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Dissociation and Activation of β-Glucuronidase[1]

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Upon dilution of highly purified β-glucuronidase, prepared from both calf spleen and liver, there occurs a considerable reduction of the specific enzyme activity, and the monomolecular reaction constant decreases immediately following dilution. This phenomenon is explained by the dissociation of the enzyme into inactive components. The dissociation of β-glucuronidase can be reversed by a number of substances which thus function as activators. Both dissociation and activation follow the Law of Mass Action. The following substances, in the order of decreasing potency, enhance β-glucuronidase activity: chitosan, protamine, cryst. bovine serum albumin, DNA from salmon milt, 1,10-diamino-*n*-decane, DNA from fish sperm, gelatin, cryst. chymotrypsin, thymus DNA, other α,ω-diaminopolymethylenes, spermine, spermidine, yeast ribonucleic acid, lysine and ornithine. The activation potency of DNA is essentially preserved after prolonged acid or alkaline hydrolysis, as well as after exhaustive enzymic degradation. Monoamino acids, and pure preparations of mononucleotides and monodesoxyribonucleotides failed to activate β-glucuronidase. The chemical nature of the activator and its activating potency are related to each other as follows: at least two basic groups per molecule are required; a greater number of basic groups per molecule produces no further effect; the presence of carboxyl groups decreases potency; the location of the basic groups in the molecule, and their distance from each other markedly influences potency, the size of the molecule being unimportant.

It is the purpose of the present paper to describe in detail our findings concerning the dissociation and activation of β-glucuronidase, based upon experiments with 1400-fold purified enzyme preparations from calf spleen[2] and with 9000-fold purified β-glucuronidase from calf liver,[3] to correlate the phenomenon of enzyme dissociation with that of enzyme activation, and to clarify the role of the activator during the course of enzyme action.

Methods and Materials

Enzyme.—The present experiments have been carried out with our most purified β-glucuronidase preparations from either calf spleen (5000 to 7000 activity units per mg. of protein),[2] or from calf liver (50,000 to 100,000 activity units per mg. of protein),[3] prepared according to our previously reported specifications. Some of the phenomena described may not be evident with less pure enzyme preparations. No significant differences have been found between the behavior of β-glucuronidase from these two sources, with regard to the phenomena reported in this paper.

Substrates.—Aqueous solutions of 0.01 *M* phenolphthalein glucuronide have been prepared from the four times recrystallized cinchonidine salt,[2] and of 0.002 *M* six times recrystallized biosynthetic *p*-hydroxybiphenyl glucuronide,[4] m.p. 183–184° (uncor.), in acetate buffer *p*H 5.0, ionic strength 0.07.

Desoxyribonucleic Acid (DNA).—If not otherwise stated, a 3.1% solution of DNA (Krishell Lab.)[2] has been used.

Dilution of the Enzyme.—To yield a *n*-fold dilution in the final digest, the stock enzyme solution was first diluted *n*/100 fold with acetate buffer (*p*H 5.0, μ 0.07); then, 0.1 ml. of this solution was added to 4.9 ml. of the same buffer containing the desired amount of activator; there will be a 2-fold dilution inherent in the activity determination.

Activity Determination.—With phenolphthalein glucuronide as substrate, previously reported conditions[2] were employed. When *p*-hydroxybiphenyl glucuronide was the substrate, 0.5 ml. of the diluted enzyme solution, containing the activator when required, was added to 0.5 ml. of substrate solution and the mixture incubated at 37°. The time of incubation was arranged so as to permit the liberation of between 2 and 40 γ of *p*-hydroxybiphenyl. The reaction was stopped by the addition of 2 ml. of 20% Na_2CO_3 solution, and the liberated *p*-hydroxybiphenyl was determined photometrically at 660 mμ after addition of 0.5 ml. of 1:1 diluted Folin–Ciocalteu reagent[5] and immersing the tube in boiling water for 1 minute. Blanks, containing enzyme, DNA and buffer, but no substrate, gave negligible values for light absorption at low enzyme and low DNA concentrations. With higher concentrations of enzyme and particularly of DNA, noticeable transmittances in the blanks were encountered which were used to correct the values obtained in the corresponding enzyme digest. The net optical density due to *p*-hydroxybiphenyl was translated into γ of this compound or into microequivalents from a previously established calibration curve.

Specific Enzyme Activity.—This is defined as the ratio of the amount of substrate hydrolyzed in one hour (expressed in either mg. or in microequivalents) to the enzyme concentration (mg. protein per ml. digest) in an 0.001 *M* substrate solution at *p*H 5.0 and 37° in a total volume of 1 ml. The enzyme concentration has been measured by determining either N (Kjeldahl), or protein with Folin phenol reagent, according to the micro-method of Lowry, *et al.*,[6] using crystallized, salt-free chymotrypsin (Worthington Biochem. Lab.) as the reference protein.

Activity Determination Immediately Following Enzyme Dilution.—One-tenth ml. of stock solution of purified calf liver β-glucuronidase (0.1 to 0.5 mg. protein per ml.) was added to various volumes of buffered (*p*H 5.0) 0.001 *M* solution of phenolphthalein glucuronide maintained at 37°, so that the time of dilution of the enzyme coincided with the beginning of enzymatic hydrolysis. At various time intervals, samples containing 2 to 25 γ of free phenolphthalein were withdrawn (0.2 to 20 ml.), 1.0 ml. of 5% trichloroacetic acid was added to each to stop the reaction and phenolphthalein content determined. Aliquots greater than 1 ml. were lyophilized and then taken up in 1 ml. of H_2O, 1 ml. of trichloroacetic acid being added to replace that lost during lyophilization, and the phenolphthalein was determined. Blanks contained an amount of substrate equal to that present in each sample.

Enzymatic Hydrolysis of DNA.—One mg. of crystallized desoxyribonuclease was added to the Mg salt of 1.5 g. of DNA, prepared by ion exchange over Amberlite IR 120, and incubated at *p*H 7.0 for 48 hours at 37° in a total volume of 60 ml. After this period, the digestion had progressed to a point where no precipitation occurred when 2 *N* HCl was added to a sample of the digest.

Five-tenths ml. of purified intestinal phosphatase (1 ml. of a 1:1000 diluted solution of this enzyme liberated 70γ P in 30 minutes at 37° from an 0.17% Na β-glycerophosphate solution at *p*H 8.6 in a total volume of 15 ml.) was added to 23.5 ml. of the desoxyribonuclease digest brought to *p*H 8.6 with a NH_4Cl–NaOH mixture, and incubated for 48 hours at 37° in a total volume of 33 ml. The *p*H of the DNA–phosphatase digest was maintained at 8.6 by repeated additions of 2 *N* NaOH. During this time, 73%

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(2) P. Bernfeld and W. H. Fishman, *J. Biol. Chem.*, **202**, 757 (1953).

(3) P. Bernfeld, J. S. Nisselbaum and W. H. Fishman, *ibid.*, **202**, 763 (1953).

(4) K. S. Dodgson, G. A. Garton, A. L. Stubbs and R. T. Williams, *Biochem. J.*, **42**, 357 (1948).

(5) O. Folin and V. Ciocalteu, *J. Biol. Chem.*, **73**, 627 (1927).

(6) O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *ibid.*, **193**, 265 (1951).

of the total P was liberated. A second analogous experiment was carried out simultaneously in the presence of 0.05 M Na_2HAsO_4.

Activation of β-Glucuronidase by Hydrolyzed DNA.—Six-tenths ml. of the acid of alkali hydrolyzate, or 0.25 ml. of the desoxyribonuclease digest, or 0.35 ml. of the DNAase plus phosphatase digest were made up to 9.9 ml. with buffer and 0.1 ml. of 1:1 diluted calf spleen β-glucuronidase was then added to each.

Materials.—Protamine (Nutritional Biochemical Corporation); crystalline salt-free chymotrypsin (Worthington Biochemical Laboratory); α,ω-polymethylene diamines (Bios Laboratories), the latter have been purified by distillation *in vacuo;* spermine and spermidine (Delta Chemical Works); and crystalline desoxyribonuclease (Worthington Biochemical Laboratory). Chitosan was supplied by Dr. Roger Jeanloz, intestinal phosphatase by Dr. Gerhard Schmidt, and pure desoxyribose mononucleotides by Dr. G. C. Butler.

Results

Effect of Dilution on the Enzyme Activity.—β-Glucuronidase activity has been measured over a wide range of enzyme concentrations, obtained by dilution of one and the same enzyme preparation. Experiments with purified calf liver β-glucuronidase (0.1–100 γ enzyme per ml. digest) have been carried out, using phenolphthalein glucuronic acid as substrate. The duration of incubation was varied from 5 minutes to 24 hours, depending on the enzyme concentration used, so that between 5 and 20 γ of phenolphthalein was liberated. The specific activity has been plotted against the logarithm of the enzyme concentration (in γ protein per ml.) (black dots, Fig. 1).

Figure 1 shows that there is a decrease of the specific enzyme activity on dilution from a maximum at very high enzyme concentrations to less than 1/10 of this value at high dilutions. Three possible explanations of this phenomen are: (1) contamination of the substrate by an enzyme inhibitor, the action of which would become increasingly important as the enzyme concentration in the digest is decreased, the inhibitor concentration in the digest remaining constant; (2) loss of the stability of the enzyme on dilution; (3) dissociation of the active enzyme into inactive components upon dilution.

Purity of the Substrate.—Table I shows that repeated recrystallizations of the cinchonidine salt of phenolphthalein glucuronide does not change the influence of dilution upon specific enzyme activity, making the presence of an inhibitor contaminant very unlikely. Cinchonidine itself which could

TABLE I

EFFECT OF REPEATED RECRYSTALLIZATIONS OF THE SUBSTRATE ON THE ACTIVITY OF CALF SPLEEN β-GLUCURONIDASE

Recryst. of substrate[a]	Specific β-glucuronidase activity[b] Dil. enzyme[c]	Concd. enzyme[d]
2 ×	2800	5400
4 ×	2900	
6 ×	2900	5500
6 ×[e]	2900	

[a] Recrystallization of the cinchonidine salt of phenolphthalein glucuronide from methanol–ethyl acetate. [b] In γ phenolphthalein per mg. enzyme per hour. [c] 1 γ purified calf spleen β-glucuronidase per ml. digest; 1-hour incubation time. [d] 20 γ purified calf spleen β-glucuronidase per ml. digest; 5-minutes incubation time. [e] With the addition of 35 γ cinchonidine per ml. of digest.

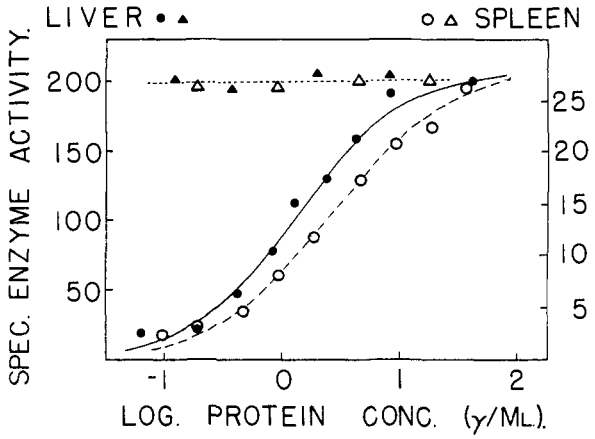

Fig. 1.—●, specific activity of calf liver β-glucuronidase, in micromoles of substrate split per mg. of enzyme per hour, with phenolphthalein glucuronic acid as substrate, no activator; ▲, same, with 300 γ of DNA per ml. of digest as activator; ○, specific activity of calf spleen β-glucuronidase, in micromoles of substrate split per mg. of enzyme per hour, with biphenylglucuronic acid as substrate, no activator; △, same, with 300 γ of DNA per ml. of digest as activator; unbroken line, theoretical curve calculated from expression 1, with 208 × E/P plotted on the ordinate and log P on the abscissa, K = 13 and n = 10; broken line, theoretical curve calculated from expression 1, with 28.2 × E/P plotted on the ordinate and log P on the abscissa, K = 4 and n = 2; dotted line, theoretical curve calculated from expression 2, with 28.2 × E/P plotted on the ordinate and log P on the abscissa, K = 4, n = 2, F = 0.3 and N = 300.

conceivably be present is without effect. Moreover, the specific activity of β-glucuronidase also decreases upon dilution when a substrate other than phenolphthalein glucuronide, namely, six times recrystallized biphenylglucuronic acid was used (Fig. 1, open circles). Biphenylglucuronic acid had been chosen for its ease of crystallization and because, once pure, it required no further treatment before using it as a substrate in contrast to cinchonidine phenolphthalein glucuronide.

Stability of Diluted β-Glucuronidase.—The decline in enzyme activity as a function of time observed at three different enzyme concentrations is shown in Fig. 2a.

Thus, the coefficient of the monomolecular reaction has been calculated from the activity values and plotted against the time elapsed since the dilution in each case was carried out. In this experiment, the time of enzyme dilution was arranged to coincide with the beginning of the enzymatic hydrolysis. It is obvious from Fig. 2a that there is an initial period during which a rapid fall of enzyme activity occurs. The latter is most significant at the highest enzyme dilution. Then, the reaction becomes nearly monomolecular, *i.e.*, after the initial stage, there is no longer observed any substantial decrease of the enzyme activity. If the apparent activity loss of β-glucuronidase was produced by a decrease in the stability of the enzyme, a progressive reduction of the enzyme activity with time should have been expected, an event which was not observed, however. It appears unlikely, therefore, that the disappearance

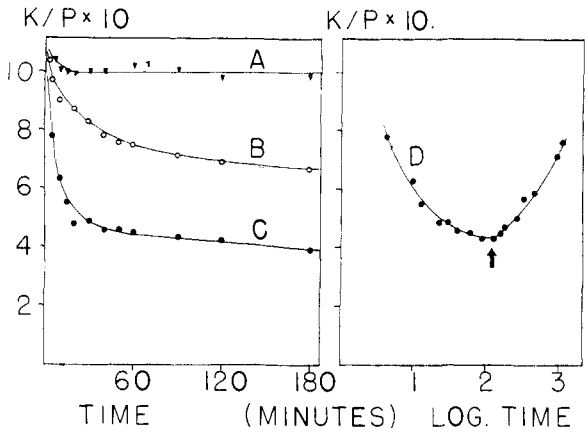

Fig. 2a and 2b.—K = the constant of the monomolecular reaction, calculated for the hydrolysis of phenolphthalein glucuronic acid by calf liver β-glucuronidase (purity 43,000 activity units per mg. of protein) when the time of dilution of the enzyme from a stock solution (2.5 mg. of protein per ml.) coincides with the beginning of the enzymatic hydrolysis (t = 0); P = concentration of enzyme protein in mg. per ml. of digest. A, 2.5 γ of enzyme per ml. of digest; B, 0.5 γ of enzyme per ml. of digest; C, 0.1 γ of enzyme per ml. of digest; D, 0.1 γ of enzyme per ml. of digest, with addition of albumin (100 γ per ml. of digest) at 120 minutes (arrow).

of the enzyme activity upon dilution is produced by an instability of the enzyme.

Dissociation of β-Glucuronidase into Inactive Components.—Reversible dissociation does provide the best explanation so far of the dilution phenomenon described. This view is supported by the fact that the experimetal data, obtained with both purified calf liver and calf spleen β-glucuronidase, using either of the two substrates (phenolphthalein glucuronide and biphenylglucuronide), fit closely theoretical dissociation curves derived from the Mass Law. This is illustrated in Fig. 1 which shows the experimental values as black dots and open circles, and the theoretical dissociation curves as unbroken or broken lines. The latter have been calculated from expression 1

$$\frac{(P - E)(nP - E)}{E} = K \qquad (1)$$

where K represents the dissociation constant and E and P are two variables, *i.e.*, the concentrations of active enzyme and of total protein, respectively; the values of E/P (specific activity) have been plotted as ordinate and log P as abscissa. Accordingly, the dissociation of β-glucuronidase upon dilution does follow the Law of Mass Action ($E \rightleftarrows X + Y$) and would yield enzymatically inactive components X and Y, the concentrations of which are represented by $X = (P - E)$ and $Y = (nP - E)$, the ratio of the total amounts of these components (dissociated + associated) being constant $nP/P = n$.

Activation of β-Glucuronidase by DNA.—From data plotted as triangles in Fig. 1 it becomes evident that, in contrast to the experiments in which no DNA was added, the specific activity of β-glucuronidase is now practically independent of the enzyme concentration. The addition of DNA, therefore, enhances the activity of dilute purified β-glucuronidase, and this effect becomes more and more pronounced as the enzyme dilution is increased, as shown by examining the distance on the ordinate between open circles (or black dots) and triangles.

The Relationship of Enzyme Activity to DNA Concentration.—The experimental values, indicated as open circles in Fig. 3, are in good agreement with theoretical dissociation curves defined by either of the expressions

$$\frac{(P - E)(nP - E + FN)}{E} = K \qquad (2)$$

$$\frac{(P - E)(nP - E)}{E} = \frac{K_1}{K_2 + FN} \qquad (3)$$

where E and P represent the concentrations of active enzyme and total protein, N that of DNA, and K, K_1, K_2, n and F constants. The curve derived from expression 2 is plotted as a solid line in Fig. 3 with E/P as the ordinate and with log N as abscissa. The curve calculated from expression 3 is not shown in the figure because of its close similarity to that of expression 2; the two curves are, however, not identical.

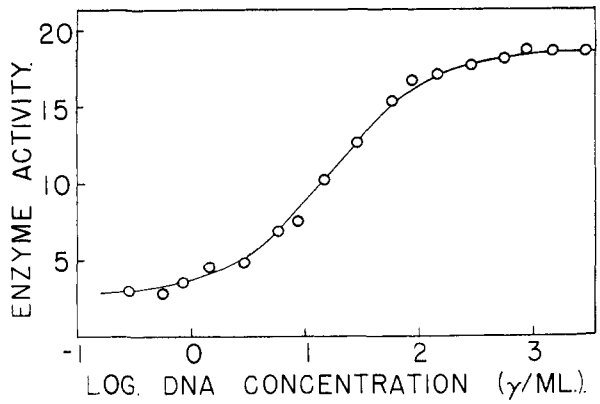

Fig. 3.—○, activity of 0.4 γ of calf spleen β-glucuronidase per ml. of digest (purity 5900 activity units per mg. of protein) at variable activator (DNA) concentrations, with phenolphthalein glucuronic acid as substrate; solid line, theoretical curve, calculated from expression 2, with 18.5 × E/P plotted on the ordinate and log P on the abscissa, where K = 4, n = 2, P = 0.4 and F = 0.3.

Referring now to some of the experiments illustrated in Fig. 1, the experimental values of specific activity of β-glucuronidase at variable enzyme concentrations in the presence of a large fixed amount of DNA (triangles) are likewise in very good agreement with the curves derived from expression 2 and 3, when P becomes a variable instead of N. Such a curve (derived from expression 2) yielding almost a straight line when FN becomes very great as compared to P, is plotted as a dotted line in Fig. 1.

Activation of β-Glucuronidase by DNA from Various Sources.—Table II provides comparative data of the activating potency of various DNA preparations from different sources, supplied by a number of commercial or research laboratories, and in addition of two ribonucleic acid (RNA) preparations.

TABLE II

ACTIVATION OF CALF SPLEEN β-GLUCURONIDASE BY DNA FROM VARIOUS SOURCES

Nucleic acid	Source	Prepared by	Specific activity of β-glucuronidase[a]
DNA	Fish sperm	Schwarz Lab., Inc.	4600
DNA	Fish sperm	Gen. Biochemicals, Inc.	4500
DNA	Fish sperm	Nutritional Biochem. Corp.	3500
DNA	Salmon milt	Krishell Lab., Inc.	6850
DNA	Thymus	Bios. Lab., Inc.[b]	1800
DNA	Thymus	Dr. G. Schmidt[c]	2800
RNA	Yeast	Nutritional Biochem. Corp.	470
RNA	Yeast	Krishell Lab., Inc.	730
None	.....	.....	290

[a] In γ phenolphthalein per mg. of enzyme per hour. [b] This DNA was only partially soluble. The optical density at 260 mμ of its solution after centrifugation was only 0.29, as compared to 6.5–7.5 for the other preparations. The concentration of this solution and hence its activation power is, therefore, lower than that of the other DNA preparations. [c] Because of the highly viscous nature of this preparation, only 0.15 mg. instead of 0.3 mg. for the other products has been used per ml. of digest.

Activation of β-Glucuronidase by Hydrolyzed DNA.—Equal volumes of 3.1% DNA solution and 2 *N* HCl (or 2 *N* NaOH) were immersed in boiling water, 10-ml. aliquots were taken and brought to *p*H 5.0 by the addition of 6 *M* sodium acetate (or 3 *M* acetic acid). The potency of DNA to activate β-glucuronidase is not essentially altered by either acid or alkaline hydrolysis up to two hours under the present experimental conditions.

Table III shows that enzymatic hydrolysis only slightly reduces the potency of DNA to activate β-glucuronidase.

TABLE III

INFLUENCE OF DIGESTION OF DNA BY DNAASE AND BY PHOSPHATASE ON ITS β-GLUCURONIDASE ACTIVATING POTENCY

Digestion of DNA by DNAase	Digestion of DNA by intestinal phosphatase	Specific activity of calf spleen β-glucuronidase[a]
−	−	6800
+	−	4350
+	+[b]	4400
+	+[c]	4250
Control, no activator added		1350

[a] In γ phenolphthalein per mg. of enzyme per hour. [b] Digested in the presence of 0.05 *M* arsenate. [c] No arsenate added.

Effect of Dialysis of Hydrolyzed DNA on its Activation Potency.—Five-ml. samples of chemically or enzymatically hydrolyzed DNA have been dialyzed for three days in cellulose sausage casings (24/32 inch diameter, obtained from the Visking Corporation, Chicago) against 50 ml. of distilled H_2O which was changed every day. The dialyzates were then combined and concentrated to 5 ml. under reduced pressure (12 mm.). Only little if any of the principle which activates β-glucuronidase diffuses through a cellophane membrane, even after strong acid, alkaline or enzymatic hydrolysis of the DNA.

Other Activators of β-Glucuronidase.[7]—Activating potency has been evaluated quantitatively, by determining β-glucuronidase activity at a low fixed enzyme concentration (0.2 γ of purified calf spleen enzyme per ml. of digest), while varying the activator concentration over a wide range (from 0.02 to 5000 γ per ml.). Plots of the specific enzyme activity as a function of the logarithm of the activator concentration are shown in Fig. 4.

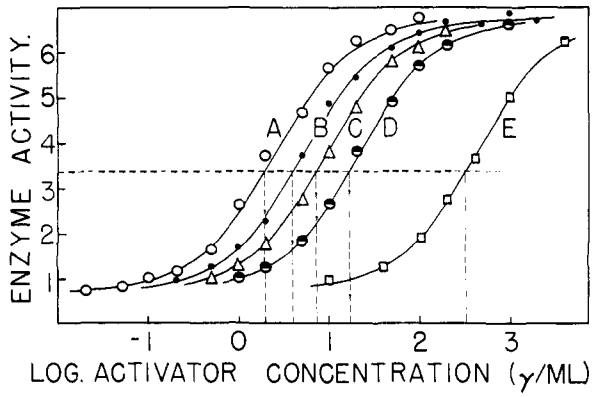

Fig. 4.—Activation of β-glucuronidase by various concentrations of chitosan (A); of crystalline bovine serum albumin (B); of 1,10-diamino-*n*-decane (C); of crystalline chymotrypsin (D); and of a boiled and filtered solution of crystalline chymotrypsin (E). The protein concentrations in curve E have been calculated as if no protein has been lost by heat denaturation; enzyme, calf spleen β-glucuronidase, 0.18 γ per ml. of digest, purity 6800 activity units per mg. of protein, 22-hours incubation time; substrate, phenolphthalein glucuronide. Enzyme activity expressed in mg. of phenolphthalein liberated per mg. of enzyme per hour. The dotted lines mark the concentrations of half activation.

The β-glucuronidase activating potency is expressed as that activator concentration (concentration of half-activation) at which the specific enzyme activity reaches one-half of the maximum value that can be obtained at very high activator levels. The substance which exhibits the lowest concentration of half-activation possesses the highest activating potency. Thus, chitosan with a half-activation concentration of 2 γ per ml. is the most powerful activator of β-glucuronidase known so far. Other activators of calf spleen β-glucuronidase yield analogous curves. Their concentrations of half-activation obtained in duplicate experiments are given in Table IV.

Certain minor disagreements between the results of duplicate experiments are indicated in Table IV by stating a range of half-activation concentrations instead of a single value. These disagreements can be explained by the magnification of the limit of error resulting from the graphical interpolation of the experimental values on a logarithmical scale.

When the activator concentration is increased above a certain level, it has been observed that, in many cases, the enzyme activity declines. In the case of the non-polymer activators, this limit is at about 0.1 *M* concentrations. Thus, for the least potent activators such as ornithine, arginine, lysine and ethylenediamine, the enzyme activity decreases on increase of the activator concentration, without reaching the activity of the fully activated enzyme.

(7) See preliminary report, P. Bernfeld, H. C. Bernfeld and W. H. Fishman, *Federation Proc.*, **12**, 177 (1953).

TABLE IV

POTENCY OF β-GLUCURONIDASE ACTIVATORS (CALF SPLEEN)

Activator		Concn. of half-activation In γ/ml.		In μmoles/ml.
Chitosan		2		
Protamine		3–4		
Cryst. bovine serum albumin		3–4		
Desoxyribonucleic acid (Kri-shell)		8.5		
Gelatin		14		
Cryst. chymotrypsin		16		
Yeast ribonucleic acid		300		
$H_2N(CH_2)_n NH_2$	$n = 10$	7	[a]	0.04[b]
	$n = 8$	60–90		0.4–0.6
	$n = 7$	200	1.5	
	$n = 6$	1300		11
(Cadaverine)	$n = 5$	250	2.5	
(Putrescine)	$n = 4$	1000		11
	$n = 3$	300	4	
	$n = 2$	3000		50
Spermine		60–80		0.3–0.4
Spermidine		300		2
L-Lysine		3000		20
DL-Ornithine		13000		100
L-Arginine		18000		100

[a] Odd numbers of C-atoms. [b] Even numbers of C-atoms.

L-Histidine exerts a very faint activation effect. No activation power has been found for glycine, glycylglycine, DL-α-alanine, β-alanine, DL-serine, DL-methionine, L-glutamic acid, hydrazine and α,α′-dipyridyl, as well as monoribonucleotides and pure preparations of monodesoxyribonucleotides.

Heat denaturation of the proteins does not entirely abolish their ability to activate β-glucuronidase. A solution of crystalline chymotrypsin (0.2% in 0.16 *M* acetate buffer, *p*H 4.5) has been heated to 100 degrees for 10 minutes, cooled, and the coagulated protein filtered off. The clear filtrate retained about 5% of its original activation potency, as compared to an equal volume of the original chymotrypsin solution. This is seen from curve E in Fig. 4, in which the plotted values of protein concentrations have been calculated as if no protein were lost upon heat denaturation. The absorption spectrum of this solution in the ultraviolet light indicated the presence of a small amount of protein.

Similar results have been obtained with heat denatured crystalline bovine serum albumin, as well as with heat denatured solutions of purified β-glucuronidase.

The Reversal of β-Glucuronidase Dissociation.—The reversibility of the dissociation of β-glucuronidase has been further demonstrated by the following experiment which is similar in design to that described in curve C, Fig. 2. When the monomolecular constant has dropped to an almost constant level as a consequence of the enzyme dissociation reaching a steady state (120 minutes after the beginning of the experiment), crystalline bovine serum albumin has been added to the digest (0.10 mg. per ml.). The results are shown in curve D, Fig. 2b and illustrate the reversal of the dissociation by a slow but constant increase of the monomolecular constant, beginning at the time of the addition of the activator.

Discussion

The Dissociation Phenomenon of β-Glucuronidase.—The evidence in favor of a dissociation of purified β-glucuronidase on dilution into inactive products ($E \leftrightarrows X + Y$) rests chiefly on the correspondence of the experimental data with theoretical curves derived from the Law of Mass Action. That this may be a general phenomenon for β-glucuronidase is indicated by the demonstration of this dissociation with the use of enzyme preparations from two different tissue sources acting on two separate substrates. The relevance of other factors such as inhibitors contaminating the substrate and of instability of the enzyme has been ruled out by experiment. No clue as to the nature of the inactive products due to dissociation is forthcoming from these experiments.

It was not possible to bring the additional evidence for the dissociation of β-glucuronidase on dilution, namely, the analysis of a dilute enzyme solution which had been dried in the frozen state and redissolved in a smaller volume, because the freeze-drying of dilute solutions of purified β-glucuronidase, even in the presence of albumin, causes a major loss in the enzyme activity.

There are two principal requirements for demonstrating the dissociation of β-glucuronidase: (1) The enzyme must be highly purified. The presence in it of certain amounts of other tissue proteins or of DNA would yield a preparation which already would be activated in the same manner as purified β-glucuronidase is in the presence of albumin, other proteins or DNA. Dilution of such an enzyme solution could, therefore, not be expected to produce a decrease of the specific activity. (2) Provided a β-glucuronidase preparation of satisfactory purity is available, the extent of dilution becomes a limiting factor in eliciting the dissociation. Thus, the inability of other workers[8] to produce a dissociation of β-glucuronidase may be explained by failure to observe these prerequisites for the dissociation phenomenon.

The Phenomenon of β-Glucuronidase Activation.—The following experimental findings support the view that the present series of activators promote the recombination of the dissociation products to reconstitute the active enzyme: (1) In the detailed study of β-glucuronidase activation by DNA, the extent of activation is a function of DNA concentration. The experimental data fit curves derived from the Law of Mass Action (Fig. 3). (2) Similar shaped curves are regularly observed with other activators (Fig. 4). (3) The velocity constant of hydrolysis, calculated for a monomolecular reaction, decreases upon dilution. It then increases upon addition of activator approaching eventually the original value (curve D, Fig. 2b). (4) In no instance has any activator elevated the specific activity of β-glucuronidase above the value obtained with undiluted enzyme. (5) No activation is observed with β-glucuronidase preparations which do not show the phenomenon

(8) E. E. B. Smith and G. T. Mills, *Biochem. J.*, **54**, 164 (1953).

of dissociation, as a consequence of inadequate purification or of insufficient dilution.

The Mechanism of β-Glucuronidase Activation.—Two possible mechanisms of β-glucuronidase activation may be advanced, according to the two mathematical expressions (expression 2 and 3) which are both in satisfactory agreement with the experimental values of specific enzyme activity at variable activator concentrations (Fig. 3).

In the case of expression 2, the role of the activator N is to favor the reaction in the direction of the formation of active enzyme E: $X + Y + N \leftrightarrows E$, by replacing one or the other of the dissociation products, the concentration of N being much higher than those of X or Y.

In the case of a type of activation represented by expression 3, the activator would promote the recombination of the dissociation products to reconstitute the active enzyme by increasing the affinity of the products of dissociation for each other, thus transforming the conventional dissociation constant, K, into a variable quantity, $K_1/(K_2 + FN)$. Here, the activator does not necessarily have to be a part of the recombined enzyme.

It is not possible to state a preference for one or other of these mechanisms on the basis of the experimental data. In a preliminary report,[9] the activation mechanism represented by expression 2 has been favored. This view was based on the demonstration of an activating effect by filtered boiled solutions of purified β-glucuronidase preparations. These findings have been confirmed. However similarly treated solutions of other proteins, such as of crystalline chymotrypsin and of crystalline bovine serum albumin, have now been found also to exhibit β-glucuronidase activating power. The activating power of the boiled proteins is due, no doubt, to the presence of heat-denatured and soluble proteins in the filtered solutions. Accordingly, the phenomenon of the activating power of filtered, boiled β-glucuronidase is not necessarily due to the presence of a coenzyme.

Influence of the Chemical Nature of the Activator on its Activating Potency.—All substances observed to activate β-glucuronidase possess two or more basic groups per molecule. Compounds with a single basic group per molecule have no activating effect, even those which bear a close resemblance to known activators, such as monoamino acids and monodesoxyribonucleotides. The optimum number of basic groups per molecule of activator, at least in the case of the aliphatic compounds, appears to be two. Thus, spermine with 10 carbon atoms and 4 basic groups and spermidine with 7 carbon atoms and 3 basic groups are slightly less active than the diamino compounds with the corresponding number of carbon atoms, deca- and heptamethylenediamine. The importance of amino groups for the activation of β-glucuronidase is clear also from findings of Beyler and Szego[10] who state that blocking the amino groups of albumin by acetylation practically destroys its activation ability.

From the study of a series of α,ω-diamino paraffins, a progressive increase in activating potency is observed with lengthening of the carbon chain in both the even and the odd numbered carbon atom series, but the increase is irregular in the diaminoparaffin series as a whole. There is an interesting analogy between the effect of the number of carbon atoms in the homologous series of compounds on their melting points, and their properties in aqueous solution, such as their ability to activate β-glucuronidase.

The important influence of the location in the activator molecule of the basic groups on the activating potency is demonstrated by the results obtained with the α,ω-diaminoparaffins. The fact that the β-glucuronidase activating effect is very low or even entirely absent in a few polybasic substances, *e.g.*, in histidine, ribonucleic acid, hydrazine and α,α′-dipyridyl, might be explained by a location of their basic groups which is unfavorable for β-glucuronidase activation.

The presence of a carboxyl group in a diamine markedly reduces its activating potency, *e.g.*, compare lysine and cadaverine, ornithine and putrescine (Table IV). Beyler and Szego[10] observed that blockage of the carboxyl groups in albumin by methylation enhanced the activating effect of albumin.

Influence of the Molecular Weight of the Activator on its Activating Potency.—The molecular weight of the activator is not a limiting factor in its ability to activate β-glucuronidase. Thus, decamethylenediamine, of low-molecular weight, exhibits on a weight per volume basis about the same activating potency as the high-molecular weight substance, DNA, and is more than twice as powerful as chymotrypsin.

This view is further supported by the failure of acid or alkali hydrolysis to reduce the activating power of DNA in spite of its degradation to substances of lower molecular weight. Purification of the activating principle from hydrolyzates of DNA by means of ion-exchange chromatography yielded a fraction which had an ultraviolet absorption spectrum and an N/P ratio essentially the same as thymidylic acid.[11] Since thymidylic acid itself is not an activator, it is believed that a polymer of thymidylic acid, perhaps in some respect related to apurinic acid[12] or an unidentified substance, is responsible for the activating power of this fraction. On account of the diversity of the chemical nature of the activators, the further characterization of this fraction did not appear to contribute much to the problem.

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