>
</tr>
<tr>
<td>Common peroneal conduction velocity, m/s</td>
<td>47.0 [45.0-51.0] (4)</td>
<td>45.0 [43.0-45.6] (7)</td>
<td>37.0 $§$ [35.5-40.7] (4)</td>
</tr>
<tr>
<td>Common peroneal motor action potential amplitude, mV</td>
<td>8.1 [4.6-10.4] (4)</td>
<td>6.5 [6.0-8.1] (7)</td>
<td>1.2 $*$ [0.6-3.1] (4)</td>
</tr>
<tr>
<td>Peroneal minimum f latency, ms</td>
<td>50.4 [47.9-51.3] (4)</td>
<td>50.1 [44.5-52.4] (6)</td>
<td>62.7 **$§$ [60.6-66.1] (3)</td>
</tr>
<tr>
<td>Sural sensory action potential amplitude, µV</td>
<td>25.2 [20.2-26.2] (4)</td>
<td>13.5 $†$ [12.1-17.3] (7)</td>
<td>2.4 $§$ [1.7-3.8] (4)</td>
</tr>
</tbody>
</table>

* p < 0.05 vs. non-neuropathic diabetic patients
** p < 0.005 vs. non-neuropathic diabetic patients
$§ p < 0.05$ vs. normal subjects
$§§ p < 0.005$ vs. normal subjects
$† p < 0.05$ vs. normal subjects

6.2.2 Sweat output measurements

Sweat output was measured with the modified ventilated-capsule sudorometer described in detail in Chapters 2 and 3. The capsule enclosed a skin area of 1.7cm². In summary, the characteristics of the silicon chip hygrometer used (Dewluxe DL20, MCM, Wetherby, Yorkshire) were high precision (±0.5°C) and stability (0–2°C per 1000 hours continuous operation), and a fast response time (1.0 s at an air flow rate of 300 ml/min) (Levy et al. 1991). Since the hygrometer measured dewpoint rather than relative humidity, the measurements were independent of environmental conditions and could be converted directly to weight of water evaporated. A computer program sampled and plotted the hygrometer
output every second and stored measurements for subsequent integration to determine total sweat output.

6.2.3 Protocol design

Experiments were performed on the volar surface of the forearm. All subjects were relaxed and semi-recumbent with the forearm supinated on the arm of a chair. Skin surface temperature was maintained at 30–33.5°C, ambient temperature at 23–25°C, and relative humidity at 40–65%. Capillary blood glucose measurements (Glucometer II/Glucox, Ames, Stoke Poges, England) were taken in all diabetic patients at the beginning and end of each test session; no patient was hypoglycaemic during the tests.

$10^{-3}$M stock solutions of the peptides (Sigma Chemical Co, Poole, England) were prepared in lactate-buffered (Hartmann’s) solution with 0.05 ml of 0.25% acetic acid, and stored at $-20^\circ$C. Methacholine bromide (Sigma) was dissolved in Hartmann’s solution alone. Peptides were thawed at room temperature and diluted in Hartmann’s solution immediately before use.

In preliminary experiments using acetylcholine in 3 normal subjects, we confirmed the findings of Clubley et al. (1978) that there were no right/left, proximal/distal or medial/lateral differences in peak or total sweat responses; three adjacent sites were therefore used on each forearm, at least 5 cm apart, in random order. The injections, in a volume of 100 µl, were given intradermally using a 0.5 ml syringe with a 27 G needle; the insertion site of the needle was outside the sweat capsule to exclude spurious recording of fluid or blood leakage. Skin evaporation rates were stable for at least 2 min before each injection; each subject then had six injections at randomly chosen sites:

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Methacholine 5 x $10^{-7}$M</td>
</tr>
<tr>
<td>2</td>
<td>VIP 3.3 x $10^{-6}$M</td>
</tr>
<tr>
<td>3</td>
<td>VIP 3.3 x $10^{-6}$M + methacholine 5 x $10^{-7}$M</td>
</tr>
<tr>
<td>4</td>
<td>SP 3.3 x $10^{-6}$M</td>
</tr>
<tr>
<td>5</td>
<td>SP 3.3 x $10^{-6}$M + methacholine 5 x $10^{-7}$M</td>
</tr>
<tr>
<td>6</td>
<td>Methacholine 5 x $10^{-7}$M</td>
</tr>
</tbody>
</table>

The first and sixth injections were methacholine alone, with the others in random order; the mean of the two methacholine responses was taken as the methacholine-induced sweat output. Sweat rate was measured for 30 min by which time most responses had reached baseline levels; it was then integrated to give total sweat output expressed in mg of water. The median
coefficient of variation for the duplicate measurements of methacholine-induced sweating was 8.6% for normal subjects (interquartile range: 4.8–22.2%) and 6.9% for diabetic patients (interquartile range: 1.7–21.5%).

6.2.4 Flare responses

Flare responses were measured 10 min after the injection of VIP and SP given alone. In three normal subjects and one diabetic subject, flare responses to the two peptides were found not to be affected by the presence or absence of methacholine. The outline of the flares were traced on a transparent plastic sheet, cut out and weighed on a balance to the nearest 0.001 mg. Flare areas were expressed in cm², by back-conversion from a calibration line produced by weighing known areas of the same plastic sheet.

6.3 Statistical analysis

Results are expressed as medians and quartiles. The changes in sweat responses after neuropeptide injections were calculated as a ratio: (sodium output after methacholine + peptide) / (sodium output after methacholine alone), to allow for the differences in methacholine-alone sweat output in different subjects. Paired differences between (methacholine + VIP), (methacholine + SP) and (methacholine alone) were analysed using the Wilcoxon test for paired samples; the Mann-Whitney U-test was used for all other comparisons.

![Figure 1](image-url)

*Figure 1:* Methacholine-induced sweat output. Neuropathic patients had markedly increased sweat output compared with non-neuropathic patients ($p=0.028$) and normal subjects ($p=0.025$)
6.4 Results

6.4.1 Sweat responses

There were no significant differences in baseline sweating between the three groups. The neuropathic diabetic patients had significantly greater median sweat production after methacholine than either the normal subjects or the non-neuropathic diabetic patients (Figure 1, Table 3). Neither VIP nor SP given alone induced sweating in any subject; there were no sweat responses to Hartmann’s solution alone in four normal subjects.

The addition of VIP or SP to methacholine significantly reduced sweat output in all groups (Table 3); both peptides reduced sweating to a similar extent in all groups, except that VIP had a smaller effect in the non-neuropathic diabetic group (16% reduction compared with 48% in normal subjects and 45% in neuropathic diabetic patients). The difference between the effect of VIP in the non-neuropathic diabetic group compared with the other two groups was significant (p=0.028 vs. neuropathic diabetic group; p=0.039 vs. normal group), as was the difference between VIP and SP in this group (p=0.014); all 8 patients showed a smaller reduction after VIP than after SP (Figure 2).

6.4.2 Flare responses

Flare responses after both VIP and SP in the neuropathic diabetic patients were significantly smaller compared to those seen in the non-neuropathic diabetic patients (VIP: p=0.01; SP: p=0.002) or normal groups (VIP: p=0.004; SP: p=0.006) (Figure 3, Table 4). There was no visible flare after methacholine alone in four normal subjects.
Figure 2: Sweat output after methacholine with either substance P (SP) (filled symbols) or vasoactive intestinal polypeptide (VIP) (open symbols). Sweat output is similar after addition of both peptides in normal and neuropathic subjects (circles, triangles, respectively), but is significantly (p=0.014) decreased after VIP compared with SP in non-neuropathic patients (p=0.014).
Table 3: Baseline sweating and sweat production after methacholine, methacholine and VIP and methacholine and SP. Sweat output is expressed as mg water. Median values [interquartile range]

<table>
<thead>
<tr>
<th></th>
<th>Normal ((n=6))</th>
<th>Without neuropathy ((n=8))</th>
<th>With neuropathy ((n=6))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal sweat rate (mg·cm⁻²·hr⁻¹)</td>
<td>0.965 [0.943-1.034]</td>
<td>0.957 [0.869-1.050]</td>
<td>1.038 [0.884-1.119]</td>
</tr>
</tbody>
</table>

Sweat output (mg) after:

<table>
<thead>
<tr>
<th></th>
<th>Normal ((n=6))</th>
<th>Without neuropathy ((n=8))</th>
<th>With neuropathy ((n=6))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methacholine + VIP</td>
<td>1.75 § [1.51-2.12]</td>
<td>2.99 § [2.00-4.06]</td>
<td>3.02 § [2.00-4.98]</td>
</tr>
<tr>
<td>Methacholine + VIP, methacholine alone</td>
<td>0.52 [0.46-0.69]</td>
<td>0.84 § [0.65-0.95]</td>
<td>0.55 [0.49-0.58]</td>
</tr>
<tr>
<td>Methacholine + SP, methacholine alone</td>
<td>0.61 [0.39-0.79]</td>
<td>0.52 [0.42-0.65]</td>
<td>0.62 [0.41-0.86]</td>
</tr>
</tbody>
</table>

* p = 0.025 vs. normal subjects, p = 0.028 vs. non-neuropathic diabetic patients
§ p < 0.05 vs. methacholine alone
¶ p = 0.028 vs. normal subjects, p = 0.014 vs. neuropathic diabetic patients

Table 4: Area of flare (cm²) 10 min after injection of VIP and SP in normal and diabetic subjects. Median values [interquartile range]

<table>
<thead>
<tr>
<th></th>
<th>Normal ((n=6))</th>
<th>Without neuropathy ((n=8))</th>
<th>With neuropathy ((n=6))</th>
</tr>
</thead>
</table>

* p = 0.01 vs. non-neuropathic patients, § p = 0.004 vs. neuropathic patients
** p = 0.002 vs. non-neuropathic patients, §§ p = 0.006 vs. normal subjects
Figure 3: Flare responses (cm²) after intradermal substance P (top panel) and vasoactive intestinal polypeptide (lower panel). The flare area induced by both peptides was significantly smaller in neuropathic patients compared with both non-neuropathic and normal subjects (Table 4).

6.5 Discussion

This study has clearly demonstrated that with progressing peripheral neuropathy, local sweat responses to methacholine increase. VIP and SP decreased methacholine-induced sweating in all groups, while VIP had a greater inhibitory effect in non-neuropathic, compared with neuropathic or normal subjects. Neuropathic patients had markedly decreased axon-reflex flare responses to the two neuropeptides.
In the patients with established neuropathy, the exaggerated methacholine sweat responses suggest denervation supersensitivity. However, since activated sweat gland size was not measured, it is not possible to exclude the presence of “compensatory hypertrophy” of upper limb sweat glands in response to lower limb denervation and hypohidrosis, as suggested by Goodman (1966). This concept, though attractive and consistent with the clinical occurrence of drenching upper body segment sweating together with lower limb anhidrosis in some diabetic patients (see Introduction, section 1.2), has not been quantified in diabetic patients. The study reported in Chapter 4 found that there was a close correlation between activated sweatspot diameter (reflecting sweat output per gland) and sweatspot density in both normal and diabetic subjects, even in those with high sweatspot density, suggesting that a combination of increased activated sweatspot density and sweat output per gland contribute to the large responses.

It is possible that the neuropathic patients had abnormal neurological function in the legs, but not in the upper limb, which was not measured in the current study. While it is probable that upper limb neuropathy was not as severe as in the legs, it is likely that they had advanced generalised neuropathy, as cardiac autonomic tests, which were uniformly abnormal, are thought to be mediated by proximal neural pathways (see Introduction, section 1.4.9.1).

Increased cholinergic sudomotor response have been noted in several other clinical studies in diabetes, though poorly quantified. Low et al. (1983), using iontophoresed acetylcholine to stimulate only axon-reflex (nicotinic) sweat responses, illustrated an excessive (“hung up”) sweat response in the arm of diabetic patient with an absent axon reflex response in the foot. He concluded that since clinically manifest neuropathy occurred later in the upper limb than in the lower limb, increased sweat responses were indicative of an earlier phase of denervation, which then progressed to absence of the axon reflex sweat response. The results of the current study, while considering muscarinic rather than nicotinic sweat gland responses, do not support this view; Ahmed and Le Quesne (1986), using Low’s Q-SART method, found the same “hung-up” response in the foot of one of their patients with advanced diabetic neuropathy. Supersensitivity to cholinergic agents is therefore a widespread phenomenon in diabetic neuropathy. In the current study, the methacholine-induced sweat responses in neuropathic subjects were, with one exception, completely separated from those of the normal subjects. The exception occurred in a 21 year old trained athlete, where sweat responses may be increased due to the phenomenon of “sweat gland training” (Sato and Sato 1983; see Introduction, section 2.5.3.10), which may in turn be caused by sweat gland hypertrophy in response to aerobic exercise.
The role of neuropeptides in eccrine sweating is poorly understood (see Introduction, section 2.4.3), but there is growing evidence that peptide actions are modulatory or enabling, in contrast to those of conventional neurotransmitters, which have been described as “mediating”. VIP receptors exist in human sweat glands (Heinz-Erian et al. 1986; Eedy et al. 1990), and VIP stimulates sweating in an isolated (i.e. denervated and devascularised) simian palmar sweat gland preparation (Sato and Sato 1987), through a slowly-acting cAMP-mediated process. This view of neuropeptides as modulatory substances is supported by the current study, in which none of the subjects showed any sweat responses to either VIP or SP when injected alone. This confirms the result obtained by Yamashita et al. (1987), who were also unable to detect sweating in response to VIP alone.

There is sparse evidence for the modulatory role of neuropeptides in sweat gland function. The modest increases in methacholine sweat responses found in response to VIP in Sato’s experimental arrangement and in Yamashita’s study contrast with the known effects of SP on the acetylcholine receptor, where it enhances the rate of desensitisation of the receptor, even, apparently, in the absence of acetylcholine (Belcher and Ryall 1977; Boyd and Leeman 1987). While no experimental studies have specifically addressed the question of the effect of VIP on acetylcholine receptors, it is possible that it also has an inhibitory action similar to that of SP, resulting in the reduced sweat responses seen with both SP and VIP in the current study. Our findings with VIP are in contrast to those of Yamashita et al. (1987), who found a 30–120% increase in methacholine-induced sweat output in four normal subjects after injection of similar concentrations of VIP and methacholine. The possible reasons for this discrepancy are not clear, and may be methodological. Yamashita et al. (1987) measured sweating with a much larger ventilated capsule (area 15 cm²) than the one used here (area 1.7 cm²). It is conceivable that VIP has inhibitory effects in high concentrations, such as would be found at the centre of our sweat capsule, while stimulating sweating in the periphery where the injected VIP would be at lower concentrations, yielding overall a slight net increase in sweating. The changes found by Yamashita et al. (1987) were indeed inconsistent between subjects, and it was suggested that the state of peripheral microvascular vasodilatation may itself modulate sweat responses (Sugenoya et al. 1988). However, we have found, in two of the normal subjects and one non-neuropathic diabetic subject, that the inhibitory effect of VIP becomes smaller at lower concentrations, down to $3 \times 10^{-9}$M; we were unable to detect any stimulatory effect. In addition, evidence against the role of the microvasculature is provided by the observation that the patients with the greatest decrease in sweat response to the peptides had the smallest flare areas; conversely, using the same sudorometer (which has a
sensitivity estimated at approximately 200 ng water vapour), we found that there was no increase in basal transepidermal water loss induced either by axon-reflex flare (SP, VIP) or by direct vasodilatation (CGRP) in normal subjects.

Apart from the increased inhibitory effect of VIP in the non-neuropathic group, there were no significant differences in the effects of VIP or SP on methacholine-induced sweating in the three groups of subjects. Sudomotor nerves do not themselves contain SP (Tainio et al. 1987), and the reduction in sweat output we have recorded is probably due not to SP acting directly on the sweat gland, but via some other effect on its micro-environment, possibly through SP-immunoreactive nerves near to sweat glands in the dermis. Interstitial oedema, caused by axon-reflex mediated extravasation (wheal formation), and resulting in mechanical obstruction of the sweat ducts, may play a role, though this cannot be the sole mechanism, as the bleb formed by the injection of methacholine alone did not result in a reduction in sweat output in neuropathic patients. The similar sweat reduction seen in normal subjects both with VIP and SP is unexpected, given that VIP-immunoreactive nerves directly innervate sweat glands, and suggests that the direct sweating effect of methacholine may in some way be inhibited by neurogenic mechanisms common to both peptides. However, the increased inhibitory effect of VIP in non-neuropathic subjects suggests a specific effect, perhaps due to upgrading of VIP receptors. Histochemical techniques, such as quantitative autoradiography of muscarinic and neuropeptide sweat gland receptors, will be required to substantiate this.

Vascular responses to VIP and SP were clearly abnormal in our neuropathic patients, as the flare responses to both were markedly reduced; however, neuropathy is not the only possible cause of a diminished flare response, as non-neurogenic (endothelium-dependent) factors are also involved in the abnormal microvascular reactivity of diabetic subjects (Westerman et al. 1987), and release of the flare mediators, for example histamine, from mast cells, may also be abnormal (Church et al. 1989).

In this study diabetic patients with the greatest deficits in neurological function assessed by standard tests also had the largest sweat responses to methacholine. This finding has been observed in the previous studies reported here. The coexistence of these two phenomena can be explained by considering two points: first, conventional neurophysiological tests, where effector organ responses are not directly observed, are unlikely to reveal hyper-responsiveness; second, potential hyper-responsiveness may be masked by deficits in conduction in long neuronal pathways. The finding of decreased sympathetic skin response in patients who may have increased pilocarpine-stimulated sweatspot densities and dynamic sweat responses to acetylcholine supports this analysis. If hyper-responsiveness is present, it
is therefore more likely to be revealed by local stimuli to effector organs like those used in the current study. However, it is clear from the discordance between the sweat responses and the flare reactions in neuropathic patients, that local responses are not uniformly affected by the neuropathic process.

In conclusion, methacholine-induced sweat responses are frequently exaggerated in diabetic patients with neuropathy, consistent with denervation supersensitivity. VIP and SP cause a reduction in methacholine-induced sweating that is similar in degree in both diabetic and normal subjects, except that non-neuropathic diabetic patients show a smaller reduction after VIP. The discrepancy between the vascular and sweating responses to VIP and SP reflects the fact that cutaneous denervation has markedly different effects on blood vessels and sweat glands. Abnormalities in both may be important in the development of diabetic foot lesions, and the mechanisms underlying these findings deserve further investigation.
7.1 Introduction

The pathogenetic mechanisms proposed in the aetiology of diabetic neuropathy appear to act at various points along the course of the peripheral nerve. For example, derangements of retrograde axonal transport involve the nerve terminals (Fink et al. 1987), while abnormal anterograde axonal transport results from abnormalities of the cell body (see Introduction, section 1.3). Microvascular abnormalities (Dyck et al. 1986) and nerve hypoxia (Low et al. 1985) are either multifocal or diffusely affect the entire length of the axon. The impact of these various processes is seen predominantly at the distal nerve terminals (Waxman et al. 1976).

Previous chapters have described methods for studying functional abnormalities of distal small fibres. The structural correlates of these functional findings are not known. The human neuronal cell body cannot be studied in vivo. Studies of sural nerve biopsies have yielded quantitative information on myelinated fibre structure (Sima et al. 1988), but the procedure is invasive and carries a small but appreciable morbidity (Dyck et al. 1984), and results of small fibre morphometry have not yet been presented.

Skin biopsy has been proposed as a simple method for investigating unmyelinated sensory and autonomic fibres, abnormalities of which are thought to underlie many symptoms of peripheral neuropathy (Johnson et al. 1981; Levy et al. 1989a; Lindberger et al. 1989) and to contribute to the development of neuropathic ulceration (Watkins and Edmonds 1983; Ahmed and Le Quesne 1986). In a preliminary study (Levy et al. 1989a) we studied semi-quantitatively the pattern of cutaneous nerve immunofluorescence to a general neuronal marker, PGP 9.5 (Thompson et al. 1983; Dalsgaard et al. 1989; Wilkinson et al. 1989). All mammalian nerves immunostain for such markers, and demonstration of the cutaneous innervation in its entirety can be achieved by the use of antibodies to them. In our earlier study, we found that PGP 9.5-immunoreactivity (IR) was markedly depleted in diabetic patients, most noticeably in those with neurophysiological evidence of diabetic neuropathy. In addition, individual nerves were found to be depleted of specific neuropeptides. The neuropeptides substance P (SP) and calcitonin gene-related peptide (CGRP) localise to sensory nerves, while neuropeptide Y and vasoactive intestinal polypeptide (VIP) are considered
immunohistochemical markers of autonomic nerves (see Introduciton, section 1.3.3). There appeared to be an association between the degree of neuropathy, as assessed by conventional small- and large-fibre tests, and the degree of PGP 9.5 and neuropeptide depletion. This study, however, was uncontrolled, and several subjects had evidence of advanced macrovascular disease. Lindberger et al. (1989) confirmed semi-quantitatively that there were decreased numbers of epidermal SP- and CGRP-immunoreactive (IR) fibres in diabetic subjects with no objective evidence of neuropathy, compared with non-diabetic controls.

The present study was designed to extend our initial observations, by standardising the skin biopsy site, and studying young subjects with no clinical evidence of macrovascular disease, classified on the basis of standard neurophysiological criteria into a group with, and a group without, evidence of small-fibre neuropathy. In addition to well-established semi-quantitative methods (Karanth et al. 1989) we have used quantitative computerised image-analysis to measure nerve immunofluorescence areas and analysed their associations with the neurological tests described in previous chapters.

7.2 Subjects and methods

7.2.1 Subjects

Three groups of subjects were studied:

<table>
<thead>
<tr>
<th>Group 1</th>
<th>Normal subjects (n=14). Exclusion criteria were as described in Appendix A. In addition, no subject taking any medication or with a history of trauma to the legs was included.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 2</td>
<td>Diabetic subjects (&quot;non-neuropathic&quot;, n=11), with normal small-fibre tests ie. Somedic Marstock warm threshold ≤8.6°C, cool threshold ≤4.7°C, and 3 normal age-related cardiovascular vagal autonomic function tests (see Chapter 1).</td>
</tr>
<tr>
<td>Group 3</td>
<td>Diabetic subjects (&quot;neuropathic&quot;, n=11), with evidence of small-fibre neuropathy ie. all had a warm thermal threshold &gt;8.6°C or cool threshold &gt;4.7°C, or both, and at least one abnormal age-related vagal cardiovascular test.</td>
</tr>
</tbody>
</table>

Clinical and neurophysiological characteristics of the subjects are given in Tables 1(a) and (b).
Table 1(a): Clinical characteristics of the three groups of subjects. Results are given as mean (range). P values refer to Mann-Whitney U-test comparisons between the non-neuropathic patients (Group 2) and the neuropathic patients (Group 3).

<table>
<thead>
<tr>
<th></th>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>28 (19–58)</td>
<td>33 (20–51)</td>
<td>44 (21–53)</td>
<td>0.2</td>
</tr>
<tr>
<td>Duration of diabetes, y</td>
<td>14 (8–30)</td>
<td>19 (5–34)</td>
<td></td>
<td>0.3</td>
</tr>
<tr>
<td>Male:female</td>
<td>10:4</td>
<td>7:4</td>
<td>9:2</td>
<td></td>
</tr>
<tr>
<td>Type 1:Type 2</td>
<td>–</td>
<td>10:1</td>
<td>11</td>
<td></td>
</tr>
</tbody>
</table>
| Glycated haemoglobin,%  
$^1$ |           | 10.9 (8.8–18.6) | 12.9 (8–17.8) | 0.2    |
| Retinopathy $^2$ | –         | 0         | 11        |        |

$^1$ Kruskal-Wallis ANOVA (Groups 1-3)
$^2$ Reference range <8.0%
$^3$ Assessed by direct ophthalmoscopy

Table 1(b): Neurophysiological characteristics of the diabetic subjects studied. Data are presented as mean (range) [number of subjects studied]. P values refer to Mann-Whitney U-tests comparing the non-neuropathic diabetic patients (Group 2) and the neuropathic patients (Group 3).

<table>
<thead>
<tr>
<th></th>
<th>Reference range</th>
<th>Group 2</th>
<th>Group 3</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Marstock warm threshold, °C</td>
<td>≤8.6</td>
<td>4.7 (3.4–7.4)</td>
<td>12.0 (5.5–16.8)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Marstock cool threshold, °C</td>
<td>≤4.7</td>
<td>3.0 (1.8–4.6)</td>
<td>9.1 (4.2–16.3)</td>
<td>0.001</td>
</tr>
<tr>
<td>Marstock heat pain threshold, °C</td>
<td>≤16.5</td>
<td>15.5 (14.0–16.5)</td>
<td>15.8 (11.0–17.9)</td>
<td>0.8</td>
</tr>
<tr>
<td>Respiratory RR variation, bpm</td>
<td>(age related)</td>
<td>22 (10–27)</td>
<td>7 (4–30)</td>
<td>0.004</td>
</tr>
<tr>
<td>Valsalva ratio</td>
<td>≥ 1.13</td>
<td>1.73 (1.09–2.13)</td>
<td>1.27 (1.19–1.58)</td>
<td>0.03</td>
</tr>
<tr>
<td>30:15 ratio</td>
<td>(age related)</td>
<td>1.20 (0.96–1.64)</td>
<td>1.08 (1.00–1.18)</td>
<td>0.07</td>
</tr>
<tr>
<td>CPMCV, m/s $^1$</td>
<td>(age related)</td>
<td>41 (32–46) [9]</td>
<td>38 (34–42) [9]</td>
<td>0.1</td>
</tr>
<tr>
<td>SSAP, $\mu V^2$</td>
<td>&gt;4.9</td>
<td>12 (7–20) [9]</td>
<td>6 (2–8) [6]</td>
<td>0.002</td>
</tr>
</tbody>
</table>

CPMCV, common peroneal motor conduction velocity
SSAP, sural sensory action potential amplitude
7.2.2 Other neurophysiological and sweat tests

Diabetic subjects had other neurophysiological tests in addition to thermal threshold and autonomic function tests (see Chapter 1): biothesiometry at the medial malleolus, and electrophysiology (common peroneal nerve conduction velocity) using standard surface EMG techniques. In addition, the three tests of sudomotor function were performed, as described in Chapters 3-5. Venous blood for glycated haemoglobin estimation (affinity chromatographic method, reference range $<8.0\%$) was taken at the time of the biopsy. Not all subjects had the full range of neurophysiological assessments.

7.2.3 Skin biopsy technique

Full-thickness skin biopsies were taken with aseptic technique from the lateral aspect of the middle one-third of the right lower leg. A standard elliptical template 1.7 cm long, 0.6 cm wide was used to mark the skin, and the skin prepared with chlorhexidine. Plain 1% lignocaine was infiltrated widely around the biopsy site. Specimens were fixed immediately after removal. The skin was closed with 2 or 3 sutures, which were removed at 14 days. The procedure was approved by the Ethics Committee of Parkside Health Authority. In order to assess reproducibility, 3 normal subjects had repeat biopsies 6-9 months after the first, taken 5 cm distal to the original biopsy and from the same leg.

7.2.4 Complications of skin biopsy

One diabetic and one normal subject developed a wound infection and dehiscence after removal of sutures. The diabetic patient (Group 2) had poor glycaemic control (glycated haemoglobin = 16.0%). In both instances written instructions relating to wound care had not been followed. Broad spectrum antibiotics were given and both healed within 3-4 weeks. In two other normal subjects, wound dehiscence occurred after removal of sutures, but healing was uneventful.

7.2.5 Histochemical methods

Specimens were fixed in Zamboni's solution for 4-6 h at room temperature, then transferred to phosphate buffered saline (PBS, 0.01 mol/l phosphate buffer, pH 7.1, containing 0.15 mol/l sodium chloride) at 4°C. Cryostat sections (10 μm) were taken up on poly-L-lysine-
coated glass slides, allowed to dry for 2 h at room temperature and then placed in PBS containing 0.2% Triton X-100 for 30 min. A modified indirect immunofluorescent staining method was used for immunostaining (Karanth et al. 1989), using antisera to PGP 9.5, VIP, SP and CGRP (Table 2). Sections were incubated in appropriately diluted primary antiserum for 16 h at 4°C, then washed thoroughly for 10 min in three fresh changes of PBS and reincubated in the primary antiserum for 3 h at room temperature. After three further 5 min PBS washes, goat anti-rabbit fluorescein isothiocyanate conjugate (FITC, 1:200, Miles Laboratories, Stoke Poges, UK) was applied for 1 h. Slides were washed again for 5 min in three fresh changes of PBS, mounted in PBS glycerol (1:9 volume/volume) and viewed using a fluorescence microscope.

Table 2: Antisera used in immunohistochemistry

<table>
<thead>
<tr>
<th>Rabbit antiserum to:</th>
<th>Source</th>
<th>Dilution</th>
<th>Absorption¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGP 9.5</td>
<td>Ultraclone, Cambridge, UK</td>
<td>1:1000</td>
<td></td>
</tr>
<tr>
<td>CGRP</td>
<td>Hammersmith Hospital</td>
<td>1:200</td>
<td>0.1</td>
</tr>
<tr>
<td>SP</td>
<td>Hammersmith Hospital</td>
<td>1:100</td>
<td>1.0</td>
</tr>
<tr>
<td>VIP</td>
<td>Hammersmith Hospital</td>
<td>1:200</td>
<td>1.0</td>
</tr>
</tbody>
</table>

¹ Concentration of synthetic antigen required to abolish immunostaining (nmol/ml of diluted antiserum)

7.2.6 Semi-quantitative (observer graded) assessment

Serial sections were cut onto four slides so that every fourth section was on the same slide and stained for the same antigen. Six sections were examined for each antigen.

All slides were screened separately by two observers who were unaware of the clinical group of the specimens, and all slides were screened within 2 days of staining to minimise natural fading of the fluorescence. The number of fibres staining for each antigen was noted separately in relation to the following regions and structures: (1) epidermis, (2) dermis, (3) blood vessels and (4) sweat glands. A third person acted as referee to ensure consistency between the observers and checked the gradings in a random 10% of the specimens.

Semi-quantitative assessments were made using the following grades:
- No fibres seen
± Sparse
+ Few
++ Moderate
+++ Abundant

7.2.7 Quantitative assessment: computerised image-analysis

Separate blocks of tissue were cut. On account of the small numbers of SP-IR fibres found in the semi-quantitative study, sections were stained only for PGP 9.5, CGRP and VIP. The system for quantitation comprised a low-light video camera (Panasonic MV1900, Osaka, Japan) mounted on a Vanox fluorescence microscope (Olympus, London, UK) and linked to an IBAS 2000 image analyser (Kontron, Watford, UK). A long pass filter of approximately 560 nm was used to reduced autofluorescence. The program for quantification was derived from that used in previous studies (Cowen et al. 1987; Gale et al. 1989; Terenghi et al. 1991). Ten digitised images of the chosen field were summed and averaged to reduce noise. Possible uneven illumination and shading at the edge of the field was automatically calibrated to the same level of the brightest part of the image, generally the centre of the field. The field image was defined by the use of interactively defined measured frames in order to establish precisely the area to be measured (for example, epidermis and subepidermal plexus), while deleting any unwanted portion of the image present on the screen (for example, dermal collagenisation, sweat gland granules, artefacts associated with non-specific background fluorescence). The image of the immunostained structures was enhanced by automatic computer process to increase the signal:noise ratio; during the process the grey/black tones of the background were darkened, resulting in an improved contrast of the white images corresponding to the immunostained structures. This was particularly important for fine nerve fibres which might otherwise be missed. A background subtraction procedure was carried out by taking the maximum grey value of the background as the threshold at which to segregate the image. The grey value for binary thresholding was maintained in the same range for all samples, in order to avoid any threshold bias. The discriminated binary image was finally measured.

For each peptide two random non-consecutive sections were analysed, and in each section three linearly-contiguous fields (× 20 objective magnification) in three skin areas — epidermis and subepidermal plexus, dermis, and sweat glands — were measured. Total field
immunofluorescent area (μm²) and field counts of the immunoreactive nerves were measured. The mean value of the total six counted fields was calculated.

7.3 Statistical analysis

Scores of 0 (-), 0.5 (+), 1 (+), 2 (++) and 3 (+++) were assigned to the observer gradings, and analysed by non-parametric methods (Spearman correlation coefficients and Kruskal-Wallis analysis of variance). In the quantitative study, most of the immunoreactive nerve areas and counts were approximately normalised by logarithmic transformation. Analysis of variance (ANOVA) was used to assess statistical differences between the three groups; if significant, further analysis of differences between pairs of groups was carried out by t-tests on the transformed data.

7.4 Results

Tables 3(a) and (b) show the results of the observer graded study, Tables 4(a) and (b) those for the quantitative study.

Table 3(a): Results of the observer graded study, presented as median (range) of the semi-quantitative scores assigned to each specimen. Group 1 = normal subjects, Group 2 = "non-neuropathic" diabetic patients, Group 3 = "neuropathic" diabetic patients

<table>
<thead>
<tr>
<th></th>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epidermis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PGP</td>
<td>2 (1-3)</td>
<td>1 (0.5-2)</td>
<td>0.5 (0-2)</td>
</tr>
<tr>
<td>CGRP</td>
<td>1 (0-2)</td>
<td>0 (0-1)</td>
<td>0 (0-1)</td>
</tr>
<tr>
<td>SP</td>
<td>0 (0-1)</td>
<td>0 (0-0.5)</td>
<td>0 (0-0.5)</td>
</tr>
<tr>
<td>Dermis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PGP</td>
<td>2 (1-3)</td>
<td>2 (0-2)</td>
<td>1 (0-2)</td>
</tr>
<tr>
<td>CGRP</td>
<td>1 (0-2)</td>
<td>1 (0-2)</td>
<td>0 (0-1)</td>
</tr>
<tr>
<td>SP</td>
<td>0 (0-1)</td>
<td>0 (0-1)</td>
<td>0 (0-1)</td>
</tr>
<tr>
<td>Sweat glands</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PGP</td>
<td>3 (2-3)</td>
<td>2 (0.5-3)</td>
<td>1 (0-2)</td>
</tr>
<tr>
<td>CGRP</td>
<td>1 (0-2)</td>
<td>1 (0-2)</td>
<td>0 (0-1)</td>
</tr>
<tr>
<td>SP</td>
<td>0 (0-0.5)</td>
<td>0 (0-1)</td>
<td>0 (0-0.5)</td>
</tr>
<tr>
<td>Blood vessels</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PGP</td>
<td>2 (0-3)</td>
<td>1 (1-3)</td>
<td>2 (0-3)</td>
</tr>
<tr>
<td>CGRP</td>
<td>0.5 (0-1)</td>
<td>0 (0-1)</td>
<td>0 (0-1)</td>
</tr>
</tbody>
</table>
Table 3(b): Kruskal-Wallis analysis of variance for the semi-quantitative study. Further analysis (Mann-Whitney U tests) of differences between pairs of groups is shown if analysis of variance showed statistically significant differences between the three groups ($p < 0.05$). Group 1 = normal subjects, Group 2 = "non-neuropathic" diabetic patients, Group 3 = "neuropathic" diabetic patients

<table>
<thead>
<tr>
<th></th>
<th>ANOVA</th>
<th>1 vs. 2</th>
<th>1 vs. 3</th>
<th>2 vs. 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epidermis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PGP</td>
<td>0.001</td>
<td>0.013</td>
<td>0.002</td>
<td>0.070</td>
</tr>
<tr>
<td>CGRP</td>
<td>0.040</td>
<td>0.218</td>
<td>0.020</td>
<td>0.216</td>
</tr>
<tr>
<td>SP</td>
<td>0.070</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dermis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PGP</td>
<td>0.002</td>
<td>0.020</td>
<td>0.002</td>
<td>0.158</td>
</tr>
<tr>
<td>CGRP</td>
<td>0.388</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SP</td>
<td>0.351</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sweat glands</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PGP</td>
<td>0.002</td>
<td>0.658</td>
<td>0.008</td>
<td>0.004</td>
</tr>
<tr>
<td>CGRP</td>
<td>0.098</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VIP</td>
<td>0.045</td>
<td>0.482</td>
<td>0.029</td>
<td>0.154</td>
</tr>
<tr>
<td>SP</td>
<td>0.791</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blood vessels</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PGP</td>
<td>0.086</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CGRP</td>
<td>0.273</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VIP</td>
<td>0.694</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 4(a): Quantitative immunohistochemistry. Results are given as mean (range). Immunoreactive nerve areas are given as mm² x 10⁶. Group 1 = normal subjects, Group 2 = “non-neuropathic” diabetic patients, Group 3 = “neuropathic” diabetic patients.

<table>
<thead>
<tr>
<th></th>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Epidermis</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PGP area</td>
<td>1020 (522-2192)</td>
<td>843 (196-1665)</td>
<td>391 (0-992)</td>
</tr>
<tr>
<td>count</td>
<td>15 (8-24)</td>
<td>11 (5-17)</td>
<td>5 (0-12)</td>
</tr>
<tr>
<td>CGRP area</td>
<td>282 (0-957)</td>
<td>235 (0-784)</td>
<td>57 (0-268)</td>
</tr>
<tr>
<td>count</td>
<td>6 (0-21)</td>
<td>4 (0-12)</td>
<td>1 (0-6)</td>
</tr>
<tr>
<td><strong>Dermis</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PGP area</td>
<td>1482 (428-2821)</td>
<td>1219 (321-2514)</td>
<td>903 (34-4076)</td>
</tr>
<tr>
<td>count</td>
<td>22 (2-53)</td>
<td>13 (4-38)</td>
<td>9 (1-25)</td>
</tr>
<tr>
<td>CGRP area</td>
<td>185 (0-710)</td>
<td>270 (0-931)</td>
<td>171 (0-590)</td>
</tr>
<tr>
<td>count</td>
<td>3 (0-8)</td>
<td>5 (0-18)</td>
<td>2 (0-8)</td>
</tr>
<tr>
<td><strong>Sweat glands</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PGP area</td>
<td>2403 (941-4337)</td>
<td>1857 (200-6320)</td>
<td>1554 (0-5318)</td>
</tr>
<tr>
<td>count</td>
<td>41 (23-83)</td>
<td>26 (0-62)</td>
<td>23 (2-53)</td>
</tr>
<tr>
<td>CGRP area</td>
<td>277 (0-849)</td>
<td>1146 (22-8975)</td>
<td>54 (0-185)</td>
</tr>
<tr>
<td>count</td>
<td>8 (0-28)</td>
<td>11 (3-26)</td>
<td>3 (0-11)</td>
</tr>
<tr>
<td>VIP area</td>
<td>759 (126-2292)</td>
<td>625 (0-1959)</td>
<td>475 (0-2889)</td>
</tr>
<tr>
<td>count</td>
<td>19 (6-44)</td>
<td>19 (0-48)</td>
<td>8 (0-29)</td>
</tr>
</tbody>
</table>
Table 4(b): Statistical analysis of quantitative immunohistochemistry. ANOVA was performed on the logarithmically-transformed data; if significant (p < 0.05), unpaired t-tests were performed across the three pairs of groups. Group 1 = normal subjects, Group 2 = "non-neuropathic" diabetic patients, Group 3 = "neuropathic" diabetic patients

<table>
<thead>
<tr>
<th></th>
<th>ANOVA</th>
<th>1 vs. 2</th>
<th>1 vs. 3</th>
<th>2 vs. 3</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Epidermis</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PGP area</td>
<td>0.001</td>
<td>0.199</td>
<td>&lt;0.001</td>
<td>0.042</td>
</tr>
<tr>
<td>count</td>
<td>&lt;0.001</td>
<td>0.142</td>
<td>&lt;0.001</td>
<td>0.003</td>
</tr>
<tr>
<td>CGRP area</td>
<td>0.062</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>count</td>
<td>0.026</td>
<td>0.289</td>
<td>0.003</td>
<td>0.150</td>
</tr>
<tr>
<td><strong>Dermis</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PGP area</td>
<td>0.028</td>
<td>0.490</td>
<td>0.022</td>
<td>0.082</td>
</tr>
<tr>
<td>count</td>
<td>0.076</td>
<td>0.146</td>
<td>0.025</td>
<td>0.229</td>
</tr>
<tr>
<td>CGRP area</td>
<td>0.691</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>count</td>
<td>0.608</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Sweat glands</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PGP area</td>
<td>0.160</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>count</td>
<td>0.063</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CGRP area</td>
<td>0.129</td>
<td></td>
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<td></td>
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<tr>
<td>count</td>
<td>0.134</td>
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<tr>
<td>VIP area</td>
<td>0.372</td>
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<tr>
<td>count</td>
<td>0.248</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 1: (Previous page). Photomicrographs of immunohistochemical preparations of epidermis. The specimens are orientated with the skin surface at the top of the photographs (original magnification × 375).

Top: PGP 9.5-immunoreactive nerves in a normal subject (left) and a neuropathic patient (right). Immunoreactive nerve fibres are seen as bright white linear structures in the subepidermal plexus and dermal papillae. In the normal specimen, two intraepidermal nerve fibres are seen penetrating almost to the stratum corneum. There is a marked decrease in these nerves in the specimen from the neuropathic patient; they also appear more fragmented and less uniform in shape and size. Three immunoreactive elements in the epidermis may represent remnants of two or three intraepidermal fibres.

Bottom: CGRP-immunoreactive fibres in a specimen from a normal subject (left) and from a neuropathic patient (right). Even in the normal specimen, CGRP-IR nerves are fewer than PGP-IR nerves. One branched intra-epidermal fibre is seen. There is almost complete absence of CGRP-IR in the neuropathic skin specimen. The single distinct nerve fibre is situated in a dermal papilla sectioned transversely.
Top: PGP 9.5-immunoreactive nerves in the basal coil of sweat glands. In the section from a normal specimen (left) these nerves form a dense, almost continuous plexus around the sweat gland ducts. They are almost completely absent in the specimen from the neuropathic subject (right), where there is also an increase in the non-specific granular staining seen within the sweat gland epithelial cells. This non-specific immunoreactivity was interactively removed before quantitative image-analysis measurements were made.

Bottom: VIP-immunoreactive fibres around sweat glands in a section from a normal subject (left) and from a neuropathic patient (right) Although VIP is the most abundant neuropeptide in sudomotor nerves, VIP-immunoreactive nerves are noticeably less prevalent than the PGP 9.5-immunoreactive fibres (see also Figure 1). The remaining nerves have (a) no specific neuropeptide-immunoreactivity, or (b) are immunoreactive for neuropeptides found in low levels in sweat gland nerves (see Introduction, section 2.2.4) or (c) are immunoreactive for as yet undiscovered neuropeptides. Granular inclusions in the neuropathic specimen are again prominent, though there is no obvious evidence of sweat gland epithelial degeneration.
7.4.1 Observer graded study (Tables 3(a) and (b))

7.4.1.1 Qualitative distribution of skin neuropeptides

Representative photomicrographs are shown in Figures 1 and 2, and dot plots of observer graded and quantitative results in Figures 3-9. The distribution of immunoreactive nerve fibres in specimens from normal subjects was similar to that previously reported (Karanth et al. 1989; Dalsgaard et al. 1989). PGP-IR was observed widely in free nerve endings in the epidermis and dermis, and in nerve endings close to hair follicles and sweat glands. Bundles of PGP-IR nerves were seen around blood vessels and arrector pili muscles. CGRP-IR fibres were found predominantly in the dermis; smaller numbers were seen extending into the epidermis and around sweat glands and blood vessels. VIP-IR was found almost exclusively in the nerves around sweat glands, though there was sparse immunoreactivity in blood vessels.

One normal subject had no detectable IR to any antigen. There were no apparent technical or clinical reasons for this finding, but in extensive experience of immunohistochemical studies of human cutaneous nerves, completely absent IR has never been encountered, and it has been assumed that there was a technical reason for this finding; the results for this subject have not been included in the analysis, though his inclusion makes no difference either to the statistical analysis or to the overall conclusions of the study. All the diabetic biopsies, even those with clinically advanced neuropathy, graded + or ± to at least one antigen.

There were more PGP-IR fibres in the sweat glands than the other regions. Specific neuropeptide immunoreactivity was lower than that of PGP 9.5 in all regions, both in number of fibres and intensity of immunostaining. Median scores for SP-IR were zero in all regions examined, and in view of the very small numbers of SP-IR fibres, quantitation of SP-IR was not undertaken.

7.4.1.2 PGP 9.5 and neuropeptide immunoreactivity

Epidermal and dermal PGP 9.5-IR were significantly depleted in non-neuropathic and neuropathic patients compared with normals (Figures 3 and 5); a similar trend was shown for sweat gland PGP 9.5-IR (Figure 7), but while there was no significant difference between normal and non-neuropathic subjects, neuropathic patients showed a significant depletion compared with non-neuropathic patients. Differences in CGRP-IR were statistically significant only in the epidermis (Figure 4), and not in the other skin regions studied. Sweat gland VIP-
IR (Figure 9) showed only borderline statistically significant differences between the three groups (ANOVA, p=0.045). Substance P showed no significant differences between any of the groups in any skin region.

7.4.2 Quantitative study (Tables 4(a) and (b))

7.4.2.1 PGP 9.5 immunoreactivity

Epidermal PGP-IR showed a progressive reduction in the three groups (Figure 3) and this finding was consistent for the observer grading and for nerve areas and numbers in the quantitative analysis. The depletion was statistically significant by ANOVA for both immunoreactive nerve area (p=0.001) and count (p<0.001). This was largely accounted for by the difference between normal and neuropathic subjects (area and count: p<0.001), but partly also by the difference between non-neuropathic and neuropathic patients (area: p=0.042; count: p=0.003). There was no significant difference in dermal PGP-IR between the three groups (Figure 5), though one neuropathic patient had an apparently elevated PGP-IR area. PGP-IR count showed a progressive and slight diminution between the three groups that was not statistically significant by ANOVA. There were similar findings for sweat gland PGP-IR (Figure 7), with one neuropathic and one non-neuropathic patient showing elevated levels, but no significant differences between the groups.

7.4.2.2 Calcitonin gene-related peptide (CGRP) immunoreactivity

Two normal subjects, three non-neuropathic patients and seven neuropathic patients had no detectable epidermal CGRP. Epidermal CGRP area decreased through the three groups, though not to a statistically significant level (p=0.062 by ANOVA; Figure 4). Epidermal CGRP-IR count showed a more marked decrease (p=0.026), with neuropathic patients having significantly lower counts than both non-neuropathic patients (p=0.003) and normal subjects (p<0.001). There were no statistically significant differences in dermal or sweat gland CGRP-IR between the three groups (Figures 6 and 8), though one non-neuropathic patient had elevated IR dermal CGRP-IR area, and two had increased nerve counts. All non-neuropathic patients had measurable sweat gland CGRP-IR counts, while 6 normals and 5 neuropaths had absent CGRP-IR. CGRP-IR counts were therefore higher in the non-neuropathic patients compared with both normal subjects (p=0.098) and neuropathic patients (p=0.008).
7.4.2.3 Vasoactive intestinal polypeptide (VIP) immunoreactivity

Sweat gland VIP-IR measurements showed marked variation (Figure 9). Statistically there were no significant differences between the three groups, either in nerve area (p=0.372) or count (p=0.248). There were 5 neuropathic patients and two non-neuropathic patients without measurable IR, but one neuropathic patient had elevated VIP-IR area, and two non-neuropathic patients elevated counts.

Figure 3:

Observer graded scores (upper panel) and quantitative immunohistochemistry (immunoreactive nerve area, middle panel and nerve counts, lower panel) in Group 1 (normal non diabetic), Group 2 ("non-neuropathic") and Group 3 ("neuropathic") subjects. This format is used for the subsequent six figures.

Epidermal PGP-IR. Observer gradings: Gp 1 vs. Gp 2 p = 0.013, Gp 1 vs. Gp 3 p = 0.002. Quantitative area: Gp 1 vs. Gp 3 p < 0.001, Gp 2 vs. Gp 3 p = 0.042. Quantitative count: Gp 1 vs. Gp 3 p < 0.001, Gp 2 vs. Gp 3 p = 0.003.
Figure 4: Epidermal CGRP-IR. Observer gradings: Gp 1 vs. Gp 3 p = 0.02. Quantitative area: Gp 1 vs. Gp 3 p = 0.031. Quantitative area: ANOVA = NS (p = 0.062). Quantitative count: Gp 1 vs Gp 3 p = 0.003.
Figure 5: Dermal PGP-IR. Observer gradings: Gp 1 vs. Gp 2 $p=0.002$, Gp 1 vs. Gp 3 $p=0.002$. Quantitative area: Gp 1 vs. Gp 3 $p=0.02$. 
Figure 6: Dermal CGRP-IR. There were no significant differences between any of the groups either in the observer graded or quantitative studies. Dermal CGRP-IR area is elevated in one non-neuropathic patient, while two non-neuropathic patients have increased dermal CGRP-IR.
Figure 7: Sweat gland PGP-IR. Observer gradings: Gp 1 vs Gp 3 \( p = 0.008 \), Gp 2 vs. Gp 3 \( p = 0.004 \). Quantitative: no significant differences. Note the elevated quantitative IR area in one non-neuropathic and one neuropathic patient.
Figure 8: Sweat gland CGRP-IR. Observer graded and quantitative studies: ANOVA = NS.
Figure 9: Sweat gland VIP. Observer grading: Gp 1 vs. Gp 3 p = 0.029. Quantitative study: no significant differences between the groups. One non-neuropathic subject had elevated VIP-IR area, and two had increased VIP-IR count.
7.4.3 Regional associations between quantitative immunohistochemistry; changes with age, physical characteristics, diabetes duration and diabetic control

In normal subjects there was a weak but statistically significant association between epidermal and dermal PGP-IR areas ($r=0.77$, $p=0.02$) but not between epidermal and sweat gland PGP-IR area ($r=0.22$, $p=0.4$). Epidermal PGP-IR area was only weakly associated with CGRP-IR area ($r=0.41$, $p=0.13$), but sweat gland PGP-IR area and VIP-IR were closely associated ($r=0.63$, $p=0.01$), confirming the visual impression that most epidermal nerve fibres are not CGRP-IR, whereas most sweat gland fibres are VIP-IR. Physical characteristics (height, weight and body mass index) showed no associations with quantitative immunohistochemical measurements.

In diabetic patients, epidermal PGP-IR was related to dermal PGP-IR ($r=0.66$, $p=0.001$), to sweat gland PGP-IR ($r=0.56$, $p=0.003$) and to epidermal and dermal CGRP-IR ($r=0.56$, $p=0.007$; $r=0.54$, $p=0.009$ respectively). Sweat gland PGP-IR and VIP-IR were, in contrast to normal subjects, not statistically associated ($r=0.37$, $p=0.11$).

Immunoreactivity showed no changes with age, either in normal or diabetic subjects, and duration of diabetes was not associated with any of the quantitative measurements. There were no significant associations with height, weight, or body mass index in diabetic patients.

All quantitative measurements correlated negatively with glycated haemoglobin; there were statistically weak associations with epidermal PGP-IR (area: $r=-0.42$, $p=0.09$; count: $r=-0.49$, $p=0.05$), dermal PGP-IR (area: $r=-0.50$, $p=0.04$; count: $r=-0.55$, $p=0.02$), and dermal CGRP-IR (area: $r=-0.44$, $p=0.08$; count: $r=-0.45$, $p=0.08$). Scattergrams of the relation between glycated haemoglobin and epidermal and dermal PGP-IR area are shown in Figure 10.

In all groups and skin regions, the correlation between immunoreactive area and counts was high ($r=0.65-0.95$) and statistically significant ($p<0.001$).
Figure 10: Scattergrams of quantitative immunohistochemistry and glycated haemoglobin at the time of the biopsy. **Top:** Epidermal PGP-IR area ($r=-0.42, p=0.09$); **Bottom:** Dermal PGP-IR area ($r=-0.50, p=0.04$). Similar weakly negative correlations were found for all other quantitative measurements.

7.4.4 Associations between quantitative immunohistochemical measurements and neurological measurements

There were no consistent changes in immunohistochemical measurements with thermal or vibratory thresholds, with any of the electrophysiological measurements, or with the cardiovascular autonomic function tests (3 tests of heart rate variation and postural systolic and diastolic blood pressure changes). Among the sweat tests, mean pilocarpine-activated sweatspot density and size (see Chapter 4) showed no associations, but transepidermal water loss rate (see Chapter 3) was significantly associated with sweat gland CGRP-IR (count)
(r=0.55, p=0.05), and with sweat gland VIP-IR (area: r=0.60, p=0.03; count: r=0.50, p=0.08). Peak sweat output was not associated with the histological measurements, but total sweat volume was associated with dermal PGP-IR (area: r=0.64, p=0.02; count: 0.65, p=0.02), sweat gland PGP-IR (count: r=0.58, p=0.04; Figure 11), CGRP-IR (count: r=0.55, p=0.05), and with sweat gland VIP-IR (count: r=0.55, p=0.05). Epidermal measurements showed the expected slight decrease with increasing latency of the sympathetic skin response (Chapter 5), but dermal and sweat gland measurements were positively associated with increasing SSR latency, reaching statistical significance with dermal CGRP-IR (count: r=0.63, p=0.04), and sweat gland PGP-IR (area: r=0.65, p=0.03; count: r=0.62, p=0.04; Figure 11).

**Figure 11:** Quantitative immunoreactivity (sweat gland PGP-IR count) and sweat function. **Top:** relationship with total sweat volume after acetylcholine iontophoresis (r=0.64, p=0.02) (Chapter 3). **Bottom:** sympathetic skin response latency (r=0.62, p=0.04) (Chapter 5)
7.4.5 Reproducibility

In order to assess variability three normal male subjects (ages 31, 35 and 45) had repeat biopsies 10–12 months after the first. Specimens were taken as in the original study, approximately 5 cm distal to the initial biopsy site, and measurements made in the same way. In addition, further sections of the original biopsy were cut, stained and measured. The results are shown in Table 5 and Figure 13.

**Table 5:** Results of repeat epidermal measurements on different sections of the first specimen (columns 1 and 2) and the second specimen (columns 3 and 4) taken approximately 1 year after the first. Mean areas of the six measured fields are expressed as mm² × 10⁶ of immunofluorescence to the general neuronal marker PGP 9.5

<table>
<thead>
<tr>
<th></th>
<th>1st specimen</th>
<th>2nd specimen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Epidermis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Subject 1</td>
<td>847</td>
<td>1268</td>
</tr>
<tr>
<td>Subject 2</td>
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</tr>
<tr>
<td>Subject 3</td>
<td>1340</td>
<td>720</td>
</tr>
<tr>
<td>Dermis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Subject 1</td>
<td>1137</td>
<td>2773</td>
</tr>
<tr>
<td>Subject 2</td>
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<td>446</td>
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<tr>
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<td>1317</td>
<td>2428</td>
</tr>
<tr>
<td>Sweat glands</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Subject 1</td>
<td>1373</td>
<td>6124</td>
</tr>
<tr>
<td>Subject 2</td>
<td>1701</td>
<td>1854</td>
</tr>
<tr>
<td>Subject 3</td>
<td>−¹</td>
<td>5205</td>
</tr>
</tbody>
</table>

¹ Sweat glands absent in the first set of sections cut from this first specimen
Figure 12: Results of quantitative PGP-IR immunohistochemistry in repeat biopsies taken from 3 normal subjects: Top (a) epidermis, Middle (b) dermis, Bottom (c) sweat glands. ◊ = subject 1, × = subject 2, □ = subject 3. Lines connect mean results of two non-contiguous specimens cut from biopsy 1 (a and b) and biopsy 2 (a and b).
Mean differences between the pairs of measurements were (×10⁻²): epidermis 0.2, 0.09; dermis 0.7, 0.8; sweat glands 2.8, 1.25. Regarding each measurement as a separate test yields the following mean coefficients of variation for the 4 measurements: epidermis 42.7%, dermis 43.7%, sweat glands 49.1%.

### 7.4.6 Characteristics of patients with high immunoreactivity

It is not possible to define precise 95% confidence intervals for a reference range when the numbers of subjects are small and non-normally distributed (N Alexander, personal communication, 1991). Definitions of what constitutes either "high" or "low" immunoreactivity are therefore arbitrary. For descriptive purposes, "high" immunoreactivity has been defined as any quantitative measurement greater than the highest measurement in the group of normal subjects. Six such patients were identified in this study. Their clinical characteristics and sweat test results are shown in Table 6.

#### Table 6: Clinical characteristics and sweat test results in patients with elevated quantitative immunoreactivity. Abnormal values are boldened.

<table>
<thead>
<tr>
<th>Subject</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
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<tbody>
<tr>
<td>Age, y</td>
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<td>26</td>
<td>28</td>
<td>45</td>
<td>44</td>
<td>38</td>
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<tr>
<td>Duration diabetes, y</td>
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<td>22</td>
<td>23</td>
<td>34</td>
<td>25</td>
<td>32</td>
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<tr>
<td>No. of elevated immunohistochemical measurements</td>
<td>5</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>TEWL, mg·cm⁻²·hr⁻¹ ¹</td>
<td>1.277</td>
<td>1.388</td>
<td>1.076</td>
<td>1.159</td>
<td>1.466</td>
<td>1.209</td>
</tr>
<tr>
<td>Peak sweating rate, mg·cm⁻²·hr⁻¹</td>
<td>17.23</td>
<td>20.99</td>
<td>16.31</td>
<td>34.48</td>
<td>15.15</td>
<td>11.27</td>
</tr>
<tr>
<td>TVol12, mg²</td>
<td>6.21</td>
<td>8.20</td>
<td>3.56</td>
<td>8.40</td>
<td>2.07</td>
<td>4.14</td>
</tr>
<tr>
<td>SSR latency, ms³</td>
<td>1798</td>
<td>2178</td>
<td>1866</td>
<td>1898</td>
<td>2030</td>
<td>2363</td>
</tr>
<tr>
<td>SSR amplitude, μV</td>
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<td>144</td>
<td>133</td>
<td>92</td>
<td>192</td>
<td>52</td>
</tr>
<tr>
<td>Sweatspot density, no./cm²</td>
<td>105</td>
<td>16</td>
<td>135</td>
<td>119</td>
<td>111</td>
<td>117</td>
</tr>
</tbody>
</table>

¹ transepidermal water loss rate
² TVol12, sweat output after acetylcholine iontophoresis
³ SSR, sympathetic skin response

Three of the six patients had one or more elevated sweat test results (peak or total sweat output after acetylcholine (Chapter 3), or increased sweatspot density after pilocarpine (Chapter 4)). One patient (no. 4) had a low SSR amplitude together with a high
acetylcholine-induced sweat output, and another (no. 2) a low sweatspot density together with a high acetylcholine-induced sweat output.

7.5 Discussion

Techniques for the histological quantification of peripheral small fibres in diabetes are of potential value, as cutaneous afferent sensory and efferent postganglionic sympathetic fibres are thought to be of importance in the generation of painful symptoms, and small fibre abnormalities are uniformly present in patients with neuropathic foot ulcers (Ahmed and Le Quesne 1986; see Introduction, section 1.4.1). The majority of neuropathological studies have quantified abnormalities of myelinated nerve fibres in sural nerve biopsies; a few studies have quantified unmyelinated fibres (eg. Behse et al. 1975), but electron microscopy is required on account of the small fibre size (0.1 – 3.0 μm) and the analyses are laborious to perform (Kanda et al. 1991). Quantitative immunohistochemistry of small fibres in skin biopsies has advantages of simplicity and potential repeatability. In addition, nerves can be studied at their most distal points, where early changes may be seen, and sensory and autonomic nerve fibres can be separately identified.

There is only one previous study of the association between quantitative neurophysiological measurements and skin histology, that of Bruce and Sinclair (1980). They measured touch detection and two-point discrimination in the finger, but were unable to show any association between these measurements and the density of Meissner corpuscles or of any other organised nerve endings in skin obtained from age-matched traumatic amputation specimens. Of necessity, the numbers studied were small, and the small variance of functional measurements found in normal subjects rarely allows significant morphological/functional correlations to be found.

Hitherto, most studies of the cutaneous innervation have used observer graded methods to assess deficits in comparison with control material. In experienced hands, observer grading is considered reliable, but there have been no systematic studies comparing observer gradings and automated quantitative methods. Quantitation may yield a more precise measurement of immunoreactive nerve areas and counts, but quantitative methods cannot yet detect differences in the intensity of immunofluorescence, a feature which may be of importance, particularly where there may be an increase in fluorescence, with or without an increase in immunoreactive nerve fibres, as shown in several animal studies of experimental diabetes (eg. Crowe and Burnstock 1988).
As with observer graded assessments, a significant problem with quantitative methods is bias which may arise from several sources, for example sampling, definition of the areas of interest, and the method used to determine the threshold at which to segregate the image (Jagoe et al. 1991). We have attempted to minimise errors due to bias by measuring linearly contiguous fields in non-consecutive sections, and by using similar thresholds for the measurement of all samples. However, the two-dimensional image-analysis used in the current study may, for example, result in over-representation of large nerve fibres, and conversely, omission of very fine fibres from the image. This may not be a significant problem when studying the superficial layers of the skin, where the terminal nerve fibres are relatively homogeneous in size. There is some supportive evidence for this view in the close correlation between immunoreactive nerve areas and nerve counts in all groups, suggesting that there are no major selective losses of nerves of particular size in diabetic neuropathy. Likewise, there were no cases where there were major discrepancies between inter-group comparisons of nerve area and counts. This is in contrast to the findings in Raynaud’s phenomenon (Terenghi et al. 1991), where CGRP- and VIP-IR nerves around sweat glands showed a significant decrease in area, but not in counts, compared with controls. Caution is required in interpreting this finding, however, on account of the small number of patients studied.

The potential biases identified in this image-analysis study may be overcome by the use of the stereological methods advocated by Gundersen et al. (1988) and Cruz-Orive (Cruz-Orive and Weibel 1990). However, these methods have not so far been validated in a tissue as heterogeneous as peripheral nerve fibres.

The results of the observer graded and quantitative analyses in this study were broadly concordant; appreciable differences in the results occurred only in the sweat glands. Statistical comparisons between the three groups of subjects gave similar results using both methods though statistically significant differences were more commonly found in the observer graded study. This may reflect the larger field area assessed by the human observer, compared with the limited size of fields used in the quantitative study. This might suggest that future studies should analyse larger numbers of fields, though this intuitive notion is not supported by the systematic study of Jagoe et al. (1991). Observer grading did not identify specimens with increased immunoreactivity; this may be due to observer bias and expectation, rather than an intrinsic inability of the eye to detect increased, rather than decreased staining, as observer graded studies have reported nerve proliferation in the skin of both experimental animals and humans (Karanth et al. 1990; Springall et al. 1991).
Our earlier pilot study (Levy et al. 1989) analysed skin specimens from a variety of sites; several of the specimens were taken from the lower limbs of patients with overt peripheral vascular disease, and the known structural effects of macrovascular disease on nerves (Chopra et al. 1968; Chopra and Hurwitz 1969; Ram et al. 1991) could not be excluded, although there is experimental evidence that unmyelinated fibres are less susceptible to the effects of ischaemia than myelinated fibres (Fujimura et al. 1991). However, a few specimens were obtained from proximal sites, for example the upper limb and abdominal wall, where ischaemic changes would not be expected, and these also showed significant depletion of PGP 9.5- and neuropeptide-IR nerves. In the current study, although all biopsies were taken from the lower leg, we minimised the possibility of macrovascular disease by choosing young subjects with no clinical abnormalities of peripheral pulses. Only one of the subjects (a "neuropathic" Group 3 patient) was a cigarette smoker. The immunohistochemical changes seen are therefore unlikely to be due to large-vessel disease.

It is more difficult to exclude the effects of microvascular disease. None of the non-neuropathic patients had Albustix-positive proteinuria, and clinical ophthalmoscopic examination was negative in all. However, the mean duration of diabetes in both diabetic groups was long, and it is likely that some of the non-neuropathic patients would demonstrate microangiopathy if more sensitive methods, such as fluorescein angiography or measurements of microalbuminuria, were used.

In the epidermis, there was a progressive diminution of the general neuronal marker PGP 9.5 and of CGRP through the three groups studied. Quantitatively there were no significant differences between the normal and non-neuropathic groups, though PGP-IR was significantly reduced in the observer graded study. Lindberger et al. (1989) counted immunoreactive nerve segments in the epidermis and found a significant depletion of CGRP- and SP-IR fibres in non-neuropathic subjects, defined using the criteria of Dyck (Dyck et al. 1985). Our non-neuropathic subjects were defined in a different way, with an emphasis on unmyelinated, rather than myelinated nerve fibre function; in addition, though Lindberger et al. (1989) studied a smaller number of patients (10) with a greater mean age (50 years), their biopsies were slightly more distal than those taken here. The use of larger numbers of subjects is likely to clarify the issue. While epidermal nerve depletion in non-neuropathic patients is therefore not a consistent finding, the changes in the neuropathic group were unequivocal.

Neuropeptide changes in the dermis and sweat glands were less consistent than those in the epidermis. Absence of sweat gland neuropeptide IR was commoner in neuropathic than normal subjects, but overall there were no significant differences between the three groups
in the quantitative study. Sweat gland IR showed differences between the observer graded and quantitative results; neuropathic and non-neuropathic patients had significantly decreased PGP 9.5-IR observer graded scores compared with normals, while the quantitative measurements showed no significant differences. Further studies will be required to resolve this discrepancy. However, it is clear that diminished sweat gland IR was not a uniform finding, in contrast to the epidermis, and there were some subjects with apparently increased neuropeptide IR, possibly signifying nerve proliferation, a process described in several experimental and at least one human study (see Introduction, section 1.3.3).

The finding of increased IR in this study should be interpreted with caution for several reasons: we studied small numbers of patients; the measurements have high variance; and there was incomplete concordance between elevated nerve area and counts in those patients with increased IR. Three out of 6 diabetic patients with increased IR had at least one supernormal value in the functional sweat tests, suggesting a relationship between increased nerve areas or counts and results of local tests of sweating function. Increased IR was found only in the dermis and sweat glands; these regions, unlike the epidermis, have an abundant capillary supply (see Introduction, section 2.2.2), which may be further increased by sympathetic denervation, and it may be this factor which causes nerve proliferation. On the other hand, microvascular occlusion and ischaemia has been shown to inhibit nerve regeneration in patients with vasculitic neuropathy (Fujimura et al. 1991). Whether nerve depletion or proliferation occurs may therefore depend on regional differences in dermal blood flow, and both processes may occur in the same patient in different skin regions at different stages of diabetes. Our earlier study (Levy et al. 1989) showed that neuropeptide Y, a vasoconstrictor peptide found in blood vessel walls, and frequently co-localised with catecholamines, was markedly reduced in skin blood vessels in diabetic patients, but this is only one of a number of neuropeptides affecting vascular reactivity, and changes in dermal blood flow in diabetic neuropathy may depend on a highly variable balance between vasoconstrictor and vasodilator substances (Cameron et al. 1991, 1992).

There is no evidence from the current study that diabetes causes differential changes in particular neuropeptides. The results for sweat gland CGRP- and VIP-IR were similar, and these changes may be related to a generalised abnormality of axonal transport, as has been demonstrated experimentally for CGRP and SP in the streptozotocin rat (DR Tomlinson, personal communication 1991). In individual skin regions, there was also a close correspondence between measurements of PGP 9.5-IR and neuropeptide-IR. PGP 9.5, one of a number of general neuronal markers (Thompson et al. 1983), is usually taken to be an indirect
indicator of structural neuronal integrity, while neuropeptide-IR is thought more closely to represent the functional integrity of nerve fibres. Our results suggest that structural and functional abnormalities in cutaneous nerves occur in parallel. There is, however, recent evidence that PGP 9.5 is a hydrolase enzyme acting on post-translational ubiquitin-protein complexes (Wilkinson et al. 1989). PGP 9.5 may therefore also be an indicator of the functional state of nerves, whose synthesis and transport may itself be altered in the diabetic nerve.

There were weak but consistent negative correlations between glycated haemoglobin at the time of the biopsy and many of the quantitative immunohistochemical measurements; all the patients were in stable diabetic control, and these results confirm that poor glycaemic control is associated with peripheral small-fibre depletion. Further longitudinal studies are required to demonstrate whether neurophysiological changes (for example the improvement in thermal discrimination thresholds found by Bertelsmann et al. (1987) with intensified insulin therapy) are reflected in small-fibre immunohistochemical measurements.

The relationship between the neurophysiological tests and the immunohistochemical findings should be interpreted with caution in small numbers of subjects. However, while there were no significant correlations with conventional neurophysiological measurements (sensory thresholds, nerve conduction studies and cardiac vagal autonomic tests), there were statistically significant correlations with some of the local tests of sudomotor function. This suggests that the effects of neuropathy may be somewhat different on efferent sympathetic nerves compared with the longer neural pathways subserving sensori-motor and cardiac autonomic function. The evidence supporting this view has been presented in earlier chapters, but the presence of some subjects with unexpectedly high immunoreactivity reinforces this view. In the test of pharmacologically-stimulated sweat output in response to iontophoresed acetylcholine, increasing sweat output was associated with increased nerve IR, while the amplitude and latency of the sympathetic skin response was inversely correlated with the immunohistochemical measurements. These contrasting results suggest that sudomotor nerves in diabetes may simultaneously demonstrate pharmacological supersensitivity and functional deficits, and both are associated with marked heterogeneity of immunoreactive nerve areas and numbers. These findings underline the importance of assessing local peripheral nerve function and structure in addition to conventional neurophysiological measurements; in the light of the functional sweating studies presented in earlier chapters, longitudinal changes in cutaneous innervation would be most appropriately followed by using tests of sudomotor function.
In summary, this study has confirmed the practicability of undertaking quantitative immunohistochemical measurements by image-analysis of standardised skin biopsies in diabetic patients. The results were similar to those of a simultaneously performed observer graded study, but offer the potential advantage of being able to detect increased, as well as decreased, immunoreactivity. The investigation, while invasive, has a low morbidity and unlike sural nerve biopsy can be repeated. It is currently the only available method for the direct quantification of dermal innervation. The variability shown in the small number of repeat biopsies in the current study may be reduced by the use of stereological techniques, though high inter-subject variability has also been frequently noted in quantitative histological studies of sural nerve morphometry (Llewelyn et al. 1991). Neuropeptide changes are seen in the absence of clinical macro- and microvascular disease. There are regional variations within the skin, with the epidermis showing the most marked depletion of immunoreactive nerve fibres, but within regions the general innervation revealed by PGP 9.5 staining appears better preserved than that of neuropeptide-IR nerves. Quantitative immunohistochemistry reflects some measures of local sweat gland function, but not conventional tests of large- or small-fibre function. Some diabetic patients have apparently increased neuropeptide IR, raising the possibility of small-fibre nerve proliferation. Although this was an inconstant finding, it is of note that it occurred only in the dermis and sweat glands, suggesting that it may be related to changes in blood flow. In a small number of normal subjects repeat biopsy had a coefficient of variation similar to that of sensory threshold testing (Fagius and Wahren 1981). The technique, together with the functional studies previously discussed, may prove of value in the longitudinal assessment of the clinical efficacy of agents whose primary effect is to induce peripheral neuronal sprouting.
Chapter 8  Portable infrared pupillometry using Pupilscan™: relation to autonomic and somatic nerve function in diabetes

8.1 Introduction

Abnormalities of pupillary size and reaction were some of the earliest clinical signs of diabetic autonomic neuropathy to be described (see Introduction, section 1.4.9.3) (Jordan 1936; Rundles 1945). Among the various deficits noted, the two commonest were miosis and a reduced light reflex. The small pupil, dilating poorly in darkness, is now thought to be due to sympathetic dysfunction, and this phenomenon has been the basis of a simple diagnostic test of pupillary abnormality in diabetes (Smith and Dewhurst 1986; Neil and Smith 1989).

Infrared pupillometry allowed dynamic responses to standard light stimuli to be studied in diabetic patients (Hayashi and Ishikawa 1979; Hreidarsson 1982). Abnormalities revealed by this method were at first thought to be partly due to abnormal stiffness of the iris in diabetes, but it is now clear that the diabetic pupil is usually normal in structure, and the pupillometric abnormalities are largely due to the small initial size of the pupil; after allowing for this, in most respects the diabetic pupil appears to constrict and redilate normally. Hreidarsson and Gundersen (1985) found the only abnormalities to be a small reduction in the response amplitude, attributed to parasympathetic damage, and reductions in the maximum constriction and redilatation velocities. Some authors have found the latency of the constriction phase of the pupillary light reflex to be prolonged in diabetes (Lanting et al. 1988), while others have not (Hreidarsson and Gundersen 1985). The pupillary light reflex is conventionally thought to represent a complex interaction between sympathetic and parasympathetic nerve supplies (Lowenstein and Loewenfeld 1950), and it is not surprising that using different methods, some studies have detected predominantly either sympathetic or parasympathetic abnormalities. The situation is further complicated by the presence of pupillary denervation supersensitivity (Hayashi and Ishikawa 1979; Sigsbee et al. 1974; Fulk et al. 1991).

The aim of the present study was to use a simple portable infrared pupillometer (Pupilscan™) to measure the dynamic pupillary light response in a group of diabetic and age-matched normal subjects, and to study its associations with other measurements of peripheral small-fibre nerve function, including cardiac autonomic function, thermal thresholds and the tests of sweating function described in previous chapters.
8.2 Subjects and methods

8.2.1 Subjects

Clinical characteristics of the 85 diabetic and 67 age-matched normal subjects are shown in Table 1 (see Appendix A). Twelve (14%) patients were Afro-Caribbean, 20 (24%) Asian (predominantly Gujerati Indians), and the remaining 53 (62%) European.

Subjects were asked not to smoke, or drink caffeine-containing drinks on the day of the test. Normal subjects with past or current retinal or ocular disease, or taking drugs which potentially interfere with autonomic function, were not included. Diabetic patients who had undergone laser treatment were also excluded, but not those with lesser degrees of retinopathy.

Table 1: Clinical characteristics of subjects. Data are presented as mean (SD) [range]

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>Diabetic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>67</td>
<td>85</td>
</tr>
<tr>
<td>Male : Female</td>
<td>32:35</td>
<td>53:32</td>
</tr>
<tr>
<td>Age, y</td>
<td>42 (12) [19-67]</td>
<td>44 (15) [18-66]</td>
</tr>
<tr>
<td>Type 1 : Type 2</td>
<td></td>
<td>43:42</td>
</tr>
<tr>
<td>Duration diabetes, y</td>
<td>12 (10) [1-34]</td>
<td>11.4 (2.9) (6.5-20.1) (n = 67)</td>
</tr>
<tr>
<td>HbA,¹</td>
<td></td>
<td>26 (37%)</td>
</tr>
<tr>
<td>Symptomatic peripheral neuropathy</td>
<td>22 (31%)</td>
<td></td>
</tr>
<tr>
<td>Symptomatic autonomic neuropathy</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

¹ Reference range <8.0%

8.2.2 Pupillometry

Pupilscan™ Version 5 (Fairville Medical Optics, Amersham, Bucks, UK) was used. It consists of a hand-held stimulating and recording device (Figure 1), connected to a video monitor and a standard IBM-compatible microcomputer with software controlling the light stimulus and a database for data acquisition and analysis. The infrared scanner scans vertically at 20 Hz.

Subjects were seated comfortably in a dark room for 5 min before testing. Tests were performed in the early afternoon and more than 2 h after insulin. The left eye was used for all tests. The operator, seated opposite the subject, gently rested the scanner against the inferior orbital bone. The position of the Pupilscan was adjusted so that the magnified pupil image was clearly defined on the monitor. This was of particular importance in Afro-
Caribbean and Asian subjects, where the pupil-iris margin was frequently difficult to distinguish by eye. A small red diode light enabled the subject to fixate at an optically infinite distance. In the model used, there was a low-intensity transverse guide-light to aid accurate positioning of the apparatus. When a steady pupil image was obtained, the white light stimulus (200 ms) was activated by releasing a switch on the stimulator; scanning continued after stimulation for 3 s. The constriction-redilatation curve was then displayed on the monitor and the data stored, or rejected if the recording was interrupted by a blink or other artifact. Valid data records were stored for later analysis. Six tracings were obtained at intervals of 1 min, and the curves averaged. Figure 2 shows the conventions used for measuring the various characteristics of the curve: several of these have been described by Hreidarsson and Gundersen (1985).

8.2.3 Measurements derived from the diameter-time curve

The following measurements were derived from the constriction-redilatation curve:

<table>
<thead>
<tr>
<th>Measurement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Constriction latency (s)</td>
</tr>
<tr>
<td>Reflex amplitude (initial diameter−minimum diameter) (mm)</td>
</tr>
<tr>
<td>Relative reflex amplitude (initial diameter−minimum diameter/initial diameter x 100) (%)</td>
</tr>
<tr>
<td>Time to minimum diameter (s)</td>
</tr>
<tr>
<td>Time to 63% redilatation (s)</td>
</tr>
<tr>
<td>Maximum constriction velocity (mm/s)</td>
</tr>
<tr>
<td>Maximum redilatation velocity (mm/s)</td>
</tr>
</tbody>
</table>

After completion of the main study, software was developed to calculate the constriction latency time, defined as the time elapsed from the beginning of the stimulus to the point where an extension of the constriction velocity regression line, calculated from pupil diameters between 0.40 and 0.60 s, intersected the mean of the first 5 diameters. This measurement was available in 44 normal subjects, whose clinical characteristics were similar to those in the initial study (mean age 42 y (SD 13)), and 76 diabetic patients (mean age 44 y (SD 15); p=0.4).
Figure 1: (Previous page). The Pupilscan device in use. The cross-wire visible at the front of the stimulation-recording unit is used by the operator for alignment. The trigger for the light stimulus is situated on the transverse hand grip and is activated by the index finger (trigger not visible in this view). The curved rest lying against the inferior orbital bone can be swivelled to allow measurements to be made from either eye. (Photograph: M Eithrington).
Figure 2: Pupilscan tracing (diameter-time plot) of a normal subject. Measurements derived from the curve are shown: RA = reflex amplitude; LT = constriction latency; TTM = time to minimum diameter; T63% = time to 63% redilatation; CV = constriction velocity; RV = redilatation velocity. For further details of the derivation of these measurements, see Methods. Relative reflex amplitude (%) was calculated as (initial diameter−minimum diameter) / initial diameter × 100.

Figure 3: Constriction latency and age in normal (●) and diabetic (○) subjects. Latency did not change significantly with age in normal subjects (r=0.08, p=0.62), but increased with age in diabetic patients (r=0.35, p=0.001); 6 had abnormal latency.
8.2.4 Other neurological measurements

Diabetic subjects had a range of other neurological tests. The percentage of patients studied with each is given:

<table>
<thead>
<tr>
<th>Small-fibre tests</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Marstock thermal thresholds (warm, cool, heat pain) (Chapter 1)</td>
<td>90</td>
</tr>
<tr>
<td>Vagal cardiovascular autonomic function tests (Chapter 1):</td>
<td></td>
</tr>
<tr>
<td>Respiratory RR interval variation with deep breathing</td>
<td>92</td>
</tr>
<tr>
<td>Valsalva ratio (average of 3 tests)</td>
<td>80</td>
</tr>
<tr>
<td>30:15 (lying:standing) ratio</td>
<td>93</td>
</tr>
<tr>
<td>Postural blood pressure change (Chapter 1)</td>
<td>87</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Large-fibre tests</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Common peroneal motor conduction velocity and action potential amplitude; sural sensory action potential amplitude (Chapter 2)</td>
<td>53</td>
</tr>
</tbody>
</table>

| Vibration perception thresholds (Chapter 1) | 80 |

<table>
<thead>
<tr>
<th>Sweat tests</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Dynamic sweat response after acetylcholine (Chapter 3)</td>
<td>93</td>
</tr>
<tr>
<td>Pilocarpine-activated sweatspot density (Chapter 4)</td>
<td>98</td>
</tr>
<tr>
<td>Sympathetic skin response (Chapter 5)</td>
<td>80</td>
</tr>
</tbody>
</table>

8.2.5 Reproducibility

Seven normal subjects, mean age 44 y (range 25-44) and 11 diabetic patients, mean age 47 y (range 26-66), chosen at random, had repeat Pupilscan measurements 2-12 weeks apart to assess the reproducibility of the method.

8.3 Statistical analysis

The following measurements were normally distributed without transformation: constriction latency, reflex amplitude, relative reflex amplitude, constriction velocity, and redilatation velocity. The remaining measurements (time to minimum diameter and time to 63% redilatation) were linearised by logarithmic transformation. After appropriate transformations, linear regression analysis was used to determine which measurements were significantly age-related. 95% confidence intervals for the normal range were calculated using standard methods. Student's t-test and Pearson correlation coefficients were used to compare transformed data in the normal and diabetic groups. Analysis of variance was used for the ethnic comparison.
8.4 Results

8.4.1 Normal subjects

The lower limit of normal (mean-2SD) for reflex amplitude was 0.54 mm, for relative reflex amplitude 16.2%, for maximum constriction velocity 2.58 mm/s, and for maximum redilatation velocity 1.34 mm/s. The upper limit of normal (mean+2SD) for time to minimum diameter was 0.91 s, for time to 63% redilatation 1.99 s, and for latency 0.33 s (Figure 4). Reflex amplitude and time to minimum pupil diameter showed weakly significant changes with age in normal subjects ($r=0.25$, $p=0.05$; $r=0.30$, $p=0.02$). Relative reflex amplitude (reflex amplitude as a percentage of the initial diameter) corrects for the normal decrease in initial and minimum pupil diameters with age, and did not change significantly ($r=0.07$, $p=0.59$). The remaining measurements also did not show significant age-related changes, including latency ($r=0.08$, $p=0.62$; Figure 4).

Mean latency measurements were significantly longer in males than females (males ($n=17$): 0.251 s (SD 0.054); females ($n=27$): 0.217 s (SD 0.039), $p=0.02$), but there were no other significant gender differences. Insufficient numbers of non-European normal subjects were tested to demonstrate any ethnic differences. Physical characteristics showed no correlations with pupillometry, with the exception of the time to 63% redilatation, which was significantly correlated with body weight ($r=0.30$, $p=0.04$).

Table 2: Comparison of pupil measurements in normal subjects and diabetic patients. Values are given as mean (SD), with $p$ values relating to $t$-test statistics, except for time to 63% redilatation and time to minimum diameter, where median, range and Mann-Whitney U-test statistics are quoted.

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>Diabetic</th>
<th>$p$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reflex amplitude (mm)</td>
<td>1.49 (0.47)</td>
<td>1.59 (0.51)</td>
<td>0.36</td>
</tr>
<tr>
<td>Relative reflex amplitude (%)</td>
<td>33.02 (8.41)</td>
<td>32.91 (9.92)</td>
<td>0.65</td>
</tr>
<tr>
<td>Time to 63% redilatation (s)</td>
<td>1.40 (0.9-2.2)</td>
<td>1.55 (1.0-3.0)</td>
<td>0.001</td>
</tr>
<tr>
<td>Time to minimum diameter (s)</td>
<td>0.78 (0.62-1.04)</td>
<td>0.79 (0.67-0.97)</td>
<td>0.20</td>
</tr>
<tr>
<td>Constriction velocity (mm/s)</td>
<td>3.40 (1.09)</td>
<td>3.82 (1.12)</td>
<td>0.36</td>
</tr>
<tr>
<td>Redilatation velocity (mm/s)</td>
<td>0.86 (0.31)</td>
<td>1.00 (0.32)</td>
<td>0.32</td>
</tr>
<tr>
<td>Latency time (s)</td>
<td>0.23 (0.05)</td>
<td>0.25 (0.06)</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>(n=44)</td>
<td>(n=76)</td>
<td></td>
</tr>
</tbody>
</table>
8.4.2 Diabetic patients

Time to minimum diameter showed a slight increase with age ($r=0.29$, $p=0.01$); there was a more pronounced age-related change in latency ($r=0.35$, $p=0.001$), but the remaining measurements showed no significant changes with age. In these randomly-selected patients there were no differences in pupillary measurements between normal and diabetic subjects except the latency, which was slightly prolonged ($p=0.05$), and the time to 63% redilatation ($p=0.001$; Table 2). Time to 63% redilatation was longer in male patients (males (median): 1.48 s; female: 1.33 s, $p=0.04$), but the gender difference in latency noted in normal subjects was not found in the diabetic patients. While as a group, pupillary measurements showed no changes with duration of diabetes, in the 43 Type 1 (insulin-dependent) patients, reflex amplitude, constriction velocity and latency all changed significantly with known duration ($r=0.48$, $p=0.002$; $r=-0.35$, $p=0.04$; $r=0.44$, $p=0.008$ respectively). Blood glucose and glycated haemoglobin at the time of the test showed no significant associations with the pupillary measurements. There were no significant ethnic differences in any of the pupillary measurements (analysis of variance). Body mass index was significantly correlated with latency ($r=0.32$, $p=0.009$), relative reflex amplitude ($r=0.278$, $p=0.016$), and time to minimum diameter ($r=0.44$, $p<0.001$), confirming results previously obtained in normal subjects (Peterson et al. 1988).

8.4.3 Associations between pupillary measurements

Many of the pupillary measurements in both normal and diabetic subjects were strongly intercorrelated, with Pearson correlation coefficients typically between 0.3 and 0.8. This was not the case for latency, which was not significantly correlated with either reflex amplitude ($r=-0.03$) or relative reflex amplitude; stronger associations, however, were found between latency and maximum constriction velocity ($r=0.29$, $p=0.07$) and maximum redilatation velocity ($r=0.45$, $p=0.007$).
Figure 4: The effect of allowing for age-related changes in pupil measurements on the correlation with respiratory RR variation. Top panel: RR variation vs. reflex amplitude \( (r=0.35, p=0.002) \). Bottom panel: RR variation vs. relative reflex amplitude \( (r=0.09, p=0.44) \).

8.4.4 Associations with other neurological measurements

It is important to take into account the age-related changes in most neurological measurements when comparisons are made (see Chapter 1). Thus while, for example, reflex amplitude was strongly correlated with respiratory RR variation \( (r=0.35, p=0.002) \), the significance of this relationship disappeared when relative reflex amplitude was considered \( (r=0.09, p=0.44; \text{ Figure 4}) \); indeed, there were no neurological measurements which showed significant correlations with relative reflex amplitude. Both the maximum constriction and redilatation velocities correlated with vibration perception thresholds at the medial malleolus (constriction: \( r=-0.36, p=0.007 \), redilatation: \( r=-0.32, p=0.003 \)). Constriction velocity correlated with respiratory RR variation \( (r=0.35, p=0.003) \), and redilatation velocity with the Valsalva ratio \( (r=-0.23, p=0.06) \), reflecting the largely parasympathetic contribution to pupillary constriction and the sympathetic contribution to redilatation. The time to 63% pupillary redilatation, also in part a sympathetic measurement, was also weakly correlated with the Valsalva ratio \( (r=-0.24, p=0.05) \). Reflex amplitude, constriction velocity and latency were
significantly correlated with postural systolic and diastolic changes in blood pressure (e.g., reflex amplitude and systolic blood pressure change: \( r = -0.42, p < 0.001 \) (Figure 5); latency and systolic blood pressure change: \( r = 0.41, p = 0.001 \)). There were no significant correlations with common peroneal motor conduction velocity and distal compound motor action potential amplitude, or with the sural sensory action potential amplitude.

![Graph](image)

**Figure 5:** The association between pupillary latency and systolic blood pressure fall; a negative fall signifies a blood pressure rise on standing \( (r = 0.42, p < 0.001) \). The relationship remains statistically significant \( (r = 0.36, p = 0.003) \) after the removal of the outlying patient with a systolic fall of 50 mmHg.

### 8.4.5 Sweating function

Among the sweat tests at the feet, sweatspot densities after pilocarpine iontophoresis and the latency and amplitude of the sympathetic skin response were not significantly associated with any of the pupillary measurements; however, dynamic evoked sweat output after acetylcholine iontophoresis showed weak, but negative correlations with constriction and redilatation velocities \( (r = -0.23, p = 0.06; r = -0.28, p = 0.006) \); Figures 6 and 7).
Figure 6: Relationship between acetylcholine-evoked sweat output at the dorsum of the foot (see Chapter 3) and pupillary constriction velocity; $r=-0.23$, $p=0.06$.

Figure 7: Relationship between acetylcholine-evoked sweat output at the dorsum of the foot (see Chapter 3) and pupillary redilatation velocity; $r=-0.28$, $p=0.006$. Increased sweat output is associated with decreased redilatation and constriction velocity.

8.4.6 Reproducibility

Median coefficients of variation for the duplicate pupillary measurements in normal subjects varied from 2.0% (range 0-9.5%) for time to minimum pupil diameter, to 12.4% (range 8.8-43.0%) for time to 63% redilatation. The corresponding results for diabetic patients were: time to minimum diameter, 2.0% (range 0-6.3%), and time to 63% redilatation, 7.2% (range
0-20.2\%). Median CV of latency was 6.1\% (range 2.6-10.7\%) in normal subjects and 5.7\% (range 0-35\%) in diabetic patients.

8.5 Discussion

In this study we have measured constriction latency, response amplitude and a representative selection of other parameters derived from the pupillary constriction-redilatation curve using a newly-developed portable infrared pupillometer, and compared them with other measures of distal and proximal autonomic and somatic nerve function. Dynamic infrared pupillometry is an established method for the investigation of ocular autonomic function, though most devices are expensive and not portable. A group in the Netherlands (de Vos et al. 1989; Lanting et al. 1991) has recently developed a binocular system for measuring pupillary latency time in the consensually reacting pupil, but measurements of other components of the pupillary light response have not been reported using this system. The Pupilscan device is relatively simple to use, though it is not capable of delivering stimuli under Maxwellian conditions ie. it cannot eliminate differences in retinal stimulation due to variations in pupil size (Hreidarsson and Gundersen 1985). In addition, in the version of the apparatus used here, initial pupil diameter was somewhat less than the dark-adapted pupil size on account of the low-intensity cross-illumination. Subtle variations between groups of subjects may not be uncovered by the measurement of necessarily arbitrary portions of a complex curve, and additional techniques, such as the use of the pupil cycle time (Clark 1988), may be of value. Conventional infrared pupillometers are capable of sampling pupil diameters at up to 100 Hz, compared with the 20 Hz used here. Finally, although the latency measurement used here might more accurately be termed "pseudo-latency", as it was derived from the intersection of two regression lines, rather than being measured directly from the constriction curve, the values obtained were very close to those quoted in studies using conventional pupillometry (Hreidarsson and Gundersen 1985). All these factors may lead to a decrease in precision of the method, though any differences have not been systematically examined. Nevertheless, even with these potential limitations, the test-retest variability of the measurements in diabetic subjects was similar to that of other tests of autonomic function (Schumer et al. 1988), and considerably lower than the variability of sensory threshold measurements (Levy et al. 1989b).

In a detailed study Hreidarsson and Gundersen (1985) uncovered only a modest reduction in the response amplitude in a large group of patients with Type 1 (insulin-dependent) diabetes.
The response latency and, allowing for the reduction in resting pupil size in diabetes, all other dynamic measurements, were found to be similar to those in non-diabetic subjects. This finding is in contrast with that of de Vos et al. (1989), who found that 78% of diabetic patients without clinical signs of neuropathy had prolonged latencies. This prevalence of abnormality is much higher than that of even the most sensitive conventional measures of distal neurological function eg. the medial plantar nerve sensory action potential amplitude (Levy et al. 1987), and suggests substantial differences in methodology. In the current study, a much lower proportion (7%) had abnormal latencies, a rate broadly consistent with the expectation that cranial nerve pathways are less frequently abnormal than long peripheral nerve pathways in a length-dependent neuropathy. Pfeifer et al. (1984) found latency to be prolonged in newly-diagnosed Type 2 (non-insulin-dependent) patients but not in Type 1 (insulin-dependent) subjects, and Lanting et al. (1988) found a weak association with glycaemic control, a finding we did not confirm in the larger group of patients studied here.

Several studies have investigated the relationship between pupillometric measurements and the results of other tests of nerve function in diabetes. Hreidarsson (1982) found an inverse correlation between pupil size and vibratory perception threshold, and Neil and Smith (1989) described correlations between dark-adapted pupil diameter and cardiovascular autonomic tests. Lanting et al. (1988) found a weak relationship between thermal perception thresholds and pupillary constriction latency, but not with vibration perception thresholds or electrophysiological measurements (H-M interval of the Hoffman reflex and common peroneal motor conduction velocity). Many of these studies, however, did not take into account the changes of both pupillary and peripheral nerve function with age. In the current study, while several neurological measurements were found to be significantly correlated with reflex amplitude, when relative reflex amplitude (which allows for age by calculating the reflex amplitude as a proportion of the initial diameter) was considered, none of the other neurological measurements retained a significant correlation.

Even when statistically significant correlations were found, their strength was in most cases very weak. An unexpected association between vibration perception thresholds, constriction and redilatation velocities was found. The reasons for this are not clear, but in studies reported in previous chapters we found that distal measurements of sweating function (pilocarpine-activated sweatspot density (Chapter 4) and sympathetic skin response (Chapter 5)) also show unexpectedly strong correlations with vibration thresholds. In this study we found that acetylcholine-evoked sweat responses at the foot were weakly negatively correlated with pupillary constriction and redilatation velocities ie. high sweat outputs were associated
with low velocities. This finding is probably due to the presence of a small number of patients showing high pharmacologically-stimulated sweat outputs together with low constriction and redilatation velocities, and is indirect evidence for the simultaneous occurrence of pharmacological supersensitivity of sweat glands to cholinergic agents and diminished neurological responsiveness to physiological (ie. non-pharmacological) stimuli. Analogous findings have been presented in Chapters 6 and 7. In contrast with the findings of Ryder et al. (1988) no significant correlations were found with pilocarpine-activated sweatspot density at the foot. While it is tempting to relate peripheral parasympathetic and sympathetic function to specific portions of the constriction-redilatation curve (see section 8.4.4), it should be noted that there is no agreement on the relative contributions of the two divisions of the autonomic nervous system to the pupillary response, and there is recent pharmacological evidence that the majority of the response, including the later part of pupillary redilatation, commonly thought to be sympathetically mediated, is transmitted through parasympathetic pathways (Heller et al. 1990). Likewise, while the cause of the increased pupillary latency time in diabetic patients is not known, Lanting et al. (1991) suggested that it was also due to an efferent ie. parasympathetic defect, and this would be in accordance with the similar rates of abnormality for latency and other pupillary measurements found in the current study. The corollary of this finding is that latency time alone is an adequate measure of pupillary function in diabetes.

The inverse correlation between cholinergically-stimulated sweat output, and the weakness of the correlations with conventional neurophysiological measurements of peripheral nerve function, has highlighted the dissociation between cranial autonomic and peripheral nerve function. The prevalence of abnormal pupillary responses was somewhat lower than that of other neurological measurements; although this may be partly related to the methodology, similar results have been found in other studies using standard pupillometry (Hreidarsson and Gundersen 1985). Our findings are consistent with the view that central nervous system autonomic pathways are less frequently affected than peripheral ones. The functional significance of abnormalities of pupillary function in diabetes is not known, but they are not likely to be of major clinical importance, unlike abnormalities of vibration perception thresholds or cardiovascular vagal function, which may be predictive of clinically significant diabetic complications or of increased mortality (O'Brien et al. 1991). Nevertheless, dynamic pupillometry using the device described in this study is a simple and reproducible measure of a proximal autonomic pathway in diabetic neuropathy.
**Conclusion**

This series of studies has characterised functional and structural aspects of small-fibre function in diabetic patients. Pharmacological stimulation of sweat glands, performed by percutaneous iontophoresis of acetylcholine and pilocarpine, results in supersensitivity to these cholinomimetic compounds in some patients with established neuropathy, though these functional measurements of sweat gland function were more often found to be diminished in diabetic patients. When selected on the basis of definite abnormalities of small-fibre function, intradermal injection of methacholine resulted in uniformly increased sweat responses. Proximal small fibre function, assessed by pupillometric measurements, was less commonly abnormal than distal sweating function. The sweat measurements correlated very weakly with other measurements of autonomic and somatic nerve function, contrasting with the generally strong correlations found between conventional measures of nerve function, which usually examine long, sometimes multi-neuronal pathways. Exaggerated pharmacologically-induced sweat responses coexist with diminished function as revealed by conventional neurophysiological tests and by decreased sweat responses when sweat glands are activated by non-pharmacological stimuli, for example the sympathetic skin response. Evidence from quantitative immunohistochemical measurements of cutaneous nerves in skin biopsies suggests that pharmacological supersensitivity is associated in some cases with increased cutaneous immunoreactivity.

**Diagnostic value**

The diagnostic value of tests in diabetic neuropathy is difficult to determine, as there are no accepted "gold standard" measurements or agreed criteria for the clinical diagnosis of neuropathy. The data for the sweat tests and the other neurophysiological measurements were therefore analysed by discriminant analysis, a procedure which fits a Gaussian distribution to each of the groups (diabetic, non-diabetic), and assigns each subject to the group which has the highest likelihood. Thus, for each subject, the probability of belonging to each group is estimated, and the subject is assigned to whichever group gives the greatest value for this probability. Table 1 summarises the results of one-factor discriminant analysis for the neurophysiological tests; results of this analysis are comparable to those of logistic regression analysis (Altman 1991).
Table 1: One-factor discriminant analysis for a selection of the tests used in the present studies.

<table>
<thead>
<tr>
<th></th>
<th>Number</th>
<th>Proportion correctly identified</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal</td>
<td>Diabetic</td>
</tr>
<tr>
<td></td>
<td>Normal</td>
<td>Diabetic</td>
</tr>
<tr>
<td><strong>Sweat tests</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TVol12</td>
<td>46</td>
<td>86</td>
</tr>
<tr>
<td>SSR latency</td>
<td>46</td>
<td>68</td>
</tr>
<tr>
<td>SSR ampl</td>
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<td>68</td>
</tr>
<tr>
<td>Sweatspot density</td>
<td>53</td>
<td>80</td>
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<tr>
<td><strong>Sensory threshold tests</strong></td>
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<td></td>
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<tr>
<td>Warm</td>
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<td>97</td>
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<td>Cool</td>
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<td>92</td>
</tr>
<tr>
<td>VPTM</td>
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<td>VPTT</td>
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<tr>
<td><strong>Autonomic function tests</strong></td>
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<td>Valsalva</td>
<td>85</td>
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<td>30:15</td>
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<td>103</td>
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<td><strong>Electrophysiology</strong></td>
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<tr>
<td>CPDMAP</td>
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<td>80</td>
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<tr>
<td>Short f</td>
<td>41</td>
<td>58</td>
</tr>
<tr>
<td>SSAP</td>
<td>41</td>
<td>74</td>
</tr>
</tbody>
</table>

TVol12, acetylcholine-induced sweat output; SSR, skin sympathetic response; VPTM, vibration perception threshold medial malleolus; VPTT, vibration perception threshold great toe; RR, respiratory RR interval variation; CPMCV, common peroneal motor conduction velocity; CPDMAP, common peroneal distal compound action potential amplitude; short f, shortest f latency; SSAP, sural sensory action potential amplitude

Based on this analysis, the discriminant value of the tests ranges from 0.51 (acetylcholine-induced sweat volume) to 0.74 (common peroneal motor conduction velocity). Further analysis, using two-factor discrimination analysis, did not produce appreciably greater discriminant values. Diagnostic sensitivity (proportion of true positives correctly identified by the test) and specificity (proportion of true negatives correctly identified) can be calculated for pairs of tests; for example, the combination of common peroneal conduction velocity and SSR latency gives a sensitivity of 71% and a specificity of 88%; similar results were obtained
for other pairs of tests. The diagnostic value of all the tests described in the current studies, therefore, is similar.

**Comparison of the three tests of sweating function; role of histological measurements of small fibres**

Given the similar diagnostic sensitivity of the three sweat tests, and that, with the exception of the SSR amplitude, they have similar reproducibility, which can be used as independent measures of sweating activity? Results for all three tests were available in 61 diabetic patients. A correlation matrix (Pearson coefficients) is given in Table 2.

*Table 2: Correlation matrix of the major indices of sweating function (see Chapters 3-5). Statistically significant associations are emboldened.*

<table>
<thead>
<tr>
<th></th>
<th>TEWL</th>
<th>TVol12</th>
<th>Peak sweating rate</th>
<th>Sweatspot density</th>
<th>Sweatspot size</th>
<th>SSR latency</th>
<th>SSR amplitude</th>
</tr>
</thead>
<tbody>
<tr>
<td>TEWL</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TVol12</td>
<td>0.011</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peak sweating rate</td>
<td>0.261</td>
<td>0.880</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sweatspot density</td>
<td>0.210</td>
<td>0.146</td>
<td>0.218</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sweatspot size</td>
<td>0.150</td>
<td>-0.078</td>
<td>0.006</td>
<td>0.350</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SSR latency</td>
<td>0.024</td>
<td>0.382</td>
<td>0.416</td>
<td>0.123</td>
<td>-0.047</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>SSR amplitude</td>
<td>-0.167</td>
<td>-0.043</td>
<td>-0.084</td>
<td>0.332</td>
<td>0.112</td>
<td>-0.199</td>
<td>-</td>
</tr>
</tbody>
</table>

After acetylcholine stimulation, the peak sweating rate correlates very closely \( r=0.88 \) with the total volume of sweat produced; this appears to be largely determined by the sweatspot density as revealed by pilocarpine stimulation, rather than the size of the sweatspots, even though there is, as discussed in Chapter 4, a significant association between mean sweatspot size and density. In turn, the mean sweatspot density is reflected weakly in the SSR amplitude (Chapter 5); neither relationship, however, is sufficiently strong to substitute for direct measurement of sweat output, and even under standardised conditions, SSR amplitude has unacceptably high intra-individual variability. The direct sweat measurement was the only one to show a significant association with peripheral sympathetic function, as measured by
postural change in blood pressure, and cranial autonomic function (pupillary constriction and redilatation velocities). Peak sweating rate after acetylcholine iontophoresis is therefore the most appropriate measure of distal sweating function; this conclusion must, of course, be balanced against the technical difficulty of performing the test, compared with the simplicity of measuring either the sympathetic skin response or sweatspot densities. Since SSR latency reflects, albeit weakly, pilocarpine-stimulated sweatspot density, and since the physiological significance of the latency of the SSR is incompletely understood, the simplest and most practical method for measuring distal sweating function is the sweatspot technique discussed in Chapter 4.

Direct histological quantitation of distal innervation has been shown to be a practicable procedure, though the image-analysis method presented here has a high variability, comparable to that of sensory threshold testing, and the method is invasive; in clinical trials large numbers of patients would need to be studied by this method in order to detect reliably small changes in innervation. By providing histological data on peripheral small fibre innervation, a comprehensive picture of structural and functional features of diabetic neuropathy can now be assembled, when used in combination with morphological data on myelinated fibres derived from sural nerve biopsy and conventional tests of large fibre function, such as electrophysiology.
Appendix A  Selection of diabetic and normal subjects

At the time of the studies, there were 1464 patients 66 years of age or under registered on our diabetic clinic database at Central Middlesex Hospital. The clinic population is drawn from a multi-ethnic area of north-west London, comprising an outer-city area (Harlesden and Willesden) and a more extensive suburban area (Wembley). The total catchment area is approximately 250,000. Two smaller diabetic clinics in the same health district, with clinic populations of approximately 300 and 600, were not included. Patients over 66 years were not included because of difficulties in obtaining adequate age-matched normal subjects for comparative purposes.

The white population contains a substantial southern Irish group, and smaller groups of central and eastern European origins. There are two large ethnic groups of Asians (largely Gujarati Indians and Kenyan Indians) and of Afro-Caribbeans. The numbers (%) of these groups on the clinic database were as follows:

<p>| | | |</p>
<table>
<thead>
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</thead>
<tbody>
<tr>
<td>European</td>
<td>612</td>
<td>42%</td>
</tr>
<tr>
<td>Asian</td>
<td>353</td>
<td>24%</td>
</tr>
<tr>
<td>Afro-Caribbean</td>
<td>382</td>
<td>26%</td>
</tr>
</tbody>
</table>

The remaining 117 (8%) consisted of smaller numbers of West Africans, Punjabi Asians and Orientals.

Fifty-four percent were male. Nineteen percent (276) were classified as type 1 (insulin-dependent) diabetics, with acute onset of diabetic symptoms under 30 years of age and with an immediate and permanent requirement for insulin therapy. Islet-cell antibody and C-peptide status were not determined. 574 patients (39%) of the whole population were treated with insulin. The median age of the clinic patients was 53 y, though the age-distribution was highly skewed towards the older age-groups; likewise, the median known duration of diabetes was 6 y, while the duration-distribution was highly skewed towards shorter durations. Total glycated haemoglobin (HbA1c) at the time of registration on the database was normally distributed, with a mean value of 11.5%, standard deviation 2.8% (reference range < 8.0%).

In view of the high proportion of type 2 (non-insulin-dependent) patients, we aimed to study a group comprising approximately one-half each of type 1 (insulin-dependent) and type 2 (non-insulin-dependent) patients.
In order to achieve approximately 100 completed neurological surveys, 180 patients were initially invited by letter to attend. Invitations were sent to randomly-selected subjects using the database listed in the order in which patients had been entered on our computer i.e. non-alphabetised. In the case of type 1 (insulin dependent) patients, every third patient was invited; for type 2 (non-insulin-dependent) patients every twelfth patient was invited. One hundred and seven patients attended, a response rate of 59%. The ethnic breakdown was as follows:

<table>
<thead>
<tr>
<th>Ethnicity</th>
<th>Number</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>European</td>
<td>65</td>
<td>61%</td>
</tr>
<tr>
<td>Asian</td>
<td>28</td>
<td>26%</td>
</tr>
<tr>
<td>Afro-Caribbean</td>
<td>14</td>
<td>14%</td>
</tr>
</tbody>
</table>

European patients were therefore over-represented in our study sample, and Afro-Caribbeans under-represented. Asian patients attended in similar proportions to their clinic representation.

Many patients made two study visits, but all tests were completed within 6 months of the first visit. It was unavoidable that with a large study population, control of seasonal factors, which may be of importance in determining sweat gland responsiveness, was impractical. The total duration of the studies was approximately 18 months.

**Selection criteria for normal subjects**

Normal subjects were recruited from hospital personnel and their relatives, and non-diabetic relatives of the study patients. We aimed to obtain at least 10 subjects in each decade from 20-60 years for each test. The following exclusion criteria were applied:

- **(a)** Current or past, established or suspected neurological disease
- **(b)** Untreated or unmonitored endocrine disease
- **(c)** Alcohol intake > 15 units per week
- **(d)** Known diabetes
- **(e)** Generalised skin disease
- **(f)** Previous lower limb fracture or current sciatica
- **(g)** Drug therapy: subjects taking any drug known to be neurotoxic were excluded, as were those currently taking antidepressant drugs with known anticholinergic activity. Beta-blocking drugs were permitted, but not vasodilators or angiotensin-converting enzyme (ACE) inhibitors.
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