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Sodium-Amino Acid Interactions in the Intestinal Epithelium

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Sodium–amino acid interactions in the intestinal epithelium

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[Plate 24]

Much evidence has accumulated during the last few years to suggest a direct coupling between the movement of sodium and amino acids across plasma membranes, a sodium gradient providing energy for the active transport of amino acid. This work is summarized and attention drawn to instances where it seems unlikely that a sodium gradient can be the only driving force involved.

An alanine dependent sodium influx and a sodium dependent alanine influx can be demonstrated to occur in intestinal epithelia. In the rabbit ileum these dependent fluxes are said to be coupled in a one-to-one fashion but, in the goldfish intestine, the ratio appears to depend on the external sodium concentration. The rate of potassium influx into the goldfish mucosa is approximately equal to that for sodium, but various attempts to demonstrate an alanine dependent potassium influx have failed. High concentrations of potassium inhibit the sodium–alanine interaction. These results with potassium are used to emphasize the specificity of the amino acid interaction for sodium.

Introduction

The initial finding that cations (Christensen & Riggs 1952) and more particularly sodium (Kromphardt, Grobecker, Ring & Heinz 1963) can affect the facility with which amino acids cross cell membranes has now been exploited in some detail. Once the widespread occurrence of such interactions became established, interest focussed on two different problems: how a sodium gradient might provide energy for the movement of amino acids against their respective concentration gradients, and what a sodium–amino acid interaction might mean in terms o molecular changes taking place within the transporting membrane. No definitive answer to either question has yet emerged in spite of an increasing interest in these problems over the last ten years.

Though the number of cells and tissues showing this type of interaction are very numerous, detailed studies as to its operation have been confined largely to the Erhlich ascites tumour cell, the pigeon erythrocyte and the intestinal epithelium. Of these three the Erhlich cell has been studied most intensively yet it is with this preparation that recent results have been obtained which question the existence of any simple relation between the influx of sodium and amino acids and the function of a sodium gradient in providing all the energy needed for the active transport of amino acids (see Christensen 1970). A more optimistic view is taken by Schultz & Curran (1970) in describing how this interaction operates in the intestinal epithelium in terms of a specific carrier mechanism with exact stoichiometry. This may be true, but it may be that further research with this tissue, whose function is inherently more complex than that of a single cell, will reveal inconsistencies similar to those now recognised for the Erhlich cell.

As a preliminary contribution to complicating the issue in the intestinal epithelial cell we should like to report some of our findings with the goldfish intestine, where we have been measuring directly the ability of sodium to augment alanine influx and of alanine to augment sodium nflux. Since the results obtained did not always correspond to those reported previously for

rabbit ileum (Schultz, Curran, Chez & Fuisz 1967; Curran, Schultz, Chez & Fuisz 1967), it would perhaps be wise first to describe in detail the tissue we used and the method chosen to study unidirectional fluxes.

Materials and methods

Fish

Goldfish weighing from 100 to 150 g were kept at 16 °C under controlled conditions of lighting for 2 to 3 weeks before use. On arrival they were placed in 2% (w/v) sodium chloride solution for 20 min to remove any surface fungus which might have been present. This treatment was sometimes repeated on two successive days. The fish were kept in aerated tap water until ready for use.

The gross appearance of the anterior intestine is shown in figure 1, plate 24. The muscle layers of the goldfish intestine are very thin compared with those of mammalian intestines and the mucosal epithelium is much simpler, having few goblet cells and no crypts. A further advantage of this tissue is seen with *in vitro* observations. It remains stable over long periods of time judged both by electrical measurements and by its ability to transport amino acids (Smith 1970). The turnover of epithelial cells is slow compared with mammalian intestine (Hyodo 1965; Leblond 1965) and its general level of metabolism is probably much lower.

Measurement of sodium and alanine uptake by the goldfish intestine

For influx experiments the anterior intestine was first rinsed with an isotonic saline solution (Krebs & Henseleit 1932) and a 10 cm length cut out and mounted in the apparatus described below.

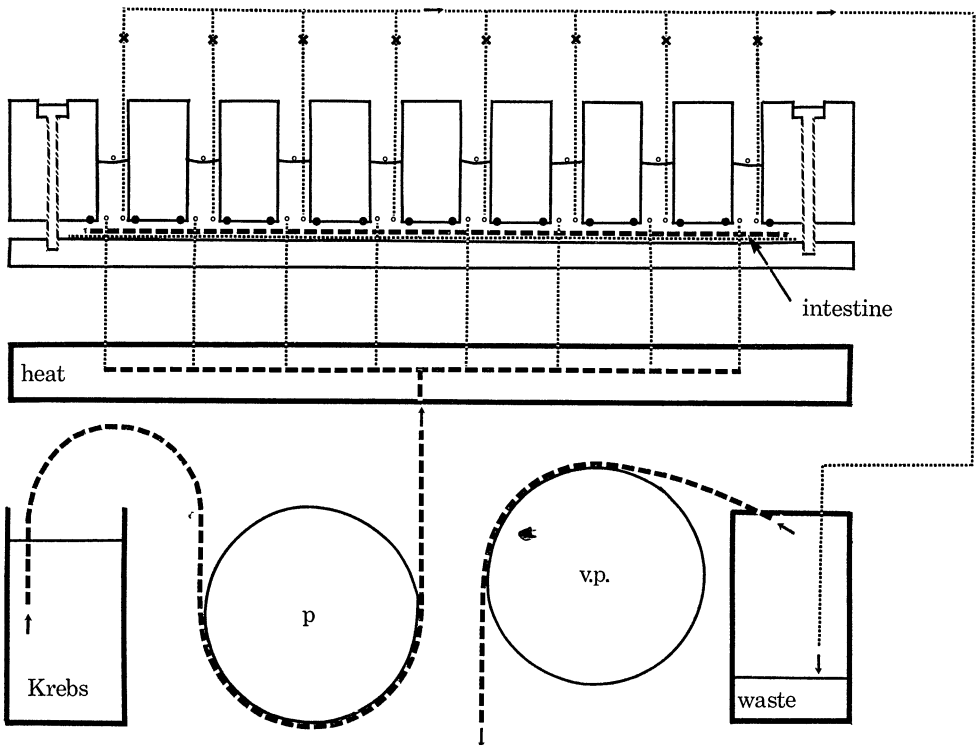

Figure 2. Apparatus for measuring alanine and sodium uptake by the goldfish intestinal mucosa. Each port contained 50 μl of Krebs–Henseleit solution heated to 29 °C, circulating at a rate of 2 ml/min per port over 0.067 cm^2 of mucosal surface. p, pump; v.p., vacuum pump.

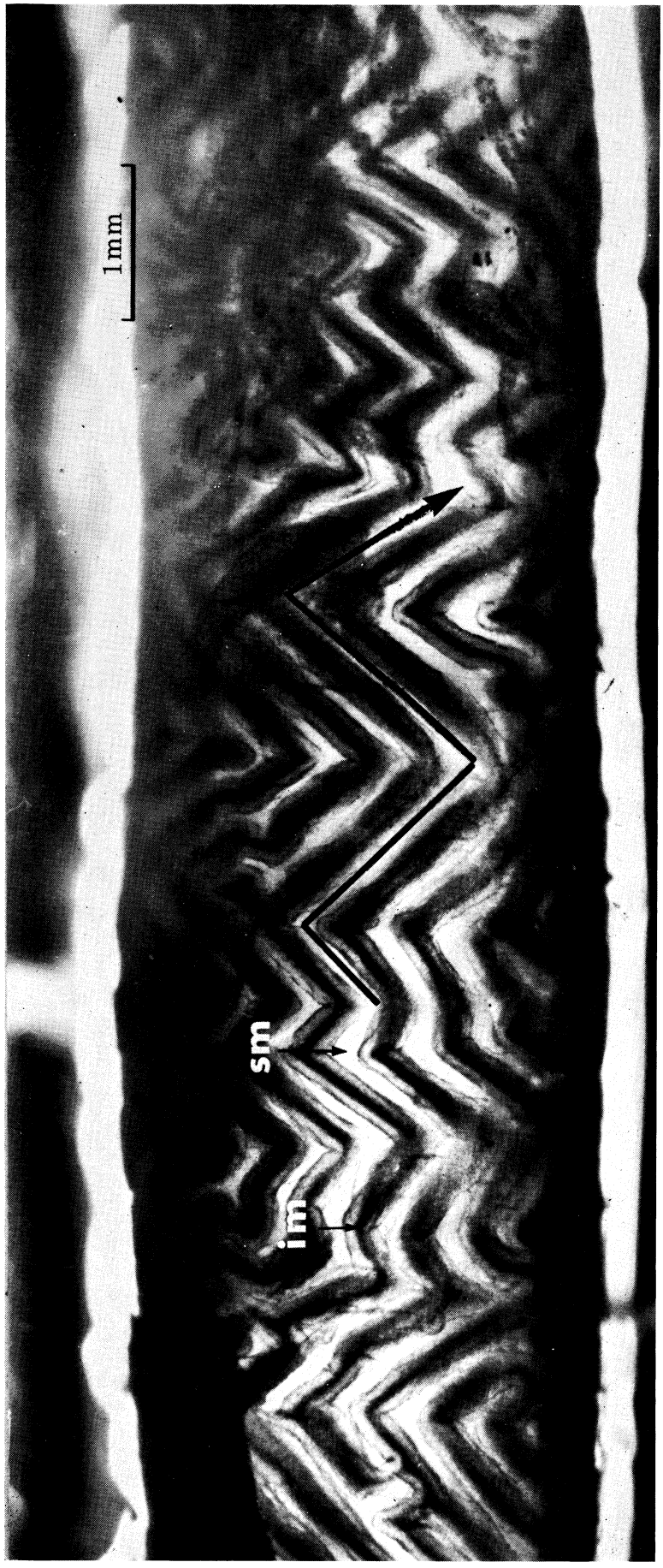

FIGURE 1. The anterior intestine of the goldfish. The intestine was filled with saline, illuminated from behind and the photograph taken with the camera focused on the near side of the intestine. The arrow shows the direction intestinal contents would take when moving down the tract. im, Intestinal mucosa; sm, smooth muscle.

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A diagrammatic representation of the apparatus is shown in figure 2. The intestine is cut open on moist plastic-backed filter paper (Benchkote, W. & R. Balston Ltd, Maidstone, Kent), so that the mucosal surface faces upwards. The paper and intestine are transferred to a perspex base plate which is then screwed to a second block of perspex containing eight small chambers provided with inlets and outlets to let medium flow over the mucosal surface of the intestine. This apparatus was modified after that described by Schultz *et al.* (1967). The present model was scaled down so that it could be applied to the goldfish intestine and solutions were pumped through each chamber so that the intestine could be washed constantly with preheated, oxygenated medium. The medium of Krebs & Henseleit (1932) was used to bathe the intestine, after gassing with 95% O_2 + 5% CO_2. This medium was used in preference to the modified high potassium Ringer of Schultz *et al.* (1967), since quite modest increases in potassium concentrations were found to inhibit both the uptake of sodium and alanine.

The intestine was allowed to equilibrate in Krebs-Henseleit medium for 30 min. The inlet to a selected port was then closed and a second outlet opened near the mucosal surface of the tissue. Medium in the port was sucked away down this second outlet and the tissue washed with 3 ml of a selected, preheated medium. The second outlet was then closed and an identical solution added containing ^{24}NaCl (20 μCi/ml) and [^{14}C]inulin (1 to 2 μCi/ml), with or without [^{3}H]alanine (5 μCi/ml). In some experiments ^{42}KCl was used instead of ^{24}NaCl. The radioactive solution was sucked away at known times afterwards and the tissue washed with 2 ml ice-cold, isotonic mannitol. The tissue was cut out and the mucosal cells disrupted in 0.3 ml 0.1 mol/l HNO_3, dioxan was added and the sample counted for ^{24}Na or ^{42}K by Cerenkov radiation in a Packard scintillation spectrometer. One week later, when the ^{24}Na or ^{42}K had decayed, concentrated scintillator was added so that the final composition of the solution corresponded to Bray's (1960) medium. Samples were then counted for [^{3}H]alanine and [^{14}C]inulin. Inulin was used as described by Schultz *et al.* (1967) to correct for the amount of alanine and ^{24}Na trapped at the mucosal surface but not taken up by the cells. Separate experiments showed inulin to be an adequate space-marker in this tissue. The recovered inulin counts showed a random variation which was independent of the time of contact with the tissue.

Results

Time course for alanine and sodium uptake by the goldfish intestine

The uptake of sodium and alanine is plotted against time of incubation in figure 3. The test solution resembled Krebs–Henseleit medium except that it contained no $NaHCO_3$. Instead the concentration of NaCl was increased to 145 mmol/l and 10 mmol/l Tris used to maintain a pH of 7.4. Alanine was present at a concentration of 1 mmol/l. The uptake of both alanine and sodium increased linearly with time during the first 60 s exposure. At longer times there was some suggestion that the rate of uptake might be decreasing. An exposure time of 1 min was therefore chosen for the remaining experiments, it being assumed that alanine and sodium would not penetrate further than the mucosal cell within so short a time. The control fluxes for sodium and alanine, measured at this time, were respectively 290 ± 29 (21) and 4.95 ± 0.52 (25) nmol cm^{-2} $min^{-1} \pm$ S.E. (number of observations).

Lines drawn to represent the uptake of alanine and sodium over the first 60 s exposure to the test solution could be extrapolated through, or very near to, the origin. This was important for two reasons; it gave independent evidence that the inulin correction was adequate and it showed that the influxes being measured took place into a single compartment.

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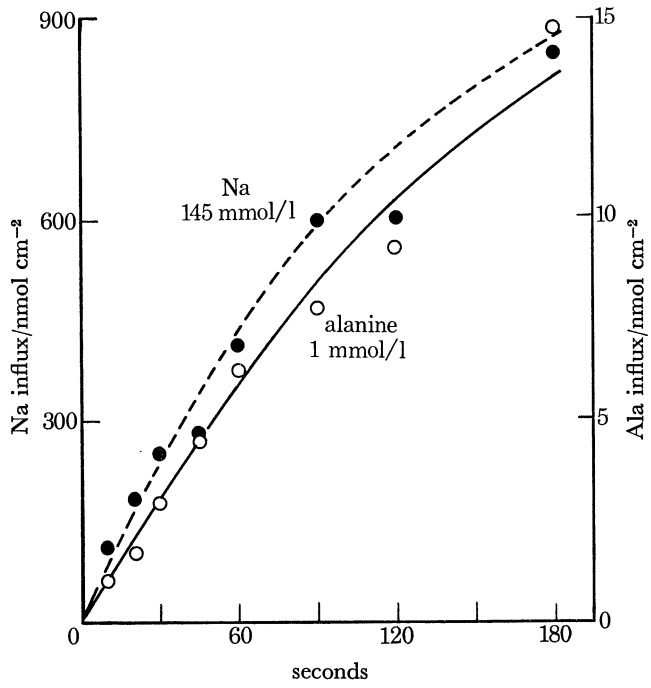

FIGURE 3. Sodium and alanine influx into goldfish intestinal epithelium. The conditions for incubation were as described in the text. ●– – –●, Uptake of sodium; ○——○, upake of alanine. Each point gives the mean of eight determinations, each determination being carried out on intestines obtained from different fish.

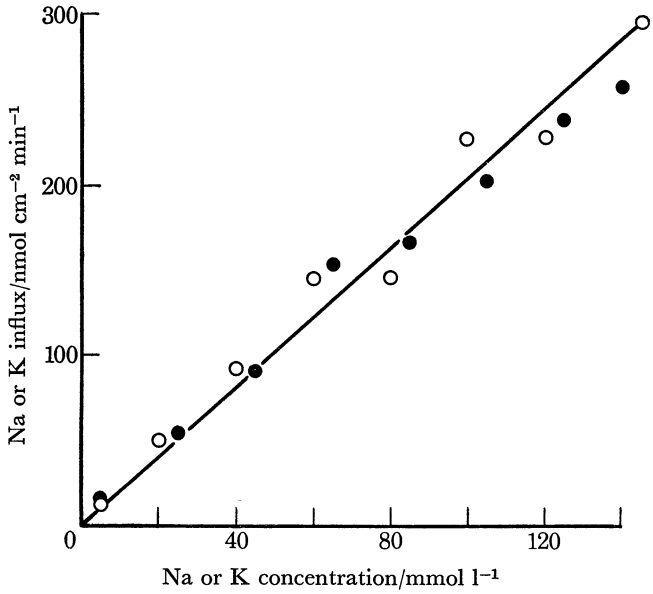

FIGURE 4. Relation between sodium and potassium uptake and the external concentrations of these cations. Solutions containing ^{24}NaCl or ^{42}KCl were presented separately to the intestinal mucosa. Choline chloride was used to maintain osmolality. ●, Uptake of potassium; ○, uptake of sodium. Values for potassium uptake give means derived from six determinations carried out on different fish. Those for sodium were collected from separate experiments designed to study the effect of sodium on alanine uptake. Each point gives the mean of at least twelve determinations.

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Dependence of sodium and potassium uptake on external cation concentration

The uptake of potassium and sodium was determined separately, at different concentrations, using 10 mmol/l Tris pH 7.4 and choline as substituting cation. The results are shown in figure 4. The uptake of sodium and potassium was a linear function of concentration over the whole range studied (5 to 145 mmol/l). The intestinal epithelium could not distinguish between sodium and potassium, both penetrated the mucosal cells with equal facility when presented separately to the mucosal surface.

The uptake of sodium was next measured in the presence of different amounts of potassium. Sodium was present in each test solution at a concentration of 20 mmol/l. Different amounts of potassium were added and lithium used as substituting cation. Separate experiments showed that lithium and choline were interchangeable substitutes for sodium in this tissue. The uptake of sodium in the absence of potassium, 44.6 ± 3.5 (22) nmol cm^{-2} min^{-1}, was reduced to 31.1 ± 2.0 (8) nmol cm^{-2} min^{-1} in the presence of 20 mmol/l potassium ($\pm$ S.E., number of observations). This inhibition was statistically significant ($P < 0.05$). Increasing the external concentration of potassium to 70 or 125 mmol/l caused no further inhibition of sodium uptake (28.7 ± 2.7 and 30.4 ± 2.1 nmol cm^{-2} min^{-1} respectively, 7 observations). It therefore seems that sodium uptake consists of at least two parts, that which is readily inhibited by and that which is not inhibited by potassium. This point was not investigated further and 5 mmol/l KCl was routinely present both during the equilibration period and during the measurement of sodium influx.

Sodium dependence of alanine uptake

The uptake of alanine was determined at four different concentrations in the presence and absence of sodium. It was possible, by using all eight ports in the apparatus, to make all these determinations on a single intestine. The results obtained, shown in figure 5, give the mean values from six such determinations. Alanine uptake from solutions containing 1 mmol/l alanine, measured in previous experiments, is also included. The double reciprocal plot gave reasonably straight lines for alanine uptake, whether or not sodium was present. Sodium increased the rate of alanine uptake at all concentrations tested, but the effect became less obvious as the concentration of alanine increased. If we take these lines to represent the kinetics for alanine interaction with a carrier in the presence and absence of sodium, then the presence of sodium can be said to increase the apparent affinity of the carrier for alanine but to leave the maximal rate of uptake unchanged. These results are very similar to those reported previously for alanine uptake by rabbit ileum (Curran *et al.* 1967).

20 mmol/l alanine was used for the remainder of the experiments to measure the augmentation of sodium influx. The effect of sodium on alanine influx at this concentration is small (see figure 5), but this concentration was chosen so that there should be enough alanine present to cause a significant increase in sodium influx. This is shown in figure 6. The results are from two typical experiments, in this case using 60 mmol/l NaCl. The sodium influx was, on average, greater in the presence of 20 mmol/l alanine and the alanine influx greater in the presence of 60 mmol/l NaCl, but the scatter was large. At least three such comparisons on intestines taken from different fish were carried out in order to calculate mean sodium-dependent alanine and alanine-dependent sodium fluxes.

Alanine could stimulate sodium uptake indirectly, if it were to be concentrated within the mucosal cells during the time allowed for measurement of influx. Though this seemed rather

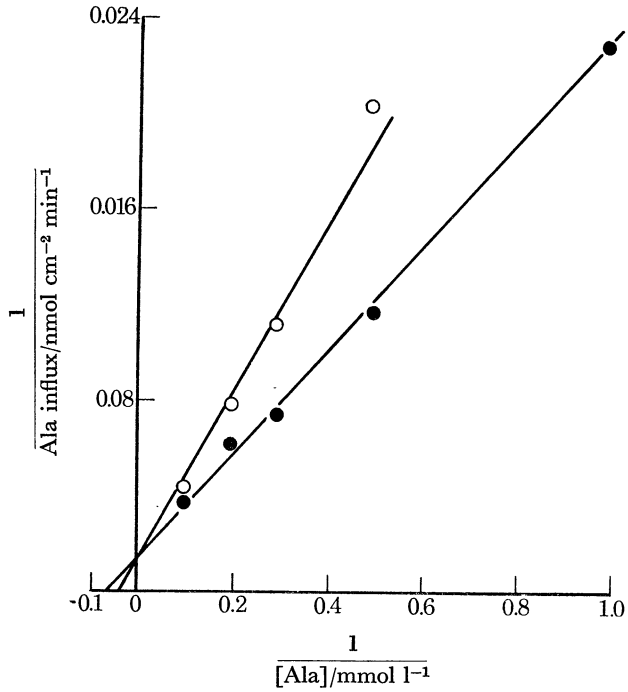

FIGURE 5. Double reciprocal plots of alanine uptake measured in the presence and absence of sodium. The test solution contained 10 mmol/l Tris, pH 7.4, with different concentrations of alanine in the presence (●) and absence (○) of 145 mmol/l NaCl. Choline chloride was used to substitute for NaCl. The conditions for incubation were as described in the text.

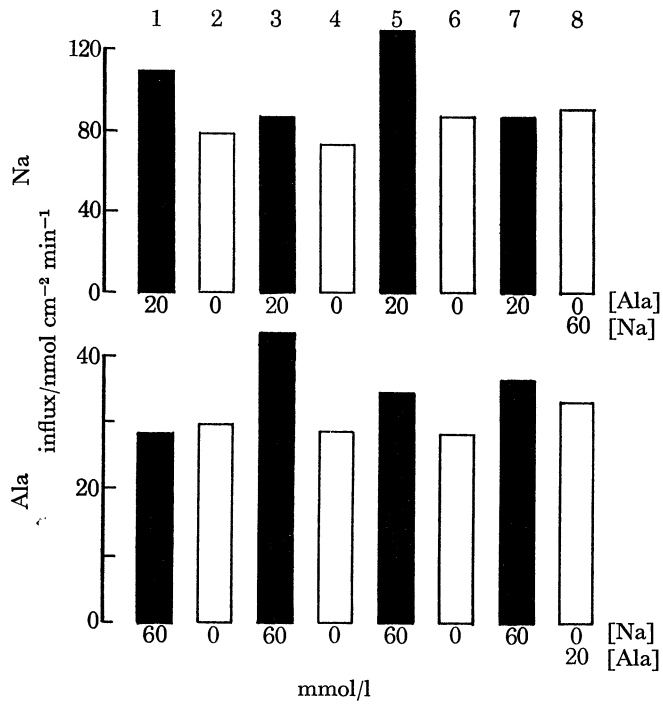

FIGURE 6. Typical experiments showing the augmentation of sodium uptake in the presence of 20 mmol/l alanine and alanine uptake in the presence of 60 mmol/l sodium. Numbers 1 to 8 refer to the ports where influx was measured. The results were obtained from two intestines, one where alanine was varied at constant sodium concentration and the second where sodium was varied at constant alanine concentration.

unlikely, control experiments were designed to test this point by measuring the effect 20 mmol/l alanine had on potassium influx. The uptake of sodium and potassium was identical when measured in the absence of alanine. It might be then that a non-specific osmotic effect by alanine would result in an increased influx of potassium as well as sodium. The results obtained are shown in figure 7. The influx of potassium fell in the presence of 20 mmol/l alanine, but the difference was not statistically significant ($P > 0.3$). This was in spite of the fact that most of the alanine entering the mucosa in the absence of potassium continued to do so in its presence. Potassium caused an inhibition of alanine influx (57.1 ± 5.7 nmol cm^{-2} min^{-1} without potassium; 47.2 ± 3.5 nmol cm^{-2} min^{-1} in the presence of 20 mmol/l KCl, 8 determinations). This reduction was small but statistically significant ($P < 0.02$). It was concluded that the augmentation alanine caused to sodium influx was a true measure of a specific sodium–amino acid interaction taking place within the microvillar membrane.

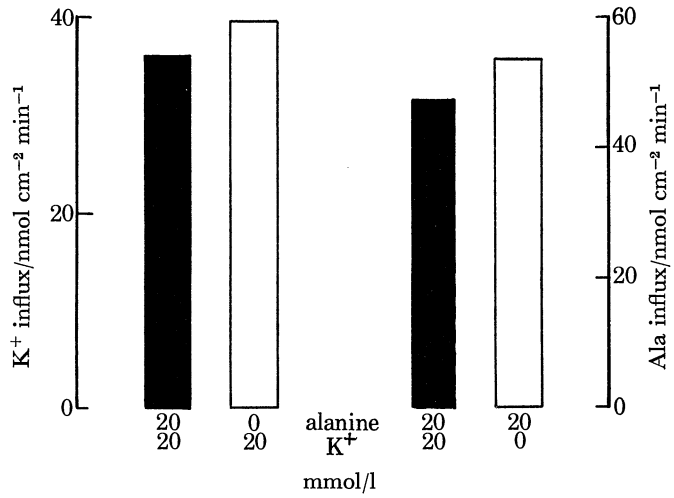

FIGURE 7. Test for potassium on alanine influx and alanine effect on potassium influx in goldfish intestine. Conditions for incubation were as described for measurements of sodium influx. Choline replaced all sodium in experiments to measure the effect of alanine on potassium influx. Lithium was used to substitute for 100 mmol/l NaCl in experiments to measure the effect of potassium on alanine influx. 20 mmol/l NaCl was present in these latter experiments. Each histogram gives the mean of eight determinations.

Interdependent uptake of alanine and sodium

Having established the selectivity of the interaction being investigated, alanine-dependent sodium and sodium-dependent alanine fluxes were measured at different external sodium concentrations. Coupling ratios for these dependent fluxes have been plotted in figure 8. The coupling ratio, alanine-dependent sodium influx/sodium-dependent alanine influx, varied from 1 at low sodium concentrations to about 5 at high sodium concentrations, the increase being linear over the sodium concentration range 5 to 40 mmol/l. There was no further increase over the sodium concentration range 40 to 80 mmol/l. It was not possible to determine coupling ratios with confidence at sodium concentrations greater than 80 mmol/l. Two values were calculated for the coupling ratio using 20 mmol/l sodium, one with choline and the other with lithium used to substitute for sodium. The values obtained, 1.8 when choline substituted and 2.3 when lithium substituted for sodium, were very similar. It was concluded that lithium and choline were equally good substitutes for sodium for this epithelium.

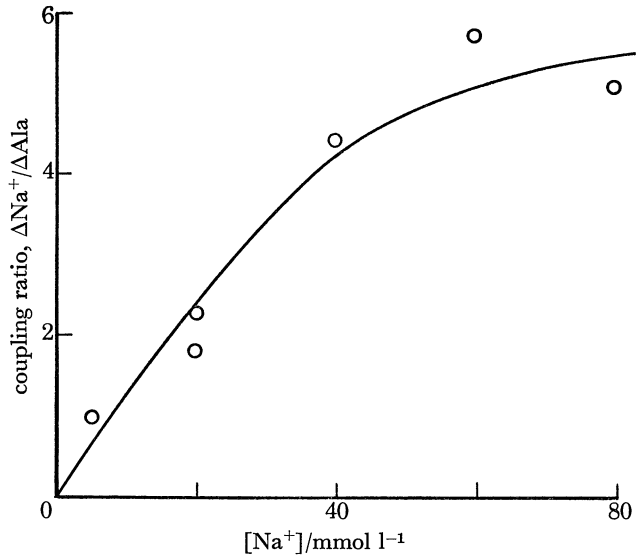

FIGURE 8. Dependence of the coupling ratio, the alanine-dependent sodium influx (ΔNa^+)/the sodium-dependent alanine influx (ΔAla), on the external concentration of sodium. Choline was used to maintain osmolality at sodium concentrations of 5, 20, 40 and 80 mmol/l; lithium was used at sodium concentrations of 20 and 60 mmol/l. Each dependent flux was calculated from at least twelve comparisons carried out on the intestines of three fish.

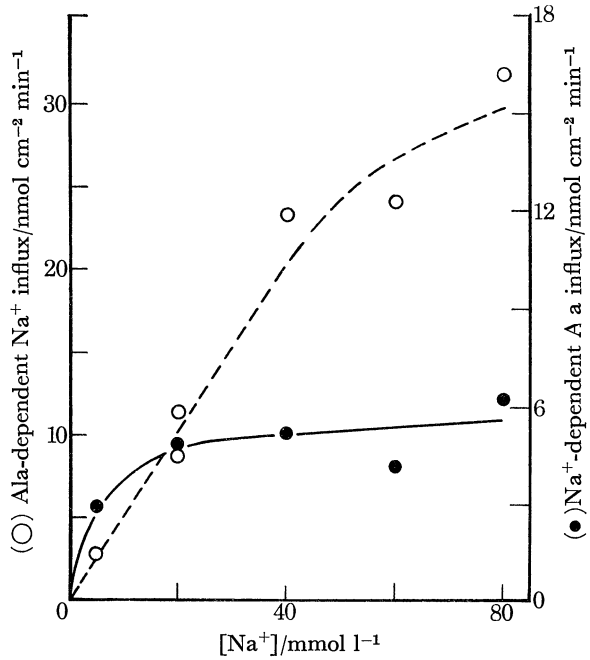

FIGURE 9. Relation of the interdependent fluxes of alanine and sodium to the external concentration of sodium. ○---○, Increase in sodium influx caused by the presence of 20 mmol/l alanine in the test solutions; ●——●, increase in alanine influx caused by the presence of different concentrations of sodium in the test solution. Cation substitutions for sodium were as described in Figure 8. Each value gives the mean of at least twelve comparisons carried out on intestines taken from three fish.

The dependence of these influxes on the external concentration of sodium is shown in figure 9. The alanine-dependent sodium influx increased linearly as the sodium concentration was raised from 5 to 40 mmol/l. Further increases in alanine-dependent sodium influx also took place with higher external concentrations of sodium but the rate of increase was not so rapid. The sodium-dependent alanine influx behaved differently from the alanine-dependent sodium influx, rising rapidly to a virtual maximum by the time the external concentration of sodium had reached 20 mmol/l.

Most of the characteristics of the coupling ratio could, therefore, be directly attributed to changes which take place in the alanine-dependent influx of sodium.

Discussion

A major disadvantage of studying transport phenomena in epithelial tissues has always been the complicated nature of the whole tissue in relation to the transporting layer of cells. This has been particularly true of the intestine and of the skin of amphibia, where the transporting epithelium comprises only a small fraction of the total tissue present. The use of short-term measurements, particularly where the epithelium is directly accessible to bathing media, allows one to overcome this difficulty. The role of supporting tissue in changing influx measurements can be ignored if the time chosen is of sufficiently short duration. One can then study the kinetics of transport into cells with a rigour normally reserved for more homogenous tissues. The technique of measuring short-term influxes, which was first described by Schultz *et al.* (1967) is now becoming widely exploited. Used in modified form it has already provided valuable information concerning the kinetics of sodium entry into the frog skin (Rotunno, Vilallonga, Fernández & Cereijido 1970; Biber & Curran 1970; Biber 1970) and toad bladder epithelium (D. R. Ferguson & M. W. Smith, unpublished data) and it will probably have still wider applications in the future.

The measurement of amino acid fluxes across the microvillar membrane assumes particular significance since it is generally agreed, with the possible exception of Gilles-Baillien & Schoffeniels (1965), that this is the site for active transport. It should be noted, however, that the present measurements of alanine influx, like those of previous workers, do not actually follow the movement of amino acids against their concentration gradients. The time of exposure is too short for such gradients to become established. This is a compromise which has to be made if one is to isolate influx from efflux phenomena. This seems justified at present, but it has been suggested that sodium can independently affect amino acid diffusion in the rabbit jejunum (Rosenberg, Coleman & Rosenberg 1965) and that sodium can increase the passive transfer of other non-electrolytes, such as acetamide and thiourea, across rat intestine (Esposito, Faelli & Capraro 1969). If these effects are taking place in the microvillar membrane one will have to re-assess the validity of the current assumptions concerning amino acid transport.

The entry of sodium into the intestinal epithelium at first sight appears to be by simple diffusion. There is no saturation of influx at high external concentrations of sodium as is seen in the frog skin and toad bladder. On the other hand, the effect of amino acids on sodium entry suggests that part at least of the sodium uptake involves an interaction with the microvillar membrane. Inhibition of sodium influx by potassium, with no amino acid present, further suggests that part of the alanine-independent sodium influx might also take place by a mechanism involving interaction with the transporting membrane.

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The suspected complexity of the means by which sodium and amino acids enter the mucosal epithelium makes it difficult to form hard and fast conclusions as to how the sodium–amino acid interaction might operate. The results of Curran, *et al.* (1967) cannot be compared directly with the present work since the method used to calculate coupling ratios was different. Nevertheless, both sets of data suggest a coupling between the influx of sodium and alanine which varies with the external concentration of sodium. In the rabbit ileum it is suggested that what is really happening is that sodium and alanine enter on a carrier in a fixed ratio of 1 : 1 and that a variable proportion of alanine enters by the same carrier but without sodium. We would suggest, in the goldfish intestine, that the situation is probably more complicated. The fact that the sodium-dependent alanine influx reaches a maximum at a lower sodium concentration than the alanine-dependent sodium influx, makes a tight coupling between the two influxes seem highly improbable. The best explanation which we could offer to explain our results in terms of a carrier-mediated interaction would be to suggest that there are two different sets of sodium-binding sites on the membrane. One set would have a high affinity for sodium and would be present when the external concentration of sodium was low. The apparent affinity of the carrier for alanine would also be low at this concentration of sodium. High concentrations of sodium increase the affinity of the carrier for alanine. It is suggested that further sodium-binding sites become exposed as the affinity for amino acid increases. These exposed sites would have to have a lower affinity for sodium to explain the observed continuing increase in alanine-dependent sodium influx seen at higher sodium concentrations. Although these seem a lot of assumptions to make in describing the interaction, they are, in fact, no more numerous than those already made to form the alternative hypothesis. These are that sodium cannot bind to the amino acid carrier in the absence of an amino acid, that all the amino acid entering the mucosal cell does so by a carrier-mediated route and that the coupled influx of sodium and alanine normally proceeds with a fixed stoichiometry. It is not the present intention to suggest that one model system is more favourable than another, but to emphasize the incomplete state of our present knowledge concerning these interactions and to suggest that simple model systems might, in the end, prove an inadequate way of describing how such a complex tissue works.

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FIGURE 1. Electron micrograph (magn. × 4600) of rabbit gall-bladder epithelium, kindly provided by Dr John M. Tormey. Fluid transport proceeds in the direction from the gall-bladder lumen (above), through the epithelium, towards the bloodstream (below, labelled 'base'). The epithelium is a single cell layer about 30 μm high, with microvilli protruding into the lumen and a large nucleus (N) located centrally in each cell. Mitochondria, the small dark objects, are particularly numerous at the luminal end of the cells, a fact whose possible significance is discussed on p. 143. Adjacent epithelial cells are separated by lateral intercellular spaces (LIS), whose luminal ends are sealed by tight junctions (J). This gall-bladder had been incubated in $NaCl + NaHCO_3$ Ringer's solution at 37 °C before fixation, so that fluid transport was proceeding at a maximal rate. Note that the lateral intercellular spaces are open.

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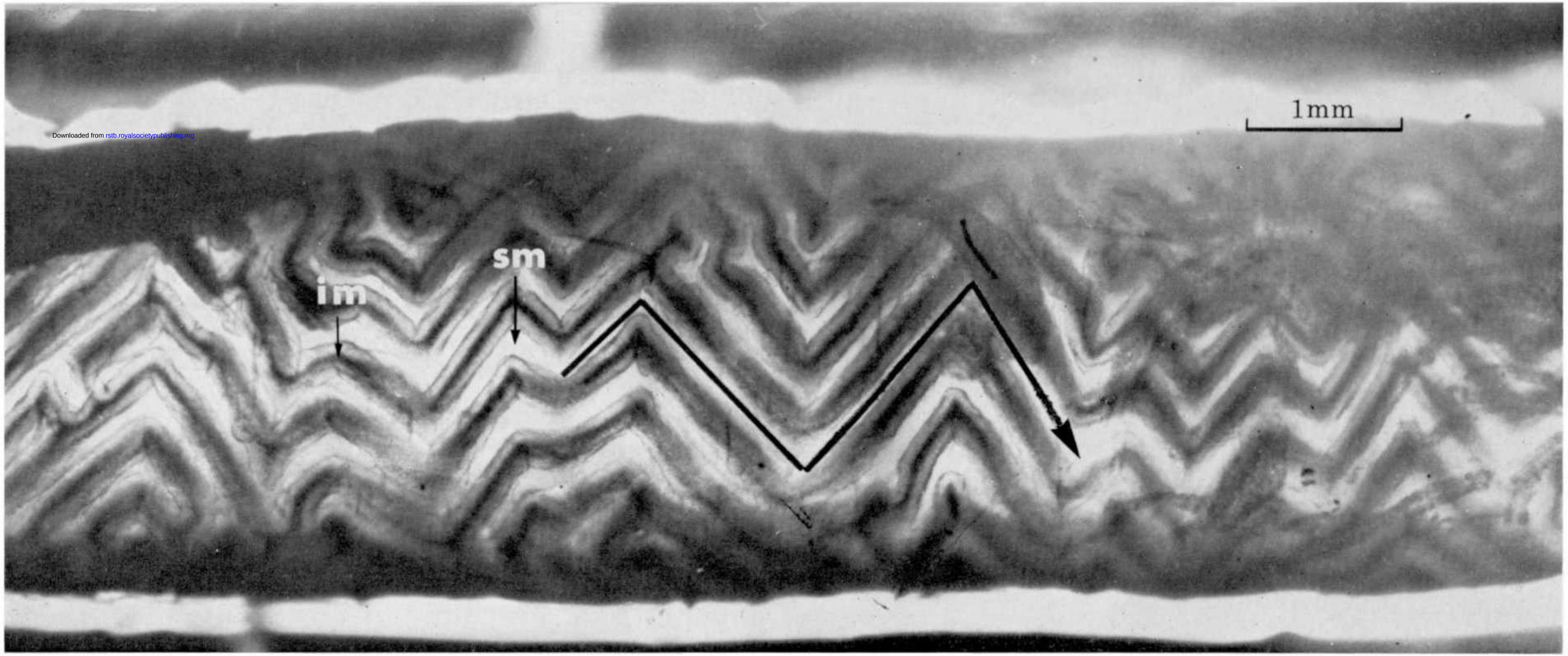

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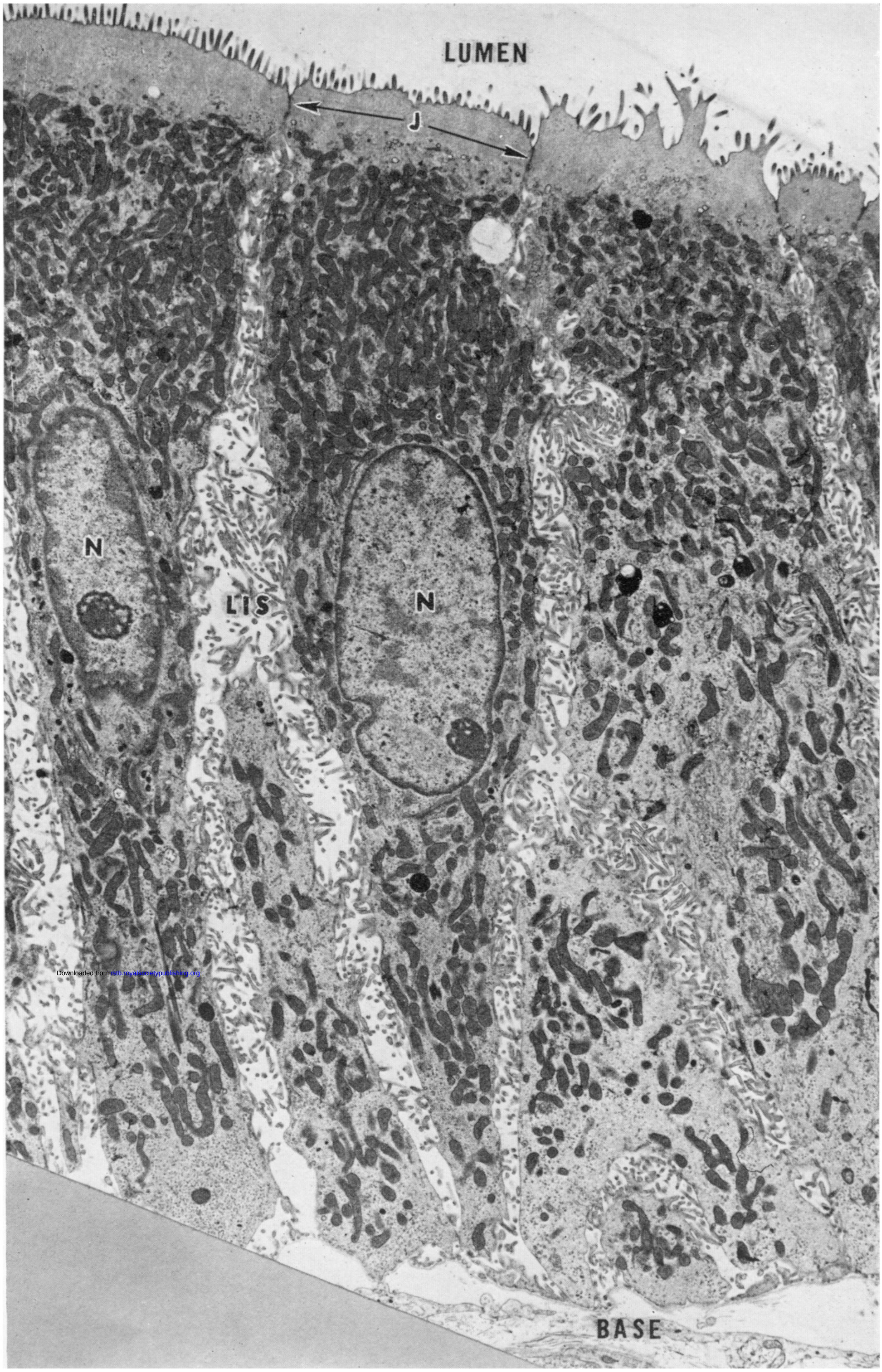

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