Indian Journal of Clinical Biochemistry 1991, **6**, 17–25

STUDY OF SOME FACTORS AFFECTING STABILITY OF N-ACETYL-β-D-GLUCOSAMINIDASE AND AMINOPEPTIDASE N IN URINE AT 37° C

K. SRI KRISHNA and A. S. KANAGASABAPATHY*
Department of Clinical Biochemistry
Christian Medical College and Hospital
VELLORE – 632 004, INDIA

Received on March 21, 1990; Revised on June 13, 1990

ABSTRACT

Little is known of conditions which influence the stability of urinary enzymes upon storage in the bladder at 37°C. Using a continuous flow system simulating *in vivo* conditions, we studied the influence of the pH of urine on the stability of two renal parenchymal enzymes N-Acetyl-β-D-Glucosaminidase (2-acetamido-2-deoxy-β-D-glucoside acetamidodeoxy glucohydrolase, NAG, EC 3.2.1.30) and L-Alanine aminopeptidase (Aminopeptidase N, AAP, EC 3.4.11.2). This continuous flow model that we have described can be employed to study the influence of pH on the stability of any renal enzyme excreted in urine. We also studied the *in vitro* effects of varying concentrations of low molecular weight regulatory metabolites such as urea, creatinine and uric acid and of some drugs excreted in urine, on the assay of these two enzymes. Urinary pH, urea content and some antibiotics seem to influence measured urinary NAG and AAP activities and we therefore express the need for caution before diagnostic interpretation of the urinary enzyme activities are made.

INTRODUCTION

Many enzymes occur in normal and pathological urines. Most urinary enzymes arise from renal cellular breakdown (1). Determinations of enzymatic activities in urine that are derived from kidney parenchyma (e.g. Lactic dehydrogenase EC 1.1.1.27, alkaline phosphatase EC 3.1.3.1, N-acetyl-β-D-Glucosaminidase EC 3.2.1.30, and aminopeptidases EC 3.4.11) or epithelial cells of the urinary tract (e.g. β-Glucuronidase EC 3.2.1.31) have been explored in the search for possible tools for the diagnosis and prognosis of urorenal disease (2). Urinary lysosomal glycosidases and peptidases have also been shown to be valuable in the prediction of acute renal allograft rejection and chronic graft outcome (3–9). In clinical pharmacology, measurement of urinary enzyme activities could help screen functional or lesional damage to kidneys by nephrotoxic drugs or to determine the relative nephrotoxic effect of a new drug compared to a standard one.

* To whom Correspondence should be addressed.

Furthermore, the assay of certain enzymes in urine may be helpful to detect inborn errors of metabolism e.g. assay of arylsulphatase A in metachromatic leukodystrophy. In principle the diagnostic value of the determinations of enzymes in urine is to be accepted, but a number of problems remain to be solved before measurement of urinary enzyme activities can be extensively employed for diagnostic purposes. The peculiar problems involved in the determination of enzyme activities in urine arise from the pH of urine which is not always conducive to the preservation of activities of some enzymes, the low protein content and the presence of high concentrations of urea, cells and possible inhibitors. Enzymes are stored at 37°C in the urinary bladder under these possibly unfavourable conditions for varying lengths of time before urine is voided, therefore possibilities of at least certain degree of inactivation of enzymes may exist. Consequently to assess the diagnostic value of any kidney parenchymal enzyme excreted in urine one has to look into the factors responsible for and extent of inactivation of the enzyme in urine upon storage in the bladder at 37°C. Reports available in literature on the subject are scanty (10, 11).

Enzymes are continuously added into urine by a constant desquamation of renal tubular cells and brush border membranes and by the continuous process of exocytosis that occurs in cells of the proximal convoluted tubule in normal as well as pathologic conditions (12,13). Therefore, we propose here an experimental model to investigate the effect of pH on the stability of two enzymes from renal tubular epithelial cells, N−acetyl−β−D−Glucosaminidase and aminopeptidase N which are useful diagnostic and prognostic markers of renal allograft rejection and ultimate graft outcome (8), however the model can be used for the study of stability in urine of other enzymes of renal origin as well.

We have also studied the *in vitro* effects of varying concentrations of urea, creatinine and uric acid on the assay of these two enzymes.

Certain drugs are known to be nephrotoxic; primary drug induced acute tubular necrosis leads to increased activities of enzymes in urine. Drugs like salicylates also cause increased desquamation of tubular cells (15). However, drugs excreted in urine can by themselves have an influence on enzyme assays, either activation or inhibition, and it is therefore pertinent to study the possible effects of varying concentrations of drugs on urinary enzyme activities *in vitro*. Knowledge on the influence of drugs on enzyme assays is lacking. We have attempted to evaluate the influence of a spectrum of drugs, used especially on renal transplant patients, on the assay of urinary NAG and AAP.

MATERIALS AND METHODS

Apparatus: Cecil CE 202 (U.K.) UV−Visible spectrophotometer; proportionating pump III from Technicon Corporation (NY, USA); Gallenkamp (U.K.) water baths; Mixing coils and flow rated pump tubing from Elkay Products (MA, USA); Clinipette pipettes from Boehringer Mannheim GmbH (West Germany).

Reagents: Alanine 4−nitroanilide and 4−nitrophenyl N−acetyl−β−D−Glucosaminide were obtained from Sigma Chemical Co., MO, USA. All other reagents were of high analytical grade.

Experimental materials: A pair of normal human kidneys were reomoved at autopsy within 12 hours after death and stored at −20°C, until use. Kidneys were also removed from bonnet monkeys (Macaca radiata) under pentabarbitone anaesthesia. Renal cortices were dissected out and homogenised at 4°C in 150 m mol/l NaCl (10 ml/gm tissue). A 4000×g supernatant served as the source of enzymes for the experiments. Pooled urines consisting of equal volumes of urine collected from healthy individuals were heat inactivated and aliquots were adjusted to pH values ranging from 4.0−9.0 at 0.5 pH intervals using Britton and Welford buffers (16).

METHODS

Enzyme assays: NAG and AAP were assayed as previously described (8, 14).

a) *Effect of pH:* The overall Continuous flow assembly system was piloted by a peristaltic pump. Calibrated manifold tubings of different diameters were used to produce different flow rates between the urine aliquots adjusted to different pH and the renal cortical homogenate kept in an ice bath. The rate of flow of the mixtures were adjusted to about 1.4 ml/min similar to the normal physiological rate of flow of urine (homogenate to urine volume ratio was 1:7.5). The two streams were joined by h3 connectors and mixing coils (kept at 37°C) and the mixtures were allowed to pass continuously into containers (that served as urinary bladders) also kept at 37°C. Aliquots of urine/homogenate mixutres were drawn immediately after mixing and at intervals of 1 hr to check for enzyme activities, over a period of 8 hours.

TABLE 1: *Drugs used in the study for their effects on in vitro NAG and AAP assays*

I.	**ANTIBIOTICS**	V.	**TUBERCULOSTATIC AGENTS**
	1) Cephalexin (12.5−1000)		1) Ethambutol (0.9−75)
	2) Cloxacillin (3.0− 250)		2) Isoniazid (0.375−30)
	3) Colistin (0.6− 50)		
	4) Doxycycline (2.5− 200)	VI.	**DIURETICS**
	5) Chloramphenicol (15.0−1250)		Furosemide (6.25−500)
	6) Gentamycin (0.1− 8.0)		
	7) NegGram (12.5−1000)	VII.	**BRONCHODILATORS**
II.	**URINARY ANTIBACTERIALS**		Salbutamol (0.05−4)
	1) Mandelamine (6.25− 500)	VIII.	**β-ADRENERGIC RECEPTOR BLOCKING AGENTS**
	2) Nitrofurantoin (1.25− 100)		Inderal (0.5−40)
	3) Septran (0.5− 40)		
III.	**ANTIMALARIALS**	IX.	**IMMUNOSUPPRESSIVES**
	Chloroquine (1.5− 125)		Prednisolone (0.06−5)
IV.	**HISTAMINE H_2 RECEPTOR ANTAGONISTS**		
	Cimetidine (7.5−600)		

Drug concentrarions in parentheses are in μg/ml assay volume.

b) *Effects of low molecular weight substances:* Renal cortical homogenates were incubated with heat inactivated normal urines (dialysed against water to remove low mw substances) to which urea was added such that the final concentration in the assay volume ranged from 10−500 m mol/l. The experiments were repeated with 5−20 m mol/l of creatinine and 2−5 m mol/l of uric acid. Enzyme activities measured before the addition of urea, creatinine and uric acid to the assay mixtures were considered 100% for each.

c) *Effects of drugs:* The effects of some drugs shown in Table 1, which are excreted in urine, on enzyme assays were assessed by incubating renal cortical homogenates in the absence and presence of varying concentrations (therapeutic to toxic levels) of each drug. Concentrations of drugs used in the assay mixtures were calculated based on the dosage and renal excretion rates of each drug (17).

RESULTS

I. *Time dependent inactivation of enzymes in urine at 37°C as a function of urinary pH:*

a) *AAP:* The time course inactivation of AAP at various pHs at 37°C, using the continuous flow system is shown in Table 2. AAP showed high degree of stability from pH 6.0−9.0. At pHs 4.5 and 5.5 the loss in activity was only 21% and 14% respectively. At pH 4.0 there was a gradual fall in activity over the course of time with about 55% activity remaining at the end of 8 hours.

TABLE 2: *Inactivation (immediate and time-course) of AAP at 37°C in urine at varying pHs*

TIME (Hours)	pH range										
	4.0	4.5	5.0	5.5	6.0	6.5	7.0	7.5	8.0	8.5	9.0
	% Original Activity Remaining *										
0	90 (1.10)	95 (2.60)	96 (3.18)	96 (1.40)	96 (0.57)	97 (0.57)	98 (1.10)	100 (2.30)	96 (1.70)	98 (2.30)	93 (2.00)
2	80 (1.20)	83 (1.67)	86 (1.33)	90 (1.10)	94 (1.27)	97 (1.38)	98 (1.20)	98 (1.80)	98 (1.80)	97 (2.08)	91 (0.70)
4	64 (2.25)	78 (1.67)	85 (3.10)	91 (2.40)	94 (2.25)	95 (1.67)	95 (1.33)	100 (2.37)	99 (1.67)	96 (0.70)	92 (1.27)
6	60 (3.90)	80 (2.20)	84 (2.37)	89 (2.42)	93 (1.90)	98 (1.20)	98 (1.73)	99 (0.70)	99 (0.63)	96 (2.19)	91 (1.33)
8	54 (2.42)	79 (0.75)	83 (1.27)	86 (2.42)	94 (1.20)	97 (0.70)	98 (2.25)	99 (1.20)	99 (1.80)	95 (0.70)	90 (3.06)
After 48 hours at 4°C	32 (4.85)	70 (3.05)	80 (2.40)	85 (2.80)	92 (1.30)	96 (1.20)	97 (0.70)	98 (0.50)	96 (1.60)	94 (1.30)	92 (1.10)

* Each value is the mean of three experiments, SEM is given in parenthesis.
Original activity was measured at pH 7.8.

b) *NAG:* Table 3 shows the time course inactivation of NAG at various pHs, using the continuous flow system. In this system, in a pH range of 5.0−7.5 NAG showed good stability with more than 90% of activity being retained. With increasing acid and alkaline pHs the loss in activity was only 15 to 20% and 15 to 48% respectively.

The homogenate/urine mixtures were stored at 4°C for two days and the enzyme activities, along with pH, were measured each day. Table 2 and 3 indicate percentage activity remaining on each day at different pHs for both NAG and AAP. A pH range of 5.5 to 7.5 and 6.0 to 9.0 for NAG and AAP respectively were found to be most conducive for retention of maximum enzyme activity.

TABLE 3: *Inactivation (immediate and time-course) of NAG at 37°C in urine at varying pHs*

TIME (Hours)	pH range										
	4.0	4.5	5.0	5.5	6.0	6.5	7.0	7.5	8.0	8.5	9.0
	% Original Activity Remaining *										
0	80 (1.85)	92 (1.21)	98 (3.10)	98 (2.80)	100 (4.2)	95 (2.50)	95 (3.40)	96 (3.90)	91 (1.10)	84 (1.70)	84 (0.70)
2	80 (1.31)	86 (1.84)	93 (1.80)	96 (2.25)	100 (2.40)	96 (3.20)	93 (1.60)	90 (2.77)	89 (2.48)	74 (3.58)	71 (2.19)
4	79 (1.27)	85 (0.75)	93 (1.10)	97 (1.20)	100 (1.60)	100 (0.98)	94 (1.10)	92 (0.50)	88 (0.70)	73 (1.85)	70 (1.67)
6	78 (0.70)	83 (1.04)	93 (0.63)	96 (1.79)	96 (1.10)	98 (0.63)	95 (0.52)	91 (1.27)	88 (2.65)	69 (2.42)	64 (1.20)
8	76 (5.40)	83 (3.64)	93 (0.63)	97 (1.27)	96 (1.79)	98 (2.54)	95 (0.58)	91 (2.78)	84 (2.48)	62 (5.14)	52 (5.37)
After 48 hours at 4°C	73 (1.79)	84 (2.25)	91 (1.10)	97 (1.27)	97 (1.33)	98 (0.63)	96 (1.10)	92 (1.96)	84 (3.70)	60 (3.06)	50 (2.40)

* Each value is the mean of three experiments, SEM is given in parenthesis.
Original activity was measured at pH 4.3.

II. *Effect of low molecular weight metabolites on NAG and AAP activities in urine:*

Figure 1 shows the inhibition by urea of the two enzymes NAG and AAP. Urea exhibited a more pronounced inhibition of NAG than AAP. A 10% inhibition of NAG was observed at an urea concentration of 50 m mol/l to nearly 40% inhibition at 500 m mol/l. At a concentration of 300 m mol/l AAP was inhibited upto 8%. These inhibitions were pH independent.

Other normal constituents of urine such as creatinine and uric acid were found not to be adversary to the *in vitro* assays of these two enzymes (data not shown).

III. *In vitro effects of drugs on urinary enzyme activities:* Out of the 18 drugs studied only 5 showed inhibitory action on either of the two enzymes (Figures 2a and 2b). There was a gradual fall in AAP activity in presence of increasing concentrations of cloxacillin, with only 33% of activity remaining at drug concentration of 250 μg/ml, while the effect

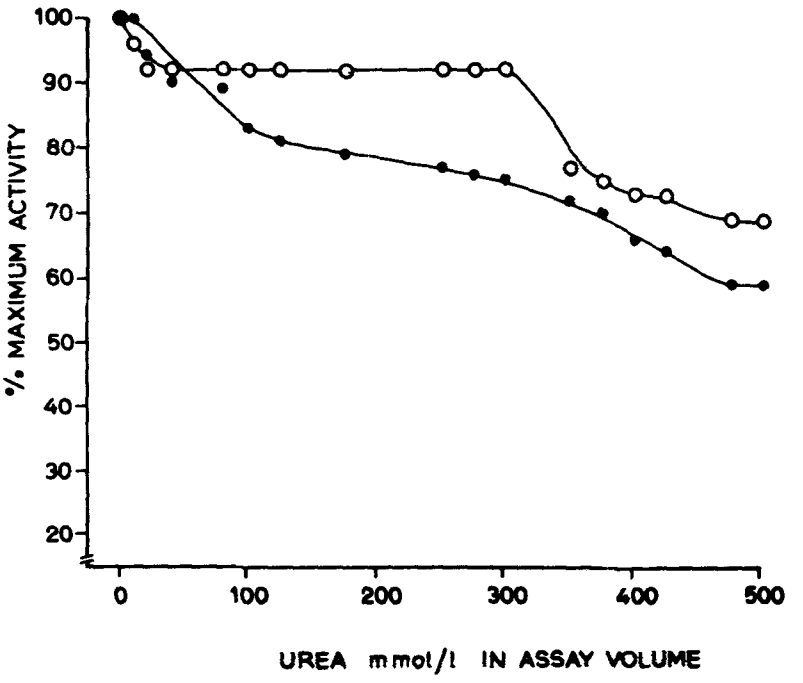
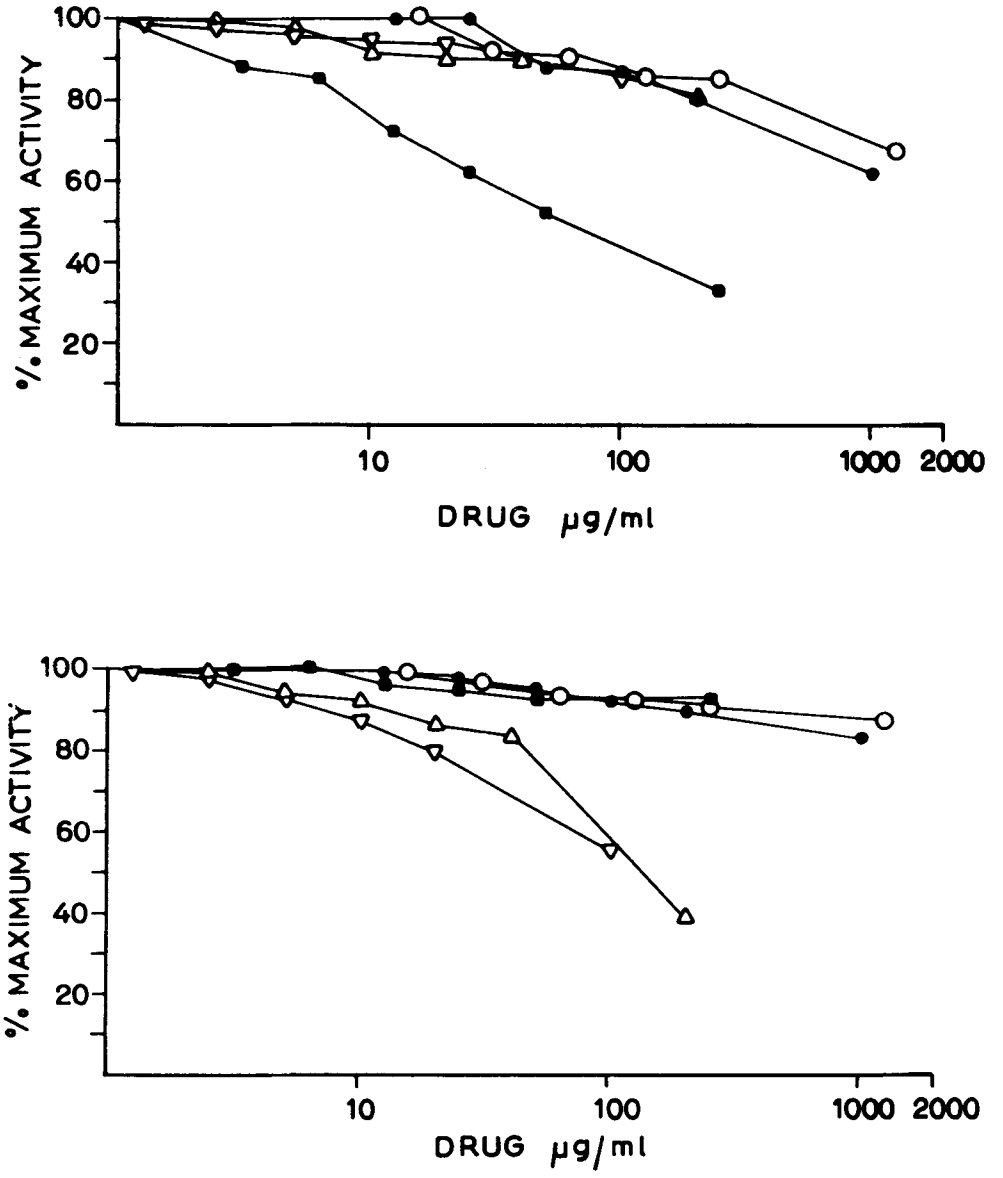


Figure 1: *Effect of increasing concentrations (m mol/l) of urea on the assay of urinary NAG* (●) and AAP (O). Each point on the graph represents the mean of three experiments.

of the drug on NAG activity was negligible. Only 39% of NAG activity was retained in presence of 200 μg/ml of doxycycline while at this concentration of the drug 81% of AAP activity was retained. Nitrofurantoin at a concentration of 100 μg/ml reduced NAG activity to 55% and AAP activity to only 86%. 62% of AAP and 83% of NAG activities remained in presence of 1000 μg/ml of cephalexin. Chloroquine at a concentration of 125 μg/ml reduced AAP activity to 67% while it had no effect on NAG activity.

DISCUSSION

Only little is known of conditions which influence the stability of urinary enzymes *in vivo*, i.e. after release from tubular cells upto voiding from the urinary bladder. For determination of reference values for urinary enzyme activities and for proper clinical interpretation it is therefore necessary to consider various factors that can affect the stability of enzymes at 37°C in the bladder. Using a continuous flow assembly that represented the functioning of the biological system more precisely than the static model employed by Jung *et al* (11), we have monitored the stability of two renal tubular enzymes NAG and AAP under varying pHs. We have compared our results with the static model of Jung *et al*. Further, the effects, on the assay of these two enzymes, of urea, and other regulatory metabolities and a spectrum of drugs that are excreted in urine, have also been studied.



Figures 2a and 2b: *Semilogarithmic plots of the effects of increasing concentrations* (µg/ml) of some drugs excreted in urine on the assay of urinary AAP (2a) and NAG (2b). Each point represents the mean of three experiments.

■ Cloxacillin
O Cephalexin
Δ Doxycycline
● Chloroquine
∇ Nitrofurantoin

Our results indicate that AAP was most stable at pH 6.0−9.0. A fall in activity was seen at lower pH range, but the fall was more gradual and less compared to what was observed with the static model. NAG retained more than 90% of the activity over a pH range of 5.0−7.5. At lower and higher pHs the loss in activity was lower compared to the static system. Thus a pH range of 6.0−8.0 appears to be most conducive for retention of maximum NAG and AAP activities. At extreme pHs the enzymes are not as pronouncely affected as has been made out by the static model. Since the pH of most urines ranges from 6.0−8.0, enzyme activities measured under standardised *in vitro* conditions should represent maximal capacities, even after storage of samples at 4°C for 2 to 3 days.

Determination of pH of freshly voided urines thus appears to be imperative and when pH values are outside the range of 6.0−8.0 either corrections should be applied or samples be best excluded from assay.

Urea significantly inhibited NAG activity above concentrations of 50 m mol/l, while AAP activity was unaffected at urea concentrations below 300 m mol/l. The inhibitions were pH independent. Since the normal excretion of urea is 100 to 500 m mol/l, the question that would arise would be whether NAG activities normally measured in urine are not maximal. In the first place, concentrations of urea at which enzymes are stored in bladder are never high enough to cause inactivation of enzymes; rather urea is only a competitive inhibitor of NAG and its inhibition is reversible. Hence, prior treatment of urines such as dialysis or gel filtration should remove inhibition by urea. In the spectrophotometric assay of NAG employed by us (14) the urine is diluted 10 fold in the final assay mixture, which reduces the urea concentration to about 50 m mol/l at the most, inhibition by urea is minimised and the need for prior treatment is circumvented. In the assay of AAP (8) sample gets diluted 6 fold and at final concentrations of lessthan 100 m mol/l urea, AAP is not affected. Moreover since these enzymes are most useful in the early diagnosis of rejection episodes after renal transplantation, and in any rejection episode the amount of urea excreted is certainly lower than what is excreted normally, possibility of measuring less than maximal enzyme activities is thus alleviated.

It is evident that some antibiotics excreted in urine inhibit NAG and AAP activities, the effect on AAP being more than on NAG. Chemical structures of these compounds show that they all contain amide groups (as in urea) which may inhibit the hydrolysis of the enzyme substrates. The clinician should therefore always be aware of the possible influence of these interfering factors while interpreting urinary enzyme values.

ACKNOWLEDGEMENT

This work (which forms part of Ph. D. thesis of Dr. K. Sri Krishna) was supported by a grant from the Christian Medical College Fluid Research Fund. We thank Dr. Anand Date for help in obtaining human autopsy material.

REFERENCES

1. WILKINSON. J.H. (1968) Enzymes in urine and kidney. DUBACH. UC (ed). VERLAG HANS HUBER. BERNE. P 207–227.
2. RAAB. W.P. (1972) Clin. Chem. **18,** 5–25.
3. WELLWOOD. J.M., ELLIS. B.G., HALL. J.H., ROBINSON. D.R., and THOMPSON. A.E. (1973) Br. Med. J. **2,** 261–267.
4. WELLWOOD. J.M., DAVIS. D., LEIGHTON. M., and THOMPSON. A.E. (1978) Transplantation **26,** 396–400.
5. WHITING. P.H., NICHOLLS. A.J., CATTO. G.R.D., EDWARD. N., and ENGLISH. J. (1980) Clin. Chim. Acta, **108,** 1–7.
6. CORBETT. R., GARDNER. G.J., KIND. P.R.N., THOMPSON. A.E., and PRICE. R.G. (1983) Clin. Chim. Acta, **128,** 141–150.
7. WHITING. P.H., PETERSON. J., POWER. D.A., STEWART R.D.M., CATTO. G.R.D., and EDWARD. N. (1983) Clin. Chim. Acta **130,** 369–376.
8. Sri KRISHNA. K., KIRUBAKARAN. M.G., PANDEY. A.P., and KANAGASABAPATHY. A.S. (1985) Clin. Chim. Acta, **150,** 69–85.
9. Sri KRISHNA. K., KIRUBAKARAN. M.G. and KANAGASABAPATHY. A.S. (1986) Clin. Chem. **32,** 201.
10. JOSCH. W., DUBACH. U.C., and STROBEL. M. (1967) Experientia **23,** 342–343.
11. JUNG. K., PERGANDE. M., SCHREIBER G., and SCHRODER. K. (1983) Clin. Chim. Acta. **131,** 185–191.
12. MAUNSBACH. A.B. (1983) in Handbook of Physiology. Section 8. Renal physiology. ORLOFF. J. and BERLINER. R.W. (Eds). Williams and Wilkins Co., Baltimore, **PP** 31–80.
13. TISHER. C. C. (1986) 'Anatomy of the Kidney' in the Kidney Vol 1, BRENNER. B.M. and RECTOR. F.C. (Eds). W.B. SAUNDERS. Philadelphia, **PP** 3–60.
14. Sri KRISHNA. K., SHASTRY. J.C.M., CHERIAN. A.M. and KANAGASABAPATHY A.S. (1985) Asian J. Cli. Sci. **5,** 61–65.
15. DUBACH U.C., (1979) 'Enzyme Tests in Urorenal Disease' in Advances in Clinical Enzymology. SCHMIDT. E., SCHMIDT. F.W., TRAUTSCHOLD. I., and FRIEDEL. R. (Eds). S. KARGER. BASEL. PP 149.
16. VARLEY. H., GOWENLOCK. A.H., and BELL. M. (Eds) (1980) Practical Clinical Biochemistry, Vol 1: General Topics and Commoner Tests. William Heinemann. London, PP 1226.
17. Compendium of Pharmaceuticals and Specialities. Published by Canadian Pharmaceutical Association (1982) Seventeenth ed. **PP** 41–615.