Br. J. exp. Path. (1981) **62**, 200

GENTAMICIN-INDUCED NEPHROTOXICITY IN MICE: PROTECTION BY LOOP DIURETICS

P. H. WHITING*, J. PETERSEN† AND J. G. SIMPSON‡

*From the *Departments of Chemical Pathology, †Therapeutics and Clinical Pharmacology and ‡Pathology, University of Aberdeen, Aberdeen*

Received for publication December 6, 1980

Summary.—Gentamicin, at doses of 50 or 100 mg/kg body wt administered daily to healthy male MF_1 mice by i.p. injection for either 7 or 10 days caused proximal tubular cell damage shown both by the urinary excretion of *N*-acetyl-β-D-glucosaminidase (NAG) and by electron microscopy. The tubular damage was maximal at 7 days. Concomitant administration of any of 3 diuretics—frusemide, bumetanide or piretanide at 5, 0·5 and 1 mg/kg body wt/day respectively—resulted in less tubular damage than that caused by gentamicin alone. This finding of protection by diuretics contrasts with those of previous studies of combination gentamicin-diuretic therapy.

GENTAMICIN, a broad-spectrum aminoglycoside antibiotic, is widely used in the treatment of serious bacterial infections due to aerobic Gram-negative bacilli. The renal handling of gentamicin involves both glomerular filtration and tubular reabsorption (Bergan *et al.*, 1973; Chiu *et al.*, 1976) and the drug has been shown to be nephrotoxic in both animals and man (Abramowicz and Edelmann, 1968; Falco, Smith and Arcieri, 1969). The renal damage, which is dose-related, is centred on proximal tubular cells which undergo a variety of structural changes (Kosek, Mazze and Cousins, 1974) with tubular enzymuria (Wellwood *et al.*, 1976), at their most severe resulting in acute renal failure.

The nephrotoxicity of gentamicin, as well as other antibiotics, has been reported to be potentiated by concurrent therapy with the diuretic frusemide (Falco *et al.*, 1969; Wilbert *et al.*, 1971; James *et al.*, 1975; Kahn, 1977; Noël and Levy, 1978; Adelman *et al.*, 1979). The present study was undertaken to determine whether combining either of 2 other loop diuretics, bumetanide and piretanide, with gentamicin would result in less toxicity than that produced by frusemide and gentamicin. Contrary to expectation, the combination of gentamicin with all 3 diuretics produced less tubular damage than that seen after gentamicin alone.

MATERIALS AND METHODS

Chemicals.—All reagents used in the study were of Analar grade where possible. Gentamicin sulphate was obtained from Nicholas Laboratories Ltd., Slough, Berkshire, frusemide and piretanide from Hoechst U.K. Ltd., Hounslow, Middlesex, and bumetanide from Leo Laboratories Ltd., Hayes, Middlesex. 4-Methyl-umbelliferyl-2-acetamido-2-deoxy-β-D-glucopyranoside was obtained from Koch-Light Ltd., Colnbrook, Bucks.

Animals.—Male MF_1 mice (42–56 days old, 25–35 g body wt) were used throughout. They were housed in metabolic cages and allowed standard mouse food and water *ad libitum* except during urinary collection periods. At the end of each experiment, the animals were killed by cervical dislocation and the kidneys removed for further study.

Treatment groups.—Each animal received a single daily i.p. injection, 0·25–0·35 ml in volume, according to the following dosage schedules.

(1) *Controls:* bicarbonate-buffered saline, pH 9·4, alone or containing frusemide (5 mg/kg

Correspondence: Dr P. H. Whiting, Department of Chemical Pathology, University Medical Buildings, Foresterhill, Aberdeen AB9 2ZD.

body wt), bumetanide (0·5 mg/kg wt) or piretanide (1 mg/kg). The diuretics were approximately equipotent at these doses.

(2) *Gentamicin:* 50 or 100 mg/kg body weight in buffer.

(3) *Gentamicin plus diuretic:* gentamicin (100 mg/kg body wt) together with either frusemide (5 mg/kg), bumetanide (0·5 mg/kg) or piretanide (1 mg/kg).

For each treatment group, 10 animals were treated for 7 days and 10 animals for 10 days: from each group of 10, 5 animals were killed 24 h after the last injection and 5 one week later.

Measurement of urinary N-acetyl-β-D-glucosaminidase (NAG) levels.—Faecally uncontaminated urine specimens diluted 1 : 5 with trisodium citrate–phosphate buffer (20mM, pH 4·3) were assayed for NAG activity and creatinine essentially by the method of Whiting, Ross and Borthwick (1979), except that the final substrate concentration was 0·5mM. A unit of enzyme activity is expressed as 1 μmole 4-methylumbelliferone (4MU) released per hour; enzyme levels are expressed as u/mg urinary creatinine.

Measurement of renal NAG levels.—Kidneys were weighed and then mechanically homogenized in 10 volumes of trisodium citrate–phosphate buffer at 5°. Cell debris was removed by centrifugation at $2{\cdot}5 \times 10^3$ *g* for 10 min at 5° and the supernatant stored at −5°. The protein content of these kidney extracts was determined by the method of Lowry *et al.* (1951). Total and heat-stable NAG activities were measured in a 1ml reaction mixture containing 15 μmoles trisodium citrate–phosphate buffer, 0·5 μmol 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-glucopyranoside and 0·05 ml kidney extract (50–100 μg protein). After a 15-min incubation at 37°, the reaction was terminated and the liberated 4MU estimated: the heat-stable NAG activity was determined using a buffer–kidney-extract mixture that had previously been incubated at 50 ± 1° for 90 min. Renal NAG activities are expressed as μmoles 4MU released/h/g wet wt.

Microscopy.—For light microscopy, blocks of kidney were fixed in 10% neutral buffered formalin and processed to paraffin wax; 5μm sections were stained with H. & E., periodic-acid–Schiff and Lendrum's Martius Scarlet-Blue. For histochemical studies, half of one kidney was snap-frozen at −70°; 6μm cryostat sections were processed for acid and alkaline phosphatase, ATPase, succinic dehydrogenase and NAG by standard methods.

For electron microscopy, $1mm^3$ blocks of renal cortex were fixed for 4 h at 4° in 2·5% glutaraldehyde in 0·1M cacodylate buffer, pH 7·2, containing 2·5mM calcium chloride, post-fixed in 1% osmium tetroxide and processed to Epon 812. Half-micron sections stained with 1% toluidine blue were used for light microscopy and orientation. Ultra-thin sections stained with uranyl acetate and lead citrate were examined in a Philips 300 electron microscope.

All of the microscopical preparations were viewed "blind" by an experienced observer.

RESULTS

Urinary excretion of NAG

Urine samples were collected daily from all experimental groups 0–6 h and 18–24 h after injection. Following diuretic administration without gentamicin, a diuresis was observed as indicated by increased urine volume, decreased urine creatinine levels and decreased body weight. Diuretics alone had no effect on urinary NAG levels over a 10-day period (Table I). NAG levels in 256 urine samples from 80 untreated or buffered-saline-injected mice were found to range from 1·4 to 4·4 u/mg urinary creatinine.

The pattern of urinary NAG excretion in the test groups was different for the 2 urine collection periods. In urine samples

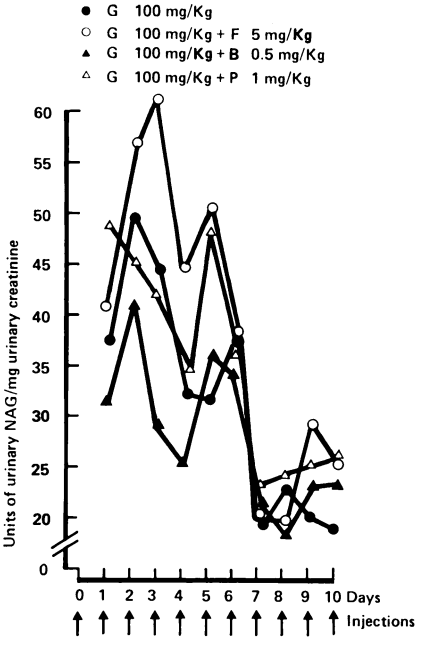


FIG. 1.—Urinary NAG excretion from 0 to 6 h during 10-day treatment with gentamicin alone or in combination with each of 3 diuretics. G = gentamicin; F = frusemide; B = bumetanide; P = piretanide.

TABLE I.—*Six-hour urinary volumes, creatinine levels and NAG activities following injection of buffered saline, frusemide, bumetanide or piretanide*

	Saline controls (10)	Frusemide (10)	Bumetanide (10)	Piretanide (10)
Urine volume (μl)	460 ± 224	1080 ± 460	1460 ± 390	990 ± 276
Creatinine (μmoles/l)	2195 ± 679	995 ± 250	1480 ± 475	1365 ± 360
NAG activity (u/mg urinary creatinine)	3·23 ± 1·57	2·72 ± 0·94	3·52 ± 0·81	3·69 ± 1·08

Results are expressed as mean ± 1 s.d. The number of determinations is given in parentheses.

TABLE II.—*Renal total NAG levels after 7 daily injections of buffered saline, gentamicin (100 mg/kg) and combinations of gentamicin with frusemide, bumetanide or piretanide*

Saline controls	Gentamicin	Gentamicin and frusemide	Gentamicin and bumetanide	Gentamicin and piretanide
(4)	(8)	(5)	(5)	(5)
2198 ± 66	3796 ± 469	4130 ± 266	3827 ± 816	3559 ± 365

Enzyme activities are expressed as μmoles 4 MU liberated/h/g/wet wt and the results shown as mean ± s.d. The number of determinations is given in parentheses.

The results in each group were compared by analysis of variance for unequal sample sizes; $P < 0.001$ for controls *vs* all other groups, with no differences between the test groups ($P < 0.2$).

collected 0–6 h after injection (Fig. 1), gentamicin at 100 mg/kg body wt produced an immediate substantial elevation in NAG excretion; levels peaked at 2 days and then declined, although they were still increased at 10 days. The combination of each of the 3 diuretics with gentamicin produced similar NAG excretion profiles to that seen after gentamicin itself. In the 18–24h post-injection urine samples (Fig. 2) there was a gradual dose-related increase in NAG enzymuria with a peak at 7 days; 1 week after the last gentamicin injection, urinary NAG levels were within the normal range. No increase in NAG excretion was observed 18–24 h after injection during the 10-day experimental period in animals treated with both gentamicin and diuretic.

Renal NAG levels

After 7 days of treatment, renal total NAG levels were increased in all test groups compared to controls; there was no statistical difference between any of the test groups (Table II). The levels of renal NAG observed in mice treated with gentamicin were dose-related (Table III). At 1 week, in the 100mg gentamicin/kg body wt group, the heat-stable form of NAG increased 5-fold, whilst the total activity doubled: a similar but less marked effect was found in the 50mg gentamicin/kg body wt group. The levels of both total and heat-stable NAG activity were lower in both groups after 10 days of gentamicin treatment, although still significantly raised.

Microscopy

Light microscopy showed little difference between control and treatment groups, although small dense granules corresponding to myeloid figures were observed in proximal tubular cells of all treatment groups. No difference was noted histochemically between the groups. Electron microscopy revealed generalized abnormalities in all treatment groups, though to varying degrees; additional focal changes were found only in animals injected with gentamicin alone. Both types of change affected only proximal tubules.

In the gentamicin-only group, the proximal tubular cells contained myeloid bodies, usually 6–10, but up to 20, in number (Fig. 3). Occasionally in large cytosegresomes, the myeloid bodies were most prominent level with or above the nucleus and they tended to increase in size towards the luminal surface of the cell. In many tubules myeloid bodies

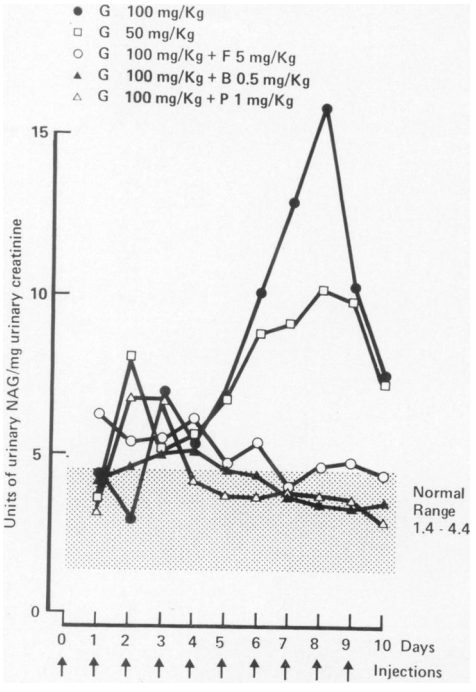


FIG. 2.—Urinary NAG excretion from 18 to 24 h during 10-day treatment with gentamicin alone or in combination with each of 3 diuretics. G = gentamicin; F = frusemide; B = bumetanide; P = piretanide.

appeared to be passing into intervillous spaces (Fig. 4). Focal changes were variable but included some mitochondria denser than normal and with loss of matrix granules, and others which were swollen and electronlucent. Affected cells also showed increased ribosomal material and prominent Golgi apparatus. More notably altered cells showed additional cytoplasmic oedema and increased lipid droplets, and some cells were completely necrotic. Although these focal changes were more common in animals killed at 7 rather than 10 days, the general minor cytoplasmic changes were present at both these times to the same extent.

The changes in the gentamicin–diuretic combination groups were less severe than those in animals given only gentamicin. Myeloid figures were common though not invariably present in proximal tubular cells, but smaller and fewer in number (up to 7): the focal severe cellular changes were never present.

DISCUSSION

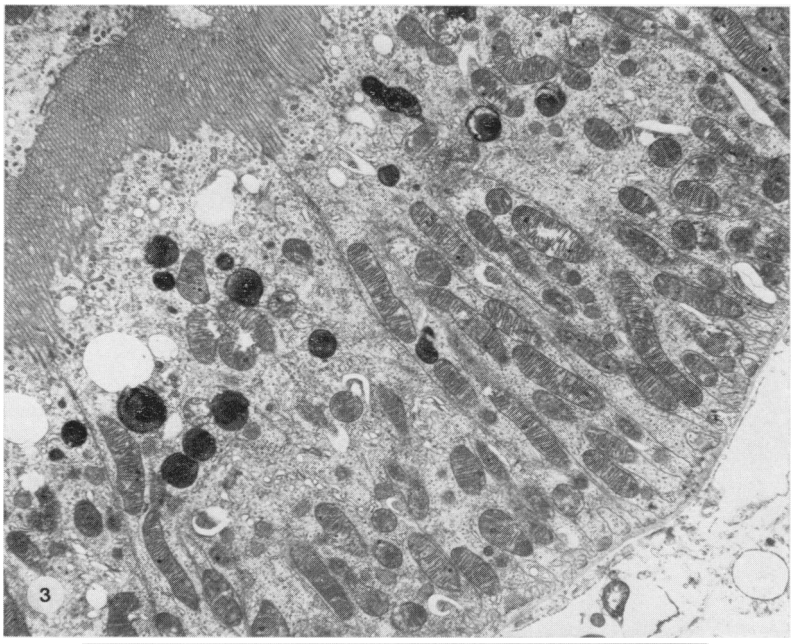
This study has shown gentamicin to be nephrotoxic in mice, with similar effects to those seen in the rat (Kosek *et al.*, 1974; Wellwood *et al.*, 1976; Houghton *et al.*, 1976). The toxic effects are centred on the proximal tubular cells which show the generalized formation of numerous lysosomal cytosegresomes and myeloid bodies, together with focal cellular necrosis. The ultrastructural changes are accompanied by increased urinary excretion of NAG, a recognized marker of tubular damage (Wellwood *et al.*, 1973). The observation that the degree of NAG enzymuria is related both to the dose of gentamicin and to the duration of treatment has been noted previously (Luft *et al.*, 1975; Wellwood *et al.*, 1976).

The concomitant administration of each of the 3 loop diuretics did not alter the magnitude of NAG enzymuria in the 6 h following gentamicin. However, the administration of diuretics did abolish the gentamicin-associated rise in NAG enzymuria in the 18–24h urine samples. Structural damage was still present in the

TABLE III.—*Effect of increasing gentamicin dosage on renal levels of NaG*

	Treatment for 7 days			Treatment for 10 days		
Gentamicin dose (mg/kg body wt)	Total NAG activity		Heat-stable NAG activity	Total NAG activity		Heat-stable NAG activity
0	1553 ± 199	(6)	241 ± 106	1686 ± 420	(4)	359 ± 53
50	2451 ± 586	(6)	608 ± 91	3230 ± 210	(4)	607 ± 24
100	3375 ± 380	(6)	1096 ± 180	2344 ± 176	(4)	643 ± 62

Enzyme activities are expressed as μmoles 4 MU liberated/h/g/wet wt and the results shown as mean ± s.d. The number of determinations is given in parentheses.



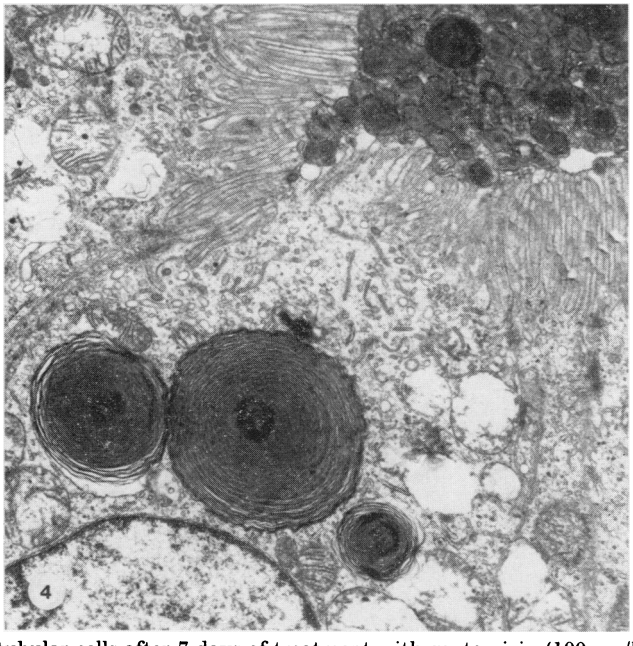


FIG. 3.—Proximal tubular cells after 7 days of treatment with gentamicin (100 mg/kg body wt). The apical portions of the cells contain numerous myeloid bodies. EM × 6000.

FIG. 4.—Proximal tubular cells after 10 days of treatment with gentamicin (100 mg/kg body wt). Three large myeloid bodies surmount the nucleus (lower left) of one cell and the intervillous space (top right) is packed with extruded myeloid bodies. EM × 11,500.

diuretic-combination groups, but was less severe than that seen after gentamicin alone. In particular, focal severe cellular damage and necrosis were only noted in the gentamicin-alone group, suggesting that these were responsible for the elevated enzymuria in the 18–24h urine samples, the generalized minor cell damage being responsible for the early enzymuria observed from 0 to 6 h in all test groups.

This finding of protection from gentamicin nephrotoxicity by concomitant diuretic administration is at variance with other accounts of combination therapy (Falco *et al.*, 1969; Wilbert *et al.*, 1971; James *et al.*, 1975; Kahn, 1977; Noël and Levy, 1978; Adelman *et al.*, 1979) and the reasons for this are not clear. Combination therapy with each of the diuretics did not prevent the increase in renal NAG levels; with the generalized structural changes present, this suggests that some gentamicin still entered the tubular cells. The sequential increase in the heat-stable NAG levels, an estimate of the β isoenzyme, with increasing dose indicates that lysosomal enzyme induction occurs as a consequence of gentamicin administration. There is already evidence that gentamicin can induce lysosomal enzymes in the proximal tubular cells of the rat kidney (Eudy and Burrows, 1972).

Although the precise mechanism of gentamicin nephrotoxicity remains unclear, the increased urine flow rates demonstrated in the present study may be responsible for the reduction in nephrotoxicity observed; this has also been suggested for the protection by frusemide in mercuric chloride-induced renal failure in the rat (Thiel *et al.*, 1976). However, the degree of hydration, rather than the diuretic, may be a more important factor in potentiating gentamicin nephrotoxicity (Chiu and Long, 1978).

The amount of gentamicin required to produce nephrotoxicity is species-dependent. At 100 mg/kg body wt, the structural changes present in the mouse are equivalent to those produced in the rat given 5 mg/kg (Wellwood *et al.*, 1976). In the present study, gentamicin was given by a single daily i.p. injection, a mode of administration shown to be less toxic than multiple injections of the same total daily dosage (Bennet *et al.*, 1979). Since all previous reports of the potentiation of gentamicin nephrotoxicity by frusemide have been made in animals relatively more sensitive to gentamicin, it may be that the protective effect of diuretics demonstrated in the present study reflects the rather minor renal damage produced in the mouse by the dosage levels employed.

This work was presented in part to the European Dialysis and Transplantation Association in Amsterdam, June 1979. We would like to thank the staff of the University Animal Department for the care of the experimental animals, the staff of the Pathology EM Unit for skilled technical assistance and Mrs A. Jack for typing the manuscript.

REFERENCES

ABRAMOWICZ, M. & EDELMANN, C. M. (1968) Nephrotoxicity of Anti-Infective Drugs. *Clin. Pediat.*, **7**, 389.

ADELMAN, R. D., SPANGLER, W. L., BEASOM, F., ISHIZAKI, G. & CONZELMAN, G. M. (1979) Furosemide Enhancement of Experimental Gentamicin Nephrotoxicity: Comparison of Functional and Morphological Changes with Actitivies of Urinary Enzymes. *J. Infect. Dis.*, **140**, 342.

BENNETT, W. M., PLAMP, C. E., GILBERT, D. N., PARKER, R. A. & PORTER, G. A. (1979) The Influence of Dosage Regimen on Experimental Gentamicin Nephrotoxicity: Dissociation of Peak Serum Levels from Renal Failure. *J. Infect. Dis.*, **140**, 576.

BERGAN, T., BRODWALL, E. K., WESTILE, L. & OYRI, A. (1973) Renal Extraction of Gentamicin and Effect of Probenecid. *Acta path. microbiol. scand.*, **241** (Suppl. Section B81), 95.

CHIU, P. J. S., BROWN, A., MILLER, G. & LONG, J. F. (1976) Renal Extraction of Gentamicin in Anesthetized Dogs. *Antimicrob. Agents Chemother.*, **10**, 277.

CHIU, P. J. S. & LONG, J. F. (1978) Effects of Hydration on Gentamicin Excretion and Renal Accumulation in Forusemide-treated Rats. *Antimicrob. Agents Chemother.*, **14**, 214.

EUDY, W. W. & BURROWS, S. E. (1972) Effect of Aminoglycosides on Renal Lysozyme in Rats. *Biochem. Med.*, **6**, 419.

FALCO, F. G., SMITH, H. M. & ARCIERI, G. M. (1969) Nephrotoxicity of Aminoglycosides and Gentamicin. *J. infect. Dis.*, **119**, 406.

HOUGHTON, D. C., HARTNETT, M., CAMPBELL-BOSWELL, M., PORTER, G. & BENNETT, W. (1976) A Light and Electron Microscopic Analysis of

Gentamicin Nephrotoxicity in Rats. *Am. J. Path.*, **82,** 589.

JAMES, G., SMITH, W., BRYANT, H. & BALAZS, T. (1975) Enhancement of Antibiotic Nephrotoxicity by Furosemide: Studies in Mice. *Toxicol. Appl. Pharmacol.*, **33,** 199.

KAHN, T. (1977) Effect of Furosemide (F) on Gentamicin (G) and Netilmycin Nephrotoxicity. *Kidney Inter.*, **12,** 527.

KOSEK, J. C., MAZZE, R. I. & COUSINS, M. J. (1974) Nephrotoxicity of Gentamicin. *Lab. Invest.*, **30,** 48.

LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. & RANDALL, R. J. (1951) Protein Measurement with the Folin-phenol Reagent. *J. Biol. Chem.*, **193,** 265.

LUFT, F. C., PATEL, V., YUM, M. N., PATEL, B. & KLEIT, S. A. (1975) Experimental Aminoglycoside Nephrotoxicity. *J. Lab. clin. Med.*, **86,** 213.

NOËL, P. & LEVY, V.-G. (1978) Toxicité Rénale de l'Association Gentamicine-Furosémide. Une Observation. *Nouv. Presse Méd.*, **7,** 351.

THIEL, G., BRUNNER, F., WUNDERLICH, P., HUGUENIN, M., BIENKO, B., TORHORST, J., PETERS-HAEFALI, L., KIRCHERTZ, E. J. & PETERS, G. (1976) Protection of Rat Kidneys against $HgCl_2$-induced Acute Renal Failure by Induction of High Urine Flow without Renin Suppression. *Kidney Intern.*, **10,** S-191.

WELLWOOD, J. M., ELLIS, B. G., HALL, J. H., ROBINSON, D. R. & THOMPSON, A. E. (1973) Early Warnings of Rejection? *Brit. med. J.*, **2,** 261.

WELLWOOD, J. M., LOVELL, D., THOMPSON, A. E. & TIGHE, J. R. (1976) Renal Damage Caused by Gentamicin: A Study of the Effects on Renal Morphology and Urinary Enzyme Excretion. *J. Path.*, **118,** 171.

WHITING, P. H., ROSS, I. S. & BORTHWICK, L. (1979) Serum and Urine *N*-Acetyl-β-D-Glucosaminidase in Diabetics on Diagnosis and Subsequent Treatment, and Stable Insulin-dependent Diabetics. *Clin. chim. Acta*, **92,** 459.

WILBERT, J. N., BURKE, J. P., BLOOMER, H. A. & SMITH, C. B. (1971) Renal Insufficiency Associated with Gentamicin Therapy. *J. infect. Dis.*, **124** (Suppl.), 148.