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STIMULATION BY ANOXIA OF ACTIVE CHLORIDE TRANSFER IN ISOLATED BULLFROG SMALL INTESTINE

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SUMMARY

1. In isolated bullfrog small intestine mounted between identical isotonic Na_2SO_4 Ringer solutions at pH 7.2, potential difference (PD) and short circuit current (I_{sc}) are largely inhibited by N_2 and by 2,4-dinitrophenol. The residual PD and I_{sc} sometimes found in the presence of N_2 is abolished by mucosal fluoride. Following moderately short exposures, the inhibitory effects of N_2 are completely reversed by O_2. Following prolonged exposure to N_2, PD and I_{sc} are only partially restored by O_2.

2. When the tissue is mounted between identical Ringer solutions in which Na^+ is completely replaced by Tris ions, PD (serosal negative) and I_{sc} are markedly increased under anoxic conditions. In the presence of metabolized sugar (glucose or fructose) these increases are sustained and are reversed by O_2. Glucose is effective whether it is present initially in the mucosal medium, the serosal medium, or both. The increase in PD and I_{sc} observed with mucosal glucose is not inhibited by phlorizin. In substrate-free media or in media containing non-metabolized or poorly metabolized solutes (3-*O*-methylglucose, sorbose or valine) N_2 elicits a transient increase only in PD and I_{sc}. In the presence of glucose, 2,4-dinitrophenol (mucosal or serosal) elicits increases in PD and I_{sc} similar to those induced by N_2. In media containing glucose, N_2 markedly increases m→s Cl^- flux but does not change s→m Cl^- flux. The increased m→s Cl^- flux under these conditions is significantly greater than the corresponding increase in I_{sc}. Part of the discrepancy between these parameters can be attributed to a net co-transport of K^+ from the mucosal to the serosal medium.

INTRODUCTION

Earlier experiments in this laboratory[1,2] showed that when isolated sheets of bullfrog small intestine are mounted in an USSING[3] chamber between identical NaCl-Ringer solutions, net mucosal to serosal Na^+ flux greatly exceeds short circuit current (I_{sc}). It was further shown that this difference can be quantitatively accounted

Abbreviations: PD, potential difference; I_{sc}, short circuit current.

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for by a net mucosal to serosal flow of Cl^- under these conditions. These experiments[2] also indicated that this net mucosal to serosal Cl^- flux is due, in part at least, to an active transport of Cl^-. The evidence for this was as follows. In Na^+-free media containing glucose, in which all of the Na^+ was replaced by Tris ions, it was found that the transmural potential difference (PD) was reversed (*i.e.* the serosal side of the preparation became negative with respect to the mucosal side, the opposite of the situation normally found in media containing Na^+) and that the I_{sc} was consistent with a net mucosal to serosal flow of negative charge. Under these conditions, both PD and I_{sc}, though small, differed significantly from zero ($P < 0.001$)[2]. It thus appeared that active transport of Cl^- by this tissue could be, to some extent, uncoupled from concomitant net Na^+ transfer. Conversely, net Na^+ flux under short-circuit conditions could be "isolated" from associated net movements of anions by replacing all the Cl^- in the bathing medium by SO_4^{2-} and adding mannitol to maintain isosmolarity[2,4]. The observation of LEVIN[5] that the effect of 2,4-dinitrophenol on PD in Cl^- media differs from its effect in sulfate solutions suggested to us that the energy requirements for active Na^+ transport and for active Cl^- transfer might not be the same.

This question is examined in the present paper, in which the effects of anoxia and of inhibitors on PD and I_{sc} in a Cl^--free Na_2SO_4 medium are compared with the effects of anoxia and 2,4-dinitrophenol on PD, I_{sc} and unidirectional Cl^- fluxes under Na^+-free conditions. Preliminary accounts of parts of this work have already been given[6,7].

METHODS

Sheets of small intestine from adult bullfrogs (*Rana catesbeiana*) were prepared and mounted between the two halves of a lucite chamber as described previously[2]. The chamber used was a modification of one used by ZADUNAISKY[8] in studies on isolated corneal tissue, with a 0.33 cm^2 circular aperture between the two halves. The fluid volume in each half chamber was 5 ml. The mucosal and serosal media were circulated and gassed continuously with 100 % O_2 or 100 % N_2 as described by USSING AND ZERAHN[3].

PD and I_{sc} were measured by methods similar to those employed by SCHULTZ AND ZALUSKY[9] with the modifications described by QUAY AND ARMSTRONG[2]. A voltage clamp device, with automatic compensation for the voltage drop due to the resistance of the bathing solution[10], was used to maintain short circuit conditions.

The Ringer solutions used were the phosphate media previously described[2]. In one of these Cl^- was completely replaced by SO_4^{2-}. In the other, Tris ions replaced Na^+. In both of them total osmolality was adjusted to 230 mosM with mannitol (65.8 mM in the Na_2SO_4 medium and 11 mM in the Na^+-free Cl^- medium). The pH of both media was 7.2 and all experiments were run at 26°. Normally, room temperature remained within $\pm$ 0.5° of this figure. When larger deviations occurred, the temperature of the medium was controlled as described elsewhere[2].

The experiments with the Na_2SO_4 medium were performed as follows. After the tissue was excised and mounted, I_{sc} was recorded every 5 min until a steady state was reached (during this time PD was recorded every 10 min). This usually required from 30 to 60 min. When sufficient measurements had been made to establish the

steady state PD and I_{sc}, the O_2 supply was changed to N_2 or the inhibitor to be tested was added, either by removing the control Ringer solution from the chamber and replacing it with one containing the inhibitor at the concentration desired, or by directly adding a small volume (usually 50 μl) of a stock solution of the inhibitor to one or both chamber compartments. Following this, measurement of PD and I_{sc} were continued as before. The experimental procedure with the Na^+-free medium was as follows. The transported solutes used (D-glucose, 3-*O*-methyl-D-glucose, and L-valine) were added to the medium in the concentrations (11, 26, and 20 mM) employed in other studies in this laboratory* (see refs. 12 and 13). The non-transported sugars, D-fructose and L-sorbose, were employed in concentrations of 11 and 33 mM, respectively. Except where otherwise indicated, these solutes were added to both the mucosal and serosal media. When they were incorporated in the medium, mannitol was omitted. With glucose and fructose this did not change the osmolarity. Media containing valine, 3-*O*-methylglucose or sorbose had higher osmolarities than the control medium.

During the first 60–90 min of each experiment the medium in both halves of the chamber was replaced several times to remove any Na^+ that leaked out of the tissue. In addition, samples of the final medium were retained and analyzed for Na^+ by flame photometry at intervals throughout the whole series of experiments. The Na^+ content did not exceed 0.3 mM in any of these analyses.

Following the initial period of rinsing and equilibration, PD and I_{sc} were allowed to attain or approach a steady state under control conditions before switching from O_2 to N_2 or adding dinitrophenol. Normally PD was serosal negative at this time. However, because of the length of time required for steady-state conditions to be established in some of the experiments with N_2, the gas phase was occasionally changed while the PD was still serosal positive.

$^{36}Cl^-$ was used to determine unidirectional Cl^- fluxes. In these experiments the tissue was allowed to equilibrate for 60–90 min in non-radioactive Ringer solution (with several changes as described above). A Ringer solution labelled with a tracer amount of $^{36}Cl^-$ was then added to either the mucosal or the serosal half chamber. Subsequently, 0.1-ml aliquots of solution were removed from both halves at suitable time intervals (15 or 20 min) and assayed for $^{36}Cl^-$ activity in a Packard series 3375 liquid-scintillation spectrometer, using Bray's solution as a scintillation medium. Flux data were calculated as described by QUAY AND ARMSTRONG[2] and are given in nequiv of negative charge transferred per cm^2 tissue per min. For direct comparison (*e.g.* in Table I) I_{sc} was converted to the same units. Analytical Reagent Grade chemicals and on-line distilled water (further purified by two passes through mixed bed ion exchangers) were used throughout.

RESULTS AND DISCUSSION

Effect of anoxia on PD and I_{sc} in Na_2SO_4 media

The results obtained for the effect of N_2 on PD and I_{sc} under these conditions

* In media containing Na^+ these correspond to a relative concentration[11] of 4, relative concentration being defined as $[S]/K'_m$, where $[S]$ is the solute concentration and K'_m is the apparent Michaelis constant for its stimulatory effect on PD and I_{sc}[4]. In the present study the same concentrations were arbitrarily adhered to.

fell into two rather well defined categories. A typical example of the first category is shown in Fig. 1. It is seen that in this experiment, following an initial period of equilibration in O_2, both PD and I_{sc} decreased rapidly to low steady-state levels during exposure of the tissue to N_2*. When this exposure was not too long (30–40 min), both PD and I_{sc} were quickly and completely restored to control values on re-admitting O_2 to the system. Thus, under these conditions, the inhibitory effect of N_2 on PD and I_{sc} appeared to be completely reversible and the viability of the tissue was well maintained.

In other experiments, the responses of PD and I_{sc} to N_2 required a much longer time to go to completion (up to 2 h in some cases) and, under these conditions, the restoration of these parameters was only partial (frequently less than 20 %). It appears therefore that prolonged exposure of the tissue to N_2 in a substrate-free Na_2SO_4 medium can result in an irreversible loss of a large part of its Na^+ transporting capacity. This is consistent with results reported for mammalian small intestine *in vitro*[15].

Effect of inhibitors on PD and I_{sc} in Na_2SO_4 media

Frequently, as shown in Fig. 1, PD and I_{sc} did not fall to zero under N_2 confirming the finding of LEVIN[5] that bullfrog small intestine has, for some time at least, a small but finite capacity for Na^+ transport in substrate-free media in the absence of O_2. This residual PD and I_{sc} was rapidly and completely abolished by addition of 0.2 mM NaF to the luminal medium. In the presence of O_2 0.2 mM mucosal F^- did not cause any clear cut inhibition but appeared to induce some electrical instability in the preparation for a time after its addition to the medium, and serosal F^- (0.2 mM) had no effect on PD or I_{sc}. LEVIN[5] reported that, in oxygenated Na_2SO_4 media, inhibition of the PD across isolated bullfrog small intestine required a relatively high concentration of F^- (50 mM). The insensitivity of this tissue to low F^- concentrations

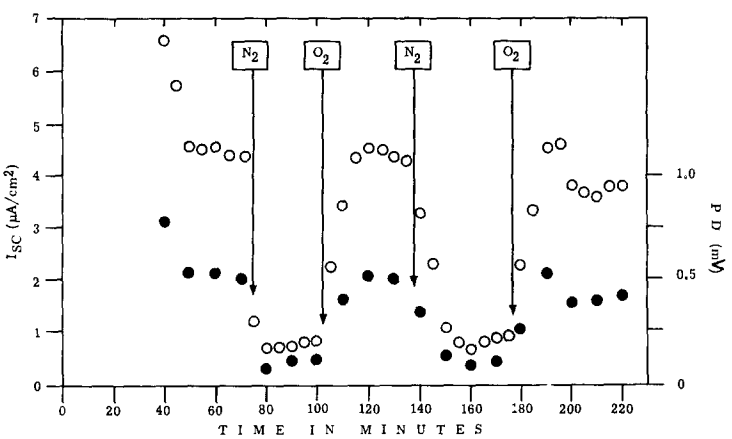

Fig. 1. Effect of short-term anoxic conditions on PD (●) and I_{sc} (○) in a substrate-free Na_2SO_4 medium.

* In all the experiments with N_2 described herein, both halves of the chamber were gassed with either O_2 or N_2. No attempt was made to study systematically differential effects of mucosal and serosal N_2 as in the recent experiments of BAKER *et al.*[14] with rat small intestine.

under aerobic conditions suggests a possible contribution to active Na^+ transfer of endogenous substrates other than carbohydrates.

When both the mucosal and serosal solutions were replaced with media containing 1 mM dinitrophenol, PD and I_{sc} were rapidly and completely abolished. After several rinsings and replacement of the dinitrophenol medium with fresh oxygenated control solutions, both PD and I_{sc} were rapidly restored. These results, together with those obtained with N_2, strongly indicate that in bullfrog small intestine, under these conditions, as in mammalian small intestine[15], net mucosal to serosal Na^+ transport depends primarily on oxidative phosphorylation as a source of energy. LEVIN[5] reported that addition of 0.5 mM ouabain to the serosal medium rapidly depressed the PD across isolated bullfrog small intestine but that mucosal ouabain was without effect. In our hands this preparation proved to be disconcertingly resistant to ouabain. In the (serosal) concentration range 0.1–1 mM, variable inhibitions, which were usually incomplete and very slow in onset, were observed. When 2 mM ouabain was added to the serosal medium, complete inhibition of PD and I_{sc} was consistently observed. Following a lag period of about 6–15 min, inhibition was usually complete within 30 min to 1 h.

Ouabain is a potent inhibitor of active cation transport in many cell species, and this effect appears to be related to the inhibition by the glycoside of a (Na^+-K^+)-dependent membrane ATPase which is directly implicated in Na^+ and K^+ transfer across the cell membrane[16,17]. When added to the fluid bathing the serosal surface of mammalian small intestine, ouabain inhibits both PD and I_{sc}, but is apparently without effect when added to the luminal medium[18,19], a finding which has been interpreted as indicating the existence of a Na^+ pump at the lateral/serosal border of the cell which is inaccessible to ouabain added to the mucosal medium[20]. Our results are consistent with this hypothesis, although the comparatively high concentration of ouabain (2 mM) required to obtain reproducible inhibitory effects suggests that bullfrog small intestine is relatively insensitive to the glycoside. This insensitivity could be intrinsic to the epithelial cells or could reflect a relatively massive binding of ouabain by tissues distal to the serosal border of these cells. Our results do not allow us to discriminate between these two possibilities. KOHN *et al.*[21] reported that serosal concentrations of ouabain as high as 1–2 mM do not inhibit the endogenous potential of rat small intestine. However, rat intestine ATPase is known to be unusually resistant to ouabain[22]. Neither anoxia nor the inhibitors examined had any significant effect on the steady-state resistance (PD/I_{sc}) of the tissue.

Effect of anoxia on PD and I_{sc} in Na^+-free media

Fig. 2 shows an experiment in which following a period of equilibration in Tris-Ringer solution containing 11 mM glucose and gassed with 100 % O_2, the gas phase in both compartments was changed to 100 % N_2. It is seen that in contrast to the situation observed in Cl^--free Na_2SO_4 media (Fig. 1), both PD and I_{sc} increased rapidly under N_2 (in a negative direction according to the convention used herein), that these increases were sustained as long as the system was gassed with N_2, and that, following restoration of the O_2 supply, these increases were to a large extent reversed. Apart from a short time lag (probably due to the time required to displace dissolved O_2 from the fluid in the tissue chamber) the effect of N_2 appeared to be virtually immediate. In other experiments with media containing glucose it was found that the

increases in PD and I_{sc} elicited by N_2 could be routinely maintained for periods up to 5 h.

In the experiment illustrated in Fig. 2 glucose (11 mM) was present in both halves of the chamber throughout. As shown in Fig. 3 a similar response of PD and I_{sc} to N_2 was obtained when glucose was present initially on one side only of the preparation. Fig. 3 further shows that mucosal phlorizin (0.1 mM) did not, during a period of 25–30 min, inhibit the response of PD and I_{sc} to N_2 in the presence of mucosal glucose.

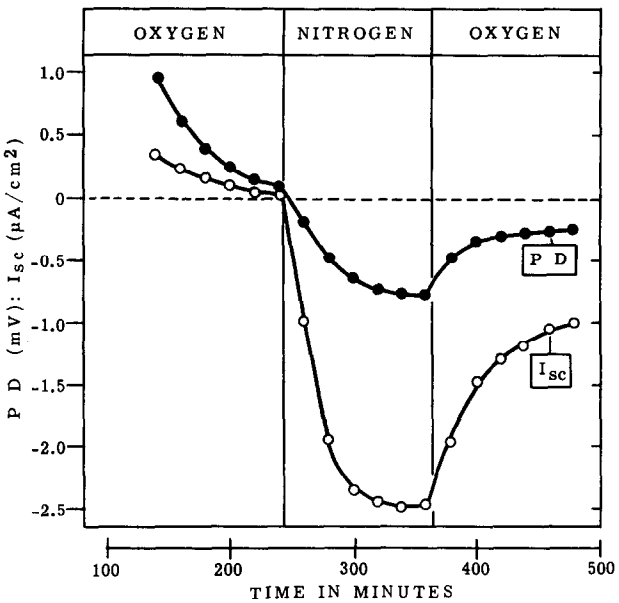

Fig. 2. Effect of N_2 on PD (●) and I_{sc} (○) across isolated bullfrog small intestine mounted between identical Tris chloride media (pH 7.2) containing 11 mM glucose. Positive values of the ordinate indicate serosal positive PD and I_{sc} consistent with net mucosal to serosal transfer of positive charge.

Fig. 4 illustrates the dependence of the N_2 stimulated PD and I_{sc} on exogenous glucose. This experiment was performed under identical conditions to those shown in Figs. 2 and 3 except that both the mucosal and serosal half chambers contained glucose-free media throughout. It is apparent that the response to N_2 of PD and I_{sc} under these conditions is transient. Following a rapid increase in negativity, both parameters declined spontaneously and fairly quickly towards the control values observed in O_2. The transient effect of N_2 under these conditions varied considerably in magnitude and duration in different experiments. We interpret these results as indicating that this response depends on utilization, under anoxic conditions, of endogenous tissue metabolic reserves and is controlled by the size of the endogenous metabolic pool in individual tissue preparations. The development of this response and its subsequent decline appears to be accompanied by irreversible damage to the metabolic machinery of the epithelial cells as shown by the fact that, once the transient increase in PD and I_{sc} in the absence of substrate had been allowed to occur and then decline (as in Fig. 4), addition of glucose to both the mucosal and serosal media consistently

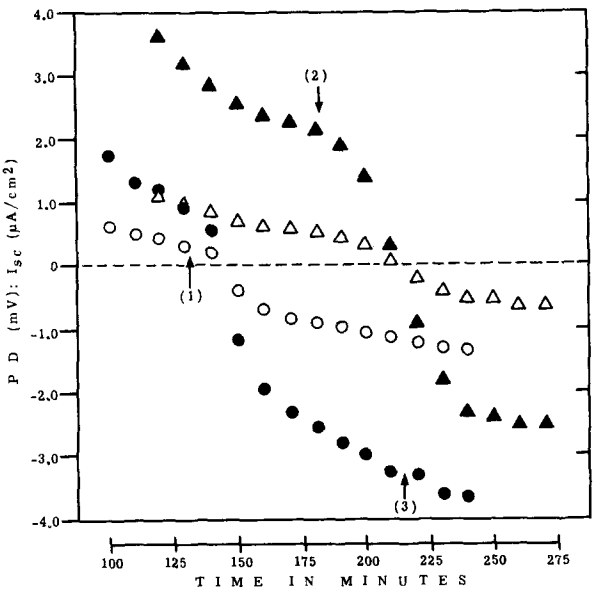

Fig. 3. Effect of N_2 on PD and I_{sc} in solutions containing 11 mM glucose on one side only of preparation. ○ and ●, PD and I_{sc} with glucose in mucosal half chamber; △ and ▲, PD and I_{sc} with glucose in serosal compartment. Arrows (1) and (2) indicate times at which N_2 was substituted for O_2 in each experiment. Arrow (3) marks time at which phlorizin (0.1 mM final concn.) was added to mucosal compartment in presence of mucosal glucose.

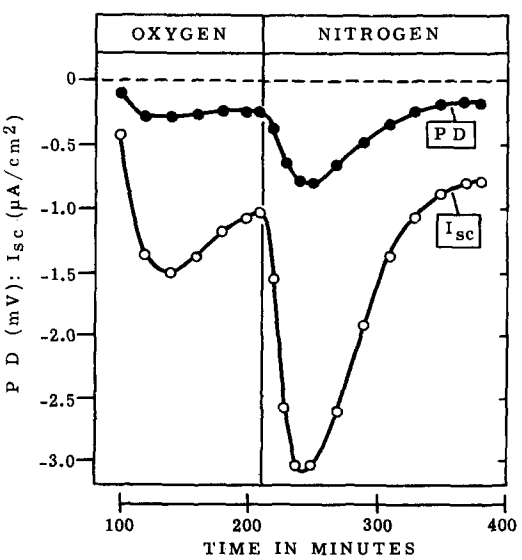

Fig. 4. Effect of N_2 on PD (●) and I_{sc} (○) in the absence of exogenous substrate. Conditions as in Fig. 2.

failed to evoke any further changes in these parameters. A similar dependence of functional integrity under anoxic conditions on an exogenous supply of metabolizable sustrate has been reported for mammalian small intestine[15].

The data of Fig. 3 strongly suggest that the glucose-dependent response of PD and I_{sc} to anoxia under the conditions of our experiments is linked to metabolic utilization of this sugar by the absorptive cells of the small intestinal epithelium and does not involve a coupled transport phenomenon similar to the well known stimulatory effect of glucose and other actively transported sugars on intestinal PD and I_{sc} in the presence of Na^+ (ref. 20). In isotonic Na_2SO_4 media (in which net mucosal to serosal Na^+ transport and I_{sc} are identical), actively transported sugars, including glucose, cause marked increases in PD and I_{sc} across isolated bullfrog small intestine. These effects are elicited only when the sugar is present in the mucosal medium and appear to depend on its ability to be transported actively across the brush-border membrane rather than on its metabolic utility to the epithelial cells[4]. Further, under these conditions, the stimulatory effect of actively transported sugars on PD and I_{sc} is abolished by mucosal phlorizin[23].

By contrast, in the present study, the stimulation by anoxia of PD and I_{sc} was obtained with either mucosal or serosal glucose and was not affected by mucosal phlorizin (Fig. 3). In mammalian small intestine *in vitro*, enhancement of certain intestinal transfer processes (*e.g.* mucosal to serosal transfer of galactose) by glucose (presumably through its stimulatory effect on cellular metabolism) has been demonstrated when glucose was present in the serosal medium only[15]. Therefore, in the present study, further evidence for the dependence of the anoxic response of PD and I_{sc} on an exogenous supply of metabolizable substrate rather than on coupled transport across the luminal membrane was sought by examining the response obtained

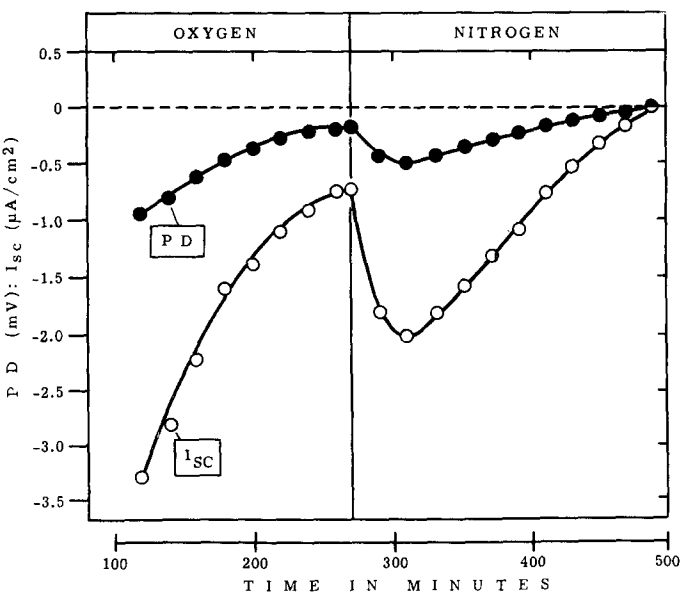

Fig. 5. Effect of N_2 on PD (●) and I_{sc} (○) in presence of 26 mM 3-*O*-methylglucose. Conditions as in Fig. 2.

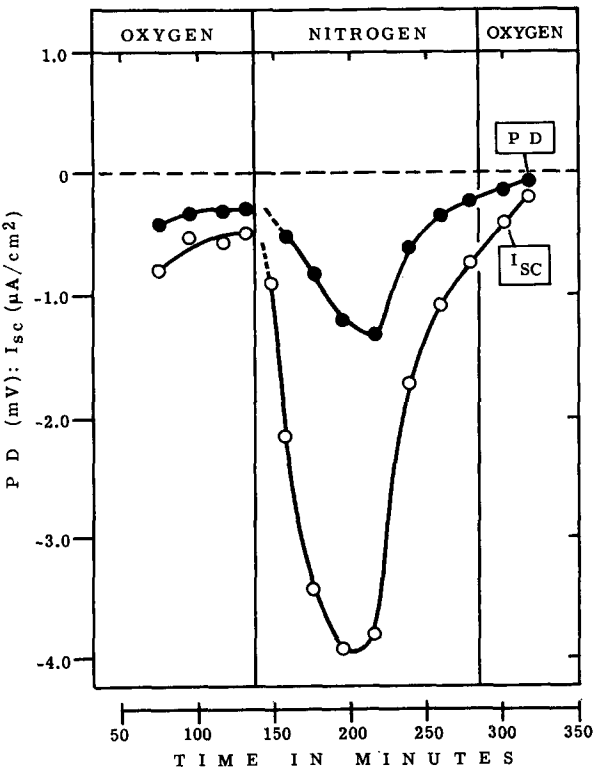

Fig. 6. Effect of N_2 on PD (●) and I_{sc} (○) in presence of 33 mM sorbose. Conditions as in Fig. 2.

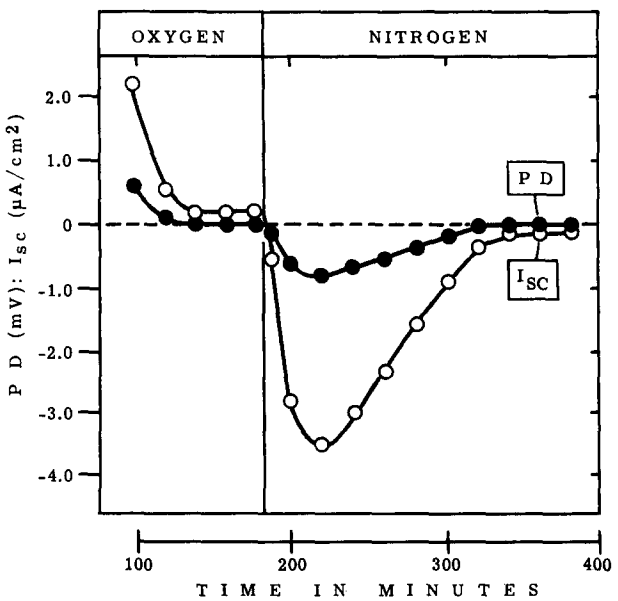

Fig. 7. Effect of N_2 on PD (●) and I_{sc} (○) in presence of 20 mM valine. Conditions as in Fig. 2.

in the presence of a variety of organic solutes. These included 3-*O*-methylglucose as an example of an actively transported but non-metabolized sugar[4], D-fructose as an example of a sugar which is metabolized but not actively transported and L-sorbose as a representative of sugars which are neither actively transported nor metabolized by the small intestine[24]. For comparative purposes, the effect of the actively transported but poorly metabolized amino acid valine[4] was also studied. In these experiments the solute under investigation was present throughout on both sides of the tissue. Examples of experiments performed with each of these solutes are shown in Figs. 5–8. Figs. 5–7 show the response of PD and I_{sc} to N_2 in the presence of 3-*O*-methylglucose, sorbose, and valine. It is evident that the results obtained in each case were essentially similar to those found in the absence of exogenous substrate (Fig. 4). There was a rapid but transient (negative) increase in PD and I_{sc} following the establishment of anaerobic conditions, both PD and I_{sc} then declining spontaneously to zero or near zero values. As was found in the absence of substrate, addition of glucose to the medium following decay of this transient increase did not elicit any further response of these parameters. By contrast, fructose (Fig. 8) elicited a sustained (negative) increase in PD and I_{sc} which was reversed when O_2 was readmitted to the system (*cf.* the effect of glucose; Fig. 2).

No significant effect of anoxia on the steady-state resistance of the tissue was found in any of the above experiments.

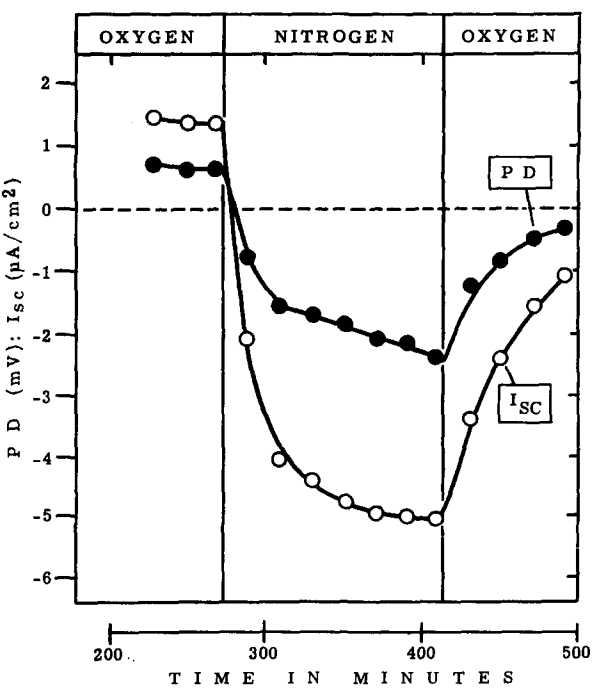

Fig. 8. Effect of N_2 on PD (●) and I_{sc} (○) in presence of 11 mM fructose. Conditions as in Fig. 2.

Effect of dinitrophenol on PD and I_{sc} in Na^+-free media

In media containing 11 mM glucose, 1 mM dinitrophenol added to either the mucosal or the serosal solution caused rapid and sustained increases in PD and I_{sc}. This is illustrated in Figs. 9 and 10. Fig. 9 shows a typical experiment in which, following equilibration with Tris chloride medium and establishment of a reasonably steady (serosal negative) PD and a corresponding I_{sc}, dinitrophenol (final concn. 1 mM) was added to the serosal medium. It is seen that, on adding dinitrophenol, both PD and I_{sc} became more negative. This increased negativity, although it decreased slowly following attainment of a maximum value, was sustained throughout the remainder of the experiment.

Fig. 10 shows that the enhancing effect of dinitrophenol on PD and I_{sc} in media containing glucose is obtained when dinitrophenol is added to either the mucosal or the serosal medium and that the effect is reversible, at least following moderately short (40–50 min) exposures to dinitrophenol. In this experiment the preparation was allowed to approach a steady state (at which time both PD and I_{sc} were near zero). Dinitrophenol (final concn. 1 mM) was then added to the mucosal medium. When the resulting changes in PD and I_{sc} had apparently reached a maximum both media were removed*, both compartments were rinsed 3 times with dinitrophenol-free medium (allowing 2 min circulation between each rinsing), and finally refilled with this medium. Following a recovery period during which PD and I_{sc} declined towards their control values, dinitrophenol was added to the serosal medium. This was followed by a second period of rinsing and recovery, a second exposure to mucosal

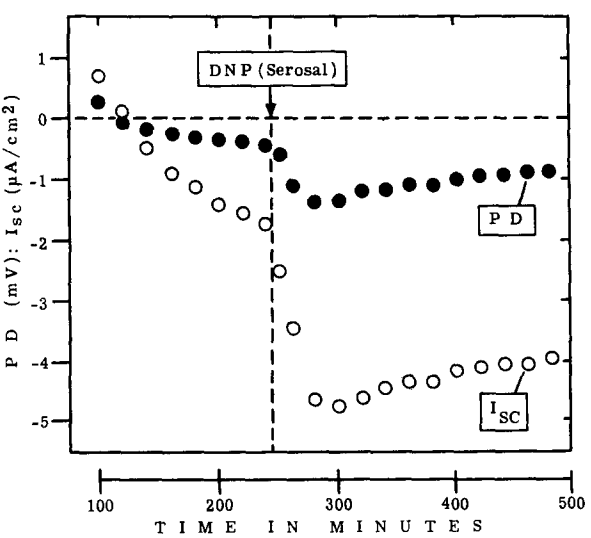

Fig. 9. Effect of serosal dinitrophenol (DNP) (1 mM) on PD (●) and I_{sc} (○) in Tris chloride medium containing glucose. Conditions as in Fig. 2.

* This was done because dinitrophenol moved across the tissue fairly readily in either the mucosal to serosal or the serosal to mucosal direction. Although no quantitative measurements of the rate of movement of dinitrophenol were made, this was evident from the appearance in the initially dinitrophenol free compartment of the characteristic yellow color of the dinitrophenol ion within a few minutes of its addition to the opposite compartment.

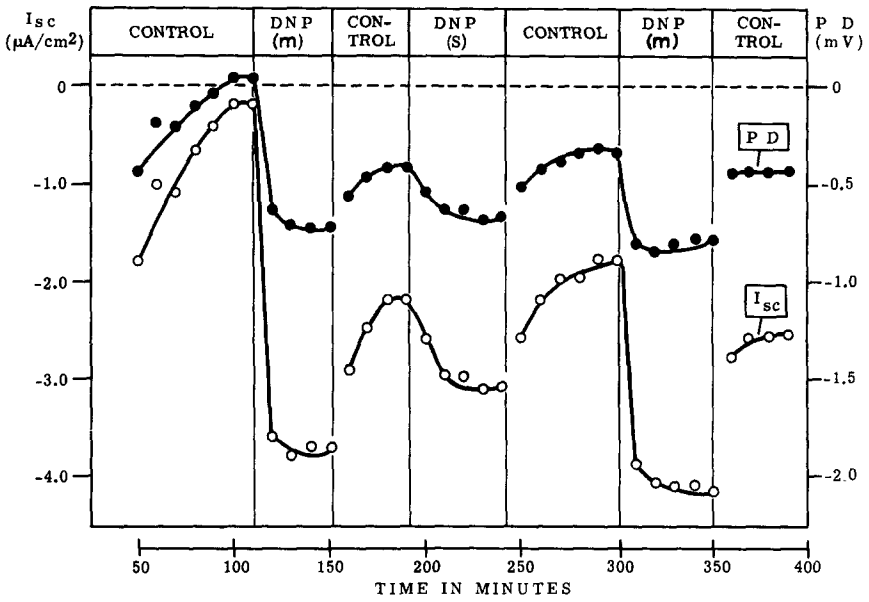

Fig. 10. Enhancement of (serosal negative) PD (●) and I_{sc} (○) by alternate additions of dinitrophenol (DNP) (1 mM final concn.) to mucosal and serosal media and reversal of these effects following rinsing with dinitrophenol (DNP)-free medium (conditions as in Fig. 2). Breaks in PD and I_{sc} curves indicate periods during which both chamber compartments were rinsed and finally refilled with dinitrophenol (DNP)-free medium.

dinitrophenol, and a final rinsing and recovery period. The stimulatory effect of dinitrophenol during the second and third exposures and its reversal on removal of this agent are clearly apparent in Fig. 10. In this figure, mucosal dinitrophenol appears to be more effective than serosal dinitrophenol. This is fortuitous. In a series of such experiments, the relative magnitudes of the mucosal and serosal responses were quite variable and no consistent differences were observed between them. As was found in the experiments with N_2, the data of Figs. 9 and 10 (and of other experiments of a similar kind) did not reveal any marked effect of dinitrophenol on steady-state transmural resistance.

Relationship between I_{sc} and Cl^- fluxes under anaerobic conditions

The increased PD and I_{sc} observed in the presence of N_2 and utilizable sugars observed in these experiments are electrically equivalent to an increased net mucosal to serosal transfer of negative charge under these conditions. They are therefore consistent with a stimulation by anoxia of the Na^+-independent mucosal to serosal Cl^- pump previously postulated for bullfrog small intestine[2]. A similar explanation can be offered for the stimulatory effect of dinitrophenol on PD and I_{sc} (Figs. 9 and 10). In the present investigation direct evidence in favor of this interpretation was sought in a series of experiments in which unidirectional transmural Cl^- fluxes were determined isotopically under short-circuit conditions, the I_{sc} being monitored simultaneously. Since in practice, only one radioisotope (^{36}Cl) of chlorine could be used, it was not possible to compare mucosal to serosal and serosal to mucosal fluxes in the same tissue preparation. The design of these experiments was therefore as

follows. The tissue was allowed to reach an electrical steady state in O_2 (with repeated changes of the medium in both chamber compartments as described above) in non-radioactive Ringer solution. Following this preliminary equilibration period (normally about 90 min) the medium in either the mucosal or the serosal compartment was replaced with one containing a tracer amount of $^{36}Cl^-$. Samples were then removed from both chambers at 15- or 20-min intervals. This was done in order to prevent the development of hydrostatic gradients across the tissue and to provide a continuous check on the constancy of the specific activity of the fluid in the initially labelled chamber. This was continued until at least 3 samples under virtually constant I_{sc} had been obtained. The O_2 supply was then replaced by N_2 and sampling was continued until at least 3 samples had been collected at constant I_{sc} under N_2. Unidirectional Cl^- fluxes for each steady-state sampling period were calculated from the increment of radioactivity in the initially non-radioactive half chamber during that period as described by QUAY AND ARMSTRONG[2].

The results obtained in these experiments are summarized in Table I. The difference in magnitude between the average serosal to mucosal and mucosal to serosal fluxes in the two groups of data shown, though quite large (particularly in O_2) are within the normal range of variation for different groups containing small numbers of animals.

Although the power of the statistical analysis is, admittedly, low (because of the relatively small number of observations), three salient points emerge from the data of Table I. First, there was no significant change ($P > 0.5$) in the unidirectional serosal to mucosal flux on changing from O_2 to N_2. Second, there was a highly significant increase ($P < 0.01$) in the corresponding mucosal to serosal Cl^- flux. Third, the increase in the mucosal to serosal flux on changing from O_2 to N_2 was significantly greater ($P < 0.05$) than the corresponding increase in I_{sc}.

In another set of experiments, evidence for an increase in the mucosal to serosal flux of Cl^- was obtained following addition of 1 mM dinitrophenol to the media on both sides of the tissue. However, insufficient data were collected in these experiments to permit statistical analysis.

TABLE I

UNIDIRECTIONAL SEROSAL TO MUCOSAL ($J_{s\to m}$) AND MUCOSAL TO SEROSAL ($J_{m\to s}$) Cl^- FLUXES IN Na^+-FREE MEDIA. EFFECT OF N_2

Groups (i) and (ii) each contain pooled data for 3 individual experiments. Numbers in parentheses are total numbers of flux determinations for each case.

Gas phase	*J (mean ± S.E.: nequiv/cm² per min)*
(i) $J_{s\to m}$	
O_2	27.5 ± 2.6 (10)
N_2	29.1 ± 1.6 (14)
$\Delta J = 1.6 \pm 1.4$: $J_{N_2} = J_{O_2}$; $P > 0.5$	
(ii) $J_{m\to s}$	
O_2	14.6 ± 1.4 (10)
N_2	23.0 ± 1.6 (13)
$\Delta J = 8.4 \pm 1.1$: $J_{N_2} = J_{O_2}$; $P < 0.01$	
$\Delta I_{sc} = 2.9 \pm 0.3$: $\Delta J = I_{sc}$; $P < 0.05$	

The results in Table I not only show that the unidirectional mucosal to serosal flux of Cl^- is increased under anoxic conditions but that this increase is significantly greater than would be predicted from the corresponding change in I_{sc}. Evidently at least one other ionic species contributes to the net transmural current under these conditions. We have not yet fully accounted for this discrepancy between net Cl^- flux and I_{sc}. However, we have obtained evidence which strongly suggests that it is due, in part at least, to a co-transport of K^+. This evidence is as follows. It was shown earlier in our laboratory[2] that net mucosal to serosal Na^+ flux in bullfrog small intestine under short-circuit conditions is drastically reduced when SO_4^{2-} is substituted for Cl^- in the medium. Similarly, because of the very small values of the (negative) I_{sc} in Na^+-free Cl^- media compared to the net flux of this ion in NaCl media, it was suggested that net Cl^- flux was also decreased in the absence of Na^+. A model involving coupled entry of Na^+ and Cl^- across the brush-border membrane was invoked to account for a reciprocal dependence of net Na^+ and Cl^- transport[2]. The present experiments show that both the mucosal to serosal and the serosal to mucosal Cl^- fluxes in Na^+-free media (Table I) are considerably smaller than those previously observed[2] in media containing Na^+, thus lending further support to the concept of an interaction between the transmural fluxes of Na^+ and Cl^-. Because of this we designed a series of experiments to test the possibility that, in Na^+-free media, K^+ might substitute to some extent for Na^+ in this co-transport process. These experiments were performed as follows.

The tissue was allowed to reach an electrical steady state under O_2 as before (with repeated changes of the bathing medium as already described) in a non-radioactive glucose medium. This was identical to that used in the experiments summarized in Table I except that it did not contain K^+. When a virtual steady state was established the O_2 supply was closed off and N_2 was admitted to the system. Changes in I_{sc} similar to that shown in Fig. 2 were obtained in the absence of K^+. When the increased I_{sc} under N_2 had steadied out the mucosal medium was replaced by an identical solution containing a tracer amount of $^{36}Cl^-$. Samples for counting were then removed from both chambers at 20-min intervals. Sampling was continued until it was estimated that at least 3–4 samples had been obtained under conditions of steady-state isotopic flux. At this time 50 μl of 0.4 M K_2SO_4 was added to each half chamber (this gave a final K^+ concentration of about 4.5 mM depending on the number of 0.1-ml samples previously removed) and sampling was continued for the remainder of the experiment.

A typical result is shown in Fig. 11. In this figure the amount of Cl^- transferred from the mucosal to the serosal half chamber, together with the concomitant I_{sc}, is plotted as a function of time. It is seen that addition of K^+ increased mucosal to serosal Cl^- transfer without appreciably changing I_{sc}. The slopes of the calculated lines of regression for steady-state Cl^- transfer before and after addition of K^+ (5.7 ± 0.4 and 7.5 ± 0.3 nequiv/min) differed significantly ($P < 0.01$). In 3 experiments of this kind the average steady-state mucosal to serosal Cl^- fluxes before and after addition of K^+ were 16.2 ± 0.4 (S.E.) and 20.6 ± 1.0 (S.E.) nequiv/cm^2 per min. The difference between these (which represents a 27 % increase in the rate of Cl^- transfer) was significant at the 0.01 level. There was no significant change in I_{sc} following addition of K^+ to the medium.

In conclusion, the results of these experiments clearly indicate that active

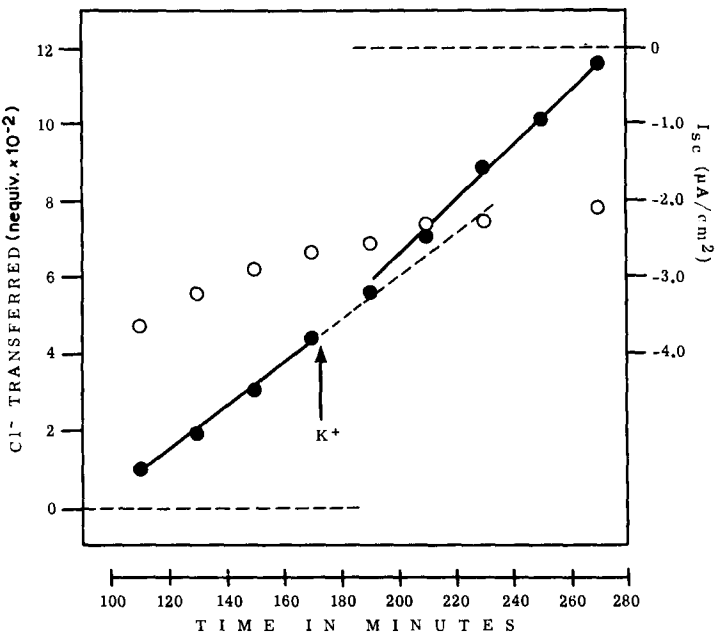

Fig. 11. Effect of K^+ (4.5 mM) on anaerobic mucosal to serosal Cl^- transfer (●) and I_{sc} (○) in presence of glucose. Lines are drawn from calculated linear regression equations for Cl^- transfer before and after addition of K^+.

mucosal to serosal Cl^- transport in bullfrog small intestine has a metabolic dependence which is quite different from that of active mucosal to serosal Na^+ transfer in this tissue. The latter appears to depend mainly on oxidative phosphorylation, being inhibited to a large extent by N_2 and dinitrophenol. In contrast to this, the present study indicates that in the presence of an exogenous supply of metabolizable substrate active Cl^- transfer is markedly increased under conditions where oxidative phosphorylation is inhibited and can be decreased again by restoring conditions favoring oxidative phosphorylation (Figs. 2 and 8). This suggests that the energy for active Cl^- transport may be directly derived from glycolytic pathways and is enhanced under conditions where the glycolytic activity of the epithelial cells is increased. We would like to speculate briefly on some possible implications of such a mechanism. In general little evidence for active mucosal to serosal Cl^- transfer has been found in mammalian small intestine *in vitro*, but there are convincing reasons for postulating its existence in a number of *in vivo* preparations[25]. If in fact active transport of Cl^- in mammalian small intestine is a similar but more labile process to that described for amphibian small intestine in the present paper, it could, because of the coupling between the transport of Na^+ and that of Cl^- previously described[2] (and further suggested by the coupling between the transport of Cl^- and that of K^+ found in this investigation) be an important regulatory mechanism in the *in vivo* absorption of salt and water by mammals. In particular, it could play a significant role in absorption under abnormal conditions (*e.g.* ischemia) where the intestinal blood supply is reduced and there may as a result be some local hypoxia. Such a mechanism could partly explain the recent finding of MAILMAN AND INGRAHAM[26] that

absorption of Na^+, Cl^- and water is enhanced in dog ileum *in vivo* following hemorrhage.

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REFERENCES

1 J. F. QUAY AND W. MCD. ARMSTRONG, *Physiologist*, 10 (1967) 286.
2 J. F. QUAY AND W. MCD. ARMSTRONG, *Am. J. Physiol.*, 217 (1969) 694.
3 H. H. USSING AND K. ZERAHN, *Acta Physiol. Scand.*, 23 (1951) 110.
4 J. F. QUAY AND W. MCD. ARMSTRONG, *Proc. Soc. Exp. Biol. Med.*, 131 (1969) 46.
5 R. J. LEVIN, *Proc. Soc. Exp. Biol. Med.*, 121 (1966) 1033.
6 G. A. GERENCSER AND W. MCD. ARMSTRONG, *Fed. Proc.*, 29 (1970) 596.
7 W. MCD. ARMSTRONG, T. K. SUH AND G. A. GERENCSER, *Physiologist*, 13 (1970) 138.
8 J. A. ZADUNAISKY, *Am. J. Physiol.*, 211 (1966) 506.
9 S. G. SCHULTZ AND R. ZALUSKY, *J. Gen. Physiol.*, 47 (1964) 567.
10 C. F. ROTHE, J. F. QUAY AND W. MCD. ARMSTRONG, *IEEE Trans. Biomed. Eng.*, 16 (1969) 160.
11 W. WILBRANDT AND T. ROSENBERG, *Pharmacol. Rev.*, 13 (1961) 109.
12 W. MCD. ARMSTRONG, D. L. MUSSELMAN AND H. C. REITZUG, *Am. J. Physiol.*, 219 (1970) 1023.
13 J. F. WHITE AND W. MCD. ARMSTRONG, *Am. J. Physiol.*, 221 (1971) 194.
14 R. D. BAKER, M. J. WALL AND J. L. LONG, *Biochim. Biophys. Acta*, 225 (1971) 392.
15 D. H. SMYTH, in W. MCD. ARMSTRONG AND A. S. NUNN, JR., *Intestinal Transport of Electrolytes, Amino Acids and Sugars*, Thomas, Springfield, Ill., 1970, p. 52 ff.
16 J. C. SKOU, *Physiol. Rev.*, 45 (1965) 596.
17 R. W. ALBERS, *Annu. Rev. Biochem.*, 36 (1967) 727.
18 M. SAWADA AND T. ASANO, *Am. J. Physiol.*, 204 (1963) 105.
19 S. G. SCHULTZ AND R. ZALUSKY, *J. Gen. Physiol.*, 47 (1964) 1043.
20 S. G. SCHULTZ AND P. F. CURRAN, *Physiol. Rev.*, 50 (1970) 637.
21 P. G. KOHN, H. NEWEY AND D. H. SMYTH, *J. Physiol. London*, 208 (1970) 203.
22 G. G. BERG AND J. SZEKERCZES, *J. Cell. Comp. Physiol.*, 67 (1966) 487.
23 G. A. GERENCSER AND W. MCD. ARMSTRONG, *Biochim. Biophys. Acta*, 255 (1972) 663.
24 R. K. CRANE, *Physiol. Rev.*, 40 (1960) 789.
25 S. G. SCHULTZ AND P. F. CURRAN, in C. F. CODE AND W. HEIDEL, *Handbook of Physiology*, Section 6, Vol. III, American Physiological Society, Washington, D.C., p. 1245 ff.
26 D. S. MAILMAN AND R. C. INGRAHAM, *Proc. Soc. Exp. Biol. Med.*, 137 (1971) 78.