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THE USE OF URINARY ENZYME MEASUREMENTS TO DETECT RENAL DAMAGE CAUSED BY NEPHROTOXIC COMPOUNDS

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Abstract—Several known and suspected nephrotoxic agents were administered to male Wistar rats and the excretion rates of 4 enzymes located largely in specific regions of the cell were measured. The excretion of lactate dehydrogenase and alkaline phosphatase in the urine was considerably increased and the severity of the renal damage appeared to be related to the enzyme activity present in the urine. The excretion of acid phosphatase and glutamate dehydrogenase was usually within normal limits or only slightly raised.

4-Aminocatechol, an abnormal metabolite of phenacetin suspected of causing kidney damage, gave normal results for enzyme excretion suggesting the absence of renal lesions in the rat.

The value of urinary enzyme measurements to detect acute kidney damage by toxic compounds and to identify the primary renal lesion is discussed.

INTRODUCTION

THERE are a number of methods available for the investigation of the anatomical and functional state of the kidneys in humans. These include radiography, biopsy and the calculation of "clearance values" for various compounds. The measurement of the clearance of urea, creatinine or vitamin B_{12} can be used to arrive at a measure of the glomerular filtration rate, and the ability of the kidney to secrete an injected compound such as *p*-aminohippuric acid can be used to determine tubular function. Values obtained for these measurements can be compared with those obtained from healthy subjects so that severe renal impairment can be detected. However, such biochemical measurements cannot be easily applied to a large number of small animals such as rats for checking the possible nephrotoxicity of a compound. Gross kidney damage can often be detected by determining the degree of proteinuria or by examining the urinary sediment but if only slight renal damage has occurred, then histological examination of the kidneys may be the only method available. This latter procedure has the disadvantage that the animal has to be killed and continuous measurements cannot therefore be made.

Disease of the kidneys in humans can be detected by measuring the activity of some enzymes excreted in urine.[1] Lactate dehydrogenase and alkaline phosphatase are the enzymes most frequently assayed in urine and these are raised in a variety of diseases of the urinary tract but neither enzyme is specific in differential diagnosis. Renal damage can also be caused by toxic compounds as well as disease, and Harrison and

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his colleagues[2] found elevated urinary levels of muramidase and ribonuclease in factory workers who showed signs of multiple reabsorption defects due to cadmium poisoning. There are also a number of reports published[3] of the release of kidney enzymes into the urine following the administration of nephrotoxic compounds to rats. It is therefore possible that urinary enzyme measurements could replace the tedious and expensive histological procedures used at present to screen for the possible renal toxicity of compounds.

The enzymes studied in the present investigation are "markers" for specific regions of the cell, so by measuring their activity in rat urine, it is hoped to discover the site of the primary lesion and to determine the extent to which the other cellular compartments are affected. Glutamate dehydrogenase is a mitochondrial enzyme, acid phosphatase is present in the lysosomes, alkaline phosphatase is the marker for the endoplasmic reticulum and plasma membrane and lactate dehydrogenase is found in the cytoplasm of soluble fraction of the cell.

Hence, by using the apparent leakage of enzymes into the urine as an index of kidney damage, it should be possible to evaluate the nephrotoxicity of compounds.

MATERIALS AND METHODS

Compounds tested for nephrotoxocity

Uranyl nitrate. This analytical grade reagent was purchased from British Drug Houses Ltd., Poole, Dorset and dissolved in sterile glass distilled water to a final concentration of 2·5 mg/ml of $UO_2(NO_3)_2.6H_2O$. Attempts to adjust the pH of the solution towards neutrality caused a precipitate to form and so the solution injected had a pH of 5·0. The compound was administered by intraperitoneal injection (1 ml/100 g).

4-*Nitrophenylaronsic acid.* This material was a gift from Professor Robinson of the Biochemistry Department, Queen Elizabeth College, London. The solution for injection was prepared in sterile glass distilled water (2·5 mg/ml) adjusted to pH 7 with sodium bicarbonate. The compound was given to the rat by intraperitoneal injection (1 ml/100 g).

4-*Nitrocatechol.* This compound was a commercial grade reagent obtained from Ralph Emmanuel Ltd., Wembley, Middlesex and administered to the rat (1 ml/100 g) as a neutral solution (1 mg/ml) in sterile glass distilled water.

4-*Aminocatechol.* Aminocatechol was prepared by the reduction of the nitro-compound. 4-Nitrocatechol (5 g) was suspended in 25 ml of water and mixed with 15 g of granulated tin. Concentrated HCl was then added in 3 portions of 25 ml each and the mixture then heated on a boiling water bath. The excess tin was removed by filtration and the filtrate was diluted with distilled water and treated with hydrogen sulphide. The solution was filtered to remove tin sulphide and the filtrate was evaporated by steam distillation until crystals of the HCl salt began to form. The solution was then left in the cold room overnight, the crystals were filtered off, dissolved in a minimum volume of water and recrystallized until a constant melting point was obtained. Yield 3·5 g, m.p. 224°.

A stock solution of 0·5 mg/ml was prepared in sterile glass distilled water and 1 ml of this was administered by intraperitoneal injection for each 100 g of rat.

Other chemicals. The sources of the reagents for the enzyme assays were as follows:

sodium pyruvate and 4-nitrophenyl disodium orthophosphate (British Drug Houses Ltd., Poole, Dorset); sodium-2-oxoglutarate (Sigma Chemical Co., London) and NADH (Boehringer Corp. Ltd., London). Other chemicals used were of analytical grade. All reagents were prepared in distilled water redistilled from Pyrex apparatus.

Collection of rat urine

Urine was collected free of faecal contamination from male Wistar rats housed in perspex restraint cages for 12 hr and prepared for assay as previously described.[4,5] Each group consisted of either 8 or 10 rats and from 6 to 8 uncontaminated urines were usually obtained.

Determination of enzyme activities

The activity of LDH,[6] alkaline phosphatase[7] and acid phosphatase[8] was determined as described in previous reports under conditions found to be optimum for the urinary enzymes.[9] Protein was determined by a Biuret method.[10]

Glutamate dehydrogenase was assayed by a method based on the rate of change of extinction at 340 nm in an S.P. 800 Unicam Spectrophotometer. All solutions were prepared in 0·067 M Sørenson phosphate buffer, pH 7·4 and adjusted to pH 7·4. Stock solutions of the reagents were dispensed into small tubes and frozen until required. $NADH_2$ was prepared fresh on the day of use.

A suitable amount of enzyme solution was mixed with 0·067 M phosphate buffer (pH 7·4) to bring the volume to 2·5 ml. $NADH_2$ (0·2 ml of 2·5 mg/ml), ammonium acetate (0·1 ml of 0·75 M) and EDTA (0·1 ml of 30 mM) were added to the above mixture and the solution warmed to 30° for 30 min. The reaction was then started by the addition of 0·1 ml of 150 mM sodium-2-oxoglutarate and the mixture transferred to the spectrophotometer. The rate of decrease in extinction at 340 nm was then measured against an appropriate blank. The conditions used were those found to be optimal for rat urine.[9]

Examination of urine for abnormal constituents

All samples of urine were examined qualitatively for the presence of glucose, protein, blood and ketone bodies using Labstix (Ames Laboratories Ltd., Stoke Poges, Buckinghamshire).

RESULTS

Normal levels of enzyme excretion in rat urine

The rate of excretion of urine and the 4 enzymes examined by normal healthy rats is given in Table 1. The upper limit of normal (ULN) of these measurements was taken as the average value plus 2 standard deviations. In all the experiments involving the administration of compounds to rats, any value greater than this (ULN) was considered to be abnormal and indicative of some degree of cellular damage. About half of the normal urines contained no measurable GDH and in the case of this enzyme, the upper limit of normal was taken as the highest value recorded in normal urine.

TABLE 1. THE EXCRETION OF URINE AND ENZYMES BY NORMAL RATS

Enzyme	No. of rats	Enzyme activity (nmoles/min) excreted per hr per 100 g of rat: Normal range (mean ± S.D.)	Upper limit of normal (mean + 2 S.D.)
Acid phosphatase	78	6·21 ± 2·74	11·7
Alkaline phosphatase	78	8·38 ± 6·06	20·5
Lactate dehydrogenase	85	3·21 ± 2·29	7·8
Glutamate dehydrogenase	60	0·14*	1·0†
		Urine volume ml excreted per hr per 100 g of rat	
Urine volume	85	0·26 ± 0·15	0·56

* Half of the normal values were zero and so standard deviation is not given.
† The highest normal value obtained.

Excretion of enzymes after the injection of test compounds

Uranyl nitrate. During this experiment, the volume of urine produced (Table 2) after injection was within the normal range in all collection periods except the 12 hr immediately after injection when it was high. The excretion rates of the enzymes are shown in Fig. 1. LDH was high in all the samples collected with a peak excretion

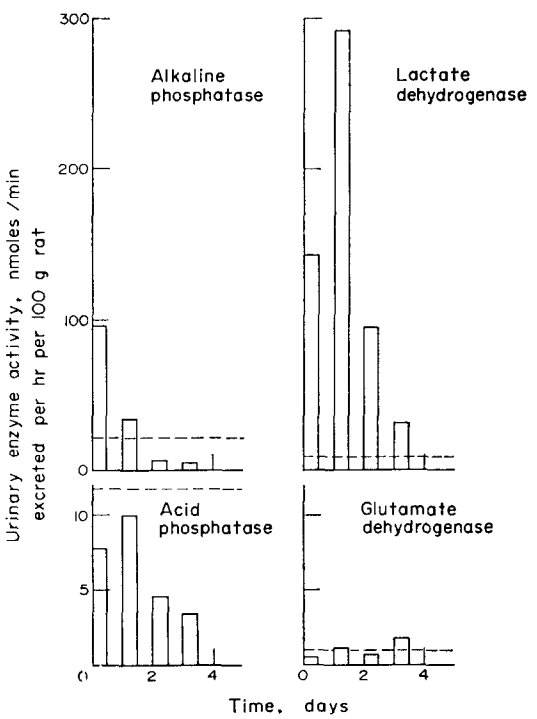

FIG. 1. The excretion of enzymes in urine following the injection of uranyl nitrate; (– – –) represents the upper limit of normal for each enzyme.

rate of about 90 times the normal average in the 24–36 hr post-injection period. Alkaline phosphatase was raised to the greatest extent in the 0–12 hr period but had returned to normal by 48–60 hr. Acid phosphatase levels in the urine remained normal throughout the period of the experiment. GDH excretion was also normal except at 48–60 hr.

TABLE 2. THE VOLUME OF URINE PRODUCED AFTER INJECTION OF THE TEST COMPOUND

Collection (hr)	Volume (ml excreted per hr per 100 g of rat ± S.D.)			
	Uranyl nitrate	4-Nitrophenylarsonic acid	4-Aminocatechol	4-Nitrocatechol
0–12	0·62 ± 0·26 (11)	0·34 ± 0·11 (10)	0·51 ± 0·25 (9)	0·40 ± 0·14 (7)
24–36	0·56 ± 0·20 (9)	0·62 ± 0·17 (7)	0·39 ± 0·15 (7)	0·32 ± 0·13 (8)
48–60	0·31 ± 0·08 (4)	0·49 ± 0·12 (6)	0·30 ± 0·08 (7)	0·18 ± 0·06 (9)
72–84	0·33 ± 0·15 (8)	0·45 ± 0·26 (7)	0·35 ± 0·14 (8)	0·26 ± 0·05 (7)
168–180	—	0·35 ± 0·16 (6)	0·42 ± 0·15 (9)	0·37 ± 0·23 (7)

Number of rats given in brackets.

All the rats injected except one died between 4 and 7 days after the injection. The sole survivor showed a lower rate of LDH excretion in all periods with a peak value at 24–36 hr which was only 7 times normal. The excretion of GDH by this rat was less than that of the other rats and the alkaline phosphatase was also normal during the urine collection periods examined.

Qualitative examination of the urine with Lasbtix showed proteinuria in all samples with the greatest amount in the 24–36 hr period. All rats also showed glucosuria which was highest at 0–12 hr and became less severe in each succeeding collection period.

4-Nitrophenylarsonic acid. All the rats excreted normal volumes of urine after injection except during the 24–36 hr period when the rats showed a slight polyuria (Table 2). LDH was elevated in all samples up to 84 hr but had returned to normal by 7 days. The peak excretion rate of LDH was in the 24–36 hr period when it was 200 times normal. The excretion of alkaline phsophatase was elevated until 36 hr post-injection but returned to normal 24 hr later. The acid phosphatase excretion was normal in all periods except from 24 to 36 hr when it was slightly raised. GDH was also above normal during this period along with the other enzymes investigated (Fig. 2).

About half of the 0–12 hr samples contained glucose and this was also present in every other sample of urine collected with the 24–36 hr sample showing the severest glucosuria. Protein was detected in all urines collected up to 84 hr and the pattern of proteinuria was similar to that found for glucose.

Despite the large excretion of enzymes, only one rat died and then not until day 7 of the experiment. This rat excreted the highest LDH and alkaline phosphatase activity during the first 12 hr collection period.

4-Aminocatechol and 4-nitrocatechol. Normal urine volumes were obtained when these compounds were injected into rats (Table 2) and only one sample gave an enzyme activity greater than normal (Figs. 3 and 4). The urines also showed only trace amounts of protein and were free of glucose and blood.

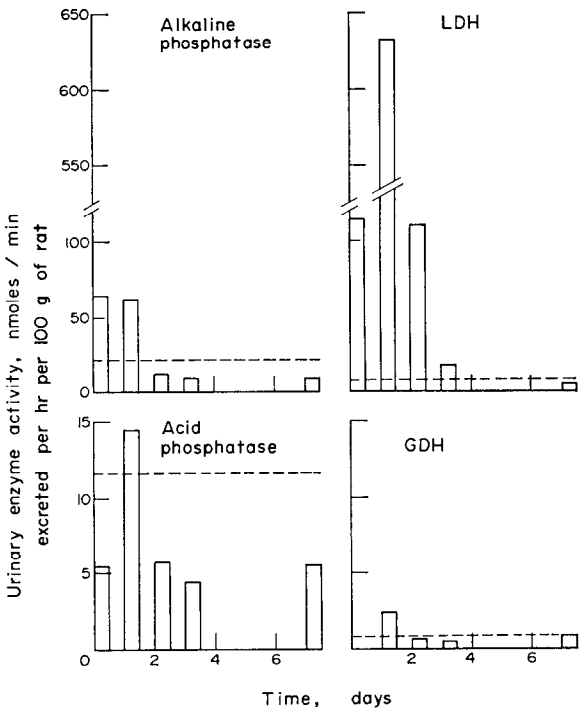

FIG. 2. The excretion of enzymes in urine following the injection of 4-nitrophenylaronic acid; (- - -) represents the upper limit of normal for each enzyme.

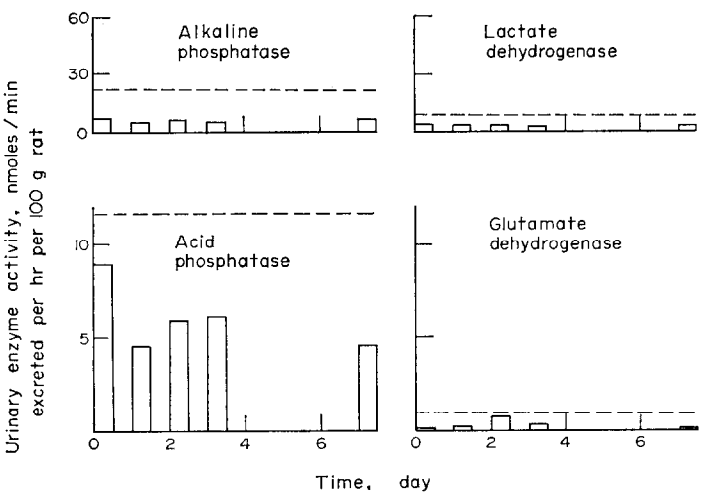

FIG. 3. The excretion of enzymes in urine following the injection of 4-aminocatechol; (- - -) represents the upper limit of normal for each enzyme.

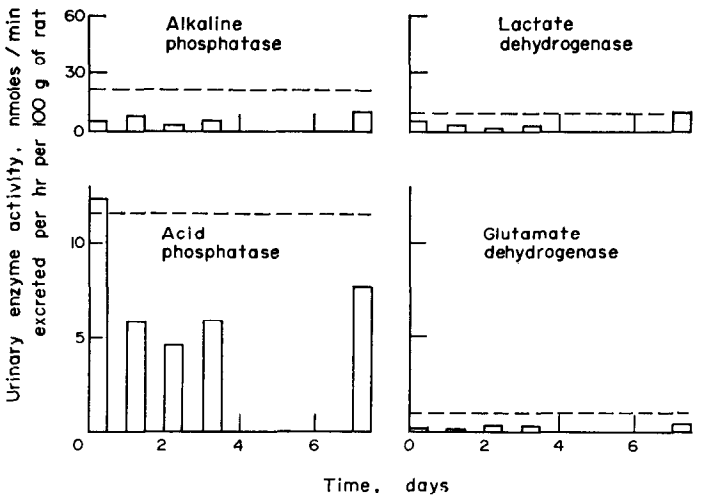

FIG. 4. The excretion of enzymes in urine following the injection of 4-nitrocatechol; (– – –) represents the upper limit of normal for each enzyme.

DISCUSSION

Uranyl nitrate is known to be a powerful nephrotoxic agent[11] which attacks the distal half of the proximal convoluted tubules.[12] The mechanism of action of this poison is not known but the uranyl ion does inhibit hexose transport[13] and the presence of glucose in all the urines collected supports this idea. Blood was absent from all urine samples suggesting that glomerular damage does not occur to any great extent.

The excretion of alkaline phosphatase is greatly increased from rats poisoned with uranyl nitrate[14] and the present study confirms this observation (Fig. 1). LDH, however, would appear to provide a more sensitive index of renal damage than alkaline phosphatase as this enzyme reached a maximum excretion of 90 times the normal average compared with only 11 times for the alkaline phosphatase. Furthermore, the LDH was still abnormal during the 72–84 hr collection period whereas the alkaline phosphatase had returned to normal by 48–60 hr. Acid phosphatase remained normal over the period of the experiment so it would appear that lysosomal rupture is not a primary event in kidney damage due to uranyl nitrate. This is similar to the earlier observation that lysosomal damage is a late event in liver injury caused by hepatoxic compounds.[15] Similarly mitochondrial injury appears to be late although there is a suggestion of some damage during the second collection period (24–36 hr).

All the rats given uranyl nitrate died between 4 and 7 days after the injection and the sole survivor had the lowest excretion rate of LDH with a peak at 24–36 hr which was only 7 times normal. This same rat excreted the smallest amount of GDH and normal amounts of alkaline phosphatase. In the case of this compound therefore, it appears that low enzyme activities in urine are related to the chance of survival.

The results for the 4-nitrophenylarsonic acid were very similar to those for uranyl nitrate except that the maximum excretion of LDH which occurred at 24–36 hr, was some 200 times greater than normal (Fig. 2). The other 3 enzymes also reached their maximum level and were all abnormal during this collection period. Robinson *et al.*[16]

injected the same dose of this compound into rats and obtained maximum excretion of a number of glycosidases during their second 24 hr collection. This agrees with the above observation that maximum damage occurs at about this time.

Despite the severe damage produced, as indicated by the very large excretion of enzymes, only one rat died and then not until the seventh day of the experiment. The one rat that died excreted twice as much LDH as the other rats during the first 12 hr after injection and the excretion of alkaline phosphatase was also higher than in the other rats. Unfortunately further samples of urine from this rat were all contaminated with faeces and were not assayed for enzyme activity. This again suggests that a relationship exists between the chance of survival and the activity of the enzymes excreted.

A minority of human adults show signs of renal lesions due to the ingestion of high doses of analgesics containing phenacetin and Prescott[17] has suggested that such lesions could be due to a genetically determined abnormality of phenacetin metabolism. Parke[18] in a discussion of this paper described the case of a girl who had cyanosis and whose urine contained 3-hydroxy-phenacetin and 3-hydroxy-4-methoxyaniline rather than the normal 2-hydroxy metabolites. 3-Hydroxy-4-methoxy aniline could give rise to 3,4-dihydroxyaniline (4-aminocatechol) which Parke showed to be extremely toxic in dogs; a dose of 10 mg/kg caused death after severe haematuria. In an attempt to test this hypothesis, 4-aminocatechol was prepared and administered to rats; however, all the enzyme excretion rates were normal (Fig. 3). There was no blood in the urine and no glucosuria or proteinuria. All of these results suggest that the compound is not nephrotoxic in contrast to the findings of Parke.[18] The discrepancy could be due to an impure preparation although 4-nitrocatechol can be ruled out since this compound gives quite normal results (Fig. 4). The most likely explanation is probably the different metabolism of 4-amino-catechol in rats and dogs.

From the results discussed, it appears that acute kidney damage by known and powerful nephrotoxic agents can be easily detected by measuring the excretion of enzymes in urine and that of the enzymes examined, the determination of lactate dehydrogenase is the most useful and sensitive index of kidney damage.

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