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EFFECT OF THE ANTIALLERGIC DRUG DISODIUM CROMOGLYCATE AND VARIOUS DERIVATIVES ON ALKALINE PHOSPHATASE

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Disodium cromoglycate (DSCG) inhibits alkaline phosphatase in a non-competitive manner, the enzyme undergoing a conformational change which is attenuated by the presence of calcium ions. The structurally related pyranone and benzoic acid are weak inhibitors of the enzyme and produce a similar conformational change. Coumarin does not induce any conformational change in the enzyme suggesting that the 4-oxo group in DSCG may be essential for its effect.

KEY WORDS: Sodium cromoglycate, alkaline phosphatase, exocytosis, calcium, enzyme inhibition, conformational change

INTRODUCTION

The regulation of several metabolic processes, such as mast cells secretion [1-7], occurs by phosphorylation and dephosphorylation processes. Alkaline phosphatase (ALP, orthophosphoric monoester phosphohydrolase, EC 3.1.3.1) may be involved in cellular regulation by means of its dephosphorylation action. In support of this view it has been observed that compounds with immunomodulating activities, e.g. bromo-levamisole inhibit [8] ALP, and membranal calcium channels, involved in these kind of activities, may have [9,10] an alkaline phosphatase component.

Disodium cromoglycate (DSCG), an inhibitor of the release of allergic mediators which affects several cellular types [11-24], blocks the transient rise in the intracellular calcium concentration and subsequent histamine liberation [25-28]. Cell-permeable derivatives of DSDCG inhibit a cytosolic kinase [29,30] and DSCG itself, even in the absence of challenge, induces phosphorylation of a M_w 78.000 protein [31], suggesting that it may stabilise the mast cell by activation of natural secretion control mechanism.

Since the mode of action of cromoglycate is not established and in view of its likely relationship with alkaline phosphatase, we have studied the inhibition of ALP by DSCG and, due to the importance of calcium in membrane cellular transport, the effect of calcium ion on this inhibitory action.

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MATERIALS AND METHODS

Intestinal alkaline phosphatases from two sources, bovine (p 8647), and chicken (p 8008), at concentrations of ~ 0.2 U/mL were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Cromoglycate (DSCG) was obtained from Fisons Limited (Loughorough, England), 2H-1-benzopyran-2-one (coumarin), 3,3-methylene bis (4-hydroxy-coumarin) (dicoumarol), from Janssen Chimica (Beerse, Belgium), 4H-pyran-4-one and benzoic acid from Aldrich Chemie (Steinheim, Germany), and calcium chloride from Sigma.

Enzymatic Activity Measurement

ALP activity was measured using a diagnostic kit provided by Sigma, which is based on the procedure described by Bowers and McComb[32] (equation (1)).

$$p\text{-Nitrophenylphosphate} + H_2O \rightarrow p\text{-Nitrophenol} + \text{Phosphate} \qquad (1)$$

The enzyme activity in the sample was calculated from ΔA at 405 nm, according to equation (2).

$$\text{ALP(U/L)} = \frac{\Delta\text{A per min} \,.\, \text{TV} \,.\, 10^3}{18.45 \,.\, \text{SV} \,.\, \text{PL}} = \Delta\text{A per min} \,.2764 \qquad (2)$$

where ΔA per min is the change in absorbance per minute at λ 405 nm; TV is the total volume (1.02 mL); SV the sample volume (0.02 mL); 18.45 is the $\varepsilon.10^{-3}$ for *p*-nitrophenol at λ 405 nm; PL the light path and 10^3 the conversion factor of units per mL to units per liter. In all measurements carbonate buffer pH 10.2±0.1 was employed, and the reaction carried out at ambient temperature.

Structural Change Measurements

Viscometry: Viscometric measurements were made using an Ubbelohde suspended level microviscometer, thermostated at 37°C, with a Viscoboy (Lauda-Konigshofen, F.R.G.). Densities were evaluated with a Gay-Lussac pyknometer and the results, obtained as falltime of liquid, were transformed into specific viscosities (η_{sp}/C) calculated by the method of Reynolds *et al.*[33], where *c* is the concentration in g/mL of protein. Measurements were carried using phosphate buffer (1/15 M) pH 7.4 and bicarbonate buffer ($2.5.10^{-2}$ M) pH 10.07.

Dilatometry: Volume changes were measured by dilatometric techniques[34] at 37°C, employing a dilatometer similar to that described by Komiyama *et al.*[35] for which the volumes of the lower and upper compartiments were 5.2 and 6.4 mL respectively. The volume change, in microliters (μL), produced by mixing protein and compound, was adjusted to mL/10^5 g of protein[36,37].

RESULTS AND DISCUSSION

Table 1 shows the high percentage inhibition of ALP activity caused by cromoglycate (DSCG), obtained using a near saturating substrate concentration ($1.6.10^{-2}$ M). The

TABLE 1
Effect of disodium cromoglycate (DSCG), dicoumarol (DICUM), coumarin (CUM), 4-pyranone (4-PIR) and benzoic acid (BENZ) on enzymatic activity of alkaline phosphatase (ALP).

COMPOUND	CONCENTRATION (M)	% INHIBITION
DSCG	$5.22.10^{-10}$	58.4
	$1.044.10^{-9}$	74.7
	$2.090.10^{-9}$	56.6
CUM	$1.6.10^{-7}$	7.5
	$3.1.10^{-7}$	17
	$6.2.10^{-7}$	12.3
DICUM	$1.6.10^{-7}$	11.7
	$3.1.10^{-7}$	21
	$6.2.10^{-7}$	9.4
4-PIR	$3.1.10^{-7}$	29.8
BENZ	$3.1.10^{-7}$	26.4

[ALP] = 0.26 U/L. A saturated substrate concentration ($1.6.10^{-2}$ M) was used. All experimental determinations are been made in duplicate with triplicate tubes.

inhibition of phosphatase activity was not directly related to drug concentration, $1.044.10^{-9}$ M producing the greatest inhibition of enzymatic activity.

The type of inhibition exhibited by DSCG was determined by a Lineweaver-Burk plot (Figure 1A) and was shown to be non-competitive with K_m and K_i values of $6.0357.10^{-3}$ M and 3.10^{-10} M respectively. This strong non-competitive inhibition suggests that the drug binds to a site other than the active site of the enzyme.

To determine whether the non-competitive inhibition by DSCG altered the conformation of ALP, viscometric measurements of the ALP-DSCG interaction were made. Figure 2 shows that changes in the specific viscosity (η_{sp}/c) of the protein occur in the presence of the drug, indicating that the enzyme undergoes a conformational change[38]. Specific viscosity decreased when the DSCG concentration was increased from 1.10^{-7} to 5.10^{-7} M, giving the greatest reduction when the [ALP]/[DSCG] value was 1-2. This change is produced at the same ratio of DSCG concentrations that produce inhibition of the enzyme (Table I and Figure 2), which suggests that the effect of DSCG on activity is closely related to its effect on the structure of the protein.

Those parts of the DSCG molecule which may be essential for activity have been examined using coumarin, dicoumarol, pyranone and benzoic acid which contain elements of DSCG structure. As can be seen from Table 1, neither coumarin nor dicoumarol inhibited ALP activity to any great extent. A weaker inhibitory effect for pyranone and benzoic acid (30 and 26.4% respectively) was observed. Possibly the pyranone group may be responsible for the effect of DSCG on ALP.

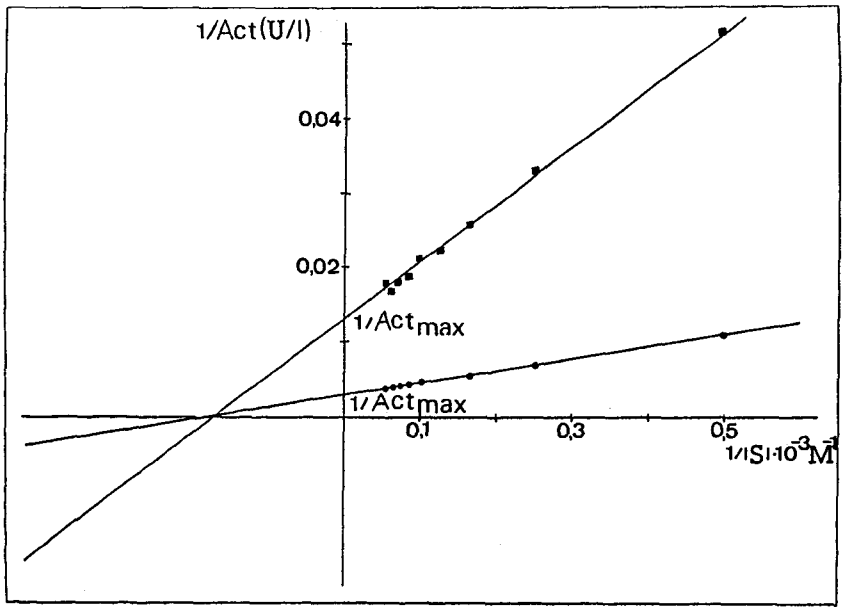

FIGURE 1A Lineweaver-Burk plot of bovine intestinal alkaline phosphatase ($6.5.10^{-10}$ M): (•) in the absence and (■) in the presence of sodium cromoglycate (DSCG) ($1.044.10^{-9}$M).

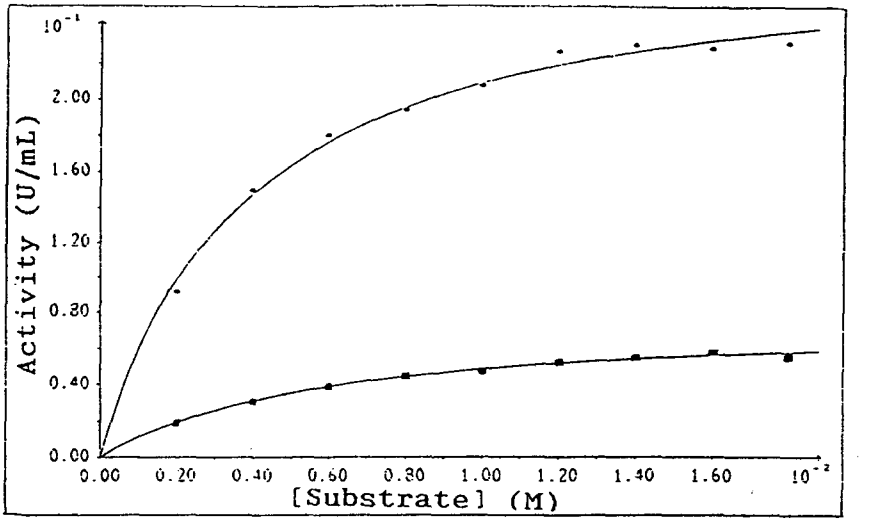

FIGURE 1B Specific activities of bovine intestinal alkaline phosphatase ($6.5.10^{-10}$ M, 0.2 U/mL): (•) in the absence and (■) in the presence of sodium cromoglycate (DSCG) ($1.044.10^{-9}$ M).

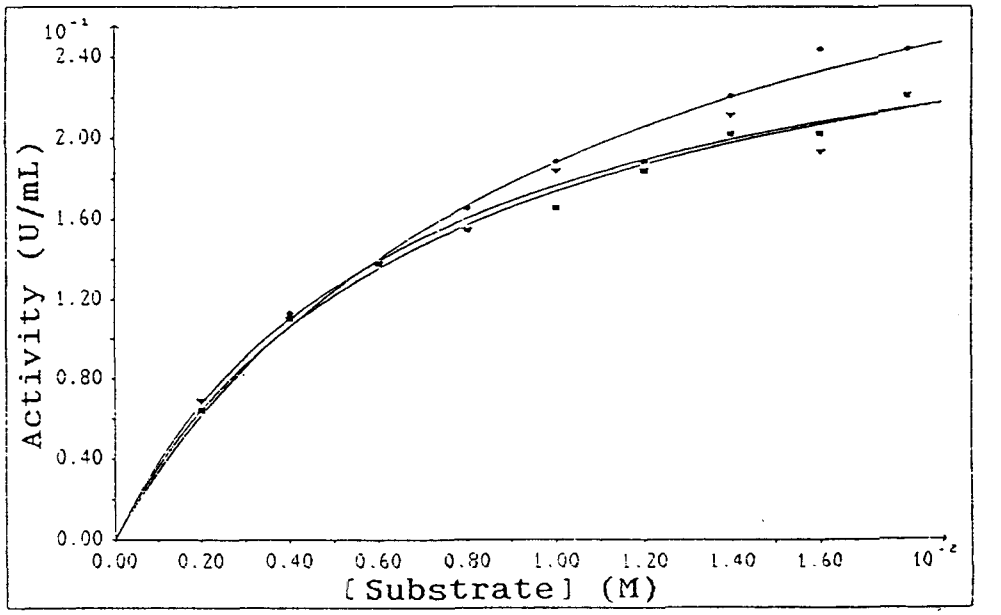

FIGURE 1C Specific activities of chicken intestinal alkaline phosphatase ($3.5.10^{-7}$ M, 0.2 U/mL):(•) in the absence, (▼) in the presence of dicoumarol ($3.1.10^{-7}$ M) and (■) in the presence of coumarin ($3.1.10^{-7}$ M).

Calcium ion produces a considerable attenuation of the effect of DSCG on ALP viscosity (Figure 2). A smaller effect of cromoglycate on enzyme activity was also noted. This attenuation is not due to a chelation of DSCG by calcium[39,40], so it may be that calcium ion stabilizes the conformation of the enzyme, antagonizing the effect of DSCG. Ca^{+2} ions would protect the enzyme from the conformational effects of the drug.

The effect of the different compounds on the conformation of the enzyme have been examined by means of dilatometric techniques and the results are shown in Figure 3. The changes of slopes obtained on studying $\bar{V}_{2C}$ variations are due to conformational changes, DSCG producing the largest conformational change as measured by the change in $\bar{V}_{2C}$ (Figure 3). Carboxylic acid chromone and pyranone alter the conformation of the enzyme (Figure 3) whereas coumarin does not induce any conformational change. It would seem from these results that the presence of the oxo group at the 4-position may be responsible for the conformational produced by DSCG on protein molecule.

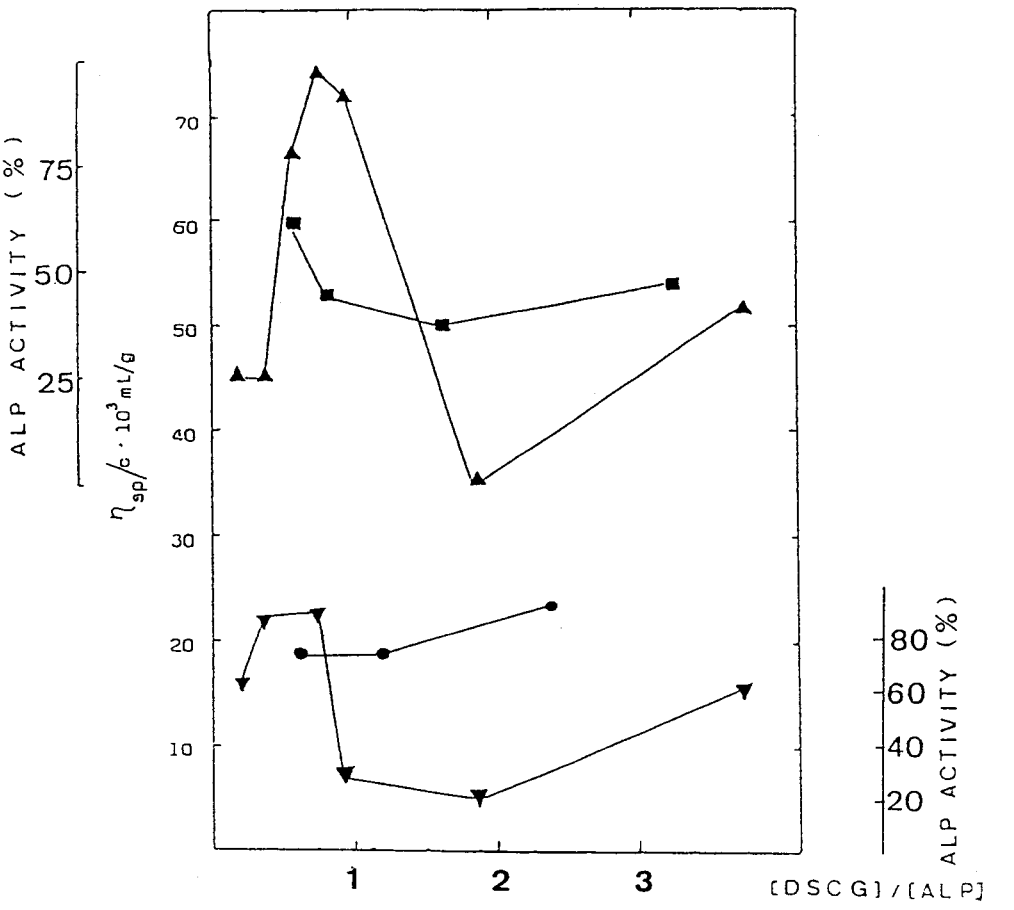

FIGURE 2 Inhibition of enzymatic activity of alkaline phosphatase (ALP) ($8.9.10^{-10}$ and $6.5.10^{-10}$M) in the presence of disodium cromoglycate (DSCG) (from $5.2.10^{-10}$ to $20.9.10^{-10}$ M) (■); and in the presence of DSCG and calcium (from $5.2.10^{-10}$ to $20.9.10^{-10}$ M) (•). Variations of ALP viscosity produced by DSCG (▲) and by DSCG with calcium (▼). All measurements were carried out at optimum conditions (37°C and pH=10) and employed equimolars cation and drug concentrations.

In the light of experimental results, we conclude that disodium cromoglycate inhibits, in non-competitive way, alkaline phosphatase activity and the effect of cromoglycate on ALP activity is closely related to its effect on the structure of enzyme. Moreover, the functional group present in DSCG essential for these effects is the pyranone 4-oxo group.

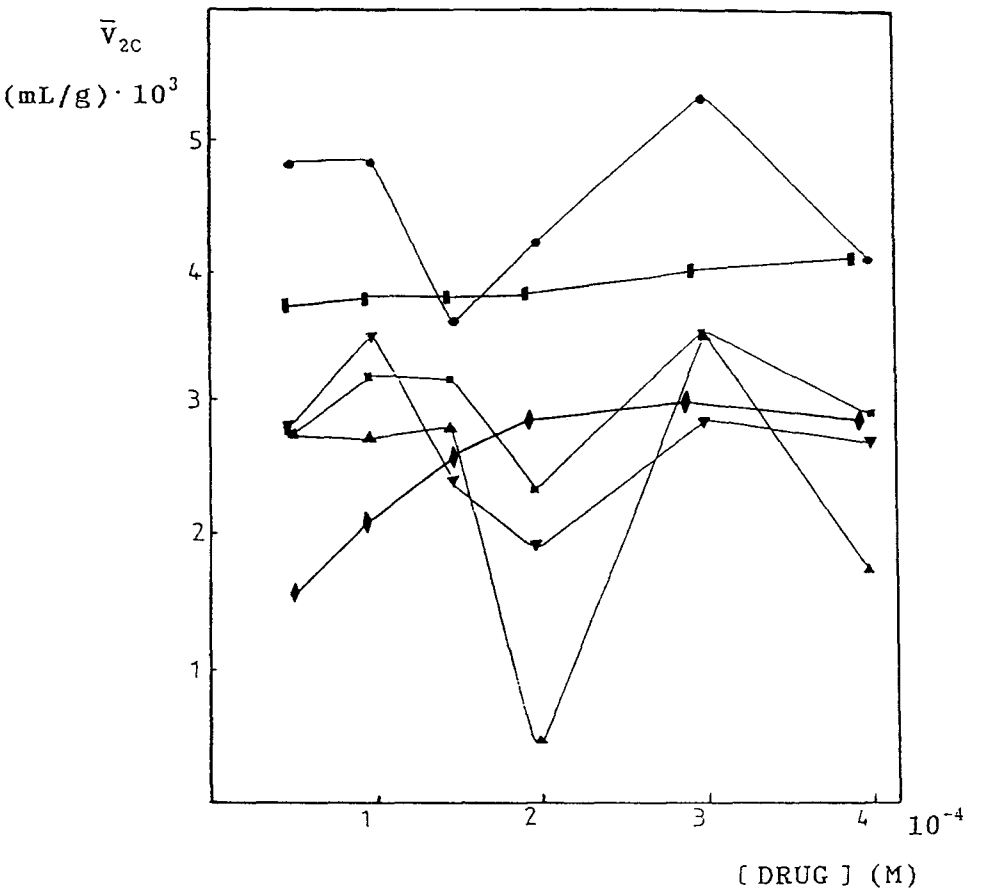

FIGURE 3 Variations of specific volume ($\bar{V}_{2C}$) for the following systems: (•) cromoglycate (from $0.5.10^{-4}$ to 4.10^{-4} M) + alkaline phosphatase ($6.25.10^{-7}$ M) (ALP); (▲) dicoumarol (from $0.5.10^{-4}$ to 4.10^{-4} M) + ALP ($6.25.10^{-7}$ M); (■) 4-pyranone (from $0.5.10^{-4}$ to 4.10^{-4} M) + ALP ($6.25.10^{-7}$ M); (▼) carboxylic acid chromone ($0.5.10^{-4}$ to 4.10^{-4} M) + ALP ($6.25.10^{-7}$ M); (♦) coumarin (from $0.5.10^{-4}$ to 4.10^{-4} M) + ALP ($6.75.10^{-7}$ M) and (■) benzoic acid (from $0.5.10^{-4}$ to 4.10^{-4} M) + ALP ($6.75.10^{-7}$ M).

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