

ANTIDIURETIC ACTION OF A CORPUS CARDIACUM FACTOR (CTSH) ON LONG-TERM FLUID ABSORPTION ACROSS LOCUST RECTA *IN VITRO*

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SUMMARY

Long-term fluid absorption (J_v) by everted rectal sacs from locusts is stimulated by both corpus cardiacum (CC) extracts and cAMP in a dose-dependent manner. This hormonal antidiuretic activity (ADH) is present in both nervous and glandular lobes of CC. This distribution is similar to that of chloride transport stimulating hormone (CTSH) but not to that of other factors previously reported from this gland. As expected if ADH were due to CTSH acting on electrogenic Cl^- transport, CC extracts also increased the electropotential across rectal sacs, and the stimulation of fluid absorption ceased in Cl^- -free salines. CC extracts also caused recta to absorb fluid against larger osmotic gradients, suggesting that the antidiuretic factor acts on the ion-dependent active transport of fluid rather than on the osmotic permeability of the rectal wall.

INTRODUCTION

Regulation of Malpighian tubule excretion by diuretic hormones in insects is now well known. There are also several preliminary reports of putative diuretic and anti-diuretic factors influencing fluid reabsorption by isolated insect recta (reviewed by Phillips, 1981, 1983) but the validity of the *in vitro* methods used in these early studies has been questioned and therefore these reports require confirmation (Phillips, Meredith, Spring & Chamberlin, 1982*b*). Control of ionic reabsorption from the locust rectum is better established. Using the voltage-clamped preparation developed by Williams, Phillips, Prince & Meredith (1978), Spring & Phillips (1980*a,b,c*; Phillips, Mordue, Meredith & Spring, 1980) demonstrated that a corpus cardiacum (CC) factor (chloride transport stimulating hormone, CTSH) acts *via* cAMP to stimulate active Cl^- reabsorption across the rectal wall. The cellular mode of cAMP action has been extensively studied by Hanrahan & Phillips (1982, 1983*a,b*).

Goh & Phillips (1978) developed an everted rectal sac preparation which reabsorbs water and ions over a 5–6 h period *in vitro* at near constant rates which are close to

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those measured *in vivo*. They showed that water absorption in the absence of osmotic concentration differences and stimulants was coupled to active absorption of Cl^- , K^+ and Na^+ from the lumen. We therefore anticipated that CTSH, by increasing ion absorption, might enhance fluid transport; however, some preliminary results were inconclusive (Phillips *et al.* 1982*b*). However, Hanrahan (1982) in our laboratory subsequently observed that higher levels of cAMP (10 mM) are required to stimulate active Cl^- reabsorption maximally in everted rectal sacs than in flat-sheet preparations (1 mM), presumably because of the small total amount of this cyclic nucleotide (e.g. 20 μl) usually placed inside these sacs. We were therefore encouraged to re-investigate the actions of cAMP and CC extracts containing CTSH on fluid absorption by everted rectal sacs, especially since we have recently elucidated the specific substrate requirements for rectal metabolism (Chamberlin & Phillips, 1982).

In this paper, we report evidence for an antidiuretic action of CTSH and cAMP. We also investigate the action of separated storage (NCC) and glandular (GCC) lobes of CC because Mordue (1970) has reported that an antidiuretic factor (ADH) is localized in the GCC, while a diuretic factor (DH) which inhibited fluid reabsorption is restricted to the NCC of *Schistocerca* and *Locusta*. The description of an antidiuretic role for CTSH, which is mostly (80%) in the storage lobe, therefore appears to conflict with the results of Mordue (1970).

MATERIALS AND METHODS

The experimental animals were 30-day-old adult female *Schistocerca gregaria*, reared as previously described (Goh & Phillips, 1978) and starved overnight. Everted rectal sacs were prepared as described by Goh & Phillips (1978) except for the saline. Sacs were filled hourly with 10 μl of fresh saline and incubated in 25 ml of saline bubbled with 95% O_2 -5% CO_2 and maintained at a 25°C. At hourly intervals for 4-5 h, weight gain and tissue volume change of rectal sacs were obtained by weighing recta before and after removing the fluid contained inside (i.e. on the haemocoel side; see Goh & Phillips, 1978). Correction for tissue volume change yields the true rate of transepithelial fluid movement. Student's *t*-test was used to establish statistical difference between means. Entire corpora cardiaca (CC), or separated NCC and GCC of this gland, were removed from adult males to avoid cyclic changes associated with female reproduction. Each tissue was homogenized three times for 30 s (Dismembrator Artex, Sonic 300) in physiological saline and centrifuged for 30 min at 24 000 *g* (Sorval Superspeed R2CB Centrifuge). All these steps were carried out at 4°C. Supernatants were stored frozen prior to assays, when they were tested in 10 μl of saline bathing the haemocoel side of rectal sacs. As a control tissue, 0.025 suboesophageal ganglion (SOG), which is several times the size of 0.5 CC, was treated in the same manner as the CC. Changes in osmolarity of the saline due to adding tissue homogenates were checked using an osmometer (Advanced Instrument Inc., Newton Highlands, Mass) and were found to be insignificant. Fresh CC or SOG extracts were added hourly to sacs after a 1-h equilibration period, unless otherwise stated.

The bathing saline (Phillips *et al.* 1982*b*) was based on ion, sugar and amino acid concentrations measured in locust haemolymph (Chamberlin, 1981; Hanrahan

1982). Osmotic concentration was adjusted to $427 \text{ mosmol l}^{-1}$ with sucrose, which is not metabolized by locust rectum (Chamberlin, 1981): pH was 7.1. In some cases the osmotic concentration of this saline was raised to $827 \text{ mosmol l}^{-1}$ with sucrose. A chloride-free saline was also prepared by replacing all chloride with nitrate. The stimulatory effect of cAMP was tested by including this agent in physiological saline on the haemocoel side.

Electropotential difference (PD) across the rectal wall was measured using two calomel electrodes in series with 3 M-KCl-agar bridges. The electrodes were connected to an high input impedance differential amplifier ($10^{12} \Omega$, 4253 Teledyne Philbrick, Dedham, Mass.). The PD was measured at the end of each hourly incubation period prior to replacing the saline within the rectal sacs.

RESULTS

Stimulation of long-term fluid transport by CC extract and cAMP

The long-term absorption of fluid over the 2nd to 5th hour is shown in Fig. 1A. The rate of uptake (J_v) was $8.3 \mu\text{l h}^{-1} \text{ rectum}^{-1}$ during the first hour (i.e. equilibration period, not shown) and dropped significantly ($P < 0.025$) to a steady-state level of $5 \mu\text{l h}^{-1} \text{ rectum}^{-1}$ for control preparations containing 0.025 SOG. The volume of rectal tissue increased by $2.8 \mu\text{l}$ during the first hour, then decreased slightly (Fig. 1B). Control J_v values were not significantly different when saline lacking SOG was present inside the sacs for the whole 5-h period (data not shown). These results are close to those previously reported by Goh & Phillips (1978). Whole CC (0.5 glands) doubled long-term fluid absorption rate to $11 \mu\text{l h}^{-1} \text{ rectum}^{-1}$ (Fig. 1A) compared with controls. Tissue volume changes were unaffected by CC extracts (Fig. 1B).

Initial measurements of fluid absorption at 30-min intervals indicated that CC extract exerted its maximum effect within the first 30 min (data not shown). Moreover, replacing this saline with CC-free saline after 10 min did not diminish the subsequent stimulatory effect, suggesting that the active factor was already absorbed onto receptor sites by this time. Based on these results, hourly measurements of fluid absorption were judged appropriate to study the CC dose-response relationship (Fig. 2). There is a good linear relationship between the logarithm of the dosage added and the increase in fluid reabsorption over a range of 0.03 to 0.50 CC: higher CC dosages did not increase J_v further (data not shown but see Table 1). In contrast, the addition of 0.1 brain without pars intercerebralis (i.e. a considerably larger amount of nervous tissue than 0.5 CC) caused only a slight but insignificant increase in fluid absorption (Fig. 2).

Since cAMP is the apparent second messenger for CTSH (Spring & Phillips, 1980a) and mimics the action of CC extracts on KCl absorption across shorted-circuited recta, we studied its effects on fluid absorption across everted sacs (Fig. 1). In comparison with controls, 10 mM-cAMP caused an 80% increase in long-term fluid absorption which was significant at the end of the fourth hour. J_v increased linearly with the logarithm of cAMP concentration (Fig. 3), reaching a maximal value at 10 mM-cAMP (i.e. in agreement with the results of Hanrahan, 1982, for stimulation of Cl^- transport across rectal sacs).

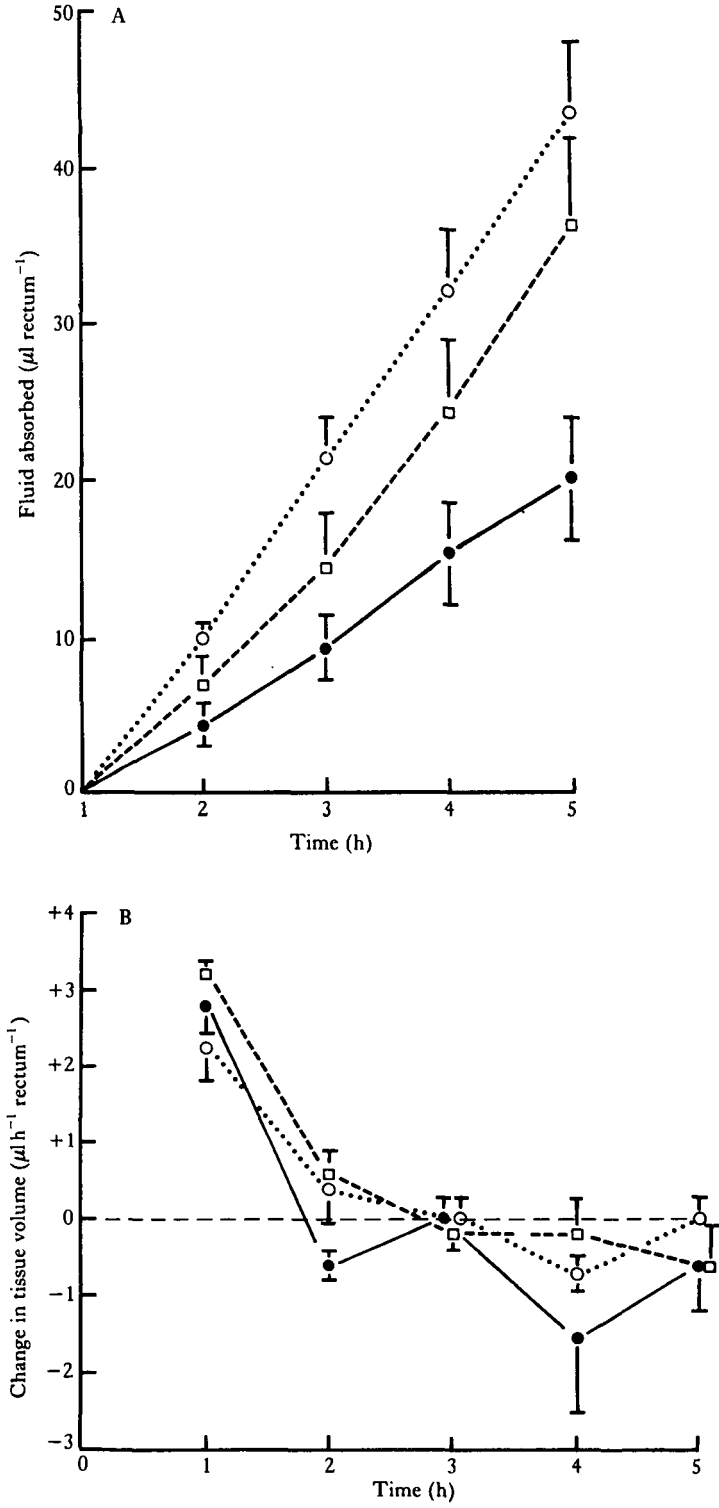


Fig. 1

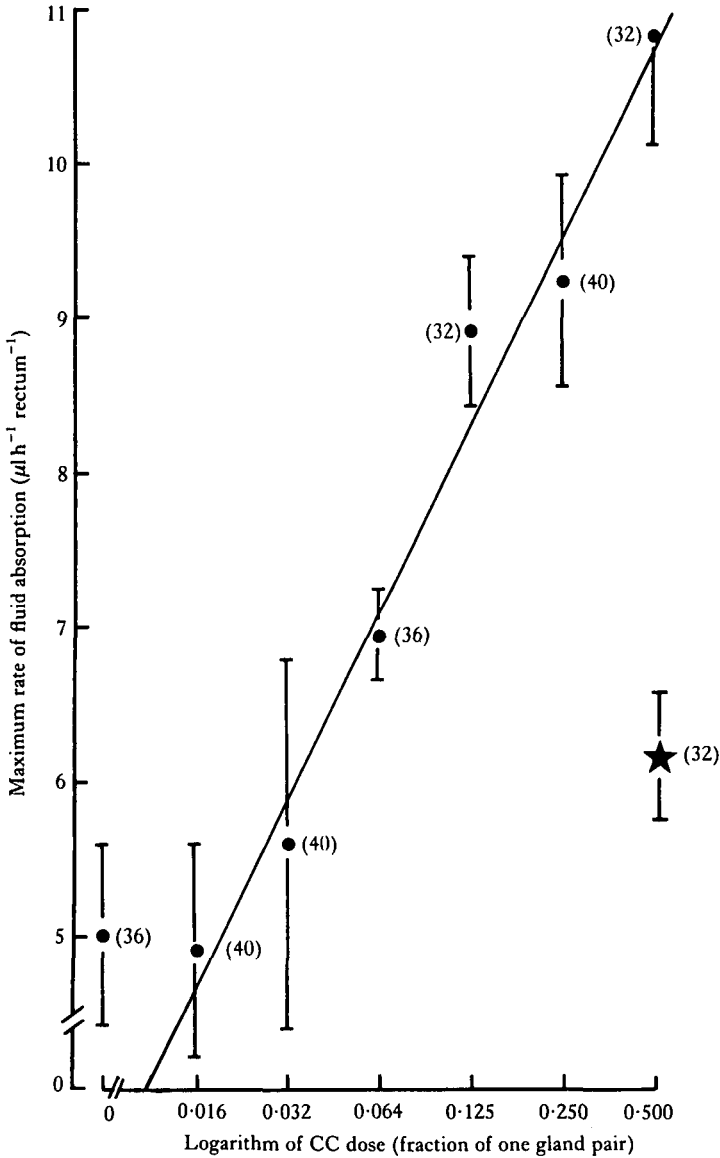


Fig. 2. Increases in fluid absorption with increasing doses of CC extract. The regression line fitted by the least squares method is expressed by $y = 4.1 \log x + 12$ ($r = 0.987$). Numbers in parentheses indicate N for each point, mean \pm s.e.m. The increase in fluid absorption was significant with addition of 0.062 CC ($P < 0.05$) and maximum increase was obtained with 0.5 CC ($P < 0.001$; higher CC dosages not shown). (★) Averaged hourly absorption rate over a 4-h period for recta filled with saline containing 0.1 brain extract without pars intercerebralis served as a control.

Fig. 1. Influence of corpus cardiacum (CC) extracts and cAMP on long-term fluid transport (J_v) across everted rectal sacs. (A) Transepithelial absorption of fluid (excluding the first hour) and (B) associated changes in tissue volume including first hour. Saline (10 µl) containing 0.5 CC (○), 10 mM-cAMP (□) or 0.025 SOG (●, control) was placed in the haemocoel compartment hourly after the first hour: mean \pm s.e.m., $N = 8, 6$ and 9 respectively. Absorption by stimulated recta became significantly greater than the controls at the end of the second hour for the CC group, but only at the end of the fifth hour for the cAMP group ($P < 0.015$ and $P < 0.050$). The averaged hourly absorption rates for the CC and cAMP groups are both significantly higher than for the control group ($P < 0.001$ and $P < 0.010$).

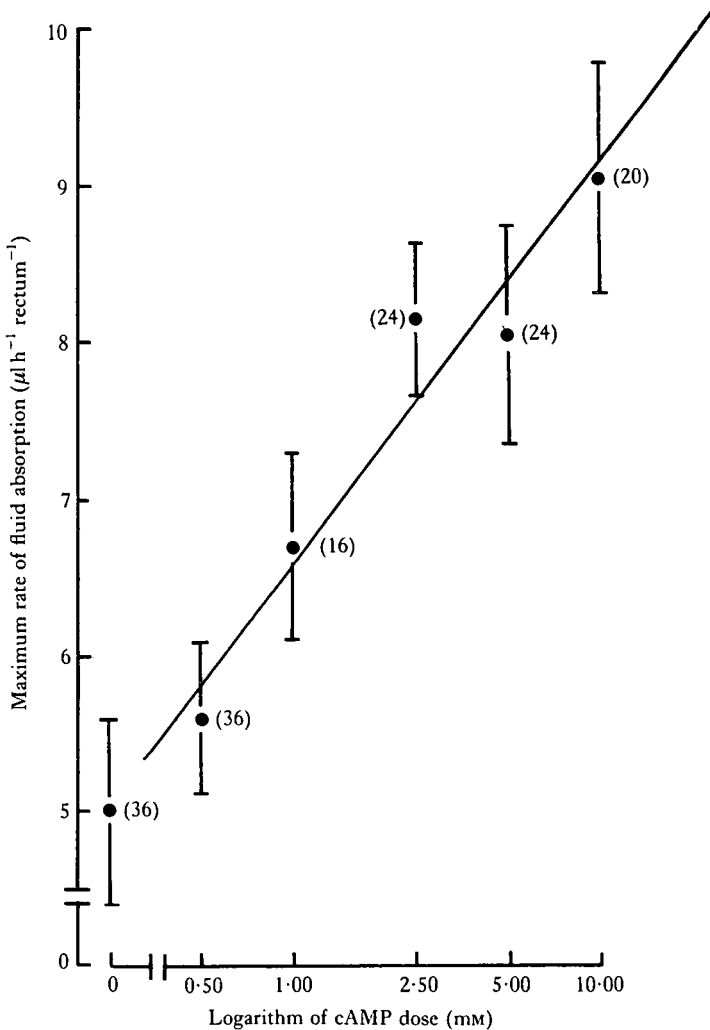


Fig. 3. Increase in long-term (4 h) fluid absorption rate with increasing doses of cAMP. The regression line fitted by the least squares method is expressed by $y = 2.5x + 6.6$ ($r = 0.967$). Numbers in parentheses indicate N for each point, mean \pm s.e.m. The increase in fluid absorption became significant when 1.0 mM-cAMP was added ($P < 0.05$) and maximum stimulation was achieved with 10 mM-cAMP ($P < 0.01$; higher dosages not shown).

Measurements of PD across stimulated recta

All previous work with CTSH has been done on flat-sheet preparations of locust rectum. If CTSH, acting *via* cAMP, is the agent in CC extracts which stimulates fluid absorption across rectal sacs, we would expect a simultaneous two- to three-fold increase in transepithelial PD, because this hormone increases electrogenic Cl^- transport and hence PD across flat-sheet preparations of recta bathed in low-K saline (Hanrahan, 1982). We measured both fluid absorption rate and PD on the same rectal sacs stimulated hourly with 0.5 CC for 4 h. Recta absorbed $9.5 \mu\text{l}$ during the initial equilibration hour, after which the addition of CC supernatant caused a J_v of $11 \mu\text{l h}^{-1} \text{rectum}^{-1}$ for the next 4 h (i.e. twice control rates shown in Fig. 1). C

extracts increased PD across these rectal sacs significantly (Fig. 4; $P < 0.01$) from 14.5 mV (haemocoel side negative) to 35 mV, in quantitative agreement with previous results from flat-sheet preparations (Spring & Phillips, 1980a). In contrast, PD for unstimulated controls decreased slightly after the first h (Fig. 4). In summary, CC stimulation of J_V correlates with increases in PD due to CTSH stimulation of Cl^- transport.

Cl^- dependence of fluid reabsorption

If increases in J_V initiated by CC extract result from CTSH acting on rectal Cl^- transport, stimulation of J_V should cease in Cl^- -free saline. Recta were bathed and

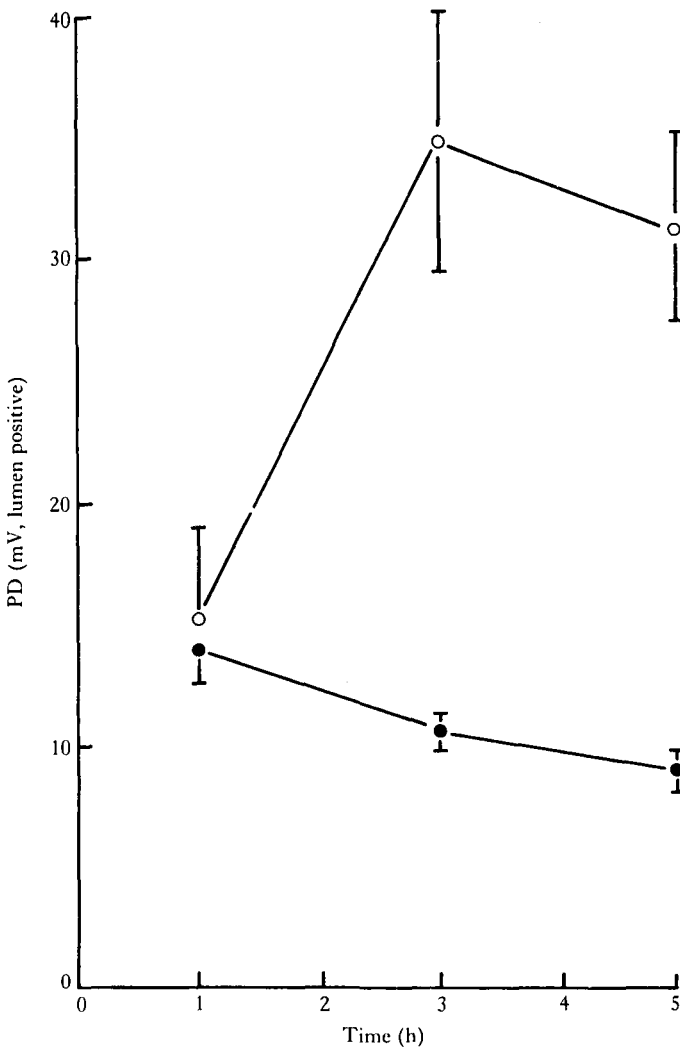


Fig. 4. Effect of CC extract on the transepithelial potential difference (PD) across everted rectal sacs. Saline containing 0.5 CC (○) or 0.025 SOG (●) was added hourly from the second hour. PD was measured at the end of the first, third and fifth hour prior to replacing the saline (mean \pm S.E.M., $N = 8$ and 6 respectively). CC extracts caused a significant increase in the transepithelial PD ($P < 0.001$).

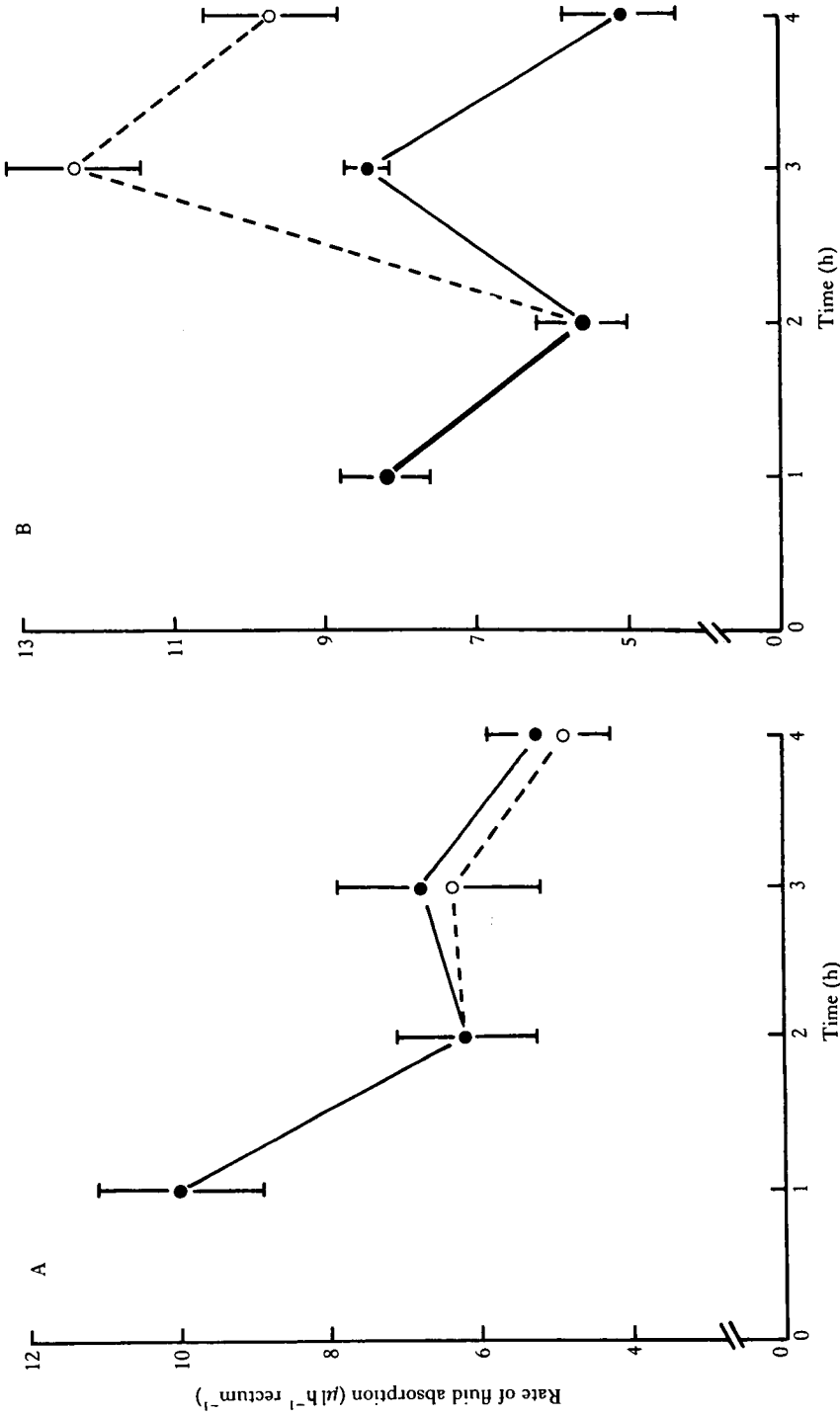


Fig. 5. The Cl-dependence of long-term fluid transport across everted rectal sacs. (A) Sacs in Cl-free saline over a 4-h period. Cl-free saline was replaced hourly in the haemocoel compartment without any extract (●), or with 0.5 CC extract from the third hour (○). (B) Sacs exposed bilaterally to Cl-free saline for the first 2 h and then during the third and fourth h with normal saline (110 mm-Cl) containing (●) 0.5 CC extract: mean \pm s.e.m., $N = 8$ (A), $N = 8-12$ (B). Transfer from a Cl-free saline to a normal saline (B) increased fluid absorption, but this increase was significantly higher for CC-stimulated than for unstimulated recta ($P < 0.001$).

filled hourly with fresh Cl-free saline over a 4-h period. From the 3rd hour, half of these recta (the unstimulated group) were filled with Cl-free saline, while the remainder (stimulated group) received Cl-free saline containing 0.5 CC extract. The rates of fluid absorption are shown in Fig. 5A. The absence of Cl⁻ in the bathing saline abolished the stimulatory action of CC extracts on fluid absorption, even at a high dosage of 1.2 CC.

A complementary experiment is shown in Fig. 5B. After a 2-h period during which recta were bathed and filled with Cl-free saline, they were re-exposed to normal saline (i.e. with Cl⁻) with or without 0.5 CC. After a decrease in absorption rate due to the lack of Cl⁻ during the first 2 h, the absorption rate increased significantly when Cl⁻ was re-added. However this increase was much greater (two-fold) in recta exposed to CC extract ($P < 0.001$). Clearly, stimulation of fluid transport by CC extract specifically requires external Cl⁻, as expected if CTSH is the stimulatory agent. Unstimulated recta are still able to transport fluid at low rates without exogenous Cl⁻, because under these conditions cation transport is known to sustain some fluid uptake (Phillips *et al.* 1982b).

Effect of separated storage (NCC) and glandular (GCC) corpus cardiacum

Is the factor stimulating fluid absorption located in both NCC and GCC, as is CTSH (Phillips *et al.* 1980), or only in the GCC with an inhibitory factor (DH) in the NCC, as reported by Mordue (1970)? Supernatants of NCC and GCC removed from the same animals were added hourly to everted sacs for 4 h at a dose of either 0.125 or 1.00 gland-equivalents in 10 μ l. The averaged hourly fluid absorption rates are shown in Table 1. Both NCC and GCC caused a significant increase in fluid absorption at both dosages ($P < 0.025$ and $P < 0.001$ respectively). These results are consistent with stimulation of fluid absorption by CTSH, but not with an inhibitory DH located in the NCC and an ADH only in the GCC (Mordue, 1970).

Fluid absorption against osmotic gradients

We have so far only considered J_v under isosmotic conditions. Phillips (1964) showed *in vivo* that J_v was linearly dependent on the osmotic concentration difference (Δ Osm) across the rectal wall. Goh & Phillips (1978) confirmed this *in vitro* using

Table 1. *Effects of neuroendocrine extracts on long-term rates* of fluid absorption by everted rectal sacs*

Treatment†	Rate of fluid absorption \pm s.e.m. (μ l h ⁻¹ rectum ⁻¹)	(N)
0.025 SOG (control)	5.0 \pm 0.59	(36)
0.125 NCC	7.7 \pm 0.77	(32)
0.125 GCC	7.2 \pm 0.66	(32)
1.00 NCC	11.3 \pm 0.40	(56)
1.00 GCC	11.9 \pm 0.36	(60)

* Second to fifth hour after removing recta from locusts, with rates measured hourly.

† Tissue extracts in 10 μ l of saline: SOG, suboesophageal ganglion; NCC, nervous (storage) lobe of corpus cardiacum; GCC, glandular lobe.

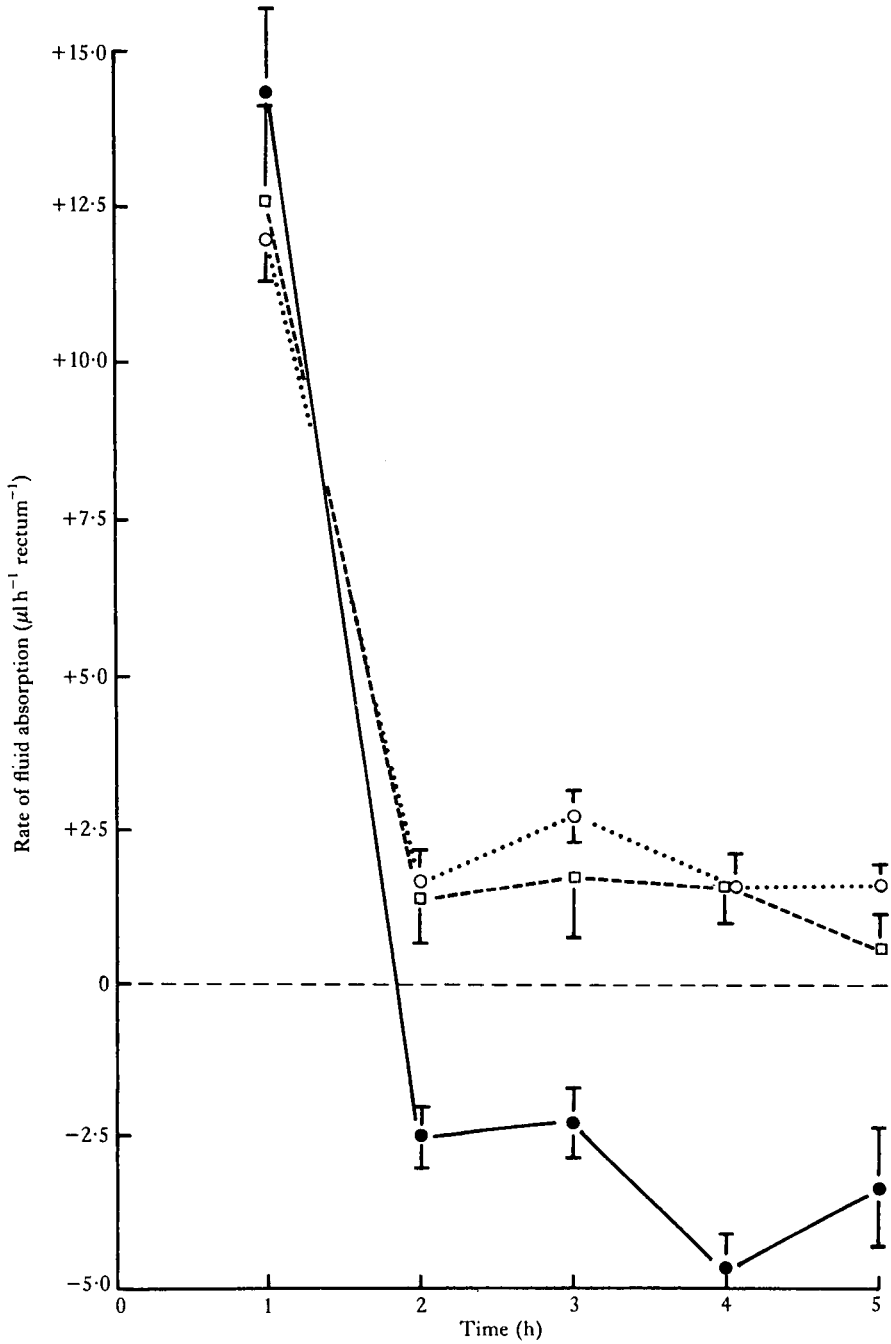


Fig. 6. The rate of fluid absorption by everted rectal sacs bathed externally in a hyperosmotic medium ($827 \text{ mosmol l}^{-1}$) after an equilibration period in isosmotic saline during the first hour. Recta were filled hourly with fresh isosmotic saline containing, from the second hour, 1 NCC (O), 1 GCC (□), or 0.025 SOG as a control (●): mean \pm S.E.M., $N = 5-6$.

unstimulated everted sacs. Because $J_v/\Delta\text{Osm}$ indicates the osmotic permeability (P_{osm}) of the rectal wall, the effect of CC extracts on this relationship should indicate whether active absorption of fluid (driven by Cl^- transport) or passive osmotic permeability of the rectal wall are changed. We measured J_v hourly for recta bathed externally in a hyperosmotic saline ($827 \text{ mosmol l}^{-1}$) and filled hourly with $10 \mu\text{l}$ of isosmotic saline containing 0.025 SOG (control), 1 NCC , or 1 GCC . This $400 \text{ mosmol l}^{-1}$ difference provoked a dramatic decrease in J_v in all three experimental groups, but especially in the control group where the direction of fluid movement actually reversed (Fig. 6). During the whole 4-h experiment, J_v of the control group remained significantly lower than that of the stimulated groups ($P < 0.001$ or $P < 0.005$). Clearly, NCC and GCC containing CTSH cause recta to absorb against much larger ΔOsm than do unstimulated controls. The increases in J_v caused by CC extracts were the same ($4\text{--}5 \mu\text{l h}^{-1} \text{ rectum}^{-1}$) when ΔOsm across the rectal wall was 0 (Fig. 1) or 400 (Fig. 6), indicating no change in P_{osm} . This suggests that the stimulant in CC acts on ion-driven active fluid absorption (as expected for CTSH), which should increase the ΔOsm against which fluid absorption can occur: increased P_{osm} would have had the opposite effect.

DISCUSSION

It was previously not possible to conclude whether CTSH acts as an ADH to stimulate fluid absorption, or strictly as an 'aldosterone-like' hormone which only increases KCl transport (Phillips *et al.* 1982*b*). The new supportive evidence that CTSH does act as an antidiuretic factor on locust rectum is as follows: (1) the neuroendocrine tissue (CC) containing CTSH doubles the rate of fluid transport whereas larger amounts of other nervous tissue (e.g. SOG) have no effect. (2) The demonstration of approximately equal amounts of antidiuretic activity in both storage and glandular lobes of CC (Table 1) is qualitatively consistent with the location of CTSH activity determined by increases in Cl-dependent short-circuit current (I_{sc} ; Phillips *et al.* 1980), but different from the distribution of other CC factors reported to date. (3) The dose-response curve for CC stimulation of fluid absorption (Fig. 2; maximum rate with 0.5 CC) is in reasonable agreement with the relationship for CC stimulation of I_{sc} , as recently measured in our laboratory (i.e. maximum I_{sc} obtained with 0.3 CC ; Hanrahan, 1982; J. Meredith & J. Proux, unpublished observations). Spring & Phillips (1980*a*) reported a somewhat more sensitive response of rectal I_{sc} to CC (i.e. 0.1 gland giving maximum I_{sc}). (4) Cyclic AMP, which mimics CTSH stimulation of KCl absorption, also stimulates fluid transport by rectal sacs (Fig. 1*A*). (5) CC extract causes an increase in transepithelial PD concurrent with the increase in fluid transport across rectal sacs, as predicted if increased fluid movement resulted from stimulation of electrogenic Cl^- transport by CTSH. (6) In support of this interpretation, the antidiuretic action of CC extracts on J_v is abolished when recta are bathed in Cl-free saline (Fig. 5), even though we have shown that NaNO_3 and KNO_3 (i.e. Cl-free) solutions can sustain some fluid transport by unstimulated recta (Phillips *et al.* 1982*b*). Clearly the increase in rectal fluid transport caused by CC extract is specifically associated with stimulation of Cl^- transport, which is also the known action of CTSH. (7) Finally, CC extracts stimulate the active ion-dependent

component of fluid absorption across locust rectal sacs against larger osmotic gradients, rather than the passive component (i.e. osmotic permeability or back diffusion, Fig. 6). This is again consistent with CTSH being the active agent. In summary, there is considerable evidence that CTSH is probably the effective stimulant responsible for the antidiuretic actions of CC extracts reported in this paper. We have run a preliminary test of the antidiuretic activity of purified CTSH by HPLC: in spite of rapid loss in biological activity, this preliminary test was positive.

The results in this paper are for long-term transfer of fluid across well-characterized rectal preparations, excluding data for the first hour when rapid decreases in fluid and ion absorption rates and large tissue volume changes are known to occur (Goh & Phillips, 1978; Phillips *et al.* 1982*b*; Williams *et al.* 1978; Fig. 1 of this study). Most earlier studies on the control of fluid absorption across insect recta were carried out on uncharacterized rectal preparations, often during the initial transient phase and without knowledge of specific incubation conditions required to sustain prolonged fluid absorption (see Phillips *et al.* 1982*b*). For example, Mordue (1970) measured fluid absorption over a 1-h period using everted rectal sacs of locusts bathed in a simple saline without oxygenation or the required metabolic substrate, proline. He reported that the NCC inhibited rectal fluid absorption (diuretic effect), whereas we consistently observed an antidiuretic effect of this tissue on long-term fluid transport. We are in agreement, however, that the GCC has an antidiuretic action on everted rectal sacs of locusts, although properties of Mordue's ADH factor and CTSH appear to be different (see Phillips *et al.* 1980). Since the DH reported in CC by Mordue is unstable (see Morgan & Mordue, 1983), this factor may have been inactive in our extracts. The VP-like DH in locust SOG (Proux, Rougon & Cupo, 1982) apparently does not influence rectal J_v , although we have still to test this in the presence of CTSH.

There is some evidence that CTSH, like DH, is released after feeding (Phillips *et al.* 1982*a*). As a working hypothesis, we therefore suggest that the combined action of these two hormones might be to increase recycling of KCl-rich fluid through the excretory system. This would serve to clear the body of waste substances ingested with or produced by metabolism of the meal. At the same time, changes in relative levels of DH and CTSH with time would ensure that excess fluid is eliminated quickly.

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