Biochemical Pharmacology, Vol. 25, pp. 913–918. Pergamon Press, 1976. Printed in Great Britain.

γ-GLUTAMYL TRANSPEPTIDASE IN THE URINE FROM AN ISOLATED RABBIT KIDNEY PERFUSED WITH AND WITHOUT DMSO

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(*Received* 26 *April* 1975; *accepted* 8 *August* 1975)

Abstract—γ-Glutamyl transpeptidase activity has been determined in the urine from isolated perfused rabbit kidneys. Kidneys were perfused for 60 min at 37° with a cell-free salt solution, and with the same solution containing 1·4, 2·1 or 2·8 M dimethylsulfoxide (DMSO). The enzyme is relatively stable when stored at 4° or at −20°, and the addition of DMSO to urine samples had no appreciable effect on enzyme stability. Analysis of urinary γ-glutamyl transpeptidase activity demonstrated time-dependent release of this enzyme by the isolated rabbit kidney perfused with the cell-free salt solution. The addition of 1·4 and 2·8 M DMSO to the solution led to significant increases in the level of transpeptidase found in the urine. There was no significant correlation between urinary enzyme levels and urine flow rates in the presence or absence of DMSO. This represents the first demonstration of a urinary form of this enzyme which is unequivocally derived from renal tissue. Unlike natural urine, the urine derived from perfused kidneys contains mostly particulate γ-glutamyl transpeptidase. The increase in particulate urinary transpeptidase and alkaline phosphatase levels with time, coupled with the significant increases in urinary transpeptidase activity during perfusion with cytotoxic levels of DMSO, suggests that pieces of renal brush border are being broken off and are appearing in the urine.

Since the early work of Hanes *et al.* [1], a great deal of interest has been focused on the enzyme γ-glutamyl transpeptidase (γ-GT). This enzyme is found in particularly high concentrations in the microsomal fraction of the renal cortex [2–4], and has also been used as an enzyme marker for the brush border [5,6]. Orlowski and Meister [7] have suggested that γ-GT may play a role in a cyclic series of enzymatic events which mediate amino acid transport. More recently, it has been proposed that γ-GT may function in an opposite direction, e.g. this enzyme may be involved in the secretion of substances from the cell [8,9].

Determination of serum levels of γ-GT has gained clinical significance as a result of the correlation between elevated serum transpeptidase activity and the involvement of both hepatic cancer [10–12] and alcoholic hepatic injury [13,14]. Urinary γ-GT levels have received much less attention, since there appears to be no correlation between urinary enzyme activity and liver damage. Recently, several investigators [15–17] have demonstrated that urinary γ-GT levels can be used as an indicator of destructive renal disease. These results are consistent with the observations of Szewczuk [4], who suggested that serum γ-GT comes from the liver, while urinary γ-GT originates in the kidney.

Previous reports from this laboratory have shown that kidney perfusion causes progressive functional and ultrastructural deterioration with time, resulting in tubular necrosis and severe glutathione depletion after 2 hr [18–20]. In view of these findings, it was of interest to determine if perfusion of the isolated rabbit kidney leads to increased levels of urinary γ-glutamyl transpeptidase with time. One method developed to determine the viability of an isolated organ has been the measurement of intracellular enzymes, such as lactate dehydrogenase, in the venous effluent and urine of stored organs [21–23]. The appearance of intracellular enzymes in either of these fluids is taken to indicate damage to epithelial cell membranes. Although it is well known that the kidney contains high concentrations of γ-glutamyl transpeptidase, the majority of which is membrane bound, there have been no reports in the literature dealing with the presence of this enzyme in the urine of isolated, perfused kidneys.

The present study was undertaken to determine the levels of γ-GT in the urine of isolated rabbit kidneys, the time course of γ-GT appearance in the urine during perfusion, the influence of urine flow rate on γ-GT activity, and the effects of perfusion with the dipolar aprotic solvent dimethylsulfoxide (DMSO) on urinary γ-GT levels. During the course of this investigation, a report appeared in the literature which had a direct bearing on our results. Stokke [24] reported that γ-GT in human urine was often inactivated when stored at −20°. He also reported that the addition of DMSO prevented freeze-inactivation. Since our urine samples were routinely frozen and stored at −20° before analysis, we felt compelled to investigate the stability of the enzyme in these urine samples under these conditions. In addition, it became necessary to ascertain whether or not DMSO had an effect on enzyme stability, since the increases reported in urinary γ-GT during perfusion with DMSO might be due to this cryoprotective effect.

MATERIALS AND METHODS

New Zealand albino rabbits of both sexes were anesthetized with urethane (1·5 g/kg) by intraperitoneal injection, heparinized (1000 units/kg), and their left kidneys were exposed by a mid-ventral incision. After surgical isolation, the kidneys were removed, flushed with oxygenated perfusate at 37° by means of a syringe at a flow rate of approximately 0·5 ml/g/min, and weighed. Kidneys were then connected to the perfusion circuit previously described [19]. The perfusion protocol was as follows: after connection to the circuit, the kidney was flushed for 10 min, while the pumping rate was adjusted to produce a flow rate through the kidney of 0·9 ml/g/min. Kidneys which did not achieve this flow rate at a pressure of 70 mm Hg or below, or which did not develop a constant urine flow during this time, were rejected from the study. After this initial equilibration period, perfusion with the standard perfusate (Solution A) was continued for 10 min, after which urine and effluent perfusate samples were taken, arterial pressure was determined, and urine and perfusate flow rates were recorded. In control kidneys, perfusion with DMSO-free Solution A was continued, and sampling was then done every 10 min thereafter for a total experimental period of 60 min. Urine flow was determined by timed collection of urine over each 10 min period, and was expressed as ml/g of tissue/min. In kidneys perfused with DMSO, the perfusate reservoir was exchanged for one containing DMSO after the first 10-min clearance period, after which the kidney was perfused for an additional 50 min with the DMSO perfusate. After the perfusion, kidneys were removed from the circuit and weighed.

The perfusate used in all experiments was a K^+-Mg^{2+}-rich solution (Solution A) of the following composition (in mM): NaCl, 96·2; KCl, 40·3; $CaCl_2$, 1·7; $MgSO_4$, 12·5; $NaHCO_3$, 11·9; dextrose, 11·1. Creatinine (10 mg/liter), *para*-aminohippuric acid (PAH) (10 mg/liter), and heparin (2000 units/liter) were also added. The perfusate contained isoxsuprine (10 mg/liter), as a vasodilating agent. The solution was oxygenated with a mixture of 97% O_2 and 3% CO_2, resulting in a final pH of 7·35 and an oxygen tension of approximately 550 mm Hg. Constant ionic concentrations were maintained in DMSO-containing perfusates by replacing solvent water with DMSO. Replacement of 10, 15 and 20% of solvent water with DMSO resulted in final DMSO concentrations of 1·4, 2·1 and 2·8 respectively. All experiments were conducted at 37°.

Urine samples were routinely stored at −20° for 2–4 weeks before analysis for γ-GT activity. In subsequent studies, fresh urine was analyzed and time-dependent inactivation of γ-GT was studied. Analysis of transpeptidase activity was performed as follows. Samples contained 20 μmoles glycylglycine and 5 μmoles L-γ-glutamyl-*p*-nitroanilide in 1·8 ml Tris–HCl (0·10 M, pH 9·0); the reaction was started by the addition of 0·20 ml urine and samples were incubated at 37° for 10 min. Renal tissue levels of γ-GT were assessed in the same manner except for the addition of 0·20 ml of diluted tissue homogenate instead of urine. The reaction was terminated by the addition of 2 ml of 2 M acetic acid and the absorbance was read at 410 nm. A standard curve was prepared with *p*-nitroaniline and values are expressed as μmoles *p*-nitroaniline formed/ml of urine/min.

Alkaline phosphatase determinations were performed according to a standard procedure [25]. *p*-Nitrophenylphosphate ($5·5 \times 10^{-3}$ M) was suspended in 5×10^{-2} M glycine buffer (pH 10·5); 1·0 ml of this solution was incubated for 30 min at 37° with 0·2 ml of urine or tissue homogenate. The reaction was terminated by the addition of 10 ml of 0·02 N NaOH and the absorbance at 405 nm was determined. *p*-Nitrophenol was used to construct a standard curve and results are reported as μmoles *p*-nitrophenol formed/0·20 ml of sample/30 min.

Lactic dehydrogenase (LDH) was determined spectrophotometrically [26], and values are reported as milliunits of activity/ml of sample.

Perfused kidney urine samples used for enzymatic determinations were obtained by combining aliquots of each of the timed urine samples collected during a perfusion experiment. Human urine was collected from healthy adult males. Rabbit urine was aspirated from the bladder of the rabbit used for the perfusion experiment. The perfused kidney was always paired with the unperfused kidney from the same animal for comparison. Tissue homogenates were made in 0·10 M Tris–HCl, pH 9·0. Cortical tissue was blotted, weighed, and a 5% (w/v) homogenate was made in a tight-fitting glass homogenizer. Tissue homogenates were spun at 1000*g* for 5 min to remove whole cells. Supernatant solutions of each type of sample were obtained by centrifugation at 100,000*g* for 45 min.

Statistical analyses were performed using the Wang 600 programmable calculator and standard Wang programs. Experimental groups were compared statistically using the unpaired *t*-test, and differences were considered significant if *t* values corresponded to P (probability) values of 0·05 or less. Linear regression analysis was also utilized, with *r* values corresponding to P values of 0·05 or less being regarded as statistically significant.

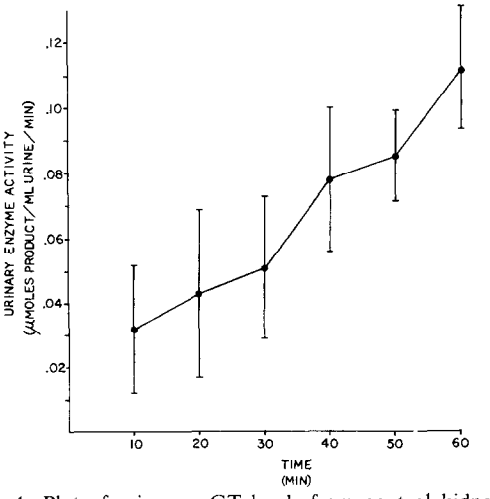

Fig. 1. Plot of urinary γ-GT levels from control kidneys vs time. Enzyme activity is expressed as μmoles *p*-nitroaniline formed/ml of urine/min. Vertical bars above and below data points represent ± S. E. M.

Table 1. Urinary levels of γ-glutamyl transpeptidase in isolated rabbit kidneys perfused with various concentrations of DMSO*

Time (min)	Urinary enzyme (μmoles product/ml urine/min) DMSO (0·0 M) (control)	DMSO (1·4 M)	DMSO (2·1 M)	DMSO (2·8 M)
10	0·032 ± 0·020	0·038 ± 0·005	0·023 ± 0·006	0·061 ± 0·025
20	0·043 ± 0·026	0·063 ± 0·009	0·065 ± 0·033	0·073 ± 0·011
30	0·051 ± 0·022	0·144 ± 0·031†	0·062 ± 0·028	0·125 ± 0·026
40	0·078 ± 0·022	0·432 ± 0·138†	0·120 ± 0·036	0·519 ± 0·123†
50	0·085 ± 0·014	0·357 ± 0·108†	0·267 ± 0·184	0·344 ± 0·093†
60	0·112 ± 0·019	0·205 ± 0·020†	0·115 ± 0·058	0·172 ± 0·030

* Values are expressed as mean ± 1 S. E. M.
† Denotes significant difference from control value.

RESULTS

Figure 1 shows the change in urinary levels of γ-glutamyl transpeptidase during the course of perfusion in the absence of DMSO. There is an obvious increase in urinary γ-GT levels throughout the course of the 60-min perfusion period. Linear regression analysis revealed a significant correlation between time and urinary levels of γ-GT ($r = 0{\cdot}56$, $P < 0{\cdot}01$). Urinary enzyme levels in control kidneys at the beginning of perfusion varied considerably, but the increases with time appeared linear in all of the samples studied ($n = 5$).

Perfusion with DMSO resulted in significant changes in urinary levels of γ-GT, when compared with controls, as shown in Table 1. Previous studies have shown that during perfusion with DMSO, this agent appears in the urine at approximately the same concentration as that found in the perfusate after 30 min [27]. DMSO (1·4 M) produced the most consistent changes in urinary enzyme levels and resulted in significantly elevated levels after 30, 40, 50 and 60 min. Perfusion with 2·1 M DMSO led to smaller, non-significant increases in urinary enzyme levels, while 2·8 M DMSO produced significant increases in γ-GT levels after 40 and 50 min of perfusion. Urinary enzyme levels were significantly correlated with time in kidneys perfused with both 1·4 M DMSO ($r = 0{\cdot}47$, $P < 0{\cdot}05$) and 2·8 M DMSO ($r = 0{\cdot}41$, $P < 0{\cdot}05$).

It was observed that perfusion with DMSO led to increases in urine flow rates. The data in Table 2 depict mean urine flow rates obtained in kidneys perfused with and without DMSO. Urine flow rates in the control group showed relatively little change during the course of perfusion. Although 1·4 and 2·8 M DMSO did cause elevated urine flow rates, there were no statistically significant deviations from control values at any time with any of the concentrations of DMSO studied. It was of interest to note that the highest levels of urinary γ-GT activity were found in conjunction with the highest values of urine flow rate. However, there was no statistical correlation, either direct or inverse, between urinary γ-GT levels and urine flow rate in control kidneys or kidneys perfused with DMSO.

The urine samples used in these studies had routinely been stored at −20° for 2–4 weeks before being assayed for γ-GT activity. In order to ascertain whether or not these samples had lost enzymatic activity because of this treatment, several experiments had to be conducted. First, these samples were assayed again, after 3–4 months storage at −20°. For these studies, samples were selected which would distinguish the effect of DMSO (Table 3) and the effect of the time of perfusion (Table 4). Since there was a linear increase in enzyme activity with time (see Fig. 1), this also represents a concentration curve.

The data presented in Tables 3 and 4 seem to indicate that there is only a slight decrease in enzymatic activity which can be demonstrated after several months of storage at −20°. Of those samples tested, only two have γ-GT values significantly lower than the initial values obtained. This finding would argue

Table 2. Urine flow rates in isolated rabbit kidneys perfused without and with DMSO*

Time (min)	Urine flow rate (ml/g/min) DMSO (0·0 M) (control)	DMSO (1·4 M)	DMSO (2·1 M)	DMSO (2·8 M)
10	0·075 ± 0·019	0·116 ± 0·011	0·091 ± 0·021	0·086 ± 0·022
20	0·084 ± 0·020	0·130 ± 0·015	0·088 ± 0·019	0·105 ± 0·023
30	0·087 ± 0·019	0·130 ± 0·020	0·080 ± 0·015	0·104 ± 0·023
40	0·086 ± 0·018	0·130 ± 0·020	0·096 ± 0·016	0·119 ± 0·022
50	0·082 ± 0·017	0·109 ± 0·009	0·096 ± 0·007	0·098 ± 0·011
60	0·074 ± 0·016	0·095 ± 0·008	0·085 ± 0·007	0·066 ± 0·011

* Values are expressed as mean ± 1 S. E. M.

Table 3. Effect of DMSO on the stability of γ-GT in the urine from an isolated perfused rabbit kidney*

DMSO (M)	γ-GT activity		Activity remaining (%)
	First determination	Second determination†	
0	0·112 ± 0·019	0·093 ± 0·014‡	82·9
1·4	0·205 ± 0·020	0·205 ± 0·034	99·9
2·1	0·115 ± 0·058	0·094 ± 0·045	81·7
2·8	0·172 ± 0·030	0·134 ± 0·018‡	78·0

* Kidneys were perfused for 60 min with Solution A which contained various amounts of DMSO and samples were collected during the last 10 min of perfusion. Results were expressed as μmoles product formed/ml of urine/min ± S. E. M.; n = 5.
† The second determination was made 90–120 days after the first determination.
‡ $P < 0.05$.

Table 4. Effect of perfusion time and enzyme concentration on the stability of γ-GT activity*

Perfusion time (min)	γ-GT activity		Activity remaining (%)
	First determination	Second determination†	
10	0·032 ± 0·020	0·028 ± 0·017	88·7
20	0·043 ± 0·026	0·034 ± 0·022	79·3
30	0·051 ± 0·022	0·043 ± 0·015	83·4
40	0·078 ± 0·022	0·059 ± 0·014	75·0
50	0·085 ± 0·014	0·073 ± 0·009	85·2
60	0·112 ± 0·019	0·093 ± 0·014‡	82·9

* Kidneys were perfused for 60 min with Solution A and urine samples were collected over 10-min intervals. Values represent μmoles product formed/ml of urine/min ± S. E. M.; n = 5.
† The second determination was made 90–120 days after the first determination.
‡ $P < 0.05$.

against the possibility that DMSO protects against freeze-inactivation and gives artificially high enzyme levels to those urine samples in which it is present.

In addition to this, however, it was necessary to demonstrate that the initial values obtained above, i.e. values from samples which had been stored for 2–4 weeks in the freezer, were not already depressed below the level found in fresh urine. For this reason, samples of fresh urine were collected at various times from one kidney, pooled, and treated in various ways.

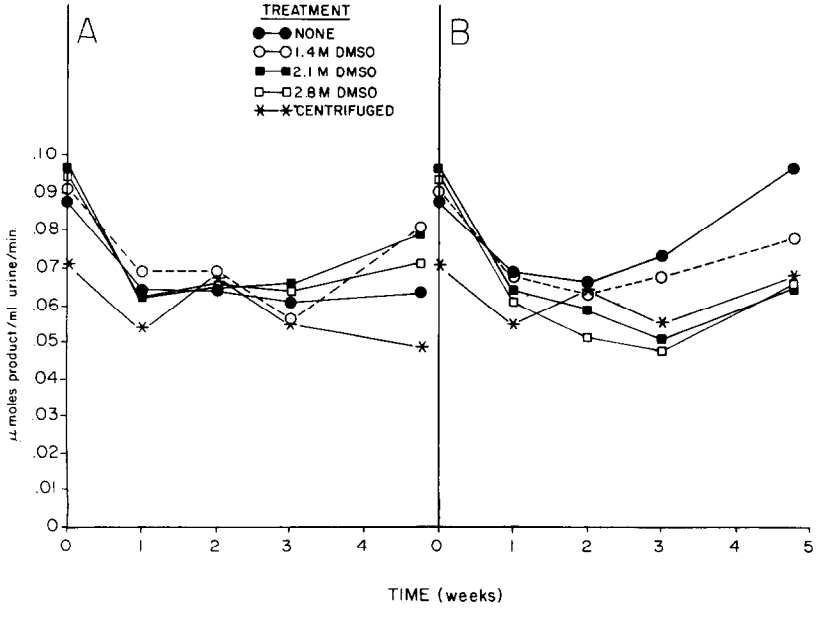

Fig. 2. Urinary γ-GT activity during storage at −20° (panel A) and at 4° (panel B).

DMSO was added to aliquots of the urine at several concentrations, while another aliquot was centrifuged at 5000 rev/min for 10 min. All samples were then assayed for γ-GT activity and divided in half. One half of each sample was stored at −20°, while the other half was stored at 4°. Subsequent analyses of γ-GT activity were run on each of these samples after 7, 14, 21 and 32 days.

The results of these studies are shown in Fig. 2, A and B. Figure 2A depicts the time course of enzymatic activity found in urine which is stored at −20°. It can be noted that the addition of various concentrations of DMSO to fresh urine has little effect on the level of γ-GT activity. Centrifugation at 5000 rev/min for 10 min does seem to reduce the level of enzyme activity somewhat. It is apparent that all of these urine samples show a loss of enzymatic activity in the first week. After this time, the γ-GT activity is relatively stable, with the individual experimental error large enough to explain any differences. We have repeated this experiment with the urine from another kidney and found an even smaller decrease over the time period tested. Urine which is stored at 4° (Fig. 2B) shows a remarkably similar pattern. These results indicate that urine samples from a perfused kidney can be stored for several weeks without significant loss of transpeptidase activity. In addition, there is no indication that DMSO, at any of the concentrations tested, caused a change in the stability of γ-GT activity in these urine samples.

These results are quite different from values reported previously for human urine; therefore, we investigated the possibility that the urine obtained from a perfused kidney contained a different form of γ-glutamyl transpeptidase than that found in human urine. The data shown in Table 5 indicate the differences between human, rabbit and perfusion-derived urine with regard to γ-GT, as well as alkaline phosphatase (AP), which is also associated with the brush border, and LDH, an intracellular enzyme. Human urine contains relatively low levels of all three enzymes and a significant fraction of each appears in the 100,000 *g* supernatant. Rabbit urine contains a considerably smaller percentage of the soluble form of γ-GT and AP. In comparison, perfused kidney urine has less than 3 per cent of its γ-GT activity, and less than 12 per cent of its AP activity in the soluble form. Also included in Table 5 are data for the enzyme distribution in renal cortical tissue of perfused and non-perfused kidneys. It is clear that perfusion urine is more similar to renal tissue, in its percentage distribution of soluble enzymes, than it is to natural urine.

Table 5. Urinary and tissue levels of γ-glutamyl transpeptidase, alkaline phosphatase and lactic dehydrogenase*

Sample	γ-GT*	AP‡	LDH§
Human urine	0·032	0·033	12
Human urine sup	0·014	0·027	12
Rabbit urine	0·048	0·025	11
Rabbit urine sup	0·005	0·005	8
Perfused kidney urine	0·084	0·160	50
Perfused kidney urine sup	0·002	0·018	23
Kidney homogenate	3·010	3·560	1793
Kidney sup	0·038	0·324	1705
Perfused kidney homogenate	2·006	2·070	1074
Perfused kidney sup	0·025	0·200	1032
10-min	0·032	0·063	73
20-min	0·041	0·096	56
30-min	0·062	0·128	50
40-min	0·114	0·178	48
50-min	0·160	0·225	32
60-min	0·200	0·231	25

* Kidneys were perfused for 60 min with Solution A and urine samples were collected over 10-min intervals. Human urine, rabbit urine, perfused kidney urine, and renal tissues were collected and prepared as described under Materials and Methods. Samples labeled 'sup' refer to supernatants from centrifugation at 100,000 *g*.

† Expressed as μmoles product/ml of urine/min or μmoles product/50 mg of tissue/min.

‡ Expressed as μmoles product/0·20 ml of urine/30 min or μmoles product/10 mg of tissue/30 min.

§ Expressed as milliunits/ml of urine or milliunits/50 mg of tissue.

These results prompted the suggestion that pieces of renal brush border are being broken off during perfusion and coming out in the urine. Previously reported light and electron microscopic findings support this contention, in that detached fragments of apical cell membranes were observed within the lumen of proximal tubules in kidneys perfused with DMSO [27]. To test this hypothesis, the appearance of γ-GT, AP and LDH as a function of time was determined in kidneys perfused without DMSO. It can be seen in Table 5 that, as γ-GT increases with time, AP does likewise. These two enzymes are both believed to be found on the brush border membrane in high concentrations. If indiscriminate tissue deterioration were occurring, one would expect to also find increasing levels of the intracellular enzyme LDH. On the contrary, the values for LDH in perfusion urine appear to decrease with time.

DISCUSSION

This report establishes for the first time the presence of γ-glutamyl transpeptidase in the urine from an isolated perfused kidney. In addition, this investigation has demonstrated that in these urine samples, γ-GT is not subject to inactivation by storage at −20° and it is not stabilized by the addition of DMSO. These findings are substantially different from those found for γ-GT in human urine [24], but this is not unexpected. This study was undertaken with an entirely different experimental design. Isolated, perfused rabbit kidneys provide the investigator with a much more uniform system than could be attained by the collection of human urine. These isolated kidneys are all perfused with the same solution and there are no hormonal or neural influences to affect renal function. With such an experimental system it is possible to easily determine the effect of an exogenous reagent (such as DMSO) on renal function, without concern for other changes that might take place in the whole animal. The disadvantage of such a system is that results obtained from an isolated organ may not reflect the situation *in vivo*.

In addition to these inherent experimental differences, it also appears that human urine contains a much higher percentage of the soluble form of γ-GT than the urine from a perfused kidney. Recently it has been suggested [28] that soluble γ-GT is associated with the secretory component of sIgA. Apparently, these perfused kidneys are not secreting sIgA, but the possibility of stimulating such activity by bacterial infection is presently being investigated. It would appear that during perfusion, pieces of the brush border membrane are being 'shed' into the renal tubule, accounting for the increasing concentration of particulate γ-GT and AP with time. The LDH levels of the perfused kidney urine are elevated initially, presumably by the ischemic damage caused by the transfer of the kidney to the perfusion apparatus, and the kidney then makes a gradual recovery.

The concentration of γ-glutamyl transpeptidase in the urine of isolated perfused kidneys appears to be independent of the urine flow rate. If γ-GT were released into the urine of perfused kidneys at a constant rate, the observed enzyme activity in urine samples would vary reciprocally with urine flow rate; higher urine flows would tend to dilute the enzyme, while lower urine flow rates would concentrate γ-GT activity. Conversely, if high urine flow rates caused an increase in the amount of tubular damage, one would expect higher urine flow rates to increase such damage and thus increase the γ-GT levels of urine. To verify the independence of urinary γ-GT levels from urine volume, we have acutely lowered urine flow rate by ureteral occlusion and by infusion of hypertonic colloid; neither of these treatments changed urinary enzyme activity when compared with control values (unpublished observation).

Previous reports from this laboratory have shown that the isolated rabbit kidney perfused at 37° undergoes progressive functional and ultrastructural deterioration with time, which results in loss of reabsorptive function and frank tubular necrosis after 2 hr [18-20]. The increasing urinary levels of γ-GT observed in the control kidneys with time may be related to this loss of tissue integrity, possibly because of damage to cell membranes. Although electron microscopic observations demonstrated good preservation of tubular cell ultrastructure after 60 min of normothermic perfusion without DMSO [20], studies of kidneys perfused with 2·8 M DMSO for the same length of time revealed conspicuous cytopathological changes in the apical plasma membranes of proximal tubular cells [27]. Even in kidneys perfused with 1·4 M DMSO, in which proximal tubular epithelium has been previously demonstrated to remain intact, increased levels of urinary γ-GT have been found; this may be due to the known effect of DMSO in increasing cell membrane permeability [29, 30]. The observation that 2·1 M DMSO did not significantly alter urinary γ-GT activity is not consistent with these findings, and cannot be explained at present. The value of this enzyme determination as a viability assay in renal perfusion studies has yet to be confirmed. Only after experiments are done which involve correlation of urinary γ-GT levels during perfusion, with kidney survival following post-perfusion reimplantation, can one interpret the effectiveness of urinary γ-GT as an indicator of tissue viability.

Acknowledgement—The authors thank Dr. Robert Sobel, Clinical Chemistry Laboratory, Eugene Talmadge Memorial Hospital, for the LDH assays, and Dr. Armand Karow, Jr. for the use of the perfusion apparatus. These studies were supported by USPHS-NIH Grant FR 5365.

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