D. M., Goldberg, B., *J. Pharm. Exp. Therap.,* 1948, v94, 249.

6. Hartman, H. A., Stitch, H. F., *Science,* 1957, v125, 445.

7. Rudberg, T., *Acta Pharmacol. Toxicol.,* 1957, v13, 233.

8. Bachuber, T. E., Lalich, J. J., Angevine, D. M., Schilling, E. D., Strong, F. M., PROC. SOC. EXP. BIOL. AND MED., 1955, v89, 294.

9. Azima, H., Grad, B., *Fed. Proc.,* 1956, v15, 17.

10. Widlocher, D., Nakajima, H., Thullier, J., *C. R. Soc. Biol., Paris,* 1957, v151, 668.

11. Selye, H., *J. Clin. and Exp. Psychopath.,* 1958, v19, 97.

12. Scholler, J., Philips, F. S., Sternberg, S. S., PROC. SOC. EXP. BIOL. AND MED., 1957, v96, 491.

13. Siegmund, E., Cadmus, R., Lu, B., *ibid.,* 1957, v95, 729.

Received March 28, 1960. P.S.E.B.M., 1960, v104.

Urinary Sulfate Ion as a Naturally Occurring Inhibitor of DNase II Activity.* (25845)

ELIZABETH C. RAUENBUSCH AND KURT I. ALTMAN (Introduced by H. A. Blair)
Division of Experimental Radiology, University of Rochester School of Medicine and Dentistry, Rochester, N. Y.

It was reported(1) that human urine inhibits its enzymatic activity of desoxyribonuclease II (DNase II) and activates DNase I slightly. The inhibitory material was dialyzable, thermostable and was not retained by anion and cation exchange resins. In the experiments to be described, an attempt was made to establish the chemical identity of the urinary inhibitor of DNase II.

Methods. 1. *Materials.* a. *Desoxyribonuclease II.* DNase II was prepared from calf spleen using the first 3 steps of the preparative procedure of Koszalka *et al.*(2). The sediment obtained in Step 3 was dissolved in a small volume of water and dialyzed overnight against running tap water at room temperature. In those experiments concerned with effect of inorganic ions on enzymatic activity, the preparation was dialyzed against 0.0001% aqueous sodium versenate. b. *Desoxyribonucleic acid.* DNA of salmon sperm was used as purchased from Calif. Fn. for Biochemical Research. c. *Urinary inhibitory material.* 890 ml of freshly voided human urine was dialyzed overnight against 1,080 ml of distilled water. The resulting 960 ml of dialysate was concentrated to 265 ml by lyophilization. In some experiments, the concentrated dialysate was passed subsequently through a column of activated charcoal (80 mesh). d. *Ashing of inhibitory material.* To determine whether the inhibitor removed by the ion-exchange resins was organic or inorganic in nature, the solid residue remaining after evaporation of solvent from the charcoal-treated urinary dialysate was ashed by heating to redness in a porcelain crucible over a Bunsen burner. The white ash so formed was dissolved in 1 N HCl and the resulting solution evaporated to dryness repeatedly, adding water after each evaporation. The residue was then dissolved in a volume of water equal to that of initial concentrated urinary dialysate. 2. *Analytical procedures.* a. *Assay of DNase II activity.* Enzymatic activity was determined by an adaptation of the procedure for DNA determination described by Schneider and Hogeboom(3). Unless stated otherwise, the following test system was employed:

Acetate buffer, 0.2 N, pH 5.2	2.0 ml
DNA, 0.4% solution	.5 "
Distilled water	1.0 "
DNase II	.2 "

This mixture was incubated for one hour at 37°C and then deproteinized with 1 ml of 2.88 M trichloroacetic acid (TCA). Simultaneously, a similar mixture, lacking DNA, was incubated and then deproteinized with TCA. To the latter deproteinized reaction mixture, 0.05 ml of a 0.4% solution of DNA

* This work was supported, in part, by a grant from the U. S. Atomic Energy Commission.

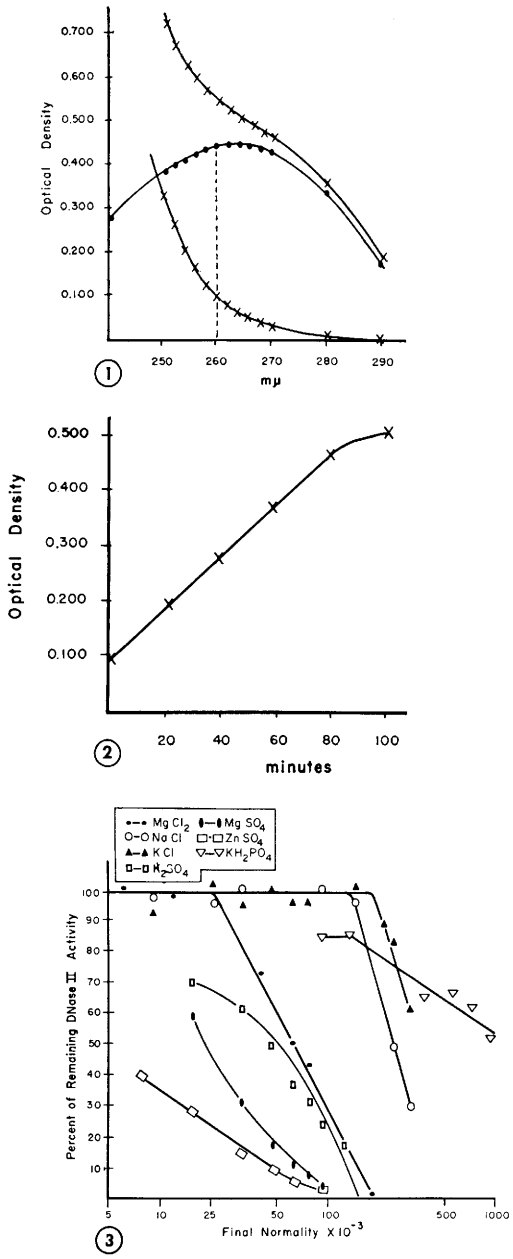

FIG. 1. Optical densities of incubation mixture as a function of wave length. Top curve represents incubation mixtures incubated with DNA. Bottom curve shows a similar plot for an incubation mixture devoid of DNA. Intermediary curve is a representation of the difference between top and bottom curves. Point of maximal difference is designated by broken guide line.

FIG. 2. Change in optical density as a function of time of incubation.

FIG. 3. Inhibitory effect of several cations and anions on activity of DNase II as a function of concentration of the inorganic ions. Concentration (normality) is plotted on logarithmic scale.

was added. The TCA-insoluble material present in both mixtures was removed by centrifugation and the supernatant solutions were diluted 20-fold with water. Subsequently, optical densities of the supernatant solutions were determined at 260 μ using a Beckman Spectrophotometer, Model DU. The enzymatic activity is expressed in terms of the difference between optical density of the complete system and that of the system devoid of DNA. The wave length used to measure changes in DNA as a result of enzymatic action was chosen on the basis of data shown in Fig. 1. In this figure, optical densities of supernatant solutions obtained after deproteinizing the reaction mixtures incubated either with or without DNA are plotted as a function of wave length for the region from 240 to 290 mμ and are represented by the top and bottom curves, respectively. The difference between these 2 optical density values for each wave length studied is shown as in the central curve. The point of maximal difference located at 260 mμ was used in DNase assays. Time of incubation was always one hour. Fig. 2 shows the relationship between change in optical density and time of incubation of the test systems. b. *Evaluation of inhibition of DNase II.* Degree of inhibition of DNase II is expressed as percent of remaining DNase II activity. The reaction mixture was incubated for one hour at 37°C. It had a composition similar to that of the complete test system for DNase II activity, but contained 1.0 ml of the urinary dialysate or of other solutions to be tested for inhibitory activity, instead of the distilled water added in the usual DNase II assay. The difference between enzyme activities in presence and absence of inhibitory material could then be evaluated in terms of percent of remaining DNase II activity. When the effect of the ionic milieu on DNase II activity was investigated, the composition of the system employed to test for inhibitory activity was modified by a) replacing urinary dialysate with 1 ml of a solution containing varying concentrations of solute, and b) by using 1.5 ml of acetate buffer instead of 2.0 ml.

Results. The effects on DNase activity of

TABLE I. Degree of Inhibition of DNase II Activity by Dialysates of Urine.

Materials tested	% of remaining enzymatic activity
Complete test system (without urinary dialysate)	100
Freshly voided urine	83
Concentrated urinary dialysate	67
Idem after treatment with charcoal	78*
Concentrated urinary dialysate†	(a) 84 (b) 76
Ashed dialysate†	(a) 79 (b) 81

In all assays 1.0 ml of solution containing inhibitory activity was used.

* This value is an avg of 3 different preparations.

† (a) and (b) represent determinations on 2 different experiments in which the dialysates are compared before and after ashing.

freshly voided urine and of concentrated urinary dialysate with or without subsequent charcoal treatment are shown in Table I; for comparison, DNase II activity without addition of inhibitory material is also given. Charcoal treatment of the concentrated urinary dialysate had no effect on inhibitor activity. Although it would appear that a considerable fraction of the inhibitory activity was lost on dialysis, this aspect remains uncertain since completeness of dialysis as well as possible interference with the inhibitor assay of other substances in crude urine could not be ascertained satisfactorily.

After charcoal treatment the inhibitory material in urinary dialysate had an affinity for certain ion exchange resins and in this respect appears to differ from dialysates not treated with charcoal since it had been observed earlier(1) that inhibitory material present in untreated dialysate passed through various ion exchange columns. The effect of charcoal treatment is probably due to removal by charcoal of substances which are exchanged more readily by the resin than is the inhibitory material. When charcoal-treated dialysate was passed in succession through 2 ion exchange columns, *i.e.*, Dowex 50 (H+) and Dowex 1 (OH–), it was observed that the effluent solution no longer inhibited DNase II.

Ashing had no detectable effect on inhibitory activity of the serum sample under the conditions of study (Table I). Ability of the inhibitory material to withstand high temperature suggests that the activity is due to inorganic ions normally present in urine. To obtain further evidence for this interpretation, inhibitory effect of various cations and anions on DNase II activity was studied. Confirming observations of Sinsheimer and Koerner (4) and of Shack(5) divalent ions had a stronger inhibitory effect than monovalent ions (Fig. 3). To obtain further quantitative information concerning concentration of ions most likely to cause inhibition in human urine, aliquots of charcoal-treated dialysates taken before and after ashing were analyzed for Ca^{++}, Mg^{++}, phosphate and sulfate.† Only sulfate ions were present in significant amounts. Although most of the sulfate ions were present in the dialysate before ashing, a small, additional amount must have been formed as a result of ashing of organically-bound sulfates or of organic sulfur compounds present in urine.

Assuming that sulfate ions are largely responsible for the inhibitory activity in urinary dialysates, one may calculate the expected degree of DNase inhibition from concentration of sulfate ions known to be present. By applying the inhibition curve obtained for K_2SO_4 (Fig. 3), the expected inhibitory activity corresponding to concentrations of sulfate found before and after ashing was calculated with the following results (average from 2 experiments):

	% of remaining enzyme activity	
	Calculated	Observed
Concentrated urinary dialysate after charcoal treatment	73	78
Ashed dialysate	86	81

The data presented above demonstrate that the order of magnitude of predicted degree of inhibition is in reasonably good agreement with average inhibition found experimentally. The experimentally obtained value of inhibition may be only an approximate value because, in the range of inhibition determined, sensitivity of the test system may be insufficient for detection of small variations in

† Analytical determinations were carried out by Schwarzkopf Microanalytical Lab., Woodside, N. Y.

inhibitor activity. Thus, inhibition of 15%-20% involves uncertainty factors of an order which would make it difficult to compare accurately the degree of inhibition obtained.

Excretion of total sulfur and phosphate (as P) in "normal" human urine is reported as approximately 1.0 g and 1.1 g, respectively, per day(6). Inasmuch as sulfate ions constitute 92% of total sulfur excreted daily(6), it is conceivable that the number of sulfate ions present in the urinary dialysate are sufficient to produce the described inhibitory effect. Quantity of sulfate ions in concentrated urinary dialysates is approximately 8.0 mg/ml or 8×10^{-2} M. This concentration of sulfate was found to inhibit DNase II effectively (Fig. 3).

The data (Fig. 3) suggest that in charcoal-treated urinary concentrates sulfate ions might be largely responsible for inhibition of DNase II. Phosphate ions probably contribute little to inhibition of DNase II in the case of the preparations used since removal of phosphate by charcoal did not affect degree of inhibition. Thus, although phosphate ions may have an inhibitory effect, their contribution to inhibition under our experimental conditions (Table I) appears to be small.

Summary. 1. Urinary dialysates were found to retain inhibitory activity toward DNase II after ashing. 2. Chemical analysis of the charcoal-treated dialysate before and after ashing indicated presence of detectable quantities of sulfate ions but not of Mg^{++} and Cu^{++}. 3. Various divalent ions, in contrast to monovalent ions, had a marked inhibitory effect on DNase II. 4. The data suggest that a major portion of urinary inhibitory activity with respect to DNase is due to presence of sulfate ions in the concentrated urinary dialysate.

1. Kowlessar, O. D., Okada, S., Potter, J. L., Altman, K. I., *Arch. Biochem. Biophys.,* 1957, v68, 231; Koszalka, T. R., Schreier, K., Altman, K. I., *Biochim. Biophys. Acta,* 1954, v15, 194.

2. Koszalka, T. R., Falkenheim, R. N., Altman, K. I., *Biochim. Biophys. Acta,* 1957, v23, 647.

3. Schneider, W. C., Hogeboom, G. H., *J. Biol. Chem.,* 1952, v198, 155.

4. Koerner, J. F., Sinsheimer, R. L., *ibid.,* 1957, v228, 1039.

5. Schack, J., *ibid.,* 1957, v226, 573.

6. Hauck, P. B., Oser, B. L., Summerson, W. H., *Practical Physiological Chemistry,* The Blakiston Co., N. Y., 1947.

Received March 30, 1960. P.S.E.B.M., 1960, v104.

Protection of Mouse Bone Marrow by Inorganic Compounds During Freezing and Thawing. (25846)

PHAN THE TRAN* AND M. A BENDER (Introduced by C. C. Congdon)
Biology Division, Oak Ridge National Lab.,† Oak Ridge, Tenn.

It has long been known that glycerol protects mammalian cells against freezing and thawing damage(1). Lovelock showed that human red blood cells are protected against hemolysis not only by glycerol but also by a number of neutral organic solutes of low molecular weight(2). He attributed freezing damage to the increase in electrolyte concentration that occurs when pure ice is formed in and around the cells, and the protective action of organic solutes to reduction of electrolyte concentration in and around the cells because of the colligative properties of solutions. However, low concentrations of inorganic ions will protect bacteria against freezing and thawing damage(3). A similar protection, if it could be demonstrated for mammalian cells, would be contrary to Lovelock's hypothesis. We tested a number of inorganic compounds for ability to protect mouse bone marrow cells against freezing and thawing

* Visiting investigator on leave from Atomic Energy Office, Saigon, Viet Nam. Present address: Atomic Energy Office, Saigon, Viet Nam.

† Operated by Union Carbide Corp. for U. S. Atomic Energy Comm.