The Excretion of Lactate Dehydrogenase in Human Urine After the Ingestion of Aspirin

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1. Cells present in normal human urine contain 5–10% of the total lactate dehydrogenase excreted. The enzyme released from these cells by ultrasonication contained a distribution of isoenzymes similar to that found in the bulk of the urine and it is suggested that these cells are the main source of urinary lactate dehydrogenase. 2. Cells were thoroughly washed before examination so it is unlikely that the enzyme found in urinary sediment was simply adsorbed. In addition, full recoveries of added lactate dehydrogenase isoenzymes LDH₁ and LDH₅ showed that adsorption did not occur. 3. Most of the cells in normal urine are of the non-squamous epithelial type and their excretion is greatly increased after the ingestion by the subject of 3 g. of aspirin. The possible origin of these non-squamous cells from the kidney is discussed. 4. Starch-block electrophoresis and relative activity measurements of lactate dehydrogenase excreted after the subject had taken aspirin show that the enzymes present in urine and cells are very similar, confirming the conclusion reached above (point 1). They have slightly more M subunits than the normal, shown particularly as an increase in isoenzyme LDH₂. The isoenzyme pattern is like that of the kidney medulla and the possible reasons for this are discussed in terms of the concentration of salicylic acid in various parts of the kidney. 5. The results confirm the previous suggestion that the kidney is the main source of urinary lactate dehydrogenase.

The presence of the enzyme lactate dehydrogenase in human urine was first reported by Klaus (1958) and Rosalki & Wilkinson (1959). Klaus (1958) demonstrated a loose correlation between proteinuria and urinary lactate dehydrogenase, and on this basis considered that the enzyme originated from the blood plasma. Rosalki & Wilkinson (1959) on the other hand suggested that the small amounts of enzyme found in the urine of healthy adults arose from the kidneys. The kinetic and electrophoretic properties of urinary lactate dehydrogenase have been examined and shown to be similar to those of the enzyme found in the kidney but different from the enzyme in the bladder, seminal fluid, kidney pelvis and plasma; the kidney therefore seems to be the most likely source of the urinary enzyme (Plummer & Leathwood, 1967). The enzyme is not identical, however, with that present in a kidney homogenate and this could be explained by considering that some cells contribute more to the urinary enzyme than others. For example, the cells lining a particular region of the renal tubule could well

differ slightly in their isoenzyme pattern from the rest of the kidney cells.

Ingestion of aspirin causes a shedding of cells into the urine (Clausen & Harvald, 1961; Scott & Denman, 1961) and Scott, Denman & Dorling (1963) showed a 10–20-fold increase in the number of cells in urine after the ingestion of 2.7 g. of acetylsalicylic acid. Clausen & Harvald (1961), who obtained similar results, considered that the cells appearing in the urine after this treatment were leucocytes, but they counted unstained cells and did not attempt to examine them more closely. Scott *et al.* (1963) found that, morphologically and in their staining characteristics, most of the cells resembled renal tubular cells. This conclusion was confirmed by Prescott (1965*a,b*), who used a peroxidase stain to identify leucocytes in the urine and showed again that the vast majority of the cells appearing in urine after ingestion of salicylate were renal tubular cells.

It was therefore decided to examine the lactate dehydrogenase present in urine and urinary sediment after the ingestion of aspirin.

A preliminary account of this work has been

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presented at a meeting of The Biochemical Society (Plummer & Leathwood, 1968).

EXPERIMENTAL

Materials. Purified lactate dehydrogenase isoenzymes LDH₁ from ox heart and LDH₅ from rabbit muscle were obtained from Boehringer Corp. (London) Ltd., London W. 5. Acetylsalicylic acid was administered as Disprin (Reckitt and Sons Ltd., Hull). Other reagents used were of analytical grade. All solutions were prepared in distilled water redistilled from Pyrex apparatus.

Collection and concentration of urine. Overnight samples of urine obtained from healthy adult males were collected in polythene containers without the addition of preservative. A measured volume of centrifuged urine, usually 100 ml., was concentrated by overnight dialysis against a 30% (w/v) solution of Carbowax 20M at 4°. The dialysis tubing was then washed free of Carbowax and dialysed for a further 4 hr. against Sørensen phosphate (67 mM) buffer, pH 7.4. The concentrate sometimes contained a jelly-like material, which was precipitated by centrifugation at 33000g for 15 min. to leave a clear pale-brown supernatant. The final volume of concentrate obtained was about 1–3 ml., involving a 33–100-fold increase in lactate dehydrogenase activity/ml. Enzyme recoveries were usually in the range 80–100%. Further examination of the urinary enzyme was carried out on this supernatant.

Cell counting. Urinary sediment from healthy adult males contains small amounts of precipitated salts, non-squamous and squamous epithelial cells, leucocytes and occasionally erythrocytes and spermatozoa. It is difficult to distinguish between non-squamous epithelial cells and polymorph leucocytes, unless a differential staining method is employed, so the method of Houghton & Pears (1957) as adapted by Prescott & Brodie (1964) was used.

The urine was thoroughly mixed and a 10 ml. sample removed and placed in a graduated centrifuge tube. One or 2 drops of acetic acid were added to clarify the sample, which was centrifuged at 3000g for 10–15 min. The supernatant was removed and 3 drops of stain were added to the sediment: the suspension was then made up to 0.5 ml. with supernatant urine and thoroughly mixed with a Pasteur pipette. Leucocytes (which stained dark blue), non-squamous epithelial cells (pale pink), erythrocytes (red), squamous epithelial cells (pink) and spermatozoa were counted in a Fuchs–Rosenthal counting chamber. At least 100 non-squamous epithelial cells were counted on two separate samples of sediment and the number of cells excreted/hr. was determined.

Large-scale collection of urinary sediment. Sediment from about 1 l. of urine was separated by centrifugation at 8000g for 30 min. The supernatant was carefully removed and the cells were washed twice with ice-cold 0.14 M-NaCl and once with Sørensen phosphate (67 mM) buffer, pH 7.4, by centrifuging at 20000g for 15 min. in the cold. The cells were finally resuspended in a small volume of phosphate buffer by gentle hand rotation in a loose-fitting Potter–Elvehjem homogenizer.

Rupture of the cells by ultrasound. A suspension of cells (10 ml.) cooled in ice was subjected to ultrasound for 8 min. with a 100 W ultrasonic disintegrator (Measuring and Scientific Instruments, London) operating at 20 kcy./sec.

and an amplitude of 2 μ m. Variations in time and amplitude were used as well as the standard procedure described above but these are described more appropriately in the Results section.

Determination of protein. A 1 ml. portion of 20% (w/v) trichloroacetic acid was added to 1 ml. of the concentrated urine and thoroughly mixed. The precipitate was removed by centrifugation and dissolved in 0.5 ml. of M-NaOH. Protein determination was carried out on this and other samples by a modified biuret method (Wootton, 1964).

Measurement of lactate dehydrogenase activity. The activity of the enzyme lactate dehydrogenase and the relative activities with pyruvate and 2-oxobutyrates as substrates were determined as described by Plummer & Leathwood (1967).

Starch-block electrophoresis. Potato starch (British Drug Houses Ltd., Poole, Dorset) was washed by decantation with distilled water, then twice with Sørensen phosphate (67 mM) buffer, pH 7.4. The buffer was decanted and the moist starch placed inside a former on a Perspex plate to give a block of 20 cm. $\times$ 10 cm. $\times$ 1 cm. Excess of moisture was removed from the block by gently blotting the surface with filter paper. Electrical connexion with the buffer solution in the electrode tank [Sørensen phosphate (67 mM) buffer, pH 7.4] was provided by wicks consisting of several thicknesses of Whatman 3MM chromatography paper. A thin sheet of polythene covered the block and a cooling tank full of ice was placed on top; the system was then equilibrated by applying a potential of 150 v for 1 hr. The enzyme material, containing an activity of 100 nmoles/min., was applied to a groove 0.2 cm. wide cut horizontally across the block 15 cm. from the anode and 1 cm. from the edge. A mixture of human haemoglobin and bovine serum albumin mixed with Bromophenol Blue was applied as a marker in a small groove parallel to that containing the enzyme. The grooves were filled with starch and the electrophoresis apparatus was placed in a refrigerator. A potential of 150 v was applied to the block for 18–20 hr. The position of the markers was carefully noted and the longitudinal portion containing these removed. The remainder of the block was then cut transversely into strips 0.5 cm. wide. Each strip was transferred to a sintered-glass funnel and washed three times with 2 ml. portions of Sørensen phosphate (67 mM) buffer, pH 7.4. Lactate dehydrogenase activity was measured on a suitable volume (0.1–2.8 ml.).

RESULTS

Ingestion of salicylate and the excretion of cells and lactate dehydrogenase. A single dose (3 g.) of acetylsalicylic acid was taken by mouth by a healthy adult male and serial 8 hr. samples of urine were collected for 4–5 days. Lactate dehydrogenase assays and cell counts were carried out on each specimen and the enzyme and cell excretion/hr. were computed. An increase in the urinary cell count was invariably seen after ingestion of the drug but the maximum count, obtained at 24–32 hr., showed individual variation from 0.3×10^6 to 2×10^6 cells/hr.

The effect of aspirin on enzyme and cell excretion by a typical normal subject is shown in Fig. 1. In

Fig. 1. Excretion of cells and lactate dehydrogenase in urine after the ingestion by the subject of 3g. of acetylsalicylate.

Table 1. Pattern of cell types excreted in six samples of normal urine

Urine	10 ⁻³ × No. of cells/hr.					
	1	2	3	4	5	6
Non-squamous epithelial cells	430	300	350	280	100	190
Squamous epithelial cells	30	18	28	14	4	10
Erythrocytes	15	—	14	—	—	—
Leucocytes	3	—	—	—	—	—
Spermatozoa	3	—	14	—	—	—
Total excretion	481	318	406	294	104	200

this case the cell count rose to a maximum of nearly 10⁶/hr. at 24–32hr., decreasing to normal values 4 days later. The cells present in normal urine were mostly of the non-squamous epithelial type (Table 1) and had the characteristic appearance of renal tubular cells as described by Scott *et al.* (1963). Ingestion of aspirin caused an increased excretion of these cells and had little effect on the other types found.

The lactate dehydrogenase activities of the supernatants from the urines were always above normal values for several days, although the change in output was less consistent than with the cells.

Urine collected at 24–32hr. after the ingestion of the drug was examined further.

Lysis of the cells. The following methods for rupturing the cells were examined: repeated freezing-and-thawing, osmotic shock, homogenization and ultrasonic disintegration. Freezing-and-thawing was unsuitable, since this treatment destroys the enzyme activity. Osmotic shock presents certain difficulties, since accurate cell counts before the treatment are precluded and also lactate dehydrogenase remains bound to the particulate fraction of a tissue in a medium of low ionic strength (Hultin & Westort, 1966). Homogenization in a Potter-Elvehjem homogenizer was also abandoned when microscopic examination revealed that few cells were broken, even after 20 vertical strokes at 5000rev./min.; this may be due to the lubricating action of the mucus-like substance often present. Ultrasonic disintegration was the method finally used to break up cells in the urinary sediment, and the conditions for the maximum rupture of the cells and the maximum yield of the enzyme were investigated.

A suspension (28.5ml.) of cells from 1.45l. of urine was prepared as described and divided into three 9.5ml. portions: A, B and C. Purified isoenzymes (0.5ml.) LDH₁ and LDH₅ were mixed with samples A and B, and 0.5ml. of phosphate buffer was added to suspension C to act as a control. The three samples were then subjected to ultrasonic disintegration at 20kcyc./sec. and an amplitude of 1μm. for up to 30min. The ultrasonication was intermittently applied since the temperature tended to rise in spite of the ice jacket. Samples (1ml.) were removed at intervals and centrifuged at 3000g for 10min.; the lactate dehydrogenase activity was determined on the supernatant and cell counts were carried out on the sediment. After about 10min. of ultrasonication, 40% of the cells appeared whole and could still be stained. After 30min., however, only about 10% of the cells remained and these were of irregular shape and vacuolated. The results of the enzyme determination before ultrasonication showed that neither isoenzyme LDH₁ nor isoenzyme LDH₅ was adsorbed by the urinary sediment. During sonication there was a progressive increase in the activity released, although the enzyme was still being released into the supernatant after 30min. exposure to the ultrasound. The treatment was therefore repeated at an amplitude of 2μm. and similar results were obtained except that release of the enzyme was complete after 8min. (Fig. 2). Exposure of the cells to ultrasound for longer than this caused no further change in activity, suggesting that the enzyme was not destroyed. Some loss of activity did occur after prolonged treatment of the samples that contained the pure isoenzymes. The

isoenzymes LDH₁ and LDH₅ were, however, stable to sonication for 8 min. and this time was used in further experiments.

Cell counts carried out after this treatment showed that no more than 20% of the original cells were present and that these appeared to be damaged on microscopic examination.

Lactate dehydrogenase activities of urine and cells. The lactate dehydrogenase present in cells represented about 5–10% of the total excretion of the enzyme in normal urine. After the ingestion of aspirin by the subject, the lactate dehydrogenase activity in both urine and cells was higher than normal, but again that present in the cells repre-

sented 6–10% of the total enzyme excreted (Table 2).

The relative activities with pyruvate and 2-oxobutyrate as substrates were measured on both supernatant urine and the centrifuged cells and the

Fig. 2. Release of lactate dehydrogenase from urinary cells and stability of added isoenzymes LDH₁ and LDH₅ during ultrasonication at 20kcyc./sec. and an amplitude of 2 μ m. O, Control; $\square$, control + isoenzyme LDH₁; Δ , control + isoenzyme LDH₅.

Fig. 3. Lactate dehydrogenase activity in fractions of normal human urine (a) and urinary cells (b) separated by starch-block electrophoresis in phosphate buffer, pH 7.4. Samples with an activity of 100 nmol/min. were placed on the block. Alb and Hb denote the positions occupied by albumin and haemoglobin markers respectively and the arrow marks the point of application. The isoenzymes are numbered 1–5.

Table 2. *Excretion of lactate dehydrogenase and cells in normal urine and that collected 24–32 hr. after ingestion of 3 g. of aspirin*

Excretion results are given as means $\pm$ s.d.

Enzyme source	No. of samples	$10^{-3} \times$ No. of cells/hr.	Lactate dehydrogenase activity (nmol/min. excreted/hr.)		Lactate dehydrogenase relative activity: 2-oxobutyrate / pyruvate	Proportion of subunits as a percentage	
			Pyruvate	2-Oxobutyrate		H	M
Normal urine	10	—	155 ± 67	138 ± 61	0.89	82	18
Normal cells	10	259 ± 117	14 ± 6	11 ± 5	0.84	81	19
Urine after salicylate	9	—	256 ± 129	210 ± 94	0.83	78	22
Cells after salicylate	9	820 ± 565	23 ± 12	18 ± 10	0.80	76	24

Fig. 4. Starch-block electrophoresis of lactate dehydrogenase excreted in urine (a) and urinary cells (b) at 24–32 hr. after the ingestion by the subject of 3 g. of acetylsalicylate. Other details are as in Fig. 3.

results are also given in Table 2. The relative activity of the cellular enzyme was lower than that found in the supernatant with both normal urine and that excreted after the ingestion of aspirin. Aspirin treatment did, however, lower the relative activity of the enzyme in both cells and urine.

Starch-block electrophoresis. Recoveries were better than those previously reported (Plummer, Elliott, Cooke & Wilkinson, 1963; Plummer & Leathwood, 1967) and were usually greater than 90% of the activity applied to the block. The phosphate buffer used in this investigation has a number of advantages over the barbitone buffer employed on previous occasions in terms of the recoveries obtained and the finer resolution. Separation of the lactate dehydrogenase into isoenzymes by starch-block electrophoresis gave a similar pattern for all the samples. About 80% of the activity was present in the fast-moving peaks 1 and 2 with less in peak 3 and only trace amounts in peaks 4 and 5. The isoenzyme patterns found in normal cells and urine were almost identical (Fig. 3), but aspirin treatment caused a slight increase in

peak 2 compared with peak 1, although the isoenzyme patterns for cells and urine were again very similar (Fig. 4). Calculation of the percentages of H and M forms present in the samples demonstrated that cells and urine excreted after aspirin treatment showed a slight fall in the percentage of the H form compared with the M form (Plummer & Leathwood, 1967). This agrees very well with the changes observed in the relative activities (Table 2).

DISCUSSION

Starch-block electrophoresis shows that the lactate dehydrogenase of human urine has an isoenzyme composition similar to that of the kidney. Kinetic measurements confirm this conclusion, so it seems likely that the kidney is the main source of urinary lactate dehydrogenase. However, the properties of the enzyme found in urine are not exactly the same as those of the lactate dehydrogenase present in the kidney cortex and medulla (Plummer & Leathwood, 1967).

One other possible source not considered previously is the cells found in urine. Rupture of isolated washed cells by ultrasound liberates lactate dehydrogenase amounting to about 5–10% of the total excreted (Table 2). Electrophoresis of this cellular enzyme on starch grains shows that the isoenzyme distribution is the same as that of the supernatant urine used in previous studies. Measurement of the relative activity against pyruvate and 2-oxobutyrate as substrates (Rosalki & Wilkinson, 1960) gives a rapid means of assessing the isoenzyme pattern of a large number of samples without recourse to the more lengthy procedure of electrophoresis (Plummer *et al.* 1963; Wilkinson & Withycombe, 1965). If the value of this ratio is near 1.0 then the extract contains predominantly isoenzyme LDH₁ whereas a ratio of 0.2 indicates the presence of the slow-moving isoenzyme LDH₅. The relative activity values of 0.89 and 0.84 for urine and cells (Table 2) agree with the results of the starch-block electrophoresis, so the cells could well be the main source of the enzyme excreted in the urine. The isoenzyme pattern (Fig. 3) and relative activities (Table 2) for the lactate dehydrogenase of urine and cells excreted after salicylate treatment are also very similar, thus confirming this suggestion.

Mucus and debris are centrifuged with the cells, so the urinary enzyme could be trapped within the mucus or adsorbed on the surface of debris or whole cells. However, the cells were thoroughly washed in 0.14M-sodium chloride and 67mM-phosphate buffer, pH 7.4, and at this pH and ionic strength no measurable adsorption occurs (Hultin & Westort, 1966; Hultin, Westort & Southard, 1966). The determination of the lactate dehydrogenase activities on the supernatants after washing showed

that only trace amounts of enzyme were present in the initial wash; these might represent adsorbed enzyme or, more likely, urine trapped in the centrifuged pellet. When the purified isoenzymes LDH₁ and LDH₅ were mixed with the sediment and the supernatant was examined for activity, no adsorption was found and recoveries of 100% of the added enzymes were obtained. Full recoveries of isoenzymes LDH₁ and LDH₅ were also obtained after mixing them with the sediment and exposing the mixture to ultrasound for 8 min., although prolonged ultrasonication did cause a greater destruction of isoenzyme LDH₅ than of isoenzyme LDH₁. Thus the lactate dehydrogenase liberated after ultrasonication almost certainly represents the enzyme present in the urinary cells.

Most of the cells found in normal human urine are of the non-squamous type (Table 1). Ingestion of aspirin by the subject causes a greatly increased excretion of these cells, which probably arise from the epithelium of the renal tubule (Prescott, 1965*a,b*; Scott *et al.* 1963), although it is not known whether they are a representative sample of all the cells lining the renal tubule. Scott *et al.* (1963) found that about 30% of the cells stained positively for alkaline phosphatase, which may indicate that some of the cells arise from the lower regions of the nephron. Some cells in the kidney are exposed to quite high concentrations of the main metabolite of aspirin, salicylic acid, and these would probably be damaged more than others. Salicylic acid, with pK_a 3.0, is present in the glomerular filtrate largely in the ionized form, so that only a small quantity is reabsorbed in the proximal tubule. Water is, however, absorbed in this region and so the concentration of the salicylic acid in the tubule is quite high. Beyond the loop of Henle the glomerular filtrate becomes more acid, so that the concentration of undissociated salicylic acid increases. The kidney is also unique in that the action of the counter-current sodium pumps in the loops of Henle causes hyperosmoticity of fluids in the renal medulla. At the tip of the renal papilla the concentration may be as much as three or four times the iso-osmotic concentration. The amount of the drug that back-diffuses from the collecting ducts is therefore very high in the medulla and papillary region of the kidney.

After ingestion of aspirin there is a rise in the lactate dehydrogenase activity both in supernatant urine and the sediment, indicating some

cellular damage (Table 2). Examination of the relative activities of the enzyme in urine and cells suggests that the enzyme is slightly different from that normally present. This is confirmed by the results of the starch-block electrophoresis, which shows a relative increase in the proportion of M subunits, mostly as isoenzyme LDH₂. The pattern thus tends towards that of the kidney medulla (Plummer & Leathwood, 1967), in accordance with the suggested distribution of the drug within the kidney.

Thus results of the investigation of normal urine and cells confirm the previous suggestion (Plummer & Leathwood, 1967) that the kidney is the most likely source of the urinary enzyme. This conclusion is supported by examination of the enzyme excreted into the urine after the ingestion of salicylate.

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REFERENCES

- Clausen, E. & Harvald, B. (1961). *Acta med. scand.* **170**, 469.
 Houghton, M. A. & Pears, B. J. (1957). *Brit. med. J.* **i**, 622.
 Hultin, H. O. & Westort, C. (1966). *Arch. Biochem. Biophys.* **117**, 523.
 Hultin, H. O., Westort, C. & Southard, J. H. (1966). *Nature, Lond.*, **211**, 853.
 Klaus, D. (1958). *Klin. Wschr.* **36**, 207.
 Plummer, D. T., Elliott, B. A., Cooke, K. B. & Wilkinson, J. H. (1963). *Biochem. J.* **87**, 416.
 Plummer, D. T. & Leathwood, P. D. (1967). *Biochem. J.* **103**, 172.
 Plummer, D. T. & Leathwood, P. D. (1968). *Biochem. J.* **106**, 55*r*.
 Prescott, L. F. (1965*a*). *Lancet*, **ii**, 91.
 Prescott, L. F. (1965*b*). *Lancet*, **ii**, 299.
 Prescott, L. F. & Brodie, D. E. (1964). *Lancet*, **ii**, 940.
 Rosalki, S. B. & Wilkinson, J. H. (1959). *Lancet*, **ii**, 327.
 Rosalki, S. B. & Wilkinson, J. H. (1960). *Nature, Lond.*, **188**, 1110.
 Scott, J. T. & Denman, A. M. (1961). *Minerva Med.* **2**, 1405.
 Scott, J. T., Denman, A. M. & Dorling, J. (1963). *Lancet*, **i**, 344.
 Wilkinson, J. H. & Withycombe, W. A. (1965). *Biochem. J.* **97**, 663.
 Wootton, I. D. P. (1964). *Micro-analysis in Medical Biochemistry*, 4th ed., p. 138. London: J. and A. Churchill Ltd.