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SALICYLATE INHIBITS HUMAN PLACENTAL SULPHATE TRANSPORT *IN VITRO*

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Abstract—The effect of salicylate on sulphate transport by the human placenta has been studied using isolated brush-border membrane vesicles and placental tissue slices. Sulphate uptake by isolated vesicles was inhibited in a dose dependent fashion ($K_I \sim 3$ mM) by salicylate. It appears that this drug blocks sulphate accumulation in a non-competitive manner. Sulphate efflux from preloaded vesicles was also found to be markedly reduced by salicylate in a non-competitive fashion. Consistent with the vesicle studies salicylate inhibited sulphate transport by placental tissue slices. The results suggest that salicylate ingestion could compromise feto-placental sulphate homeostasis. In addition we have found that the aspirin-like drug, flufenamic acid, inhibits sulphate transport by isolated microvillus membrane vesicles.

Sulphate is an essential nutrient required by the feto-placental unit for a number of sulphate-dependent conjugation reactions and biosynthetic pathways. With this in mind several groups have attempted to elucidate the mechanism of sulphate transport across the placenta using isolated membrane vesicles [1–3]. The major route for sulphate transport across the brush-border membrane of the human placental syncytiotrophoblast (the first barrier between the maternal and fetal circulations, respectively) is an anion-exchange process which shares many characteristics with anion-exchange mechanisms of other tissues [4]. Thus, sulphate transport by this membrane displays saturation kinetics, is electroneutral and is strongly inhibited by the stilbene sulphonate DIDS (4,4′-diisothiocyanatostilbene-2,2′-disulphonic acid). A recent study by Bustamante *et al.* [5] has revealed that the brush-border membrane sulphate carrier exhibits a marked asymmetry for trans-stimulation which may account for the finding of Cole *et al.* [6] that the concentration of sulphate is higher in the fetal than the maternal circulation.

In view of the similarity between the properties of placental sulphate transport and that of other cell types Shennan and Boyd [7] investigated the effect of salicylate on sulphate transport by isolated placental brush-border membrane vesicles because it had previously been shown that this drug inhibits anion-exchange by human red blood cells [8, 9]. The authors found that salicylate at high therapeutic and toxic concentrations (e.g. see Ref. 10) markedly reduced sulphate accumulation by placental vesicles. They suggested that a pertubation of placental sulphate metabolism may partly explain (in addition to an inhibition of prostaglandin synthesis) the observation that chronic salicylate ingestion during pregnancy leads to prolonged gestation, complicated deliveries and significantly reduced weights at birth [11–13].

The present study was initiated in an attempt to confirm and extend the preliminary observation of Shennan and Boyd [7] that sulphate transport is inhibited by salicylate. Therefore, we have examined the effect of salicylate on the bidirectional movement of sulphate across the isolated brush-border membrane and on sulphate movement from whole placental tissue. In addition we have investigated the effect of flufenamic acid, an aspirin-like drug, on placental sulphate transport since this drug is also known to be a very potent inhibitor of red cell anion transport [14, 15].

MATERIALS AND METHODS

Preparation of brush-border membrane vesicles. Normal-term human placentae were stored at 4° after delivery of the baby. The process of preparing microvillus membrane vesicles, according to the method of Boyd and Lund [16] as modified by Shennan *et al.* [17], was normally commenced within 90 min. This method of vesicle preparation enriched the brush-border marker enzyme alkaline phosphatase from 0.35 ± 0.07 μmol P_i released/mg protein/min in the original preparation to 8.2 ± 1.22 μmol P_i/mg protein/min in the vesicle preparation. Membranes were suspended in a medium containing 0.4 mM K_2SO_4, 300 mM sucrose and 10 mM KOH-Hepes, pH 7.4. The vesicles were stored at −80° prior to use.

Vesicle sulphate transport assay. $^{35}SO_4^{2-}$ was used to assay sulphate transport (both influx and efflux) by microvillus membrane vesicles. All experiments were conducted at room temperature (20 ± 1°). The potassium ionophore valinomycin, at a concentration of 10^{-6} M, was routinely used to clamp the electrical potential across the vesicle membrane. Since the intra- and extra-vesicular potassium concentrations were equal in all experiments the vesicle membrane potential would have effectively been clamped to 0 mV. Sulphate transport was assayed using the ion-exchange column method developed by Gasko *et al.* [18] as modified by Boyd and Shennan [2]. The principle employed in separating intra- from extravesicular isotope is the use of a column containing an ion-exchange resin. When an aliquot

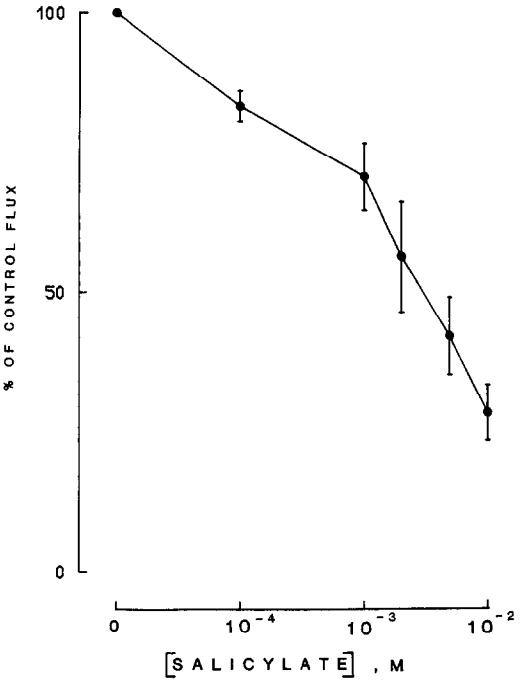

Fig. 1. Dose–response curve of salicylate inhibition of SO_4^{2-} uptake by placental brush-border membrane vesicles. Salicylate was added to the incubation media as the sodium salt. The incubation media also contained 0.4 mM K_2SO_4, sucrose (adjusted over the range 280 to 300 mM to maintain osmolarity) and 10 mM KOH-Hepes pH 7.4. Each point represents the mean ± SE of three determinations.

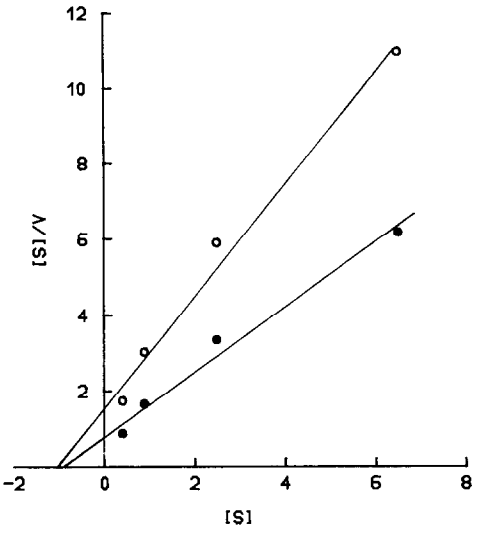

Fig. 2. Hanes–Woolf plot ($[S]/V$ vs $[S]$) of SO_4^{2-} uptake by human placental brush border membrane vesicles in the presence (○) and absence (●) of 5 mM salicylate. Uptake was measured in media containing 0.4–6.5 mM SO_4^{2-} sucrose (concentration adjusted to maintain osmolarity) and 10 mM KOH-Hepes, pH 7.4. Each point represents the mean of three or seven determinations.

containing vesicles and medium is added to the top of such a column extravesicular isotope will be rapidly sequestered by the resin; thus, the isotope which appears in the eluent flowing from the column will be that which is retained by the vesicles. In the present study, Pasteur pipettes filled with a strongly basic anion exchange resin (Dowex, chloride form, dry mesh 50–100, 8% cross linked) were used to separate intra- from extravesicular isotope.

SO_4^{2-} *influx experiments.* $^{35}SO_4^{2-}$ was used to measure SO_4^{2-} uptake by microvillus membrane vesicles. Sulphate influx experiments were generally initiated by adding 50 μL of vesicles to 200 μL of incubation medium which contained $^{35}SO_4^{2-}$ and inhibitors as required. The precise composition of the incubation media are given in the figure legends. At pre-determined times 200-μL aliquots of the vesicle and incubation medium mixture were applied to the ion-exchange column. This was immediately washed into the column with 2.0 mL of an ice-cold solution containing 300 mM sucrose and 10 mM KOH-Hepes, pH 7.4. The eluent was collected in scintillation vials and prepared for counting by adding 10 mL of liquid scintillation cocktail.

SO_4^{2-} *efflux experiments.* Vesicles were loaded with $^{35}SO_4^{2-}$ by incubation for 60–90 min at 20° in a medium containing 0.4 mM K_2SO_4, 300 mM sucrose and 10 mM KOH-Hepes, pH 7.4. Following this period of loading the vesicle suspension was applied to a column containing anion-exchange resin and the column was washed with an appropriate volume (2.0 mL) of solution containing 300 mM sucrose and 10 mM KOH-Hepes, pH 7.4. This procedure, which effectively removes extravesicular isotope, allows the eluent containing the vesicle (pre-loaded with $^{35}SO_4^{2-}$) to be collected and immediately split into aliquots. The efflux assay was initiated by adding 200 μL of such pre-loaded vesicles to 1.0 mL of incubation medium. At pre-determined times 200-μL aliquots were removed and the quantity of isotope remaining within them was determined using the ion-exchange column assay described above. The rate constant of sulphate efflux, K, was calculated using the following equation:

$$K_t = \ln\left(\frac{S_t}{S_\infty} - 1\right),$$

where S_t = intra-vesicular sulphate at time t and S_∞ = intra-vesicular sulphate at infinite time (taken to be 120 min).

Sulphate transport by placental tissue slices. Placental slices were prepared according to Ref. 19. Particular care was taken to ensure that the tissue was kept moist at all stages during the dissection. A piece of cotton thread was secured to each of the tissue fragments which were then incubated for approximately 90 min at 20° in a solution containing 135 mM NaCl, 5 mM KCl, 5 mM glucose, 0.1 mM Na_2SO_4 and 10 mM Tris–BES pH 7.4 (+10 μCi/mL $^{35}SO_4^{2-}$). Following the loading period the tissue slices were subsequently transferred through a series of tubes containing 2.0 mL of radioactive-free solutions at 2 min intervals. At the end of the experiment the tissue was allowed to stand in 4 mL of distilled water for at least 16 hr in order to leach

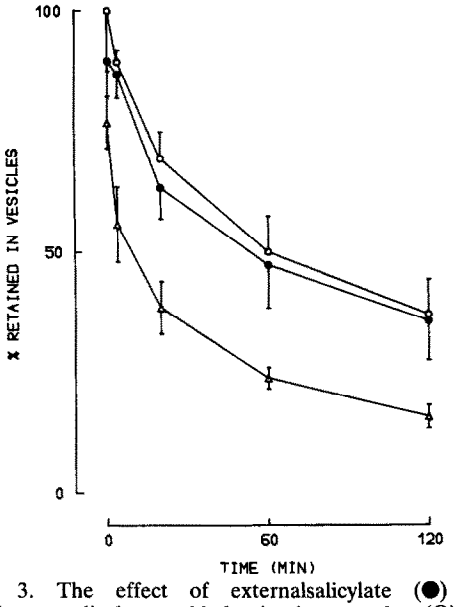

Fig. 3. The effect of externalsalicylate (●) and sulphate + salicylate added simulataneously (○) on sulphate efflux from placental brush-border membrane vesicles. The control medium (△) contained 0.4 mM K_2SO_4, sucrose (concentration adjusted to maintain osmolarity) and 10 mM KOH-Hepes, pH 7.4. Salicylate and SO_4^{2-} were added to give a final concentration of 10 and 30 mM, respectively when required. Each point represents the mean ± SE of four determinations.

out the isotope remaining in the tissue. The fractional release of $^{35}SO_4^{2-}$ was calculated for each period: this was taken as the ratio of the amount of the isotope lost from the placental slices per minute to the arithmetic mean isotope content.

Statistics. Data are expressed as means ± SE of mean. Differences were assessed by Student's paired or unpaired *t*-test as appropriate and were considered significant when $P < 0.05$. Each experiment was conducted using vesicles or slices prepared from separate placentae.

RESULTS

Influence of salicylate on sulphate uptake

To verify that salicylate inhibits sulphate uptake by human placental brush-border membrane vesicles we tested the effect of increasing the medium salicylate concentration over the range 0.1 to 10 mM on sulphate uptake measured after 2 min of incubation under equilibrium conditions. Figure 1 shows that the drug inhibited $^{35}SO_4$ uptake in a dose-dependent fashion with a K_I (i.e. the concentration inhibiting half of the sulphate uptake) of approximately 3 mM. This confirms the findings of Shennan and Boyd [7].

In order to determine the effect of salicylate on the kinetic parameters (i.e. K_t and V_{max}) of SO_4^{2-} accumulation we measured the uptake from media containing varying amounts of SO_4^{2-} in the presence and absence of 5 mM salicylate. A Hanes–Woolf transformation of the experimental data is shown in

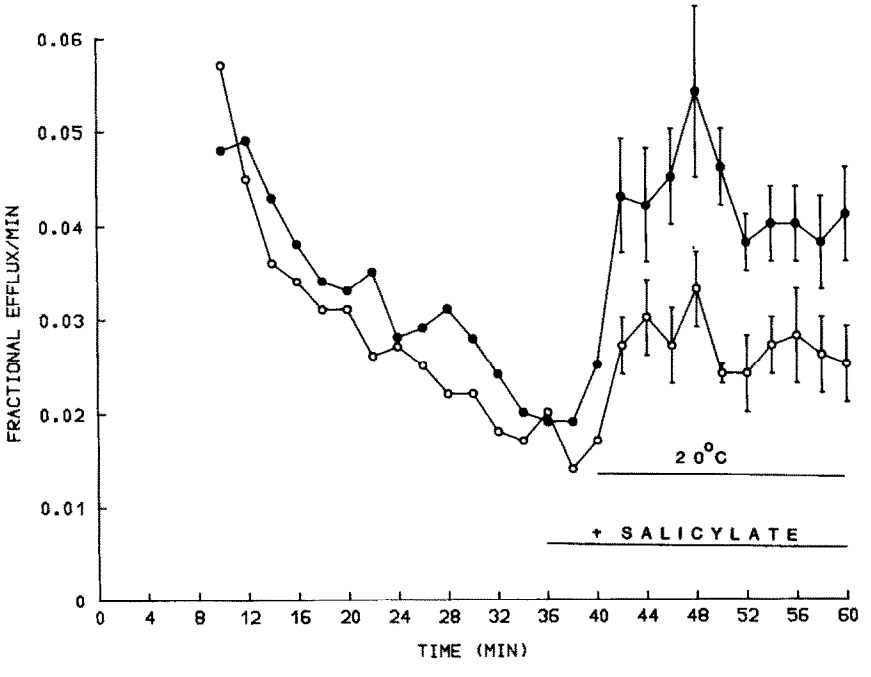

Fig. 4. The effect of temperature in the absence (●) and presence (○) of salicylate on sulphate efflux from placental tissue slices. The incubation medium contained 135 mM NaCl, 5 mM KCl, 0.1 mM Na_2SO_4, 5 mM glucose and 10 mM Tris–BES, pH 7.4. The period during which the temperature was maintained at 20° is indicated by the upper bar; for the rest of the experiment the temperature was 4°. Salicylate (sodium salt) was used at a concentration of 10 mM when required (as indicated by lower bar). Each point represents the mean ± SE of four determinations. For the sake of clarity error bars up to $t = 40$ have been omitted.

Fig. 2. The V_{max} of SO_4^{2-} influx was reduced from a value of 1.18 ± 0.09 to 0.68 ± 0.05 nmol/mg protein/2 min by the inclusion of salicylate in the medium (N = 7, P < 0.001, paired *t*-test). In contrast the K_t of uptake was not significantly changed (0.96 ± 0.11 vs 1.07 ± 0.09 mM). Thus, it appears that salicylate is acting as a non-competitive inhibitor.

Influence of salicylate on sulphate efflux

On the basis that sulphate efflux and influx across the brush-border membrane share a pathway for transport [20] we predicted that sulphate egress from vesicles preloaded with $^{35}SO_4$ should be inhibited by salicylate. To test this we examined the effect of 10 mM salicylate added to the incubation medium. Figure 3 shows that sulphate efflux, plotted as the percentage of sulphate remaining in the vesicles as a function of time, was markedly reduced by salicylate. Figure 3 also illustrates that the addition of 30 mM SO_4^{2-} (which is known to reduce the inhibition of SO_4^{2-} efflux by DIDS, a competitive inhibitor [21]) did not relieve the salicylate inhibition of SO_4^{2-} efflux. The calculated efflux rate constants were 0.0336 ± 0.0012 (control), 0.0152 ± 0.0043 (+salicylate) and 0.0150 ± 0.0032 (+salicylate and 30 mM SO_4).

The effect of salicylate on SO_4^{2-} efflux from placental tissue slices

We next sought evidence to establish if sulphate transport by tissue slices prepared from term human placentae is inhibited by salicylate. Studies on ion transport by mammary tissue slices have shown that testing the potency of drugs on the temperature-sensitive portion of solute efflux is a good way to readily unmask inhibitory effects (e.g. see Ref. 22). We decided to use this approach to see if salicylate retards sulphate efflux from placental slices. It was necessary in the first instance to establish that SO_4^{2-} release is temperature dependent. Figure 4 shows that the fractional loss of sulphate was approximately doubled upon transferring the tissue from a medium maintained at 4° to one at 20°. Figure 4 also depicts the effect of salicylate (10 mM) on the temperature-sensitive portion of efflux. It is clear that the inclusion of salicylate in the incubation medium attenuates the increase in the fractional efflux of SO_4^{2-} found with raising the temperature.

The effect of flufenamic acid on sulphate transport

We decided to test the effect of flufenamic acid (as flufenamate) on sulphate transport by human placental brush-border membrane vesicles. Figure 5 shows the effect of this compound tested over the range 0.001 to 1 mM on sulphate uptake. It is clear that flufenamate inhibits sulphate accumulation in a dose-dependent fashion with a K_I of approximately 0.1 mM. We also examined the effect of flufenamate on the kinetic parameters of sulphate influx (Fig. 6). The V_{max} of transport was decreased by flufenamate from a value of 0.74 ± 0.05 to 0.47 ± 0.06 nmol/mg protein/2 min (N = 3, P < 0.01, paired *t*-test) whereas the K_t of uptake was not significantly altered (1.58 ± 0.18 mM vs 2.10 ± 0.76 mM). Flufenamate, like salicylate, is acting as a non-competitive inhibitor.

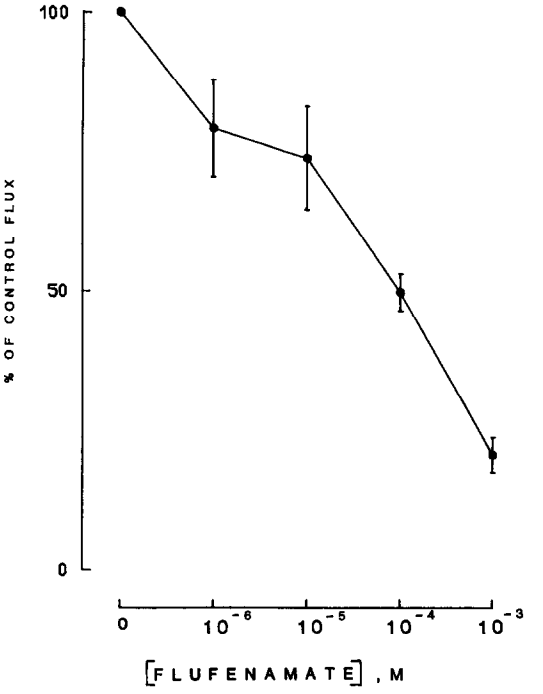

Fig. 5. Dose–response curve of flufenamic acid inhibition of SO_4^{2-} uptake by placental brush-border membrane vesicles. The incubation media contained 0.4 mM K_2SO_4, sucrose (adjusted to maintain osmolarity) and 10 mM KOH-Hepes, pH 7.4. Flufenamic acid was added over the range 0.001 to 1 mM. Each point represents the mean ± SE of three determinations.

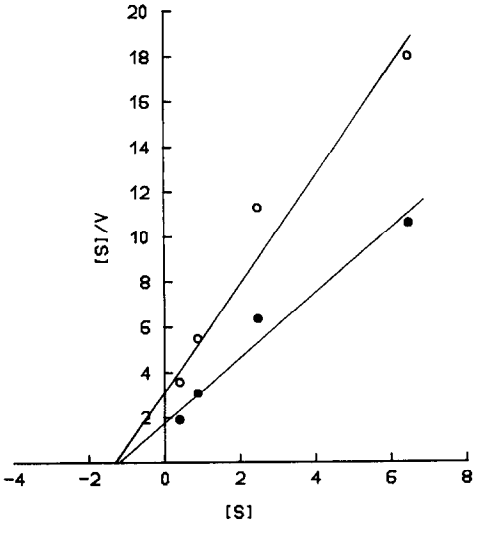

Fig. 6. Hanes–Woolf plot ([*S*]/*V* vs [*S*]) of SO_4^{2-} uptake by human placental brush-border membrane vesicles in the presence (○) and absence (●) of 0.1 mM flufenamate. Uptake was assayed in media containing 0.4–6.5 mM SO_4^{2-}, sucrose (concentration adjusted to maintain osmolarity) and 10 mM KOH-Hepes, pH 7.4. Each point represents the mean of three determinations.

Figure 7 shows that flufenamate at a concentration of 1 mM added to the incubation medium reduces the rate of loss of sulphate from preloaded vesicles. In this set of experiments the rate constant of efflux (min^{-1}) was reduced from a control value of 0.0402 ± 0.0009 (±SE, N = 3) to 0.0246 ± 0.0009 (±SE, N = 3) by flufenamate.

DISCUSSION

The experiments described in this paper were designed to further investigate the effect of salicylate on placental sulphate transport. The results confirm the earlier observation of Shennan and Boyd [7] that salicylate markedly inhibits sulphate accumulation by vesicles prepared from the maternal facing surface of the human placenta. In addition we have also found that salicylate reduces the rate of sulphate efflux from placental brush-border membrane vesicles. Moreover, the results suggest that salicylate inhibits placental sulphate transport in a non-competitive fashion. Consistent with the vesicle studies we have also found that salicylate inhibits SO_4^{2-} egress from human placental tissue slices which on the basis of the large surface area of the microvillus membranes are assumed to give a measure of brush-border membrane permeability (see Ref. 23).

Although the detrimental effects due to regular salicylate ingestion during pregnancy can probably be mainly attributed to an inhibition of prostaglandin synthesis [11–13] the present results strengthen the claim of Shennan and Boyd [7] that salicylate at high concentrations may also exert its action by interfering with placental ion transport. In this connection it is interesting to note that Cl transport (via an anion exchange pathway) by placental brush-border membrane vesicles is also inhibited by salicylate [17]. Given that SO_4 and Cl transport across the brush-border membrane is linked to the movement of HCO_3^- ions [1, 4, 24, 25] it can be predicted that inhibition of ion transport by a large concentration of salicylate could affect fetal acid–base balance. It is interesting to note that Wieth and Brahm [9] have proposed that an inhibition of red cell anion-exchange may explain some of the respiratory aspects of salicylate poisoning.

We have found that the aspirin-like drug flufenamic acid which is a potent blocker of red-cell anion transport [14, 15] also inhibits human placental brush border membrane sulphate transport. In addition, niflumic acid which is similar in structure to flufenamic acid has also been found to markedly reduce placental sulphate transport (Shennan, unpublished). Thus, it is tempting to suggest that all structurally related aspirin-like drugs at high concentrations may also be capable of acting as placental ion transport inhibitors in addition to having an effect on prostaglandin synthesis.

In summary, the results indicate that salicylate at a high concentration could compromise fetal and placental sulphate homeostasis. Therefore, this study serves as a reminder of the potential hazard of salicylate (and possibly other salicylate-like drugs) to the feto placental unit.

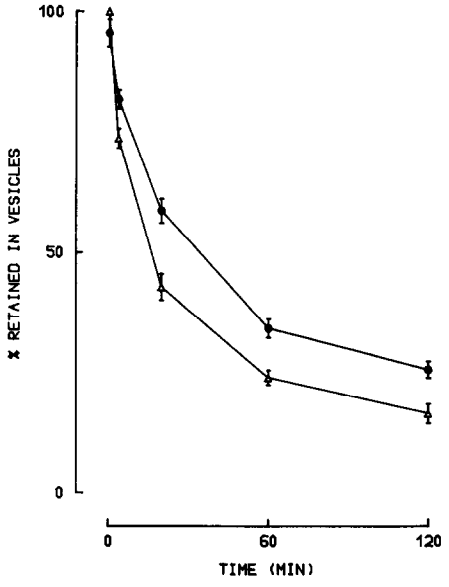

Fig. 7. The effect of flufenamic acid (●) on sulphate efflux from placental brush-border membrane vesicles. The control medium (△) contained 0.4 mM K_2SO_4, 300 mM sucrose, 10 mM KOH-Hepes, pH 7.4, ±1 mM flufenamic acid. Each point represents the mean ± SE of three determinations.

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