REFERENCES

1. E. Goldstein, G. W. Jordan and M. R. MacKenzie, *A. Rev. Pharmac. Toxic.* **16**, 447 (1976).
2. G. M. Green, G. T. Jakab, R. B. Low and G. S. Davis, *Am. Rev. respir. Dis.* **115**, 479 (1977).
3. E. Goldstein, in *Pulmonary Macrophages and Epithelial Cells*, ERDA Symposium Series 43 (Eds. C. L. Sanders, R. P. Schneider, G. E. Dagle and H. A. Ragan), p. 382. Technical Information Center, Energy Research and Development Administration, Springfield, VA. (1977).
4. D. E. Gardner and J. A. Graham, in *Pulmonary Macrophages and Epithelial Cells*, ERDA Symposium Series 43 (Eds. C. L. Sanders, R. P. Schneider, G. E. Dagle and H. A. Ragan), p. 1. Technical Information Center, Energy Research and Development Administration, Springfield, VA. (1977).
5. G. Franken, H. Lüllmann and A. Siegfriedt, *Arzeneimittel-Forsch.* **20**, 417 (1970).
6. R. Lüllmann-Rauch and G. H. Reil, *Toxic. appl. Pharmac.* **30**, 408 (1974).
7. D. Heath, P. Smith and P. S. Hasleton, *Thorax* **28**, 551 (1973).
8. Z. Hruban, A. Slesers and I. Aschenbrenner, *Toxic. appl. Pharmac.* **26**, 72 (1973).
9. M. J. Reasor, R. A. Koshut, M. J. McNulty and M. A. Trush, *Toxic. appl. Pharmac.*, **52**, 497 (1980).
10. D. B. Drath, A. Harper, J. Gharibian, M. L. Karnovsky and G. L. Huber, *J. cell. Physiol.* **95**, 105 (1978).
11. D. B. Drath, M. L. Karnovsky and G. L. Huber, *J. appl. Physiol.: Respirat. envir. Exercise Physiol.* **46**, 136 (1979).
12. J. B. L. Gee and A. S. Khandwala, in *Respiratory Defense Mechanisms* (Eds. J. D. Brain, D. F. Proctor and L. Reid), p. 927. Marcel Dekker, New York (1978).
13. B. D. Cheson, R. L. Christensen, R. Sperling, B. G. Koher and B. M. Babior, *J. clin. Invest.* **58**, 789 (1976).
14. P. R. Miles, V. Castranova and P. Lee, *Am. J. Physiol.* **235**, C103 (1978).
15. E. H. White and R. B. Brundrett, in *Chemiluminescence and Bioluminescence* (Eds. W. D. McElroy and B. Glass), p. 183. The Johns Hopkins Press, Baltimore (1961).
16. R. C. Allen and L. D. Loose, *Biochem. biophys. Res. Commun.* **69**, 245 (1976).
17. P. Stevens, D. J. Winston and K. Van Dyke, *Infect. Immun.* **22**, 41 (1978).
18. K. Van Dyke, M. Trush, M. Wilson, P. Stealey and P. Miles, *Microchem. J.* **22**, 463 (1977).
19. G. E. Hatch, D. E. Gardner and D. B. Menzel, *Pharmacologist* **20**, 276 (1978).
20. M. J. Reasor, R. A. Koshut and V. Castranova, *Expl molec. Path.* **31**, 297 (1979).
21. G. W. Snedecor and W. G. Cochran, *Statistical Methods*, p. 130. Iowa State University Press, Ames (1967).
22. H. Lüllmann, R. Lüllmann-Rauch and O. Wassermann, *Biochem. Pharmac.* **27**, 1103 (1978).

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Interaction between arsenic and alloxan-induced diabetes—effects on rat urinary enzyme levels*

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General kidney dysfunction has been detected previously in patients afflicted with pathological renal conditions by monitoring the presence of aberrant urinary enzyme patterns [1–4]. The microvascular changes associated with diabetes mellitus result in clinically important effects on the kidneys as well as on other organs. Diabetic patients have various degrees of nephropathy and of elevated urinary enzyme excretion, when compared to non-diabetic subjects [5, 6]. Increased urinary excretion of lyosomal enzymes is presumed to reflect secondary renal tubular involvement and to serve as a sensitive indicator of the onset of diabetic nephropathy [7]. In addition, experimental animals given either nephrotoxins [8–11] or an inducer of diabetes [12] have been found to produce elevated urinary enzyme levels. Enzymes with characteristic subcellular locations may be selected to indicate the site of the primary event and to determine the extent to which the various cellular compartments are involved [9].

The effects of environmental agents on urinary enzyme levels in diabetes have not been studied. Some environmental agents that accumulate in the kidney, however, also produce effects on normal renal function [13]. Identification of an interaction between the renal lesions of diabetes and exposure to an environmental toxin is potentially significant with respect to the interpretation of clinical findings and assessment of renal damage. The alteration of urinary enzyme levels is used in this study as a convenient, non-invasive, non-destructive method for assessing such an interaction.

This investigation is focused on the interaction between an oral exposure to chronic low doses of an environmental nephrotoxin and the diabetic state. The model system studied is the alloxan-diabetic rat with previous exposure to low levels of sodium arsenate (As^{5+}) in the drinking water. Alloxan has been shown previously to produce damage specifically in the insulin-secreting β-cells present in the pancreas of laboratory animals [14, 15]. The inability of the affected islet tissue to secrete insulin provides a model for inducing experimental diabetes and studying the ontogeny of the diabetic state. If the dosage of alloxan is higher than that required to damage the β-cells, definite effects may be observed on other tissues [16–19]. The levels of several key lysosomal enzymes (acid phosphatase and *N*-acetyl-β-D-glucosaminidase) and non-lysosomal enzymes (alkaline phosphatase, lactate dehydrogenase and L-glutamate oxaloacetate transaminase) from these animals were mon-

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Table 1. Activities of selected enzymes in urines from normal and alloxan-diabetic rats exposed to arsenic in the drinking water for 3 weeks

	Specific activity*					
	Lactate dehydrogenase	L-Glutamate-oxalo-acetate transaminase	Alkaline phosphatase	Acid phosphatase	β-Glucuronidase	*N*-Acetyl-β-D-glucosaminidase
Normal						
Control	159 ± 38	156 ± 31	544 ± 114	245 ± 38	65.2 ± 33.5	369 ± 79
Arsenic	145 ± 50	138 ± 25	787 ± 107†	306 ± 92	38.1 ± 21.3	381 ± 110
Alloxan-diabetic						
Control	197 ± 26	184 ± 53	541 ± 136	602 ± 126†	38.7 ± 14.4	275 ± 65
Arsenic	412 ± 17†‡	685 ± 104†‡	823 ± 234	969 ± 105†‡	154 ± 79†‡	714 ± 62†‡

* Values are specific activities given as the mean ±1 S.E.M. for duplicate determinations on three to six separate urine samples. Enzyme specific activities are expressed as nmoles per min per milligram of urinary protein (mU/mg).
† Statistically significant difference from control (two $P < 0.10$) based on the two-sided U test [25].
‡ Statistically significant difference from normal arsenic (two $P < 0.10$) based on the two-sided U test [25].

itored in the urine. In addition, β-glucuronidase levels were monitored.

Adult male Charles River CD rats were given access to a casein-based purified diet and to deionized drinking water containing 0 or 40 ppm of arsenic as sodium arsenate ($Na_2HAsO_4 . 7H_2O$) for a period of 3 weeks. The 40 ppm dose of arsenic was chosen because no mortality or overt signs of toxicity occurred among these animals at this level [20]. After 3 weeks, one-half of the 0 ppm rats and one-half of the 40 ppm rats were made diabetic by injecting 40–50 mg/kg of alloxan monohydrate (freshly prepared and administered via the tail vein while depressing the renal area). This dose and method of administration have been used successfully in inducing the diabetic state in the rat [21]. Injected alloxan disappears from the blood within 3–5 min. Two days after injection, urines were collected, free of fecal contamination from diabetic and normal rats, for 24 hr in restraining cages kept on ice. There were no significant differences in the urine volumes collected during this period. All animals had access to water (0 or 40 ppm As^{5+}) during the collection period. The data in Table 1 are from animals that were not fed during the collection period.

After the 24-hr collection period, aliquots of urine were processed as described previously by Kuwahara *et al.* [12], before measuring the enzymes. Serum glucose levels were checked with Clinistix and then quantitated by the glucose oxidase method [4]. The alloxan-diabetic rats had serum glucose concentrations of more than 30 mM, which is 5-fold higher than the normal levels. Multistix were used to check urinary levels qualitatively; no changes other than in glucose content were detected. The arsenic level in rat urine after a 6-week oral exposure to 40 ppm was 6–10 mg/l (for a 24-hr urine collection) as determined by proton-induced X-ray analysis. The final concentration of arsenic in the enzyme assays would be less than 3–5 μM. In each experiment, all assays were performed with urines from three separate animals per group and in duplicate at 37°. The spectrophometric enzyme assays were described in detail previously as follows: lactate dehydrogenase (EC 1.1.1.27) and L-glutamate-oxaloacetate transaminase (EC 2.6.1.1.) [22], alkaline phosphatase (EC 3.1.3.1) and acid phosphatase (EC 3.1.3.2) [4], β-glucuronidase (EC 3.2.1.31) [23], and *N*-acetyl-β-D-glucosaminidase (EC 3.2.1.30) [12]. Enzyme activities are expressed as nanomoles of substrate utilized per minute per milligram of urinary protein (mU/mg). The protein content was measured using the method Lowry *et al.* [24].

The effects of a 3-week oral arsenic exposure on normal and alloxan-diabetic rats, as reflected in urinary enzyme levels, are given in Table 1. Alkaline phosphatase, an enzyme usually associated with the tubular brush border, was the only enzyme found to be increased significantly in the normal, arsenic-treated animals. Alloxan diabetes produced a statistically significant effect on the excretion of acid phosphatase, an enzyme usually associated with the lysosomes. This effect was increased further by the arsenate treatment. β-Glucuronidase, an enzyme localized in both microsomes and lysosomes, was increased significantly by these combined treatments.

In addition, each of the other enzymes measured (*N*-acetyl-β-D-glucosiminidase, lactate dehydrogenase and L-glutamate-oxaloacetate transaminase) was markedly elevated only in the alloxan-diabetic rats that were exposed previously to oral arsenic. Both the lysosomal enzymes (acid phosphatase and *N*-acetyl-β-D-glucosaminidase) and the non-lysosomal enzymes (lactate dehydrogenase and L-glutamate-oxaloacetate transaminase) were affected. This pattern of elevation was observed whether the animals were fasted or fed during the collection period.

Arsenic is one of the more common trace elements in the environment. Substantial concentrations of arsenic are present in coal and fossil fuels [26], and increased utilization of these energy sources can be expected to release even

larger quantities of this element into biological eco-systems. There are two common forms of inorganic arsenic in the environment, arsenite (As^{3+}) and arsenate (As^{5+}). Arsenite is considered the more toxic of the two [27], but arsenate is more common [28, 29]. The kidney is a target for arsenic toxicity as it provides the major route of arsenic excretion from the body [30] and accumulates this element to high levels [31, 32]. Recent studies in our laboratories have assessed the *in vivo* effects of chronic oral exposure to arsenate on the rat kidney [33].

Although diabetic acidosis can kill rapidly and is, therefore, the most fearful complication of the disease, it is now less important as a cause of disability than the results of changes in the blood vessels. While many parts of the body are affected, clinically important microvascular effects are produced in the kidney, retina, nervous system, and skin. The exact causes of these changes are unknown at present. The kidneys usually have microvascular changes on biopsy during the preclinical stage or very early after the disease is diagnosed [34]. Glomerulosclerosis with renal failure is the most common cause of death in juvenile diabetes. However, the earliest time of appearance of proteinuria was at the ninth year of known disease [35, 36].

In summary, there are numerous clinical and experimental reports which describe elevated urinary enzyme levels associated with diabetes and also with kidney dysfunction [4–6, 28, 34]. The most striking finding of the current study, however, is the marked, statistically significant increase in urinary enzyme levels of the alloxan-diabetic animals that were exposed previously to oral arsenic. The arsenic, alloxan-diabetic interaction in rats appears to reflect an exacerbation of the nephropathy associated with diabetes. The value of these data lies in the recognition of the necessity to re-evaluate clinical findings from the diabetic and prediabetic population which is also exposed to environmental nephrotoxins.

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REFERENCES

1. R. Sandman, R. M. Margules and S. L. Kountz, *Clinica chim. Acta* **45**, 349 (1973)
2. R. J. Price, N. Dance, B. Richard and W. R. Cattell, *Clinica chim. Acta* **27**, 65 (1970).
3. N. Dance, R. G. Price, W. R. Cattell, J. Lansdell and B. Richards, *Clinica chim. Acta* **27**, 87 (1970).
4. H. U. Bergmeyer, *Methods of Enzymatic Analysis*, Vol 1–3. Academic Press, New York (1974).
5. F. Belfiore, L. LoVecchio, E. Napoli and V. Borzi, *Clin. Chem.* **20**, 1229 (1974).
6. F. M. Bomback, S. Nakagawa, S. Kumin and H. M. Nitowsky, *Diabetes* **25**, 420 (1976).
7. D. Maruhn, K. Rosenstiel, K. Hennewig and D. Paar, *Z. klin. Chem. klin. Biochem.* **12**, 5 (1974).
8. K. Nomiyama, A. Yamamoto and C. Sato, *Toxi. appl. Pharmac.* **27**, 484 (1974).
9. P. J. Wright and D. T. Plummer, *Biochem. Pharmac.* **23**, 65 (1974).
10. B. B. Kirschbaum and H. B. Bosmann, *Chem. Biol. Interact.* **8**, 207 (1974).
11. W. E. Stroo and J. B. Hook, *Toxic appl. Pharmac.* **39**, 423 (1977).
12. M. D. Kuwahara, S. A. Lyons, P. D. Rosenblit and R. P. Metzger, *Proc. Soc. exp. Biol. Med.* **153**, 305 (1976).
13. G. E. Schreiner, in *Structural Basis of Renal Diseases* (Ed. E. L. Becker), p. 703. Harper & Row, New York (1968).
14. J. S. Dunn, H. L. Sheehan and N. G. B. McLetchie, *Lancet* **i**, 484 (1943).
15. J. S. Dunn, E. Duffy, M. K. Gilmour, J. Kirkpatrick and N. G. B. McLetchie, *J. Physiol., Lond.* **103**, 233 (1944).
16. M. G. Goldner and G. Gromori, *Endocrinology* **33**, 297 (1943).
17. C. J. Diaz, R. Picatoste, E. L. Garcia and M. M. Pleguezuelo, *Revta clín. esp.* **31**, 15 (1948).
18. C. T. Test, H. T. Ricketts, P. E. Steiner, E. S. Peterson, H. A. Lints and N. Tupikova, *Proc. Soc. exp. Biol. Med.* **78**, 21 (1951).
19. S. Ramfjord, *Am. J. clin. Path.* **22**, 745 (1952).
20. B. A. Fowler, J. S. Woods and C. M. Schiller, *Envir. Hlth Perspec.* **19**, 197 (1977).
21. M. L. Halperin, *Biochem. J.* **124**, 615 (1971).
22. C. M. Schiller and R. Walden, *Devl. Biol.* **60**, 130 (1977).
23. G. W. Lucier and O. S. McDaniel, *Biochim. biophys. Acta* **261**, 168 (1972).
24. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
25. A. Goldstein, *Biostatistics: An Introductory Text*, p. 55. Macmillian, New York (1964).
26. B. A. Fowler, in *Advances in Modern Toxicology 2: Toxicology of Trace Elements* (Eds. R. A. Goyer and M. A. Mehlman), p. 79. Hemisphere Publishing, Washington, DC (1977).
27. B. L. Vallee, D. D. Ulmer and W. E. C. Wacker, *Archs envir. Hlth* **21**, 132 (1960).
28. R. S. Braman and C. C. Foreback, *Science* **182**, 1247 (1973).
29. D. B. Frost, *Fedn Proc.* **26**, 194 (1967).
30. F. T. Hunter, A. F. Kip and J. W. Irvine, *J. Pharmac. exp. Ther.* **76**, 207 (1942).
31. J. M. Ginsburg, *Am. J. Physiol.* **208**, 832 (1965).
32. M. R. Mayers, *Archs ind. Hyg.* **9**, 384 (1954).
33. M. M. Brown, B. C. Rhyne, R. A. Goyer and B. A. Fowler, *J. Toxic. envir. Hlth* **1**, 505 (1976).
34. P. K. Bondy, in *Textbook of Medicine* (Eds. P. B. Beeson and W. McDermoth), p. 1175. W. B. Saunders, Philadelphia (1967).
35. H. C. Knowles, in *Diabetes in Juveniles, Modern Problems in Pediatrics* (Ed. Z. Laron), Vol. 12, p. 297. S. Karger, New York (1975).
36. J. M. Court and M. Dunlop, in *Diabetes in Juveniles, Modern Problems in Pediatrics* (Ed. Z. Laron), Vol. 12, p. 307. S. Karger, New York (1975).