A NOTE ON THE DETERMINATION OF β -GLUCURONIDASE ACTIVITY IN URINE

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Interest in urinary β -glucuronidase has recently developed from the work of Boyland, Wallace, and Williams (1955), who found high levels of this enzyme in the urine of patients with carcinoma of the bladder. The method used by these authors was modified from that of Talalay, Fishman, and Huggins (1946). After a careful study of the following points, however, it was found necessary to reinvestigate and modify this method.

Adjustment of the pH Value

Adjustment of the pH value is important for two reasons.

(1) Before incubating the urine with the acetate buffer-substrate mixture, it must be adjusted to the correct pH, since its buffering capacity may exceed that of the acetate buffer, and hence the final pH of the mixture will be closer to that of the original urine. The acetate buffer itself must not be less than 0.1 M to be of an effective concentration in undiluted urine. After accurate adjustment using a pH meter, β -glucuronidase activity was determined at pH 4.5 and at pH 5.2 in over 250 24-hour collections of normal and pathological urines. Also the enzyme activity was investigated over the complete pH range of 3.6 to 5.6, using acetate buffer in a number of normal and pathological urines, with and without dilution of urine. In the majority of these cases the enzyme activity at pH 5.2 in undiluted urine was higher than that at pH 4.5. The difference in activity at the two pH values in some pathological cases was considerable. On diluting the urine, and especially in the presence of added protamine, the activity in normal urine was optimal at pH 4.5.

(2) At the end of the incubation period, when alkaline buffer solution was added to develop the phenolphthalein colour, the adjustment of pH is again critical. The colour production is maximum between pH 10.4 and 11.2, but is very unstable

in solutions which are more alkaline than pH 10.6. Hence the importance of an efficient buffer solution, the addition of which to the mixture will always produce a final pH within the range 10.4 to 10.5 where the maximum colour is combined with stability. Glycine buffer used by previous workers was found unsatisfactory for this purpose, while the sodium carbonate solution described below proved to be much more efficient.

The Substrate Concentration

The Km for urinary β -glucuronidase with phenolphthalein glucuronide substrate was found to be 0.00108, thus the optimum substrate concentration is slightly over 2 mM. Hence the considerable variation in enzyme activity at different substrate concentrations. In the Fishman unit, which has been commonly used for expressing this enzyme activity in blood plasma and certain other body fluids, 1 mM substrate concentration was used. It was thought advisable, for the sake of comparison, to use the same substrate concentration (mM).

Enzyme Activity in Undiluted and Diluted Urine

The presence of certain β -glucuronidase inhibitors in urine has already been shown (Abul-Fadl, 1957). The activation of the urinary enzyme by protamine after removing the inhibitory effect by dilution or dialysis was also described. Thus, in order to determine the total amount of enzyme excreted, it would be necessary to test the activity in diluted urine at pH 4.5 and in the presence of protamine. A dilution of 10 was found to be adequate in most cases to overcome the inhibitory effect. In order to determine the enzyme activity which is effective in the bladder, the undiluted urine adjusted to the correct pH should be used with the minimum possible volume of concentrated buffered substrate.

Contaminants in Urine

Infection.—Specimens of sterile normal urine were inoculated with *Escherichia coli*, diphtheroids, *B. proteus*, *Staphylococcus pyogenes*, streptococci, and *Streptococcus faecalis*.

The glucuronidase activity was determined at pH 4.5 and 5.2 in dilute and undiluted, infected and sterile control urines, which had been incubated at 37° C. for 24 hours, then filtered clear and adjusted to the correct pH.

Diphtheroids did not seem to produce any change on the urinary β -glucuronidase activity, while staphylococcus and streptococcus infections caused an apparent increase (15–20%) in the enzyme activity of undiluted urine measured at both pH 4.5 and at 5.2. No significant change, however, could be detected when the enzyme activity was measured in diluted urine. *E. coli*, on the other hand, produced an apparent decrease (about 15%) in enzyme activity at pH 4.5 in undiluted urine, but produced an increase (about 50%) in the β -glucuronidase activity at pH 5.2. With *B. proteus* infection, however, the enzyme activity at pH 4.5 was markedly decreased (30–50%); while that at pH 5.2 was less affected (10–20% decrease). This enzyme destruction by *B. proteus* might be attributed to the high alkalinity produced by this organism (pH 9.3). *Streptococcus faecalis* seemed also to cause some decrease (about 10%) of the urinary β -glucuronidase activity at pH 4.5 and 5.2.

Cellular Matter.—Pus cells did not seem to affect the β -glucuronidase activity of filtered urine. Fishman, Springer, and Brunetti (1948) found that human erythrocytes contained little or no β -glucuronidase activity. Other cellular matter, such as epithelial cells, in urine was found to contain variable levels of β -glucuronidase activity which, sometimes, were extremely high.

Non-cellular Contaminants.—Diluted blood plasma was found to contain at least five times as much β -glucuronidase activity as undiluted urine. Serous exudates from inflamed areas were found to be very high in β -glucuronidase activity, and, as contaminants in urine, would contribute to elevated enzyme levels. Post-operative specimens of urine showed, in most cases, very high β -glucuronidase activity. Bile, ammonium salts, etc. (Abul-Fadl, 1957), were found to inhibit the enzyme activity in undiluted urine, but their effect was overcome by dilution. Antibiotics did not seem to have a direct inhibitory effect on the urinary enzyme *in vitro*, but their administration

(especially the terramycin group) caused a considerable diminution in β -glucuronidase excretion in urine.

Method

Acetate Buffer Solutions.—The following were used:

2M Buffer pH 5.2.—Crystalline sodium acetate A.R. ($C_2H_3O_2Na \cdot 3H_2O$), 43.5 g., and 4.6 ml. glacial acetic acid A.R. are dissolved in 100 ml. distilled water.

2M Buffer pH 4.5.—Crystalline sodium acetate, 26.1 g., and 12 ml. glacial acetic acid are dissolved in 100 ml. distilled water.

0.05M Buffer pH 4.5 Containing 0.05% Protamine Sulphate.—Protamine sulphate, 50 mg., is dissolved in 20 ml. distilled water, 2.5 ml. of the above 2M acetate buffer solution, pH 4.5, is added and the volume made up to 100 ml. with distilled water.

In each case the pH should be checked with a pH meter and solutions kept in the cold, preserved with chloroform.

M/50 Substrate Solutions.—Crystalline phenolphthalein glucuronide (Sigma), 50 mg., is dissolved in 5 ml. distilled water and kept in the refrigerator. This solution was kept for over three months without deterioration.

Concentrated Sodium Carbonate Solution pH 10.5.—One hundred and fifty millilitres of 20% sodium hydroxide solution are added to 11.8% sodium bicarbonate solution and the pH adjusted to 10.5 using a pH meter.

Standard Phenolphthalein Solution.—Phenolphthalein, 18 μ g./ml. (1 ml. = 1 unit), is dissolved in 100 ml. ethanol, then diluted to 1 litre with distilled water. A calibration curve (0.5–2 units) should be prepared with each new batch of the above sodium carbonate solution.

The complete 24-hour urine collection, preserved with benzene-thymol and kept in the cold, is subjected to a preliminary analysis of volume, specific gravity, pH, and tested for albumin and any other expected abnormality. The pH in a clear filtered specimen is adjusted to 5.2 using a pH meter if possible. Another specimen is adjusted to pH 4.5. All estimations are made in duplicate.

Enzyme Activity in Undiluted Urine.—A carefully measured quantity, 0.05 ml., of M/50 substrate solution and 0.05 ml. of 2M acetate buffer of the appropriate pH are introduced into a clean stoppered tube and 1 ml. of urine adjusted to the correct pH is added. Two blank tubes are used, one containing no urine but 0.1 ml. of buffered substrate and 1 ml. distilled water, the other containing 1 ml. of urine alone. After incubation for 18 hours in a water-bath at 37° C., the contents of the two blank tubes are mixed together and 2 ml. sodium carbonate solution pH 10.5 is added to each of the test and blank tubes. The volume in each tube of the test is then made up to 4 ml. with distilled water, mixed, and centrifuged if necessary to remove any precipitate or turbidity. The test is read against the blank on a spectrophotometer at 550 μ m. The activity is expressed

in units, 1 unit liberating 1 μ g. phenolphthalein from 1 mM substrate per hour at 37° C.

Enzyme Activity in Diluted Urine.—M/50 substrate, 0.05 ml., is introduced into a stoppered tube and 1 ml. urine, adjusted to pH 4.5 and diluted 10 times with 0.05M acetate buffer containing protamine, is added. After incubation for 18 hours at 37° C., 1 ml. carbonate solution is added and the procedure is completed as above.

Normal Values.—Analysis conducted on 25 healthy individuals of both sexes ranging from 5 to 47 years of age gave the following values.

	Units/ml. Urine		
	Undiluted Urine		Diluted Urine
	pH 4.5	pH 5.2	
Range ..	0.52-2.36	0.45-3.2	1.1-7.5
Average ..	1.65	1.73	3.5

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