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# pCMBS-induced swelling of dogfish (*Squalus acanthias*) rectal gland cells: role of the $Na^+$,$K^+$-ATPase and the cytoskeleton

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**(1) 0.1–1.0 mM *p*-chloromercuribenzene sulfonate (pCMBS) and some other organic mercurials produce a swelling of slices of dogfish shark (*Squalus acanthias*) rectal glands, with an uptake of cell $Na^+$ and a loss of $K^+$. In contrast, 1 mM *N*-ethylmaleimide (NEM) does not swell rectal gland cells (RGC), while affecting cell cations. (2) The slow entry of [$^{203}$Hg]pCMBS is linearly related to its external concentration (10 $\mu$M–1 mM) and a small accumulation of pCMBS (apparent gradient about 3) in the cells occurs in 2 h. Cell $^{203}$Hg rapidly washes out of the cells (fast rate constant 0.153 · $min^{-1}$; slow rate constant 0.0067 · $min^{-1}$), and this efflux is accelerated by 1 mM dithiothreitol. Thus, a major portion of pCMBS inter-acts rather loosely with cell components. (3) pCMBS and NEM share: (a) a negligible effect on the efflux of $^{86}Rb^+$ and of [$^{14}$C]urea; (b) a gradual inhibition of the cell $Na^+$,$K^+$-ATPase activity. (4) NEM as well as agents lowering cell glutathione accelerate and increase the pCMBS-induced cell swelling. Conditions inhibiting the $Na^+$,$K^+$-ATPase (ouabain, absence of $Na^+$) have the same effect. (5) pCMBS, but not NEM produce a disappearance of the F-actin-phalloidin fluorescence independent of cell volume changes, particularly at the basolateral RGC membrane. (6) The data are consistent with the following set of events: (a) pCMBS (but not NEM) affects the cell membrane by increasing the efflux of the cell osmolyte taurine (Ziyadeh et al. (1988) Biochim. Biophys. Acta 943, 43–52 and unpublished data); (b) on entry into the cells, pCMBS and NEM interact with cell -SH, including those of the $Na^+$,$K^+$-ATPase; this action produces the observed changes in cell cations. Also, pCMBS, but not NEM, decrease F-actin at the membrane; (c) the inhibition of the $Na^+$,$K^+$-ATPase activity together with the decreased resistance of the cell membrane to stretch (absence of F-actin) produces the observed pCMBS-induced cell swelling by osmotic forces (intracellular non-diffusible anions).**

## Introduction

It is generally taken that organic mercurials specifically interact with protein thiol groups (see, e.g., Ref. 1, 2). Cell membrane -SH are considered to be the primary target of mercurials.

In renal cortical cells, hydrophilic, diuretic and non-diuretic organic mercurials, e.g., esidrone, mersalyl, chlormerodrin [3] or mercaptomerin [4] produce a marked cellular swelling. While a primary effect of these mercurials on the membrane permeability was postulated, the extrusion of $Na^+$ from the cells, and the accumulation of $K^+$ not being greatly affected [3,5], other investigators [4] considered a primary mercurial effect on the sodium pump. The dogfish shark (*Squalus acanthias*) rectal gland cells (RGC) secrete NaCl and have transport properties analogous to the renal thick ascending limb [6,7]. The pCMBS-induced massive swelling of RGC prompted a detailed analysis of the mechanism by which the mercurial affects cell volume maintenance. One specific effect of the mercurial on RGC (basolateral) membrane, i.e., an increased leak for the major RGC osmolyte, taurine, has been described previously [8].

This study was designed to elucidate the mechanism of the pCMBS-induced RGC swelling by comparing

Abbreviations: RGC, rectal gland cells; pCMBS, *p*-chloromercuribenzene sulfonate; NEM, *N*-ethylmaleimide; DTT, dithiothreitol; Cl-DNB, 1-chloro-2,4-dinitrobenzene; DMF, dimethylfumarate; Thiolyte MQ, monobromo-trimethylammoniobimane; DIDS, 4,4′-diisothiocyano-2,2′-stilbenesulfonic acid.

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effects of some other thiol agents, particularly NEM. Such comparison was desirable in the light of previous studies showing that pCMBS and another thiol reagent, NEM, differ in their effects on the erythrocyte membrane [9]. It will be shown here that in addition to the pCMBS effect on the taurine efflux, the mercurial targets primarily two processes: It gradually inhibits the $Na^+$,$K^+$-ATPase, and produces changes in the organization of the cytoskeleton (F-actin). The concurrence of both effects provides an explanation of the pCMBS-induced RGC swelling.

## Methods and Materials

Experiments were carried out in July–August at the Mt. Desert Island Biological Laboratory, using dogfish sharks (*S. acanthias*) caught in the Frenchman's Bay. The methods employed for the preparation of 0.3 mm thick slices of the rectal gland, incubation of the tissue in vitro (15 °C, air + 1% $CO_2$ as gaseous phase) and most analytical procedures were given previously [10–12]. The tissue of several sharks was pooled whenever a large number of experimental points was required.

*Media.* The standard elasmobranch incubation medium (cf. Ref. 10) contained (mM); $Na^+$ (180), $K^+$ (5.0), $Cl^+$ (285), $HCO_3^-$ (8), $Ca^{2+}$ (2.5), $Mg^{2+}$ (1.2), phosphate (1), sulfate (0.5), urea (350), glucose (5), osmolarity, 900 mosM (pH 7.5) when gassed with 1% $CO_2$ in air. Modifications: Chloride-free, gluconate saline: all $Cl^+$ was equivalently replaced by gluconate; $Li^+$ saline: all $Na^+$ was equivalently replaced by $Li^+$. For the measurement of $^{86}Rb^+$ fluxes, 1 mM $Rb^+$ replaced the equivalent of $K^+$ in the standard saline. For the determination of the extracellular space, the respective media contained [$^3$H]poly(ethylene) glycol (PEG, 1 mg/ml, 1 $\mu$Ci/ml).

*Incubation.* For each condition, 4 or 5 slices were incubated in 5 ml medium. The blotted tissue was weighed wet and after drying at 95 °C overnight. The dry tissue was extracted 48 h with 0.1 M $HNO_3$, and in the extract $Na^+$, $K^+$, $Cl^-$ as well as [$^3$H]PEG was determined. From these data, the following values were assessed: Tissue water ($H_2O_t$, kg/kg dry wt.); intracellular water ($H_2O_i$, kg/kg dry wt.), after correction for the extracellular space; tissue electrolytes (mequiv./kg dry wt., or mequiv./kg $H_2O_i$ (mM).

The activity of the $Na^+$,$K^+$-ATPase was evaluated by the 'zero-time' influx of $^{86}Rb^+$ (4 min uptake) [11,12]. The values are given in nmol $^{86}Rb^+$ uptake/kg dry wt. per min. It has been shown previously that about 90% of this uptake is inhibitable by 0.5 mM ouabain [12].

*Efflux measurements.* The methods employed for the measurement of $^{86}Rb^+$ from tissue preloaded with the label has been given [10,12]. The same procedure was used to follow the efflux of [$^{14}$C]urea and [$^{203}$Hg]-pCMBS. From the efflux curves the respective rate constants were assessed.

*Uptake of [$^{203}$Hg]pCMBS.* Tissue was incubated in salines containing 1 mM labeled pCMBS (0.1 $\mu$Ci/ml). At the end of the experiment, the tissue was first rinsed in ice-cold medium without pCMBS for 2 min in order to remove extracellular mercurial. The blotted slices were weighed wet and dry, and the tissue was digested 24 h in 1 M NaOH. In this extract, the label was determined.

All radioactivity was measured by scintillation spectrometry in a Packard Tricarb Model 3000 instrument.

Measurements of tissue glutathione (reduced + oxidized) were kindly carried out by Dr. N. Ballatori, employing the method of Griffith [13].

*Statistical evaluation.* Means ± S.E. for typical experiments are given. The significance of differences between controls and experimental values was assessed by Student's *t*-test. Each experimental result was verified by at least two separate experiments. In some cases, the variations between individual fishes permitting, the results of several experiments were pooled.

*Morphology.* Methods employed for the preparation of samples for light and electron microscopy, as well for the localization of F-actin were as described [12].

*Materials.* [$^{203}$Hg]pCMBS, $^{86}Rb$, [$^{14}$C]urea and [$^3$H]poly(ethylene glycol) were obtained from the Amersham Corporation, IL, U.S.A. All other reagents were commercial preparations of the highest available purity.

## Results

*The swelling effect of pCMBS*

Fig. 1 shows that pCMBS produces a marked increase in the tissue water of rectal gland slices. From the data of $H_2O_t$ at 2 h shown in this figure, the values of $H_2O_i$ were calculated and found to have practically doubled from 2.48 to 4.19 kg cell $H_2O$/kg dry wt. This cell swelling was associated with a loss of tissue $K^+$ and an uptake of $Na^+$ (and $Cl^-$); the mercurial thus depolarized the cells. In most experiments, the swelling was not linear: little swelling in the first hour was followed by a subsequent rapid uptake of $H_2O$, and a new steady state was reached in about 3 h. On the other hand, the pCMBS effect on tissue cations was clearly evident already in the first hour of incubation.

The swelling effect of various organic mercurials was examined. Table I shows considerable differences in the degree of swelling produced by the tested agents. The hydrophilic, relatively little permeant pCMBS and mersalyl had a more pronounced effect in 2 h than the more lipophilic *p*-hydroxymercuribenzoate and phenylmercuriacetate. Such differences in the effect of organic mercurials have also been noted in, e.g., erythrocytes [14] and have led to the postulate that while the slowly

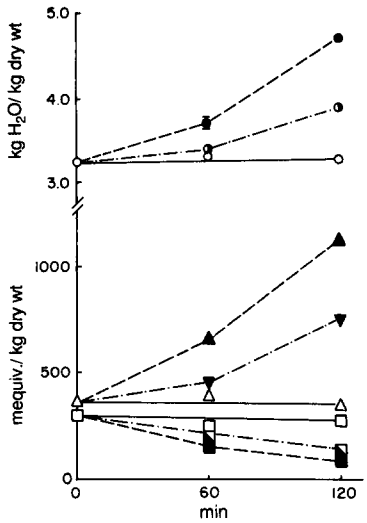



Fig. 1. Time course of pCMBS effect on tissue water and cations in rectal gland slices. Groups of slices were incubated in standard elasmobranch saline aerobically (1% $CO_2$ in air) at 15°C, without (control, open symbols) and with 0.5 and 1 mM pCMBS (partially or fully closed symbols). $H_2O_t$ (kg/kg dry wt.): ○, ◑, ●; tissue Na (mequiv./kg dry wt.): △, ▼,▲; tissue K (mequiv./kg dry wt.): □, ◪, ■. Mean values ± S.E., $n = 5$.

permeant pCMBS exerted its effect primarily by interacting with thiol groups in the cell membrane, the lipophilic mercurials, after passing the membrane, interacted with intracellular thiols, thus reducing their membrane effects.

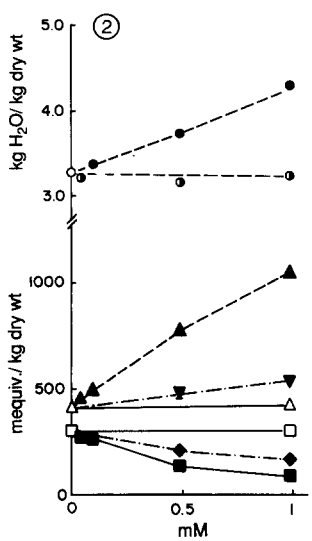
The swelling effect of pCMBS on tissue water and cations could be detected at 0.1 mM mercurial; a significant ($P < 0.02$) swelling effect of 0.1 mM mersalyl was also seen. The experiment shown in Fig. 2 also demonstrated that NEM, an agent covalently interacting with thiol groups, had practically no effect on cell water while producing significant losses of tissue $K^+$ and uptake of $Na^+$. Thus both thiol agents differ in their effect on cell volume, while having similar effects on the ionic distribution. These experiments, however, suggested that both pCMBS and NEM might increase the permeability of the RGC membrane to cations, thus producing a loss of cell $K^+$ and an uptake of $Na^+$. Such putative effect of pCMBS (and NEM) on the RGC membrane can only be minor as shown in Fig. 3; the fast efflux component of $^{86}Rb^+$ was actually decreased by the presence of pCMBS in the washout medium by 19 ± 2% (S.E., three experiments). No significant effect

TABLE I

*The swelling effect of various organic mercurials on rectal gland cells*

Groups of slices were incubated 120 min in standard elasmobranch saline without (controls) and with 1 mM mercurials. Mean values of tissue $H_2O_t$ (kg/kg tissue dry wt.) and cations (mequiv./kg dry wt.), ± S.E. (four analyses) are given.

| Mercurial | $H_2O_t$ (kg/kg dry wt.) | mequiv./kg dry wt. | |
|---|---|---|---|
| | | $Na^+$ | $K^+$ |
| Control | 3.17 ± 0.07 | 445 ± 26 | 310 ± 20 |
| pCMBS | 4.80 ± 0.24 | 1439 ± 56 | 79 ± 3 |
| *p*-Hydroxymercuribenzoate | 4.05 ± 0.18 | 1027 ± 74 | 156 ± 6 |
| Mersalyl | 5.40 ± 0.09 | 1561 ± 53 | 125 ± 19 |
| Phenylmercuriacetate | 4.16 ± 0.12 | 1033 ± 56 | 143 ± 8 |



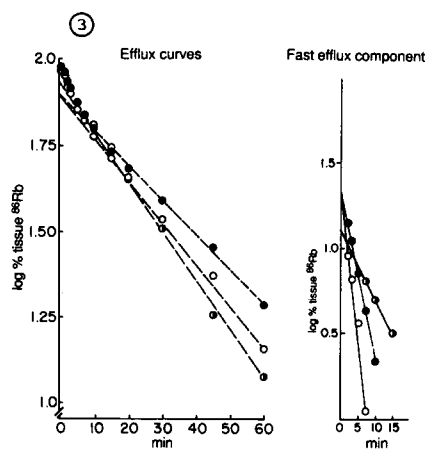




Fig. 2. Effect of varying concentrations of pCMBS and NEM tissue water and cations in slices of the rectal gland. The tissue was incubated for 2h in salines without (control) and with the thiol agents (0.5 and 1 mM pCMBS or NEM). $H_2O_t$ (kg/kg dry wt.): no addition, ○; NEM, ◑; pCMBS, ●. Tissue cations (mequiv./kg dry wt.); Na: control, △; NEM, ▼; pCMBS, ▲. K: control, □; NEM, ◆; pCMBS, ■. Mean values ± S.E., $n = 4$.

Fig. 3. Effect of pCMBS on $^{86}Rb^+$ efflux from slices of the rectal gland. A portion of the tissue was first loaded with $^{86}Rb^+$ (0.2 μCi/ml) by incubation for 45 min in standard elasmobranch saline containing 1 mM $Rb^+$ (replacing 1 mM $K^+$), and the efflux of the label was then followed by the washout technique [12], without (control, ○), and with 1 mM pCMBS, ●. Another group of tissue was first incubated for 2 h in saline containing 1 mM pCMBS, and only then loaded with $^{86}Rb^+$ and the label was washed out as above, in the presence of the mercurial (◑).

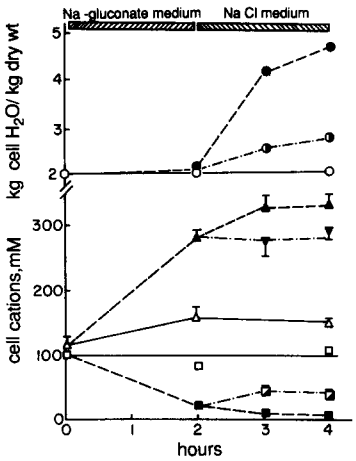



Fig. 4. Effect of pretreatment of tissue with 1 mM pCMBS on cell volume, and the action of DTT. Groups of tissue were first incubated in sodium gluconate saline without (control) and with 1 mM pCMBS. Subsequently, the tissue was transferred into regular elasmobranch saline without the mercurial, in the absence and with 2 mM DTT. Mean values ± S.E. ($n = 4$) of $H_2O_t$ (kg/kg dry wt.) and tissue cations (mequiv./kg dry wt.) are given. $H_2O_t$: control, ○; pCMBS, ●; DTT, ◑. Na: control, △; pCMBS, ▲; DTT, ▼. K: control, □; pCMBS, ■; DTT, ◪.

of pCMBS on the efflux of urea or influx of $^{22}Na^+$ were seen (details not given). Similar results were obtained when the tissue was first incubated 2 h in the presence of either thiol agents, and the efflux of $^{86}Rb^+$ and [$^{14}$C]urea was then followed (details not given). With respect to the pCMBS effect on the fluxes of $^{86}Rb^+$ or [$^{14}$C]urea, the (basolateral) membrane of RGC differs from, e.g., the erythrocyte [2,14] or liver cell membrane [15]. The alternative possibility, i.e., pCMBS affecting the sodium pump, thus producing the observed effects on cell cations, will be analyzed below.

Two properties of the pCMBS-induced swelling of the tissue were noted (details not given). A slight increase in the swelling effect was seen when lowering the pH of the incubating media to pH 6.48, as compared to incubation at pH 7.28 and 7.99. This may indicate some involvement of the system regulating cell pH. Also, pCMBS produced swelling of slices in the absence of $Na^+$ in the incubating media (Li-saline) (see below); $Na^+$ is thus not is not required for the swelling.

*The interaction of pCMBS with tissue components*

The possibility of mercurial binding to tissue components was explored. In the experiment shown in Fig. 4, cells were first incubated in a sodium gluconate medium without (control) and with pCMBS; under these conditions, the low permeability of the cell membrane to gluconate [12] prevented the swelling effect of pCMBS, while the mercurial effect of the cation distribution was evident; in this experiment the Nernst $K^+$ potential (close to the membrane potential, cf. Ref. 10) dropped from 68 ± 2 mV (control) to 20 ± 13 mV. On subsequent transfer of the tissue to pCMBS-free standard elasmobranch saline, the swelling effect of the mercurial persisted. Such result might suggest binding of pCMBS by some cell components, thus manifesting its effect even in the absence of the mercurial in the incubation media. It should also be noted that 1 mM DTT greatly reduced this swelling, but did not markedly reverse the cationic distribution. On increasing the DTT concentration to 2 mM, some reversal of the pCMBS effect was seen in that a reuptake of $K^+$ (from 128 ± 8 mequiv./kg dry wt. after pCMBS treatment to 221 ± 15 mequiv. in the presence of DTT), and a loss of $Na^+$ was found (details not given). Such result would indicate that a blocking of the $Na^+$,$K^+$-ATPase activity by pCMBS could in part be reversed by DTT.

*Interaction of [$^{203}$Hg]pCMBS with RGC.* The possibility of mercurial binding to cell components was explored further using [$^{203}$Hg]pCMBS. Fig. 5 shows that RGC take up pCMBS rather slowly in a concentration-dependent manner (inset). At 1 mM pCMBS, the uptake decreased with time and may have tended towards a steady state. From values of cell pCMBS it may be computed that an accumulation of the mercurial by the cells has taken place; the apparent concentration of pCMBS in tissue exceeded that in the medium by a factor of 3. Studies on the rate of [$^{203}$Hg]pCMBS uptake (details not given) failed to indicate evidence of a carrier-mediated mercurial uptake in that no indication for a saturating mechanism was found. Pretreatment of the cells with NEM did not decrease pCMBS uptake. Also, 0.1 mM DIDS inhibited the uptake of [$^{203}$Hg]pCMBS only by about 20% (mean of two experiments), suggesting that pCMBS uptake does not preponderantly enter

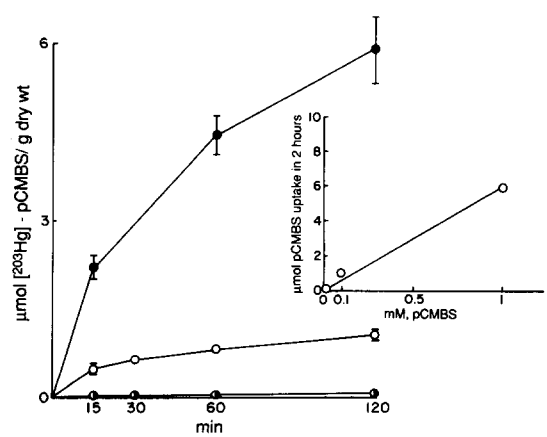



Fig. 5. Uptake of [$^{203}$Hg]pCMBS by rectal gland slices. Groups of tissue were incubated in standard elasmobranch saline containing 0.01 (◑), 0.1 (○) or 1 (●) mM labeled pCMBS (0.07 µCi/ml). After incubation, the tissue was rinsed for 2 min in ice-cold non-labeled saline, blotted, weighed wet and dry, and the label was determined in tissue digested with 0.1 M NaOH. Data are given in µmol [$^{203}$Hg]pCMBS taken up/g tissue dry wt. Mean values of three analyses. (Inset) Plot of [$^{203}$Hg]pCMBS taken up by the tissue in 120 min vs. concentration of the mercurial in the medium.

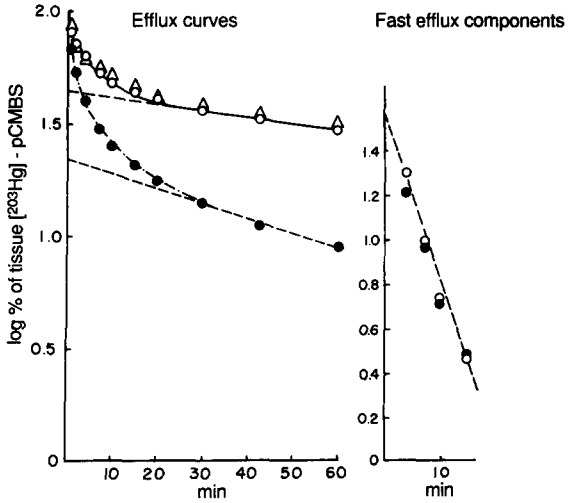



Fig. 6. Efflux of [$^{203}$Hg]pCMBS from slices of dogfish rectal gland. Groups of tissue were preincubated 1 h in 10 ml saline containing 1 mM labeled pCMBS (0.2 $\mu$Ci/ml). The efflux of the label from the tissue into standard elasmobranch saline without (control, ○), with 1 mM dithiothreitol (●) or 20 $\mu$M metallothionein (△) was then followed. (Left panel) Efflux curve: broken line indicates the linear portion of the curve corresponding to the slow efflux rate constant. (Right panel) Computed fast efflux component.

the cells via the anionic exchange system [16]. The absence of external $Na^+$ ($Li^+$- or $K^+$-salines) had no significant effect on pCMBS uptake.

As opposed to the binding of mercurials with renal cortical cells [3,17], the putative interaction of pCMBS with cellular component(s) in RGC is not firm. As shown in Fig. 6, tissue preloaded with [$^{203}$Hg]pCMBS released more than 60% of the label in 60 min of a washout experiment. Thus, the rate of the mercurial efflux is similar to that of, e.g., $K^+$ [10,12]. This efflux of the label was greatly accelerated by the presence of 1 mM DTT; in 60 min, more than 90% of the mercurial was released. The rather bulky metallothionein had no effect on pCMBS efflux. An analysis of the efflux curves demonstrated two distinct cellular components. DTT increased only the rate constant of the slow efflux component (from $0.0067 \cdot min^{-1}$ to $0.023 \cdot min^{-1}$) whereas the fast efflux components were practically identical ($0.153 \cdot min^{-1}$ in the control; $0.197 \cdot min^{-1}$ in the presence of DTT). Such data would be consistent with pCMBS being slowly released from an intracellular pool (after interaction with cell non-protein and protein thiol groups), and the dissociation of the pCMBS-thiol components would be the rate-limiting process for the PCMBS efflux across the cell membrane, as reflected by the fast rate constant. The possibility that pCMBS exited from the cells as a complex with an intracellular thiol cannot be dismissed.

The above data suggested that a major target for the swelling effect of pCMBS should be sought at the intracellular level, not only at the membrane.

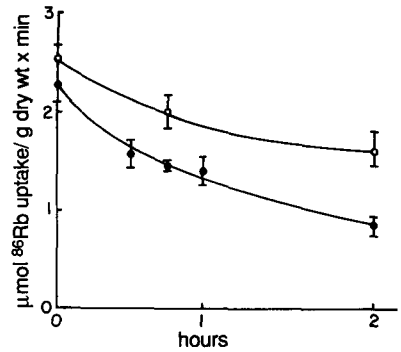



Fig. 7. $Na^+,K^+$-ATPase inhibition by pCMBS. Groups of slices were incubated for 0 to 2 h in elasmobranch saline containing 1 mM $Rb^+$, without (control, ○), and with (●) 1 mM pCMBDS. The 4-min (zero-time) uptake of $^{86}Rb^+$ (0.5 $\mu$Ci/ml) was then measured. Mean values, in $\mu$mol $^{86}Rb^+$ uptake/g dry wt. per min of pooled data from three experiments, ±S.E., are given.

*Effect of pCMBS and NEM on the cellular $Na^+,K^+$-ATPase*

Since pCMBS did not markedly increase the $^{86}Rb^+$ (and $^{22}Na^+$) fluxes (see above), an alternative mechanism was considered. Organic mercurials are known to be potent inhibitors of the $Na^+,K^+$-ATPase, interacting with thiol groups of this enzyme [18]. As shown in Fig. 7, pCMBS gradually inhibited the $Na^+,K^+$-ATPase. Prolonged incubation of the tissue in $Na^+$-saline in the presence of 1 mM pCMBS did decrease the activity of the $Na^+,K^+$-ATPase, as measured by the zero-time uptake of $^{86}Rb$. At 2 h incubation, the inhibition was close to 50% ($P < 0.05$, 4 experiments). Identical effects of 1 mM mersalyl and NEM were also found (Table II); NEM (cf. Ref. 18) was a more potent inhibitor than the mercurials, possibly because of its more rapid entry into the cells. In this, and in most other experiments, the zero-time $^{86}Rb^+$ uptake in controls was practically constant (or slightly diminished) for the duration of the experiments (2 h). The fact that the inhibition by both types of thiol agents increased with time is consistent with the view that the membrane permeability of these

TABLE II

*Effect of mersalyl and NEM on the zero-time $^{86}Rb^+$ influx into RGC*

Groups of slices were incubated 4 min at 15°C in standard elasmobranch saline labelled with 1 mM $^{86}Rb^+$ (1 $\mu$Ci/ml) without (controls) and with 1 mM thiol agent: immediately after slicing and after preincubation of the tissue for 2 h (without (control) or with the thiol agent. Mean values of label influx ($\mu$mol/g dry wt. per min), ±S.E. (five analyses).

| Tissue | Thiol agent | Control | Experimental |
|---|---|---|---|
| Fresh | mersalyl | – | – |
| Preincubated | mersalyl | 2.21±0.16 | 1.17±0.03 ** |
| Fresh | NEM | 1.63±0.07 | 1.74±0.10 |
| Preincubated | NEM | 1.49±0.07 | 0.43±0.08 ** |

** $P < 0.01$ vs. control.

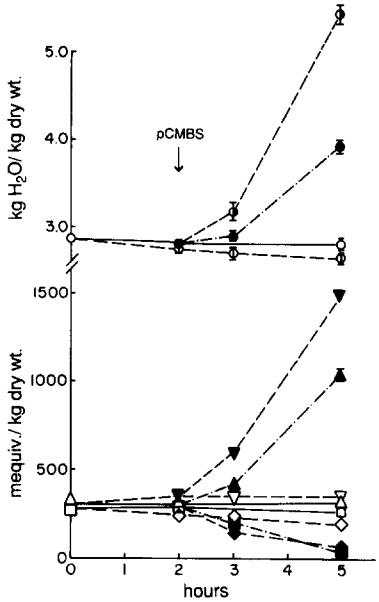



Fig. 8. Pretreatment of tissue with agents affecting cell glutathione affects the pCMBS-induced cell swelling. Tissue was first incubated for 2 h without (control) and with the glutathione agent, 1 mM Cl-DNB; portions were then transferred to identical media without and with pCMBS (1 mM) and incubated for further 60 and 180 min. Mean values for $H_2O_t$ (kg/kg dry wt.) and cations (mequiv./kg dry wt.), +S.E. (four measurements) are given. $H_2O_t$: ○, control; ⊙, Cl-DNB; ●, pCMBS; ⊙, pCMBS+Cl-DNB. Tissue Na: △, control; ▽, Cl-DNB; ▲, pCMBS; ▼, pCMBS+Cl-DNB. Tissue K: □, control; ◇, Cl-DNB; ■, pCMBS; ◆, pCMBS+Cl-DNB.

agents is rather slow; also, intracellular thiol groups might compete with the $Na^+$,$K^+$-ATPase for the thiol agents. It should be mentioned that 1 mM pCMBS had no effect on substrate oxidation by the cells, measured by the formation of $^{14}CO_2$ from universally $^{14}C$-labeled acetate (details not given).

The observation that pCMBS and NEM inhibit the $Na^+$,$K^+$-ATPase offers a reasonable explanation for the effect of these agents on the cation distribution seen in Figs. 1, 2 and 4.

*Effect of various thiol agents on the pCMBS-induced swelling of RGC*

The possibility that thiol agents might compete with pCMBS for intracellular -SH was tested further. If this were the case, pretreatment of the tissue with thiol agents would be expected to reduce the swelling effect of pCMBS (cf. Ref. 19). The following agents were tested: NEM, and reagents selectively interacting with cell glutathione [20], i.e., Cl-DNB [20], DMF [21] and thiolyte MQ [22]. Preliminary experiments showed that the cell GSH plus GSSG level (1.5 mM, mean of two experiments) was reduced by all of the above substances to not measurable values (less than 0.1 mM). The results of these experiments are given in Fig. 8 and Table III.

It will be seen that all the above agents actually markedly increased the swelling effect of pCMBS; the

TABLE III

*Effect of agents affecting cell glutathione on the swelling effect of pCMBS*

The conditions of the experiment were as given in the legend to Fig. 8. Concentrations of agents: NEM, 1 mM; DMF, 10 mM; Thiolyte MQ, 20 μM. Mean values, ±S.E., for tissue water and cations are given only for one time point, after incubation of the tissue without and with pCMBS for 3 h.

| | Tissue | | |
|---|---|---|---|
| | $H_2O_t$ (kg/kg dry wt.) | mequiv./kg dry wt. | |
| | | $Na^+$ | $K^+$ |
| Control | 2.72±0.04 | 308± 8 | 268± 7 |
| NEM | 2.97±0.09 | 555±20 | 110± 9 |
| DMF | 2.67±0.04 | 312±14 | 240± 8 |
| Thiolyte MQ | 2.79±0.05 | 350±12 | 270± 8 |
| PCMBS | 3.77±0.15 | 907±50 | 93± 5 |
| NEM+pCMBS | 5.72±0.17 | 1638±87 | 40± 3 |
| DMF+pCMBS | 4.51±0.09 | 1135±51 | 51±13 |
| Thiolyte MQ+pCMBS | 4.02±0.05 | 1021±25 | 82± 4 |

relatively smaller effect of thiolyte may well be due to the lower permeability of the cell membrane for this reagent [22]. It should be also be noted that the agents selectively reducing cell glutathione per se did not affect tissue water and cation distribution. Thus, thiol agents which alone do not produce cell swelling accelerate the pCMBS-induced cell swelling. In the light of the above information on the swelling effect of pCMBS the data

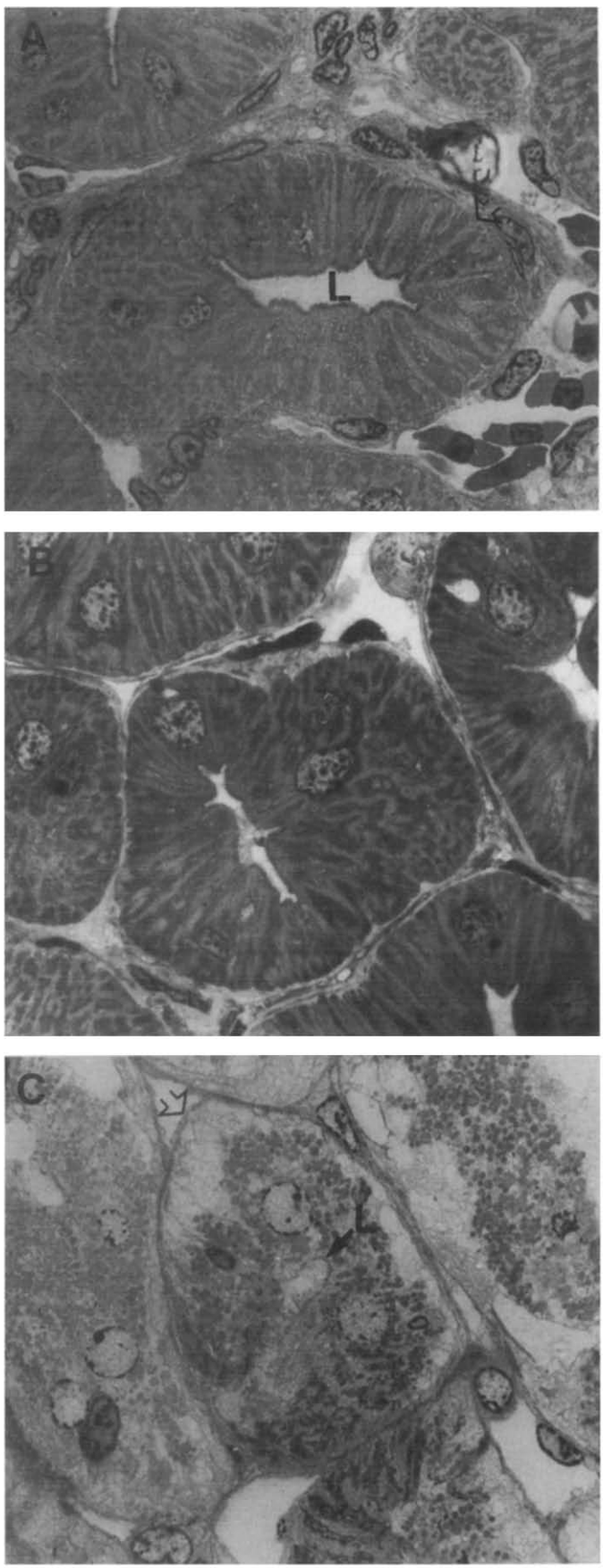
TABLE IV

*Effect of inhibition of $Na^+$,$K^+$-ATPase activity on the swelling effect of pCMBS*

Tissue was incubated 3 h in standard salines (controls) or under conditions inhibiting $Na^+$,$K^+$-ATPase (0.5 mM ouabain, Li-saline), with or without 1 mM pCMBS. Mean values, ±SE, of tissue $H_2O$ (kg/kg dry wt.) and cations (mequiv./kg dry wt.), $n = 4$.

| | Tissue | | | |
|---|---|---|---|---|
| | $H_2O_t$ (kg/kg dry wt.) | mequiv./kg dry wt. | | |
| | | $Na^+$ | $K^+$ | $Li^+$ |
| Exp. 1 | | | | |
| Control | 3.01±0.05 | 335± 8 | 275±9 | - |
| Ouabain | 3.29±0.06 | 694±11 | 81±6 | - |
| pCMBS | 3.93±0.04 | 937±23 | 57±5 | - |
| Ouabain +PCMBS | 4.59±0.09 ** | 1312±68 | 53±3 | |
| Exp. 2 | | | | |
| Control (Na saline) | 2.99±0.04 | 325± 9 | 367±6 | - |
| Li saline | 3.34±0.03 | 9± 4 | 70±1 | 495± 8 |
| Na saline +pCMBS | 3.33±0.12 | 906±40 | 73±2 | - |
| Li saline +pCMBS | 4.72±0.06 ** | 14± 7 | 63±6 | 900±16 |

** $P < 0.01$ of pCMBS vs. experimental condition+pCMBS (for $H_2O_t$ only).




indicate that reduction of cell -SH permits a more rapid inhibition of the $Na^+,K^+$-ATPase by cell pCMBS.

*Effect of inhibitors of the $Na^+,K^+$-ATPase on the pCMBS-induced cell swelling*

Data given in Table IV further substantiate the above conclusion. Ouabain, or the absence of external $Na^+$ (Li-saline) had little effect on tissue water [23,12] but significantly increased the pCMBS-induced swelling.

The data also clearly show that the pCMBS-induced cell swelling is produced by a mechanism which cannot be solely due to the action of the mercurial on the $Na^+,K^+$-ATPase.

*Effect of pCMBS on cell morphology and F-actin arrangement*

Organic mercurials are known to produce a disruption of the cell cytoskeleton (F-actin and spectrin), thus affecting the properties of cell membranes [24]. Hence, the effect of pCMBS on the morphology and F-actin arrangement in RGC was examined.

As shown in Fig. 9, exposure of RGC to pCMBS results in a major alteration of RGC morphology. The observed effects coincide, temporally, with cell volume changes. The most obvious change occurs at the basolateral pole of the cells, with the appearance of clear 'lakes' for expanded regions of the cytoplasm. This disappearance of a clear intercellular zone between basolateral foldings of the RGC membrane is seen throughout the tubule as well. Although the apical membrane appears to be intact, the lumina of all the tubules contain debris and evidence of membrane blebs. This is indicative of some disruption also of this membrane domain.

An analysis of the localization of F-actin with rhodamine-conjugated phalloidin showed that changes in this component of the cytoskeleton occur in concert with the morphological changes (Fig. 10). In control sections, F-actin is associated with the plasma membrane defining the basolateral and apical surfaces. This pattern of F-actin was unchanged at one hour of exposure to pCMBS. After 3 h exposure, almost no phal-

Fig. 9. Light micrographs of shark rectal gland. Magnification 760×. (A) Section of slice incubated 3 h in control saline. The tubule organization is intact. The basolateral folds (arrow) can be seen between each cell as an area of less dense staining. The apical surface of microvilli are also detectable in the lumen (L). (B) Section of slice exposed to 1 mM pCMBS for 1 h. The organization of the tubule cells is similar to controls with the exception of some debris in the lumen. (C) Section of rectal gland slice incubated in the presence of 1 mM pCMBS for 3 h. The tubule organization is disrupted. The basal compartment of the RGC is expanded into clear areas ('lakes') relatively devoid of organelles (arrow) and there is no clear pattern to the intracellular space as seen in A and B. The lumenal surface can be detected (L) and the microvilli appear altered.

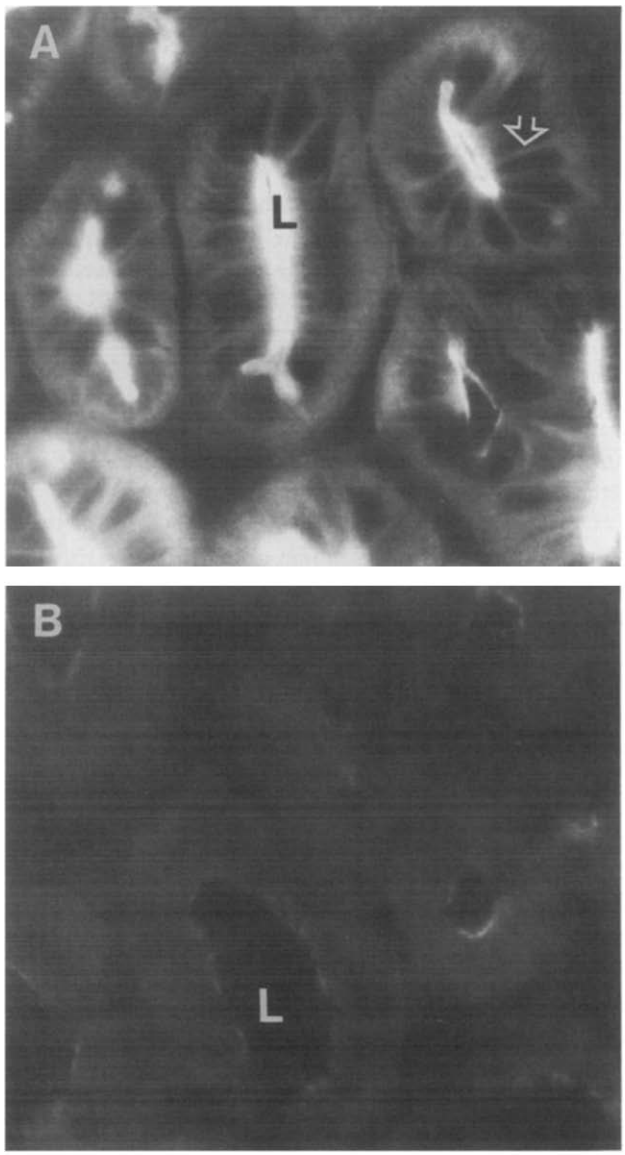



Fig. 10. Localization of F-actin in shark rectal gland cells. Magnification 520×. (A) Section of slice incubated for 3 h in control saline: Bright fluorescent linear pattern defines the basolateral membranes of the intercellular spaces (arrow). This is due to actin filaments associated with the membrane folds in this region. The lumenal surface (L) is also brightly fluorescent due to the abundance of F-actin in the microvilli of this membrane surface. (B) Rectal gland after exposure to 1 mM pCMBS for 3 h: the pattern associated with the basolateral folds is absent and there is only a faint positive fluorescence at the lumenal (L) surface.

loidin-associated fluorescence is seen along the basolateral cell pole. In some cases a low level of fluorescence was seen associated with the apical membrane while in adjacent tubules no fluorescence in this region could be detected. Mersalyl (0.5 mM, 2 h exposure) produced alterations in cell structure similar to those seen with pCMBS (details not given). This mercurial action was found to be independent of cell volume: Fig. 11 shows cells incubated in sodium gluconate medium with an F-actin distribution as seen in Fig. 10, controls; cells treated in sodium gluconate medium plus pCMBS did not swell (see Fig. 4) but showed practically the same loss of F-actin associated fluorescence compared to the action of the mercurial when cell-swelling occurred (Fig. 10).

As opposed to mercurials, no effect of NEM on RGC morphology was seen, even when the tissue was incubated for 5 h (Fig. 12A). Agents affecting cell glutathione levels (Cl-DNB, DMF or thiolyte) also did not affect cell morphology. However, when combined with pCMBS, the morphology was more severely altered then in controls with pCMBS alone. This point is demonstrated by comparing Figs. 12B and C for Cl-DNB. These results are consistent with data on cell volume (Fig. 8 and Table III).

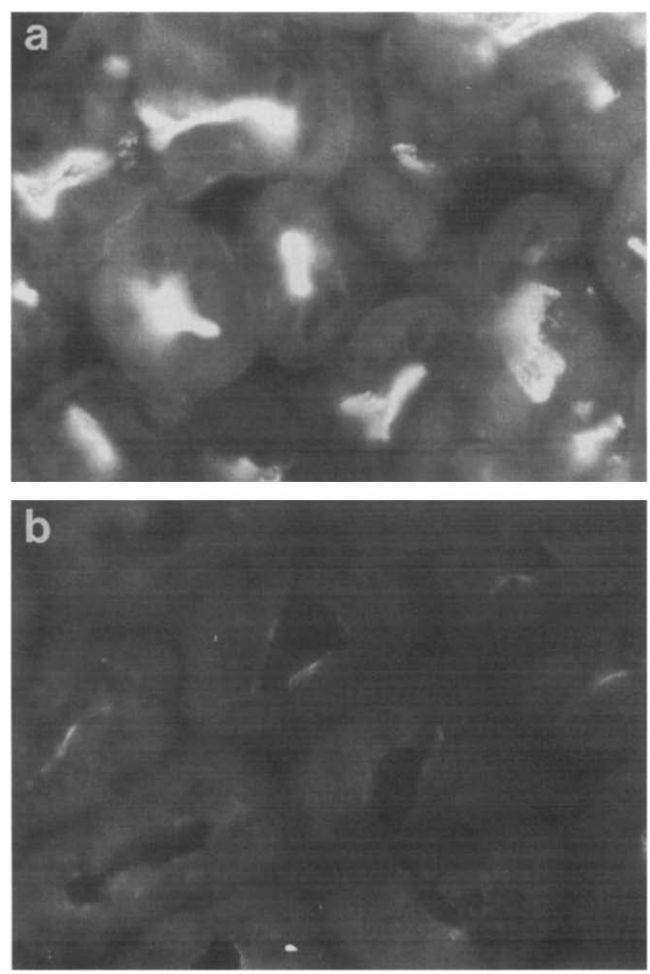



Fig. 11. Localization of F-actin in rectal gland slices exposed to sodium gluconate medium (a) and sodium gluconate plus 1 mM pCMBS (b). Magnification 430×. The amount of fluorescence, indicating a reduction in the amount of F-actin in the cells, is greatly reduced after exposure to pCMBS and is most clearly seen along the lumenal membrane.

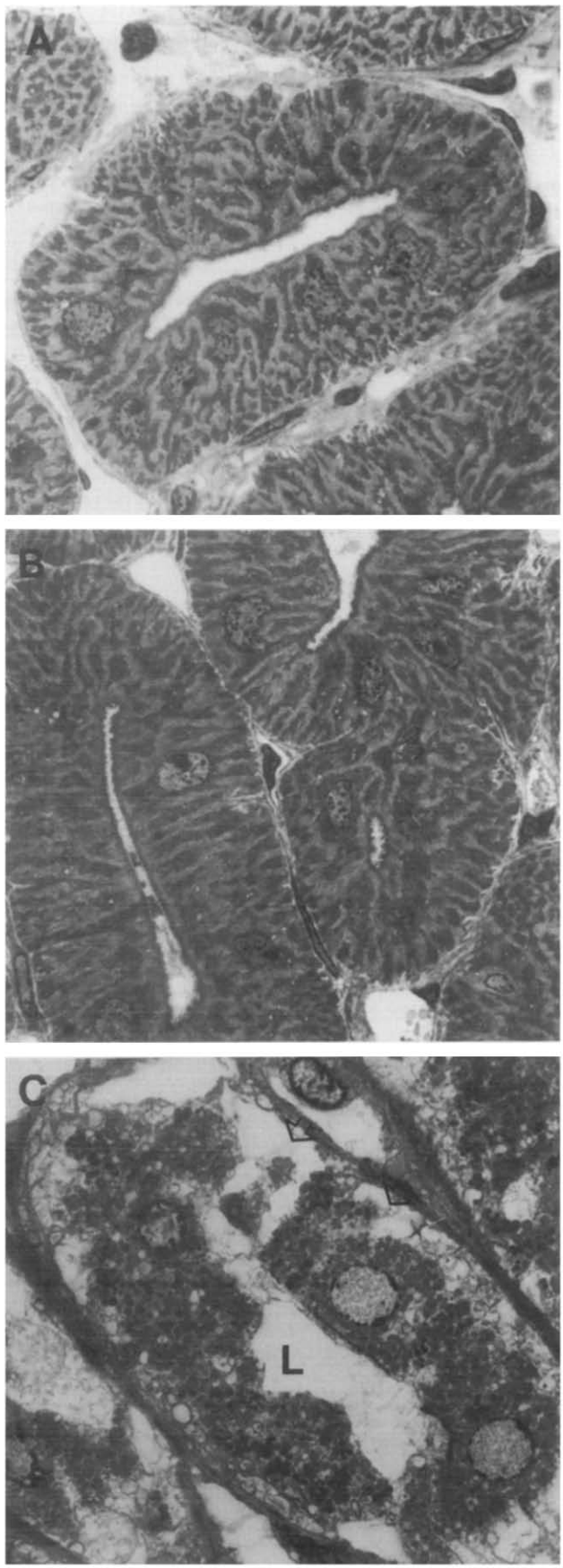



Fig. 12. Light micrographs of shark rectal gland. Magnification 800×. (A) Section from slice incubated in the presence of 1 mM NEM for 5 h; the morphology is preserved (cf. control, (Fig. (A)). (B) Section of slice incubated in the presence of 1 mM Cl-DNB for 5 h; again, the morphology is identical with controls. (C) Slice incubated in saline with 1 mM Cl-DNB for 2 h and then in Cl-DNB plus 1 mM pCMBS for 3 h: the tubule cells are markedly disrupted. The basal surface is expanded (arrows), as with pCMBS alone (cf. Fig. 9C), and in some cases appears to be detached from the lining of the tubule. The lumen (L) can be easily identified but the surface microvilli are disrupted.

## Discussion

pCMBS, as well as other organic mercurials tested, produced a swelling of RGC, associated with an uptake of $Na^+$ and a loss of $K^+$ (Figs. 1 and 2). This effect on cell cations took place even under conditions when cell-swelling was prevented (Fig. 4). Thus, the mercurials acted on the mechanism(s) which maintain a balanced state of cell cations, i.e., the passive permeability of the RGC membrane to these cations, and/or the operation of the sodium pump. On the other hand, NEM, a thiol reagent covalently interacting with -SH, did not affect cell volume while producing changes in cell cations similar to those of pCMBS (Fig. 2). Since it has been shown in erythrocytes that NEM does not bind to the site of water transport inhibited by pCMBS (Ref. 9; cf. also Ref. 24), a comparison of the effects of both thiol reagents was deemed necessary to elucidate the mechanism by which pCMBS produces RGC swelling.

The mechanism of pCMBS action on cells has been most thoroughly studied in erythrocytes [14,2,25]. Here, pCMBS produces its effect by an interaction with band 3 and 4.5 proteins of the cell membrane [24–26]; of these two sites, band 3 protein appears to be more relevant for the phenomena discussed here [24,25]. Several points distinguish the action of pCMBS on RGC from that in some other cells. (1) Thus, as opposed to erythrocytes [14,2], pCMBS did not significantly affect the steady-state efflux of $^{86}Rb^+$ and of [$^{14}$C]urea (Fig. 3 and text). In liver cells of the skate, mercurials also increased the membrane permeability to $K^+$ [15]. (2) The pCMBS entry into RGC was slow (Fig. 5), as in erythrocytes [4]. However, in contrast to red blood cells [16], the pCMBS entry into the cells did not show evidence of employing a carrier-mediated mechanism. In particular, DIDS barely inhibited the pCMBS entry, suggesting that the membrane anion exchange system is not involved in the process. (3) Still more surprising was the observation that most of the mercurial was not firmly bound by cell components, as shown in Fig. 6. It should be pointed out that a swelling effect of pCMBS was seen even in the absence of the mercurial in the incubating media, after the tissue has been pretreated with pCMBS (Fig. 4), suggesting some interac-

tion of the mercurial with a component involved in the regulation of cell volume. This swelling could be prevented by enhancing the removal of cell pCMBS with DTT. (4) Lastly, the enhancing effect of NEM, as well as some other thiol reagents (Fig. 8, Table III) was also unexpected.

The rationale of the present analysis of the pCMBS effects of RGC is based on three major observations. Organic mercurials: (1) accelerate the efflux of the major cell osmolyte, taurine [8]; (2) gradually inhibit cell $Na^+,K^+$-ATPase activity (Fig. 7, Table II); and (3) produce a reorganization (depolymerization?) of F-actin (and possibly other cytoskeletal proteins) (Fig. 10 and 11). Of these three sites of pCMBS action, NEM shares only the inhibitory effect on the $Na^+,K^+$-ATPase.

*Cell osmolytes and the effect of pCMBS*

pCMBS produces a rapid loss of the major osmolyte, taurine [8]. This property appears to be selective for the mercurial, since NEM had no such effect (unpublished data). The cells compensate for the loss of osmolytes by a net uptake of external electrolytes [28]. This may in part be responsible for the cation changes seen particularly under conditions when cell-swelling was prevented in sodium gluconate media (Fig. 4). Thus, in one such experiment, the sum of bulk cations ($[Na^+]_i + [K^+]_i$) in the controls was $151 \pm 2$ mM, a value somewhat lower than in controls incubated in regular elasmobranch saline (215 mM, cf. [24,28]); particularly, $[K^+]_i$ was significantly lower (contribution of the $Cl^-$ potential?). After incubation in sodium gluconate saline in the presence of pCMBS, ($[Na^+]_i + [K^+]_i$) significantly increased ($P < 0.001$) to $278 \pm 4$ mM. However, the Donnan ratio of $K^+$ decreased in the course of such experiment indicating a cell membrane depolarization from some $68 \pm 2$ mV to $20 \pm 13$ mV. This result already suggested that pCMBS also produced a partial inhibition of the $Na^+,K^+$-ATPase.

The effect of pCMBS on the osmolyte levels of RGC per se is insufficient to explain cell swelling. This follows from data showing that cells first incubated in high $K^+$ media, when a major loss of osmolytes also takes place [28], the cells practically fully recover their original volume when transferred to sodium media free of osmolytes [24].

*The pCMBS effect on the sodium pump*

The pCMBS-induced inhibition of the $Na^+,K^+$-ATPase activity developed gradually (Fig. 7), reflecting the slow entry of the mercurial (Fig. 5). The fact that no significant ATPase inhibition was seen immediately when the tissue was brought into contact with the mercurial appears to indicate that pCMBS interacted with thiol groups of the enzyme at the inner face of the membrane.

An inhibition of the $Na^+,K^+$-ATPase is insufficient per se to produce massive swelling of RGC. This is borne out by previous observations on the effect of ouabain [24,12], as well by data in Table IV, and by Fig. 2 and Table II, showing that NEM, while inhibiting the $Na^+,K^+$-ATPase, does not produce cell-swelling.

Inhibition of the $Na^+,K^+$-ATPase being one of the factors of pCMBS-induced swelling of RGC, it might be predicted that an increase in such inhibition should also enhance the swelling effect of the mercurial. This prediction is borne out by experiments given in Fig. 8 and Tables III and IV. Agents reducing the level of cell glutathione (which had no effect on cell water and cations), would increase the cellular pool of the pCMBS because of the absence of a sink for the mercurial; thus the inhibition of the $Na^+,K^+$-ATPase was enhanced, leading to an increase in both the rate and level of RGC swelling. Also, blocking of the ATPase by NEM (Table II), ouabain or absence of $Na^+$ (Table IV) increased the pCMBS-induced RGC swelling.

*pCMBS effect on the cytoskeleton*

Another major site of pCMBS action is F-actin (and possibly other components of the cytoskeleton). pCMBS has been found to produce a depolymerization of erythrocyte F-actin and the tetrameric spectrin [25,29,30] as well as of muscle F-actin [30]; in contrast, NEM had no such effect [25]. Also in blood platelets, organic mercurials affect the proteins of the cytoskeleton [31]. These observations are fully consistent with data on RGC reported above (Fig. 10, 11). pCMBS gradually produced a disappearance of the selective fluorescence at the basolateral membrane of RGC, formed on interaction of F-actin with phalloidin derivatives (Fig. 10). The pCMBS effect may well be reversible on removal of the mercurial; this would follow from the observations that DTT, while depleting mercurial from the cells, prevented cell swelling (Fig. 4). However, the high degree of microscopic cell disorganization by pCMBS (Fig. 9C) might indicate additional toxic effects of the mercurial.

The possibility that the observed changes in F-actin organization might be the result of cell swelling per se, rather than reflecting the pCMBS effect, is excluded by the following observations: (a) pCMBS produced a disappearance of F-actin fluorescence also under conditions when cell swelling was prevented by incubation in sodium gluconate media (compare Figs. 10 and 11, and Fig. 4); (b) it has been shown previously that cell swelling did not affect F-actin reorganization elicitated by high-$K^+$ media [12]; (c) conversely, cell swelling produced by hypotonicity (urea-free media, Ref. 12) or propionate [11] did not affect the organization of F-actin.

A change in the amount or organization of F-actin, particularly that portion which interacts with the cell

membrane, would be expected to decrease the restraint of the cell membrane to osmotically-induced stretch, thus permitting cell swelling. We have previously reported [12] that high external $K^+$ alters the amount of F-actin in the rectal gland cells, and this effect was associated with massive cell swelling. However, agents producing a dissociation of the cytoskeleton per se do not produce cell swelling. Thus, in spite of the cytoskeleton breakdown in high-$K^+$ media, or in the presence of pCMBS, no cell-swelling took place when osmotic forces were not exerted on the membrane, e.g., in gluconate media (Ref. 12, and Fig. 4 above). Experiments to be published elsewhere provided further support for such view: Rectal gland cells respond to changes of external osmolarity at constant external $Na^+$ as osmometers (*N*-methylglucamine sulfate as the relatively impermeant osmotic agent), showing a linear relationship when plotting the cell volume against the reciprocal of external osmolarity. Conditions which were shown not to affect the F-actin localization in the cells (e.g., ouabain, Li-media, propionate media) [11,12] also did not change the slope of the plot. On the other hand, conditions affecting the F-actin organization in rectal gland cells, i.e., high external $K^+$ (KCl or potassium gluconate media, Ref. 12) or pCMBS (Figs. 10, 11) significantly increased the increment of cell volume change as a function of media osmolarity.

A disappearance of F-actin-phalloidin fluorescence in high-$K^+$ media [12], in the presence of pCMBS (Fig. 10, 11) and in hypotonic media [32] also increase the efflux of the osmolyte taurine. A role of the cytoskeleton in the water and urea transport in red blood cells has been postulated [25]. At the present state of our knowledge, the possibility cannot be excluded that the pCMBS-induced efflux of osmolytes is actually brought about by changes in the cytoskeleton.

The presented data thus substantiate the view that the pCMBS-induced RGC swelling requires the concurrence of two effects of the mercurial, i.e., (a) inhibition of the $Na^+$,$K^+$-ATPase, which produces the osmotic forces acting on the membrane; and (b) a dissociation of cytoskeletal elements (F-actin), permitting the massive swelling of the cells.

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