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BICARBONATE SECRETION AND NON-Na COMPONENT OF THE SHORT-CIRCUIT CURRENT IN THE ISOLATED COLONIC MUCOSA OF *BUFO ARENARUM*

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SUMMARY

1. In the isolated colonic mucosa of *Bufo arenarum*, under special circumstances, there is a variable fraction of the short-circuit current (0–38 %) that is unaccounted for by either the Na or the Cl and bicarbonate transmembrane net fluxes.

2. The hypothesis that a special kind of bicarbonate transport may account for the non-Na component of the short-circuit current was investigated. According to this, bicarbonate ions formed within the membrane await transport towards the mucosal solution within a compartment that does not undergo isotopic exchange with the serosal bathing solution. This kind of transport may be detected by a lowering of mucosal specific activity of bicarbonate but would not be revealed by the classic method of comparing the difference between the unidirectional fluxes with the short-circuit current.

3. The specific activity of bicarbonate was determined in the inside solution (initially bicarbonate-free) of ten normal and four everted colonic sacs incubated in an external medium (reservoir) containing a constant specific activity of bicarbonate. Comparison between membrane-to-internal solution bicarbonate flux and non-Na component of the short-circuit current was carried out in two different ways: (*a*) by measuring the remaining short-circuit current in Na-free medium and (*b*) by determining simultaneously the Na net flux.

4. Whatever the value of the short-circuit current and its non-Na component, there is no reduction of the specific activity of the bicarbonate appearing in the inside solution of the everted colonic sacs.

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5. In the normal sacs there is a reduction of the specific activity of bicarbonate which accounts for a membrane-to-mucosa bicarbonate flux which parallels the variations of the non-Na component of the short-circuit current although quantitatively representing only 68–87 % of it.

6. There is no systematic decrease in the rate of reduction of the mucosal specific activity of bicarbonate in successive experimental flux periods; this excludes a slow equilibration of the intracellular bicarbonate with serosal bicarbonate.

7. Other possible explanations of the present results are discussed, as well as the availability and hydration rate of metabolic CO_2 necessary to account for this kind of bicarbonate transport.

INTRODUCTION

Experiments reported in the previous paper (Lew, 1970) show that, in the absence of electrochemical gradients, the Na net flux across the colonic mucosa of *B. arenarum* does not account for all the short-circuit current. The magnitude of the non-Na component of the short-circuit current varies greatly among different animals and, in a single membrane, it usually declines with time. The factors that control its presence and initial magnitude as well as the nature of the ions involved are unknown.

Cl and bicarbonate ions have been considered but their net isotopic flux did not differ significantly from zero under short-circuit conditions in which the non-Na component as determined with a Na-free choline Ringer solution, before and after the experimental flux periods, ranged between 7 and 33 % of the short-circuit current. These results rule out the Cl ions as possible candidates but not necessarily the bicarbonate ions. A secretory mechanism for bicarbonate has been proposed (Lew, 1970) which is compatible with the observation. If bicarbonate ions formed within the cell are transported towards the mucosal side of the membrane without undergoing isotopic exchange with the bicarbonate ions contained in either bathing solution, their transport, even active and electrogenic, would not be revealed by the classic method of comparing the net fluxes of the ionic species across the membrane with the short-circuit current.

The experiments reported in this paper were designed to test the possibility that transport of this kind may explain at least part of the non-Na component of the short-circuit current.

METHODS

Experimental approach. The method by which this kind of transport may be detected is to measure the lowering of specific activity on the side of the membrane towards which the ions are carried. An important point in the original hypothesis is

that the bicarbonate ions must be continuously formed within the cell and their formation coupled in such a way to their transport that they cannot exchange with bicarbonate ions coming from the serosal bathing solution. A necessary consequence would be the lowering of the specific activity of the mucosal bicarbonate.

If a solution containing [^{14}C]bicarbonate of known specific activity is placed in contact with the serosal surface of the colonic mucosa and a bicarbonate-free solution in contact with the mucosal surface, all the bicarbonate appearing in the mucosal solution must proceed either from the serosal solution or from the membrane. If the specific activity of the bicarbonate present in the mucosal solution after a given time is the same as in the serosal solution, it all came from this solution; but if its specific activity is lower, part of it originated within the membrane. A similar argument applies to the situation in which the radioactive solution is placed on the mucosal side and the bicarbonate-free solution on the serosal side. If the only outcome of these experiments is the reduction in specific activity of the mucosal bicarbonate, a necessary condition in support of the proposed model is met. However, as other possibilities, which will be discussed later, may lead to a similar result, this result alone is not sufficient.

It is also necessary that the absolute value of the diluting bicarbonate flux from the membrane towards the mucosal solution should be equal to the difference between Na net flux and the short-circuit current. An immediate consequence is that in the absence of that difference, no reduction of the mucosal bicarbonate specific activity should be detected. This comparison can be carried out in two different ways: (*a*) by measuring the reduction in specific activity of the mucosal bicarbonate in the absence of Na ions and with the membrane under short-circuit conditions, and (*b*) by measuring both the net Na flux and the bicarbonate efflux in the same short-circuited mucosa. In the first case it is necessary to use a very unstable choline-[^{14}C]bicarbonate solution, which is highly inconvenient. It was therefore decided to compare the short-circuit current with the net Na flux and bicarbonate efflux calculated from the isotopic fluxes and change in specific activity of these ions in the same short-circuited colonic membrane. The term efflux will be used throughout this paper to refer to the flux of a molecular or ionic species from the interior of the epithelial cell outwards, whether to the serosal or mucosal surface.

The contribution of the CO_2 efflux is not expected to produce any kind of asymmetry in the dilution of the bicarbonate specific activity since it is assumed that both the serosal and mucosal borders of the epithelial cell have a very high and similar permeability to CO_2 and that the rate-limiting process in the CO_2 efflux is its metabolic production rate and its hydration rate.

The participation of CO_2 from the solutions bathing the preparation in the unidirectional fluxes is also unlikely to produce any dilution of specific activity, as it should have the same specific activity as the bicarbonate. But if the contribution of the CO_2 flux to the over-all ^{14}C serosa to mucosa flux is so high that the proportion of the eventual diluent bicarbonate becomes too small, then measurement of the dilution of the specific activity of bicarbonate may become affected by a large experimental error. However, when the radioactive solution is saturated with 100 % O_2 the bicarbonate efflux required to explain at least 50 % of the non-Na component of the short-circuit current is about a half or even more of the unidirectional combined CO_2 + bicarbonate flux (Lew, 1970).

The accuracy of the estimate of the change in bicarbonate specific activity rests upon the correct determination of the activity and the final bicarbonate concentration in the originally bicarbonate-free solution. The maximum bicarbonate flux that can be expected is about 1·5 μ-equiv/cm^2 hr. In order to get final concentrations of bicarbonate that could be measured with low relative errors it is convenient to

increase the area of exposed membrane or the incubation time or both. As some 'time' experiments are needed to study the possibility of a slowly exchanging bicarbonate compartment, it is more convenient to reach reasonable values of bicarbonate concentration even within 1 hr periods by increasing the membrane area and simultaneously decreasing the volume of the nominally bicarbonate-free solution.

A convenient procedure is, therefore, to use a colonic sac prepared from the entire colonic mucosa, in which the internal solution is always the bicarbonate-free one. While the external solution acts as a reservoir, the volume of the internal solution can be made arbitrarily small (within experimental limitations). Normal and everted sacs may be used to test the bicarbonate efflux into both mucosal and serosal solutions.

Experimental procedure. The procedures for selecting and killing the toads and for preparing the membranes and the composition of the Ringer solutions have already been described (Lew, 1970).

Female toads were used mainly within the first 2 weeks of captivity. For the preparation of the normal sacs, once the dissection of the muscular layer was completed, the distal end of the mucosal sac was tied with linen thread and the whole colonic membrane, hanging from the glass cannula, mounted in the flux apparatus (see below). When everted sacs were used, after the muscle sheet had been peeled off, the glass cannula was introduced inside the colonic lumen until it reached the distal end. This end was tied with linen thread at the constriction in the cannula; the upper part of the colonic sac was then gently drawn over the ligature, leaving the everted sac suspended from the cannula.

The apparatus used to measure the potential difference, short-circuit current and Na and bicarbonate fluxes of the normal and everted colonic sacs was a slightly modified version of the apparatus described by Barry, Smyth & Wright (1965). The main modification consisted in the use of a silver sheet, 1 mm thick, as the external current electrode. It was applied against the wall of the cylindrical glass tube (25 mm internal diameter) that contained the external nutrient solution. A silver wire, 1·5 mm diameter, was used as the internal current electrode. It was fixed in such a way as to act as the geometrical axis of symmetry of the system. The potential electrodes were agar–saline fine-tipped bridges with their ends within 2 mm of the membrane. In this way, the condition of uniform current density through the membrane is satisfied (Ussing & Zerahn, 1951). Both current electrodes were electrolytically coated with AgCl.

The true short-circuited current was calculated by the method of Clarkson & Toole (1964). The current–voltage relation was plotted before and immediately after mounting the membrane in the apparatus. All the experiments reported in the present paper were run while keeping the potential difference across the membrane at 0 mV (corrected value). The short-circuit current was recorded every 5 or 10 min. The initial volume of saline inside the sacs, whether normal or everted, was 2 ml. and the volume of the external solution was between 50 and 160 ml. Fluid changes inside the sacs were estimated with a standard gravimetric technique. The combined weight of the cannula, the inside current electrode and the sac was determined before and after filling or emptying of the sac at the beginning and at the end of each experimental period. The weighing procedure lasted about 45 sec. Corrections for evaporation of fluid from the external surface during the weighing, fluid retained in the dead volume of the system, variation in the degree of drying of the external surface (by gently blotting with a wet filter paper) and evaporation of fluid from the inside, were derived from dummy experiments. The absolute error in the weighing was 1 mg and the error in the whole procedure, 12 mg. This represents a maximum relative error of 1·2 % in the estimation of the internal volume. A further maximum

of 1 % arises from the assumption that the specific gravity of the saline solutions equals 1 (presence of mucus).

Whereas the external solution was continuously stirred by a gentle flow of 100 % of O_2, the internal solution was saturated with 100 % O_2 before being introduecd inside the sac and was not stirred except at the beginning and at the end of the experimental period by circulating an air bubble left at the top of the cannula.

Two main groups of experiments were done:

(1) Four 'time' experiments with normal sacs and four with everted sacs, in which the bicarbonate concentration and ^{14}C radioactivity in the internal solution was determined every hour over 2–4 hr, 2 ml. fresh solution being replaced each time. The concentration of bicarbonate in the external solution was 30 mM and the specific activity of the [^{14}C]bicarbonate was 55 μc/m-mole. The internal solution was initially bicarbonate-free. The short-circuit current was first recorded half an hour after the sac was mounted. Before and after each experimental period the normal sacs were exposed for 15 min to a Na-free, bicarbonate-solution outside and to a Na-free, bicarbonate-free solution inside, and the short-circuit current before and after replacing the Na was recorded. The same was done to the everted sacs, but reversing the place of the solutions.

(2) In six final flux experiments, with normal sacs, the unidirectional fluxes of Na were measured using ^{22}Na and ^{24}Na for each unidirectional flux, in the same short-circuited colonic mucosa. At the same time the dilution of specific activity of bicarbonate in the inside solution was determined in the same way as reported for the first group of experiments. The experimental period lasted 4 hr and samples for determination of radioactivity and bicarbonate concentration were withdrawn at the end of this experimental period.

Radioactive assay. The ^{14}C activity in samples containing labelled bicarbonate as the only radioactive isotope was counted as already described (Lew, 1970). In the flux experiments, the external (serosal) solution contained initially ^{22}Na and [^{14}C]-bicarbonate and the internal (mucosal solution), ^{24}Na. The specific activity of ^{22}Na in the external solution was about 0·01 μc/m-mole (approximately 0·001 μc/ml.) and the specific activity of ^{24}Na in the internal solution was about 5–10 μc/m-mole (approximately 0·5–1·0 μc/ml.). The specific activity of [^{14}C]bicarbonate in the external solution was 55 μc/m-mole in the 'time' experiments and 19 μc/m-mole in the 4 hr flux experiments. In this way, the errors involved in the correction for contaminant isotopes were minimized and the relevant activity was always a substantial proportion of the total activity in the sample. At the end of the 4 hr experimental period both solutions contained the three isotopes. The internal solution had to be analysed for ^{22}Na, ^{24}Na and ^{14}C, and the external one for ^{24}Na. $^{24}Na + ^{22}Na$ in the external solution were counted without interference from ^{14}C activity in a well scintillation counter; the ^{22}Na contaminant activity was subtracted by counting the same sample at different time intervals. The activities of ^{22}Na, ^{24}Na and ^{14}C in the internal solution were determined separately by using a well-type scintillation counter and a gas flow counter and correcting with a calibration factor for the relative efficiencies of ^{22}Na with the two methods. The activity of ^{24}Na was estimated by counting the same samples at different times; its value was subtracted from the ^{22}Na and ^{14}C activities. The reliability of the correction factor was tested by extracting the [^{14}C]-bicarbonate from a diluted sample containing the three isotopes with a gentle stream of 95 % O_2 + 5 % CO_2 saturated with water vapour. The effluent gas was bubbled through two flasks containing alkali before reaching the atmosphere. At least 95 % of the ^{14}C radioactivity was recovered in the first flask. The remaining ^{24}Na and ^{22}Na activities were within 2 % of their calculated value, using the correction factor. The specific activity of the ^{24}Na inside the sacs decreased by about 7–10 % in 4 hr in the

normal sac experiments. The mucosa to serosa Na flux was calculated assuming a linear change in the ^{24}Na internal specific activity between the initial and final value.

Chemical methods. The final Na concentration inside the sacs was estimated by flame photometry. The bicarbonate ion concentration was measured in duplicate with a manometric Warburg unit. A 0·5 ml. sample was made up to 2 ml. with distilled water. 1 ml. was placed at the bottom of a Warburg flask which contained 0·5 ml. lactic acid in the side arm. The reaction was started by pouring the lactic acid on to the sample under an atmosphere of 95 % O_2 + 5 % CO_2. The manometer together with the flask was gently stirred for about 100 sec. The change in volume was recorded at atmospheric pressure on the manometer. The whole procedure was carried out at 37° C. With this method it is possible to reach a sensitivity of 6–8 manometric divisions per milli-equivalent per litre of bicarbonate concentration using 1 ml. of sample.

Before and after each sample determination, 1 ml. standard solution of comparable bicarbonate concentration (within 5 m-equiv/l. before and 1 m-equiv/l. after) was assayed in the same way. Comparison with the Van Slyke manometric method for concentrations above 10 mM gave an agreement within 1 %.

Flux calculations. The unidirectional Na fluxes were calculated from the proportion of isotope crossing the membrane, the Na concentration and the volume of the solutions on each side of the membrane. The bicarbonate flux corresponding to the dilution of the specific activity inside the sac, was calculated from the formulae

$$\frac{F_1}{F_2} = \frac{S_o}{S_i} - 1, \tag{1}$$

$$F_1 + F_2 = c_i v_i, \tag{2}$$

in which

F_1 amount of bicarbonate (in μ-mole) that has originated within the membrane and entered the inside solution during one experimental period;

F_2 amount of bicarbonate (in μ-mole) that crossed the membrane from the outside solution to the inside solution during a single experimental period;

S_o, S_i specific activity of [^{14}C]bicarbonate outside and inside, at the end of the collection period, respectively.

$c_i v_i$ amount of bicarbonate (in μ-mole) present in the inside solution at the end of the experimental flux period (estimated from the final bicarbonate concentration, c_i, and the final internal volume, v_i).

The fluxes calculated in this way can be expressed in μ-equiv (or μ-mole) of ion per hour if the total flux is divided by the time interval between sample collections expressed in hours. This value can be compared with the average short-circuit current over the same period. The estimation of the area of membrane is not relevant for that comparison but may be of interest if one wants to compare the absolute values of the fluxes in the sac and plane membrane experiments. A rough estimate of the area of the colonic sac was obtained from the value of its weight by using the formula

$$S = 43 \times w,$$

in which S is the surface of the membrane in cm^2 and w is the weight of the empty and dried colonic sac in g, measured at the end of the experiment. The factor was obtained assuming that the specific gravity of the tissue is 1·1 and that the average thickness is 0·021 mm (microscopic average of thirty freeze sections corresponding to eight membranes).

Preliminary experiments with the colonic sac (not reported here) showed that this preparation behaves in the same way as a plane sheet of colonic mucosa in relation

to all the characteristics mentioned in the previous paper (Lew, 1970): spontaneous variations of the potential difference and short-circuit current, response to drugs and to changes in the composition of the bathing solutions. Effects due to lack of stirring of the inside solution and to topographical differences of transport activity (the upper or proximal part of the colon is about twice as active as the distal part although qualitatively similar) may account for the differences in the absolute value of the fluxes expressed per unit area in the sac and plane membrane experiments.

RESULTS

Table 1 gives the results of the two groups of 'time' experiments in which the specific activity of bicarbonate in the inside solution was measured over 2–4 1 hr flux periods in four normal and four everted colonic sacs. The specific activity of bicarbonate in the inside solution is expressed as a fraction of the specific activity in the external solution. The membrane-to-inside solution bicarbonate flux, calculated from the specific activity quotient, as described in the Methods section, is compared with the remaining fraction of the short-circuit current when both solutions are transiently replaced by Na-free solutions before and after each experimental flux period. These results show that

(1) whatever the value of the short-circuit current and the non-Na component, there is no reduction of the specific activity of the bicarbonate appearing in the inside solution of the everted sacs;

(2) in the normal sacs there is a reduction of the specific activity of bicarbonate with the following characteristics: (*a*) the amount of unlabelled CO_2 + bicarbonate transfer necessary to account for the reduction in specific activity is always less than the non-Na component (between 68 and 87 % of it); (*b*) it is absent when the non-Na component is less than 8 % of the total short-circuit current; (*c*) the diluting bicarbonate represented a proportion which ranged from zero to 56 % of the total bicarbonate within the sac at the end of each experimental period; (*d*) there is no systematic decrease in the rate of reduction of the mucosal specific activity of bicarbonate in successive experimental periods; this excludes a slow equilibration of the intracellular bicarbonate with serosal bicarbonate; (*e*) 'diluting' bicarbonate appears to be 'extra' bicarbonate, since the final amount of bicarbonate present inside the sacs when the specific activity is reduced is always higher than in the everted sacs or when there is no significative difference between short-circuit current and net Na flux (Expt. no. 3, normal sac).

Previous experiments (Lew, 1970) on colonic mucosa mounted as a flat sheet and confirmed in experiments with colonic sacs showed that a concentration of 5 mM bicarbonate in the serosal solution is sufficient to restore at least 50 % of the short-circuit current with the same proportion of Na

TABLE 1. Reduction of the specific activity of bicarbonate in the inside solution of normal colonic sacs of *Bufo arenarum*. Four normal and four everted colonic sacs were incubated at room temperature for two to four 1 hr experimental flux periods. The external Ringer solution contained (mM): Na^+, 110; K^+, 2·5; Cl^-, 79·5; HCO_3^-, 30; $H_2PO_4^{2-}$, 1·33; SO_4^{2-}, 0·5; Ca^{2+}, 1·25; gluconate, 2·5; glucose, 5·5. The specific activity of [^{14}C]bicarbonate was 55 μc m-mole^{-1}. The internal solution was initially bicarbonate-free. Its volume was always 2 ml. at the beginning of each experimental flux period. The external solution acted as a reservoir and its volume varied between 50 and 160 ml.

The potential difference across the membrane was kept at 0 mV. The short-circuit current was continuously recorded. The mean value given in the Table corresponds to the average of eight to twelve measurements performed during each experimental flux period. Before and after each experimental flux period the sac was exposed on both sides to a Na-free, choline solution, and the remaining short-circuit current under these conditions was recorded during 15 min. The average of four values, two before and two after each period, is given in the Table.

The other values given in the Table were measured or calculated as described in the Methods section

Expt. no.	Period	Mean short-circuit current (μ-equiv $\times$ hr^{-1})	Short-circuit current in Na-free medium (μ-equiv $\times$ hr^{-1})	Final internal bicarbonate concentration (mM)	Relative specific activity of bicarbonate Inside / Outside	Bicarbonate flux from membrane to inside solution (μ-equiv $\times$ hr^{-1})	Bicarbonate flux from outside to inside solution (μ-equiv $\times$ hr^{-1})	Final internal volume (g = ml.)	Estimated area of the sac (cm^2)
					Normal sacs				
1	1	19·2	5·8	5·2	0·44	4·0	3·2	1·388	10·3
	2	21·3	6·4	6·7	0·46	4·5	3·8	1·230	—
	3	16·1	4·1	4·4	0·47	3·5	3·1	1·497	—
	4	14·0	3·6	4·5	0·54	3·1	3·7	1·520	—
2	1	32·9	6·2	7·4	0·46	4·9	4·1	1·211	17·1
	2	36·6	6·2	7·7	0·54	4·2	5·0	1·198	—
3	1	22·6	1·1	3·6	0·98	0·1	5·7	1·612	16·0
	2	19·3	1·3	3·6	0·96	0·2	5·4	1·575	—
	3	15·6	0·8	2·4	1·02	—	4·2	1·733	—
4	1	31·5	5·0	5·7	0·49	3·9	3·7	1·322	11·2
	2	26·5	7·2	7·1	0·49	5·0	4·9	1·400	—
	3	23·0	6·2	7·5	0·51	5·3	5·5	1·440	—
	4	21·7	6·3	7·0	0·54	5·3	6·1	1·627	—
					Everted sacs				
1	1	1·23	0·10	2·3	1·02	—	4·7	2·061	11·8
	2	0·81	0·10	2·7	1·03	—	5·9	2·170	—
2	1	3·1	0·3	2·6	0·95	0·3	4·9	2·001	14·3
	2	4·2	0·2	2·2	0·99	—	4·4	2·010	—
	3	7·2	0·4	2·7	0·93	0·4	5·2	2·075	—
3	1	17·1	5·0	1·9	1·01	—	4·0	2·117	12·8
	2	12·0	4·3	1·9	0·98	0·1	3·8	2·057	—
4	1	12·3	3·6	2·2	1·00	—	4·5	2·066	15·7
	2	14·7	4·2	2·9	1·01	—	5·9	2·053	—

and non-Na components as is observed in the presence of 30 mM bicarbonate (fully activating concentration). In the everted sac experiments, the serosal bicarbonate concentration increased with time and so did the short-circuit current, and, presumably, its two components. However, under no circumstances was the specific activity of the internal bicarbonate significantly different from that in the external (mucosal) solution. There was a small increase in the volume of the internal solutions in the everted sacs, in contrast to the bigger decrease in the internal volume in the experiments with normal sacs. The direction of the fluid movement follows qualitatively the Na net movements.

Table 2 presents the results of six flux experiments in which the Na and non-Na components of the short-circuit current were estimated by measuring simultaneously the unidirectional fluxes of Na as well as the bicarbonate serosa to mucosa flux and internal specific activity in single normal colonic sacs. The results clearly show the parallel behaviour of the non-Na fraction of the short-circuit current and the bicarbonate efflux computed from the reduction of the mucosal specific activity. They also emphasize the existence of a small and variable unexplained fraction of the short-circuit current. Whereas the proportion of the short-circuit current corresponding to the non-Na component varied between 0 and 36 %, the fraction of the short-circuit current accounted for by the bicarbonate efflux varied only from zero to 24 %. Although the influence of the bicarbonate concentration in the bathing solutions upon the unidirectional bicarbonate fluxes has not been investigated, the mucosa to serosa 'back-flux' of bicarbonate, carrying a proportion of diluting bicarbonate ions from the mucosal bicarbonate pool, seems to be the obvious explanation for this unaccounted fraction of the short-circuit current.

DISCUSSION

Three main conclusions can be drawn from the present experiments:

(1) There is bicarbonate + CO_2 compartment in the epithelial cell layer of the isolated colonic mucosa of *Bufo arenarum* which is isolated isotopically from bicarbonate + CO_2 present in the serosal bathing solution;

(2) bicarbonate ions proceeding from this compartment appear in the mucosal solution;

(3) the cell to mucosa bicarbonate flux (efflux) parallels the variations of the non-Na component of the short-circuit current.

These results support the hypothesis that an electrogenic bicarbonate transport of the kind proposed in the previous paper (Lew, 1970) may account for most of the non-Na component of the short-circuit current observed in the colonic mucosa of *B. arenarum* under special conditions.

However, this is not the only mechanism that can be proposed to explain the present results and two other explanations deserve further discussion.

A mechanism similar to that described by Leaf (1959) as operating for lactate in the toad bladder will first be considered. According to this, the bicarbonate ions formed within the cell would accumulate there and diffuse

TABLE 2. Comparison between the non-Na component of the short-circuit current and the bicarbonate flux from membrane to mucosal solution in six normal colonic sacs of *Bufo arenarum*. Six normal short-circuited colonic sacs were incubated at room temperature for 4 hr and the unidirectional fluxes of Na were measured at the same time as the serosa to mucosa bicarbonate flux as described in the Methods section. The composition of the Ringer solutions was as described in Table 1 except for the specific activity of the external bicarbonate, which was 19 μc/m-mole. The bicarbonate flux from the membrane towards the mucosal solution was calculated from the reduction in the mucosal bicarbonate specific activity relative to the serosal one

Expt. no.	Mean short-circuit current (μ-equiv $\times$ hr^{-1})	Na net flux (mucosa to serosa)	Final bicarbonate concentration in the mucosal solution (mM)	(A)*	(B)†	Difference (A − B)
1	33·1	24·1	25·4	27·2	17·6	9·6
1	19·0	13·7	21·7	28·1	22·0	6·1
3	25·6	16·3	21·0	36·5	24·1	12·4
4	27·3	22·8	19·9	16·5	11·4	5·1
5	16·7	16·3	9·6	2·5	1·2	—
6	21·1	21·3	10·2	−0·9	1·6	—

* Percent of the short-circuit current unaccounted for by the Na net flux.
† Percent of the short-circuit current corresponding to bicarbonate flux from membrane to mucosal solution.

outwards through the serosal and mucosal membranes with different permeabilities to bicarbonate, thus explaining the observed asymmetry (accumulation–diffusion theory). The experimental design of the sac experiments makes the detectable bicarbonate transport always proceed downhill in relation to the serosal bicarbonate concentration. However, the facts (i) that the short-circuit current is completely independent of the mucosal bicarbonate concentration and (ii) that the short-circuit current remaining in the absence of Na ions in the bathing solutions is also independent of the level of the bicarbonate in the mucosal solution (Lew, 1970) show that the components of the short-circuit current are unaffected by the bicarbonate gradient across the mucosal membrane. This is obviously incompatible with the accumulation–diffusion theory.

The secretion of hydrogen ions towards the serosal solution being the

primary electrogenic process, followed by diffusion of bicarbonate ions towards the mucosal surface, is an alternative explanation, at first sight compatible with the present results. The main objection to this theory is that the bicarbonate ions diffusing from the cell interior towards the mucosal solution should remain isolated from the serosal CO_2 pool since they have to account for the dilution of the specific activity of the mucosal bicarbonate. The permeability of the whole membrane to CO_2 seems to be, at least, two orders of magnitude higher than the permeability to bicarbonate ions. Cooperstein & Hogben (1959) studied the unidirectional fluxes of bicarbonate + CO_2 in isolated colonic mucosa of *Rana catesbeiana* with the potential difference clamped at two different values: 0 and 45 mV. They found no net flux at 0 mV and a net flux along the electrical gradient (mucosa to serosa) of 0·34 μ-equiv/cm^2 at 45 mV. If the flux of bicarbonate + CO_2 is composed of a neutral flux of CO_2 and an anionic flux of bicarbonate, then the asymmetry in the unidirectional fluxes at 45 mV can be entirely attributed to bicarbonate ions. From a calculated ratio of 6 for the unidirectional fluxes of bicarbonate at 45 mV using Ussing's equation (Ussing, 1949) the over-all unidirectional flux of bicarbonate + CO_2 of 1·9 μ-equiv/cm^2 hr may be partitioned into a CO_2 flux of 1·5 μ-equiv and a bicarbonate flux of 0·4 μ-equiv. Since the equilibrium concentration of CO_2 is one twentieth that of bicarbonate (at pH about 7·4) and its contribution to the over-all flux is four times bigger than that of bicarbonate the membrane must be at least 80 times more permeable to CO_2 than to bicarbonate ions. This figure may be even greater in the colonic mucosa of *Bufo arenarum* since the unidirectional fluxes of bicarbonate + CO_2 (Lew, 1970) at 0 mV and pH 8·1, i.e. when the CO_2 equilibrium concentration is about one hundredth of the bicarbonate concentration, are very similar to those reported by Cooperstein & Hogben (1959) at a pH about 7·5, i.e. at higher CO_2 concentrations. Application of the Ussing equation may not be justified, however, since local changes of pH along the flux path and interaction with other ionic passive fluxes (counter ions flowing in the same direction or ions of the same charge flowing in opposite directions) may also affect the passive movements of CO_2 or bicarbonate through the colonic mucosa. It might be argued that the transmembrane flux of bicarbonate + CO_2 is entirely extracellular, but this is unlikely, since CO_2 diffuses (Longmuir, Forster & Chi-Yuan Woo, 1966) much faster than O_2 and N_2, which seem to have ready access to the intracellular compartment from either side of the membrane. If the serosal border of the epithelial cell is therefore not impermeable to CO_2 molecules, diffusing bicarbonate ions should be expected to equilibrate isotopically with the serosal bicarbonate + CO_2 pool. The existence itself of an isolated bicarbonate compartment represents a difficult localization problem. Metabolic formation of CO_2 within

the epithelial cell coupled with hydration and electrogenic transport of bicarbonate ions at the mucosal surface provide the most satisfactory explanation of the present results. Indirect evidence suggesting that metabolic CO_2 is compartmentalized in a particular way is provided by the fact that no dilution of the specific activity of the serosal bicarbonate could be detected as might have been expected if membrane CO_2 had entered the serosal solution in the experiments with everted sacs. For this hypothesis to be tenable two further questions ought to be answered: (*a*) is the metabolic production of CO_2 big enough to sustain a bicarbonate transport of the required magnitude and (*b*) is the spontaneous hydration rate of CO_2 enough to provide the required amount of bicarbonate?

We are not aware of any direct measurement of the rate of production of CO_2 from metabolism in amphibian colonic mucosa. In any case, it would be very difficult to estimate the contribution of the superficial epithelial cell layer to the over-all metabolism of the tissue except for the fraction of it that depends on Na transport. Maffly & Coggins (1965), however, have made such measurements in the toad bladder. They related short-circuit current to extra CO_2 production and found a figure of about 30 μl. CO_2 formed per hour. They do not give the value of the area of membrane used in their experiments but if it is assumed that the ratio of 13·6 Na ions transported per molecule of CO_2 produced is valid for the colonic mucosa too, and that the Na-dependent CO_2 production represents about 50 % (approximately their value) of the over-all CO_2 formed within the epithelial cell, it is possible to estimate a minimum amount of CO_2 that might have been formed in 1 hr/cm^2 of membrane. This turns out to be just about 0·5 μ-equiv. This is, however, a very rough approximation and, since the bicarbonate transport itself is dependent on aerobic metabolism (Lew, 1970), the value just calculated might be a gross underestimate. In fact, the Na-transport-dependent CO_2 production cannot be the only or main source of the transported bicarbonate since, when Na is replaced by choline in the bathing solutions, the remaining short-circuit current can be maintained for 3 or more hours (Lew, 1970). The spontaneous hydration rate of CO_2 is a relatively slow process, with a rate constant close to $3{\cdot}7 \times 10^{-2}$ sec^{-1} (Edsall, 1966–67, 133·2 hr^{-1}). In order to calculate the hydration rate of the metabolic CO_2 one has to estimate its concentration in the isolated compartment. This is obviously impossible but even under favourable conditions the spontaneous hydration rate can hardly account for the hydration of a high proportion of the metabolic CO_2 as required to sustain the highest observed rates of bicarbonate transport. Although hardly a proof, the action of Diamox is the only evidence that enzymic hydration of CO_2 is necessary to sustain part of the short-circuit current (Lew, 1970).

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