TRANSPORT ACROSS THE CROP WALL IN *HELIX ASPERSA*.

A thesis presented for the degree of

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by

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CONTENTS

Section 1 - SUMMARY

This project was carried out to elucidate the ion transport properties of the crop of Helix aspersa. An examination was made of each of the following: changes in ion concentrations; the effects of inhibitors; and glucose and amino acid transport. Short circuit current and transepithelial resistance were measured in order to achieve this. The major findings of the study were:

1 The most unusual feature of the crop epithelium is the fact that the short circuit current (SCC) could be either positive or negative under standard control conditions. In most, although by no means all, epithelia studied to date, the SCC would be basal side positive, and would always have this polarity under almost any conditions. In Helix the mean control SCC was 1.1 $\mu A/cm^2$, the range being -79.3 to 60.2, with respect to the basal side. The mean SCC is equivalent to a net ion transport rate of 6.8×10^{-4} $\mu Equiv/cm^2/min$. Under sodium free conditions as control the mean SCC was 2.4 $\mu A/cm^2$ with the range being -13.0 to 29.8 $\mu A/cm^2$. The mean SCC is equivalent to a net transport rate of 1.46×10^{-3} $\mu Equiv/cm^2/min$. Under sodium and chloride free as control the mean SCC was -1.0 $\mu A/cm^2$, the range being -24.3 to 8.7 $\mu A/cm^2$. Under these conditions the mean SCC is equivalent to a net ion transport rate of -6.2×10^{-4} $\mu Equiv/cm^2/min$.

2 After a period of stabilisation, under standard bathing conditions, the crop was found to have a transepithelial potential difference that could be either positive or negative with respect to the basal side. The mean PD was -0.03mV, but the PD ranged from -6.5 to 4.9mV. Under sodium free conditions as control the mean PD was -0.2mV and the range was -2.8 to 1.6mV. With both sodium and chloride substituted as the control the mean PD was 0.3mV with the range being -2.1 to 5.6mV.

3 The mean transepithelial resistance, under standard bathing conditions, was 67.9 ($\pm$ 3.1) ohm cm^2 (n=158).

4 The magnitude of the mean resting potential difference, short circuit current and resistance were consistent with those of a classic "leaky" epithelium.

5 Sodium was required for the maintenance of a large transepithelial potential difference. Sodium substitution in the bathing solutions resulted in the greatest change in the SCC of any of the ion removal/substitution experiments, the direction of the change depended on what side the sodium was substituted. Apical substitution resulted in a greater change in the measured SCC than did basal. When it was apical the SCC became more negative and when it was basal it became more positive. The SCC also became significantly more

negative when removal was from both sides.

6 The SCC became significantly more negative when potassium was removed from both sides, or from the apical side only. The SCC became significantly more positive when potassium was removed only from the basal side. The greatest changes were obtained with apical removal and snails with an initially positive SCC showed the largest change. The opposite was true for basal removal. These responses were similar to those found for sodium substitution. The changes in the SCC upon potassium removal required the presence of both sodium and chloride in the bathing medium.

7 Upon the substitution of chloride on both sides of the tissue there was a significant change in the SCC, with the basal side becoming more positive. This change was sodium independent and remained unaffected by the presence of ouabain. Under normal bathing conditions the response was only significant in starved snails. The greatest changes were obtained when the SCC was initially positive.

8 The removal of magnesium was tested under sodium free as well as sodium plus chloride free condition as control. It resulted in a significant increase in the SCC when removal was from the basal side only, that is the basal

side became more positive. The greatest changes were observed when the SCC was initially positive. These changes were independent of both sodium and chloride.

9 Calcium removal was tested under standard and sodium plus chloride free conditions. Removal from both sides resulted in the basal side becoming significantly more positive. However when sodium and chloride were absent the basal side became significantly more negative. Under sodium plus chloride free conditions the largest change in the SCC was when calcium was removed from the apical side, the basal side became more negative.

10 Ouabain, which has been universally accepted as inhibiting the Na^+/K^+ATPase, was highly effective when added basally at a concentration of 10mM. Nevertheless, the SCC was not eradicated but was made more negative. The greatest changes were obtained when the SCC was initially negative. These results are consistent with the presence of a Na^+/K^+ATPase in the basolateral membrane.

11 Piretanide, a loop diuretic related to the compounds furosemide and bumetanide, is also an inhibiter of $Na^+/K^+/2Cl^-$ co-transport. In the crop epithelium, when it was added only apically, at a concentration of approximately 1mM, the SCC became significantly more

negative. The greatest change in the SCC was obtained from snails where it had been initially positive. Basal addition resulted in no significant change in the SCC. When considered in conjunction with the potassium free results it was deduced that there was a triporter located in the apical membrane.

12 Use of sodium thiocyanate, an inhibitor of chloride transport, resulted in the basal side becoming significantly more positive only when added basally. There was no significant change in the SCC when added apically. This indicated the possibility of a primary chloride transport system located basolaterally. It should be noted that the existence of this system elsewhere remains the subject of controversy.

13 The crop epithelium seems to have potassium conductance channels on both sides of the tissue. The barium ion is a known inhibitor of potassium channels and when it was added to each side of the tissue in turn the SCC changed significantly. Apical addition resulted in the basal side of the epithelium becoming significantly more positive. Addition just on the basal side resulted in the SCC becoming significantly more negative that is the basal side of the tissue became more negative. When added apically only but under potassium free conditions, there was a significant decrease in the SCC This is in

the opposite direction to that observed under normal control conditions.

14 Amiloride made the SCC significantly more negative when added at a concentration of 0.5mM to the apical side of the epithelium. The greatest changes in the SCC were observed when it was initially positive.

15 The crop epithelium has the ability to transport organic substances, for example D-glucose and glycine. Both of these significantly made the SCC more negative when added apically. This direction of change is unexpected since if they utilise sodium dependent systems then one would expect the SCC to become more positive since sodium absorption would also increase. However the more negative SCC observed is sodium dependent since there is no change under sodium free conditions. The more negative SCC could be due either to an associated increase in cation secretion or to anion absorption. The glucose could also be utilised inside the cell and used to power other transport processes that would decrease the SCC. There was also a significant change in the SCC when D-glucose was added to the basal side, the mean SCC became more positive. The addition of glycine to only the basal side had no effect on the mean SCC.

16 D-glucose and glycine used different carriers, these

having different saturation concentrations. There was no competitive inhibition taking place, as was illustrated by the outcome of the addition of firstly a maximal concentration of either D-glucose or glycine apically and then of the other substrate apically, which brought about a further change in the short circuit current.

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Section 2 - INTRODUCTION

The questions to which this work seeks answers are; 1/ what are the ion transport processes present in the crop wall epithelium and to what extent are these revealed by the use of specific inhibitors and ion removal/substitution experiments? 2/ what are the similarities and differences between the crop epithelium and other transporting epithelia from other invertebrates and from mammals? 3/ to what extent organic substances affect the transport across the crop epithelium?

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Little has been published on ion transport across the crop epithelium of *Helix aspersa* or other Pulmonate molluscs, though Dean *et al.* (1987) have noted neutral, imino and basic amino acid transport in *Helix* intestine. This introduction describes first the relevant anatomy of the *Helix* digestive system and then some very relevant studies on the mantle epithelium of *Helix* and the gut epithelium of another gastropod mollusc, *Aplysia*. It was expected that the crop of *Helix* might show some of the properties of these two. Transepithelial potentials in both *Helix* mantle and *Aplysia* gut seem to be dominated by active chloride transport, so a section then follows on chloride transport in the epithelia of some other animals.

At this stage it may be helpful to anticipate some of the findings of this study in order to note some of the peculiarities of the Helix crop epithelium. Notably, the transepithelial potential varies unpredictably in polarity as if determined by a variable balance of two opposing processes. The potential is small (i.e. a few mV) and declines for a while after the epithelium is first set up in artificial saline. It was hoped that this effect could be prevented but this did not prove possible. Also a small potential remains even after the removal of sodium, potassium and chloride from the artificial salines used to bath the crop epithelium. The addition of D-glucose or glycine apically resulted in the short circuit current becoming more negative, the reverse of the effect seen in most other epithelia.

Because of the variable polarity of the electrical potential, it is essential to keep in mind the particular convention adopted in this thesis for describing it.

Throughout this thesis the polarity of the PD is always quoted with respect to the basal surface, i.e. a negative PD means that the basal side is negative with respect to the apical side. The polarity of the SCC is taken as that of the PD before application of the current. The direction of change in the SCC is quoted as being either in a negative direction or in a positive one regardless of the initial polarity of the SCC.

Amongst the pulmonates there is considerable variation in the general anatomy of the digestive system. However, in stylommatophora, for example Helix aspersa, and in basommatophora, for example Lymnaea stagnalis, the gross anatomy can be divided into a number of distinct regions. The mouth leads into the buccal cavity containing the jaw, odontophore and radula, this being surrounded by a complex arrangement of muscles all of which make up the buccal mass. Paired salivary glands open via ducts into the rear of the buccal cavity. Food passes from the buccal cavity into the oesophagus and then into the greatly enlarged crop. Stylommataphora have a short oesophagus and large crop while, conversely, basommataphora have a long oesophagus and a short crop. Food then passes into the stomach which has ducts that lead to the two lobes of the digestive gland. Faeces form in the style sac, anterior to the stomach where they are consolidated and transported along the intestine and rectum to be discharged at the anus.

The faeces often have the same colour as the food and contain large pieces of apparently undigested material, which would seem to indicate that digestion is inefficient, although utilisation of ingested food appears to be very high. Mason (1970) has shown that Helix aspersa has a mean assimilation of 53.5% for ingested natural food materials. After feeding, food can remain in the crop for up to three days. Burton (1983) found that in inactive Helix pomatia the concentration

of sodium and potassium in the crop fluid was similar to that of the haemolymph. After feeding, however, the concentration of potassium in the crop fluid may rise to thirteen times the haemolymph value, whereas the sodium level falls.

The crop itself is a thin walled structure with the lining epithelium being of the columnar type and containing patches of ciliated cells and many uniformly distributed mucous cells. The crop lumen in stylommataphora is usually filled with a brown or red fluid, before eating known as crop juice. This juice contains a number of different enzymes which will break down a wide range of substrates (Table 2.1). It is also possible that bacteria in the crop juice contribute to the enzyme content but digestion of fats, proteins and polysaccharides certainly starts here. This results in the release of soluble material, especially fatty acids which are then absorbed through the crop wall. Absorption from the crop itself has only been confirmed for Agriolimax reticulatus (Walker, 1972). Walker showed that labelled galactose, glucose and glycine were apparently taken up by membrane transport as they seemed to be diffused throughout the general cytoplasm.

There do not, however, appear to be any studies on ion transport across the crop of Helix aspersa itself. Dean, Barber and Ponz (1987) using H.aspersa "intestine" have demonstrated mediated amino acid transport. Neutral amino

ENZYME	Substrates tested
Amylase	Starch, glycogen, amylase, amylopectin
Cellulase	Degraded and native cellulose
α-glucosidase	Sucrose, maltose, melezitose methylgalactoside
β-glucosidase	Salicin, amygdalin, cellobiose, gentiobiose, p-nitrophenyl-β-glucoside
Trehalase	Trehalose
α-galactosidase	Melitose, methyl glucoside raffinose
β-galactosidase	Lactose
α-D-fucosidase	p-nitrophenyl-α-D-fucoside
β-D-fucosidase	p-nitrophenyl-β-D-fucoside
Xylanase	Xylan
Laminarinase	Laminarin
Licheninase	Lichenin
Alginase	Alginic acid, sodium alginate
Chitinase	Degraded chitin
Pectinase	Pectin
Mannanase	Mannan
Glucanase	Glucan
Steroid sulphate	Oestrogen, cortico-steroid sulphates
Cathepsin	Casein
Protease	Peptone, casein, fibrin, haemoglobin
Gelatinase	Gelatin
Lipase	Olive oil, tween

Table 2.1 Enzymes reported in the crop juice of pulmonates (from Runham, 1975)

acids show mediated transport as well as simple diffusion. The mediated transport is both sodium dependent and independent, the former being more important. Dean et al. also found a system for imino acids and one for basic amino acids. The imino acid system is totally sodium dependent, whereas for basic amino acids there seems to be a sodium independent high affinity system as well as a sodium dependent low affinity system. The neutral amino acids seem to share a common carrier since they show mutual inhibition, and under sodium free conditions transport still occurs although it is quantitatively less than sodium dependent transport. Entry occurs against a concentrate gradient and hence both systems are active. The transport systems for imino and basic amino acids can also be shared by neutral amino acids, but the transport systems for neutral amino acids are not shared by imino and basic amino acids. Imino acid transport seems to depend on sodium, in contrast to the transport of basic amino acids which does not. Burton (1966, 1968a, 1983) has studied the effects of feeding and hydration on the concentrations of sodium and potassium in the crop fluid and haemolymph in Helix, see above.

Enyikwola and Burton (1983) and Enyikwola (1987) found using Helix mantle and Achatina fulica anterior intestine that the potential difference was unaffected by ouabain and by the removal of sodium, potassium, calcium or magnesium. The removal of chloride from the shell side in Helix mantle and

the mucosal side in Achatina fulica intestine reduced both the potential difference and the short circuit current; thiocyanate and furosemide had a similar effect. The magnitude of the potential difference was also dependent on the presence of mucosal stirring, this being relevant when looking at the results from Helix crop. Enyikwola surmised that electrogenesis is due to chloride transport.

A major area of discussion has centred around the question of the existence of a primary ATP dependent chloride pump. Several authors have obtained evidence both in favour and against the existence of such a pump. However, the most relevant studies are those on the sea hare Aplysia californica. Gerencser (1978, 1980, 1983, 1988, 1990) and Gerencser and Hong (1977) proposes that a sodium-independent chloride pump is to be found in the intestinal epithelium of Aplysia. Unlike most vertebrate intestinal preparations, when Aplysia intestinal epithelium is bathed in a sodium containing medium, the serosal surface is negative relative to the mucosal surface. This has been attributed to the net transfer of chloride ions from the mucosal to the serosal side. However, the action of serosal ouabain indicates the involvement of the Na^+/K^+-ATPase. There seem to be at least two ions transported, namely chloride and probably sodium (Gerencser 1978), with the chloride transporting mechanism being more vigorous than that of its sodium equivalent so that there is a net transport of negative charge from mucosa to

serosa. According to Gerencser most of the short circuit current could be attributed to net chloride transport from mucosa to serosa, with a small part being attributable to sodium transfer. There would seem to be a contradiction in this statement since the effects of a net chloride flux and a net sodium flux in the same direction cannot be additive since the ions are of opposite charge.

Although chloride transport seemed at first to require sodium (Gerencser 1978), in a subsequent study Gerencser (1983) demonstrated that chloride transport is at least partially independent of Na^+/K^+-ATPase activity. In a sodium free medium the recorded chloride flux from mucosa to serosa was stable for two to three hours and the electrical orientation was still serosally negative. However the magnitude of mucosa to serosa flux was significantly higher in the sodium free medium. This outcome implies that net active chloride absorption can occur whether or not sodium is present. On the addition of D-glucose to the mucosal solution, in the presence of sodium, the net chloride flux from mucosa to serosa increased. Gerencser proposed a model for this in which linked sodium and sugar entry depolarised the mucosal membrane which in turn lowered the electrochemical gradient opposing extracellular to intracellular chloride transport. Then the chloride pump would increase its activity to accommodate the increase intracellular chloride concentration. This increase in the pump's activity would result in an increase in the

serosal negative transmural potential difference. Comparable results have also been obtained using mucosal glycine. These results lead to the hypothesis of non-coupled transport of sodium and chloride across the mucosal membrane, with an active extrusion mechanism for chloride being located in the basolateral membrane. However, Gerencser did not provide any explanation of why the chloride entry would outstrip the increased glucose-induced sodium entry.

Moran and Garretson (1988) have disputed the existence of primary chloride transport in Aplysia californica, and have provided evidence that both sodium and chloride absorption are increased when sugars are present in the mucosal bathing solutions. The application of barium to the mucosal side resulted in a depolarisation of the apical membrane and an increase in the transepithelial resistance, indicating that it is blocking an apical membrane potassium conductive pathway. They concluded that under normal conditions the apical membrane of the proximal intestine is potassium, and not chloride, conductive. Furthermore, they suggested that the experiments of Gerencser were performed in the oesophagus, and not in the intestine as was reported, and that the sugar stimulated ion transport is no different to that found in vertebrates. In addition, they proposed that since the mucosal addition of D-glucose increased the short circuit current, but had no effect when sodium was substituted for by tetramethylammonium, that the stimulation must be due to

increased rheogenic sodium absorption. Finally, this study showed no evidence of D-galactose increasing the net chloride flux under short circuit current conditions. From these findings the differences from Gerencser's conclusions can be attributed to several factors, the most likely of which being that Gerencser was indeed using the oesophagus, and not the intestine as he stated, since the results obtained by Moran and Garretson (1988) using the oesophagus were very similar to those that Gerencser had obtained. This explanation is also supported by histological studies which illustrate the parallels between the intestine studies performed by Gerencser and the oesophagus work performed by Moran and Garretson. There are definite uncertainties and puzzles in Gerencser's work, for example the role of sodium in the SCC where Gerencser changes his mind in later papers but with no supporting explanation.

Hanrahan and Phillips (1983) have also proposed the existence of a chloride pump, but located in the apical membrane of locust rectum epithelium. The pump is not sodium or HCO_3^-/CO_2 dependent and is also insensitive to the normal inhibitors of chloride transport. However it is stimulated by the presence of luminal potassium. There is no evidence of electrogenic chloride transport being driven by sodium, potassium or bicarbonate gradients and it has been shown to be insensitive to furosemide and ouabain. Although no evidence of the energy source was presented the activating effects of

potassium on the kinetics of the chloride flux and on the electromotive force developed by the apical chloride pump are indicative of enzyme activation.

White (1988) used salamander small intestine and concluded that there was more than one pathway for apical chloride entry; with one third being furosemide sensitive and thus involving the $Na^+/K^+/2CL^-$ cotransporter; and one third of the chloride influx being sodium independent. This suggested the possibility of more than one carrier of chloride which resulted in net chloride transport from the apical to the basal side.

Duffey et al. (1979) using winter flounder intestine and Epstein et al. (1973) using teleost gill, provided further evidence for chloride dependent electrogenesis. However both these studies showed that the chloride dependent electrogenesis was also dependent on sodium. This raises the possibility of the existence of an Na/Cl or Na/K/2Cl cotransporter as has been found in a number of other epithelia, for example flounder intestine (Musch et al., 1982); TALH (Gregor and Schlatter, 1981); and amphibian distal tubule (Oberleithner, Guggina and Giebisch, 1983). Gradmann (1988) obtained strong evidence for chloride translocation by a primary ATPase in the marine alga Acetabularia. Gradmann found that by forcing the pump to operate in reverse, by using low external chloride concentrations, ATP was synthesised and

he claimed that this provided strong evidence for a membrane located ATPase tranlocating chloride.

However, Bonting (1988) states that there is no evidence for a primary chloride-ATPase located in the epithelial membrane of rabbit gastric mucosa. He also studied other epithelia where a claim for an anion-sensitive ATPase has been made, these were pancreas, kidney, gill and erythrocytes and also found no evidence to support the existence of such an ATPase. There is a high risk of mitochondrial contamination in F_1-ATPase activity when trying to separate epithelial homogenates and Bonting concluded that the length of time taken to separate them could influence the eventual outcome. He found that after sixteen hours there were three peaks: a mitochondrial peak; a peak with the highest anion ATPase activity; and a membrane peak with high sodium/potassium ATPase activity. However after eight hours there were only two peaks, with no membrane peak being evident. This could explain why some investigations suggested a plasma membrane location for the anion ATPase. In addition, electronmicroscopic evidence indicates that the middle peak consists mainly of mitochondrial fragments.

A number of other transport processes are also fairly well established within invertebrate epithelia. The sodium/potassium ATPase seems to be as widespread in invertebrates as it is in vertebrates. Hanozet et al. (1992)

have demonstrated amino acid transport in lepidopteran midgut, using a potassium driven cotransporter that could also use sodium, albeit at a reduced efficiency. Also among certain insect epithelia and other tissues there appears to be a unique potassium-ATPase. Harvey et al. (1983) proposed a model that had the potassium pump located in the apical membrane of goblet cells. Indications of this apical location include fluxes against an electrochemical gradient; close association of mitchondria with the apical membrane and portasomes on that membrane. Once again, the membrane does not seem to be of mitochondrial origin, since using density gradient centrifugation it is present in plasma membrane enriched fractions (Wieczorek, 1982). Nor is the pump sensitive to ouabain inhibition and thus it is not just a masked sodium/potassium ATPase. This pump seems to be present in several potassium transporting epithelia, including midgut, salivary glands, malpighian tubes, sensory sensilla and rectum. Wieczorek states that the pump is electrogenic (fly labellum) and that it may be important for the generation of receptor currents.

Section 3 - METHODS AND MATERIALS.

Experimental animals.

Helix aspersa were mostly obtained from a commercial supply company. They were kept in a large tank and at room temperature prior to experimentation. Initially the snails were in a state of aestivation, but they could be aroused by removing the epiphragm and moistening the exposed snail beneath. Once the snail was active it was placed in a smaller container which held either shallow water only or water and food. The snails were active for at least 24 hours before experimentation.

It has been shown (Burton, 1966) that complete rehydration takes place within two hours in Otala lactea and Helix aperta with a return to standard and constant ionic concentrations in the haemolymph. Thus 24 hours would be more than sufficient to allow complete rehydration in H. aspersa.

"Fed" snails were allowed to eat at will from a store of porridge oats for at least 24 hours before the experiment. Food could still be present in the crop up to 72 hours after feeding had been stopped. "Starved" snails however were given only water, again at least 24 hours before use.

Isolation of the crop.

The crop is best removed through the floor of the lung. To expose the lung the shell is removed by blunt dissection and this in turn exposes the respiratory mantle. This is then cut away to reveal the floor of the lung. A small incision is then made and the pressure within the haemocoel forces the crop through the hole. The crop can then be cut out.

The remainder of the dissection was performed in standard physiological saline with the aid of a dissecting microscope. The salivary glands, which loosely cover the crop, were removed using forceps and scissors. The crop was then cut along its length to produce a flat sheet of tissue. This was then washed using standard saline to remove any remaining food or crop juice. Great care had to be taken to ensure that the basal side was touched as little as possible by the crop's contents.

The crop was then mounted in the modified Ussing chamber, complete with both thin layers of muscle. Both side of the preparation were bathed by the pre test experimental bathing solutions and all electrical connections were made. After the crop was mounted, it was left for a priod of one hour to allow the tissue to stabilise. This resulted in the potential difference and the short circuit current being stable before any experimentation was started, (see below, Results section

4; Initial setup and the electrical characteristics of the epithelium).

Apparatus.

The crop was mounted between the halves of a modified Ussing chamber. This consisted of two identical perspex halves each of volume 1.8 mls and with a circular connecting "window" between them of 0.2 cm^2 where the crop was placed (Fig 3.1). The chambers were held together by a strong clamp.

The crop was bathed on both sides by snail saline. The solution on the apical side was stirred by a plastic paddle powered by a small electric motor. Stirring was continuous throughout the whole experiment to keep the bulk solution moving and to reduce the unstirred layer next to the apical surface.

Voltage and current were measured using two pairs of silver/silver chloride electrodes, the voltage pair being matched. The pair used to measure the voltage across the crop (p.d.) were connected to the bathing solutions using saline bridges. These were plastic catheters containing standard bathing solution. The more commonly used 3M KCL was not used as it was feared that leakage into the normal bathing solution might be significant and influence the characteristics of the crop. The recording electrodes were connected to a digital

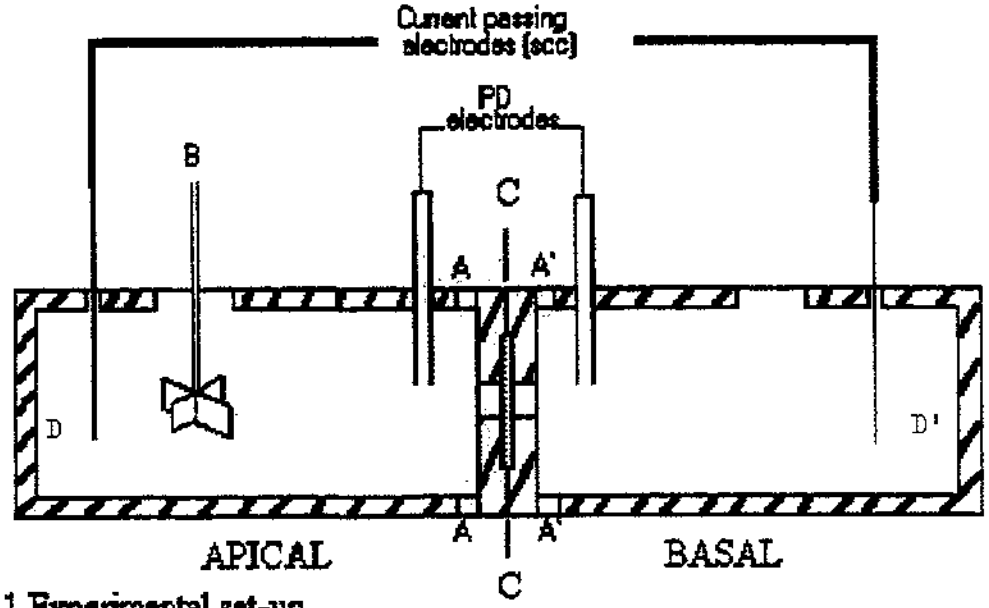


FIG 3.1 Experimental set-up.

KEY

AA' -Clamp holding two halves
B -Apical stirring
C -Epithelium held at C

voltmeter (Morrison and Sinclair, 1979). This could also be used to cancel any electrode potential using the variable D.C. offset before the experiment and also on occasion after the bathing solutions were changed. Another pair of silver/silver chloride wires was used to pass current through the crop and was connected to a second digital meter. This set-up is essentially the same as that used by Ussing and Zerahn (1951) in their experiments on frog skin.

Bathing solutions.

The standard solution used to bathe the crop was a snail physiological saline solution with an ionic composition within the physiological range for the haemolymph. The concentrations are correct for hydrated, starved snails, but after feeding the concentrations of calcium and potassium in the haemolymph rise. The same standard solutions were nevertheless used for both fed and starved snails. The compositions of the various solutions used in the experiments can be seen in Table 3.1. The solutions were made using glass distilled water and were buffered by 5mM HEPES (N-2-hydroxyethypiperazine-N-2-ethanesulphonic acid) and the pH adjusted to 7.8.

The sodium-free solution was prepared using non-sodium salts e.g. HEPES and NMDG. Inhibitors such as ouabain, thiocyanate and barium chloride were added by Pasteur pipette at their

SOLUTION	CaCl2	NaCl	MgSO4	KCl	Calcium Gluconate	Choline Chloride	Mannitol	Hepes Buffer	K2SO4	Na2SO4	CaSO4	NMDG
STANDARD	6	70	3	3	-	—	—	5	-	—	-	—
Sodium Free	6	—	3	3	-	70	—	5	-	—	-	—
Chloride Free	-	—	3	-	6	—	18	5	1.5	35	-	—
Potassium Free	6	70	3	-	-	—	—	5	-	—	-	—
Calcium Free	-	70	3	3	-	—	10	5	-	—	-	—
Magnesium Free	6	70	-	3	-	—	6	5	-	—	-	—
High Potassium	6	70	3	40	-	—	—	5	-	—	-	—
Na and Cl Free	-	—	3	-	6	—	140	40	1.5	—	-	—
K and CL Free	-	—	3	-	6	—	3	5	-	35	-	—
Na, Cl and Mg Free	-	—	-	-	6	—	70	5	1.5	—	-	—
pH Experiments												
Solution A	-	—	-	-	-	—	140	—	1.5	—	6	—
(500uls of A)+ Soln B	-	—	-	-	-	—	—	—	-	—	-	40
(500uls of A)+ Soln C	-	—	-	-	-	—	—	40	-	—	-	—
Solutions B and C mixed to give desired experimental pH												

TABLE 3.1 Composition in mM of experimental solutions

final concentrations, to the chamber, having been already dissolved in the bathing solutions. For introduction of the test solution, the whole solution bathing the crop was changed, the chamber having first been washed using some the test solution. In the case of piretanide, a number of options were tried to overcome its low solubility. The use of 3M methyl alcohol as a solvent was found to be inappropriate as the alcohol itself had marked effects on the SCC. Another option tried was changing the pH of bathing media to 9.2. Again the required amount of piretanide did dissolve, but this pH would be unnaturally high. The adopted option was simply to dissolve as much of the required weight in normal saline, to give a concentration as close to 1mM as possible. If the test solution was agitated and then left to stand for a while most of the piretanide would dissolve. Only a few crystal remained and so the effective concentration was almost the desired 1mM.

Electrical parameters.

The equipment used to measure the transepithelial potential difference and also to pass current through the crop has been described under Apparatus. The potential difference (p.d.) was measured in millivolts (mV). The current or short circuit current (SCC) was measured in microamperes per square centimetre of tissue ($\mu A/cm^2$). The SCC was the current flowing in the external circuit between electrodes D and D'

(Fig 3.1), required to reduce the p.d. across the epithelium to zero. The transepithelial resistance could be determined from the current/voltage ratio since the p.d. and the SCC were shown to be linearly related. To refine this measurement, and also when either the p.d. or the SCC was close to zero, other readings were taken under closed circuit conditions. From these the resistance could be calculated. The true tissue resistance was determined by subtracting the fluid resistance from the total measured resistance. Open circuit conditions were the normal state except when short circuit current and resistance were being measured. The electrical parameters were measured every two minutes.

Presentation of data and statistical methods.

Since the initial polarity of the SCC could be either positive or negative the description of the data could have proven troublesome. It was decided at the outset to analyse the magnitude of the change between the "pre-mean" and the "post-mean". The pre-mean was the mean of the measurements taken before the test solution was added at time zero and the post-mean the mean of the values after the solutions were changed. Since in the majority of cases the direction of change was the same, it did not matter if the relative starting points, at time zero, were different. In this way any significant difference between the pre (standard) solution

and the post (test) solution could be easily shown. The actual analysis was performed using a paired one tailed Student's t-test, the results being presented as the mean plus and minus the standard error (S.E.).

Throughout this thesis the polarity of the PD is always quoted with respect to the basal surface, i.e. a negative PD means that the basal side is negative with respect to the apical side. The polarity of the SCC is taken as that of the PD before application of the current. The direction of change in the SCC is quoted as being either in a negative direction or in a positive one regardless of the initial polarity of the SCC.

Correlations

The results were examined to see whether there was any relationship, for any one set of experiments, between the magnitude of each experimental change in the SCC and the initial value. (In illustration of the rationale, consider a hypothetical preparation in which chloride transport is an unusually dominant property, giving a very negative SCC; removal of chloride should make the SCC less negative to an extent that is much greater than average.) Some of the high correlation coefficients are due to a single outlying data point and are therefore of uncertain significance. The probability values attributed to the correlation coefficients

are based on the assumption of normal distributions. More data would be useful, therefore, but this analysis was carried out after the experimental work had been completed. The Figures are useful in another way, however, in that they illustrate, for each kind of experiment, the variability in change that is otherwise summarised as a standard error.

Section 4 :- RESULTS

The direction of change

Changes in the short circuit current upon the addition or change of solutions could be either positive or negative regardless of the initial polarity. Under most test conditions, once the test solution was added, the direction of change was the same in all the experiments although the magnitude of the changes varied. Therefore the results are quoted as the magnitude as well as the direction of change. In a number of experimental groups the initial polarity and the starting values had a significant effect on the magnitude of the change (see below).

The structure of the epithelium.

The crop itself is a large fluid filled sac usually yellow or brown in colour (Fig 4.1). It's inner surface is thrown up into a series of large folds, but it can be distinguished from the oesophagus by its larger size. The epithelium lining the crop is derived from the lining of the oesophagus and is composed mainly of columnar cells containing many granules (Figs 4.2, 4.3 & 4.4). Upon staining it can be seen that these cells are interspersed with goblet type cells (Fig 4.3). The epithelium also contains an intense P.A.S. staining brush

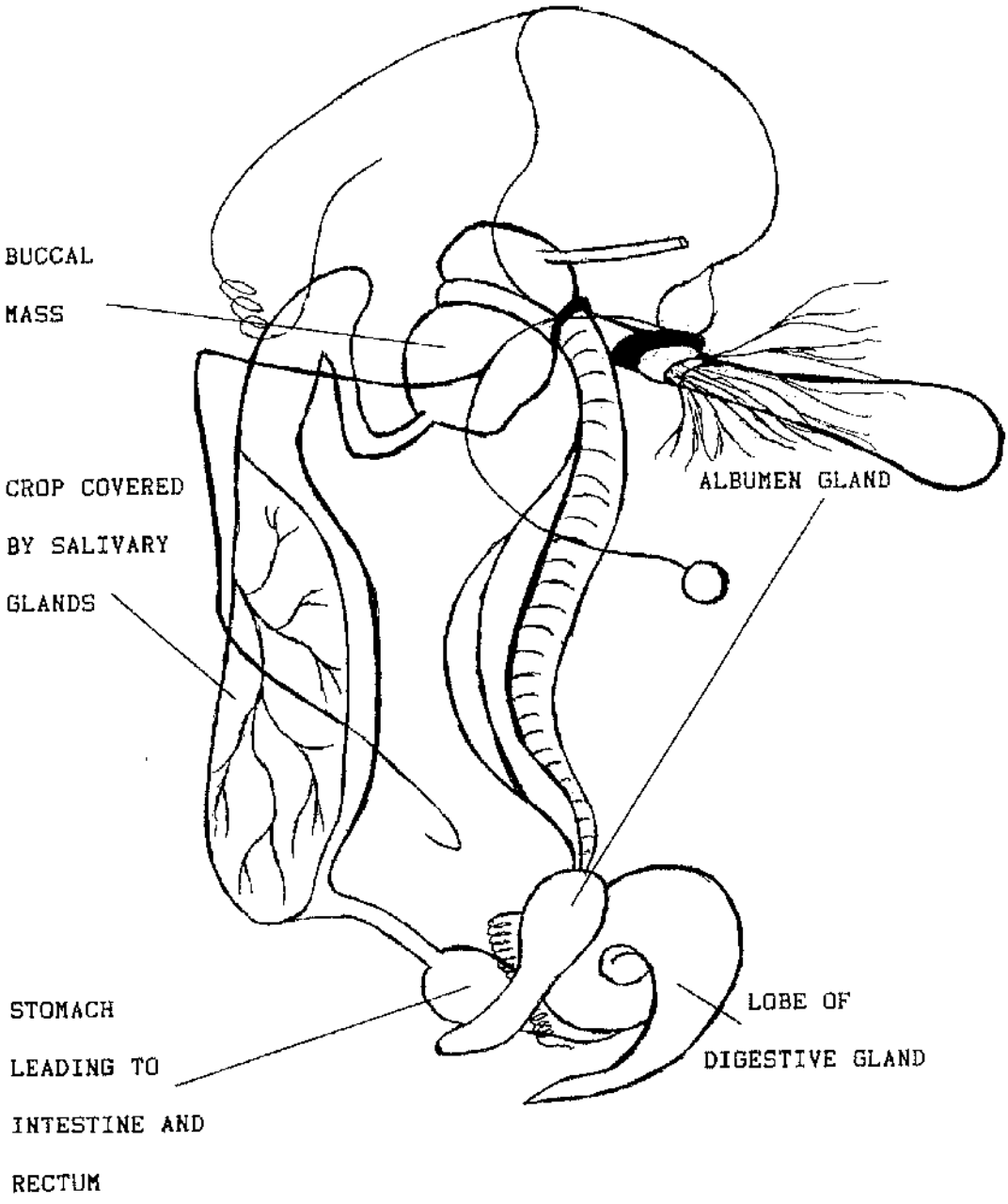


FIG 4.1 General anatomy of Helix digestive system.

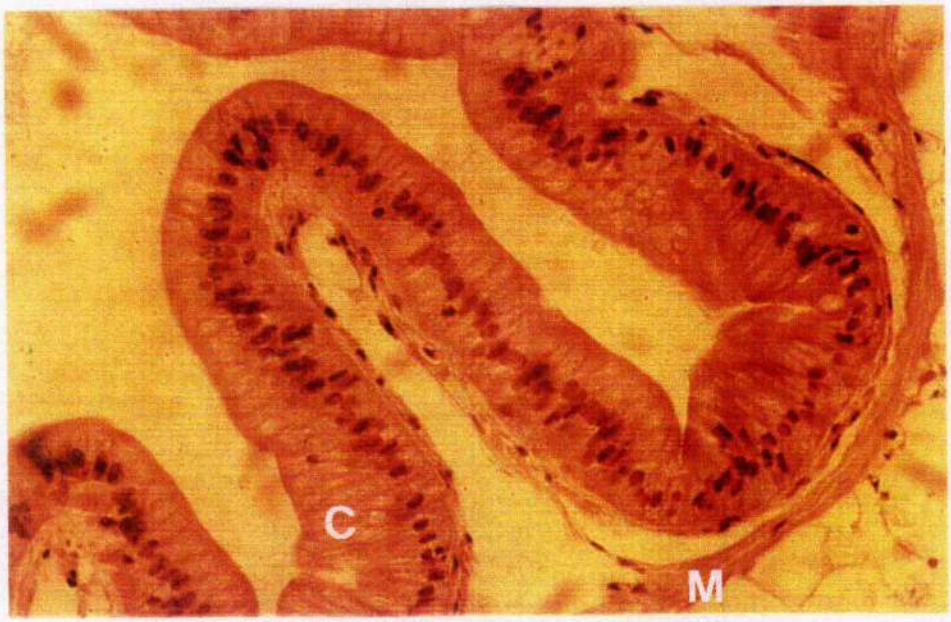


FIG 4.2 Photomicrograph of a semithin section of the crop wall of Helix aspersa. Stained with H&E. M= supporting layers, C= columnar epithelial cells. Magnification X 1000.

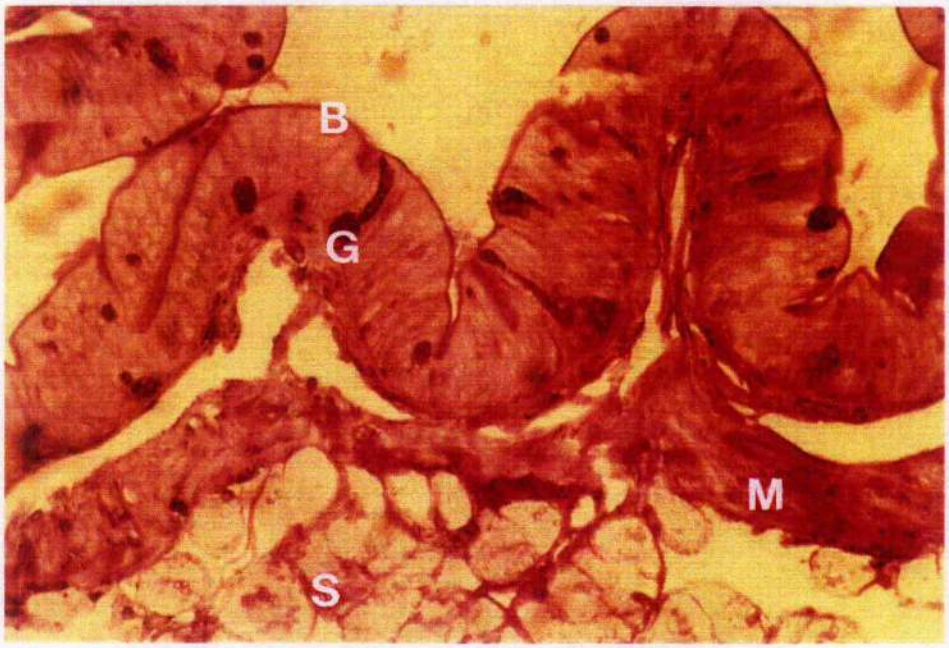


FIG 4.3 Photomicrograph of a semithin section of the crop wall of Helix aspersa. Stained with PAS. B= brush border, M= supporting layers, S= salivary gland tissue, G= mucus/goblet type cell. Magnification X 1000.

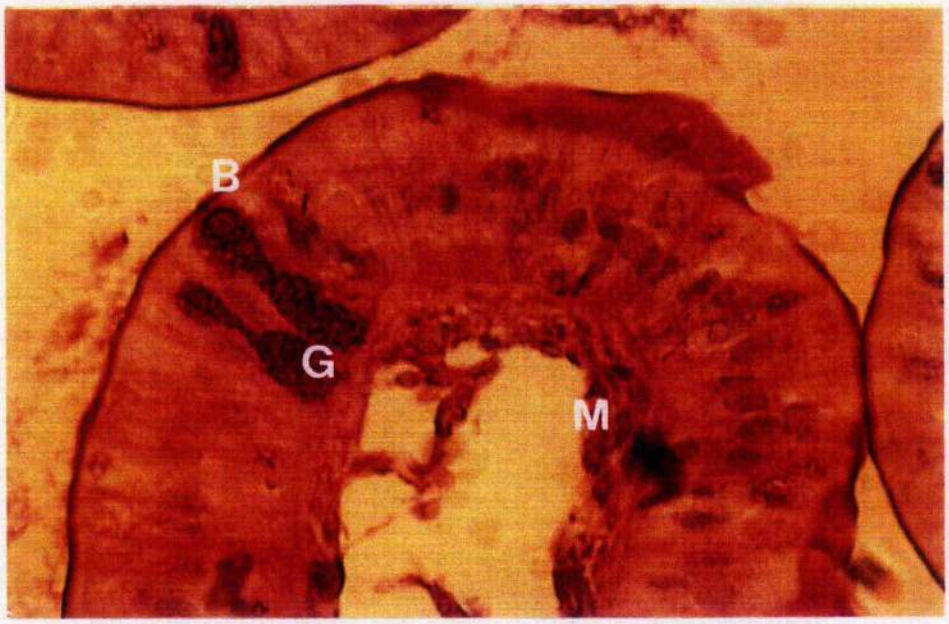


FIG 4.4 Photomicrograph of a semithin section of the crop wall of Helix aspersa. Stained with PAS. B= brush border, M= supporting layer, G= mucus/goblet type cells. Magnification X 1200.

border and a lighter basal membrane (Fig 4.4). Underlying the epithelial cells are two thin layers of muscle.

Initial setup and the electrical characteristics of the epithelium

It was found that apical stirring was very important for the maintenance of a stable SCC during experimentation. Regardless of the initial polarity of the SCC, when stirring was stopped the SCC moved towards zero, sometimes reversing. (So in this rare respect the direction of change was different for initially positive and initially negative SCC.) In snails where the SCC was initially positive the mean change after cessation of stirring was -15.7 ($\pm$6.4)$\mu A/cm^2$, n=6, from a mean of 32.8 to 17.2 $\mu A/cm^2$. In snails where it was negative the change was 18.0 ($\pm$6.9)$\mu A/cm^2$, n=5, from a mean of -27.5 to -9.5. Both these changes were significant (Fig 4.5). The reason for the change is presumably an increase in the size of the "unstirred layer" immediately next to and in the brush border.

In four experiments the solutions were stirred on both sides of the epithelium. When serosal stirring was stopped there was no significant change in the SCC, the mean change being only -0.1$\mu A/cm^2$ (Fig 4.6), from 1.7 to 1.6 $\mu A/cm^2$. There was no significant change in the transepithelial resistance when basal stirring was stopped ($p>0.1$). Once the tissue was

The effect of stopping apical stirring on the SCC. Apical stirring was absent in the period marked by the bar. Each point represents the mean value, (plus or minus the SE), of 6 experiments where the SCC was initially positive and 5 experiments where it was initially negative.

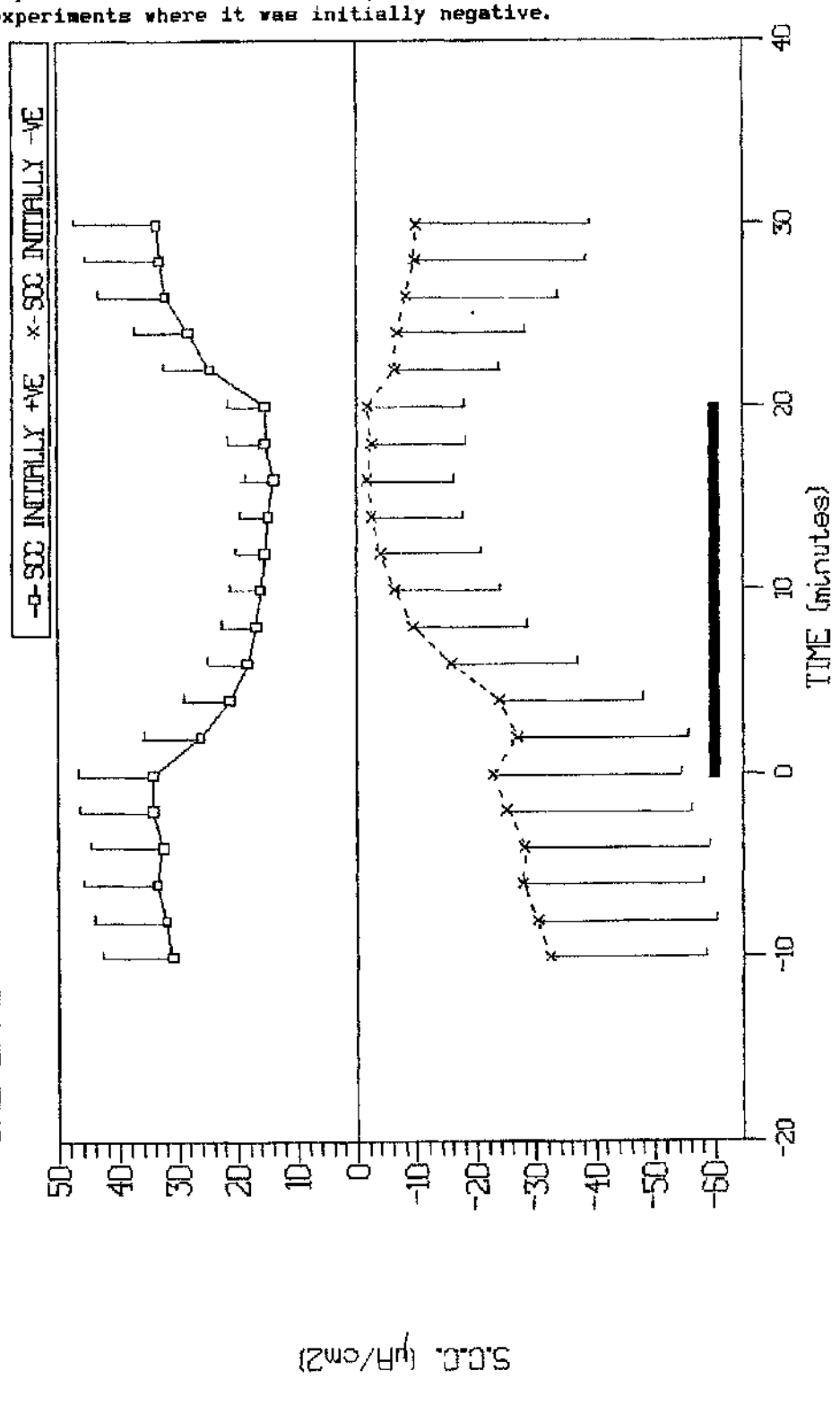


FIG 4.5

THE EFFECTS OF APICAL STIRRING

The effect of stopping basal stirring on the SCC. Basal stirring was absent in the period marked by the bar. Each point represents the mean value of 4 experiments (± the SE).

FIG 4.6

BASAL STIRRING

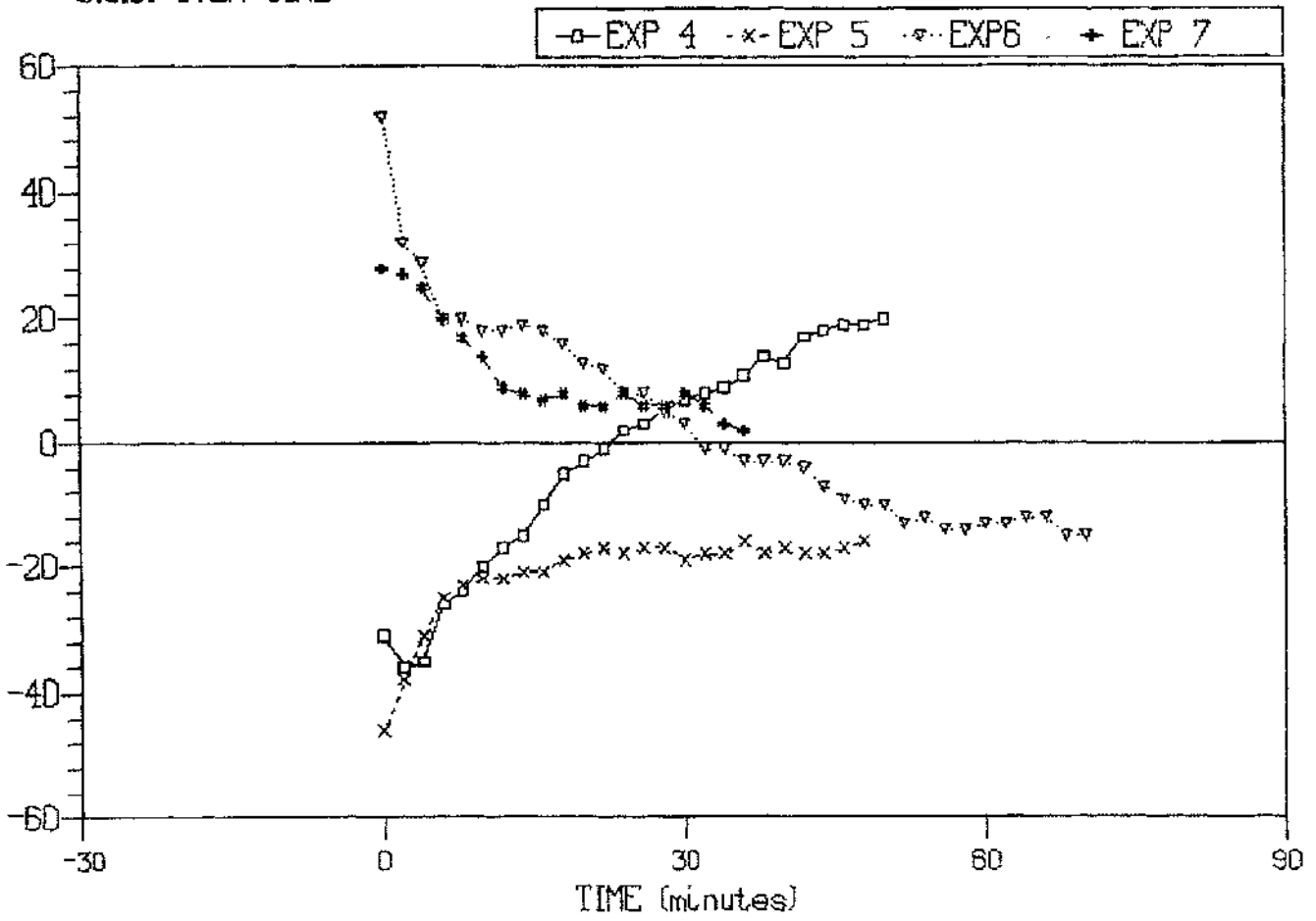
mounted in the modified Ussing chamber the SCC seemed to exhibit two phases. First it moved rapidly towards zero although it did not reach it. This phase lasted approximately twelve minutes after the time the epithelium was set up. The second phase was a more gradual change over the next thirty five minutes (Fig 4.7, n=4). In two of the experiments the polarity of the SCC reversed during this second phase, in the others it did not. The transepithelial resistance remained the same throughout both phases. Under standard conditions the resting SCC, i.e. after phase two, could be either positive or negative.

The state of feeding prior to experimentation had a significant effect on polarity. In fed snails, under standard control conditions, the SCC was positive more often than in starved snails (Table 4.1). For fed snails the mean SCC is equivalent to net transport rates of 6.3×10^{-3} μEquiv/cm^2/min and -6.2×10^{-3} μEquiv/cm^2/min respectively for snails with positive and negative polarities. For starved snails the corresponding means are 7.6×10^{-3} μEquiv/cm^2/min and -8.3×10^{-3} μEquiv/cm^2/min respectively.

The current voltage relationship was found to be linear over a wide range (Fig 4.8). This means that the resistance across the epithelium could be found from the open-circuit potential difference and the SCC, provided these were large enough. The mean transepithelial resistance was 67.9 ohm cm^2 (S.E. was

FIG 4.7

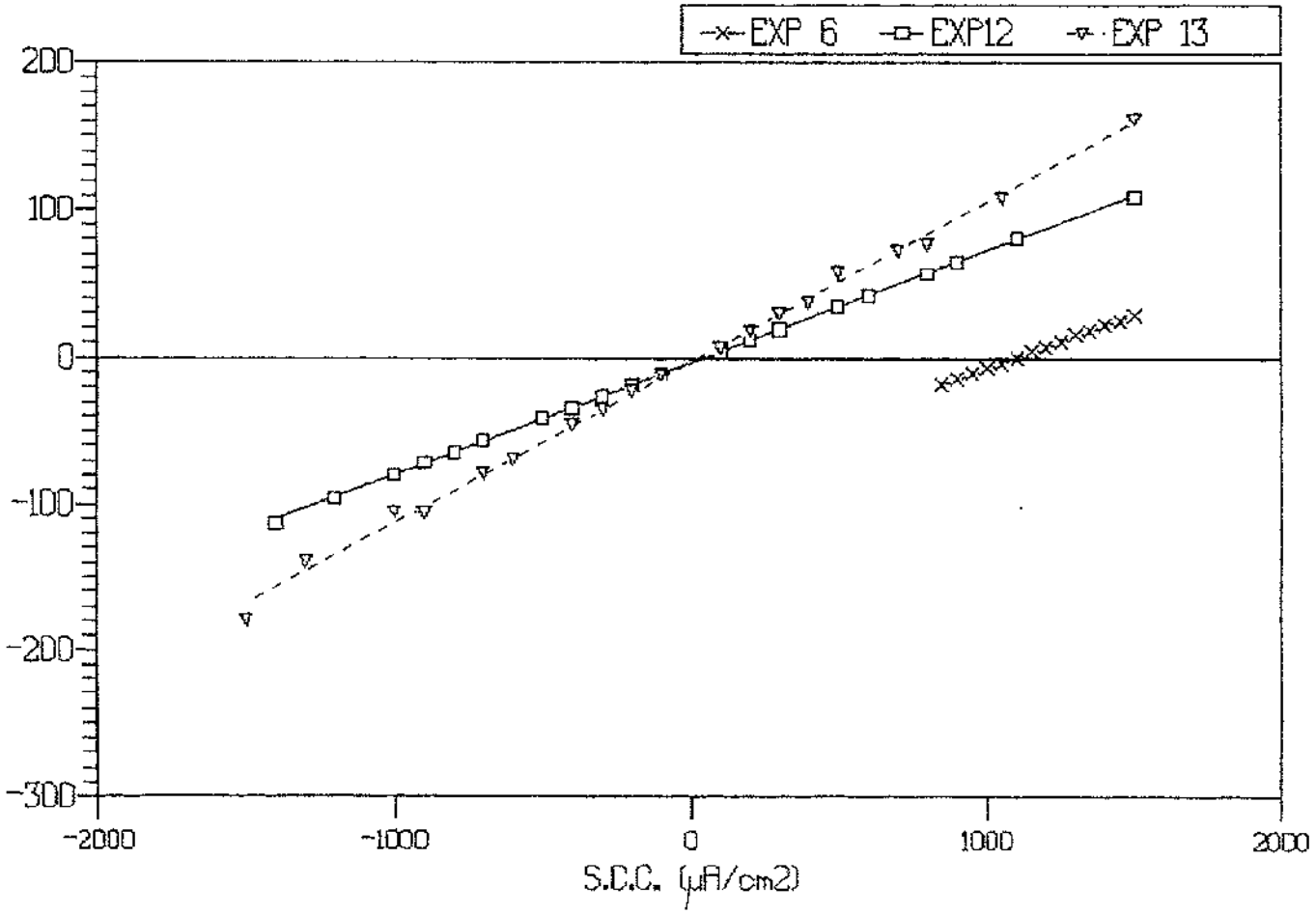
CHANGES IN THE RESTING S.C.C. OVER TIME



Standard Conditions		(± SE)
Mean SCC	1.03μA/cm²	(± 1.0)
Mean PD	-0.03mV	(± 0.03)
Mean Resistance	67.9Ωcm²	(± 3.1)
Initially +ve SCC Mean Resistance N=89	60.1Ωcm²	(± 3.1)
Initially -ve SCC Mean Resistance N=67	72.2Ωcm²	(± 4.0)
Fed Snails		
Mean positive SCC	10.1μA/cm²	(± 1.6)
% positive	64.5	
Mean negative SCC	-9.9μA/cm²	(± 2.3)
% negative	35.5	
Total N	93	
Starved Snails		
Mean positive SCC	12.3μA/cm²	(± 1.8)
% positive	45.7	
Mean negative SCC	-13.4μA/cm²	(± 2.1)
% negative	54.3	
Total N	70	

Table 4.1-SCC, PD and Resistance
normal control conditions.

FIG 4.8
CURRENT/VOLTAGE
RELATIONSHIP



3.1 ohm cm^2). In the two groups that were initially positive and initially negative the average resistances were not significantly different ($p>0.05$).

INHIBITORS

Ouabain

In order to test whether ouabain had the effect of abolishing the SCC, it was added at a concentration of 10mM to the basal side of the epithelium. However, in all of eight experiments this made the SCC more negative (Fig 4.9). The mean change was -9.3 ($\pm$4.1) $\mu A/cm^2$ ($n=8$, $p>0.05$). There was a correlation between the initial SCC and the change, the change being much greater when the initial polarity of the SCC was negative ($r=-0.86$, $n=8$, Fig 4.9A). The maximal change was obtained between two and six minutes and once this level was achieved there was no further change in the SCC over the remainder of the test period. There was no recovery evident in any of the experiments once the bathing solutions were returned to standard conditions. The transepithelial resistance increased significantly when ouabain was present, the mean change being 41.2 ($\pm$18.6) ohm cm^2 ($n=5$, $p<0.05$).

The effect of 10mM ouabain (basally only) on the SCC. Ouabain was present in the period marked by the bar. Each point represents the mean value (± SE) of 8 experiments.

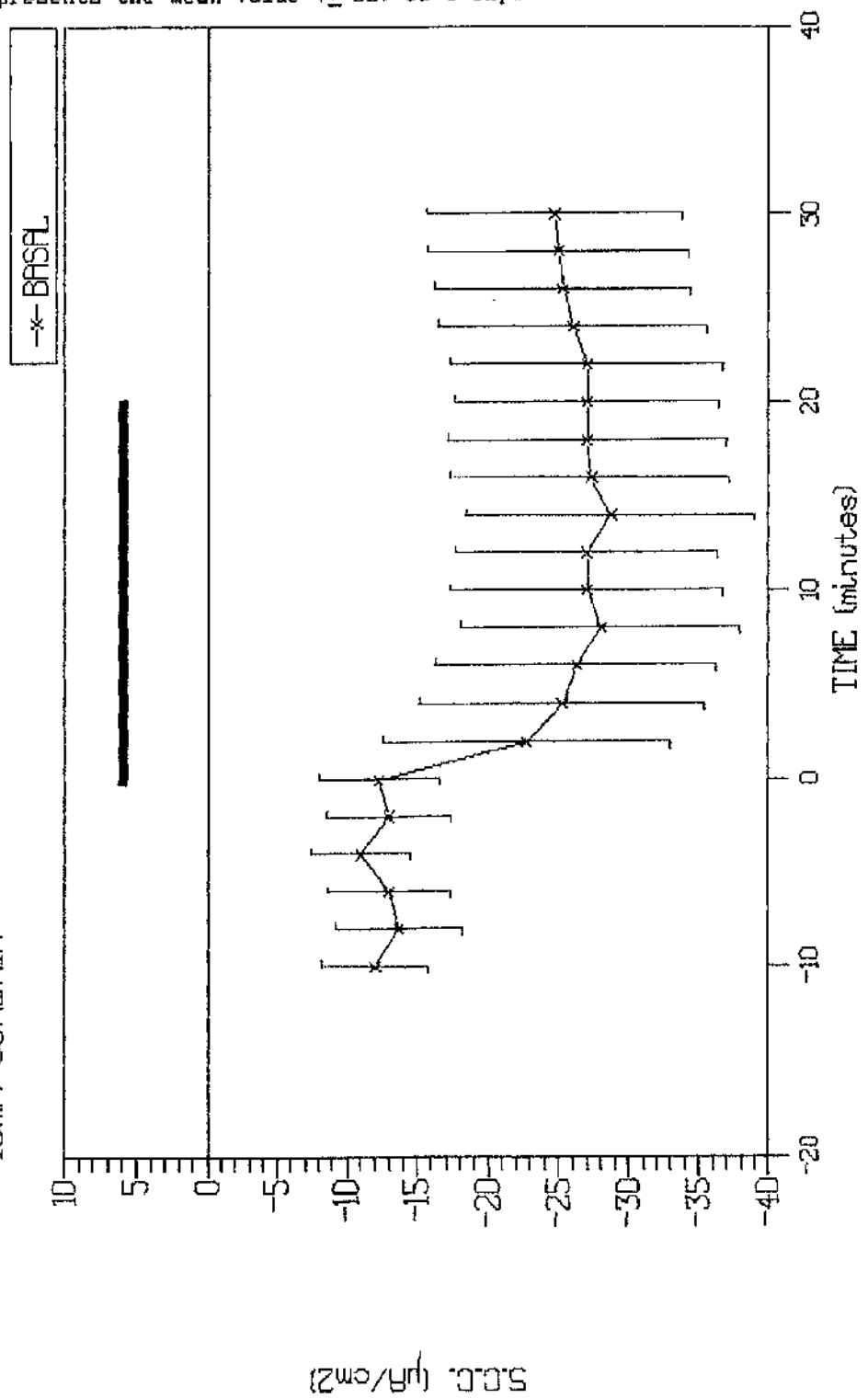
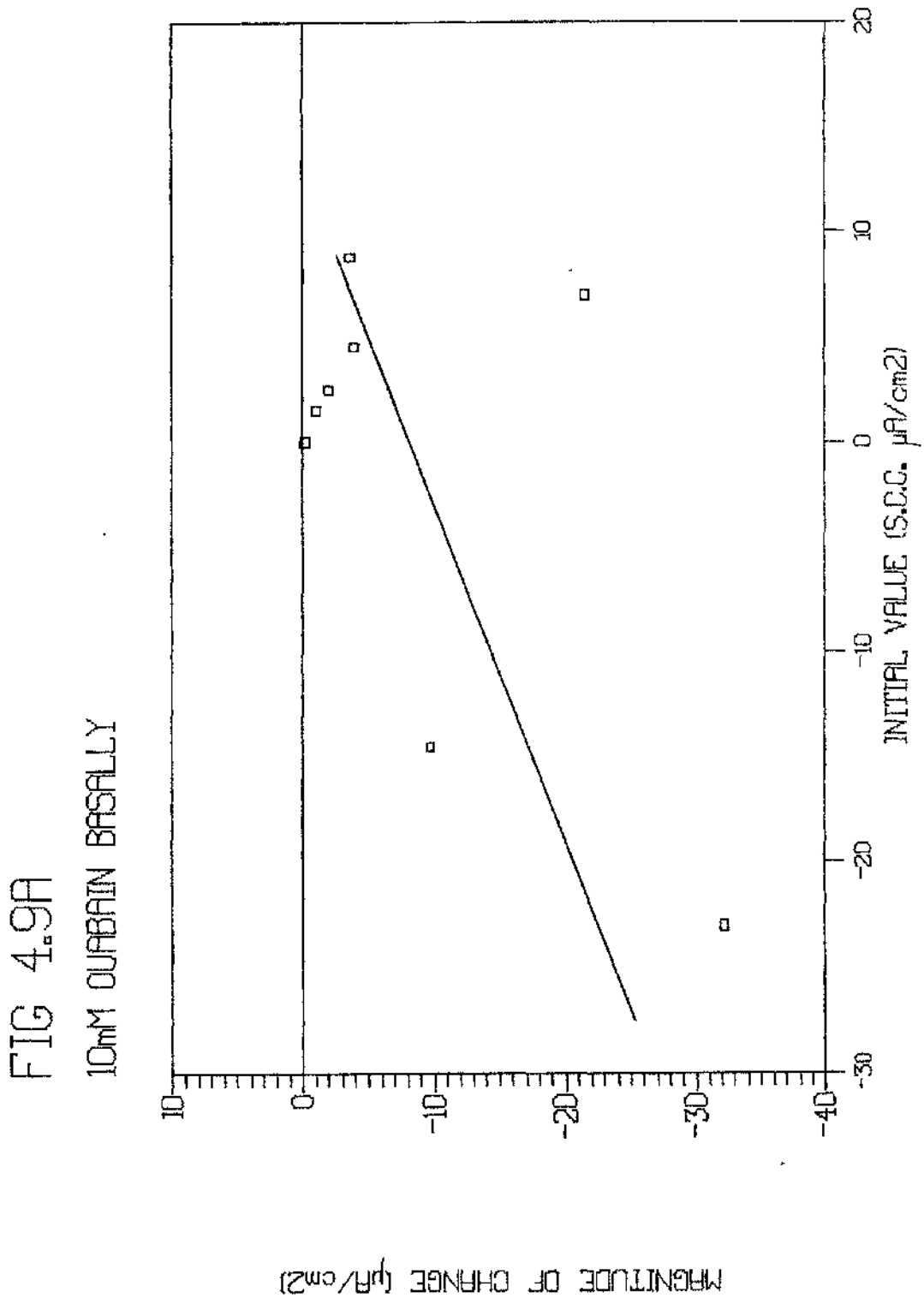


FIG 4.9
10mM OUABAIN

The correlation between the initial SCC and the magnitude of change in the SCC with 10mM ouabain present on the basal side only. Each point represents one experiment. The line is a best fit linear regression line.



Piretanide

To test for the presence of coupled sodium/potassium/chloride co-transport across the crop wall epithelium, piretanide was added at a concentration of approximately 1mM to either the apical side only or the basal side only. Because of its low solubility, a few crystals remained undissolved. As an alternative method of addition, piretanide was dissolved fully in methyl alcohol, but methyl alcohol was found to have a possible effect on the SCC. Results using alcohol are not included here therefore. Piretanide was added apically only and basally only under otherwise normal bathing conditions. When added apically there was a significant change in the SCC (Fig 4.10), the SCC becoming more negative. The mean change was -4.1 ($\pm$2.2) $\mu A/cm^2$ (n=8, $p<0.05$). In seven of the experiments the maximal change was observed within four minutes; the other was within ten. Thereafter there was no further change. When piretanide was present, the mean decrease in resistance of -18.3 ($\pm$15.1) ohm cm^2 (n=6, $p>0.1$) was not significant.

The addition of piretanide just basally resulted in no significant change in the SCC (Fig 4.10). The mean change was only 0.1 ($\pm$1.9) $\mu A/cm^2$ (n=7, $p>0.1$). On return of standard bathing solution the large positive change in the mean SCC, seen in Fig 4.24 was due to only one experiment. The remaining six showed no change such on removal of piretanide.

The effect of 1mM piretanide on the SCC. Piretanide was present in the period marked by the bar. Each point represents the mean value (+ or - SE), with n=8 for apical addition only and n=7 for basal addition only.

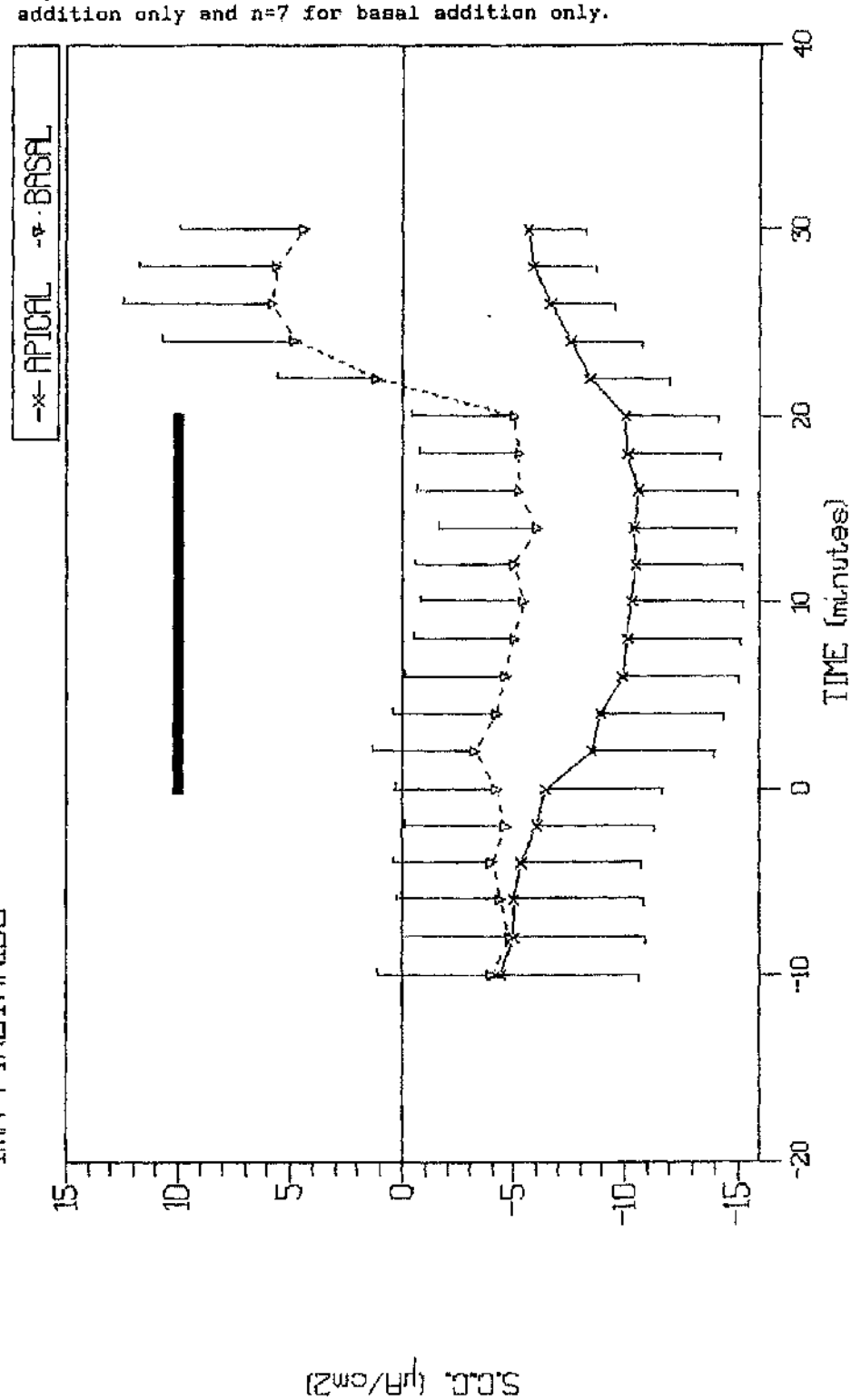


FIG 4.10
1mM PIRETANIDE

There was no significant change in the resistance during the experiments, the mean change being 1.1 (±5.1) ohm cm^2 (n=7, p>0.1).

Sodium thiocyanate.

In order to test whether thiocyanate had the effect of stopping primary chloride transport across the crop epithelium, it was added either apically only or basally only at a concentration of 10mM. The addition of sodium thiocyanate to the apical side only did not significantly affect the mean SCC (Fig 4.11), the mean change being -1.2 (±1.1) $\mu A/cm^2$ (n=8, p>0.1). However, although in half of the experiments there was no real change evident, in the others there was a more pronounced change that was still not significantly different from the control values. The changes were maximal at two minutes. These two groups corresponded to four fed and four starved snails respectively. The fed snails showed a mean negative change in the SCC of only -0.1 (±0.1) $\mu A/cm^2$ (n=4, p>0.1); with the starved snails the mean negative change in the SCC was -2.3 (±2.2) $\mu A/cm^2$ (n=4, p>0.1).

The transepithelial resistance did increase a little, but significantly, during the test period. The mean change was 6.0 (±2.0) ohm cm^2 (n=7, p<0.05).

The effect of 10mM sodium thiocyanate on the SCC. Sodium thiocyanate was present in the period marked by the bar. Each point represents the mean value (+ or - SE), with n=8 for apical addition only and n=7 for basal addition only.

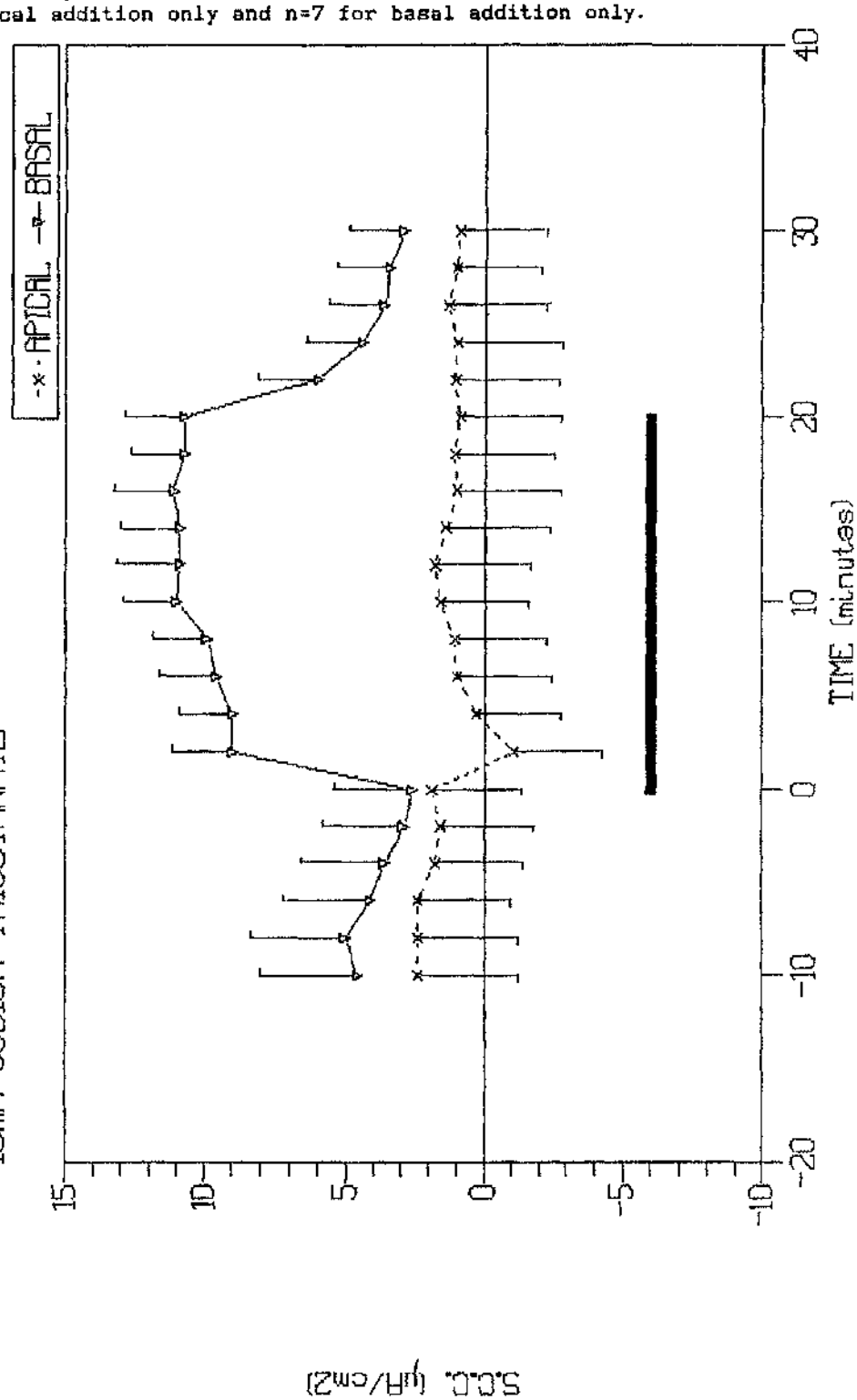


FIG 4.11

10mM SODIUM THIOCYANATE

When sodium thiocyanate was added to the solution bathing on the basal side only, there was a significant positive change in the SCC (Fig 4.11). The mean change was 6.5 ($\pm$1.8) $\mu A/cm^2$ (n=7, $p<0.01$). There was a seemingly significant correlation between the magnitude of the initial SCC and the size of the change recorded, r=-0.786 (n=7, Fig 4.11A), but this was mainly due to one result. The maximum increase was obtained within two minutes in six of the experiments, in the other it was at four minutes. There was no further change, in all but one case, after this time. This one experiment showed a slight gradual increase over the remainder of the test period. When the thiocyanate was removed from the bathing solution there was a full recovery, in all but two cases, which showed none. As with apical addition, basal addition also resulted in a significant increase in the resistance. The mean increase was 12.0 ($\pm$4.7) ohm cm^2 (n=5, $p<0.05$), from a pre-mean of 88.0 to a post-mean of 100.0 ohm cm^2.

Barium chloride.

Barium, a known inhibitor of membrane potassium conductive channels, was added at a concentration of 5mM to the apical side only and to the basal side only, in order to test for potassium conductive channels in Helix crop epithelium. It was also added apically only but under potassium free conditions as standard.

The correlation between the initial SCC and the magnitude of change in the SCC with 10mM sodium thiocyanate present on the basal side only. Each point represents one experiment. The line is a best fit linear regression line.

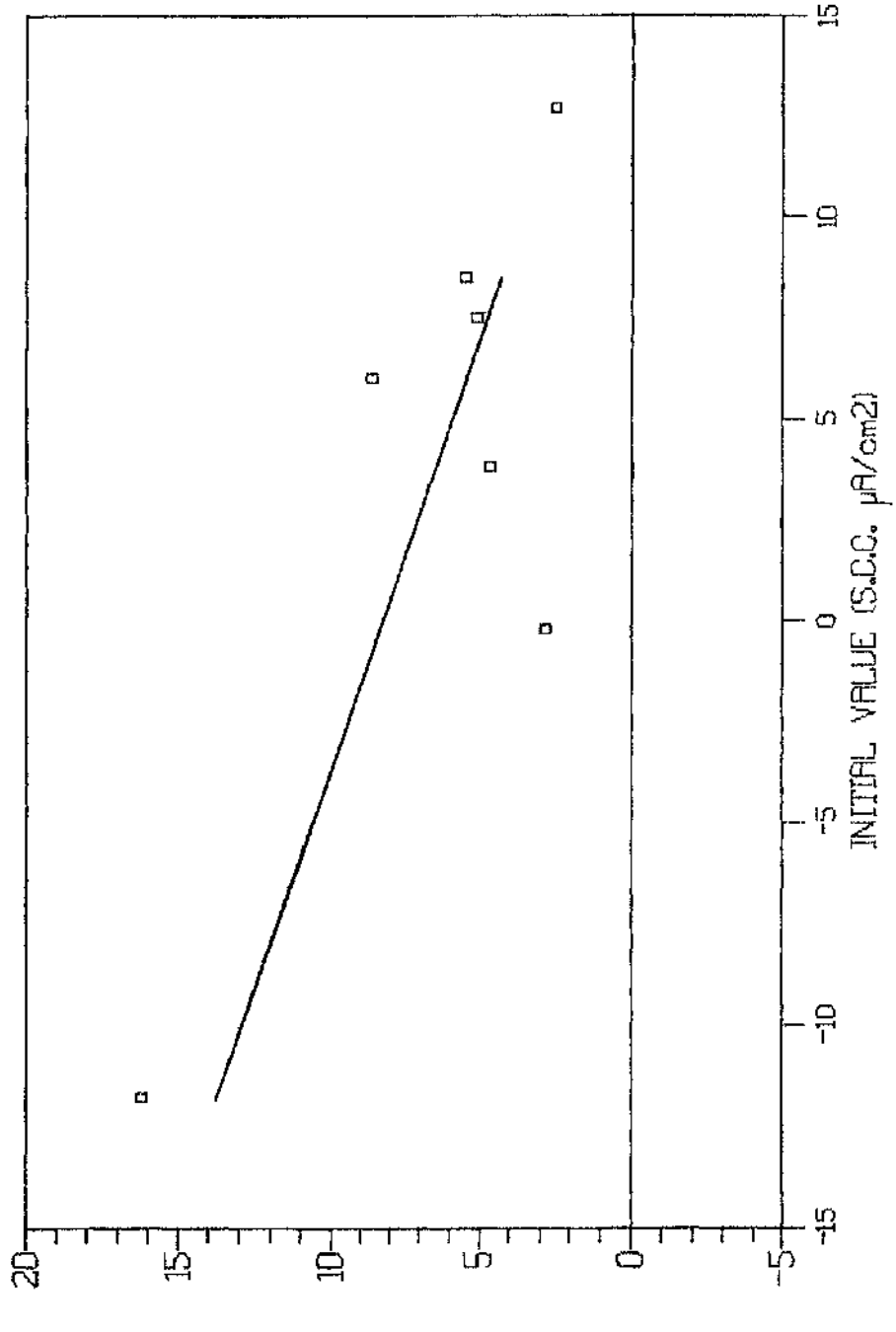


FIG 4.11A

10mM NaSCN BASALLY

Apical addition, under normal bathing conditions, resulted in a significant positive change in the SCC (Fig 4.12). The mean change was 16.8 ($\pm$6.0) $\mu A/cm^2$ (n=5, $p<0.05$). The largest changes were seen in experiments where the control SCC was initially negative. However the correlation between the initial SCC and the magnitude of change was not significant ($r=-0.512$, n=5). The maximum change was observed between two and ten minutes, with the majority near two minutes. Only one experiment showed any change after this time, a continuing gradual decrease in the SCC. In most cases there was no recovery in the SCC when barium was removed from the bathing solution. However two experiments showed a full recovery, one to levels greater than those at rest. There was no significant change in the resistance across the epithelium when barium was present. The mean change was a fall of -8.8 ($\pm$4.3) ohm cm^2 (n=4, $p>0.05$).

Basal addition also resulted in a significant change in the SCC, although in the opposite direction (Fig 4.12). The mean change was -13.4 ($\pm$4.0) $\mu A/cm^2$ (n=5, $p<0.05$). The initial polarity of the SCC had no effect on the size of the change. The time to maximal response was again quite varied, from two to eight minutes with the longer times predominating. There was no further change, in most cases, once this level was attained. In one experiment there was a gradual positive change back towards the resting pre test values, although these were not achieved. In four of the experiments there was

The effect of 10mM barium chloride on the SCC. Barium chloride was present in the period marked by the bar. Each point represents the mean value (+ or - SE), with n=5 for apical addition only and n=5 for basal addition only.

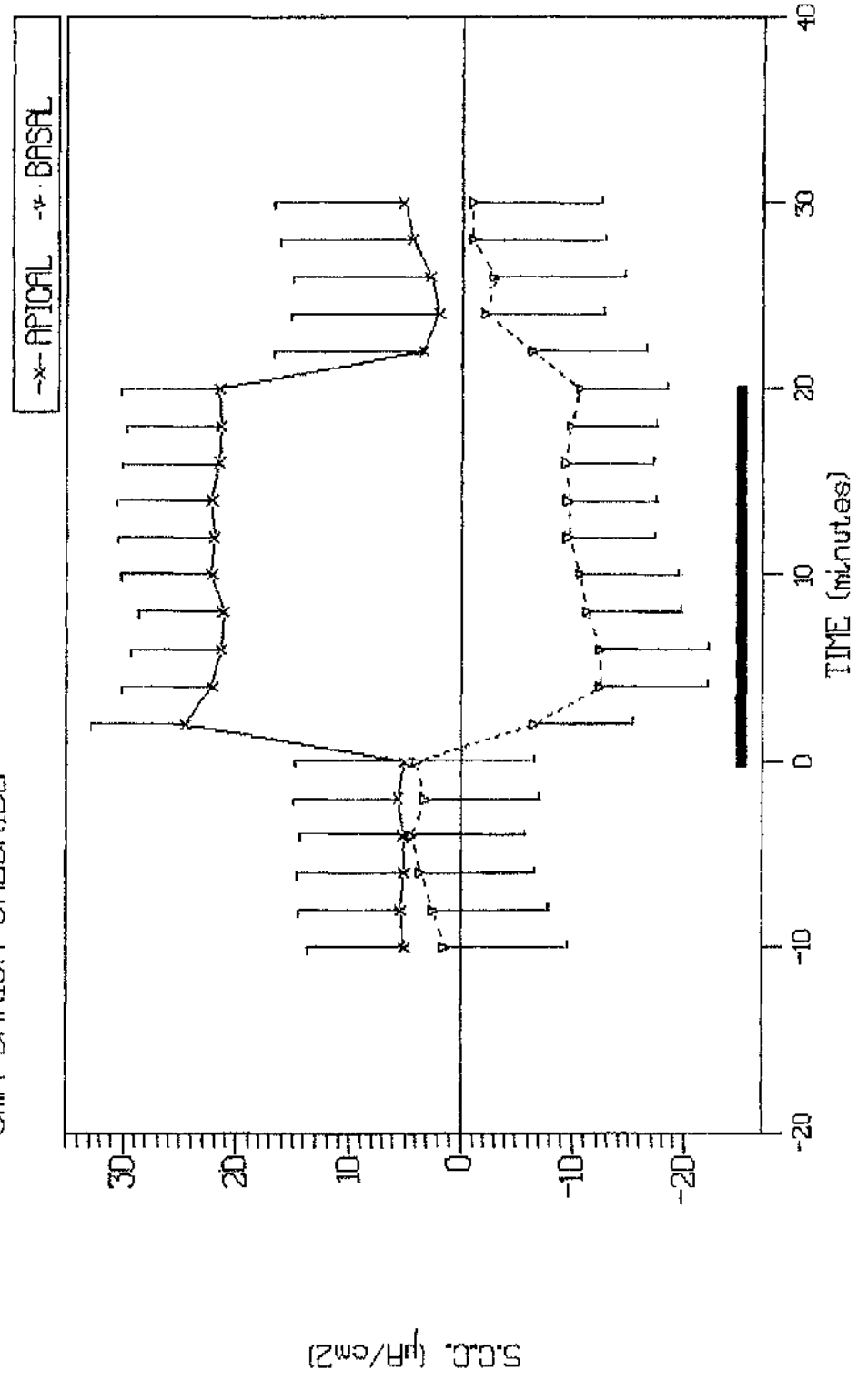


FIG 4.12

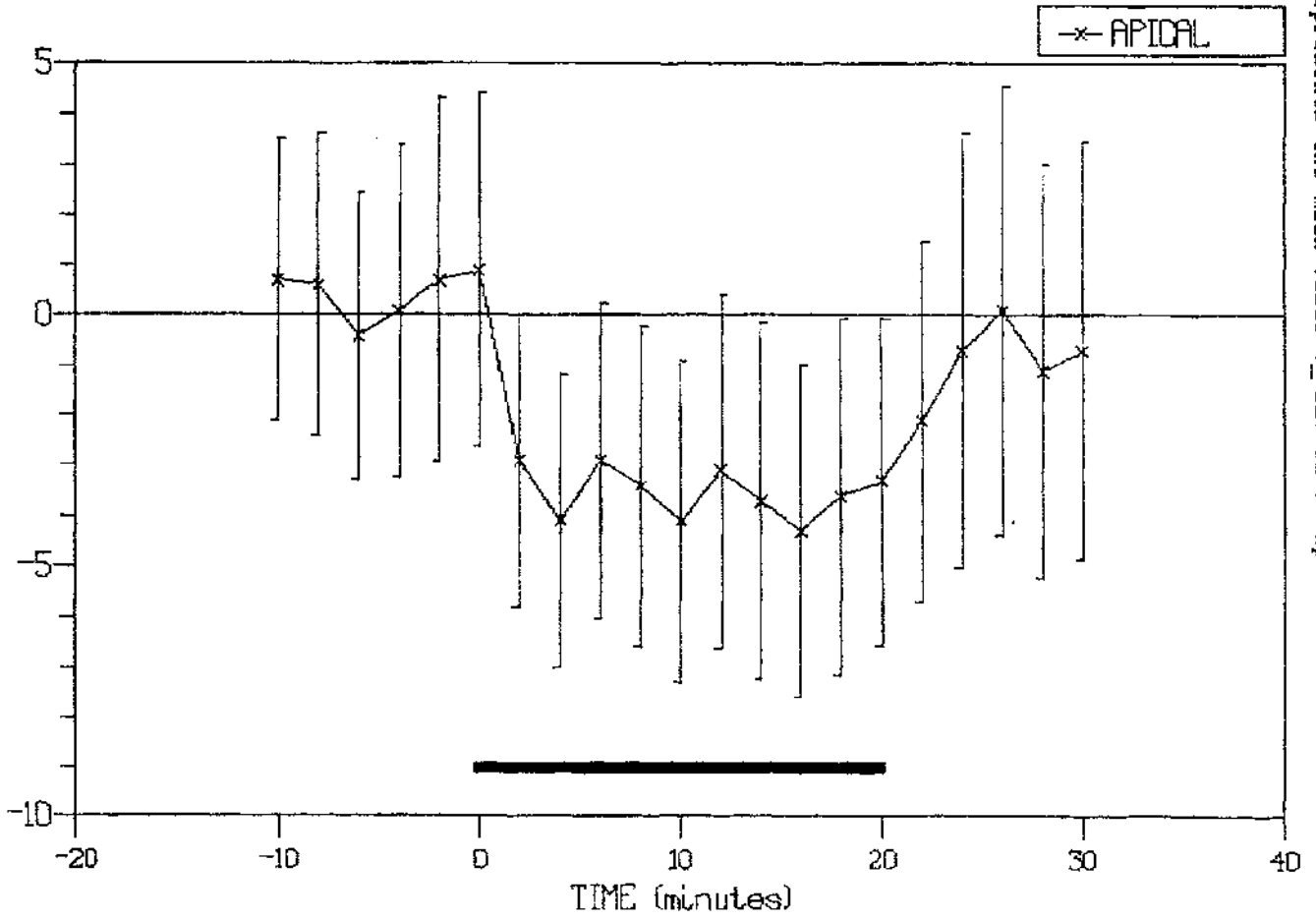
5mM BARIUM CHLORIDE

some degree of recovery when the barium was removed from the basal solution. Three showed a full recovery, with one only recovering partially. The other experiment showed no recovery. There was again no significant alteration in the resistance across the epithelium, the mean change being 20.6 ($\pm$29.7) ohm cm^2 (n=4, $p>0.1$) an increase from 71.0 to 91.6 ohm cm^2.

When barium chloride was added to the apical side of the tissue, but under potassium free conditions, there was a significant change in the SCC but in the opposite direction to that observed under normal bathing conditions (Fig 4.13). The mean was -4.1 ($\pm$0.9) $\mu A/cm^2$ (n=7, $p<0.01$). The initial polarity made no significant difference to the degree of change. The maximal change was observed between two and twelve minutes, with most of the changes being between two and four minutes. There were no further effects in any of the experiments. When standard bathing conditions were restored four experiments showed no recovery and the other three had a full recovery, quite gradual in one case. In this series of experiments there was a significant increase in the resistance when barium chloride was present. The mean change was 13.5 ($\pm$6.1) ohm cm^2 (n=6, $p<0.05$), an increase from 74.5 to 88.0 ohm cm^2.

FIG 4.13

5mM BARIUM CHLORIDE
(POTASSIUM FREE AS STANDARD)



The effect of 10mM barium chloride (apically only) on the SCC, under potassium free conditions as control. Barium chloride was present in the period marked by the bar. Each point represents the mean value (± SE) of 7 experiments.

Amiloride.

In order to test whether amiloride had any effect on the SCC, which could indicate the presence of sodium conductive channels or sodium/hydrogen exchange, it was added at a final concentration of 0.5 mM apically only under normal bathing conditions (Fig 4.14). This resulted in a significant change in the SCC in the negative direction. The mean change was -4.8 ($\pm$2.5) $\mu A/cm^2$ (n=8, $p<0.05$). The fall in the SCC was largest when the control was initially positive, the magnitude of the change being correlated to the magnitude of the initial SCC, r=-0.899 (n=8, Fig 4.14A). In five of the experiments the SCC changed gradually over twelve to sixteen minutes to a maximum that was then maintained until the end of the test period, but once the maximum was reached in only six minutes. The two remaining experiments showed no real change in the SCC. Upon the restoration of standard bathing conditions, only one experiment showed a full recovery. The rest showed no recovery. There was a significant increase in the resistance when amiloride was added to the apical solution. The mean increase was 15.0 ($\pm$6.1) ohm cm^2 (n=7, $p<0.05$), from 86 to 101 ohm cm^2.

Ion removal and substitution

Bearing in mind the results from the inhibitor experiments,

The effect of amiloride (apically only) on the SCC. Amiloride was present in the period marked by the bar. Each point represents the mean value (± SE) of 8 experiments.

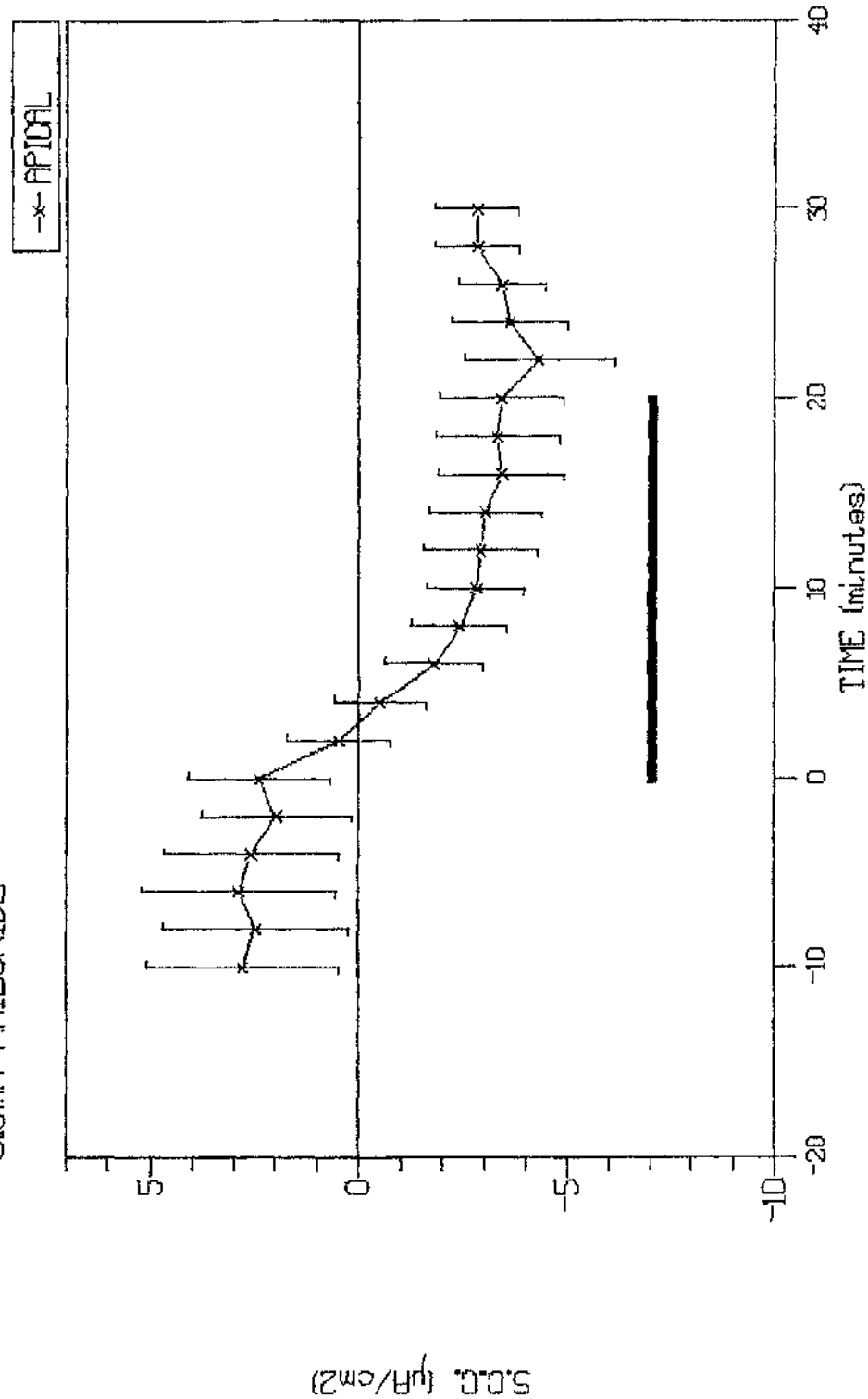
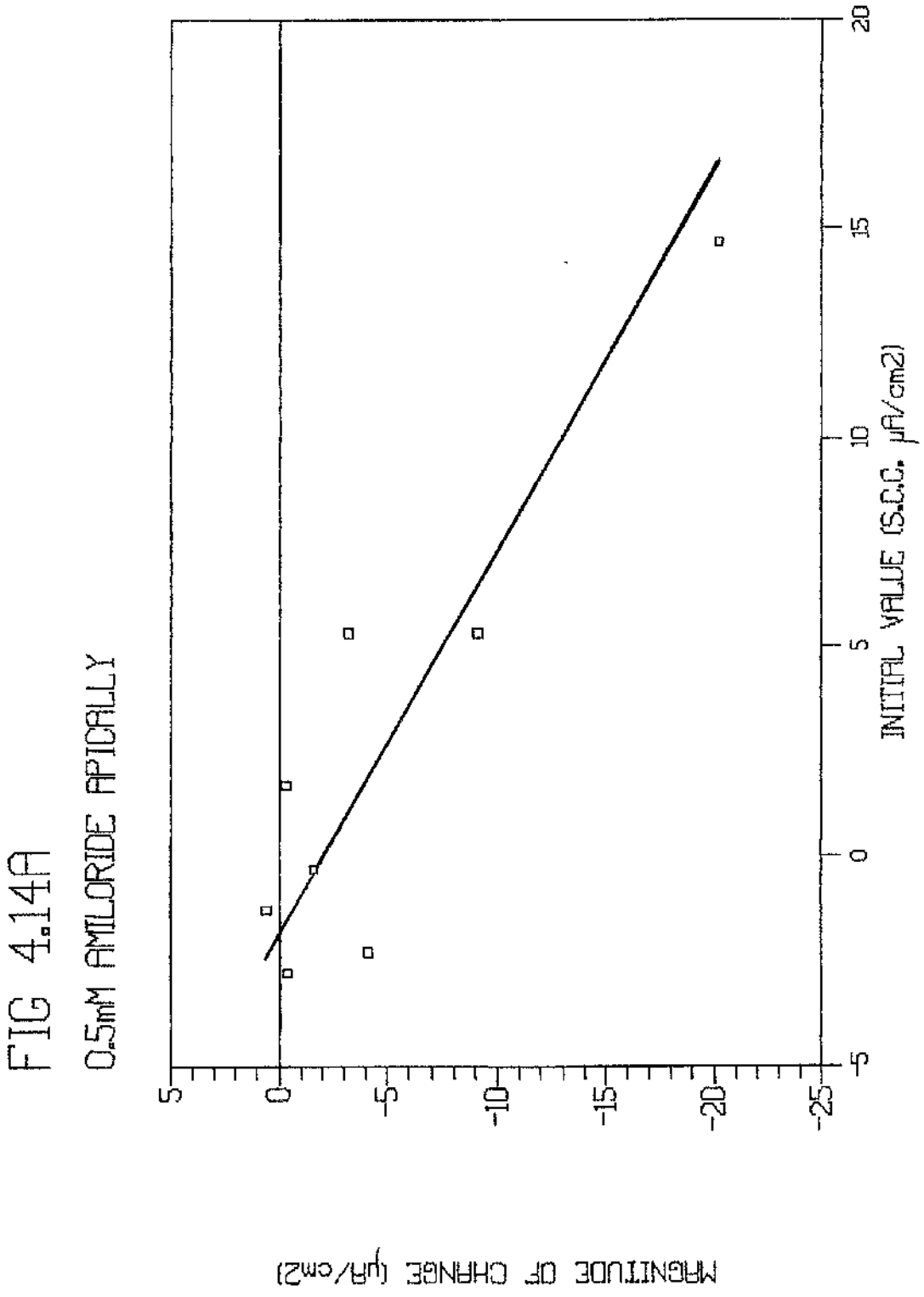


FIG 4.14

0.5mM AMILORIDE

The correlation between the initial SCC and the magnitude of change in the SCC with amiloride present on the apical side only. Each point represents one experiment. The line is a best fit linear regression line.



the ion replacement experiments were preformed to see if they would provide further evidence in favour of the transport processes already suggested by the use of the inhibitors. To achieve this, some of the experiments were performed with other ions already absent in the bathing media in an attempt to mimic the conditions in the presence of the inhibitors. A number of ion replacement experiments were also carried out with sodium and chloride absent in the standard bathing media in an attempt to elucidate the nature of the residual SCC.

Sodium free

The substitution of sodium by choline^{+} had a significant effect on the SCC. Upon substitution on both sides of the epithelium (Fig 4.15), the SCC became more negative, the mean change being -14.2 ($\pm$4.0) $\mu A/cm^2$, (n=6, p=0.01). This corresponds to a change in net transport of -8.8 $\mu Equiv/cm^2/min$. This could be due to either a decrease in cation absorption or anion secretion, either of which would result in a decreased SCC. In all experiments the maximal effect was seen within four minutes, but there was a partial recovery over the next sixteen minutes (Fig 4.15). When sodium was returned to the bathing solutions there was a complete recovery to the pre-test SCC values, in all but one experiment. The substitution effect and its magnitude were independent of the pre-test polarity of the SCC. The mean transepithelial resistance increased insignificantly from 70.5

The effect of sodium free conditions on the SCC. Sodium was substituted in the period marked by the bar. Each point represents the mean value. N=6 for substitution on both sides (± SE).

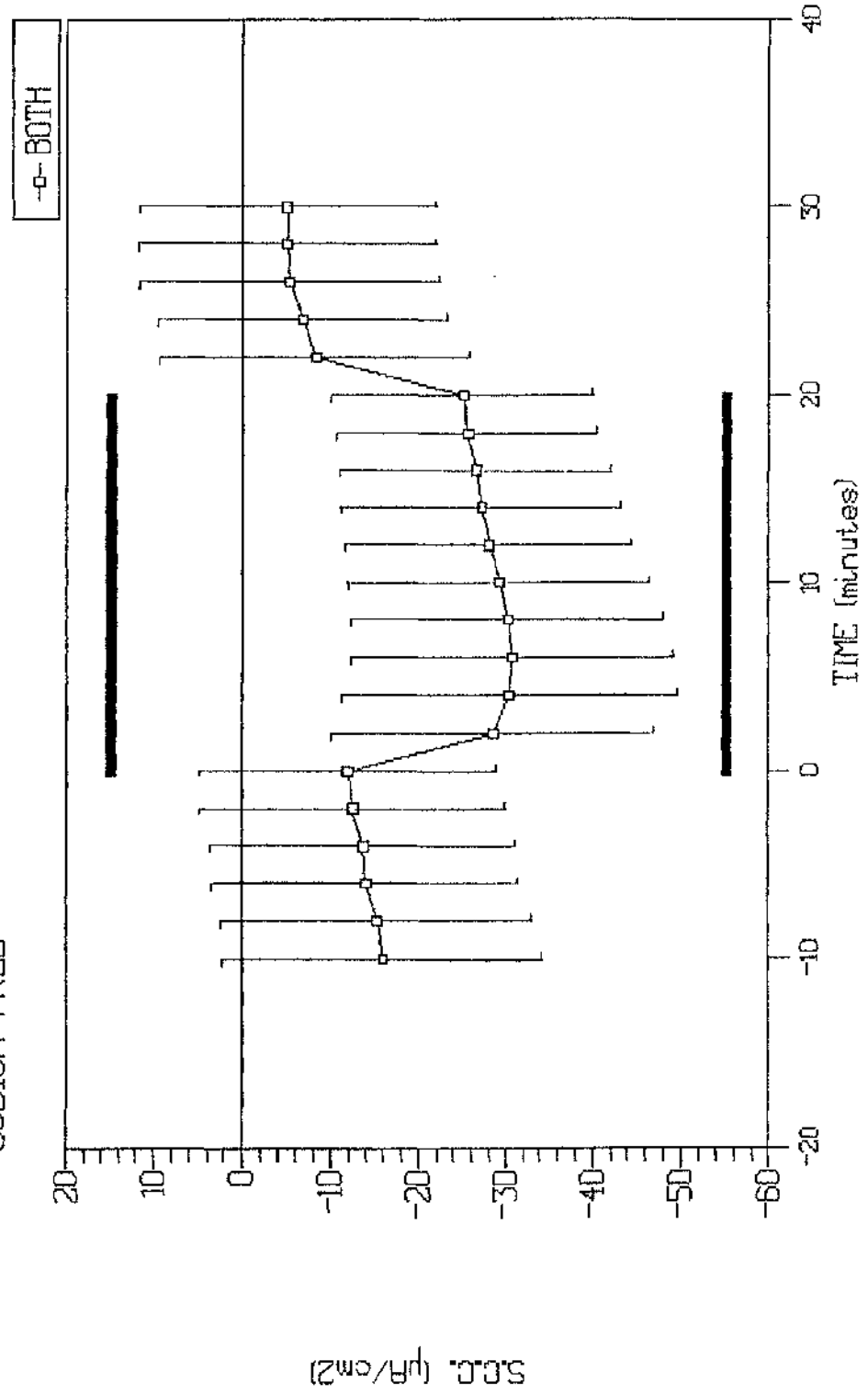


FIG 4.15

SODIUM FREE

to 73.2 ohm cm^2 over the test period.

When sodium was substituted on the apical side only (Fig 4.16), this also resulted in a significant change, the SCC always becoming more negative. The mean change was -18.8 ($\pm$2.6) $\mu A/cm^2$ (n=7, p=0.01). The maximum change was obtained within four to eight minutes. Upon the restoration of apical sodium, there was a complete recovery in the SCC in all cases, and in two experiments there was an overshoot to values twice those of the pre-test ones and these values were maintained. The resistance did not significantly alter during this period, the mean increase being 33.9 ohm cm^2 (n=7, p>0.5), from a pre-mean of 77 ohm cm^2 to a post-mean of 111 ohm cm^2. Although this increase is relatively large, in two of the experiments there was actually a slight decrease in the resistance, the mean being -5.5 ohm cm^2, which resulted in the overall mean increase not being significant.

Basal substitution (Fig 4.16) also resulted in a significant change in the SCC. The SCC became more positive, the mean change being 12.2 ($\pm$1.9) $\mu A/cm^2$ (n=7, p<0.01). Again the maximal effect was seen within eight minutes and in most cases this was maintained throughout the test period with partial recovery in only one experiment. Once sodium was returned to the serosal solution, there was a full recovery of the SCC in 5 of 7 cases. However, one experiment showed an overshoot to twice the control values, while another one showed a recovery

The effect of sodium free conditions on the SCC. Sodium was substituted in the period marked by the bar. Each point represents the mean value (+ or - SE). N=7 for apically only and n=7 for basally only.

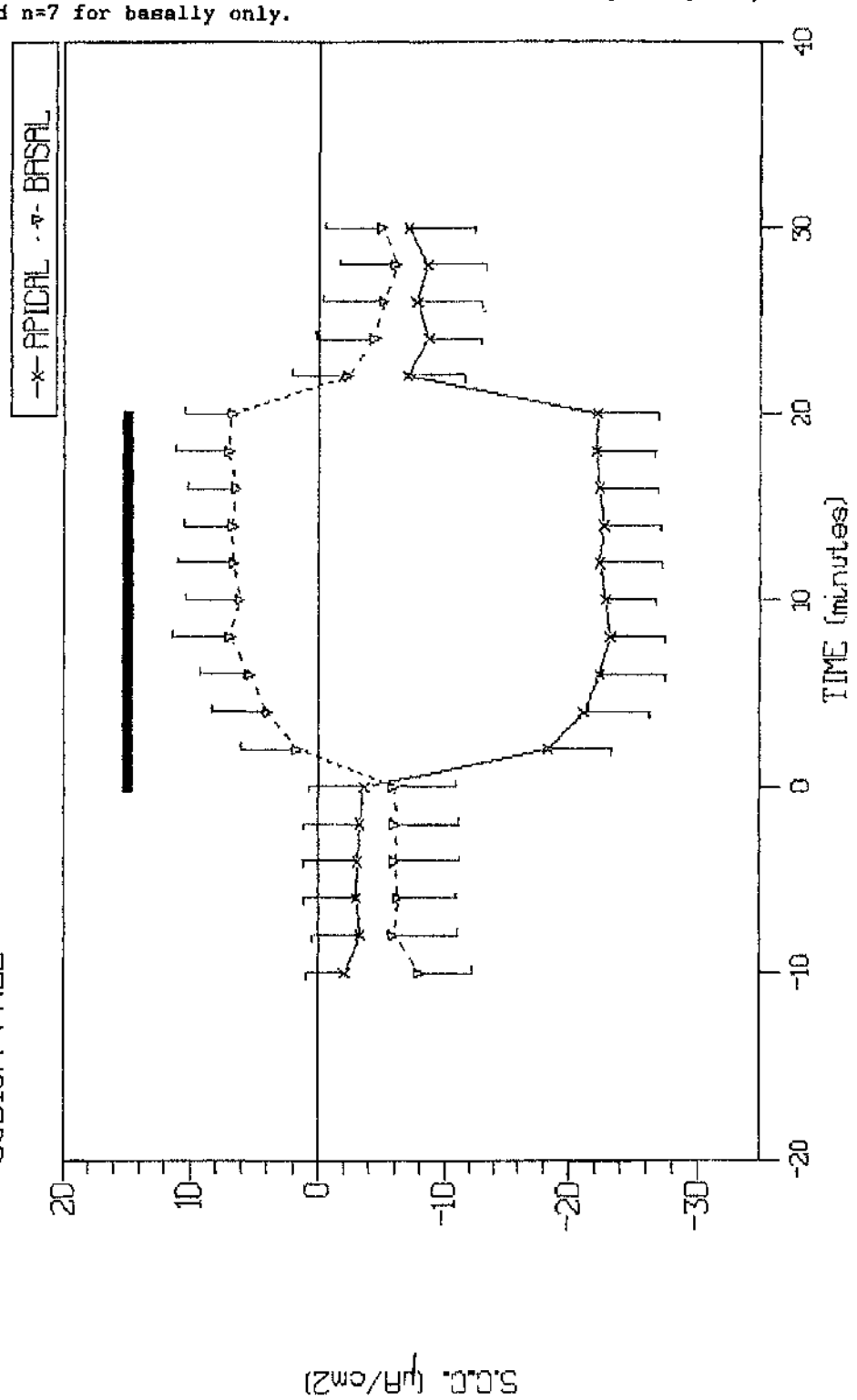


FIG 4.16
SODIUM FREE

of only 75%. The resistance across the tissue under sodium free conditions on the serosal side decreased significantly. However the mean change was only -9.6 ($\pm$4.7) ohm cm^2, (n=7, $p<0.05$). Due to the small S.E. this change is statistically significant whereas the larger change observed under apical sodium-free conditions is not.

Potassium free.

The removal of potassium from the bathing solutions had significant effects on the S.C.C that were similar to that seen for sodium, although not as marked.

When potassium was removed from the solutions bathing both sides of the epithelium, the SCC became significantly more negative (Fig 4.17) the mean change being -4.9 ($\pm$1.1) $\mu A/cm^2$, (n=6, $p<0.01$). The maximal change was obtained between two and ten minutes and in five of the experiments this level was maintained throughout the test period. In the other experiment there was a gradual recovery towards pre test levels although the SCC did not reach these. On restoration of the potassium there was usually no recovery in the SCC, although in two experiments there was complete recovery. During the test period, with potassium removed from the bathing solution, the mean increase in the transepithelial resistance was 45.0 ($\pm$19.5) ohm cm^2 , this change being

The effect of potassium removal on the SCC. Potassium was absent in the period marked by the bar. Each point represents the mean value (± SE), with n=6 for removal from both sides.

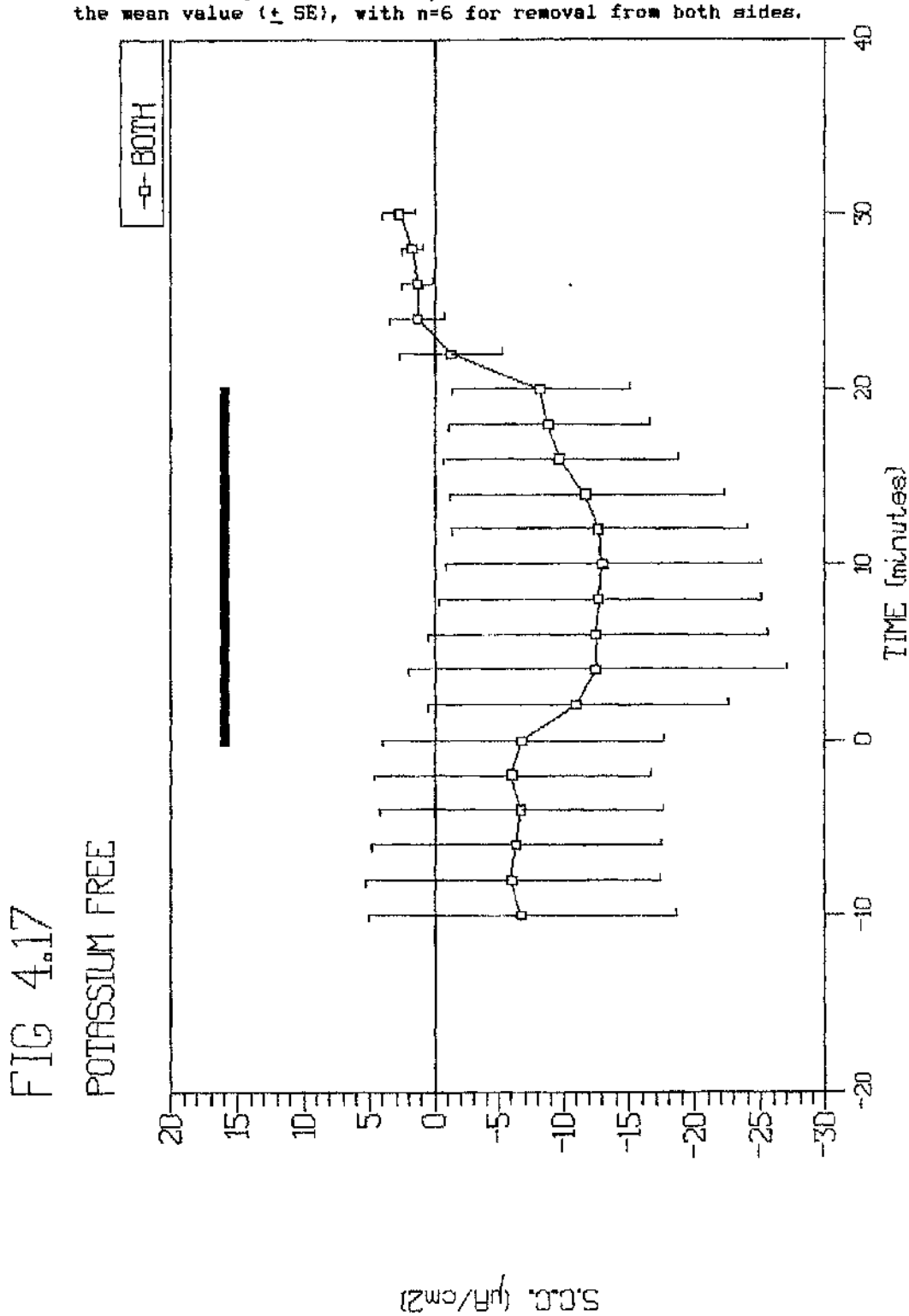


FIG 4.17

POTASSIUM FREE

significant (n=5, $p<0.05$). The mean resistance increased from 60 to 105 ohm cm^2.

The removal of potassium from the apical side of the epithelium (Fig 4.18) resulted in a significant negative change in the SCC, the mean change being -11.5 ($\pm$4.0) $\mu A/cm^2$ (n=5, $p<0.05$). There was a significant correlation between the magnitude of the initial SCC and the magnitude of the change with r=-0.84 (n=5, Fig 4.18A). The maximal change was obtained between two and ten minutes. In two of the five experiments there was only a partial recovery over the remainder of the test period. A similar situation to that found with potassium free on both sides was observed when potassium was returned to the mucosal side. The recovery ranged from none to a full recovery, most showing at least a 50% recovery. There was no significant change in the transepithelial resistance when potassium was removed from the apical side only. There was a mean increase of 13.7 ($\pm$6.4) ohm cm^2 (n=4, $p>0.05$).

Basal removal of potassium resulted in a change of similar magnitude to that for apical removal, although in the opposite direction (Fig 4.18). The change was 10.2 ($\pm$5.0) $\mu A/cm^2$ (n=7, $p<0.05$) and was therefore significant. The greatest changes in the SCC were from experiments where it had been initially negative; the mean change was 22.0 $\mu A/cm^2$ compared to only 1.3 $\mu A/cm^2$ where the SCC was positive. Indeed, there was a

The effect of potassium removal on the SCC. Potassium was absent in the period marked by the bar. Each point represents the mean value (+ or - SE), with n=5 for apical removal and n=7 for basal removal.

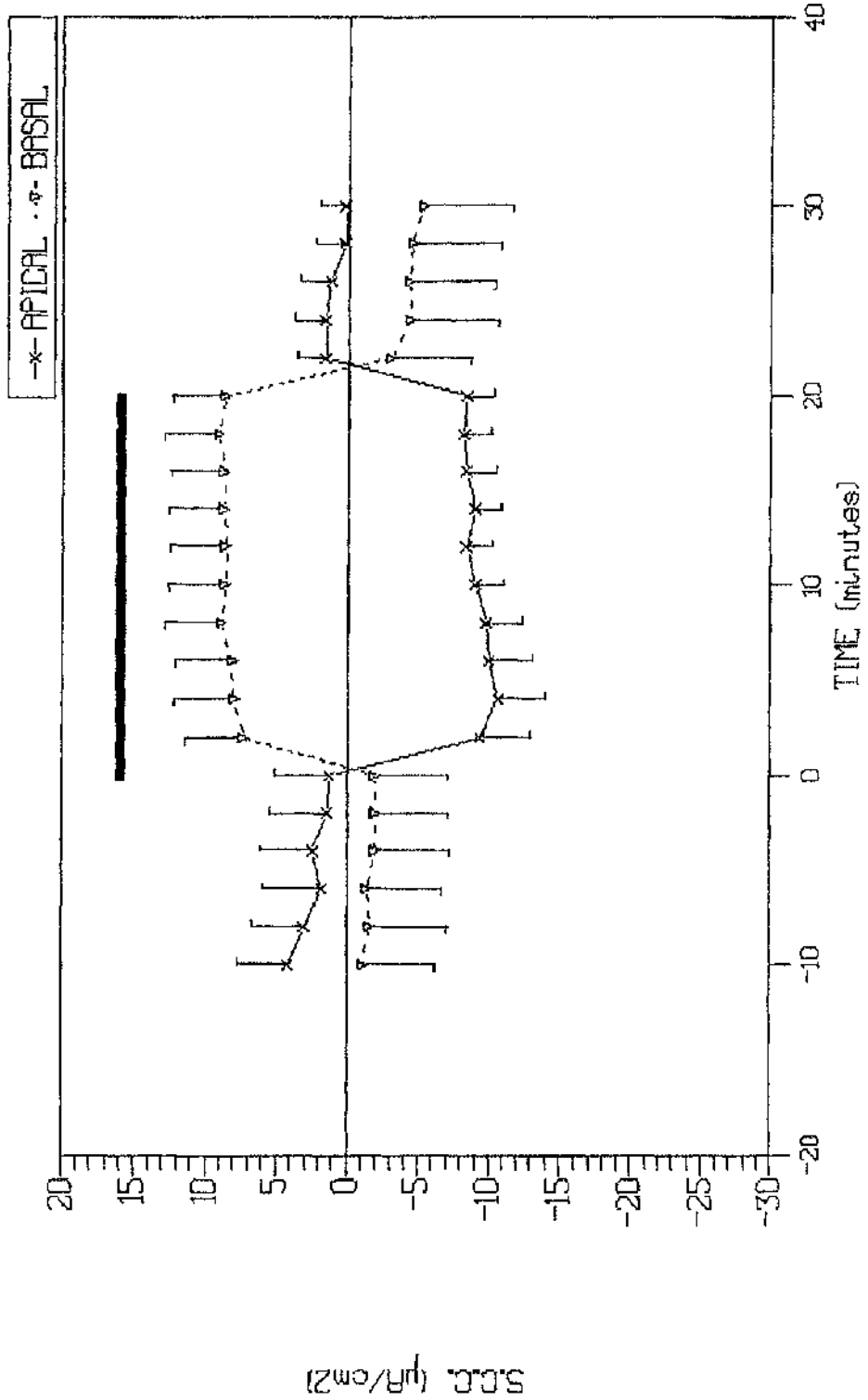


FIG 4.18
POTASSIUM FREE

The correlation between the initial SCC and the magnitude of change in the SCC upon potassium removal apically only. Each point represents one experiment. The line is a best fit linear regression line.

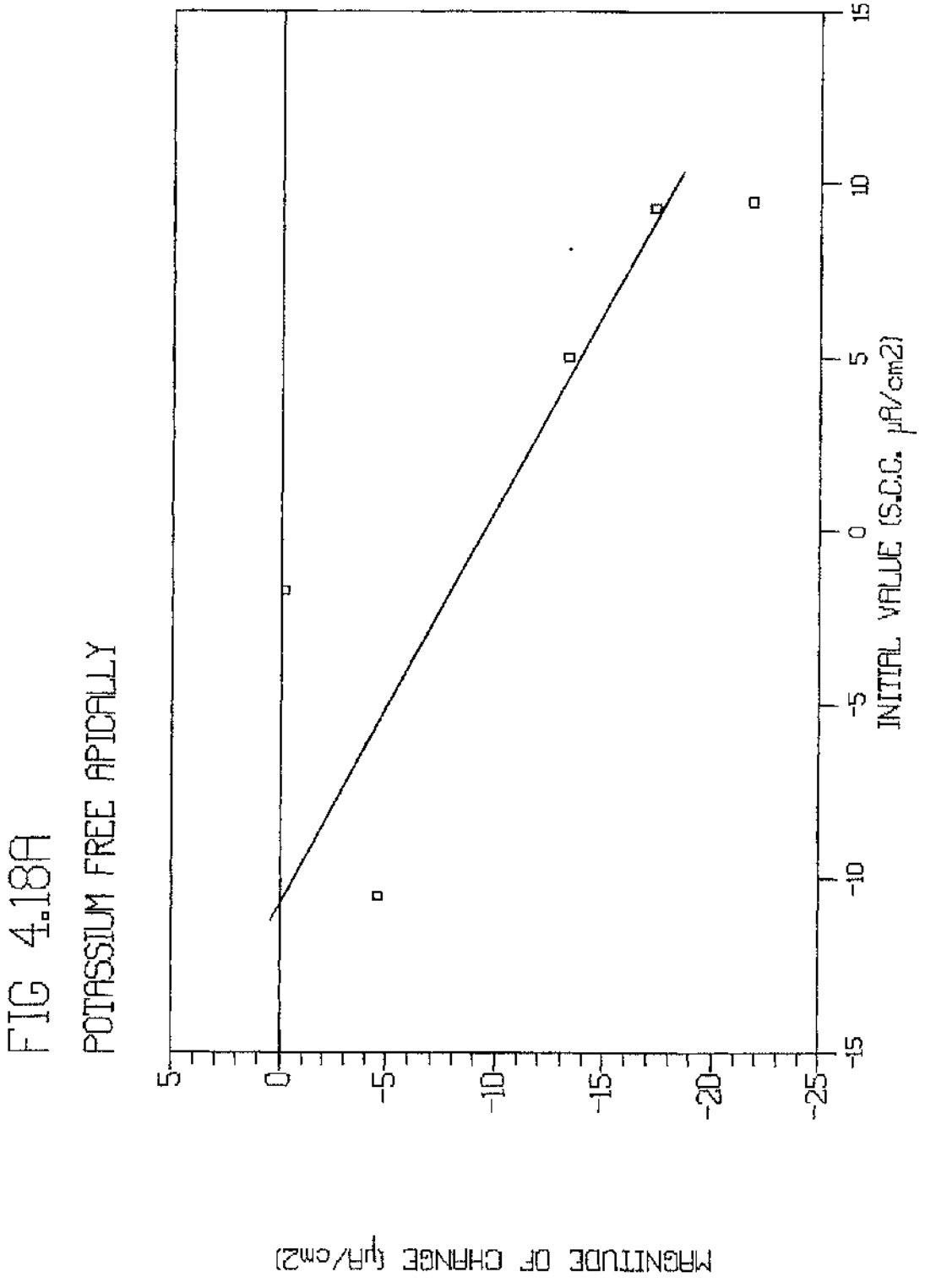


FIG 4.18A

POTASSIUM FREE APICALLY

significant correlation between the initial SCC and the magnitude of the change, the r value being -0.72 (n=7, Fig 4.18B). The approximate maximum change occurred within two to ten minutes and was maintained in five out of the seven experiments. In the other two, one exhibited a partial recovery, whilst the other showed a further gradual increase in the SCC over the remainder of the test period. In most cases there was no recovery evident once potassium was returned to the basal solution, but in two there was an almost complete recovery and one finished with twice the resting SCC. The transepithelial resistance was unaffected by basal removal of potassium, the mean change being 8.3 ($\pm$5.2) ohm cm^2 (n=7, $p>0.05$).

Potassium was also removed from both sides of the epithelium under sodium-free, chloride free and sodium plus chloride free conditions as control. Under just sodium-free conditions there was on average a non-significant change in the SCC (Fig 4.19), i.e. -2.1 ($\pm$1.3) $\mu A/cm^2$, (n=8, $p>0.05$). However in five of the experiments there was a clear decrease in the SCC. This ranged from -8.3 to -0.9 $\mu A/cm^2$, the mean being -4.1 $\mu A/cm^2$. These 5 snails were all starved. In the remaining three, all fed, there was no change in the SCC. When a change was obtained, it was within six minutes of potassium removal and was always maintained. When potassium was returned to the bathing solutions there was once again a range of responses from no recovery to full recovery. However in most cases

The correlation between the initial SCC and the magnitude of change in the SCC upon potassium removal basally only. Each point represents one experiment. The line is a best fit linear regression line.

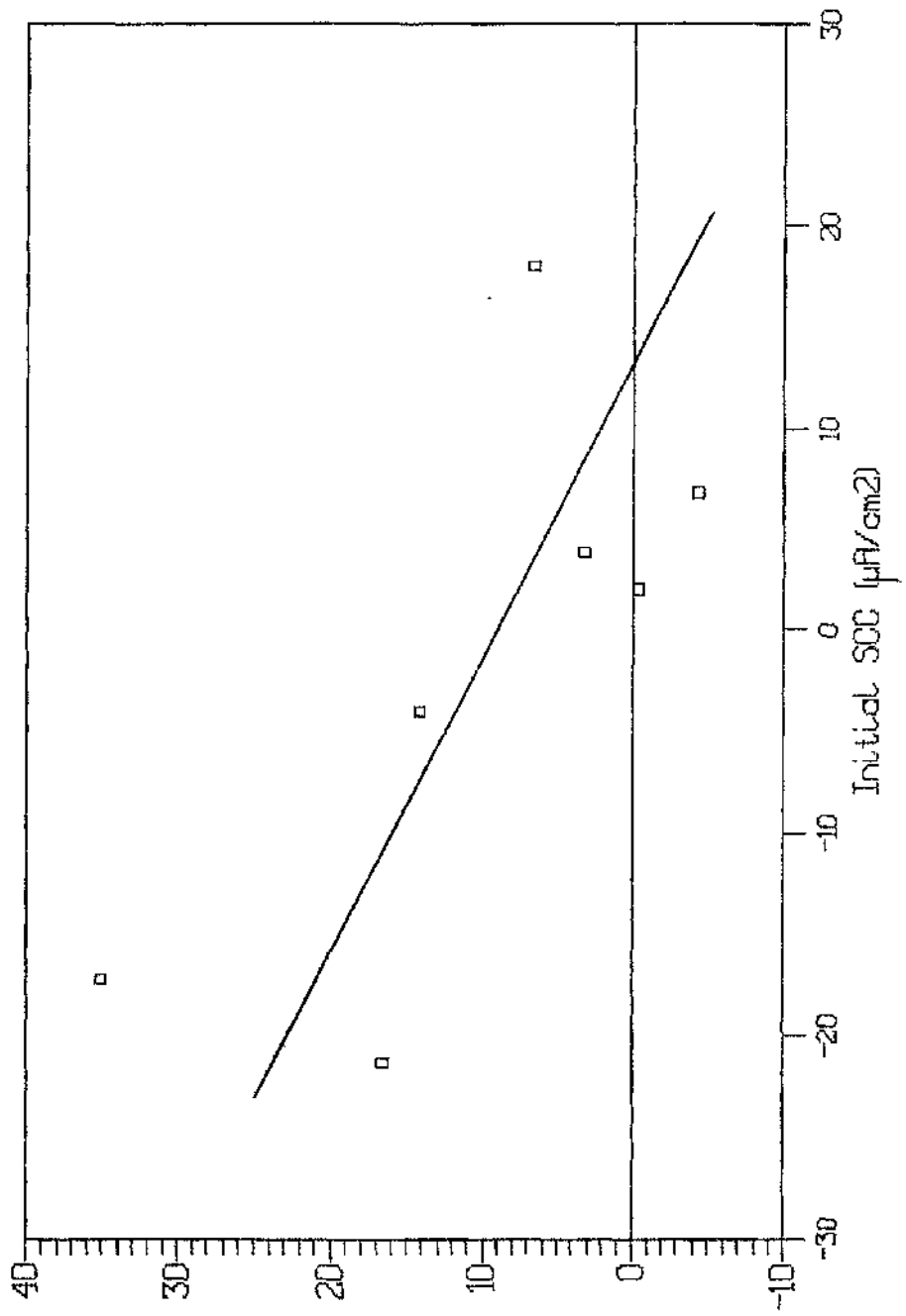


FIG 4.18B
K+FREE BASALLY

The effect of potassium free conditions (both sides), under sodium free conditions as control, on the SCC. Potassium was absent in the period marked by the bar. Each point represents the mean value of 8 experiments (± SE).

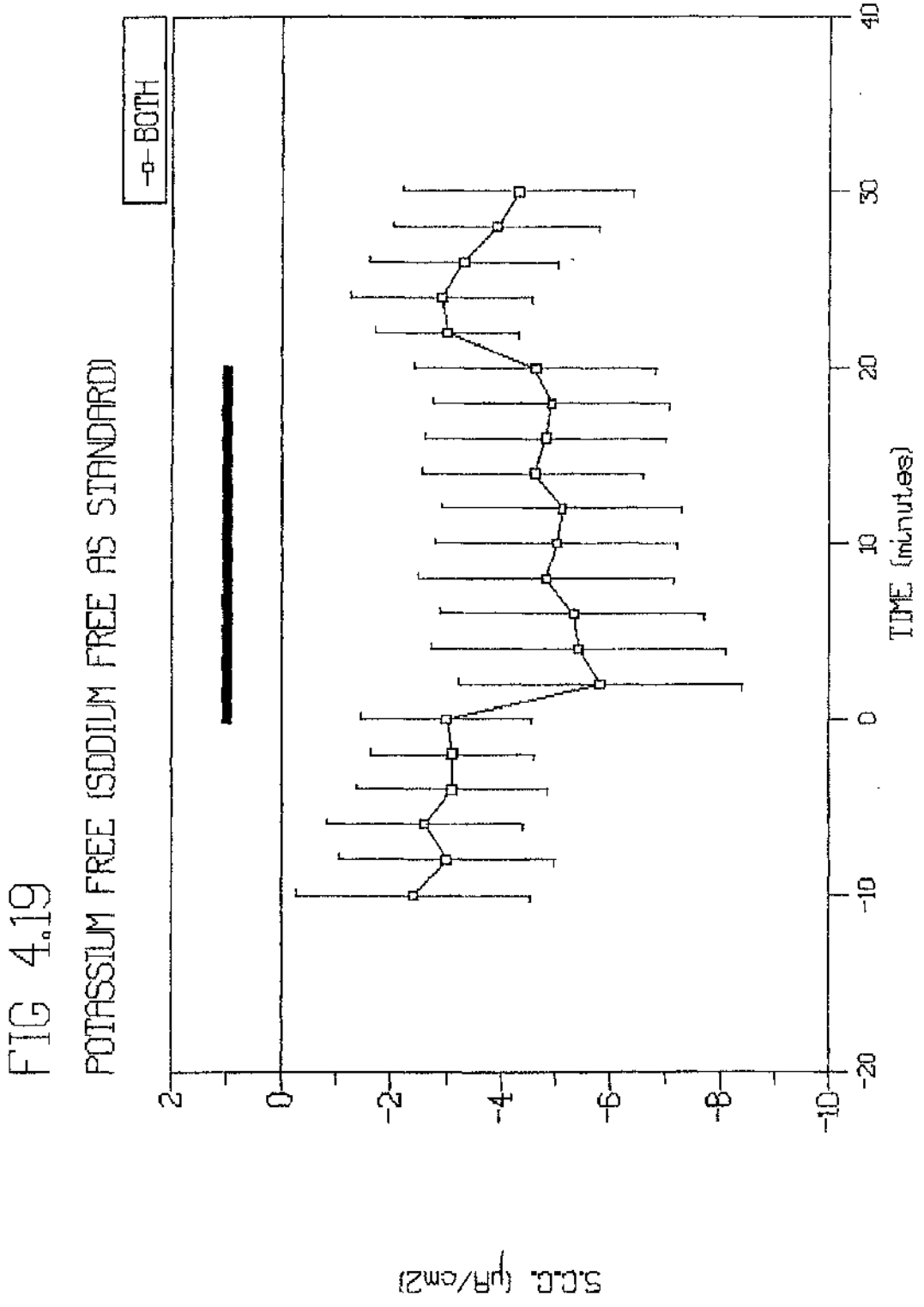


FIG 4.19

POTASSIUM FREE (SODIUM FREE AS STANDARD)

there was no recovery evident. The size of the change elicited upon potassium removal had no effect on the degree of recovery that was observed once potassium was returned to the bathing solutions. There was no significant change in the resistance during potassium free conditions; there was a mean increase of 9.0 ($\pm$5.4) ohm cm^2 (n=5, p>0.05), from 88 to 97 ohm cm^2.

The results were similar for potassium removal under chloride free conditions as standard. The mean change of 4.3 ($\pm$2.4) $\mu A/cm^2$ (Fig 4.20) was not significant (n=4, p>0.1). However in two of the experiments there was quite a large change in the SCC (5.6 and 10.6 $\mu A/cm^2$), occurring within six minutes and maintained throughout. The initial polarity of the SCC does not seem to have affected the size of the response since the mean change was 3.9 $\mu A/cm^2$ for a positive SCC compared to 5.6 $\mu A/cm^2$ when negative. There was no recovery in the SCC after potassium was returned to the bathing medium. There was no significant change in the resistance, the mean change being -15.3 ($\pm$9.2) ohm cm^2, (n=4, p>0.1).

With sodium and chloride free conditions as standard, when potassium was removed from both sides of the tissue there was no significant change in the mean SCC (Fig 4.21), i.e. only -0.2 ($\pm$0.4) $\mu A/cm^2$ (p>0.1).

The effect of potassium free conditions (both sides), under chloride free conditions as control, on the SCC. Potassium was absent in the period marked by the bar. Each point represents the mean value of 4 experiments (± SE).

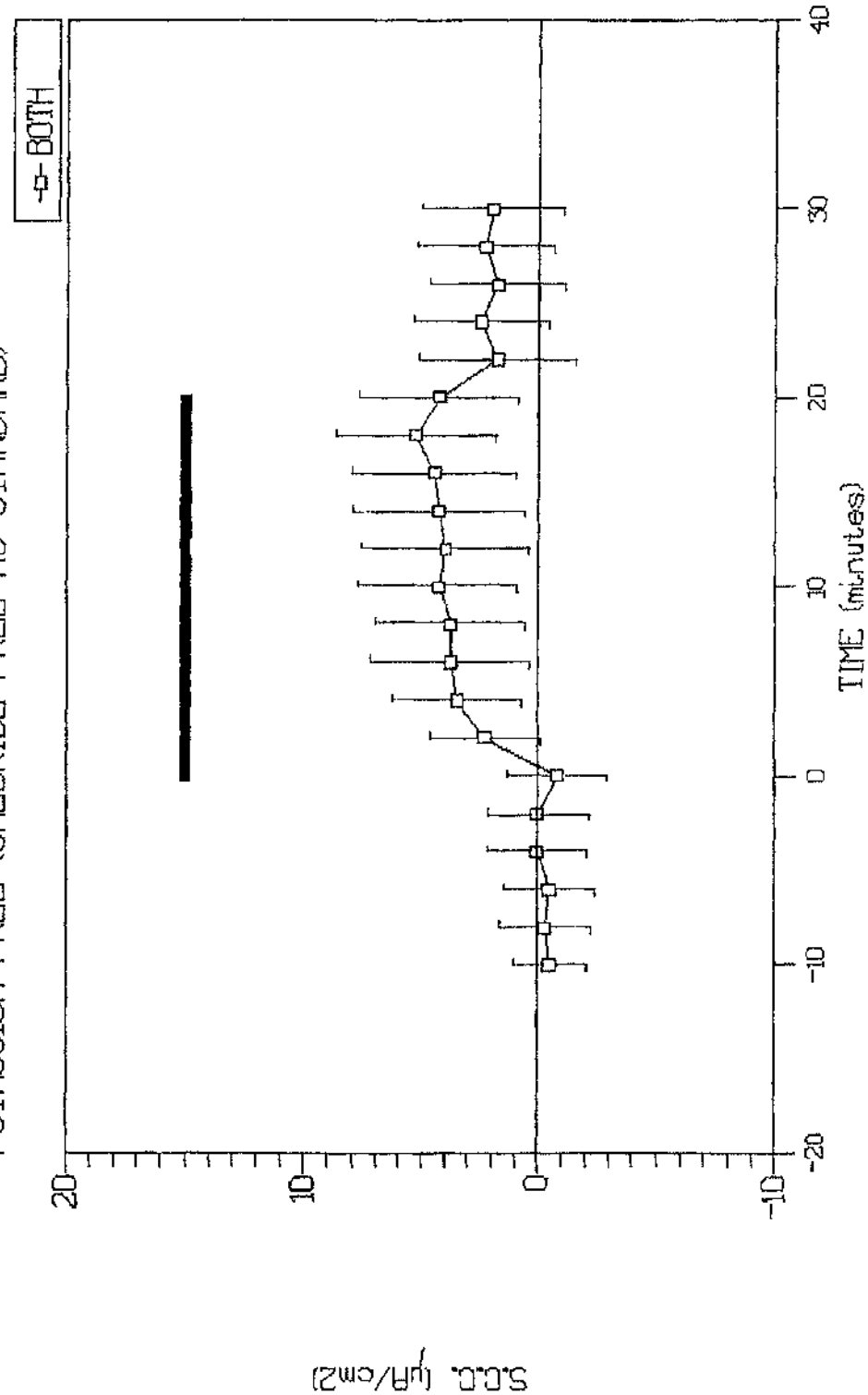


FIG 4.20

POTASSIUM FREE (CHLORIDE FREE AS STANDARD)

The effect of potassium free conditions (both sides), under sodium and chloride free conditions as control, on the SCC. Potassium was absent in the period marked by the bar. Each point represents the mean value of 7 experiments (± SE).

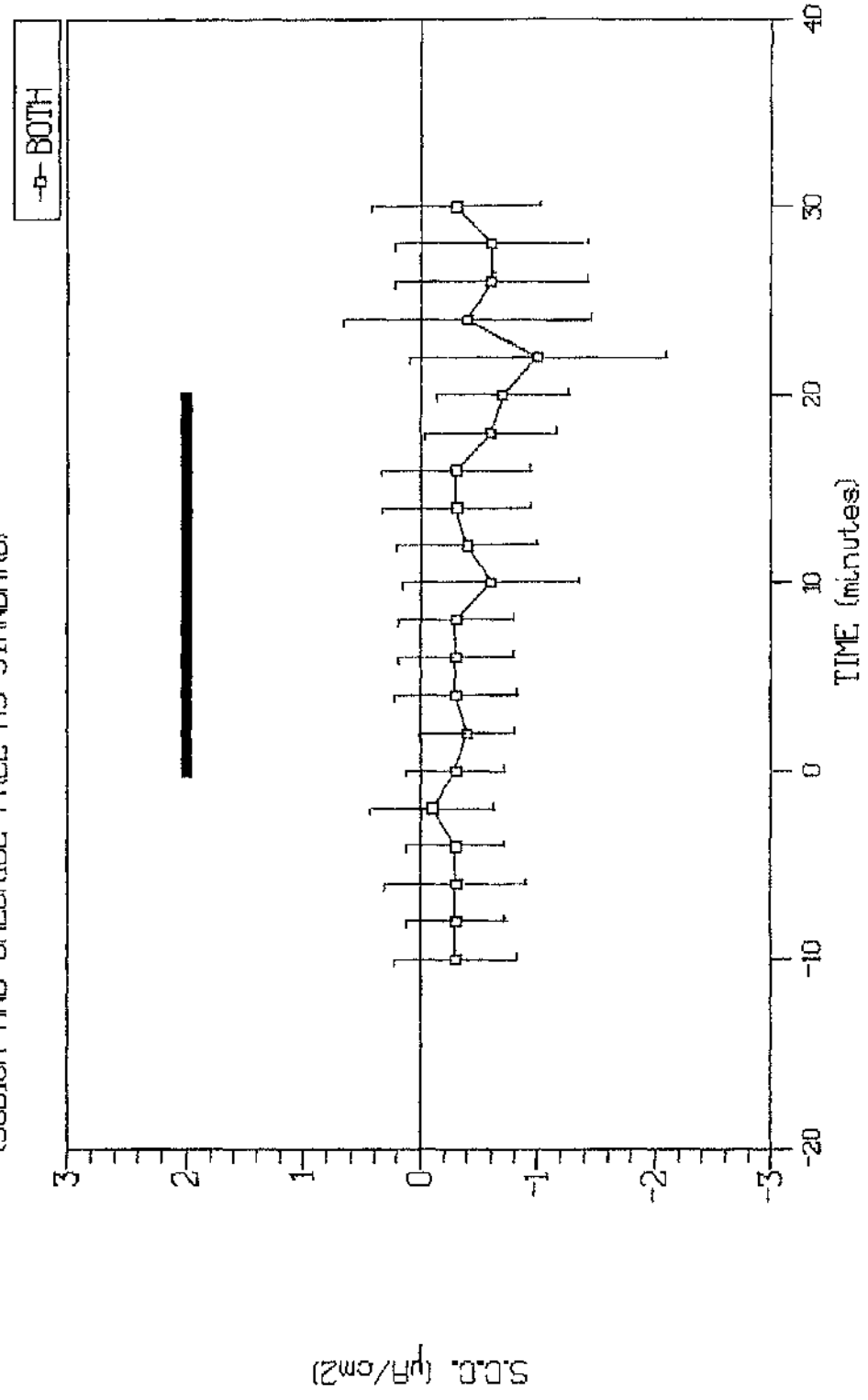


FIG 4.21
POTASSIUM FREE
(SODIUM AND CHLORIDE FREE AS STANDARD)

Calcium free

Calcium was removed from both sides of the tissue under normal as well as sodium plus chloride free conditions. It was also removed from the apical side only as well as the basal side only under sodium plus chloride free conditions as standard. Upon removal from both sides of the epithelium, under normal bathing conditions, there was a significant change in the SCC in the positive direction (Fig 4.22). The mean change was 5.1 ($\pm$1.3) $\mu A/cm^2$, ($n=7$, $p<0.01$). This increase occurred within eight minutes of calcium removal and in five out of the seven experiments this degree of change was maintained. In the other two there was a further gradual increase in the SCC and also a marked spike in the SCC both when calcium was removed and when it was then eventually replaced. The other five experiments did not show this. The correlation between the initial SCC and the magnitude of the change in the SCC, $r=-0.470$ ($n=7$), was not significant. There was a full recovery in all but one experiment when calcium was returned. This one experiment was one of those that exhibited a spike and gradual increase in the SCC. There was no significant difference between the resistances before and after calcium removal, the change being 6.26 ($\pm$8.54) ohm cm^2 ($n=7$, $p>0.1$).

The response observed when calcium was removed from both sides, but under sodium plus chloride free conditions as standard, was the opposite of that reported above. There was

The effect of calcium free conditions (both sides) on the SCC, under standard conditions as control. Calcium was absent in the period marked by the bar. Each point represents the mean value of 7 experiments (± SE).

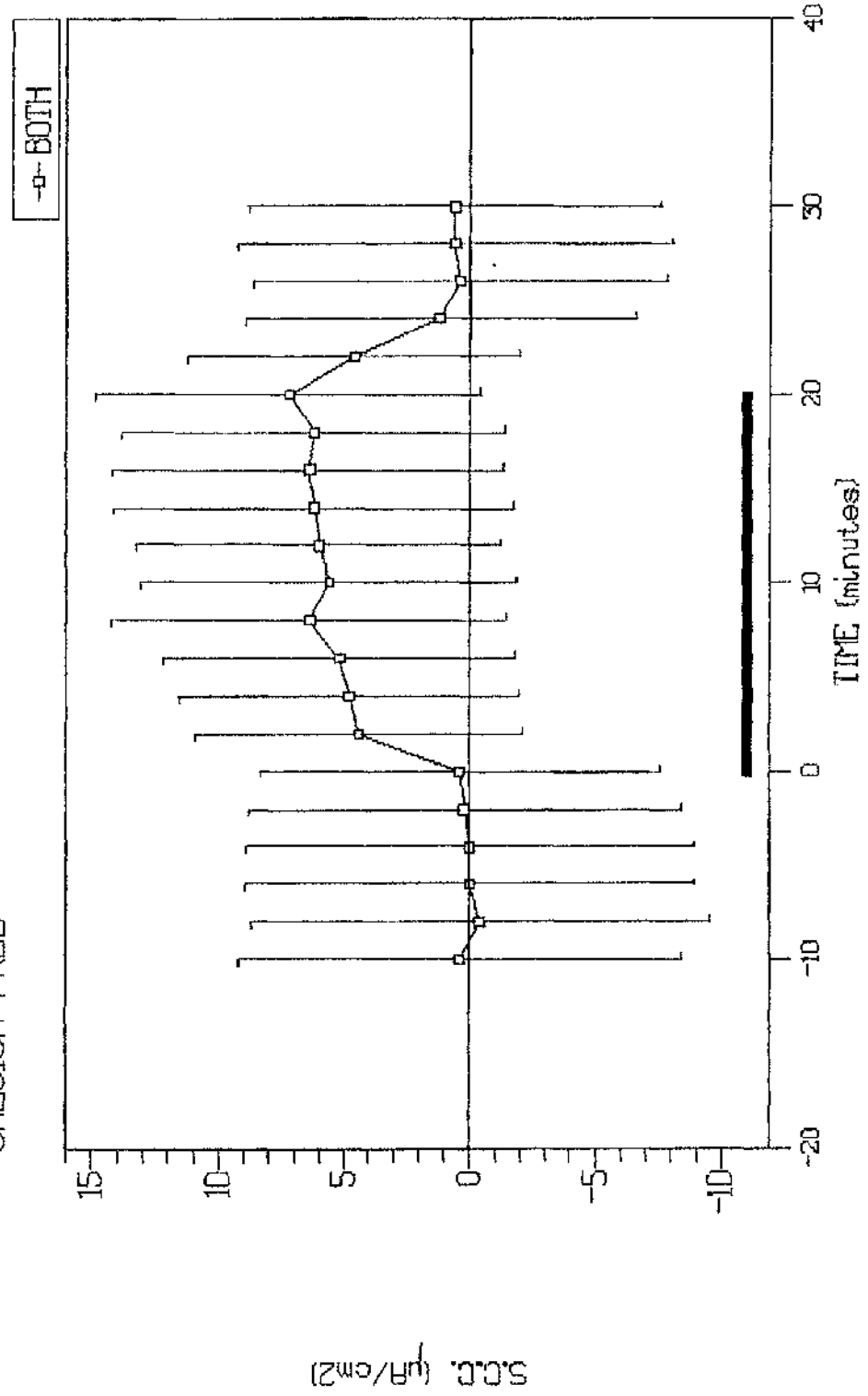


FIG 4.22
CALCIUM FREE

a significant decrease, of -1.6 (±0.48) $\mu A/cm^2$, in the mean SCC (Fig 4.23), significant at the $p<0.01$ level (n=7). The greatest change was obtained within ten minutes and in all of the experiments this remained steady until calcium was returned to the bathing solution. There was no correlation between the magnitude of change and the initial SCC. Upon restoration of the calcium, there was a full recovery in five out of the seven experiments, the other two showing no recovery at all. There were no spikes evident in the SCC when calcium was removed or returned. The transepithelial resistance showed a significant increase when calcium was removed from the bathing solution, the mean change being 141 (±12.7) ohm cm^2 (n=6, $p<0.001$), from a mean of 429 to 570 ohm cm^2.

Calcium was also removed from the apical side only, under sodium and chloride free conditions as standard. This resulted in a significant decrease in the SCC (Fig 4.24). The mean change was -4.9 (±0.9) $\mu A/cm^2$. This was significant at the $p<0.005$ level (n=7). The maximal change in SCC was obtained between two and ten minutes after calcium removal and in no experiment was there any further change. There was no real difference in the magnitude of the change when comparing the polarity of the SCC; the mean decrease was -5.5 $\mu A/cm^2$ when negative compared to -4.4 $\mu A/cm^2$ when positive. Once calcium was returned to the mucosal solution there was a complete recovery to pre-test levels in all but one

The effect of calcium removal on the SCC, under sodium and chloride free conditions as control. Calcium was absent in the period marked by the bar. Each point represents the mean value (± SE), with n=7 for removal from both sides.

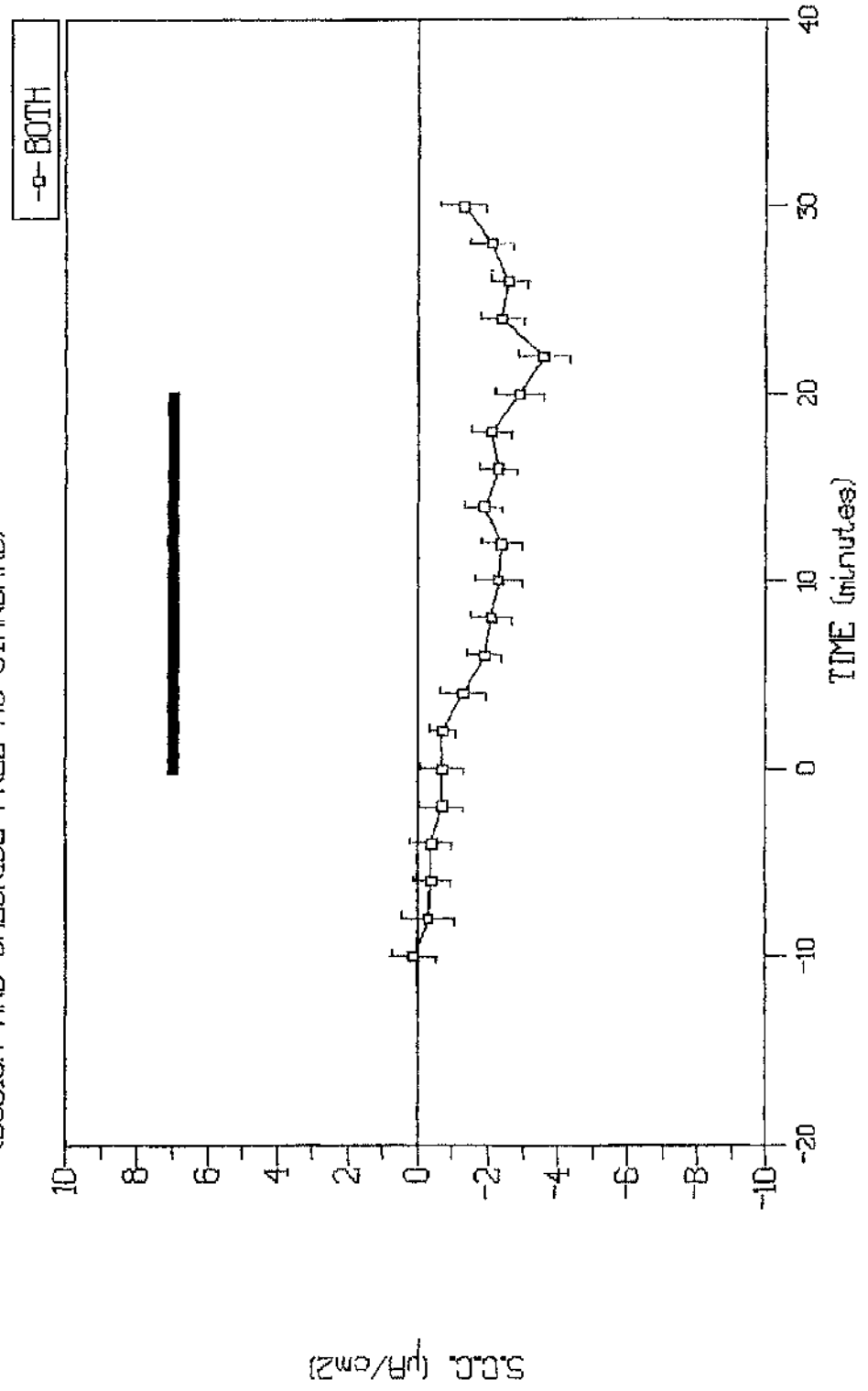


FIG 4.23
CALCIUM FREE
(SODIUM AND CHLORIDE FREE AS STANDARD)

The effect of calcium removal on the SCC, under sodium and chloride free conditions as control. Calcium was absent in the period marked by the bar. Each point represents the mean value (+ or - SE), with n=7 for apical removal and n=7 for basal removal.

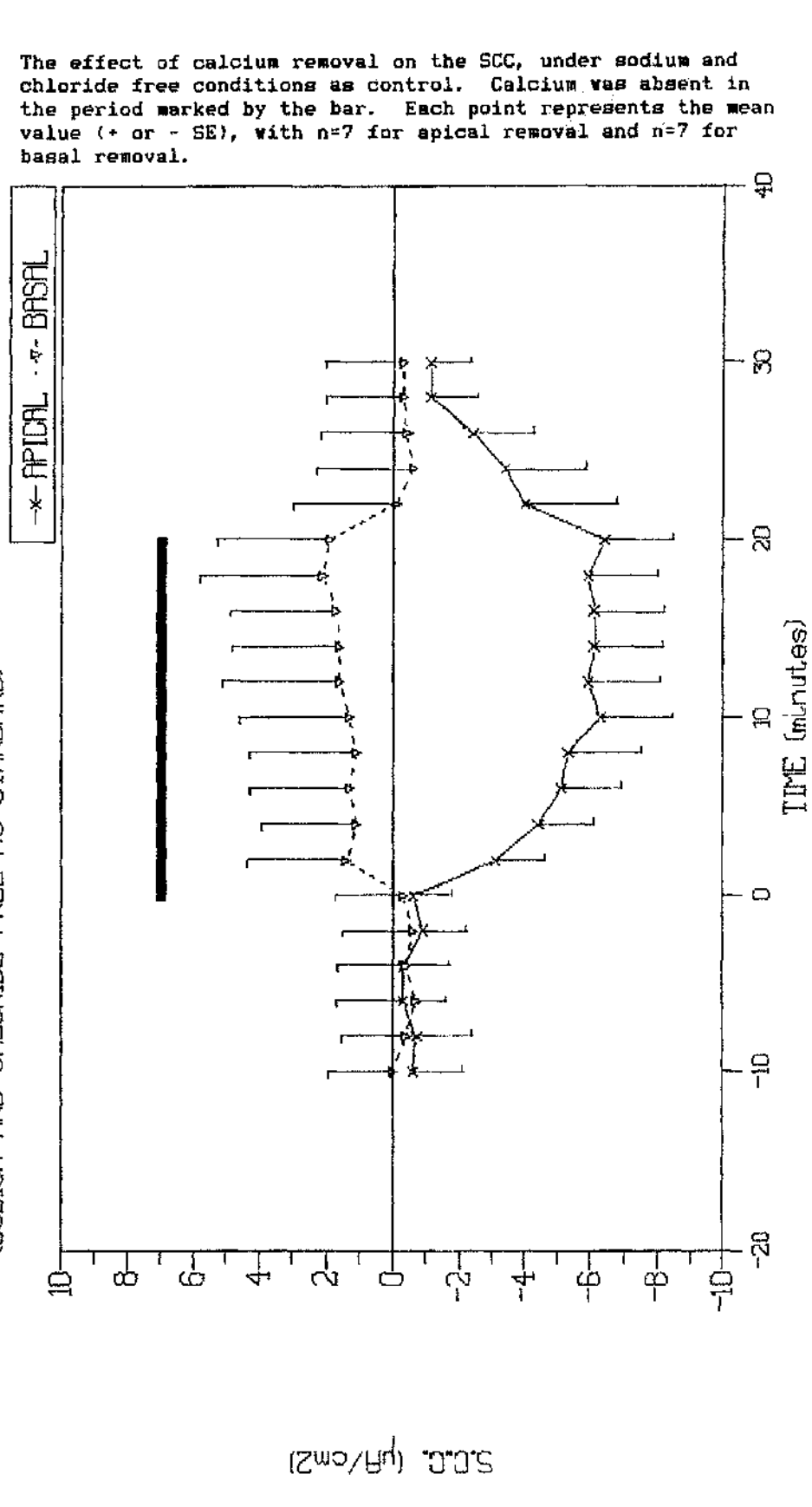


FIG 4.24
CALCIUM FREE
(SODIUM AND CHLORIDE FREE AS STANDARD)

experiment; this one showing no recovery at all. There was no significant change in the resistance, the mean change being only -0.5 (±15.1) ohm cm^2 (n=6, p>0.1).

When calcium was removed from the basal side only, under sodium plus chloride free conditions, there was a significant change in the SCC, but in the opposite direction from that found for mucosal removal (Fig 4.24). There was an increase of 3.8 (±0.7) $\mu A/cm^2$, (n=7, p<0.001). Again the response was observed within ten minutes and in five out of the seven preparations there was no further change. The remainder showed a further gradual increase in the SCC over time. Again there was no correlation between magnitude of change and initial polarity of SCC. Once calcium was returned there was a full recovery in the SCC except in one experiment where none at all was seen. One other showed a recovery to a level that was twice those seen before any change in the bathing solution. There was no significant change in the resistances pre and post test. The mean change was 5.3 (±38.1) ohm cm^2, (n=6, p>0.1).

Magnesium free

The standard conditions under which magnesium was removed from the bathing solutions were either sodium free or sodium plus chloride free.

Magnesium was removed from both sides of the epithelium under sodium free conditions as standard. There was no significant change in the SCC (Fig 4.25). The mean change was -0.2($\pm$0.7) $\mu A/cm^2$ (n=8, p>0.3). There was no significant change in the resistance when magnesium was removed from the bathing solution. The mean change was 0.7 ($\pm$6.4) ohm cm^2 (n=6, p>0.4). However when the SCC was initially negative the resistance decreased upon magnesium removal, the mean change being -3.3 ohm cm^2 (n=4). When the SCC was initially positive (n=2), the resistance increased, by an average of 8.5 ohm cm^2. However there was no significant difference between these two means (p>0.1).

The removal of magnesium apically only and basally only was performed under sodium plus chloride free conditions as standard. After apical removal there was no significant change in the mean SCC (Fig 4.26), the change being a decrease of -0.3 ($\pm$1.5) $\mu A/cm^2$ (n=8, p>0.4). There was no relationship evident between the size of individual changes and either the initial polarity of the SCC or the state of feeding prior to experimentation.

There was no significant change in the resistance during magnesium free conditions, the mean change being 62.9 ($\pm$35.2) ohm cm^2, (n=6, p>0.05). Again this increase in the resistance, although large is not statistically significant due to the large S.E. In five out of six experiments there

The effect of magnesium removal on the SCC, under sodium and chloride free conditions as control. Magnesium was absent in the period marked by the bar. Each point represents the mean value (± SE), with n=8 for removal from both sides.

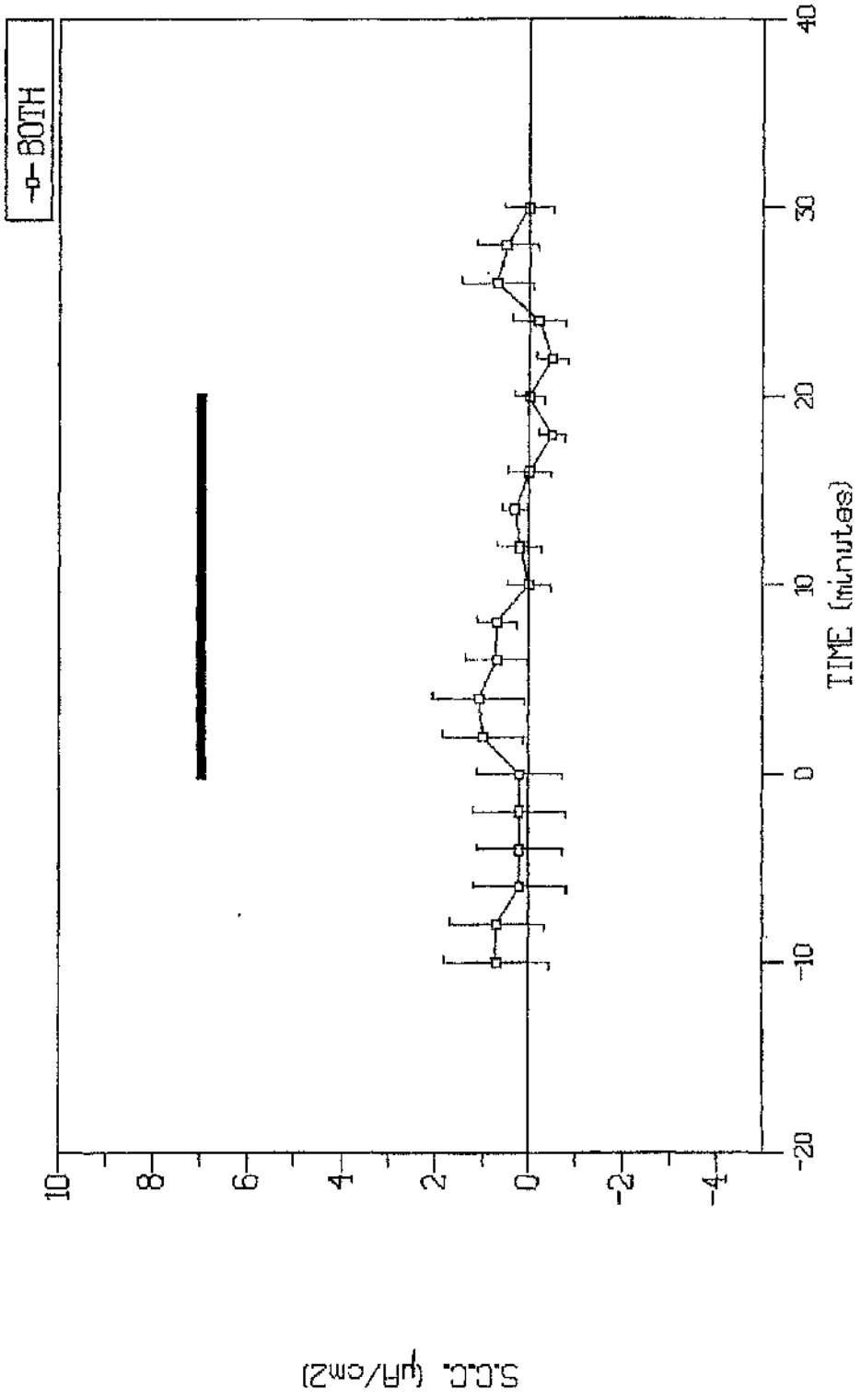


FIG 4.25
MAGNESIUM FREE
(SODIUM AND CHLORIDE FREE AS STANDARD)

The effect of magnesium removal on the SCC, under sodium and chloride free conditions as control. Magnesium was absent in the period marked by the bar. Each point represents the mean value (+ or - SE), with n=8 for apical removal and n=8 for basal removal.

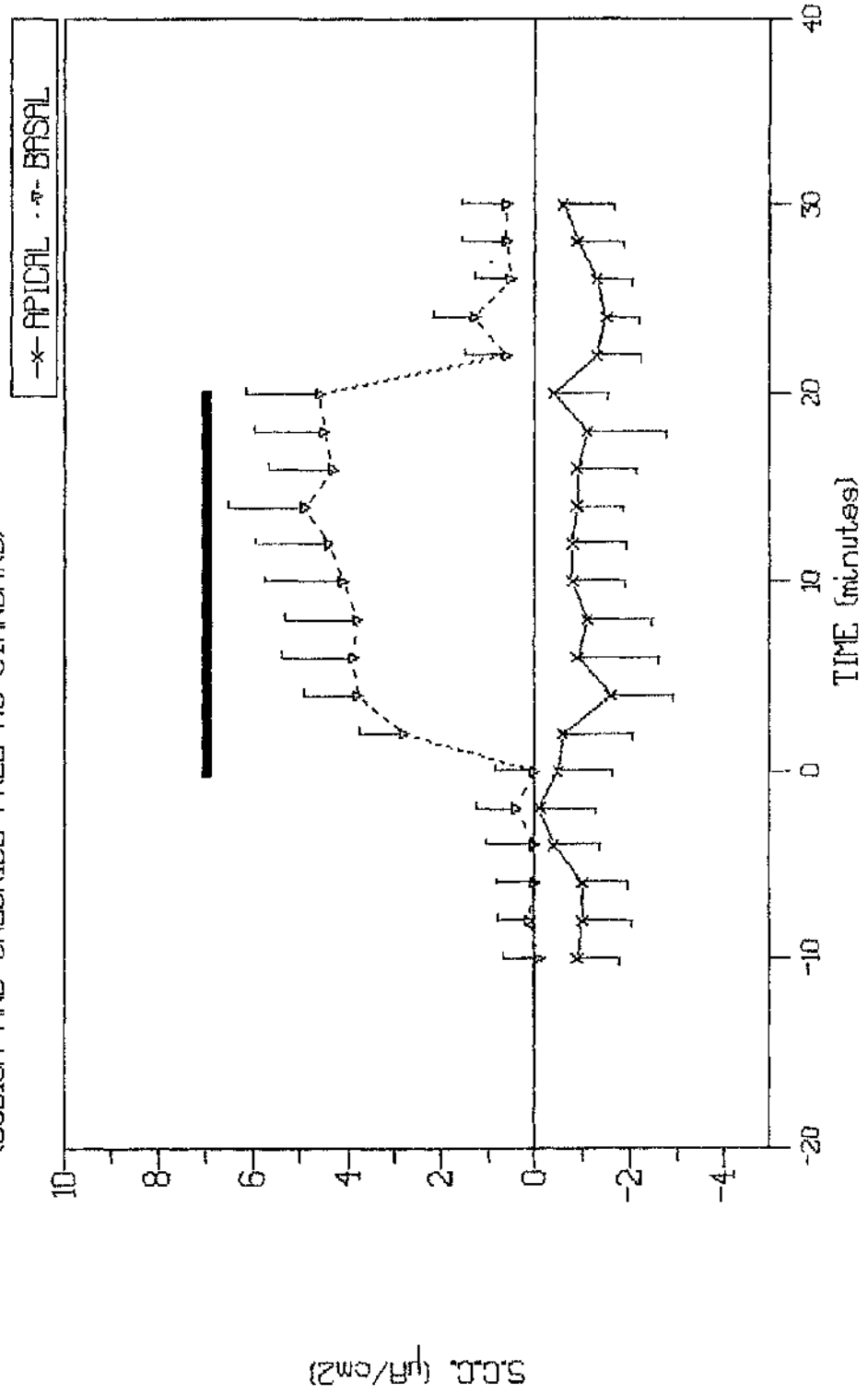


FIG 4.26
MAGNESIUM FREE
(SODIUM AND CHLORIDE FREE AS STANDARD)

was an increase in the resistance, but in the sixth it decreased.

Unlike magnesium removal from both sides or from the apical side only, basal removal did result in a significant change in the SCC (Fig 4.26). In all experiments the maximal change in the SCC was obtained between four to six minutes, there being no further change and there was always a complete recovery upon the restoration of magnesium. The mean SCC became more positive by 4.0 ($\pm$0.8) $\mu A/cm^2$, (n=8, $p<0.01$). The mean change in the resistance during the experiments was not significant, being a decrease of -127.0 ($\pm$128.3) ohm cm^2, (n=7, $p>0.1$) from a mean of 443 to 316 ohm cm^2. There was no correlation between the size of the changes in the resistance or either the initial polarity or the size of the SCC.

Chloride free

The substitution of chloride by sulphate and mannitol on both sides of the epithelium under normal conditions resulted in two different responses depending on whether the snail had been fed prior to experimentation. In fed snails there was a slight change in the negative direction in the mean steady SCC that was not significant (Fig 4.27) the mean change being -3.3 ($\pm$2.0) $\mu A/cm^2$ (n=8, $p>0.05$). However in two of the experiments there were larger negative changes, these being

The effect of chloride substitution (both sides) on the SCC. Chloride was absent in the period marked by the bar. Each point represents the mean value (+ or - SE) of 8 experiments for fed snails and 8 experiments for starved snails.

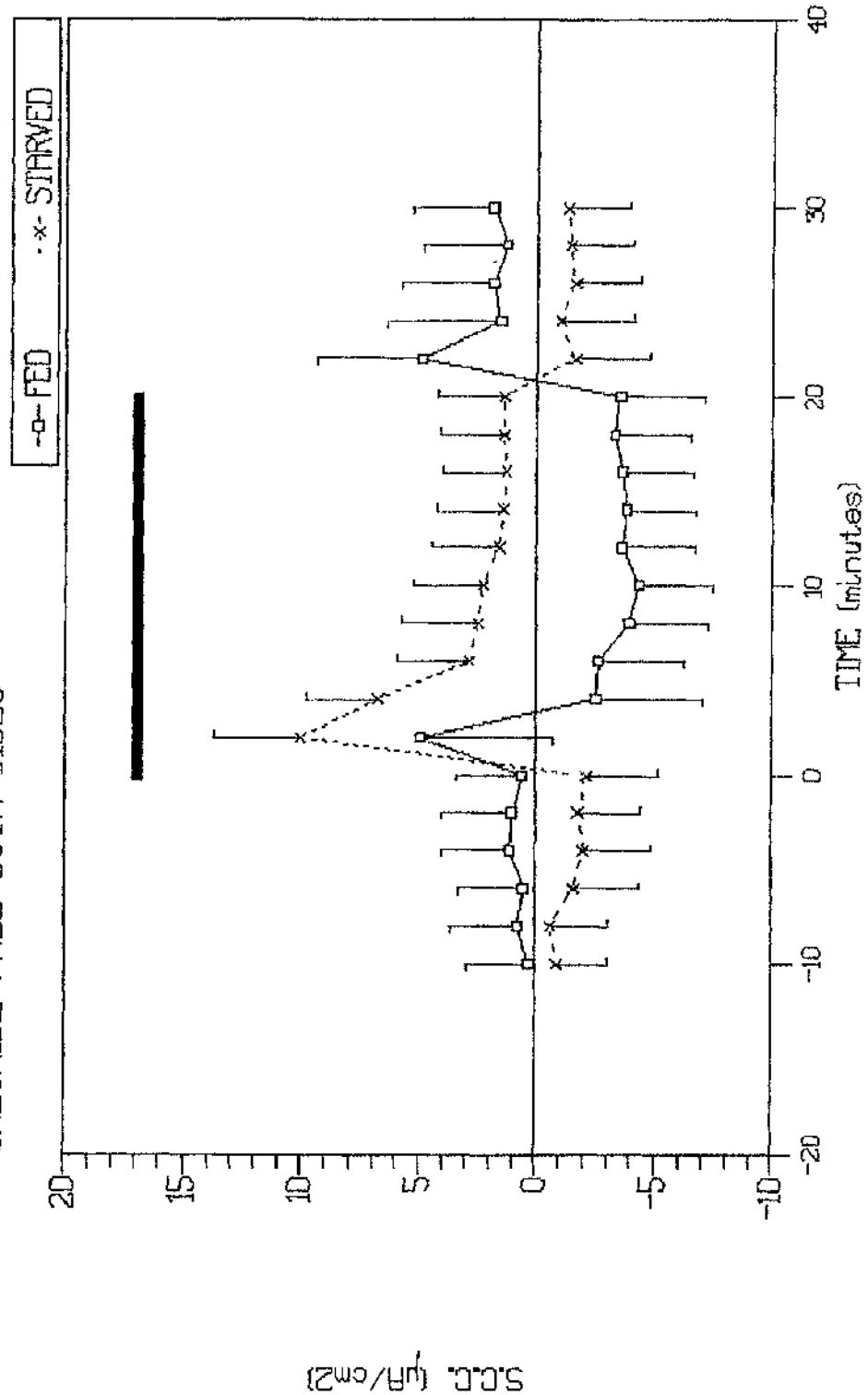


FIG 4.27
CHLORIDE FREE BOTH SIDES

-8.6 and -15.1 $\mu A/cm^2$ respectively. The remaining experiments showed no change in the SCC upon chloride substitution. The transepithelial resistance increased significantly during the test period, the mean change being 61.0 ($\pm$20.3) ohm cm^2, (n=7, $p<0.05$) from 80 to 141 ohm cm^2 upon chloride substitution.

Interestingly, the response is the opposite for starved snails, (Fig 4.27). There is a significant change in the positive direction upon chloride substitution. The mean increase being 4.6 ($\pm$1.4) $\mu A/cm^2$ (n=8, $p<0.01$). There was no significant correlation between the initial SCC and the size of the change observed upon chloride substitution r=-0.058; n=8). All but one experiment showed an initial large, but temporary, increase in the SCC when chloride was substituted. The SCC became stable within eight to ten minutes but at less positive values. However, in all cases these were more positive than the resting values and they were maintained throughout the remainder of the test period. Once the chloride concentration was restored to normal all the experiments showed a complete and full recovery.

Considering both starved and fed snails, there is no significant correlation between the magnitude of change and the initial SCC, r=-0.164 (n=16). There was no significant change in the resistance during the experiments, the mean change being an increase of 26.9 ($\pm$18.2) ohm cm^2 (n=7, $p>0.05$). There was no reverse (negative) spike observed in

any of the experiments in either fed or starved snails immediately after chloride was restored to the bathing media.

Chloride was also substituted on both sides as well as the apical side and the basal side only but under sodium free conditions as standard. When substituted on both sides there was a significant change in the SCC in the negative direction (Fig 4.28). The mean change was -3.6 ($\pm$1.7) $\mu A/cm^2$ (n=7, $p<0.05$). The initial polarity of the SCC had no obvious effect on the size of the response. In all of six experiments using starved snails there was a negative change in the SCC. The one fed snail showed a positive change. This is a similar situation to that found using normal bathing conditions, although the directions of change are opposite. In all the negative change experiments the maximum value was obtained within two minutes and there was then a gradual increase in the SCC over time. Once chloride was restored there was a full recovery in all cases. Interestingly, there was a positive spike in the SCC when chloride was returned, but not when it was removed from the bathing solution. There was a significant increase in the epithelial resistance when the chloride was substituted. The change was 217 ($\pm$30.7) ohm cm^2 (n=7, $p<0.001$), from 86 to 304 ohm cm^2.

When chloride was substituted apically only, under sodium free conditions as control, the SCC became significantly more negative (Fig 4.29). The mean change was -11.7 (2.4) $\mu A/cm^2$

The effect of chloride substitution on the SCC, under sodium free conditions as control. Chloride was absent in the period marked by the bar. Each point represents the mean value (± SE) with n=7 for removal from both sides.

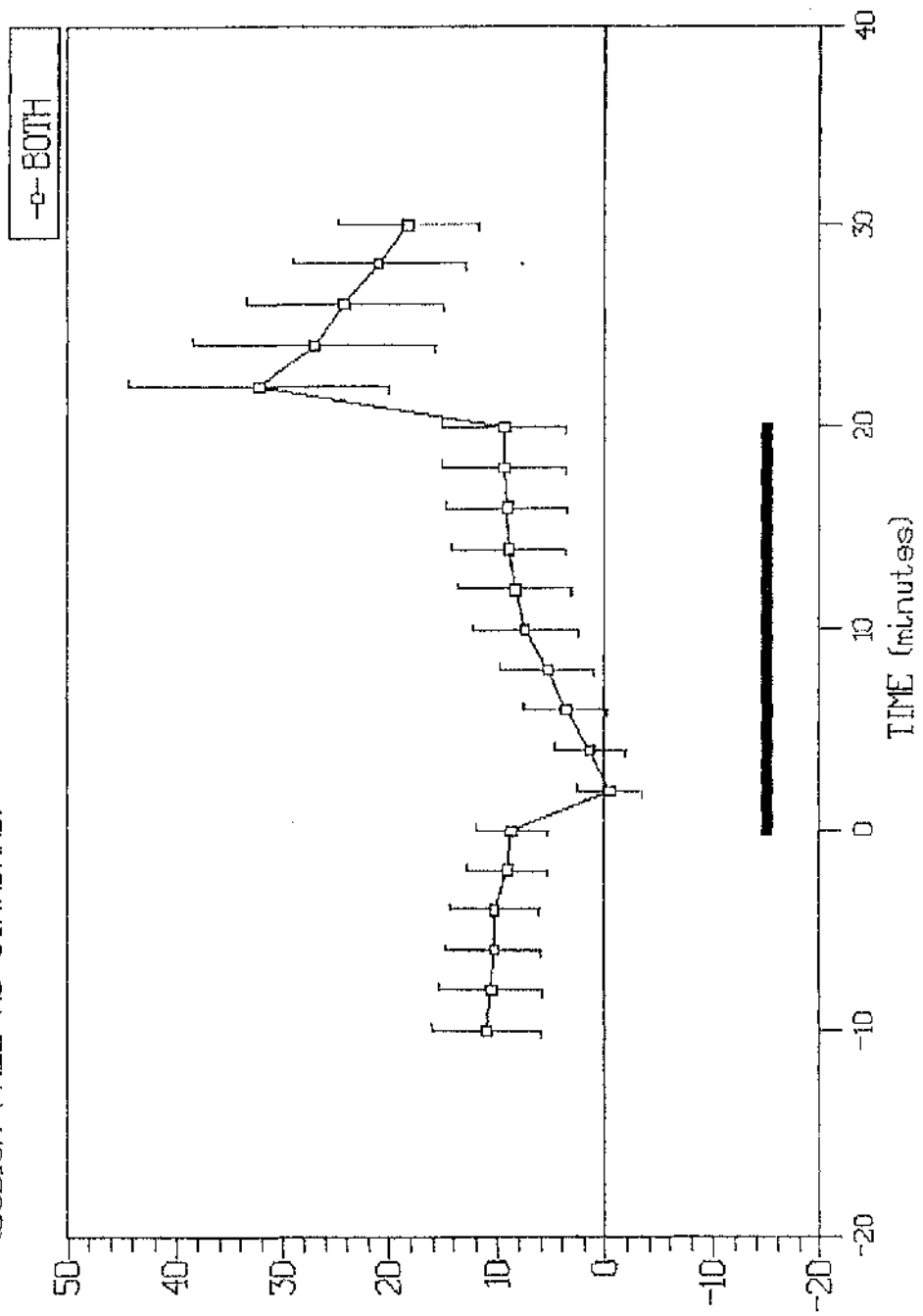


FIG 4.28
CHLORIDE FREE
(SODIUM FREE AS STANDARD)

The effect of chloride substitution on the SCC, under sodium free conditions as control. Chloride was absent in the period marked by the bar. Each point represents the mean value (+ or - SE), with n=6 for apical removal and n=5 for basal removal.

FIG 4.29
CHLORIDE FREE
(SODIUM FREE AS STANDARD)

(n=6, $p<0.005$). The maximal change was obtained within six minutes and in five of the experiments this level was maintained throughout the test period. The remaining experiment showed a gradual rise in the SCC towards the control level, although it did not reach it. There was a significant correlation between the size of the initial SCC and the size of the change, $r=-0.763$ (n=6, Fig 4.29A). Once chloride was restored to the bathing solutions there was a full recovery in all experiments. There was a significant increase in the resistance when chloride was substituted apically only. The mean increase was 74.9 ($\pm$13.7) ohm cm^2 (n=6, $p<0.005$), from 109 to 184 ohm cm^2.

There was a significant change in the SCC when chloride was substituted basally only under sodium free conditions as control (Fig 4.29). The SCC became more positive, the mean change being 6.5 ($\pm$1.5) $\mu A/cm^2$ (n=5, $p<0.01$). There was a wide variation in the time before the maximal change was reached, i.e. four to twelve minutes. Thereafter there was no further change in the SCC in any of the experiments. There was a significant correlation between the initial SCC and the magnitude of change, $r=-0.886$ (n=5, Fig 4.29B). Again there was a wide variety in the recovery achieved once the chloride was restored. In most cases there was a recovery to a level that was twice the resting values, for initially negative SCC, or one that was half the resting values if the SCC was initially positive. The others showed partial to full

The correlation between the initial SCC and the magnitude of change in the SCC upon chloride substitution apically only, under sodium free conditions. Each point represents one experiment. The line is a best fit linear regression line.

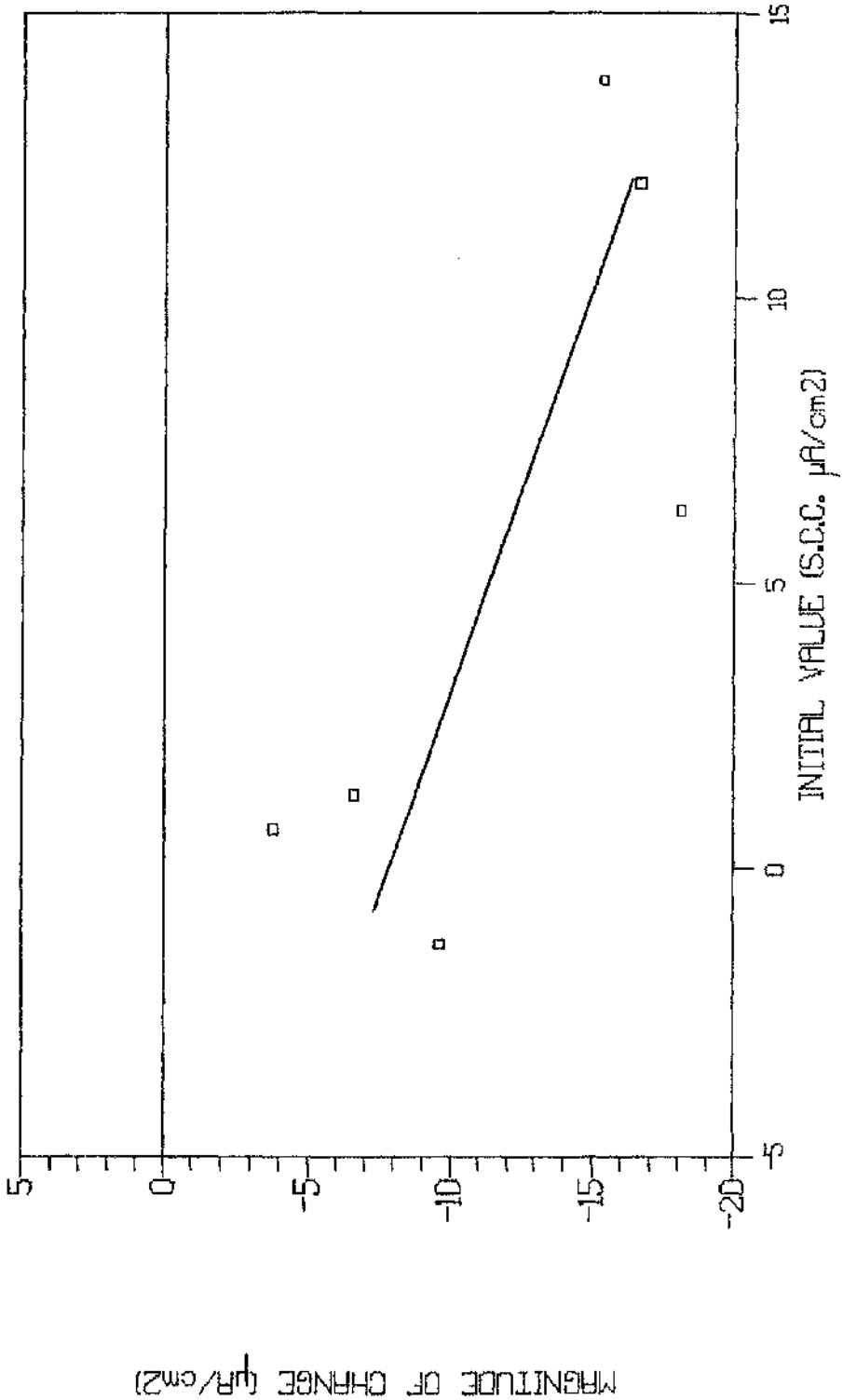
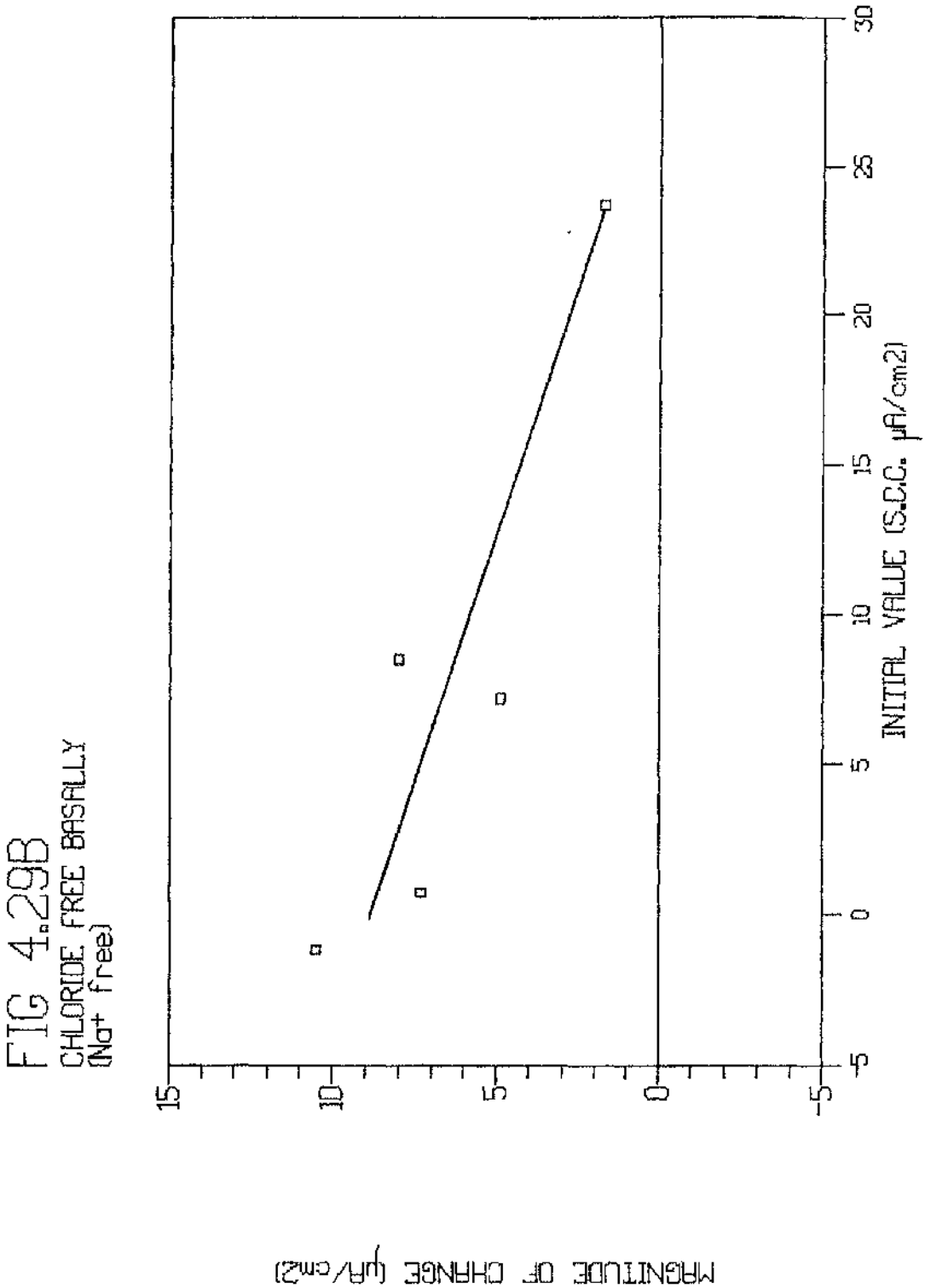


FIG 4.29A
CHLORIDE FREE APICALLY
(Na+ free)

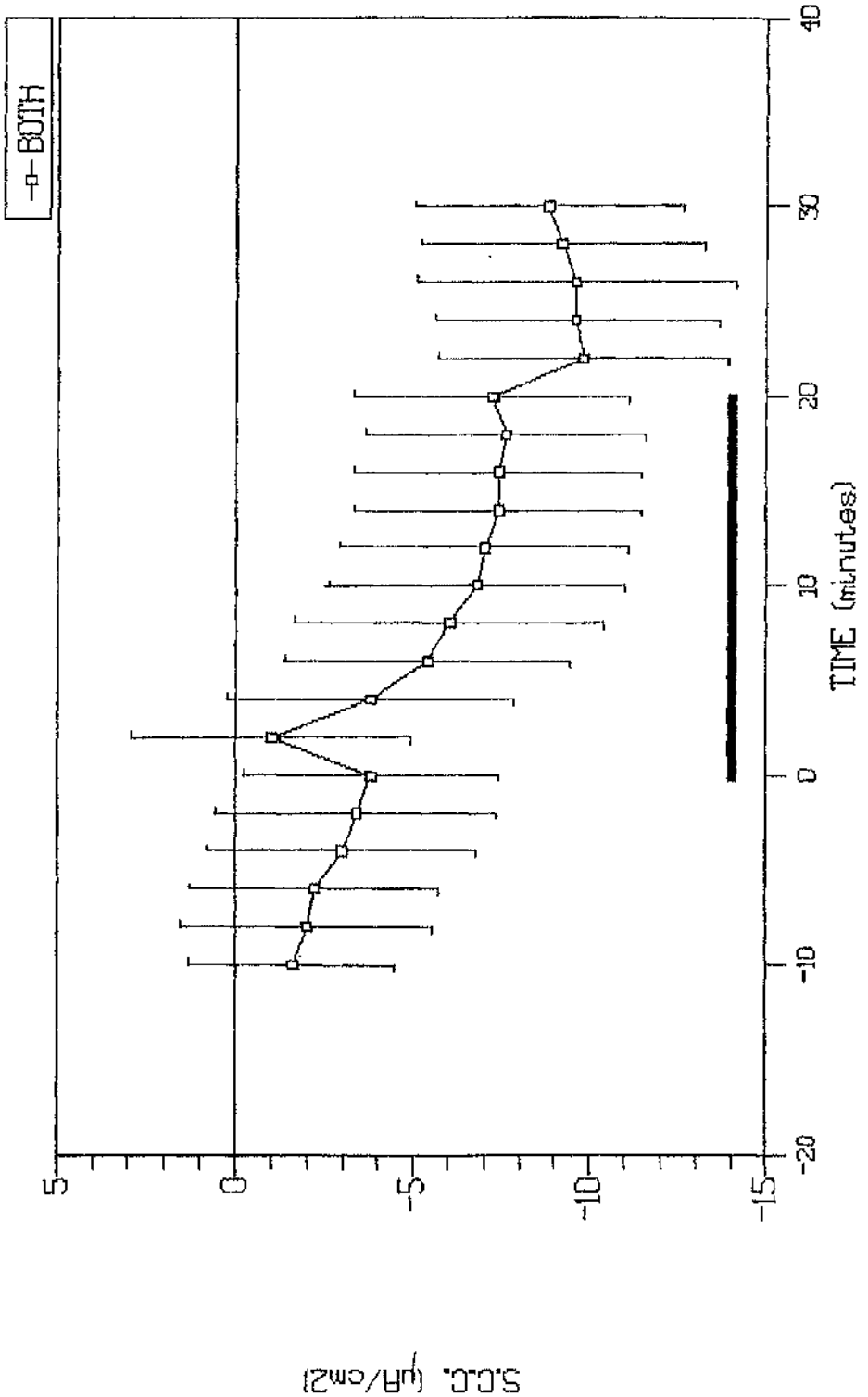
The correlation between the initial SCC and the magnitude of change in the SCC upon chloride substitution basally only, under sodium free conditions as control. Each point represents one experiment. The line is a best fit linear regression line.



recovery. Again there was a significant increase in the resistance during chloride free conditions, the mean change was 82.6 ($\pm$3.5) ohm cm^2 (n=4, $p<0.001$). This was independent of the initial polarity of the SCC.

Chloride was also substituted on both sides under normal conditions, but with ouabain present at a concentration of 10mM basally only. Under these conditions there was a significant change in the SCC in the negative direction (Fig 4.30), the mean change was -3.0 ($\pm$1.0) $\mu A/cm^2$ (n=5, $p<0.05$). This change was similar to that obtained under sodium free conditions (-3.6 $\mu A/cm^2$) and this would be expected since both treatments should abolish sodium transport (see below). The initial polarity of the SCC had no effect on the size of the decrease. The maximal change in the SCC was obtained between eight and ten minutes and in all cases there was no further change over the remainder of the test period. In two of the experiments there was a positive spike when the chloride was removed, but the rest did not show this. When the concentration was returned to normal there was no recovery evident in any of the experiments. As with most of the other chloride free experiments there was a significant increase in the transepithelial resistance when chloride was substituted on both sides under these conditions. The mean change was 45.7 ($\pm$16.1) ohm cm^2 (n=4, $p<0.05$).

The effect of chloride substitution (both sides) on the SCC, with ouabain present as control. Chloride was absent in the period marked by the bar. Each point represents the mean value (± SE) of 5 experiments.



Organic substances that are likely to be transported.

D-glucose and glycine were added to the bathing media to see what effects, if any, they had on the SCC and also to see if the crop responded in the usual way to organic substances.

D-Glucose.

The transport of D-glucose is usually linked to sodium transport. D-glucose was added apically only and basally only under normal conditions and also apically only but under sodium free conditions as standard. The saturation curve was also examined.

The addition of 10mM D-glucose to the apical side only resulted in a significantly less positive SCC (Fig 4.31). The mean change was -6.1 ($\pm$2.6) $\mu A/cm^2$ (n=8, $p<0.05$). The largest changes were observed in experiments where the control SCC was initially positive and there was a significant correlation between the initial SCC and the size of the change, $r=-0.879$ (n=8, Fig 4.31A). These changes are in the opposite direction to those found in other epithelial preparations, e.g. frog duodenum and rat small intestine, and not what should occur from a simple increase in sodium transport. In most of the experiments (n=6) there was a maximal, and sustained, change between two and eight minutes. The other experiments showed a continuous gradual change over the complete test period.

The effect of 10mM D-glucose on the SCC. Glucose was present in the period marked by the bar. Each point represents the mean value (+ or - SE), with n=8 for apical addition only and n=8 for basal addition only.

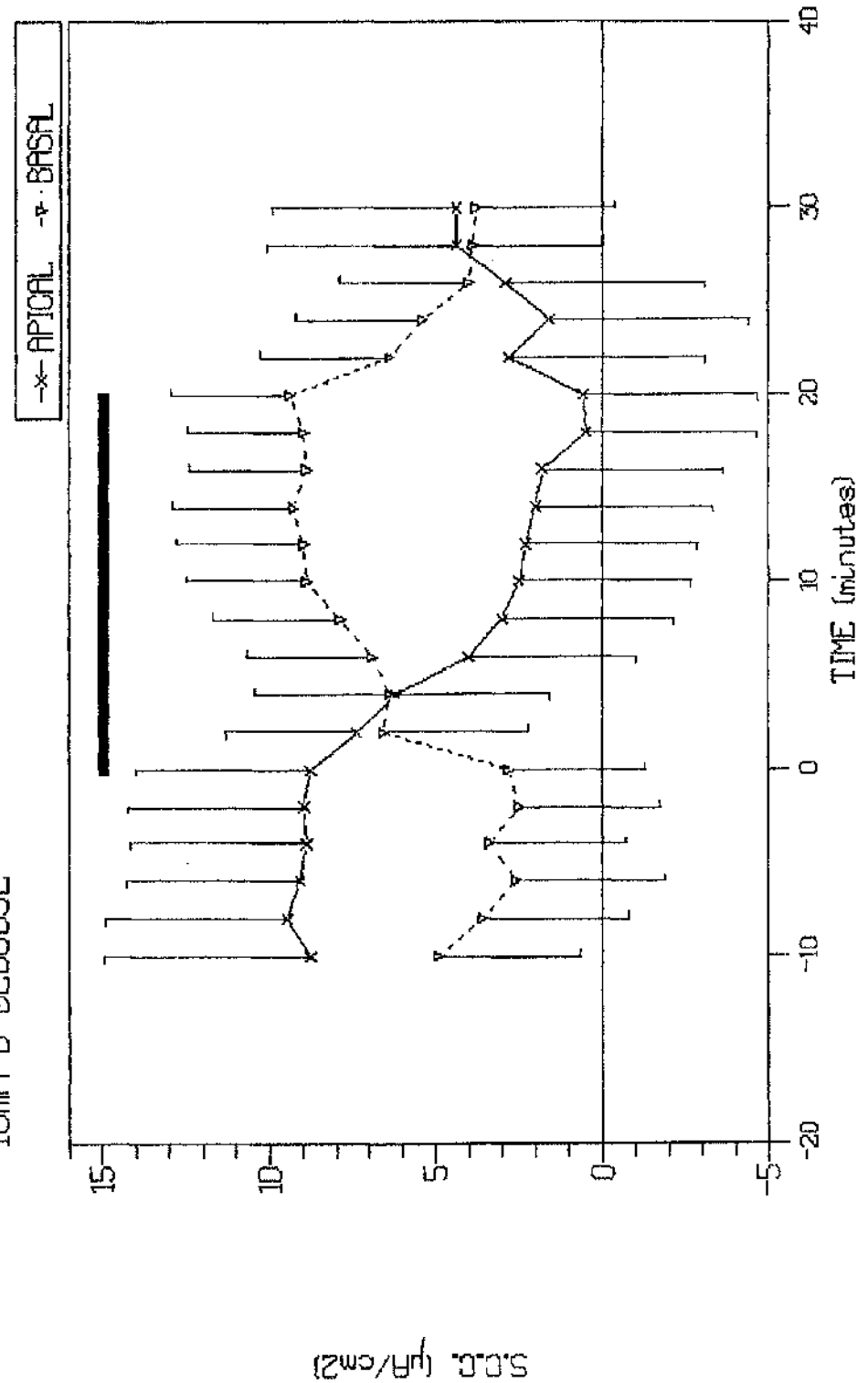


FIG 4.31

10mM D-GLUCOSE

The correlation between the initial SCC and the magnitude of change in the SCC with 10mM D-glucose present on the apical side only. Each point represents one experiment. The line is a best fit linear regression line.

There was a split in the responses once D-glucose was removed from the bathing solution. In most cases there was no recovery evident, while in three there was a full recovery, two immediate and one more gradual. There was no significant change in the transepithelial resistance when D-glucose was present apically. The mean change was -1.3 ($\pm$2.4) ohm cm^2 (n=8, $p>0.1$), from 65.5 to 64.2 ohm cm^2.

Basal addition also resulted in a significant change in the SCC (Fig 4.31). The mean SCC became more positive, the change being 4.9 ($\pm$1.1) $\mu A/cm^2$ (n=8, $p<0.01$). However the correlation between the SCC and the magnitude of change obtained on the addition of D-glucose was not significant ($r=-0.582$, n=8). The maximal change was obtained between two and fourteen minutes, the majority between eight and fourteen minutes. This could reflect the fact that the epithelium still had its supporting muscle and connective tissue backing. This would decrease the access to the epithelial cells and so result in longer times for the changes to appear. No further change was observed once the maximum value was reached. There was a full recovery in all but one experiment once the D-glucose was removed from the bathing solution. This one experiment showed no recovery.

There was no significant change in the average resistance during the test period. The mean change was 10.0 ($\pm$6.2) ohm cm^2 (n=6, $p>0.05$), from 71 to 81 ohm cm^2.

To examine whether the change caused by D-glucose added apically was sodium dependent, 10mM was added apically but under sodium free conditions on both sides as standard. This resulted in no significant change in the SCC (Fig 4.32). The mean change was only -0.8 ($\pm$0.5) $\mu A/cm^2$ (n=8, $p>0.05$). In five of the experiments there was no real change evident. In three others there was a small decrease, up to -3.1 $\mu A/cm^2$, that was maximal at four minutes. The resistance was not significantly changed during the experiments. The mean change was 17.9 ($\pm$11.1) ohm cm^2 (n=7, $p>0.05$).

A saturation curve was obtained for D-glucose by adding increasing concentrations to the mucosal solution under otherwise normal conditions. The concentrations were 0, 10, 20, 30 and 40mM (Fig 4.33). The results obtained are summarised in Table 4.2. There was no further increase in the SCC between 30 and 40mM. It can be concluded that the carrier was approximately saturated at 30mM. In all the experiments the maximal change was obtained between two and six minutes with no further change after this time. From the average dose response curve (Fig 4.33), the concentration giving the half maximal decrease in the SCC is approximately 12mM.

In two of the experiments 10mM glycine was also added to the mucosal solution, after the final addition of 40mM D-glucose. This resulted in a further negative change in the SCC in both cases of -7.4 $\mu A/cm^2$ and -8.8 $\mu A/cm^2$.

The effect of 10mM D-glucose (apically only) on the SCC, under sodium free conditions as control. Glucose was present in the period marked by the bar. Each point represents the mean value (± SE) of 8 experiments.

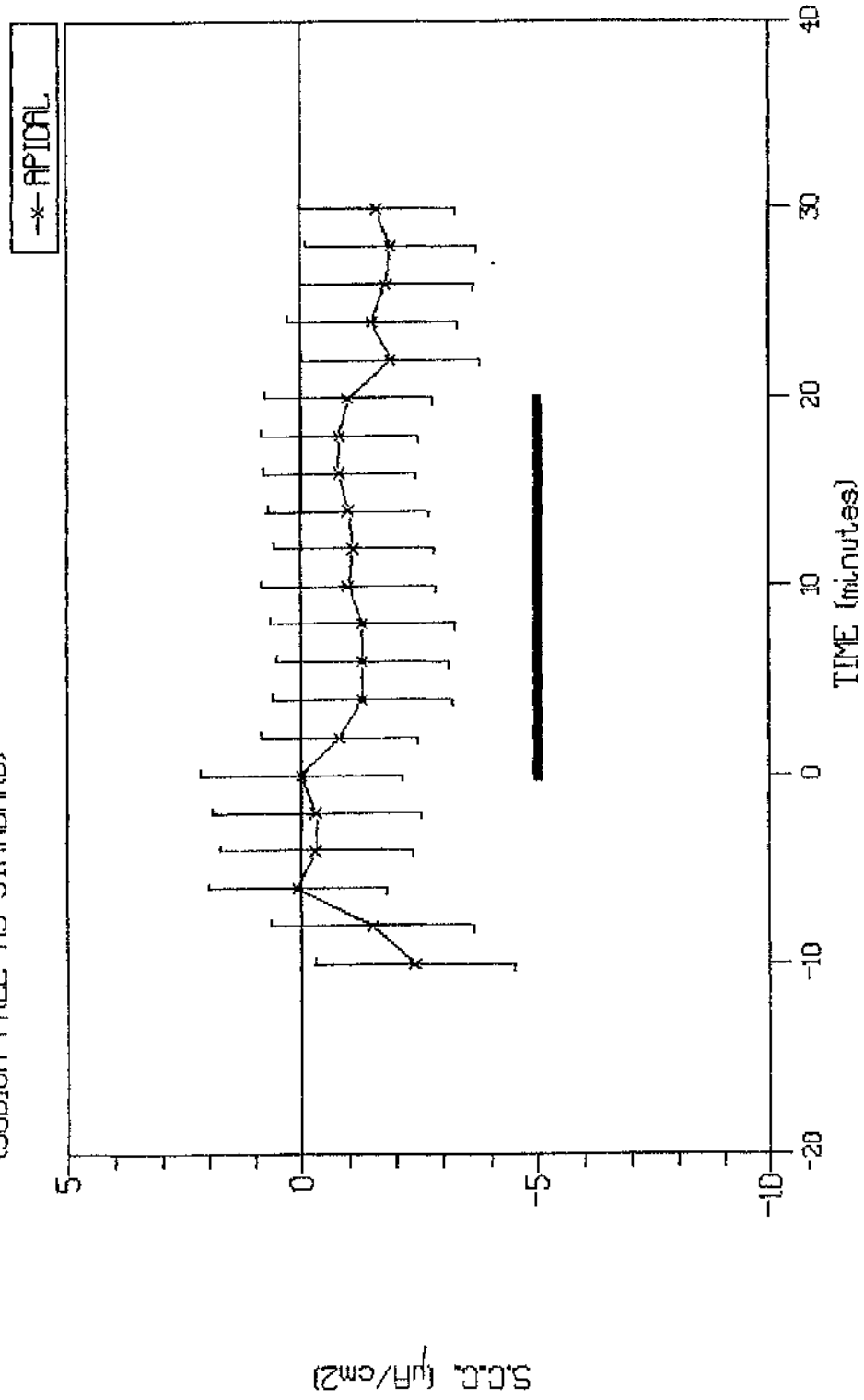


FIG 4.32
10mM D-GLUCOSE
(SODIUM FREE AS STANDARD)

The mean saturation curve for apical D-glucose addition. Each point represents the mean of 4 experiments, to help with clarity the standard errors are not shown (see table 4.2 for errors).

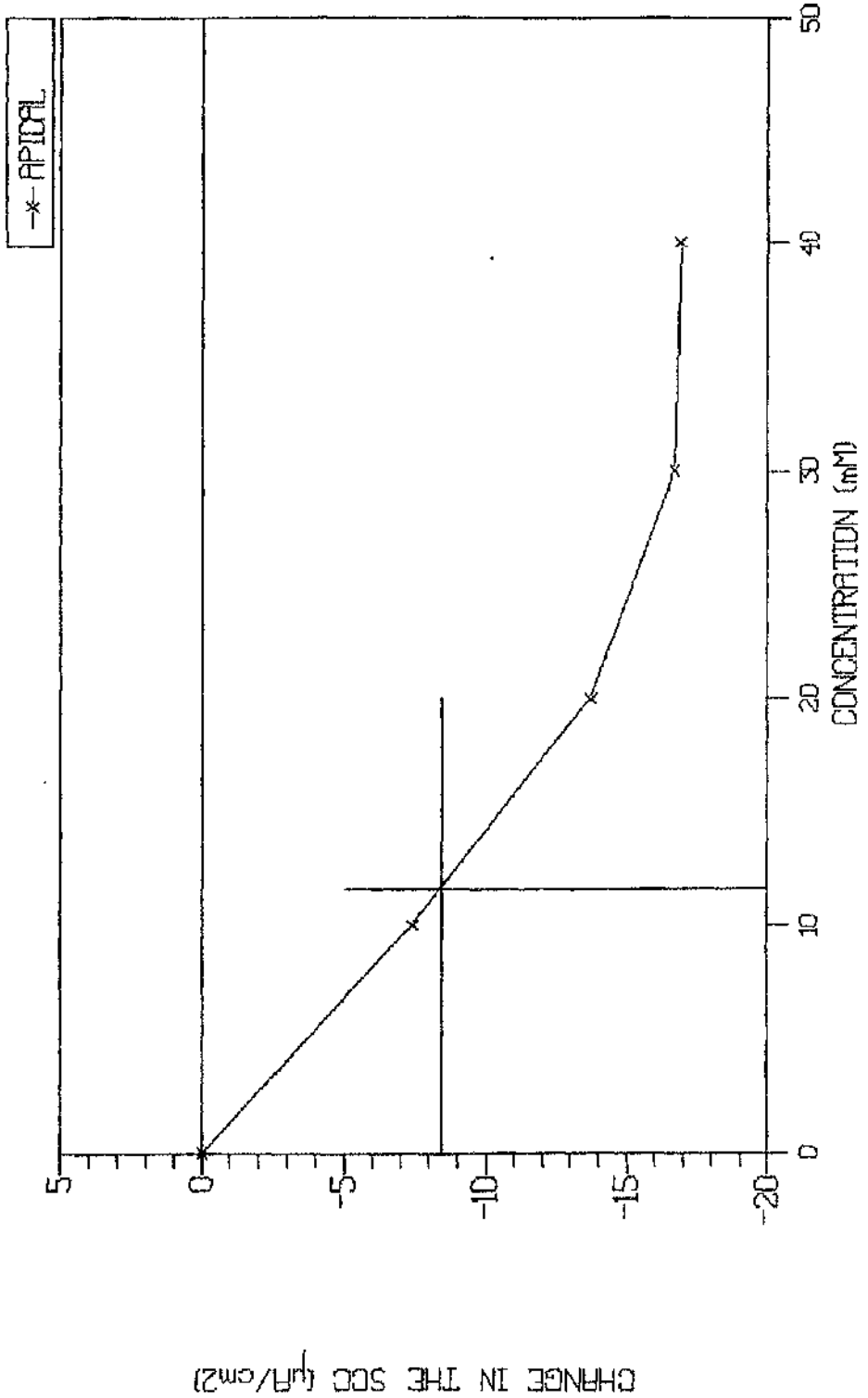


FIG 4.33
DOSE RESPONSE CURVE FOR D-GLUCOSE

Glycine.

Glycine is known to be transported via sodium dependent systems in a number of epithelia, e.g. frog duodenum. To examine its transport in Helix crop it was added at a concentration of 10mM to the apical side only and the basal side only, under normal bathing conditions. It was also added apically only but under sodium free conditions as standard. A saturation curve was also obtained by adding concentrations of 0, 10, 20, 30, 40 and 50mM to the apical side only under normal bathing conditions.

Apical addition (10mM) resulted in a significantly more negative SCC (Fig 4.34). The mean change was -7.5 ($\pm$0.9) $\mu A/cm^2$ (n=9, $p<0.001$). The initial response on addition varied such that five experiments, all having initially negative SCC, exhibited a slow decrease in the SCC throughout the whole test period, in that it became even more negative. The remaining four however showed a maximal decrease within two to ten minutes, after which this level was maintained. There was also a variation in the response once glycine was removed from the bathing solution. In most cases (n=5) there was at least some recovery in the SCC. Four had a full recovery within ten minutes with one only recovering partially. The remaining four experiments exhibited no recovery. There was no significant change in the transepithelial resistance when glycine was present in the

The effect of 10mM glycine on the SCC. Glycine was present in the period marked by the bar. Each point represents the mean value (+ or - SE), with n=9 for apical addition only and n=8 for basal addition only.

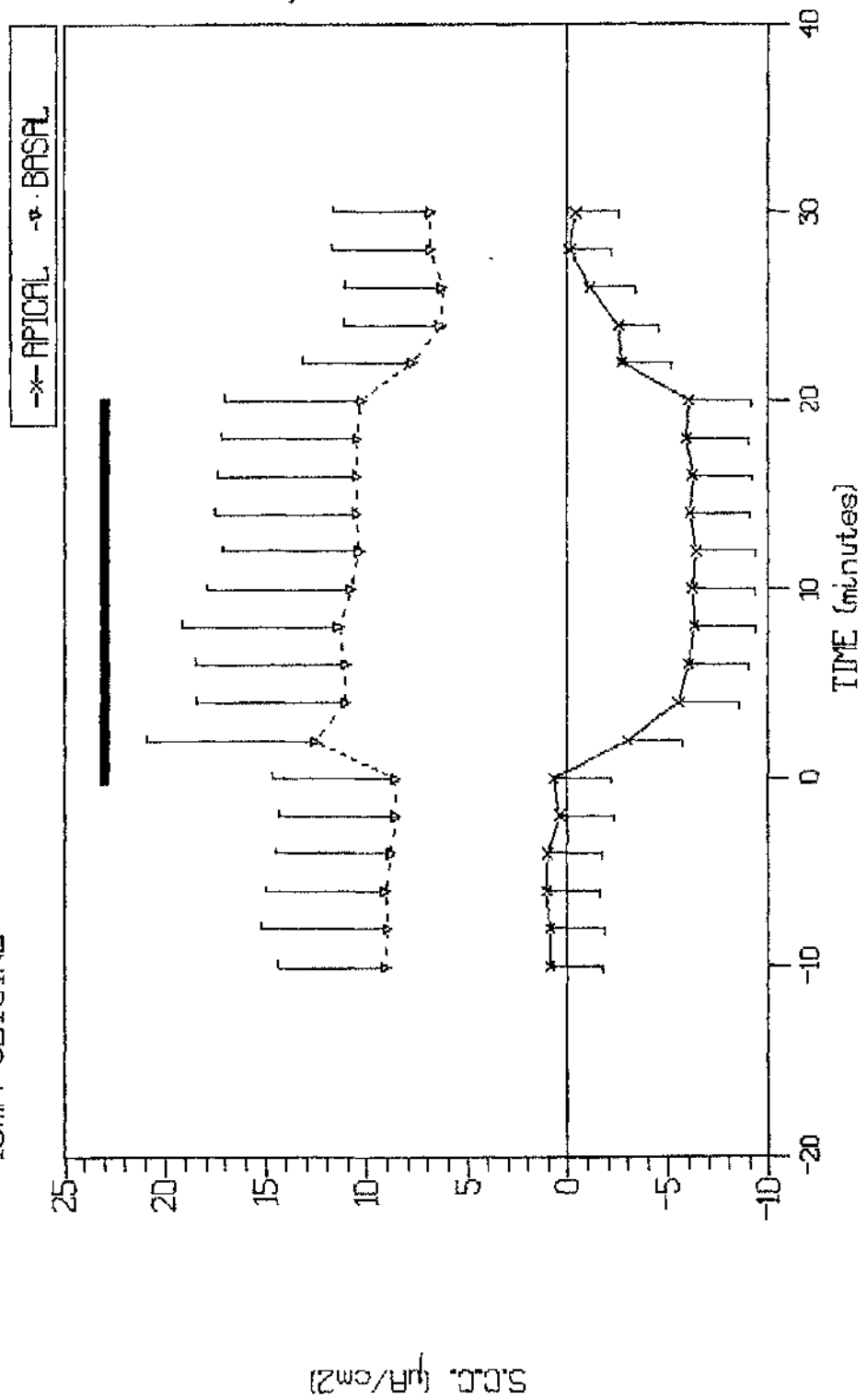


FIG 4.34
10mM GLYCINE

bathing solution. The mean change was only -2.9 (±6.3) ohm cm^2 (n=9, p>0.1).

The addition of glycine to the basal side of the epithelium did not result in any significant change in the mean SCC (Fig 4.34), though there was sometimes an increase as with glucose. The mean positive change was 2.0 (±1.5) $\mu A/cm^2$ (n=8, p>0.1). There was no real change observed in six of the experiments. The other two showed a maximal increase at two and eight minutes with one then having a gradual fall in the SCC. The other had no subsequent change. There was a full recovery in the SCC in both cases once the glycine was removed from the basal side. The resistance increased significantly during the test period. The mean change was 5.4 (±2.0) ohm cm^2 (n=7, p<0.05). There was no correlation between the SCC and the size of the resistance increase.

Glycine was also added apically only, but under sodium free conditions as standard. This was to examine whether sodium was required for the changes observed under normal bathing conditions. Only a small change was observed (Fig 4.35). The mean became more negative on average by -1.4 (±1.1) $\mu A/cm^2$ (n=10, p>0.1). Again the size, and in this case the direction, of change were dependent on the polarity of the control SCC, there being a significant correlation between the initial SCC and the magnitude of change, r=-0.813 (n=10). In those that did show any change there was a great variation in

The effect of 10mM Glycine (apically only) on the SCC, under sodium free conditions as control. Glycine was present in the period marked by the bar. Each point represents the mean value (± SE) of 10 experiments.

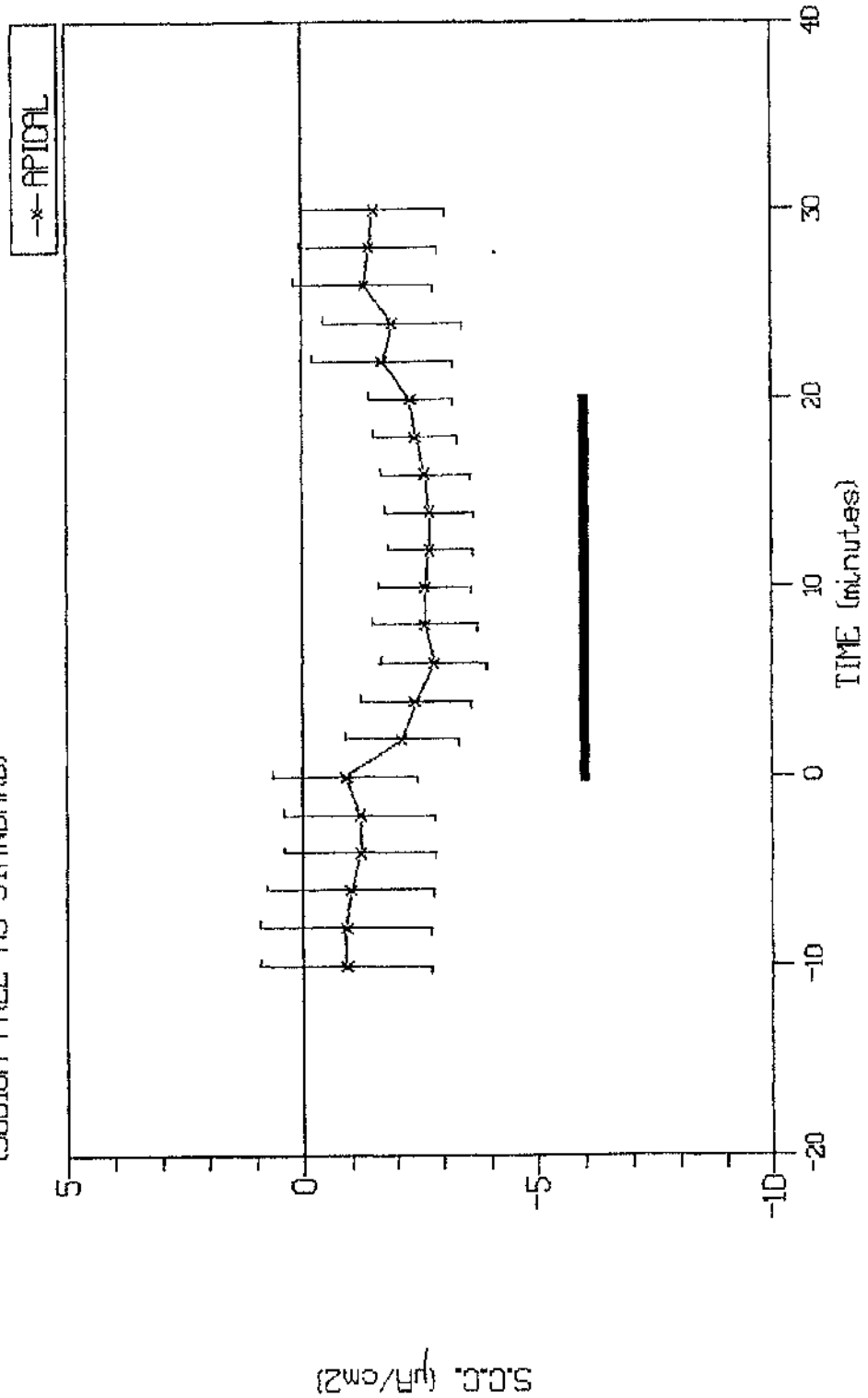


FIG 4.35
10mM GLYCINE
(SODIUM FREE AS STANDARD)

the response once the glycine was removed from the bathing solution. This ranged from no recovery (n=2) to eventual full recovery (n=3). The remaining experiment showed only a partial recovery. There was a significant increase in the resistance, the mean change being 10.0 ($\pm$4.5) ohm cm^2 (n=7, $p<0.05$).

A saturation curve was also obtained for glycine by adding varying concentrations to the mucosal solution only (Fig 4.36). The results are summarised in Table 4.2. There was minimal increase in the SCC between the addition of 40mM and 50mM and so it is reasonable to assume that the carrier was virtually saturated at 40mM. The maximal changes were obtained between two and eight minutes with the majority observed between two and four minutes. The concentration of glycine which gives half maximal change in the mean SCC was approximately 13.0 mM as calculated from the average dose response curve (Fig 4.36).

The mean saturation curve for apical glycine addition. Each point represents the mean of 4 experiments, to help with clarity the standard errors are not shown (see table 4.2 for errors).

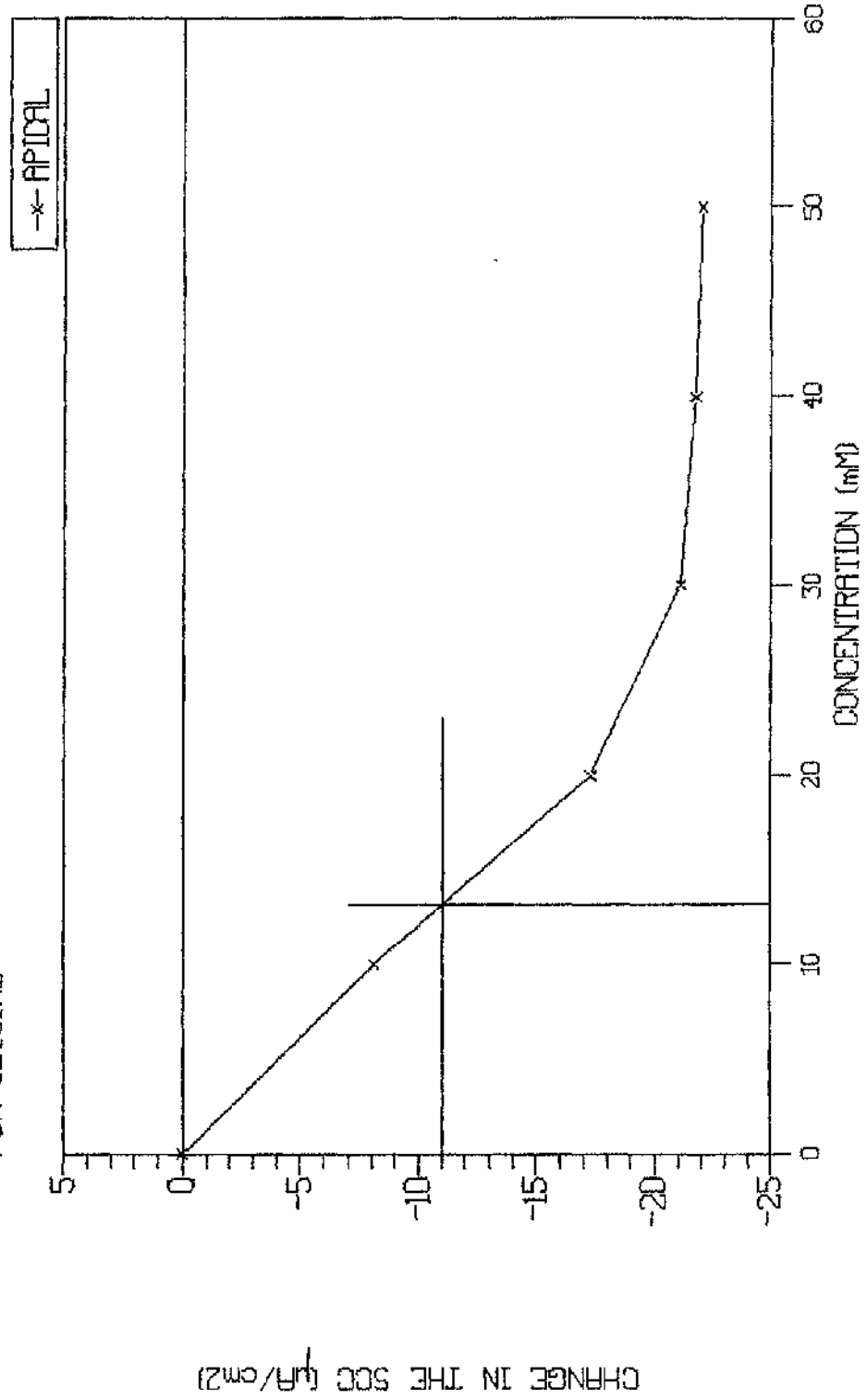


FIG 4.36
DOSE RESPONSE CURVE FOR GLYCINE

Saturation Curves		Mean SCC	S.E.	N
D-glucose	0mM	0.0μA/cm²	0	4
	10mM	-7.4μA/cm²	.15	4
	20mM	-13.7μA/cm²	.5	4
	30mM	-16.7μA/cm²	.8	4
	40mM	-16.9μA/cm²	.8	4
Glycine	0mM	0.0μA/cm²	0	4
	10mM	-8.1μA/cm²	.45	4
	20mM	-17.3μA/cm²	.55	4
	30mM	-21.1μA/cm²	.9	4
	40mM	-21.7μA/cm²	.9	4
	50mM	-22.0μA/cm²	.95	4

Table 4.2 Data for dose response curves for D-glucose and Glycine

Section 5 - DISCUSSION

General features of the crop epithelium in Helix

Before discussing ion transport in Helix aspersa in detail some important general features of the crop epithelium should be noted. The isolated crop epithelium, when bathed on both sides by a physiological saline free of organic substances, exhibits a spontaneous potential difference of about a millivolt. The epithelium is of the "leaky" sort with a low transepithelial resistance. This low resistance results in a low potential difference in spite of a quite high SCC.

When first set up, always with stirring of the apical solution, the PD and SCC could be either positive or negative. These would then move towards zero and a steady state. This is unusual since in most epithelial preparations bathed with normal physiological saline the PD and SCC would be of constant sign (or polarity). This is a complicating, but interesting feature of the preparation. This could be accounted for in a number of ways. There could, for example, be net sodium transport sometimes one way and sometimes the other. This could perhaps be achieved by different cells. The histological work would support this since the epithelium seems to be composed of two cell types, although one type predominates. Could it be that both cations and anions are

both actively transported in the same direction with a variable balance between them? It was hoped that suitable manipulation of the physiological saline might have allowed the study of two such separable systems.

The major limitation of the SCC method is its inability to measure actual ion fluxes. It only gives an indication of net flux and even then a number of ions could be involved. In effect this means that there could be huge sodium and chloride transport, linked or not, which partially or exactly cancel. This would result in a small SCC being observed at high ion transport rates. It should be noted that a SCC of zero does not mean that there is no ion transport.

The stable SCC and PD under control conditions were dependent on the state of feeding prior to experimentation, SCC being more often negative in starved snails than in fed. This effect is similar to that observed for chloride-free conditions in starved snails, discussed below. These two facts are probably linked since starved snails are much more sensitive to chloride substitution than are fed snails. The polarity of the SCC is always quoted with respect to the basal surface, such that a "negative SCC" means that the basal side of the epithelium is negative with respect to the apical side. The direction of change in the SCC is quoted as being in either a negative or positive direction. This is regardless of the initial polarity of the SCC.

The control conditions also have an important effect on the stable SCC. Under sodium free conditions the mean stable, initially positive, SCC was 7.5μA/cm^2 and the mean initially negative SCC was -4.2μA/cm^2 compared to 10.9μA/cm^2 and -11.8 μA/cm^2 (Table 4.1) for standard control conditions. Chloride substitution also seems to influence the SCC. The effect was greater if the SCC was initially positive than when negative since the mean positive SCC was 2.1μA/cm^2 under sodium and chloride free conditions. The mean negative SCC was essentially unchanged being -5.1μA/cm^2. In other words, although the removal of both sodium and chloride affect the stable SCC, other ions must also contribute towards the SCC since there is a residual but significant SCC when both sodium and chloride are absent from the bathing media.

Initial decline in the SCC

The time course of the SCC after the epithelium was initially mounted was observed to have two phases. There was a initial rapid decay which lasted up to twelve minutes. This phase took the SCC towards zero, regardless of the initial polarity of the SCC. It was then followed by a more stable state where the decay was more gradual. This second phase lasted more than thirty-five minutes (Fig 4.7). During this phase the polarity of the SCC reversed in two of the experiments. These two phases have been reported in other epithelial preparations, e.g. Manduca sexta (Blankemayer, 1976),

Hyalophora cecropia (Harvey and Nedergaard, 1964) and Antheraea pernyi (Wood, 1972). Cioffi and Harvey (1981) observed a 60% decrease in the SCC during the first rapid decay phase. In Helix the decay is similar during the initial phase. Cioffi and Harvey found that the first and second phases were thirty and ninety minutes long respectively. In Helix they lasted up to twelve and thirty five minutes respectively. The initial phase has been attributed to potassium movement in Antheraea pernyi. Wood (1972) suggested that it was not due to a change in the ability to transport potassium, but was due to a loss of potassium from the tissue after it was mounted. In Helix when the initial set up was under potassium free conditions as control (n=3) there was still a two-phase decay in the SCC. However the initial phase was more pronounced. In two of these experiments the size of the decay, on average 90% of the starting value, was also much greater than in standard saline. This phase only lasted four minutes and ten minutes in the other. This is consistent with potassium loss from the tissue since under potassium-free conditions the electrochemical gradient for ion movement out of the epithelial cells would be increased. The initial decline in the SCC when the preparation is first set up could be due to a number of things. Is the preparation being deprived of a hormonal influence, of a metabolic substrate or even of a substance that is transported along with sodium, e.g. glucose? We will discuss the subject of transported organic substances later.

As also discussed later, zero SCC does not imply zero transport, but a balance of anion and cation transport. This complicates the interpretation of "a decay towards zero".

Stirring

Apical stirring is important in the maintenance of a stable SCC (Fig 4.5). When stirring was stopped the SCC moved towards zero. Enyikwola and Burton (1983) also found that apical stirring was required for a stable SCC in Helix pomatia mantle epithelium. The need presumably relates to the unstirred layer immediately next to the apical surface of the epithelium where there is a deep brush border. Thus the concentrations of transported solutes could be very different at the apical cell surface as compared with the bulk solution. The unstirred layer has been demonstrated in other epithelial preparations (Winnie, 1973, Lukie et al., 1974, Enyikwola, 1987). Basal stirring has no significant effect on the stable SCC (Fig 4.6). This is presumably because the crop epithelium was mounted complete with its supporting basal muscle layers. It proved impossible to remove these layers without causing significant damage to the epithelium. They may, therefore, have delayed the effects of changes in the basal solution.

RESISTANCE

The transepithelial resistance is a fairly uncomplicated

matter and can be dealt with relatively easily. The mean transepithelial resistance of 68 ohm cm^2 is that of a classic "leaky" epithelium. It lies midway in the range reported by Fromter and Diamond (1972) of 6-113 ohm cm^2 and is not unlike the resistance in, e.g., Necturus proximal tubule (80 ohm cm^2) and rat jejunum (30 ohm cm^2). The resistance is low when compared with those of "tight" epithelia, e.g. toad urinary bladder (800 ohm cm^2) and frog skin (2000 ohm cm^2). Moran and Garretson (1988) give values of 14 ohm cm^2 for Aplysia oesophagus and 67 ohm cm^2 for proximal intestine. Gerencser (1982) gives a range of 20-30 ohm cm^2 for the resistance of A. juliana intestinal epithelium. The low resistance implies that there is a large paracellular shunt pathway through which most of the current could pass. Frizzell and Schultz (1972) have shown that 85-90% of the current passed across the rabbit ileum was carried via the shunt pathway. The resistance is greatly raised when chloride is substituted by sulphate. This is regardless of the side on which the substitution takes place. Sulphate being a much larger and less permeant anion cannot move easily through the junctional complexes between the cells. This results in an increase in the transepithelial resistance.

Other factors which change the resistance are potassium-free conditions on both sides of the tissue; calcium-free on both sides and ouabain applied basally. Why ouabain should increase the resistance is unknown. Since its action is

cellular, on the sodium pump, and not paracellular, it should have no large effect on the resistance. None of the other inhibitors studied had any significant effects on the resistance. Calcium and potassium only affect the resistance when removed from both sides of the epithelium. This could be explained if potassium and calcium can carry significant current between the cells despite their low concentrations. However, it could be due to changes in the fixed charges on the junctional complexes themselves which could decrease the paracellular flux of other ions.

Inhibitors

Ouabain

In the crop addition of this inhibitor to the basal side of the epithelium resulted in a more negative SCC (Fig 4.9). Ouabain is accepted as inhibiting the sodium pump and its action basally in the crop indicates that the sodium pump is situated in the basolateral membrane, as is usual. Sodium is usually absorbed electrogenically in the apical to basal direction, making the basal side positive. The addition of ouabain would be expected to inhibit this and so the basal side would become more negative, as was found. Thus, in Helix crop, sodium absorption may be expected to be achieved by the activity of the Na/K pump.

Amiloride

Amiloride is an inhibitor of sodium/hydrogen exchange, although its action is not specific, since it will also block sodium conductive channels and at high concentrations inhibit the sodium pump. It is also known to block negatively charged junctional channels, and calcium channels in nerve and muscle. Nevertheless its action apically in the snail crop epithelium at least suggests the presence of sodium conductive channels in the apical membrane (Fig 4.14). Its actual effects are consistent with inhibition of sodium absorption from the apical to the basal side since the SCC becomes more negative, the mean change being -4.8 $\mu A/cm^2$. The significant increase in the resistance is also consistent with the reduction of ion permeability (both through and between the cells). Sodium transport would not be totally abolished by amiloride since the supposed Na/K/2Cl cotransporter would remain unaffected (see below). This would account for the fact that the decrease in the SCC is less for amiloride addition than for basal ouabain addition or for apical sodium removal.

Piretanide

Piretanide, a "loop" diuretic related to bumetanide, was effective when added to the apical solution bathing the crop epithelium (Fig 4.10), but there was no change in the SCC when added basally (Fig 4.10). Upon apical addition the SCC became

more negative by -4.1 $\mu A/cm^2$. This is consistent with a reduction in net cation movement, probably sodium, from the apical to the basal side. The "loop" diuretics like furosemide, bumetanide and piretanide are known to inhibit linked sodium/chloride/potassium transport (Schlatter et al., 1983, McGahan et al., 1977). For a review of Na/K/CL cotransport see Palfrey and Rao (1983) and for sodium chloride coupled transport see Frizzell, Field and Schultz (1979). This triporter has been shown to be present in a large number of epithelia in both the vertebrates and invertebrates. The action of piretanide is compelling evidence for the apical location of a Na/K/2Cl triporter in the snail crop epithelium. In the crop there are two routes of sodium entry into the epithelial cells one via the triporter and the other via conductance channels. Since apical piretanide would only inhibit sodium entry via the triporter the actual change in the SCC would be less than that for ouabain, which is what was observed.

Barium chloride

Both the apical and basal membranes of the crop epithelium would seem to be potassium conductive. The barium ion has been shown to block potassium conductance via conductance channels in a number of preparations. Nagel (1979) and Neilson (1979) showed this for the inward, basolateral membrane of frog skin In the seahare Moran and Garretson

(1988) concluded, from their barium and high potassium experiments, that the apical membrane of the intestinal epithelium was potassium conductive, since these conditions resulted in a large depolarisation of the apical membrane P.D.. In Helix crop barium added to only one side or the other resulted in a significant change in the SCC (Fig 4.12). This is consistent with potassium conductance channels being present in both sides of the epithelium. Apical addition resulted in a slightly greater effect on the SCC than did basal. Apical addition made the SCC more positive, by 16.8 $\mu A/cm^2$ whereas basal addition made it more negative, by a mean of -13.4 $\mu A/cm^2$. This tends to suggest that although potassium can move in or out of the epithelial cells across either membrane the net flux is actually from the basal to the apical side. It is unknown whether the 5mM concentration of barium chloride that was used in these experiments would block all the available potassium conductance channels. Barium would also result in the cellular potassium concentration rising if the sodium pump was stimulated. This could explain the results obtained using barium apically under potassium free conditions (Fig 4.13), the mean SCC became more negative (instead of more positive, as above) by -4.1 $\mu A/cm^2$. Under potassium free conditions the sodium pump would be inhibited and there would also be no net potassium flux across the epithelium. This in turn could result in a net leak of cellular potassium out through the membrane conductance channels. If more leaked out apically than basally the apical

solution would become more positive with regard to the basal side and the SCC would therefore become more negative. If, however 5mM barium does not block all the channels a small leak would be expected and so a smaller negative change in the SCC would be anticipated, which is what was observed.

Thiocyanate

There is evidence in the Helix crop for primary chloride transport partially linked to sodium absorption. The active step is across the basal membrane. Thiocyanate, which is an inhibitor of active chloride transport (Gerencser, 1984; Epstein et al., 1973), had no significant effect when added apically (Fig 4.11). The SCC became significantly more positive when it was added basally however (Fig 4.11). The more positive SCC observed (by 6.5 $\mu A/cm^2$) is consistent with the inhibition of chloride transport from the apical to the basal side; in this case sodium would move across from the apical to the basal side without any accompanying chloride transport in the same direction. Thus relatively more net positive charge would move to the basal side, so increasing the SCC. The situation is similar in another pulmonate, namely Aplysia where there is primary chloride transport from the apical to the basal side with the active step being located in the basolateral membrane.

ION TRANSPORT

Discussion of the ion substitution/removal results is made easier by reference to the diagrammatic model of Figure 5.1. This shows likely movements just of sodium, potassium and chloride through the basal and apical membranes of the crop epithelium in the absence of glucose and amino acid. It is based on the inhibitor results in *Helix*, but also partly on the results, but not necessarily interpretations, of Gerencser on *Aplysia*. In the basal membrane are a representative sodium pump and chloride pump, shown by the sensitivity of the crop to ouabain and thiocyanate respectively and also a potassium channel to allow recycling of potassium transported into the cell in exchange for sodium, as shown by the sensitivity to barium. On the apical side is a triporter that reflects the sensitivity to furosemide (and, in *Helix*, piretanide). There is also a potassium channel as postulated by Moran and Garretson (1988) for *Aplysia* intestine and shown in *Helix* by the action of apical barium. Furthermore, there is an apical conductive chloride channel as postulated by Moran and Garretson for *Aplysia* oesophagus and also an apical sodium conductive channel that reflects the sensitivity to amiloride.

Shown beside the arrows are the relative rates of transport expressed in terms of the letters k, m, n and p, on the assumption of long-term mass balance for sodium, potassium and

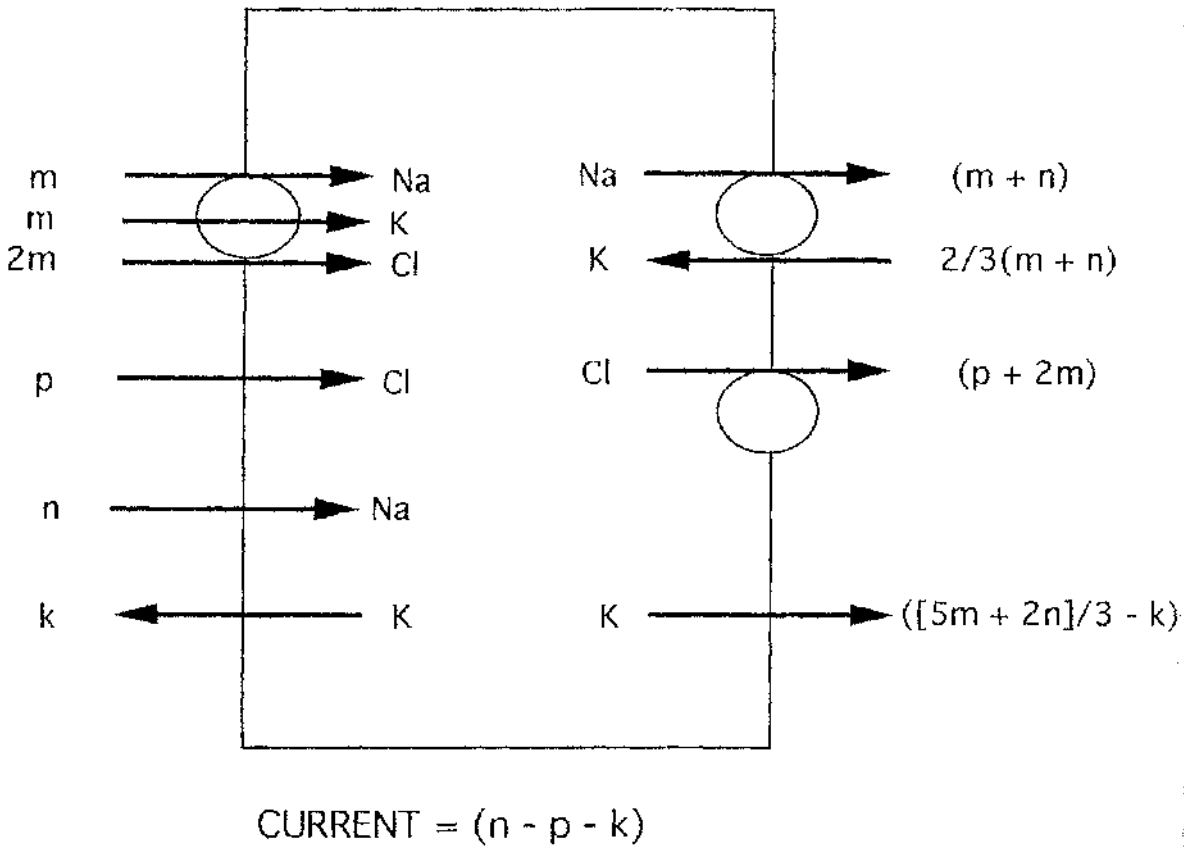


FIG 5.1 Proposed cell model for ion transport across the crop wall epithelium in *Helix aspersa*.

(When predicting correlations from the model the potassium efflux can vary between (5m+2n)/3 and zero, with the triporter present, and between 2n/3 and zero, with the triporter removed. The SCC=(n-p-k) and so, therefore, the limits of the SCC are (n/3-p-5m/3) and (n-p), with the triporter present, and (n/3-p) and (n-p), with the triporter removed).

chloride. The letter m stands for the relative rate of transport via the triporter, n for passive apical entry of sodium, k for passive apical efflux of potassium and p for passive apical entry of chloride. It is assumed that the sodium pump exchanges two potassium ions for three sodium, as is usual.

Such a model, whether or not correct in every detail, can be useful in ordering one's thoughts on the various kinds of evidence. Moreover, because of its semi-quantitative (algebraic) content, there is the possibility that it can also suggest explanations for the correlations found, in particular experiments, between changes in SCC and initial values. The model does however neglect other possible transport e.g. calcium, and although it is a simplification it helps one think about the results and correlations.

The crop of Helix aspersa was generally believed to be little more than a storage area for food before it continued into the posterior intestine, stomach and digestive gland. However the presence of a large diversity of enzymes in the crop juice and the presence of a potential difference across the crop wall epithelium, indicating ion transport, shows this is not the case. The structure (e.g. microvilli and basal invaginations) of the epithelium suggests a transport function.

In many epithelial preparations the spontaneous

transepithelial potential difference can be attributed mainly to the active electrogenic transport of one ion. In the vertebrates this is usually sodium although its transport can be linked to the movement of other ions, most commonly chloride (Epstein et al., 1973). The overall net movement is of sodium from the mucosa to the serosa which in itself results in the serosa being positive with respect to the mucosa. Other ions can account for the electrical potential in other preparations so that this is sometimes reversed, as in the thick ascending Loop of Henle. In the seahare A.californica the serosa is negative with respect to the mucosa (Gerencser, 1981,1984). This has been shown to be due to net electrogenic chloride transport which is sodium independent. There was however a small net residual flux of sodium from mucosa to serosa under chloride free conditions, but this was quantitatively much less than the usual net chloride flux from mucosa to serosa. In Aplysia Gerencser reported that the SCC could be accounted for mainly by the net chloride flux, with the net sodium flux, in the same direction, only accounting for a small part of the total SCC. However, the movement of sodium in the same direction as chloride, would not have an additive effect on the SCC as suggested by Gerencser. It would make the SCC less negative and not more so. White (1988) has also reported electrogenic chloride transport, in the small intestine of the salamander Amphiuma, rendering the blood (basal) side negative. However the influx of chloride is dependent on the luminal presence of

both sodium and potassium, since the absence of these ions in the luminal medium significantly reduced the net flux of chloride across the epithelium. The dependency of the chloride flux on sodium and potassium would tend to indicate the involvement of the sodium/potassium/chloride triporter. In H.aspersa crop the situation does not seem to be so simple since the basal side of the epithelium can be either positive or negative under normal control conditions. There are a number of ions involved in this and these will be discussed below.

Sodium

Sodium is the most important ion required for the maintenance of a stable SCC. Removal of sodium from the bathing solutions resulted in the greatest changes observed for any of the experimental conditions. The results obtained using Helix crop are consistent with sodium movement in the usual direction, i.e. from the apical to the basal side, as one would expect on functional grounds in Helix. However the stable SCC is not abolished upon sodium removal. Sodium substitution on both sides of the epithelium resulted in the SCC becoming more negative (Fig 4.15). This was always so, despite the fact that the starting SCC ranged from 61.7 to -51.2 $\mu A/cm^2$. This is what would be expected if sodium was moving across the tissue towards the basal compartment. Since under sodium free conditions this movement would stop, by

inhibiting the triporter and sodium entry via the conductance channels, and so the flow of positive charge across the tissue would fall and the SCC would become more negative.

Apical (i.e. extracellular) sodium is more important in electrogenesis than is basal since its substitution results in a greater change in the SCC (Fig 4.16). This is expected since apical removal would totally prevent sodium absorption, again by inhibiting the triporter and conductance channels, whereas basal removal will just change the driving force for sodium absorption that would favour sodium transport and so make the SCC more positive, as observed (Fig 4.16). So again this is consistent with sodium transport being achieved by the universally present Na/K ATPase which is situated in the basolateral membrane. However, the removal of sodium from only one side of the tissue would result in the creation of paracellular diffusion potentials which would tend to add to the response obtained for either apical free only or basally free only.

There was a link between sodium and organic transport. D-glucose and glycine were both shown to require sodium in the external bathing solution if they were to have any effect on the SCC (Fig 4.32 and 4.35). This is discussed below.

Potassium

As stated previously, other ions are involved in the maintenance of a stable P.D. and so therefore SCC. In the invertebrates there is evidence for primary ATP-dependent potassium transport, i.e. not the sodium pump, for example in Lepidopteran larvae midgut (Harvey et al., 1983), and in the proboscis of Protophormia terraenovae (Wieczorek, 1982). In the above cases net potassium transport is from the basal to the apical side of the cell. There is an active step, located at the apical membrane.

In Helix crop there is no evidence for purely primary transport of potassium such as found in Lepidopteran midgut. Potassium removal did however result in significant changes in the SCC (Fig 4.17 & Fig 4.18). These were similar to those obtained on removing sodium. Thus the direction of change was the same, regardless of the initial polarity for similar experimental conditions, e.g. sodium-free apically and potassium-free apically etc. However the changes upon potassium removal were not as pronounced. Removal from both sides of the tissue should inhibit the basal sodium pump and as a result inhibit the net sodium flux from the apical to the basal side. This would result in the SCC becoming more negative, as was observed the mean change being -4.9 $\mu A/cm^2$ (Fig 4.17).

Results of purely apical-only and basal-only removal (Fig 4.18) do not agree with an effect just on the sodium pump, as can be seen from the model Fig 5.1. Under apical potassium-free conditions little effect might be expected since no inhibition of the sodium pump should result. This would mean that the SCC should remain relatively unchanged. However the result was a more negative SCC, the mean SCC became more negative by -11.5 $\mu A/cm^2$. Sodium entry across the apical membrane seems, however, to be at least partially dependent on the presence of potassium and chloride in the apical bathing solution, via the Na/K/2Cl cotransporter (see above). Thus under apical potassium free conditions there would be inhibition, at least partially of sodium and chloride movement to the basal side which would then become more negative. This would result in a more negative SCC. Apical removal would also result in a potassium gradient from the basal to apical side with, therefore diffusion of potassium between the cells. Also this would tend to drive potassium to the apical side which in itself makes the SCC more negative, and so should increase sodium extrusion from the cell into the basal compartment, via the sodium pump. This would result in a more positive SCC. However, as stated above, sodium entry would also be inhibited and so, presumably, the net effect would be a more negative SCC as found. This could also explain why potassium removal does not have as large an effect on the SCC as does sodium removal, presumably since potassium removal inhibits sodium movement but does not totally stop it.

With basal removal, the mean SCC became more positive by 10.2 $\mu A/cm^2$. Potassium entry into the cell and its transport across the apical membrane would be inhibited. Also potassium recycling across the apical membrane would be reduced (see above) with an increase in the apical to basal transfer. Potassium would also diffuse between the cells towards the basal side. The net result would be an increase in the positive charge moving to the basal side which leads to a more positive SCC, as observed. This is regardless of the initial polarity of the SCC.

As mentioned above potassium entry into the epithelial cells seems to be at least partially dependent on the presence of sodium in the medium. Under sodium free conditions potassium removal from both sides of the tissue resulted in no significant change in the SCC (Fig 4.19). Fron the model this would be expected since under sodium free conditions the removal of potassium would have no further effect on the sodium pump or the triporter. However the effects of potassium removal were dependent on the presence of chloride in the bathing medium. If potassium entered the cell purely on the sodium pump, then under chloride free conditions as standard (Fig 4.20), potassium removal should still result in a significant change in the SCC. There was a more positive SCC upon potassium removal, under chloride-free conditions, although it was not significant. Such an effect would be expected to occur if inward chloride transport were inhibited.

It is less likely to be due to the inhibition of outward sodium transport, which there is no evidence for, or to stimulation of inward sodium transport. There was also no significant change in the mean SCC when potassium was removed from both sides of the tissue under sodium chloride free conditions as control (Fig 4.21).

Chloride

There is no convincing evidence for primary active transport of chloride in vertebrate epithelial preparations, chloride transport being linked to the downhill movement of sodium, itself dependent on the basolateral sodium pump. Therefore chloride transport can be thought of as secondary active. The transport systems for chloride included the NaCl cotransporter and the Na/K/2Cl triporter (Epstein et al., 1973; Frizzell et al., 1979; Palfrey and Rao, 1983). In the invertebrates, however, a number of studies have concluded that there is ATP dependent chloride transport (Hanrahan and Phillips, 1983; Gerencser 1978,1981,1983; Gerencser and White 1980; White 1988). This has been shown to be responsible for the negative SCC recorded across the epithelium in these studies. The SCC in Helix mantle is also dependent on chloride (Enyikwola and Burton 1983; Enyikwola 1987).

The removal of chloride from only one side of the epithelium has to be treated with caution since the large electrochemical

gradient that is established presumably results in a diffusion potential.

Chloride removal from both sides of the epithelium would inhibit net apical to basal chloride transport by inhibiting the apical triporter and also the chloride conductance channels. Sodium absorption would continue via the basal sodium pump, entering apically via the sodium channels rather than the triporter. The net result would be a more positive SCC (Fig 4.27), the actual mean change being 4.6 $\mu A/cm^2$. Interestingly, this response was only observed in starved snails; there was no significant change in the SCC in fed snails (Fig 4.27). It is unclear why feeding removes the response to chloride free conditions. If p is low in fed snails (see above) then this could explain the lack of a significant change in the SCC upon chloride removal since no effect should be seen if there is no chloride flux across the epithelium. It would seem reasonable to assume that the effect is on the chloride transport system since the state of feeding does not seem to affect the responses obtained for any other experimental condition, although the SCC was more often positive in fed than starved snails. It would seem unlikely that the effect of chloride free conditions, in fed snails, is on sodium transport since the actual direction of change in the SCC was wrong.

Under sodium free conditions as control the responses elicited

by bilateral chloride removal are different -i.e. the SCC became significantly more negative (Fig 4.28). Harvey et al. (1983) have shown that potassium transport, or the step across the luminal membrane of M.sexta, is achieved by a potassium pump. However calcium and other alkaline earth metals can substitute for potassium when the external potassium concentration is low. If such a system were present in Helix this could explain the results for chloride free under sodium free conditions as control (Fig 4.28). The removal of chloride would inhibit the residual net flux of potassium across the epithelium, calcium could then substitute for the potassium and so a net flux of calcium would be the result. This flux would be from the basal to the apical side carrying a small positive charge in that direction, thus the basal side would become more negative which results in the SCC decreasing, as was observed.

These experiment were also performed with ouabain present in the control solutions (Fig 4.30). The results should be similar to those for sodium free conditions as control since ouabain would inhibit sodium transport via the sodium pump. Sodium absorption could still continue via the triporter. The removal of chloride would inhibit this absorption and a more negative SCC would be the result. However the sodium that did enter via the triporter could not then exit the cell via the sodium pump. The mean SCC became more negative by -3.0 $\mu A/cm^2$.

However, even if diffusion potentials are considered, then the chloride free experiments are still difficult to explain (Fig 4.29). Apical substitution only, under sodium free conditions as standard should result in an electrochemical gradient being established such that the basal side would be much more positive than the apical side. However this is not the case as the SCC became more negative, by -11.7 $\mu A/cm^2$. The opposite is true for basal substitution in that the gradient should be from the apical to the basal side, with the basal side being more negative. Again this is not what was observed since the SCC became more positive, by 6.5 $\mu A/cm^2$. However, a possible explanation could be that there is a leak of chloride from the saline filled recording electrodes that results in the observed changes.

RESIDUAL SCC IN THE ABSENCE OF SODIUM AND CHLORIDE

Calcium

These experiments, as well as the magnesium free experiments (see below), were performed to try and find the ions responsible for the residual SCC in the absence of sodium and chloride.

Calcium itself is known to be transported across epithelia.

Membrane systems responsible for this include sodium/calcium exchange, usually across the basolateral membrane. This utilises the favourable gradient generated by the sodium pump. Calcium transport may also be independent of sodium (Schoenmakers and Flik, 1992).

Under NaCl free conditions as control the transport of sodium and chloride ions should stop, by inhibiting the sodium pump, the triporter and the basal chloride pump. The removal of calcium under these conditions resulted in the SCC changing significantly (Fig 4.23 & Fig 4.24), depending on what side the calcium was removed. The observed changes are consistent with calcium transport occurring from the apical to the basal side of the epithelium. Calcium removal from both sides would inhibit this transport and so the basal side would become more negative as would the SCC (Fig 4.23) as was observed, the mean change being -1.6 $\mu A/cm^2$. This is also what happened on apical removal, again the transport from the apical to the basal side would be inhibited and so the SCC would become more negative (Fig 4.24). The mean change was -4.9 $\mu A/cm^2$. Basal removal would result in a decrease in the concentration gradient opposing net transfer to that side. The transfer rate would increase with more positive charge moving across to the basal side, itself becoming more positive. This would result in a more positive SCC, as observed (Fig 4.24), the mean change was 3.8 $\mu A/cm^2$. Diffusion through the paracellular pathway could contribute to the effects seen in

the presence of concentration gradients but not to the SCC on bilateral removal.

For other epithelia, calcium in the external medium has been shown to have a regulatory effect on chloride transport across the apical membrane. Frizzell et al. (1979) concluded that increasing the external concentration of calcium caused a concomitant increase in the chloride flux across the apical membrane in a number of different epithelia. They postulated that it could be via cAMP or even as simple as the calcium binding to exposed negative sites on the apical membrane, thus reducing the repulsion to the chloride ions. This would result in more chloride crossing the membrane, with greater net transport. Hanrahan and Phillips (1983) also found that chloride transport was stimulated by calcium, via increased cAMP levels, in locust rectum epithelium.

In Helix the removal of calcium from both sides of the epithelium, under normal bathing conditions, makes the SCC more positive, the mean change being 5.1 $\mu A/cm^2$. This is consistent with an inhibitory effect on apical to basal chloride transport (Fig 4.22).

Magnesium

In Helix all magnesium-free experiments were performed under sodium chloride free conditions, again in an attempt to find

what was responsible for the residual SCC. Under these conditions the removal of magnesium from both sides, and from the apical side alone, did not alter SCC significantly (Fig 4.25 & Fig 4.26). This would tend to point to there being no magnesium transport that is not sodium or chloride dependent. However removal from the basal side only did result in a significant more positive SCC (Fig 4.26), the mean change being 4.0 $\mu A/cm^2$. This could be due to passive leak between the cells but the concentration of magnesium is small. However there would be a diffusion gradient created that would favour the movement of magnesium to the basal side, which would make the SCC more positive, as was observed. Since removal from both sides and apically only, had no significant effect, it is unlikely that basal removal is acting through any transcellular magnesium transport. If it was, one would have expected to see something similar for removal from both sides. Perhaps it could have an effect on the transport of other ions. The only available ions are potassium, calcium, hydrogen, hydroxide and the substitutes for the sodium and chloride. The removal of the magnesium basally could result in the SCC becoming more positive if calcium transport from the apical to the basal side increased (as discussed above). The net result would be a more positive basal side and SCC, which would explain what was observed.

GLUCOSE AND AMINO ACIDS

The standard salines lack organic compounds. This was partially to simplify the system. The findings in the Helix crop epithelium were that both D-glucose and glycine caused a significantly more negative SCC (Figs 4.31 and 4.34), when added apically. If these substrates were being transported solely along with sodium, then there should be an increase in the sodium absorption which would result in a more positive SCC and serosa. This is obviously not the case. These findings in the Helix crop epithelium are in agreement with Gerencser's using A.californica intestine. In the crop, after feeding, the concentration of potassium rises and that of sodium falls (see above). If the absorption of glucose is sodium dependent, could the glucose raise ATP concentrations and so open ATP-gated potassium channels? This would lead to potassium secretion which would result in a more negative SCC, as was observed. However the ingested plant material is high in potassium and so the increased potassium concentration after feeding is more probably from the food. The response elicited by D-glucose and glycine are however dependent on sodium being present in the bathing medium, since sodium free conditions abolished any response (Figs 4.32 and 4.35). The change in the SCC required glycine apically, and no change was observed when the addition was from the basal side. However, with D-glucose, basal addition did significantly change the SCC: it became more positive, with a mean change of 4.9

$\mu A/cm^2$. This suggests that the glucose is either being transported basally to apically into the crop or that it is being used to power some other basal system which results in a more positive SCC.

The majority of transport systems for sugars and amino acids in the vertebrates are sodium dependent. In the invertebrates there seems to be a number of systems, both sodium dependent (Dean, Barber and Ponz, 1987) and independent (Gerencser, 1978,1983). Dean,Barber and Ponz have reported the existence of different systems for neutral and basic amino acids as well as one for imino acids in H.aspersa intestine (Dean, Barber and Ponz, 1987). The neutral and basic amino acids also seem to have sodium independent systems. Gerencser reported that in A.californica intestine sugars and amino acids stimulate chloride as well as sodium transport which resulted in the serosa becoming more negative. This was consistent with chloride absorption being stimulated more than sodium absorption. These findings have been disputed by Moran and Garretson (1988) who could find no difference in the response of A.californica intestine to sugars as compared with that of the vertebrates. They concluded that the large negative SCC was due to a sodium diffusion potential across the paracellular pathway and that sugar transport was totally sodium dependent.

D-glucose and glycine use different transport systems in the

crop. With the SCC stimulated maximally by D-glucose (40mM), the addition of 10mM glycine resulted in a further negative change in the SCC. This latter change was comparable in size to that produced by 10mM glycine when no D-glucose was present. Although the two substances both produce the same end result of a more negative serosa, they must achieve this by different systems. So in the crop the net flux of chloride from the apical to the basal side must increase relative to that of sodium, so decreasing SCC. This could be achieved by the electrical coupling of sodium and chloride across the apical membrane. As proposed by Gerencser (1978) linked sodium+sugar entry across the mucosal membrane would depolarise the membrane and thus would lower the electrochemical gradient opposing chloride transport. Chloride could then move into the cell more easily by means of the electrical coupling. The increase in cellular chloride would stimulate the basal chloride pump which would increase its activity, so pumping more chloride out into the serosal compartment. This would result in the SCC decreasing, as is observed. The sodium entering apically would exit the cell basally via the sodium pump.

From the D-glucose and glycine saturation curves, Figs 4.33 and 4.36, the concentrations giving the half maximal rate of transport, in Helix, for D-glucose was approximately 12mM and approximately 13mM for glycine. Gerencser (1978), for A.californica intestine, gives a Km of 16mM for D-glucose and

a Km of 37mM for glycine (1981). Moran and Garretson (1988), studying galactose, do not give a Km value for either A.californica intestine or oesophagus.

The correlation between the initial SCC and the magnitude of the change

In several experimental procedures a strong correlation was found between the initial SCC and the change observed in the SCC. When significant, the correlations are generally negative. The observed relationships can be explained in terms of the proposed transport systems. If the SCC is initially negative then this is presumed to be due to relatively more negative charge, probably chloride, than positive charge, probably sodium, moving from the apical to the basal side. Positive charge can also move towards the apical side which would have the same effect on SCC as chloride moving basally.

The correlation are discussed and predicted in terms of the diagrammatic model Fig 5.1 which includes sodium, chloride as well as potassium, but no other ions. The negative correlations can be predicted with the triporter either present or absent in the model.

Potassium

The removal of potassium from either the apical side only (r=-0.836) or the basal side only (r=-0.721) both yielded strong correlations between the initial SCC and the magnitude of the change in the SCC (Figs 4.18A and 4.18B). With potassium removal apically the SCC became more negative, especially if the initial SCC was positive. The direction of change is in accordance with diffusion potential. If the SCC is initially positive, from the model there should be little chloride transport and p would be near 0. The depolarisation of the cell, upon apical potassium removal, would speed up the chloride transport (p would increase) making the SCC more negative, adding to the diffusion effect. If the SCC was initially negative then we can imagine no sodium transport to begin with (n=0). Now remove apical potassium: the sodium effect would be irrelevant. As to changes in k, it is unclear what the effect would be. Actually the effect might be to stimulate sodium transport from zero, making the SCC more positive (counteracting diffusion effect). Also, the effect of hyperpolarisation on the chloride transport would be to slow it (decreasing p). This would make the SCC less negative, offsetting the diffusion effect again.

Potassium free basally would inhibit the sodium pump, reducing sodium absorption, but would probably have no effect on chloride absorption. This would result in a more negative

SCC, as observed. If the initial SCC was positive then we can imagine there being no chloride transport (p=0). The removal of basal potassium would affect the second mechanism (sodium pump). However there is also no chloride transport. So, aside from the diffusion effect, the SCC should move to zero, becoming less positive. This offsets the diffusion effect.

Chloride

Chloride free conditions apically only or basally only, under sodium free conditions, both yielded a correlation between the magnitude of the initial SCC and the magnitude of the change in the SCC (r=-0.763 and -0.886 respectively). Under sodium-free control conditions, as there should be no sodium transport, an initially positive SCC would be due to either other cations moving towards the basal side or anions moving to the apical side. Similarly a negative SCC would be due to either cations moving apically or anions moving basally.

Chloride free conditions apically (Fig 4.29A) would result in an electrochemical gradient that would favour positive charge moving towards the basal side and negative charge moving to the apical side. This would result in the SCC becoming more positive. However, this is not what was observed, as the mean SCC became more negative. From the model chloride free apically, under sodium free conditions, should result in p=0, m=0 and n=0, so is the negative change due to potassium moving

apically, i.e. k? It is easier to explain the negative correlation using the model. If the SCC was initially negative then under sodium free conditions as standard m=0 and n=0 with p being high. The more negative the SCC, the greater p would be. If chloride was removed from the apical side then this would make the SCC less negative. However, if the SCC was initially positive, there would be little or no chloride flux basally and p would also be approximately 0. If chloride was then removed apically there would be less of an effect on the SCC. There would be a smaller change in the SCC. Thus the correlation is still negative, as calculated.

The same is true for chloride free basally only (Fig 4.29B). Again the observed positive change in the SCC is in the opposite direction to that predicted from the model. Under sodium free conditions as standard, m=0 and n=0. However p is not zero. So with chloride removed basally p should increase, with the SCC becoming more negative. This is not what was observed. Again could the more positive SCC be due to changes in k? Again using the model it is possible to predict a negative correlation, such as was found. An initially negative SCC would have a high chloride flux from the apical to the basal side with p being high. The more negative the SCC was initially the greater p would be. The removal of chloride basally would increase the flux of chloride basally, so p would also increase, with the SCC becoming slightly more negative. With the SCC initially positive there would be

little chloride flux to the basal side and p would be approximately 0. Chloride free basally would increase the chloride flux, with p becoming high. This would result in the SCC becoming much more negative than for an initially negative SCC, where p would have been high to start with. The predicted correlation is therefore negative, as was found.

As in the main discussion, see above, the effects of such a large electrochemical gradient created by the removal of chloride from only one side of the epithelium has to be treated with caution.

Sodium thiocyanate

Only basal addition yielded a correlation (r=-0.786), although this was really due to one outlying point. With sodium thiocyanate present basally the preparation can be thought of as being the same as chloride free both sides since the thiocyanate would stop chloride transport. With an initially negative SCC, p would be high. The thiocyanate would be expected to reduce this to approximately 0. The SCC would then be dominated by n which could possibly increase. The SCC would therefore become less negative. If the initial SCC was positive then p would be approximately 0, with n being high. The thiocyanate would be less effective since p was already low. The change in the SCC would therefore be less. Again this would yield a negative correlation, as was found (Fig

4.11A).

Amiloride

At the concentration used (0.5mM), amiloride would block sodium conductive channels. In Helix there are two proposed methods of cellular sodium entry at the apical membrane. One is via the triporter and the other is via sodium conductive channels. Addition of amiloride apically would block sodium movement from the apical to the basal side via the apical channels (i.e. n=0), thus reducing the net sodium flux but not stopping it. From the model if the SCC was initially positive then n would be high and p low. Reduction of n to zero would make the SCC more negative. If, instead, the SCC was initially negative, then n would be low and p would be high. The addition of amiloride would then have little or no effect since n would already be near 0. The SCC would, therefore not change very much. This again would yield a negative correlation, as was found (r=-0.899, Fig 4.14A).

D-glucose

Only apical addition, under normal control conditions, yielded a correlation between change and initial SCC (r=-0.879, n=8, Fig 4.31A). Again there is one outlying point. However, if the correlation coefficient is recalculated, ignoring this point, it is still significant (r=-0.68, n=7). If the SCC is

initially positive then from the model n>p. The addition of D-glucose would increase both n and p, with p increasing more (discussed above). The increase in n would result in an increase in the activity of the sodium pump and potassium entry would also rise. So the net transport of potassium to the apical side would also increase, since there is now a favourable electrochemical gradient in that direction. So the SCC would become more negative. From the model an initially negative SCC would presumably have a low n and high p. The addition of D-glucose would again increase both n and p, with p still being greater than n. The increased sodium would exit the cell via the sodium pump, increasing its activity, so potassium entry would again rise. The electrochemical gradient for potassium would favour recycling across the basal membrane, which presumably would increase, but this would have no net effect on the SCC. This would result in a less negative change in the SCC when the SCC was initially negative than when it was initially positive, which is what is observed. It also yields a negative correlation.

Note that these "predictions" take no account of changes in membrane potential, intracellular concentrations and the effects of concentration gradients across the epithelium. The predictions were worked out on the basis that variations in the original SCC were due to variations in k (apical potassium loss). However, the variations in the initial SCC are likely also to be due to variations in the balance of sodium and

chloride transport. Consideration of these also leads to the prediction of negative correlations.

APLYSIA

Active transport by part of the digestive tract has been extensively studied in another gastropod mollusc, namely the sea hare, Aplysia, mainly by Gerencser and co-workers. It is appropriate to survey some of the findings for comparison with Helix, since the result obtained using the crop epithelium are similar in many respects, to those reported in Aplysia. Not all details are yet clear, and there are some inconsistencies to be noted. Gerencser describes the epithelium he studied as coming from the "intestine". Moran and Garretson (1988) argue that it was the oesophagus that was being studied (see below), but this does not reduce the relevance of the results here.

Graph of net chloride flux against SCC

The data for the chloride flux, in the absence of glucose and amino acid, comes from four references Gerencser (1977, 1978a & b and 1981a). This gives five points in all (Fig 5.2 and Table 5.1). This gives a fair straight line of gradient 1.0, such that $-SCC = J_{Cl} - 38$ approximately ($\pm$ scatter). This is explainable if J_{Na} were approximately constant at 40. Two data pairs for sodium agree with that; a third has J_{Na} at ca 62. Gerencser seems to see the SCC as $J_{Cl}+J_{Na}$ instead of

The fluxes of chloride and sodium, both in the presence and absence of glucose/glycine, plotted against the SCC. The fluxes and SCC are given as n-equiv/cm^2/min (from Gerencser).

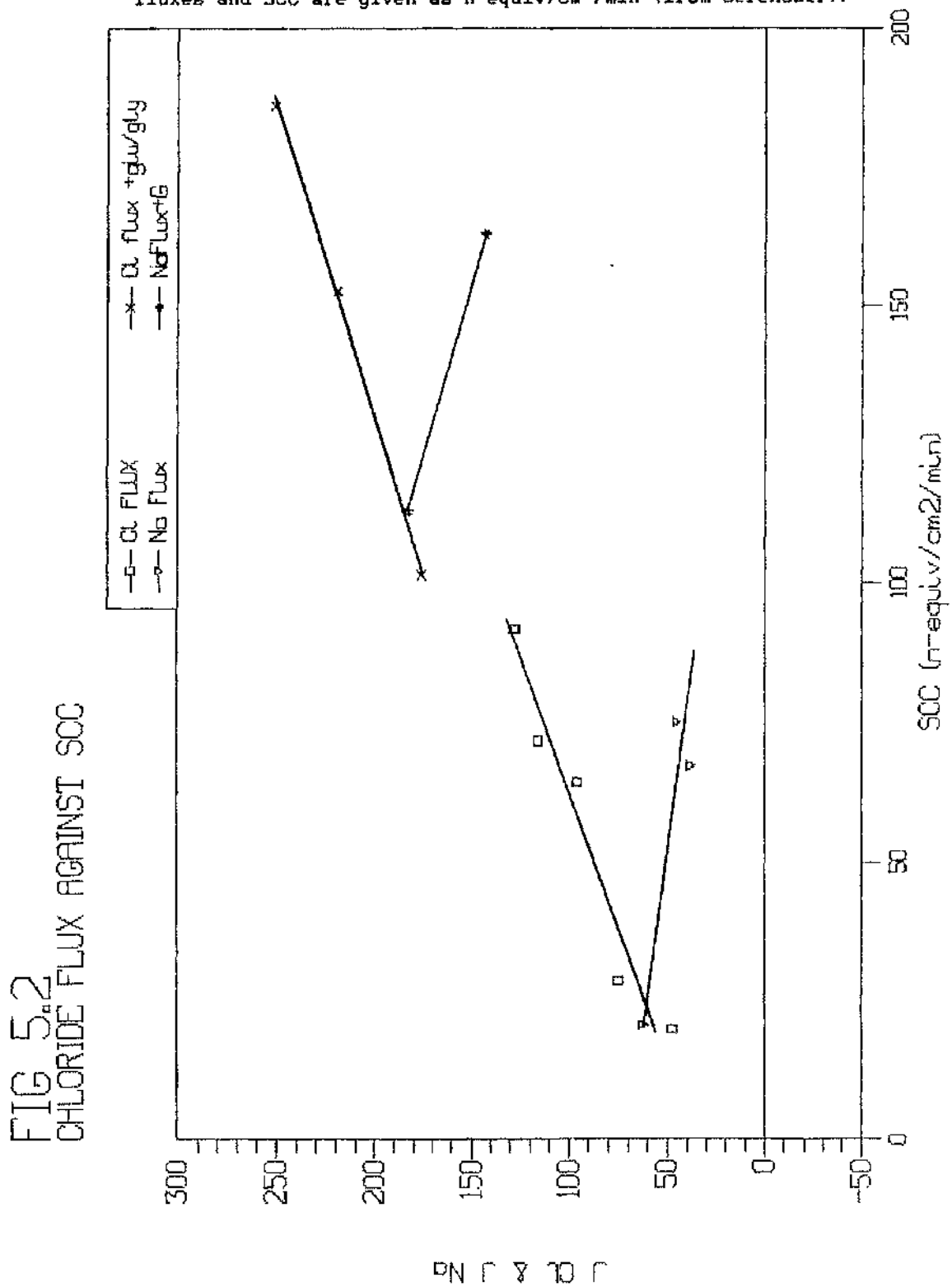


FIG 5.2
CHLORIDE FLUX AGAINST SCC

PAPER	Substrate	Chloride Flux	Sodium Flux	SCC
Gerencser & Hong (1977). Comp. Biochem Physiol., 58A: 275-280	Glucose Glucose	115.7 218.7	45 182.7	71.7 75.1 152.7 112.8
Gerencser (1978) Comp. Biochem. Physiol., 61A(2): pp209-212	Glucose Glucose	75.2 250.2	38.1 142.8	28.6 67.3 185.9 162.9
Gerencser (1978) Comp. Biochem. Physiol., 61A(2): pp203-208		127.8	62.1	91.8 20.5
Gerencser (1981) Am. J. Physiol., 240: R61-R69	Glycine	96.2 47.6 175.7		64.3 19.7 101.4

TABLE 5.1 Sodium and chloride fluxes in artifical seawater media (From Gerencser and Lee 1977 & Gerencser 1978A & B, 1981. Fluxes are given as n-equiv/cm2/min).

$J_{Cl}-J_{Na}$, there being more than one reference to this. Also he gives no consideration to potassium fluxes, though he postulates potassium entry via the sodium pump. For the above result we can postulate basal exit of the potassium pumped in, so there would be no effect on the SCC.

J_{Cl} is close to the SCC in sodium free solution (Gerencser 1984), so is chloride entering apically through channels? Yet ouabain abolishes the SCC and most of J_{Cl} (Gerencser 1981a). This would seem to be a contradiction in that both sodium free conditions and ouabain should result in much the same effect on the SCC.

There are three data points for chloride and two for sodium after the addition of glucose or glycine (Gerencser 1977, 1978a & b and 1981a). The SCC, J_{Cl} and J_{Na} all increase greatly (Table 5.1). The three data points suggest another straight line, of gradient 1.0, such that $-SCC \approx J_{Cl} - 85$ approximately. However, the two values of J_{Na} are not near 85, but are 143 and 183. So I suspect that one needs to postulate, for example, a potassium flux from the basal to the apical side after glucose addition. Certainly the results make no sense in terms of sodium and chloride only.

It is important to note that the addition of glycine apically restores the SCC and chloride flux in the presence of ouabain (Gerencser, 1981a). Presumably a little sodium enters with

the glycine, resulting in depolarisation of the cell interior, but is not transported out. There should be no sodium flux in the presence of ouabain. The depolarisation would favour greater chloride entry, and so the SCC and chloride flux would recover. In other words chloride entry outpaces sodium. Gerencser and White (1980) also showed that the addition of glucose apically lead to depolarisation and to an increase in chloride activity. However, Gerencser himself acknowledges that if chloride enters the mucosal side faster than sodium, then there should be hyperpolarisation (Gerencser 1981b), which is not the case. This is somewhat of a "paradox".

Another area of confusion is the role of sodium in the maintenance of the SCC. Gerencser and Hong (1977) reports that sodium is required for the SCC in A. juliana. However, in later papers, he states that in A. californica sodium is not required for the SCC (Gerencser 1981a, Gerencser & Lee 1985). It should be noted that these result do come from different species. This is further complicated by the results obtained using furosemide. In four different papers it was used at three different concentrations. At a concentration of 10^{-6} M there was inhibition of the SCC upon mucosal addition, but none upon serosal addition (Gerencser 1981). At 10^{-4} M there was inhibition when addition was mucosal or serosal (Gerencser 1978). At 10^{-3} M there was a 34% inhibition of Cl ATPase activity of membrane material, 10mM thiocyanate gave the same result. In the absence of sodium 10^{-3} M furosemide had no

effect on either side (Gerencser 1984). This suggests an important apical triporter. Also that sodium should be needed for chloride transport. However, the triporter is electroneutral, so for there to be a SCC, there would have to be some other apical ion movement. Perhaps potassium ions exit apically. This does not fit the above evidence that the SCC = $J_{Cl} - J_{Na}$, could the cotransporter co-exist with sodium and chloride channels?

The addition of thiocyanate to the apical bathing solution resulted in the inhibition of the SCC under sodium free conditions as control (Gerencser 1984). There was 32% inhibition for 0.1 mM, 85% for 1 mM and 100% for 10 mM thiocyanate. There was little or no effect on the SCC when addition was to the basal side. However, for the three concentrations the total number of experiments was only four. Gerencser proposed that the action of thiocyanate was either on the highly chloride conductive mucosal membrane or on the intracellular aspect of the active extrusion mechanism for chloride located in the basolateral membrane. The former would be consistent with the blockage of mucosal chloride conductive channels.

Gerencser has provided evidence for the existence of basal ATPases (Gerencser, 1985 and 1988). The results also support a normal basal Na^+-ATPase in Aplysia, since basal ouabain leads to the inhibition of the mucosal to serosal sodium flux.

Under sodium free conditions as control the SCC was equal to the net flux of chloride (J_{Cl}). Both the SCC and J_{Cl} were inhibited by thiocyanate and acetazolamide, but not by ouabain. Gerencser explained this as inhibition of a basal Cl^--ATPase. He arrived at the basal location by reasoning that the intracellular chloride electrochemical gradient is less than that in the extracellular medium, therefore chloride would enter the cell across the mucosal membrane down this favourable gradient. However, to exit basally against this gradient the chloride would have to be pumped out, so the pump would be located basally. Cl-ATPase activity was of non-mitochondrial origin and was of the E_1-E_2 type and not the F_0-F_1 type, due to it being sensitive to vanadate.

Gerencser (1981b) has shown that diffusion potentials could account for the observed transepithelial p.d. in the presence of an ion concentration gradient across the epithelium. In the presence of ouabain, with sodium and chloride transport inhibited, there was no p.d. across the tissue in the absence of sodium and chloride gradients. However, on replacing sodium or chloride in the mucosal or serosal bathing medium there was a p.d. This showed symmetrical electrical properties in that reversing the mucosal and serosal bathing solutions gave a p.d. of opposite polarity but approximately the same magnitude (Gerencser, 1981b). Gerencser provides two reasonable interpretations for the above observations. 1/ The main permeation pathway is across the tight junction, i.e.

like a single membrane. 2/ The main pathway is across the cells with the mucosal and serosal membranes having identical relative permeability coefficients. Diffusion potentials may be much higher in the marine species than in Helix, however, since normal ion concentrations are much higher.

Moran and Garretson (1988) have disputed Gerencser's conclusions for Aplysia intestine. They studied mainly the anterior intestine of Aplysia, but also the oesophagus. Of these it is the latter that matches Gerencser's "intestine", both physiologically and histologically.

In the intestine they found that mucosal galactose made the SCC more positive, but not in the absence of sodium. This is the same as vertebrate gut. The apical membrane was potassium and not chloride conductive, since the addition of barium or a high potassium concentration apically resulted in a large depolarisation of the apical membrane electrical potential difference. There was also only a very small net chloride flux that was not affected by the addition of galactose.

They found, using the oesophagus of A.californica, that there was certainly a luminal chloride conductive pathway. However, luminal galactose made the SCC more positive, the reverse of what Gerencser found. There was also a large negative SCC and a chloride flux. They did not however, try and see what effects galactose had on the chloride flux. So from the above

conclusions, together with their histological findings, Moran and Garretson concluded that sugar stimulated ion absorption was no different to that in vertebrate intestine.

Thoughts on the "paradox"

Both Gerencser and Moran and Garretson see a problem in Gerencser's explanation that mucosal glucose results in an increase in the entry of sodium which leads to depolarisation and to greater chloride entry. The point is that if chloride enters faster than sodium, then there should be hyperpolarisation. However, if we only consider the basal membrane, then under SCC conditions the two membrane potentials must be equal. We know that chloride leaves basally faster than does sodium, and we can assume that potassium enters and leaves equally. This then implies depolarisation. Presumably then chloride transport is stimulated more by a rise in intracellular concentration than is sodium transport. This would explain the greater increase in net chloride flux and the more negative basal side upon the addition of mucosal glucose or glycine in Aplysia.

Section 6 - CONCLUSION

The crop of H.aspersa was long thought of as little more than a storage sac for food before it passed into the stomach and digestive gland. However, the presence of a transepithelial potential difference shows that there must be some active transport across the epithelium lining the crop.

The ability of the crop epithelium to produce an electrical potential that can be either positive or negative under normal physiological conditions is unusual. The main route of ion movement, in life, may however be paracellular as shown by the low transepithelial resistance. However, under SCC conditions and with no concentration gradients there would be no net paracellular movement. It will be mainly sodium, chloride and potassium, in life, that will move through the "tight" junction since these ions are at a much higher concentration than the others. In life the potassium concentration in the crop juice can exceed that of sodium, especially after feeding.

The main transport systems involved in the active movement of ions across the crop epithelium (Fig 5.1) are: 1/ the ubiquitous sodium-pump which is located on the basal side of the cells. This is revealed by the action of ouabain when added basally. 2/ The Na/K/Cl electroneutral cotransporter located on the apical membrane. This is suggested by the

action of piretanide. 3/ A basally located "chloride" pump as in Aplysia, suggested by the action of thiocyanate when added basally. The action of amiloride, when added apically only, also suggests that there are sodium conductive channels located in the apical membrane.

Sodium and chloride, as the ions at highest concentration, have the greatest effect on the SCC when they are substituted. The epithelium has the ability to absorb both sodium and chloride. The cotransporter provides a route of entry into the cell for both sodium and chloride. The sodium pump then moves sodium across the basal membrane and into the basal compartment. Sodium and chloride can also enter across the apical membrane through their conductive channels. Chloride leaves the cell using a chloride ion pump also on the basal membrane. Potassium has two routes of active entry into the cell, one via the cotransporter from the apical side and the other via the sodium pump from the basal side. The epithelial cells are also potassium conductive with channels located on both the apical and basal sides, unlike Aplysia where only the apical membrane has conductive channels and these are only chloride conductive. In Helix barium has an effect on the SCC when added to either side of the tissue which indicates the presence of the channels on both sides. This could explain how the crop can exhibit either a positive or negative PD and SCC under normal physiological conditions.

Potassium that enters the cell can either leave apically or basally. In the former case this results in the net movement of positive charge to the apical side, and, combined with the net movement of the other ions, results in a negatively charged basal side. With the latter, when it exits basally, there is, net movement of positive charge to the basal side and so this becomes more positively charged. In Aplysia Gerencser has found that the SCC can be attributed mainly to the net basal movement of chloride.

The proposed model has the ability to produce either a positively or negatively charged basal side. Using the accepted stoechiometry for the known transport systems the crop epithelium should in theory be able to produce a greater negative charge than positive. This is the case for the mean SCC under normal physiological conditions, -11.8 $\mu A/cm^2$ (n=71) compared to 10.9 $\mu A/cm^2$ (n=92), and also for the largest negative and positive SCC, -79.3 $\mu A/cm^2$ compared to 60.2 $\mu A/cm^2$. From the model (Fig 5.1) the SCC = (n-p-k), with the theoretical limits of the SCC being (n/3-p-5m/3) and (n-p). It is obvious that the SCC would be dependent on the balance between the fluxes of sodium, potassium and chloride.

The response to organic substances is also unusual. The SCC becomes more negative, which is the opposite of the conventional response, when addition is to the apical side. The fact that the responses elicited are removed upon sodium

substitution shows that the systems are nevertheless sodium dependent. This is different to the effects that Gerencser has found in Aplysia. There, although the SCC became more negative upon glucose or glycine addition, the response was still obtained under sodium free conditions. Gerencser's explanation that the increased cellular entry of sodium, stimulated by the glucose or glycine, results in an even greater increase in the chloride entry, is possible (see above). This would result in a more negative SCC. The situation is similar in Helix.

Overall the actual net flux of ions across the crop epithelium of H.aspersa is not known, although it can be inferred from the effects of ion removal or substitution from only one side of the tissue. These ion fluxes could also be quantified by radioactive ion flux studies.

Section 7 - REFERENCES.

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