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BALKAN (ENDEMIC) NEPHROPATHY AND A TOXIN-PRODUCING STRAIN OF *PENICILLIUM VERRUCOSUM* VAR *CYCLOPIUM*: AN EXPERIMENTAL MODEL IN RATS

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Summary Cultures of an isolate of *Penicillium verrucosum* var. *cyclopium*, obtained from stored maize in an area of Balkan (endemic) nephropathy—Vratza, Bulgaria—has consistently induced renal tubular lesions when force-fed to rats for 20 days. The lesions, confined to the lower reaches of the proximal convoluted tubules (pars recta and junctional zone), closely resemble the tubular changes in patients with Balkan nephropathy. Preliminary evidence suggests that this nephrotoxin-producing strain of *P. verrucosum* var. *cyclopium* may be implicated in the aetiology of Balkan nephropathy.

Introduction

BALKAN (endemic) nephropathy, first described in the 1950s, is a remarkable disease on several counts.¹⁻⁷ Its basic clinical picture is one of slowly progressive renal failure rarely accompanied by signs of sodium retention or hypertension. There is a clear association between the frequency of Balkan nephropathy and that of tumours of the renal pelvis, ureters, and bladder. It seems to be confined to localities in Yugoslavia, Romania, and Bulgaria, close to the Danube and its tributaries. Within the rural areas of this region there may be more than 20 000 cases of the disease.

The aetiology of Balkan nephropathy is unknown. No constant correlation has been established between the disease and trace elements in the local soil and water. There is no evidence to implicate bacteria such as β -haemolytic streptococci, *Leptospira icterohaemorrhagiae*, or *Brucella melitensis*. A tentative claim has been made for arborviruses and, more recently, a coronavirus.⁸ An attempt by Dimitrov⁹ to incriminate fungi in 1960 gave inconclusive results, but the possible role of mycotoxins in the aetiology of Balkan nephropathy was reappraised by Barnes¹⁰ and Akhmeteli.⁵

Investigation into a possible mycotoxic cause was begun in May, 1971, and preliminary observations have been published.^{6 11} The main finding has been the highly significant correlation between mortality from the disease and the occurrence of excessive rainfall during the harvest periods (August–October) of the previous two years. This is taken to indicate that in such years the environmental conditions at harvest and in the early period of storage of the crops—notably, of grain—would favour the growth of saprophytic fungi such as penicillia, aspergilli, and fusaria, and hence the formation of mycotoxins. Examination of the fungal flora of foodstuffs from the endemic areas revealed a “normal” range of species for similar crops grown elsewhere in temperate regions and analytical tests failed to show the presence of aflatoxin, citrinin, or ochratoxin, although zearalenone was found in maize samples. Subsequent studies have shown that, in one endemic village in the Slavonski Brod area of Yugoslavia, some 8–20% of the foodstuffs have ochratoxin A present (Krogh, P., Hald, B., Plestina, R., Ćević, S. unpublished)—a fungal toxin which is known to produce renal changes in pigs and poultry in Scandinavia.^{12 13}

We present here a combined mycological and pathological study which indicates that one strain of *Penicillium verrucosum* var. *cyclopium*, isolated from maize grown in the endemic area, induces in rats renal tubular lesions which closely resemble the changes found in patients with Balkan nephropathy.

Materials and Methods

Collection of Food and Culture of Fungi

Fresh material for fungal cultures was collected by P.K.C.A. during visits to five districts in the endemic area—the Slavonski Brod and Bijelina districts of Yugoslavia (May, 1971), Slavonski Brod (June, 1972), the Vratza district of Bulgaria (November, 1972), and the Oravita and Turnu Severin districts of Romania (June, 1973). Food samples and other materials were obtained from houses and farms and placed in plastic or paper bags or in sterile universal bottles. When appropriate, air-drying was carried out as quickly as possible, or the samples were kept at 5°C. Macroscopical and direct microscopical examinations were carried out under stereo and high-power microscopy to determine the amount and type of fungal growth. Isolations were made from both surface-sterilised and unsterilised materials by plating out on Czapek-Dox agar (containing 0.05 mg chlortetracycline/ml) for fungi, and on half-strength nutrient agar for actinomycetes. Incubation was at 26°C for up to three weeks. Stock cultures were kept on Czapek-Dox agar under sterile mineral oil. Details of the isolates tested and their origin are given in table 1.

Cultures for toxicity testing were grown in Roux flasks containing 100–200 ml of yeast extract–sucrose medium (YES medium, containing 20% sucrose and 2% Difco yeast extract, in distilled water). Sterilisation was by Seitz filtration. The inoculum consisted of conidial suspensions in sterile distilled water (without a wetting agent) adjusted to a final concentration in the medium of 1×10^6 conidia/ml. Incubation was at 26°C for 8–21 days. Harvesting was carried out in an exhaust protective cabinet by pouring off the remaining culture fluid, homogenising the mycelium for 1–2 minutes in a Waring blender and mixing the homogenate and the culture fluid before dispensing in 25 ml universal bottles. These were deep-frozen at –20°C.

Conduct of Experiments

48 male Lac P rats were used, aged five to six weeks and in-

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TABLE I—SPECIES, STRAINS, AND SOURCES OF FUNGI TESTED FOR TOXIN PRODUCTION

Culture ref. no.	Fungus species	Substrate	District and country
N.159/4	<i>Penicillium verrucosum</i> var. <i>cyclopium</i> (Westling) (Samson, Stolk, and Hadlok)	Mouldy garlic (<i>Allium sativum</i> L.)	Slavonski Brod, Yugoslavia.
N.202/14	<i>Penicillium verrucosum</i> var. <i>cyclopium</i> (Westling) (Samson, Stolk, and Hadlok)	Fermenting plums (<i>Prunus domestica</i> L.)	Vratza, Bulgaria
*N.202/34	<i>Penicillium verrucosum</i> var. <i>cyclopium</i> (Westling) (Samson, Stolk, and Hadlok)	Mouldy maize grain endosperm (<i>Zea mais</i> L.)	Vratza, Bulgaria
N.202/7	<i>Penicillium expansum</i> Link	Rotting quince (<i>Cydonia oblonga</i> Mill.)	Vratza, Bulgaria
N.202/10	<i>Penicillium expansum</i> Link	Mouldy rose hip (<i>Rosa</i> sp.)	Vratza, Bulgaria
N.260/17	<i>Penicillium</i> sp.	Mouldy garlic (<i>Allium sativum</i> L.)	Turnu Severin, Romania
N.202/16	<i>Phoma</i> sp.	Diseased paprika (<i>Capsicum annuum</i> L.)	Vratza, Bulgaria
N.202/35	<i>Fusarium oxysporum</i> Schlect.	Mouldy maize grain endosperm (<i>Zea mais</i> L.)	Vratza, Bulgaria

* Toxin-producing isolate

initially weighing 150–160 g. They were fed on a standard 41B cubed diet and water *ad libitum*. Rats in the test groups were given the culture homogenate suspended in 3–5 ml sterile water; animals in the control groups were given equivalent volumes of sterile water. In both groups, test and control materials were given by force-feeding 5 days in each week for four weeks. The animals were killed with ether 30 to 32 days after the beginning of the experiments. Full necropsies were made and tissues from parenchymal organs—liver, spleen, kidneys, heart, lungs—were fixed in buffered formalin and processed for light microscopy. 4 μ m paraffin sections were stained with periodic-acid/Schiff (P.A.S.) and other standard histological techniques

The following additional investigations were made with rats fed with one of the cultures of *P. verrucosum* var. *cyclopium*, isolated from mouldy maize collected from Vratza, Bulgaria (see table 1):

(i) Pairs of rats were kept in metabolism cages for 24 h for collection of urine samples. Fluid intake was measured during this time. In addition to qualitative testing of the urine specimens for the presence of protein, excess reducing substances, and glucose, volume, osmolality, pH, and protein concentration were measured. The pattern of protein excretion was examined by electrophoresis on cellulose acetate after concentration of the protein to approximately 30 g/l by dialysis in Amicon microconcentrators. The pattern of aminoacid excretion was examined by two-dimensional paper chromatography. The techniques were similar to those that have been employed in the study of tubular proteinuria.¹⁴

(ii) Autoradiographs were prepared from the kidneys by coating 2 μ m 'Epon' sections with Kodak 'AR 10'. Tritiated methylthymidine (Radiochemicals, Amersham) was injected intraperitoneally at a dose of 100 μ Ci, specific activity 21 Ci/mmol. The rats were killed one hour later. The exposure times ranged from one to five weeks.

(iii) For electron microscopy the kidneys were perfused *in situ* with 4% glutaraldehyde in cacodylate buffer.¹⁵ Blocks of tissue were trimmed and fixed in 2% osmium tetroxide. The tissues were embedded in epon and thin sections were stained with uranyl acetate and lead citrate.

Results

Mycology

During four trips to Yugoslavia, Bulgaria, and Romania between 1971 and 1973, P.K.C.A. visited five endemic areas, 19 villages, and 63 premises. Some 163 samples of foodstuffs and other materials were collected,

and from these 208 colonies of fungi or actinomycetes were obtained. The 8 isolates finally tested for animal toxicity came from the 73 obtained in pure culture and were selected with a view to covering as wide a range of both suspected foodstuffs and predominant fungi as possible with such a restricted number. *Penicillium verrucosum* var. *cyclopium* (a well-known toxin producer) was isolated 34 times and was the commonest species found. *P. expansum*, which produces patulin, was isolated twice. Various pycnidial fungi related to the *Phoma* sp. isolated produce the mycotoxin responsible for lupinosis in sheep and cattle, and many *Fusarium* spp. (20 isolates) produce toxic trichothescenes and zearalenone.

Oral administration of culture homogenates from 7 of the 8 fungal isolates listed in table 1 produced no abnormal effects in the rats during life and no macroscopic or microscopic changes in any of the organs subsequently examined. The cultures giving negative results comprised 5 of the penicillia and 1 each of the *Phoma* sp. and *Fusarium oxysporum*. By contrast, cultures of one of the three isolates of *P. verrucosum* var. *cyclopium* (see table 1) invariably induced striking abnormalities in renal structure in all the animals tested.

Morphological Changes

Rats fed with these culture homogenates gained steadily in weight and appeared healthy. At necropsy, the kidneys were macroscopically normal apart from slight hydronephrosis which occurred equally in occasional test and control animals. The kidneys showed no capsular thickening, no scarring, and no loss of parenchyma. The renal papillae, pelves, and ureters were normal (except in the animals with slight hydronephrosis), and no naked-eye changes were seen in the bladder.

In the light microscope, abnormalities were confined to the kidneys; other parenchymal organs, and the ureters and bladder, were histologically normal. The renal lesions were consistently localised to the lower reaches of the proximal convoluted tubules (pars recta and junctional zone) in the vicinity of the corticomedullary junction and the outer medulla. The tubular epithelium was grossly abnormal with distended, irregularly shaped cells, lacking the normal brush-border and con-

taining nuclei two to three times larger than normal (fig. 1). These nuclei were strikingly pleomorphic, showing swelling, fragmentation of chromatin, and pyknosis. Several mitoses were present, tending to be patchily distributed. Degenerate epithelial cells often protruded into the lumen, sometimes becoming detached and filling the lumen with cell debris. In several fields, certain affected tubules had retained parts of their normal epithelium so that abnormal lining cells were interspersed with well-preserved epithelium. The tubular basement membrane and the local reticulin framework were unchanged, and there was no intertubular fibrosis. A few tiny foci of interstitial inflammatory cells were present but these were not related to the tubular lesions; similar interstitial collections were found in the control rats. Other segments of the renal tubules, and the glomeruli, showed no abnormalities. Intrarenal blood-vessels were normal.

The findings with autoradiography and electron microscopy will be reported in detail elsewhere, but the salient features are as follows. The autoradiographs showed small numbers (<1/high-power field) of tritiated-methylthymidine-labelled nuclei in or near the damaged proximal tubules. Most of the labelled epithelial cells seemed either normal or slightly enlarged; no nuclear labelling was seen in the grossly enlarged or dysplastic cells. Electron microscopy confirmed the location of the lesion in the epithelial cells lining the lower segment of the proximal convoluted tubules (pars recta and junctional zone). Two main patterns of epithelial degenera-

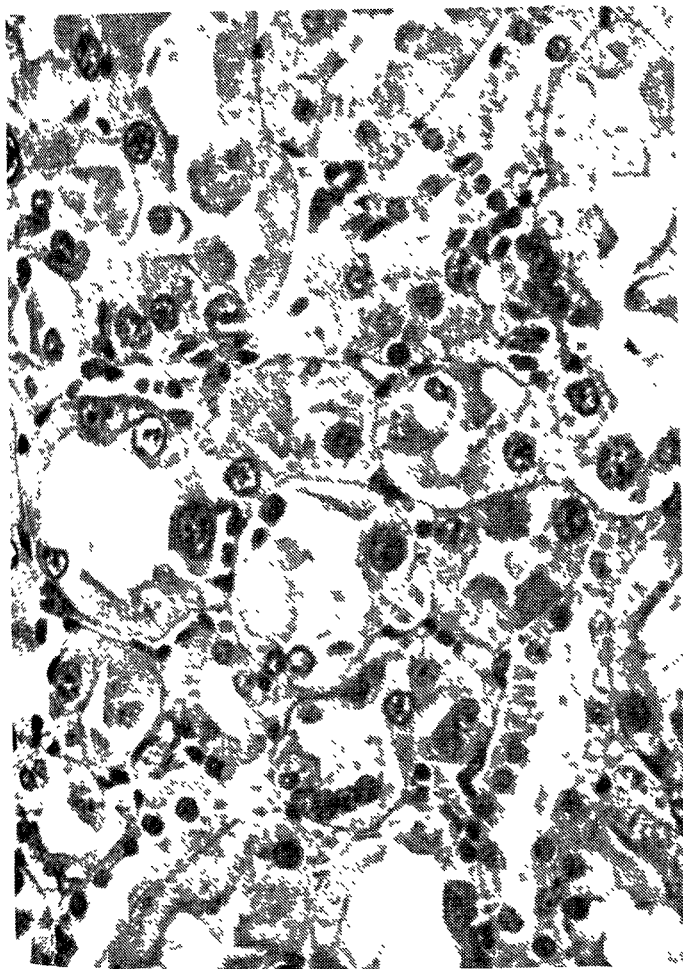


Fig. 1—Kidney of rat fed with isolate from *P. verrucosum* var. *cyclopium*, 30 days.

Cartucomedullary junction, with many abnormal cells in the proximal convoluted tubules. Note intense nuclear pleomorphism. P.A.S. $\times 380$.

tion were seen (figs. 2 and 3). Some cells showed pronounced pyknosis, cytoplasmic vacuolation, densely staining mitochondria, and loss of microvilli in the brush-borders. The cells were commonly shed into the



Fig. 2—Electronmicrograph of a proximal tubular cell in the pars recta showing the cell in a phase of necrosis.

The chromatin of the nucleus (N) is condensed, the nuclear envelope is dilated, and the cell sap is vacuolated. $\times 3750$.

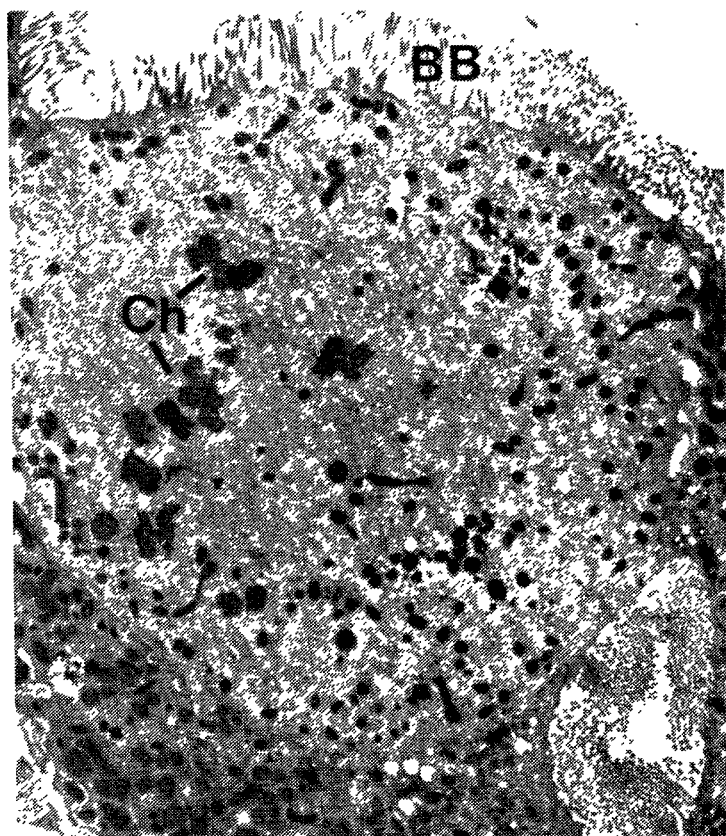


Fig. 3—Electronmicrograph of a mitotic proximal tubular cell in the pars recta region.

The number of microvilli in the brush-border (BB) is reduced. Ch=chromosomes. $\times 3000$.

TABLE II—URINARY PROTEIN EXCRETION (mg/24 h)*

Time (days)	Test	Control
1	2	2
3	1	2
8	7	9
10	6	15
15	13	13
21	13	13
22	6	12
24	8	14

* Measurements made on pooled 24 h urine samples.

tubular lumen. The other major abnormality consisted of large tubular cells, apparently in metaphase, with abnormal mitochondria (condensed matrix and widened intracrystal spaces), and reduced or absent microvilli. In addition, large well-preserved cells were seen in which the only abnormality was the presence of a considerably enlarged, irregularly shaped nucleus. A few of these cells extended a short way up into the more proximal parts of the tubule. Probably the large cells represent the population that was labelled with tritiated methylthymidine in the light autoradiographs. No ultrastructural changes were seen in the loops of Henle, the distal convoluted tubules and the collecting ducts, or in the glomeruli.

In control animals, all parts of the nephron seemed normal in the light and electron microscope. Sparse collections of chronic inflammatory cells were occasionally seen in the interstitial tissues. There was slight dilatation of the pelvicalicine system in the few rats with early hydronephrosis.

Urine Analyses

All analyses were made on pooled samples of urine, collected from 2 rats over a 24-hour period. Total protein excretion, recorded as mg protein/24 h., is shown in table II. Rats in both groups showed a comparable trend towards an increase in total protein excretion between days 1 and 24. The electrophoretic analyses of the urinary proteins excreted on days 1 and 3 showed poorly differentiated patterns with components spread between fast alpha-1-globulin and fibrinogen positions as judged by reference to human serum run alongside. The electrophoretic patterns of the urine protein that was excreted after day 3 were characterised by a predominance of proteins with mobilities approximating to that of the alpha-2 and alpha-1 globulins of human serum, albumin again being present only in trace amounts. Despite variations in these latter patterns, with sometimes an alpha-2 or alpha-1 band predominating, and sometimes both being present in approximately equal concentrations, no consistent differences emerged between the test and control groups. Tests for glucose and other reducing substances were negative in all specimens and the aminoacid chromatograms showed no significant differences between test and control groups. A comparably wide range of urine osmolality was found in test and control animals.

Discussion

Penicillium verrucosum var. *cyclopium* commonly produces toxins,^{16 17} some of which have been chemi-

cally characterised. They range from the neurotoxic penitrems, the hepatotoxic and nephrotoxic ochratoxin A and cyclopiazonic acid, and the possible carcinogen penicillic acid. It has been stated¹⁸ that "the major toxic compound being produced appears to differ from one toxigenic strain to another", and the present findings—with nephrotoxic effects demonstrated with only one of the three isolates of *P. verrucosum* var. *cyclopium*—is compatible with this view. The renal lesions are unusually specific in their morphology, and they may perhaps be due to a hitherto unrecognised nephrotoxin produced by this isolate.

There are several reasons for proposing that the nephrotoxin-producing strain of *P. verrucosum* var. *cyclopium* may be implicated in the aetiology of Balkan nephropathy.

Firstly, *P. verrucosum* var. *cyclopium* was the commonest mould found in the foodstuffs from all the endemic areas visited.

Secondly, the maize grain from which the nephrotoxin-producing strain was isolated came from a cob stored on a farm in a Bulgarian village near Vratza where the incidence of Balkan nephropathy is very high.

Thirdly, all rats force-fed with culture homogenates developed striking morphological changes in the lower part of the proximal convoluted tubules (pars recta and junctional zone) near the corticomedullary junction. The location and morphology of these lesions are almost identical to those described in many patients with Balkan nephropathy.^{1-7 19} In man, the major feature is the damage sustained by the tubules with relative sparing of the glomeruli. Specific glomerular lesions, including thickening of the basement membrane, have been described more recently but their incidence and (in particular) their time of onset are disputed;^{5 7 20 21} in any case, the central importance of the tubular lesions in man is shown by the well-documented abnormalities in renal function.^{1-7 22 23}

Interpretation of urine analyses in the rats is difficult because of the background level of low-molecular-weight globulins in the urine of both treated and control animals, but certain negative features are noteworthy: the virtual absence of albuminuria, the absence of either detectable or abnormal amounts of materials which are normally reabsorbed in the proximal convoluted tubule (glucose and other reducing substances, aminoacids), and the absence of polyuria and lowered urine osmolality. These findings are compatible with a lesion localised to the lower reaches of the proximal convoluted tubule.

The postulated causal association between this nephrotoxin-producing strain of *P. verrucosum* var. *cyclopium* and Balkan nephropathy is at present only tentative. Given the common occurrence of *P. verrucosum* var. *cyclopium* in stored foodstuffs in the endemic area, it is likely that closer examination of more isolates of this and other species will reveal further nephrotoxin-producing strains. Non-fungal aetiological agents may be involved, and different combinations of aetiological agents may act synergistically in different parts of the endemic area.

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PLASMA-MYOINOSITOL CONCENTRATIONS IN URÆMIC NEUROPATHY

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Summary In a series of patients with chronic renal failure managed conservatively, the rise in the plasma-*myo*-inositol (myoinositol) concentration has been found to be related to depression of sural-nerve conduction velocity. There was no correlation with motor-nerve conduction velocity in the peroneal nerve, or with either of these variables in a series of patients receiving chronic hæmodialysis. Despite the negative correlation with sural-nerve conduction velocity, there was no correlation between the plasma-*myo*-inositol concentration and the presence of peripheral neuropathy as assessed clinically. It is concluded that hypermyoinositolæmia may depress nerve conduction velocity, but there is no evidence that it is responsible for the development of uræmic polyneuropathy.

Introduction

URÆMIC polyneuropathy is an important consequence of chronic renal failure, being present in about 50% of

patients treated conservatively¹ and in 20% of a recently analysed group of cases treated by periodic hæmodialysis.² The cause remains unknown, but the improvement that can be produced by adequate hæmodialysis,^{2, 3} or more effectively by renal transplantation,⁴ suggests the action of a retained metabolite or, in view of the variability of the clinical features of the neuropathy, possibly more than one metabolite.⁵ The occurrence of polyneuropathy is not correlated with the plasma urea or creatinine levels. Various substances including methylguanidine⁶ have been advanced as possible candidates, but none has been convincingly implicated. Claims have been made that substances in the "middle molecular weight" range may be involved.^{7, 8} Clements et al.⁹ have proposed that hypermyoinositolæmia may be responsible. Plasma-*myo*-inositol (myoinositol) levels are known to be raised in chronic renal failure. These workers¹⁰ found that experimental hypermyoinositolæmia in rats produces a reduction in nerve conduction velocity similar to that observed in human uræmic polyneuropathy.

In this study, we have measured the plasma-*myo*-inositol concentration in patients with chronic renal failure and have related this to the results of nerve-conduction studies and to the occurrence of clinically evident neuropathy.

Patients and Methods

17 patients with severe chronic renal failure not receiving treatment with periodic peritoneal dialysis or hæmodialysis were investigated. Observations were also made on 50 cases receiving regular hæmodialysis. Both groups were questioned about symptoms of neuropathy and examined neurologically. Cases with other possible causes of neuropathy were excluded.

Motor nerve conduction velocity was measured in the peroneal nerve with surface recordings over the extensor digitorum brevis muscle and stimulation of the nerve at the knee and ankle. Sural-nerve conduction was examined antidromically by stimulating the nerve percutaneously at midcalf level and recording from needle electrodes inserted alongside the nerve at the ankle. In both instances, 200 µs stimuli were delivered from a Devices isolated stimulator. The recordings were suitably amplified and displayed on a Telequipment storage oscilloscope. For the recording of the sural nerve action potentials, 150 responses were averaged on a Medelec AVM6 averager. Skin temperature over the lower leg at the time of recording was 32–34°C.

In the non-dialysed patients, blood samples were taken in the fasting state for the estimation of myoinositol. In the cases receiving treatment by hæmodialysis, a random blood sample was obtained at the time nerve conduction was examined.

Myoinositol was assayed by gas-liquid chromatography by a method based on that of Lewin et al.¹¹ To 2.5 ml of plasma was added 250 µl of 0.02% methyl- α -D-mannopyranoside as internal standard followed by 250 µl of 60% w/v trichloroacetic acid to remove the proteins. The supernatant was adjusted to pH7 with approximately 0.5 g (Dowex 1-X8) resin in the hydroxide form. Aliquots (1.0 ml) were evaporated to dryness at 60° and stored in a desiccator pending silylation with 0.5 ml of anhydrous pyridine/hexamethyldisilazane/trimethylchlorosilane, 5/2/1 (by volume). The samples were shaken for four hours and stored at 4° overnight before gas-liquid chromatography with a 150 cm long, 3 mm inside diameter column of 2% OV1 on (Gas Chrom W) and a Pye-Unicam gas chromatograph with temperature programming.

Results

The distributions of the values for creatinine clear-