Effects of Proteins and Polynucleotides on the Activity of Various Hydrolases

By M. J. PALMIERI and O. KOLDOVSKÝ

Division of Biochemical Development and Molecular Diseases, Children's Hospital of Philadelphia and Department of Pediatrics, University of Pennsylvania School of Medicine, Philadelphia, Pa. 19146, U.S.A.

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The effect of various macromolecules on the activity of several hydrolases was studied. Dilution of partially purified acid β -galactosidase from ileal mucosa of suckling rats resulted in a decrease of specific activity. The relationship between specific activity and dilution of the enzyme suggests a dissociation of the enzyme. This could be prevented by addition of several proteins tested. However, addition of DNA to the assay mixture for acid β -galactosidase caused an inhibition. This inhibition could be prevented by addition of proteins. Other polynucleotides and tRNA also exert an inhibitory effect that is prevented by albumin, but nucleotides have no effect. This inhibition occurs maximally at a low pH (3.0–4.0); no inhibition is observed at pH 5.5. A similar pH-dependent inhibition by DNA was also found with various other acid hydrolases.

Acid β -galactosidase isolated from the intestinal mucosa was found to be unstable during storage; however, the addition of serum albumin was used to prevent this instability (Asp & Dahlqvist, 1968; Kraml *et al.*, 1969).

Highly purified preparations of several other enzymes (e.g. crystalline β -amylase and β -glucuronidase) were reported to lose activity on dilution (Walker & Whelan, 1960; Bernfeld & Fishman, 1950; Bernfeld *et al.*, 1953). As with acid β -galactosidase these enzymes could be protected in dilute solution by the addition of serum albumin.

The present paper reports attempts to stabilize acid β -galactosidase from the ileal mucosa of suckling rats during purification. The protective effect of serum albumin was confirmed, and a study was made of the effects of other proteins in stabilizing this enzyme from dilution. Further, since DNA was found to be highly efficient in activating calf liver β -glucuronidase (Bernfeld *et al.*, 1953), its effect on acid β -galactosidase was investigated. Unlike β -glucuronidase, acid β -galactosidase activity was inhibited by DNA. This phenomenon was analysed in detail and effects on several other hydrolases were compared.

Experimental

Materials

p-Nitrophenyl α - and β -D-galactopyranoside, *p*-nitrophenyl β -D-glucuronide, *p*-nitrocatechol sulphate, *p*-nitrophenyl *N*-acetyl- β -D-glucosamide, β -glucuronidase (bovine liver, B grade) and DNA (salmon sperm) were purchased from Calbiochem,

Los Angeles, Calif., U.S.A. Maltose was obtained from J. T. Baker Chemical Co., Philipsburg, N.J., U.S.A., and Glucostat from Worthington Biochemical Corp., Freehold, N.J., U.S.A. Bovine serum albumin (fraction V), sheep haemoglobin (2 $\times$ crystallized) and sheep γ -globulin (fraction II) were obtained from Pentex Inc., Kankakee, Ill., U.S.A. Bovine pancreas insulin (crystalline), AMP, ADP, ATP, 3':5'-cyclic AMP (adenosine 3':5'-cyclic monophosphate), UMP, UTP, uridine and adenosine were purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A. Poly(A), poly(C), poly(G), poly(I,C), poly(U₃,C) and poly(U₃,G) were obtained from Miles Laboratories, Elkhart, Ind., U.S.A. *Escherichia coli* tRNA was obtained from General Biochemicals, Chagrin Falls, Ohio, U.S.A.

Methods

Enzymic activities. Partially purified acid β -galactosidase was prepared from rats. Suckling rats (10–14 days old) of the Sprague–Dawley strain were killed by decapitation, the distal third of the small intestine was divided along its length, excised and then homogenized in cold, twice-distilled water (180–200 mg wet wt./ml) in a Waring Blender. An acetone-dried powder was prepared as described by Morton (1955), and 1 g was extracted overnight (16–18 h) in 100 ml of water at 5°C with constant stirring. The suspension was then centrifuged at 13500g for 30 min at 2°C. All subsequent centrifugations were performed for 30 min (unless otherwise indicated) at this temperature and centrifugal force.

The supernatant (pH 6.5) was fractionated with

solid $(\text{NH}_4)_2\text{SO}_4$. The protein that precipitated between 40 and 60% saturation was suspended in 10 ml of cold, twice-distilled water.

Enough cold acetone to give a final concentration of 37% (v/v) was added. After standing at 0°C for 30 min the suspension was centrifuged. The precipitate was discarded and the supernatant was adjusted to 57% saturation by the addition of cold acetone. The suspension was then centrifuged and 5 ml of 0.05 M-sodium-potassium phosphate buffer, pH 7.0, was added to the precipitate. This was again centrifuged for 45 min, and the resulting supernatant was used without further purification. The specific activity of this fraction was 5 times that of the initial extract; 50 μmol of *p*-nitrophenyl β -D-galactopyranoside (final concentration 5 mM) was hydrolysed/h per mg of protein in citrate buffer, pH 3.5. The enzyme preparation was stored at -40°C and was used within 6 weeks.

β -Glucuronidase from bovine liver was obtained commercially and was used without any further purification. Mucosal homogenates of the ileum of 12-14-day-old rats killed on the same day were used as a source of α -galactosidase and arylsulphatase. Homogenates of kidney and liver of 13-day-old rats were assayed for *N*-acetyl- β -D-glucosaminidase activity. The microvilli fraction of enterocytes from jejunal homogenates of adult rats was prepared as described by Forstner *et al.* (1968) and served as a source of maltase.

Enzyme assays. Acid β -galactosidase was assayed as described by Koldovský *et al.* (1969) by using *p*-nitrophenyl β -D-galactopyranoside as the substrate (final assay concentration 5 mM). The reaction mixture consisted of 2 vol. of buffer, 1 vol. of substrate solution and 1 vol. of enzyme solution. All assays were carried out in sodium citrate buffer, pH 3.5, the final assay concentration being 0.05 M. The appropriate enzyme dilutions were made in deionized water, and they were always added to the buffer within 2 min after dilution. After preincubation of the tubes with buffer and enzyme for 2 min, the reaction was initiated by the addition of substrate. Incubations were performed at 37°C for 15-60 min.

The reaction was stopped and the colour developed by the addition of 2 vol. of 1 M- K_2CO_3 . The extinction at 420 nm was measured and the quantity of *p*-nitrophenol liberated was calculated from a standard curve prepared with free *p*-nitrophenol.

Arylsulphatase activity was determined by a modification of the method of Danovitch & Laster (1969). The assay mixture was composed of 2 vol. of 0.5 M-sodium acetate buffer, pH 5.5, 1 vol. of homogenate prepared in water and 1 vol. of substrate solution (0.02 mM-*p*-nitrocatechol sulphate). The reaction was carried out at 37°C as described above, and stopped by the addition of 2 vol. of 5% (w/v) trichloroacetic acid. The mixture was centrifuged at 1600g for

10 min, and 1 vol. of the supernatant solution was added to 1 vol. of 5 M-NaOH. The extinction at 520 nm was determined and the amount of *p*-nitrocatechol liberated was calculated from a standard curve prepared with free *p*-nitrocatechol.

β -Glucuronidase and *N*-acetyl- β -glucosaminidase activities were determined at 37°C as described previously (Koldovský, 1971; Koldovský & Herbst, 1971). A similar assay mixture was used for the determination of α -galactosidase. Generally, the assays were carried out as described above, and the

Fig. 1. Effect of time and enzyme concentration on the activity of β -galactosidase

(a) Time-dependency of acid β -galactosidase activity (57%-acetone-dried fraction; see the Experimental section) at various dilutions of enzyme (μg of protein/ml of assay mixture): ●, 54.0; ○, 13.5; △, 3.9. (b) Dependency of β -galactosidase activity (same preparation as above) on the concentration of enzyme. Assay time: ●, 30 min; ○, 45 min; △, 30 or 45 min, in the presence of serum albumin (1 mg/ml of assay mixture).

Fig. 2. Activity of acid β -galactosidase (57%-acetone-dried fraction) in the presence of various concentrations of different proteins with and without DNA

Incubation was for 30min and the concentration of enzyme protein was $2.25\mu\text{g/ml}$ of assay mixture. ●, Albumin; ○, γ -globulin; Δ , haemoglobin; ▲, insulin. (a) $100\mu\text{g}$ of DNA/ml of assay mixture; (b) $10\mu\text{g}$ of DNA/ml; (c) no DNA.

mixtures were composed of 2 vol. of buffer, 1 vol. of enzyme solution and 1 vol. of substrate solution. The final concentrations of substrates were 5.0mM, 2.5mM and 5.0mM respectively. The final concentration of sodium acetate buffer (β -glucuronidase) and of sodium citrate buffer (α -galactosidase and *N*-acetyl- β -glucosaminidase) was 0.05M, and the pH of each buffer is given in the figure legends. After the reaction had been stopped with 1M- K_2CO_3 the liberated *p*-nitrophenol was determined by measuring the E_{420} .

Maltase activity was assayed as described by Dahlqvist (1964). Sodium acetate buffer of various

pH values was employed at a final concentration of 0.05M. Before the assay of glucose with Glucostat the pH of the mixture was adjusted to 7.0 by the addition of 1.5 vol. of 0.6M-sodium phosphate buffer, pH 7.0, as described by Koldovský *et al.* (1966).

Determination of protein. The method of Lowry *et al.* (1951) was used. Standards of fresh serum albumin were used throughout.

Effect of DNA and other nucleic acid constituents. In the experiments where the effect of these molecules was to be examined, 0.2 vol. of a stock DNA solution (see figure legends) was added to the assay mixture to give a final concentration of one-twentieth of the

initial concentrated stock solutions. The addition of DNA or its constituents did not alter the pH of the assay mixture.

Protein studies. The respective proteins were dissolved in 0.1M-sodium citrate buffer, pH 3.5. In experiments where both the effect of proteins and nucleic acid constituents were examined, protein was always added first.

Results

Relationship between activity and amount of acid β -galactosidase

Various dilutions of partially purified acid β -galactosidase were assayed for activity. The amount of endogenous protein/ml of assay mixture varied from 2.7 to 54.0 μ g. The activity was linear with time up to 60 min (Fig. 1*a*), but not with the concentration of enzyme (Fig. 1*b*). Sigmoid concentration-dependence curves were observed whether the assay was carried out for 30 or 45 min. The addition of albumin (1 mg/ml) prevented this phenomenon, and the specific activity was then independent of the enzyme concentration.

The question arose whether acid β -galactosidase activity could be stimulated when proteins other than serum albumin were employed. Therefore acid β -galactosidase was assayed in the presence of globulin, insulin and haemoglobin at different concentrations, and the results are presented in Fig. 2(*c*). As the concentration of exogenous protein increases, there is a concomitant increase in enzyme activity. (It should be noted that the diluted enzyme fraction contained 2.25 μ g of endogenous protein/ml of assay mixture.)

Effect of DNA

Since Bernfeld *et al.* (1953) demonstrated that, at pH 5.2, DNA in addition to other macromolecules activated β -glucuronidase, acid β -galactosidase was assayed in the presence of DNA. In preliminary experiments it was found that DNA inhibited rather than stimulated acid β -galactosidase activity; simultaneous addition of albumin (1 mg/ml of assay mixture) prevented this inhibition by DNA.

A quantitative investigation on the effect of albumin, γ -globulin, haemoglobin or insulin on DNA inactivation was carried out (Figs. 2*a* and 2*b*). The inhibition by DNA became more apparent at low protein concentrations; albumin and haemoglobin were more effective than insulin and γ -globulin in maintaining enzymic activity at the lower concentrations. When the DNA concentration was increased tenfold, the quantity of protein necessary to activate the enzyme was also increased 10 times (Fig. 2*a*).

The effect of pH on DNA inhibition was examined, and the effect of albumin at various pH values was also

tested. Fig. 3 demonstrates the activation of acid β -galactosidase by albumin at various pH values. As the pH increased the effect of albumin became less pronounced. When DNA was added to the assay (either 10 or 1 μ g/ml), there was a 60–80% inhibition at pH 3.5. As the pH was increased this inhibition was relieved, and at pH 5.0 the values were comparable with those obtained in the absence of albumin.

The inhibitory action by DNA on galactosidase activity was not specific for this compound, since the polynucleotides listed in the Experimental section exerted the same effect at pH 3.5, when added at

Fig. 3. Dependency of the activity of acid β -galactosidase (57%-acetone-dried fraction) on pH in the presence and absence of DNA and serum albumin

The incubation time was 30 min. (a) $\circ$, 1 μ g of DNA/ml of assay mixture; $\bullet$, 100 μ g of DNA/ml; Δ , 10 μ g of DNA + 1 mg of albumin/ml; (b) $\circ$, no albumin; $\bullet$, 1 mg of albumin/ml.

Fig. 4. Activity of various enzymes at different pH values in the presence and absence of DNA

○, No DNA; ●, 100 μ g of DNA/ml of assay mixture. (a) β -Glucuronidase from bovine liver, 100 μ g of protein/ml, 60min assay; (b) α -galactosidase from a crude homogenate of the ileal mucosa of suckling rats, 125 μ g of protein/ml, 30min assay; (c) maltase from microvilli of jejunal enterocytes of adult rats, 1.6 μ g of protein/ml, 30min assay; (d) arylsulphatase from a crude homogenate of the ileal mucosa of suckling rats, 40 μ g of protein/ml, 45min assay; (e) *N*-acetyl- β -glucosaminidase from a crude homogenate of kidney of 13-day-old rats, 43 μ g of protein/ml assay, 50min assay; (f) *N*-acetyl- β -glucosaminidase from a crude homogenate of liver of 13-day-old rats, 114 μ g of protein/ml, 60min assay.

10 μ g/ml. Again the addition of serum albumin prevented this action, and at pH 5.5 no inhibition by these compounds was observed. Various nucleosides and nucleotides had no inhibitory effect on the enzyme activity.

Finally, other acid hydrolases were also examined to determine whether they were similarly influenced by DNA. As Fig. 4 shows, at low pH values, DNA inhibited the activities of β -glucuronidase from bovine liver, α -galactosidase and arylsulphatase from the ileal mucosa of suckling rats and *N*-acetyl- β -glucosaminidase from both the kidney and liver. In addition, maltase activity from a purified microvilli fraction of the jejunal mucosa of adult rats was inhibited at acid pH. This effect disappeared at about pH 5.0. Unlike the other enzymes *N*-acetyl- β -glucosaminidase was activated at pH 5.5.

Discussion

Bernfeld *et al.* (1953) showed that the decrease in specific activity of a highly purified fraction of β -glucuronidase was due to a dissociation of the enzyme into inactive components. The observed curves of the enzyme concentration versus acid β -galactosidase activity suggest that a similar mechanism of dissociation of the active enzyme may take place. It was most pronounced when the added protein concentration was decreased from 36 to 16 μ g/ml. Below 10 μ g/ml a flattening of the curve was observed, and this was attributed to the presence of endogenous protein (2.25 μ g/ml).

The time-dependency curves of acid β -galactosidase indicate that the degree of dissociation does not increase further during the assay, i.e. dissociation occurs on dilution, whereupon an equilibrium be-

tween active and inactive enzyme is immediately established.

An unexpected phenomenon was the inhibition of acid β -galactosidase and other acid hydrolases by DNA and other polynucleotides. In all cases the maximal inhibition by DNA was exerted at low pH values, and at pH 5.0 the inhibition was not present.

The commercial preparation of β -glucuronidase used in our studies was not activated by DNA at pH 5.5, as described by Bernfeld *et al.* (1953). Since the crude fraction had a low specific activity as compared with the preparation used by Bernfeld *et al.* (1953), it is possible that the degree of purification was a decisive factor in the activation mechanism. Nevertheless, DNA did exert a stimulatory effect on *N*-acetyl- β -glucosaminidase from both the liver and kidney at pH 5.5.

The activation of acid β -galactosidase by different proteins as well as the inhibition by DNA showed that these two effects were dependent on the concentration of protein and DNA. Further, the latter were related to the concentration of enzyme, i.e. the enzyme protein and the accompanying proteins present in the preparation. Thus the inhibitory action by DNA and the activating effect of albumin and other proteins could not be detected in preparations of low specific activity.

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References

- Asp, N.-G. & Dahlqvist, A. (1968) *Biochem. J.* **110**, 143
- Bernfeld, P. & Fishman, N. H. (1950) *Science* **112**, 653
- Bernfeld, P., Bernfeld, H. C., Nisselbaum, J. S. & Fishman, N. H. (1953) *J. Amer. Chem. Soc.* **76**, 4872
- Dahlqvist, A. (1964) *Anal. Biochem.* **7**, 18
- Danovitch, S. H. & Laster, L. (1969) *Biochem. J.* **114**, 343
- Forstner, G. G., Sabasin, S. M. & Isselbacher, K. (1968) *Biochem. J.* **106**, 381
- Koldovský, O. (1971) *Arch. Biochem. Biophys.* **142**, 378
- Koldovský, O. & Chytil, F. (1965) *Biochem. J.* **94**, 266
- Koldovský, O. & Herbst, J. (1971) *Biol. Neonatorum* **17**, 1
- Koldovský, O., Heringová, A. & Jirsová, V. (1966) *Biol. Neonatorum* **10**, 241
- Koldovský, O., Asp, N.-G. & Dahlqvist, A. (1969) *Anal. Biochem.* **27**, 409
- Kraml, J., Koldovský, O., Heringová, A., Jirsová, V., Kácl, K., Ledvina, M. & Pelichová, H. (1969) *Biochem. J.* **114**, 621
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265
- Morton, R. K. (1955) *Methods Enzymol.* **1**, 34
- Walker, G. J. & Whelan, W. J. (1960) *Biochem. J.* **76**, 264