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Effects of Triton X-100 on Acid Phosphatases with Different Substrate Specificities

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Summary. The effect of Triton X-100 on the activities of acid phosphatases from wheat germ, potato and human prostate was tested using β-glycerophosphate, p-nitro-phenyl phosphate and naphthol AS BI phosphate as substrates. There was little effect on β-glycerophosphatase activity at the concentrations of Triton X-100 tested. However at low concentrations of the detergent there was a stimulation of the activities of p-nitrophenyl phosphatase and naphthol phosphatase which were inhibited with the higher concentrations. Triton X-100 was found to enhance colour production between naphthol AS BI and fast red violet LB.

Further evidence is presented confirming the presence of more than one acid phosphatase from each of the sources employed.

Introduction

Triton X-100 has been used extensively by biochemists to disrupt lysosomal membranes and release the bound acid hydrolases contained within the lysosomes (for example DE DUVE, 1959). The use of the detergent has been extended to histochemical assays, though with inconclusive results (HOLT, 1959; BITENSKY, 1962; GAHAN, 1965). In all of these experiments acid phosphatase activity was assayed with β-glycerophosphate as substrate. Recently, naphthol AS monophosphate esters have been adopted as substrates for the histochemical demonstration of acid phosphatase. Preliminary reports have indicated that Triton X-100 acts as an activating agent when using these substrates for biochemical assays (ALLEN *et al.*, 1964; MAGGI, 1965). It was of importance to know therefore, if during the demonstrations of the lysosomal localization of acid phosphatases, the Triton X-100 was (a) actually causing a change in the lysosomal membranes, or (b) activating the enzyme molecules, or (c) affecting the chemical reaction involving the naphthol AS salts. The present communication concerns the findings of such an investigation.

Materials, Methods and Results

Acid Phosphatase Preparation

Samples of wheat germ and potato acid phosphatases (Sigma Ltd.) were used without further purification. Human prostates obtained at prostatectomy were used as a source of acid phosphatases which were prepared according to the method of SCHMIDT (1955).

The purity of the samples was tested by electrophoretic separation on polyacrilamide gel columns at pH 8.6 for 30 minutes, at 20° C and 5 m.amp/column. The columns were washed for 60 minutes in frequent changes of 0.05 M acetate buffer pH 5.2 at 4° C.

Protein sites were visualised with 0.1% nigrosin in 1% acetic acid.

Enzyme activity was localized by incubating the polyacrilamide gel columns for either 3 or 18 hours at 37° C in the medium of either GOMORI as modified by HOLT (1959) or of BURSTONE (1958).

Wheat germ acid phosphatase when separated on polyacrilamide gel revealed two bands of naphthol AS BI phosphatase and five bands of β-glycerophosphatase. When separating potato acid phosphatase on polyacrilamide gel, there were two bands of naphthol AS BI phosphatase and four bands of β-glycerophosphatase. Prostate acid phosphatase yielded five bands of β-glycerophosphatase and three bands of naphthol AS BI phosphatase.

Biochemical Assay of Acid Phosphatase Activities

(i) *β-Glycerophosphate.* The reaction medium contained 0.04 M sodium β-glycerophosphate ([B.D.H.] Analar grade) and 0.5 mg of wheat germ and potato phosphatases, and a standard aliquot of prostate phosphatase in a total volume of 2.5 ml of 0.2 M acetate buffer pH 5.0. Incubations were performed in triplicate for 15—20 minutes at 37° C. Aliquots were tested colorimetrically for inorganic phosphate using ferrous sulphate as the reducing agent (Dr. J. PALMER, personal communication). Boiled enzyme preparations served as controls. To test the effect of the detergent, Triton X-100 was dissolved in 0.2 M acetate buffer (v/v) replacing acetate buffer in the above reaction mixture so that the final concentrations of Triton X-100 were 0.1, 0.5 and 1%.

Triton X-100 had no effect on the release of phosphate from β-glycerophosphate by both potato or wheat germ phosphatase, although a 10% increase resulted from its addition to the prostate phosphatase reaction medium (Table 1).

Table 1. *Effect of concentrations of Triton X-100 on acid phosphatase activities using β-glycerophosphate, p-nitro-phenyl phosphate and naphthol AS BI phosphate as substrates. Results are given as optical density readings at 640 mμ for β-glycerophosphate, 550 mμ for naphthol AS BI phosphate and 405 mμ for p-nitrophenyl phosphate*

Acid phosphatase	Substrate	Boiled enzyme	Boiled enzyme + 1% Triton	Concentration of Triton X-100			
				none	0.1%	0.5%	1.0%
Wheat germ	β-glycerophosphate	0.028	0.028	0.124	0.123	0.122	0.122
	p-nitro-phenyl phosphate	0.085	0.086	0.425	0.500	0.511	0.440
	naphthol AS BI phosphate	0.017	0.019	0.078	0.369	0.284	0.122
Potato	β-glycerophosphate	0.034	0.034	0.112	0.126	0.110	0.115
	p-nitro-phenyl phosphate	0.108	0.108	0.479	0.535	0.500	0.475
	naphthol AS BI phosphate	0.004	0.004	0.031	0.197	0.099	0.036
Prostate	β-glycerophosphate	0.035	0.033	0.288	0.288	0.307	0.319
	p-nitro-phenyl phosphate	0.198	0.198	0.569	0.656	0.702	0.702
	naphthol AS BI phosphate	0.006	0.004	0.019	0.189	0.129	0.085

(ii) *p-Nitrophenyl Phosphate.* The reaction mixture contained 5.5×10^{-3} M p-nitrophenyl phosphate and 0.1 mg of wheat germ and potato phosphatases, and a standard aliquot of prostate phosphatase in a final concentration of 2 ml of 0.1 M acetate buffer pH 5.0. Boiled enzyme preparations served as controls. Triton X-100 was tested on enzyme activity at final concentrations of 0.1, 0.5, and 1.0%. Triplicate samples were incubated for 10 minutes at 37° C, the reaction being stopped by the addition of 4 ml 0.1 N. NaOH, and the optical density of the resultant solutions was determined spectrophotometrically at 405 mμ.

Triton X-100 caused an increase of 12—17% in the activity of the acid phosphatase preparations (Table 1).

(iii) Naphthol AS BI Phosphate as Substrate. The reaction mixture contained 0.08 mg or 0.4 mg of naphthol AS BI phosphate (Sigma Ltd.) dissolved in 0.5 ml dimethyl formamide, and 0.1 mg or 0.05 mg wheat germ and potato phosphatases, and a standard aliquot of prostate phosphatase preparation in a final volume of 2.0 ml of 0.2 M acetate buffer pH 5.0. Incubations were performed for 10 minutes, the reaction being stopped by the addition of 0.2 ml 1.0 M Tris. Free Naphthol AS BI was assayed by adding 0.1 ml of freshly prepared 0.1% aqueous solution of Fast Red Violet LB and a colour allowed to develop for 15 minutes. 0.2 ml of 85% trichloroacetic acid was added, the sample was allowed to stand for 15 minutes prior to colour extraction with 3 ml of 40% n-heptanol in tetrachloroethane (v/v). End-product was estimated spectrophotometrically at 550 mμ (MAGGI and CHNG, 1966).

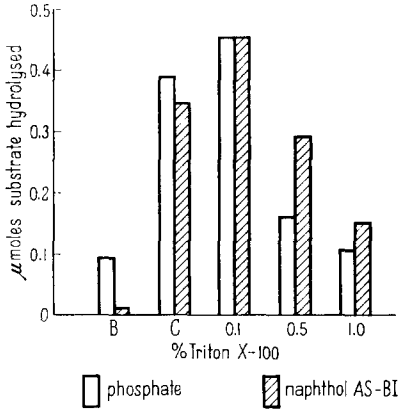

Fig. 1. μ moles of P_i or naphthol AS BI released from 15 μ moles naphthol AS BI phosphate by 0.5 mg wheat germ phosphatase in 0.05 M acetate buffer pH 5.0, in the presence and absence of various concentrations of Triton X-100. *B* Boiled enzyme +1.0% Triton X-100; *C* no Triton X-100

For the determination of free phosphate, 0.4 mg of substrate and 0.5 mg of wheat germ phosphatase was employed in the reaction mixture, the free phosphate being assayed by the method of CHEN *et al.* (1956), using ascorbic acid as the reducing reagent.

The effect of Triton X-100 was assayed either (i) by simultaneous incubation of various concentrations of the detergent in the reaction medium, or (ii) by pre-incubating the enzyme preparations for 10 minutes at 37° C in the presence of various concentrations of Triton X-100, or (iii) by incubating some enzyme samples in the presence or absence of the various concentrations of detergent followed by the addition of various concentrations of detergent at the end of the incubation (Tables 1 and 2). There was a large increase in the amount of naphthol AS BI estimated when the enzyme was incubated simultaneously with 0.1% and 0.5% Triton X-100 (Fig. 1), but the amount of liberated phosphate was not increased so much in the presence of the detergent (Fig. 1).

Table 2. *Effect of pre-incubating wheat germ acid phosphatase in the presence of different concentrations of Triton X-100 using naphthol AS BI phosphate as substrate. Readings are given as optical density units at 550 mμ*

Boiled enzyme +1% Triton	Control	Concentrations of Triton X-100 during pre-incubation		
~		0.1%	0.5%	1.0%
0.013	0.154	0.179	0.222	0.169

The addition of Triton X-100 at the end of the incubation, whether performed in the presence or absence of the detergent, consistently increased the intensity of colour development in the assay for naphthol AS BI (Fig. 2).

The pre-incubation of the enzyme in the presence of Triton X-100 yielded similar results to those concerning simultaneous incubation.

Effect of Triton X-100 on Coupling of Naphthol AS BI and Fast Red Violet LB

Concentrations of Triton X-100 ranging from 0.1—5% (w/v) were added to 0.005% naphthol AS BI in 0.2 M acetate buffer pH 5.0. Colour development with Fast Red Violet LB, colour extraction and estimation were performed as described above.

All concentrations of Triton X-100 tested caused an increase in the intensity of colour production between naphthol AS BI and Fast Red Violet LB (Fig. 2).

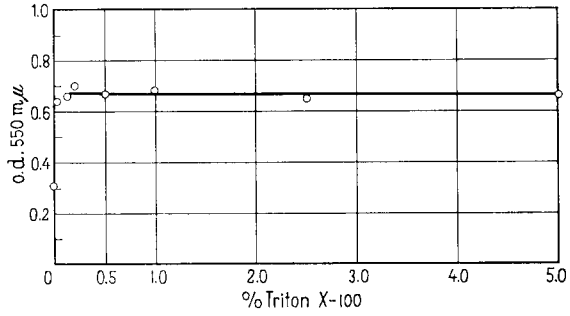

Fig. 2. Effect of various concentrations of Triton X-100 on the colour intensity of naphthol AS BI — Fast Red Violet LB complex. The reaction mixture contained 82 mμ moles of naphthol AS BI and excess of Fast Red Violet LB

Discussion

Triton X-100 had little or no apparent effect upon the activity of acid phosphatases from different sources when β-glycerophosphate was used as a substrate. However, when p-nitrophenyl phosphate was used as substrate, low concentrations of Triton X-100 activated the reaction whilst higher concentrations (0.5—1%) appeared to be inhibitory. The observed activation (Table 1) is in agreement with the findings of Tsuboi *et al.* (1955) who attributed this effect to a protective action of the detergent on the enzyme during the incubation.

The results obtained when using naphthol AS BI phosphate as substrate are more complex and may be considered in three parts. The first involves the enhancement of colour production between naphthol AS BI and Fast Red Violet LB in the presence of Triton X-100 which was similar at all concentrations of detergent between 0.02 and 5%. This may have been due to the Triton X-100 increasing the solubility of the naphthol AS BI in the aqueous medium so facilitating a more rapid rate of coupling between the base and diazonium compound, or this may have been due to an effect on the naphthol AS BI — Fast Red Violet LB complex so increasing the observed colour.

Although there was no difference in the effects of the detergent on the complexing of naphthol AS BI and Fast Red Violet LB when concentrations were increased from 0.02 to 5%, maximum colour development occurred at a concentration of 0.1% when the substrate was incubated together with the detergent and enzyme; the colour development then decreased with increasing concentrations of detergent

(Fig. 1). These data suggest that there is a stimulation of enzyme activity comparable to that obtained with p-nitrophenyl phosphate as substrate, and confirmed by the measurable release of phosphate (Fig. 1). At higher concentrations there was an inhibition of enzyme activity, again, comparable to that observed when using p-nitrophenyl phosphate as substrate. No such effects were observed when using β-glycerophosphate as substrate.

Naphthol AS compounds are being used more extensively in cytochemistry (LOJDA *et al.*, 1964; FISHMAN *et al.*, 1964; MAGGI, 1965; MAGGI and CHNG, 1966) and an activating effect of the detergent on the naphthol AS BI phosphatases has already been reported (ALLEN *et al.*, 1965; MAGGI, 1965). Triton X-100 has also been used in biochemical studies (DE DUVE, 1959) to "activate" lysosomes by altering the structure of the membranes of these particles and causing a release of the enzyme. The use of Triton X-100 in histochemical studies can result in an apparent activation of phosphatase activity at particulate sites in tissue sections. The activation observed in both biochemical and histochemical studies may be due to an effect of the detergent on the lysosomal membranes so making the enzyme more available to the substrates, but in the case of the substrates p-nitrophenyl phosphate and naphthol AS BI phosphate, there would also appear to be a direct activation effect upon the enzyme molecule itself. Moreover, the Triton X-100 appears to directly affect the complexing of the naphthol AS BI and the Fast Red Violet LB diazonium salt. It would seem that care must be exercised in the use of this detergent in studies of lysosomal enzymes.

The different responses of the phosphatases from various sources to the three substrates employed in the presence and absence of Triton X-100 was suggestive of the presence of more than one phosphatase in each of the enzyme preparations, a fact supported by the results of the polyacrilamide gel electrophoresis studies showing a number of protein bands responding to β-glycerophosphate and naphthol AS BI phosphates. That more than one phosphatase activity is present in a tissue has been shown by NEIL and HORNER (1962) in guinea pig liver; MAGGI *et al.* (1966) and LUNDIN and ALLISON (1966) in a variety of animal tissues; and GAHAN and MCLEAN (1967) in root tips of *Vicia faba*. In the present study, it was observed that only one band from each preparation was active with both substrates and it is possible therefore, that the Triton X-100 was affecting one naphthol AS BI phosphatase but not the several β-glycerophosphatases.

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Literature

ALLEN, S. L., J. M. ALLEN, and B. M. LICHT: Effects of Triton X-100 upon the activity of some electrophoretically separated acid phosphatases and esterases. J. Histochem. Cytochem. **13**, 434—440 (1965).

BITENSKY, L.: The demonstration of lysosomes by the controlled temperature freezing-sectioning method. Quart. J. micr. Sci. **103**, 205—209 (1962).

BURSTONE, M.: Enzyme histochemistry and its application in the study of neoplasms. New York: Academic Press 1958.

CHEN, P. S., T. Y. TORIBARA, and H. WARNER: Microdetermination of phosphorous. Analyt. Chem. **28**, 1756—1758 (1956).

DUVE, C. DE: Lysosomes, a new group of cytoplasmic particles. In: Subcellular particles (ed. T. HAYASHI), p. 128—159. New York: Ronald Press 1959.

FISHMAN, W. H.: Rat skeletal muscle β-glucuronidase localization by postcoupling technique using naphthol AS-BI-β-D-glucosiduronic acid and Fast Dark Blue R. J. Histochem. Cytochem. **12**, 306—308 (1964).

GAHAN, P. B.: Histochemical evidence for the presence of lysosome-like particles in root meristem cells of *Vicia faba*. J. exp. Bot. **16**, 350—355 (1965).

—, and J. MCLEAN: Acid phosphatases in roots of *Vicia faba*. Biochem. J. **102**, 47—48P (1967).

HOLT, S. J.: Factors governing the validity of staining methods for enzymes, and their bearing upon the Gomori acid phosphatase technique. Exp. Cell Res., Suppl. **7**, 1—27 (1959).

LOJDA, Z., B. VEČEREK, and H. PELICHOVÁ: Some remarks concerning the histochemical detection of acid phosphatase by azo-coupling reactions. Histochemie **3**, 428—454 (1964).

LUNDIN, L. G., and A. C. ALLISON: Acid phosphatases from different organs and animal forms compared by starch-gel electrophoresis. Biochim. biophys. Acta (Amst.) **127**, 527—529 (1966).

MAGGI, V.: Biochemical evidence for the presence of two acid phosphatases in the coagulating gland of the mouse. Biochem. J. **96**, 12 P (1965).

—, and W. L. CHNG: A study of acid phosphatase in mouse kidney using naphthol AS BI phosphate as a substrate. Histochemie **7**, 267—273 (1966).

—, L. M. FRANKS, and A. W. CARBONELL: Histochemical study of a number of hydrolases, including a new acid phosphatase, in various tissues of men and mice. Histochemie **6**, 305—312 (1966).

NEIL, M. W., and M. W. HORNER: The acid p-nitrophenyl phosphatase activity of adult guinea-pig liver subcellular fractions. Biochem. J. **84**, 32 P (1962).

SCHMIDT, G.: Acid prostatic phosphatase. In: Methods in enzymology (ed. S. P. COLOWICK and N. O. KAPLAN), vol. II, p. 523—530. New York: Academic Press 1955.

TSUBOI, K. K., and P. B. HUDSON: Acid phosphatase III. Specific kinetic properties of highly purified human prostatic phosphomonoesterase. Arch. Biochem. **55**, 191—205 (1955).

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