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The effect of sodium salicylate on the release of acid phosphatase activity from rat liver lysosomes *in vitro*

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Salicylate, in concentrations up to 10mM, does not inhibit the activity of the acid phosphatase liberated from rat liver lysosomes. The release of the enzyme from lysosomal preparations incubated with the drug depends on the pH of the incubation medium, the duration of incubation and the concentration of salicylate. It is concluded that salicylate does not influence lysosomal stability *in vivo*.

The release of lysosomal enzymes may play an important part in several types of cell injury (de Duve, 1964). It has often been stated that one or more phases of either acute or chronic inflammatory reactions may be mediated by the intracellular release of such enzymes. An interesting corollary to this view is that anti-inflammatory drugs may act by interfering with either the release or the activities of the liberated enzymes. Duthie (1963) was the first to suggest this mechanism of action for salicylate and several workers have studied the *in vitro* effects of the drug on lysosomal preparations and enzymes. The results appear to be confusing and contradictory. Salicylate and acetylsalicylate have been reported to decrease the release of enzymes and hence stabilize the lysosomes (Miller & Smith, 1966; Tanaka & Iizuka, 1968), to have no effect (Weissman, 1968; Robinson & Willcox, 1969; Ennis, Granda & Posner, 1968), to increase the liberation of enzymes (Lee & Spencer, 1969; Brown & Schwartz, 1969) and to inhibit the activities of the free enzymes (Anderson, 1968). Differences in experimental conditions may explain, at least in part, these discrepancies and the most important variables seem to be the concentration of the salicylate, the pH and the time of incubation. We have therefore investigated the effects of 0 to 4mM salicylate on the release of lysosomal enzymes over the pH range 5·0 to 7·0 using periods of incubation up to 4½ h.

EXPERIMENTAL

Preparation of lysosomal suspension

Male Wistar rats, weighing between 300 and 500 g, maintained on M.R.C. cube diet No. 41, were killed by cervical fracture. The liver was removed, weighed and minced with scissors in ice-cold 0·25 M sucrose. The sucrose solution was replaced twice to remove as much blood as possible, and the liver homogenized in 10 vol of the sucrose medium using one stroke of a glass homogenizer and a Teflon pestle. The homogenate was centrifuged for 10 min at 3500 *g* to remove nuclei, cell debris and heavy mitochondria. This and subsequent centrifugations were done at 2°. The supernatant was centrifuged for 10 min at 25000 *g* and the lysosomal pellet washed with 5 vol of ice-cold sucrose and finally resuspended in 100 ml of the 0·25 M sucrose (lysosomal suspension).

Effect of salicylate on activity of free acid phosphatase

The acid phosphatase was liberated from the lysosomal suspension by the addition of sufficient Triton X-100 to give a final concentration of 0·2% (v/v). The membranes and any intact lysosomes were removed by centrifugation for 10 min at 25000 *g* and the supernatant used for the subsequent experiments. The triton X-100 was not removed at this stage because it was found to have no effect on the activity of the acid phosphatase. The reaction mixture contained sodium salicylate, 0–10mM; sufficient sodium chloride to give a final sodium ion concentration of 10 mM and 0·8 mg/ml *p*-nitrophenyl phosphate (Sigma Chemical Co., St. Louis) in 0·05 M acetate —0·25M sucrose buffer, pH 4·8. The reaction was started by the addition of 3·0 ml of the reaction mixture to 0·1 ml of the lysosomal supernatant, which had been preincubated for 30 min at 37° with either 0·1 ml distilled water or with 0·1 ml sodium salicylate (0–10 mM). The phosphatase assay incubation was performed at 37°, and stopped after 20 min by addition of 1·0 ml ice-cold N NaOH. The *p*-nitrophenol was estimated by measuring the absorption at 410 nm in a Unicam SP 800 spectrophotometer.

Effect of salicylate on release of acid phosphatase activity from the lysosomal suspension

Each reaction mixture contained 1 ml of the lysosomal suspension; 1 ml of a solution containing 0·25 M sucrose and 0·1 M cacodylate buffer at either pH 5·0, 5·5, 6·0 or 7·0; and either sodium salicylate 0 to 4 mM (experimental mixtures) or sufficient sodium chloride to give a final sodium ion concentration equal to that contributed by the sodium salicylate (control mixtures). The mixtures were prepared at 2°, and at zero time were transferred to a waterbath at 37° and incubated with shaking. At appropriate time intervals, up to 4½ h, four experimental and four control mixtures were taken from the incubation bath, cooled on ice and immediately centrifuged at 30 000 *g* for 10 min to remove intact lysosomes and membranes. The supernatants were separated and stored at 0° before being assayed for phosphatase activity. This was performed by taking 0·25 ml aliquots of each supernatant, the reaction being started by the addition of 3·0 ml of a 0·8 mg/ml solution of *p*-nitrophenyl phosphate in 0·25 M sucrose —0·05 M acetate buffer, pH 4·8. The mixtures were incubated for 20 min at 37°, the reaction stopped by adding 1·0 ml of ice-cold NaOH and the absorption at 410 nm measured as described above. Similar experiments were made in which the total acid phosphatase activity of the lysosomal suspension was liberated by 0·2% v/v Triton X-100 and the release of the phosphatase in the presence of salicylate was expressed as a percentage of the values obtained with triton.

RESULTS

Analysis of the results obtained when the enzyme preparation was incubated with salicylate, in concentrations up to 10 mM, and subsequently assayed in the presence of similar concentration of the drug showed no significant ($P = 0{\cdot}02$) inhibition of the free lysosomal acid phosphatase from the control value of 100 ± 2·5%. Experiments in which salicylate was present only during the assay procedure also showed no significant inhibition.

The results of representative experiments concerned with the release of the enzyme from the lysosomal suspensions are given in Table 1. In this Table the values are expressed as percentages of the acid phosphatase activity released by Triton X-100 which has been taken to represent the total enzyme activity originally present in the lysosomes. If the release of acid phosphatase activity is regarded as an index of the

Table 1. *Effect of* 4 mM *salicylate on the release of lysosomal acid phosphatase.* Experimental details were as described in the text. The values, expressed as the percentages of the enzyme activity released by Triton X-100, are given as means of four separate experiments. In the control experiments the mean acid phosphatase activity released by the Triton X-100 was 11·2 μ mol *p*-nitrophenol/mg lysosomal protein in 20 min. The results have been analysed by Students *t*-test, the minimal acceptable level of significance being taken as P = 0·02. (C, control mixture; S, mixture containing 4 mM salicylate).

pH of incubation mixture	Time of incubation (min)									
	30		60		90		180		270	
	C	S	C	S	C	S	C	S	C	S
5·0	5·6	10·0*	22·7	29·6*	41·7	43·2	58·7	58·7	—	—
5·5	1·5	2·3*	7·2	11·4*	22·2	31·6*	55·6	58·1*	—	—
6·0	0·4	0·7*	1·1	1·3*	3·1	4·2*	25·1	32·7*	—	—
7·0	—	—	—	—	2·6	3·0	7·4	8·1	19·8	24·4

* Significant difference between the control and salicylates values

stability of the lysosomes then the pH of the incubation mixture is an important factor. Thus at pH 5·0 approximately 25% of the enzyme activity is released after 1 h incubation whereas at pH 5·5 this is reduced to 7% and is only about 1% at pH 6·0. A long period of incubation is required to cause a release of the enzyme at pH 7·0, e.g. only 7% being liberated after 3 h. The effects of 4 mM salicylate in increasing the release of the enzyme parallel the effects of the pH on the lability of the lysosomal suspensions. The drug is most effective when maximum release of the enzyme is occurring under the corresponding control conditions. This is illustrated in Fig. 1 in which the results for the release of acid phosphatase from the lysosomal suspensions in the presence of 4 mM salicylate are plotted against time of incubation for each of the pH values studied. In

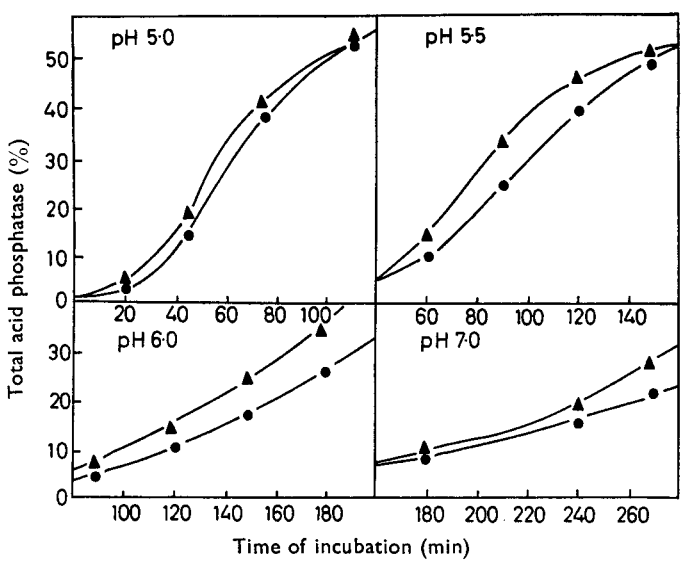

FIG. 1. Effects of salicylate on the release of acid phosphatase from rat liver lysosomes at different pH values. Experimental conditions as in Table 1. ●, control. ▲, 4mM salicylate.

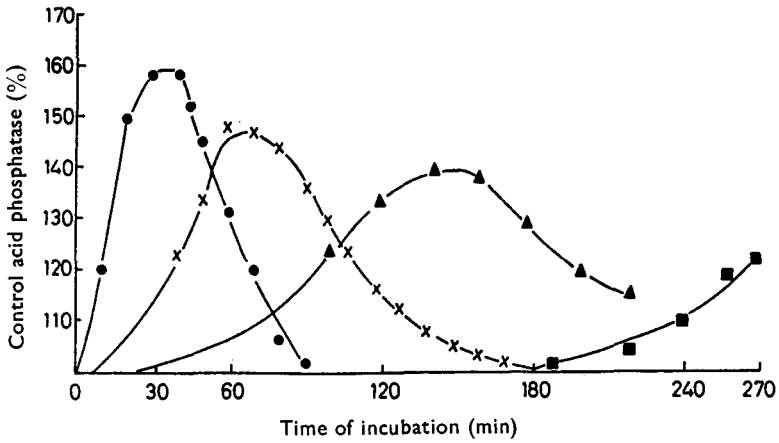

FIG. 2. Effects of varying the pH of the incubation medium on the release of acid phosphatase from rat liver lysosomes in the presence of 4mM salicylate. The data, expressed as percentages of the corresponding control values has been calculated from experimental results. ●, pH 5·0; X, pH 5·5; ▲, pH 6·0 and ■, pH 7·0.

Fig. 2 the results in the presence of salicylate have been expressed as percentages of the corresponding control values and plotted against time of incubation for each pH value. A possible stabilizing action of low concentrations of salicylate could not be studied at neutral pH because little, if any, phosphatase was liberated in several hours. The effects of varying the drug concentration was therefore investigated at pH 6 because the lysosomes were reasonably stable at this pH compared to more acid conditions (Fig. 1). The results in Table 2 show that the only effect of salicylate was to increase the release of acid phosphatase and this became significant at drug concentrations of mM and above.

Table 2. *Effect of varying the salicylate concentration on the release of acid phosphatase from rat liver lysosomes incubated at* pH 6·0 *for* 140 *min.* Experimental details were as described in the text and the results are expressed as in Table 1 except that standard deviations are included.

Salicylate (mM)	Enzyme activity (% of that released by Triton)	*P*
0	10·9 ± 0·7	—
0·5	12·1 ± 1·0	0·1
1·0	12·2 ± 0·3	0·02
2·0	13·0 ± 0·7	0·005
5·0	15·3 ± 0·4	0·001

DISCUSSION

The release of lysosomal enzymes may play an important role in the development of acute and chronic inflammatory reactions (Lack, 1966). It has been suggested that the later stages of rheumatoid joint disease may involve the disruption of intra-articular lysosomes causing the release of hydrolase enzymes which degrade the protein-polysaccharide complexes of cartilage (Ennis & others, 1968). In this situation the proteolytic cathepsins (Barrett, 1969) may be of particular importance but lysosomes

contain many other hydrolases including β-glucuronidase, aryl sulphatases and acid phosphatase (Weissmann, 1967). Acid phosphatase is frequently used as a "marker enzyme" to assess lysosomal damage, both *in vivo* and *in vitro*, because it is localized almost exclusively in the particles, its release parallels that of the other lysosomal hydrolases and its activity may be easily measured. Thus the high activities of acid phosphatase in rheumatoid synovial fluid (Smith & Hamerman, 1962) have been interpreted as evidence for an increased breakdown of lysosomes in the disease. The estimation of the release of the enzyme from lysosomal suspensions is an accepted technique for assessing the integrity of the particles *in vitro*.

Several workers have attempted to explain the actions of anti-inflammatory drugs in terms of interaction between the drugs and lysosomal enzymes. Two possible mechanisms are concerned. The first assumes that the drugs inhibit the activities of the released enzymes. Anderson (1968) reported that aspirin inhibited lysosomal cathepsins and acid phosphatase, the latter enzyme activity being inhibited by about 30% by 1 mM and 67% by 2 mM acetylsalicylate. This has not been the experience of other workers (Tanaka & Iizuka, 1968; Miller & Smith, 1966) and we find salicylate concentrations, up to 10 mM, do not inhibit lysosomal acid phosphatase. Sodium salicylate and aspirin are equally effective in the treatment of acute rheumatic fever and rheumatoid arthritis (Woodbury, 1965) and as anti-inflammatory agents in many acute inflammatory responses in experimental animals (Wilhelmi, Gdynia & Ziel, 1968). There are differences in analgesic potency between aspirin and sodium salicylate (Lim, 1966) and in the antagonism of these drugs to bradykinin-evoked reactions (Collier, 1969) and their effects on the adhesiveness of blood platelets (O'Brien, 1968) but there is no reason to differentiate the two drugs in terms of anti-inflammatory action. Thus any *in vitro* effects of salicylate on lysosomal enzymes should be equivalent to those of acetylsalicylate.

The second mechanism relating the anti-inflammatory activity of salicylate to lysosomal enzymes suggests that the drugs stabilize the lysosomal membrane and hence decrease the liberation of the hydrolases in response to the initial inflammatory insult. Miller & Smith (1966) claimed that acetylsalicylate concentrations of 0·1 mM, and above, decreased the release of acid phosphatase from rat liver lysosomes. The lysosomal suspensions were labilized by incubating at 37° but the pH of the incubation mixtures was not recorded. The importance of this factor was stressed by Tanaka & Iizuki (1968) who reported that variable results were obtained with lysosomal suspensions at neutral pH due to the presence of inorganic salts. Using tris-acetate buffer-sucrose medium at pH 7·4, an ethanolic solution of acetylsalicylate, and a heavy lysosomal fraction, they reported that drug concentrations of 0·5 to 5 mM progressively and significantly decreased the release of acid phosphatase into the medium. In contrast, at pH 5·0, they reported that mM acetylsalicylate caused a slight increase in the release of the enzyme and that this was "drastically accelerated" if the drug was used in a non-buffered medium. The apparent stabilization of lysosomes by acetylsalicylate or salicylate at or about neutral pH has not been confirmed. Weissmann (1968) reported no effect of mM salicylate at pH 6·8, Robinson & Willcox (1969) could not detect any effect of mM acetylsalicylate or salicylate at pH 7·4 and the present results (Table 1) show that 4 mM salicylate does not alter the rate of release of acid phosphatase at pH 7·0 during prolonged incubation. In contrast, the labilizing action of salicylate at pH 5·0 has been observed by several workers with the reservation that

in vitro drug concentrations at mM and above are necessary for the effect to become evident. Thus acetylsalicylate and salicylate concentrations from 0·001 to 1 mM have been reported to have no effect on the release of acid phosphatase and other hydrolases from lysosomal suspensions at pH 5·0 to 6·0 (Ennis & others, 1968) whereas 1–2 mM concentrations of the drug cause an increased release of enzyme under these conditions (Lee & Spencer, 1969; Brown & Schwartz, 1969). The present work shows firstly that decreasing the pH of the incubation mixture increases the lability of the lysosomes when this is assessed in terms of the rate of release of acid phosphatase (Table 1; Fig. 1). Furthermore the effect of salicylate in causing an increased release of the enzyme parallels this lability, becoming more pronounced as the pH of the incubation mixture is reduced (Fig. 2). This effect also increases as the drug concentration is increased (Table 2).

It must be concluded that salicylate does not stabilize lysosomes *in vitro* at neutral pH and that the only interaction between the drug and the subcellular particles is an increased release of acid phosphatase at acid pH. This latter effect is dependent on the pH of the incubation medium, the time of incubation and the concentration of salicylate. A direct interaction between salicylate and either lysosomal membranes or the lysosomal enzymes cannot be the mechanism by which the drugs exert their experimental and clinical anti-inflammatory actions.

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