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THE PRODUCTION OF A HYDROGEN ION GRADIENT ACROSS THE ISOLATED FROG SKIN
QUANTITATIVE ASPECTS AND THE EFFECT OF ACETAZOLAMIDE

M. G. EMILIO*, M. M. MACHADO AND H. P. MENANO
Laboratory of Physiology, Gulbenkian Institute of Science, Oeiras (Portugal)
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SUMMARY

1. The production of a H^+ gradient across the isolated frog skin was qualitatively and quantitatively studied in pieces of ventral skin mounted in Ussing-type chambers and maintained in CO_2-free conditions.

2. Acidification of the outer bathing medium and alkalinization of the inner bathing medium were evaluated either by pH measurement or by the pH-stat technique.

3. An asymmetry of the two phenomena was manifested by a different behaviour in time and by a greater amount of base added to the inner chamber as compared with the amount of acid added to the outer chamber.

4. Acidification was determined by the extrusion of H^+ generated intracellularly.

5. The dependence of acidification on the presence of O_2 and its suppression by a carbonic anhydrase inhibitor suggest that the H^+ are provided by the intracellular hydration of metabolic CO_2.

6. A fraction of the added base could be stoichiometrically related to the acid added.

INTRODUCTION

When a sheet of frog skin is mounted between two half chambers containing a small amount of Ringer's solution, the pH of this solution becomes more acid at the epidermal side and more alkaline at the corium side[1,2]; the difference gradually developed reaches 2 pH units when the leg skin of *Rana pipiens*[3] is mounted as a bag with unbuffered electrolyte solution.

Acidification of the outer medium has been attributed to the diffusion and subsequent hydration of metabolic CO_2; essentially, this process would be the same as that which operates in the intact animal, subserving the respiratory function of CO_2 elimination[4]. On the other hand, recent studies on the "normal" skin secretions of the intact frog indicate that these secretions are slightly alkaline (pH range between

* Present address: School of Biological Sciences, University of East Anglia, University Plain, Norwich, NOR 88C, England.

7.3 and 7.8), and that the skin is able to control the pH of its epidermal surface between limiting pH values of 6 and 8 (refs. 3, 5, 6).

This work was designed to determine quantitatively the acid-base changes that take place across the isolated abdominal skin of the frog and to study the mechanisms involved in the process. It has been possible to observe that acidification of the liquid bathing the outer face of the skin is not wholly dependent on the elimination of CO_2 as such towards this liquid, thus implying some form of transport of H^+ across the outer barrier. The relationship between acidification and alkalinization and the effects of a carbonic anhydrase inhibitor are also discussed.

METHODS

Frogs of the species *Rana ridibunda* Pallas were kept in a cold chamber (4–8°), half immersed in running tap water, for periods of 1–10 days. After an animal had been double-pithed, a piece of skin from the belly was dissected and mounted in chambers of the type utilized by USSING AND ZERAHN[7]. These chambers were designed to admit a combined pH electrode (Radiometer GK 2026C) near each face of the skin. They were also provided with four Ringer agar bridges, so that the potential difference across the skin could be measured and short-circuited by an external current. The area of exposed skin was 7.1 cm^2.

The Ringer's solution utilized to bathe the two sides of the skin had the following composition, which was regularly checked: 116 mM NaCl, 2.4 mM KCl, 0.12 mM Tris, with an osmolality of 0.220; all the substances were reagent grade.

Special care was taken to obtain a reasonable pH stability with that small buffer concentration. The solution was prepared with distilled boiled water on the day before the experiment and aerated with CO_2-free air through the night and right up to the moment of being utilized. (CO_2-free air was obtained by pumping the air, successively, through a flask containing 5 M NaOH, a soda-lime filter and a flask of distilled water.) The pH of the solutions was adjusted to the required levels with 0.1 M HCl or NaOH. Penicillin (10^6 I.U./l) and, in some experiments streptomycin (0.5 g/l), were added to avoid the development of microorganisms, as this could interfere with pH changes. In these conditions, pH stability was assured within 0.05 pH unit. pH values were monitored by means of Radiometer equipment, pHM4 and PHM27. Standard buffer solutions were prepared according to the N.B.S. techniques[8], and pH values were obtained with a precision of at least 0.05 pH unit.

Two main types of protocol will be described, which were adapted to the experimental requirements: in the first group of experiments, the pH variations were measured at both surfaces of the skin, as well as the overall potential difference and the short circuit current; these will be called "free run experiments". In the second group, designated as "titration experiments", the pH variations were compensated by a titration procedure; potential difference and short circuit current were measured as in the former group.

Free run experiments

The skins were mounted in the chambers with 6 ml of Ringer's solution in each side; stirring and aeration were maintained by bubbling CO_2-free air. Two combined pH electrodes were introduced near the epidermal (outer) and the corium (inner) surfaces of the skin, and the pH values were read at regular intervals or continuously

registered by means of a Heathkit recorder. The potential difference was checked by a Solartron digital voltmeter, and the short circuit current was injected and measured through a Universal Avometer. The preparations were kept in open circuit conditions except for the few seconds required to measure the current, at intervals of 20–30 min. As the pH electrodes were in the path of the short circuit current, there was an interference over the pH readings when short circuiting conditions were introduced, and so pH determinations were not considered during these periods.

Titration experiments

For the second part of this study, results were obtained from experiments based on the pH-stat technique[9]. Two titration procedures were successively utilized. First, the chosen pH value was maintained by delivering standard Ringer's solutions with conveniently adjusted pH values to both sides of the chamber by means of two magnetic valves controlled by Radiometer TT1 titrators. The amount of fluid within the chamber was kept constant by the overflowing of the solutions, and these were collected under liquid paraffin, at measured intervals (between 3 and 10 min), and weighed. The buffer capacity of the solutions was daily verified by titration with 0.01 M HCl and NaOH. Alternatively, 1 mM HCl and NaOH were used as titrants and were delivered from calibrated burettes mounted over the chamber (Fig. 1). There was then no overflowing of the fluid, but the volume of added titrant was not allowed to exceed 0.7 ml, in order to avoid further dilution errors. When that limit was reached, the chamber was emptied and refilled with fresh solution.

Further details about the protocols will be described in the following section.

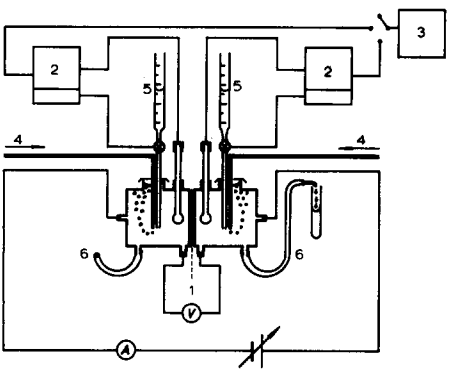

Fig. 1. Diagram of the set up utilized for the titration experiments. The skin (1) is mounted between two half-chambers. Combined pH electrodes are connected to pH meter-titrator assemblies (2) which regulate the amount of titrant fluids (5) added to the solutions. When Ringer's solutions are utilized as titrants, an overflowing system (6) is used. The air inlets (4), the recording system (3), and the circuits for measuring the overall potential difference and the short circuit current are also shown.

RESULTS

Free run experiments

When the building up of a pH gradient across the isolated frog skin was followed by continuously monitoring the pH of the bathing solutions, a similar pattern emerged for each of the twenty skins used. At the corium side, a progressive increase of pH

was immediately recorded, gradually tending towards a steady value after 3 or 4 h. At the epidermal side three stages could be distinguished: an initial period of some 30 min, during which no pH change was seen; a second stage, characterized by the beginning and gradual increase of the rates of acidification, and a last stage of decreasing rates of pH change. Typical pH curves from one of these experiments are shown in the left side of Fig. 2.

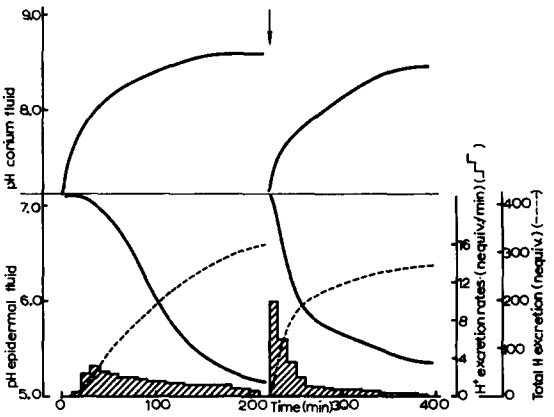

Fig. 2. Typical free-run experiment. pH changes of the fluids bathing the skin are represented by the thick lines (corium fluids in the upper part of the figure, and epidermal fluids in the lower part). At the arrow, the solutions were substituted by fresh Ringer. The calculated H^+ excretion rates and the respective cumulative plots are also shown in the lower part of the figure.

After producing a stable pH gradient between their two surfaces, the skins maintained it for long periods, even when the preparations were left mounted overnight. In no instance were we able to detect a reversal of this gradient. No effects could be noticed on the time evolution of the potential difference and short circuit current which could be attributed to the gradual alteration of the pH conditions of the bathing media. When these conditions were suddenly altered by emptying and refilling the chambers with fresh Ringer (pH 7.0), after the spontaneous pH gradient had been installed, the electrical parameters of the skin were again not affected.

The maximal and minimal pH values obtained in these experiments are shown in Table I, as well as the final values of the potential difference and short circuit current. The pH differences between the two sides were often larger than 3 pH units. There was no apparent correlation between the pH gradient attained by each skin and its electrical parameters.

The observation that no further pH changes took place after 3–4 h might suggest a rundown of the skin's capacity to produce them. In order to test this hypothesis, after a stable pH gradient had been reached the chambers were rinsed and refilled with fresh Ringer (pH 7.0 $\pm$ 0.1). Under these conditions, the skins were again able to produce a pH gradient, though smaller and with a different pattern (right side of Fig. 2). Acidification started without any appreciably delay and the decrease in pH values was very rapid. At the corium side, the rates of pH increase in the first few minutes were not so high as the corresponding increases in the first experimental period. This experiment could be reproduced several times on the same skin, the maximal pH gradient being successively smaller. These results show that the process

TABLE I

MAXIMAL H^+ GRADIENT ACROSS FROG SKINS, IN FREE-RUN OPEN CIRCUIT CONDITIONS

The values given for the potential difference (PD) and short circuit current (s.c.c.) were measured when the maximal H^+ gradient was recorded.

Expt. No.	*pH*			*PD (mV)*	*s.c.c. (μA)*
	Epidermal side	*Corium side*	*Gradient*		
1	4.70	8.80	4.10	50	260
2	5.08	8.45	3.37	25	210
3	5.12	8.62	3.50	25	100
4	5.12	8.55	3.43	42	200
5	5.15	8.72	3.57	24	80
6	5.20	8.40	3.20	38	200
7	5.22	8.80	3.58	40	90
8	5.25	8.90	3.65	40	200
9	5.30	8.45	3.15	58	200
10	5.35	8.58	3.23	30	100
11	5.36	8.70	3.34	42	170
12	5.40	9.10	3.70	30	90
13	5.55	8.88	3.33	20	70
14	5.60	8.60	3.00	70	300
15	5.68	8.65	2.97	36	200
16	5.70	8.65	2.95	46	200
17	5.85	8.80	2.95	10	100
18	5.85	8.15	2.30	45	150
19	6.02	8.00	1.98	42	170
20	6.15	8.85	2.70	35	130

changes with time, as judged from the decreasing pH gradients obtained, but they also show that the capacity of the skins to produce acid–base changes is not definitely impaired after reaching limiting pH values.

In order to calculate the quantity of acid necessary to produce the observed pH decrease, titration curves of the solutions taken from the outer chambers were drawn and compared with curves obtained by titration of fresh Ringer's solution. As the curves were indistinguishable, it seemed reasonable to assume that the buffer capacity of the outer fluids remained constant and that the titration curve on the initial Ringer could be used to calculate the amount of acid which must be excreted by the skin to obtain a given pH decrease in those fluids.

The curves of the cumulative increase of H^+ in the epidermal fluid as calculated by this method, are drawn in the example of Fig. 2; the respective rates were calculated from the slopes of these cumulative curves. Maximal rates were obtained after about 20 min and then decreased as the pH value fell; when the initial pH conditions were reset, H^+ addition was resumed at higher rates and rapidly decreased to zero.

An identical approach to the indirect determination of base increase at the corium side was not possible because the titration curves of the corium fluids were different from those obtained from fresh Ringer.

As described under METHODS, the experimental conditions were chosen in order to minimize CO_2 trapping in the bathing fluids. However, the hypothesis that extrusion of metabolic CO_2 as such might be responsible for the acidification of the

epidermal fluid had to be tested. This was done in a group of experiments, by measuring the pH of samples taken from the epidermal chamber at hourly intervals after 30 min aeration with CO_2-free air, and after keeping them in a boiling water bath for 20–30 min. These procedures would remove CO_2 or any volatile acid, so that the pH of the samples would return to the initial values. It was observed in a total of 25 determinations that the pH remained constant or had but a small rise after the aeration of the samples; no further rise occurred after the boiling procedure, the final pH being usually lower.

Another attempt was made to detect CO_2 by microtitration of the solutions that had been in contact with the skins. In a group of five experiments, samples of 4–5 ml of the solutions taken from the chambers at hourly intervals were brought to pH values less than 3.0 and kept for 20–30 min under aeration with CO_2-free air; the samples were then alkalinized back to their original pH. The difference between the quantities of added acid and alkali was a measure of the amount of CO_2 liberated from the sample. The reagents (0.01 M HCl and NaOH) were titrated beforehand and delivered through calibrated microsyringes assembled to a micrometer. No CO_2 was detectable by this method in the solutions taken from the epidermal chamber; on the other hand, appreciable amounts of CO_2 were present in the samples from the corium chamber (Table II). This was probably due to the passage of tissue HCO_3^- towards the inner side of the chamber, and may account for the variation of the buffer capacity of the corium fluids.

TABLE II

DETERMINATIONS OF CO_2 BY MICROTITRATION OF RINGER'S SOLUTIONS BATHING THE CORIUM OF FROG SKINS AFTER FIVE CONSECUTIVE FREE-RUN PERIODS OF 1 h

Results are expressed as μmoles/7.1 cm^2 of skin.

Period	CO_2				
Expt. No.	*1*	*2*	*3*	*4*	*5*
1	0.55	0.52	0.54	0.86	0.70
2	0.51	0.43	0.40	0.58	0.65
3	0.43	0.26	0.29	0.20	0.62
4	0.46	0.25	0.2	0.28	0.32
5	0.47	0.2	0.2	0.2	0.55

Titration experiments

A direct way of measuring the rates of addition of acid (H^+) and base (B^-) to the bathing solutions was provided by the continuous titration of these fluids to a determined pH level. The results obtained from the two methods described earlier were identical.

Relations between acid and base titration rates. A set of experiments was designed to study the relations between acidification and alkalinization. Titration at pH 7.0 on both sides of the chamber was started as soon as the skin was able to acidify the solution on the epidermal side. The rates of base increase were not determined in the initial phase of these experiments before acidification occurred. The chambers were rinsed and refilled with fresh Ringer (pH 7.0) before the titration was started.

Fig. 3 shows the time evolution of the mean rates of titration of H^+ and B^- simultaneously detected in eleven experiments. The mean rates of H^+ titrated at the epidermal side show an initial small rise to a maximal value, and then they smoothly decrease for the next 2 or 3 h. Allowing for the individual variation, one can consider a plateau for the H^+ production rates during which 6–10 nequiv/min of H^+ are added to the solution bathing the outer face of the epithelium. The time-course of these events can be compared with the evolution of the acidification process in "free run" conditions. In both conditions there was a lag of 20–30 min between the setting of the preparation and the moment when the acidification started, followed by a rise in H^+ excretion rates. However, at steady pH conditions the H^+ excretion rates were maintained for a rather long period, before showing a trend to decrease, and no appreciable change was detected on the net H^+ addition by changing the Ringer's solutions in the chambers. Finally, the absolute values found for the maximal rates were higher in pH-stat conditions than in the "free run" experiments.

The rates of base titrated at the corium side were always consistently higher than the simultaneous H^+ rates, and the process of alkalinization could be followed for much longer periods. The difference between the simultaneous B^- and H^+ rates was maximal at the beginning of the experiments and gradually decreased to a nearly constant value. Disregarding for the moment the values obtained during the first 80 min, the parallel evolution of these two rates suggests that the total base titrated, $(B^-)_{tot}$, includes a fraction that is closely related to the hydrogen ion titrated, (H^+), and another one, independent and approximately constant, $(B^-)_{ind}$. This can be expressed by the relation:

$$(B^-)_{tot} = A(H^+) + (B^-)_{ind}$$

where A is a stoichiometric factor relating the B^- and H^+-linked fractions.

Dividing the former expression by (H^+) we obtain

$$\frac{(B^-)_{tot}}{(H^+)} = A + \frac{(B^-)_{ind}}{(H^+)}$$

In 13 experiments, in which the same protocol was followed, the cumulative

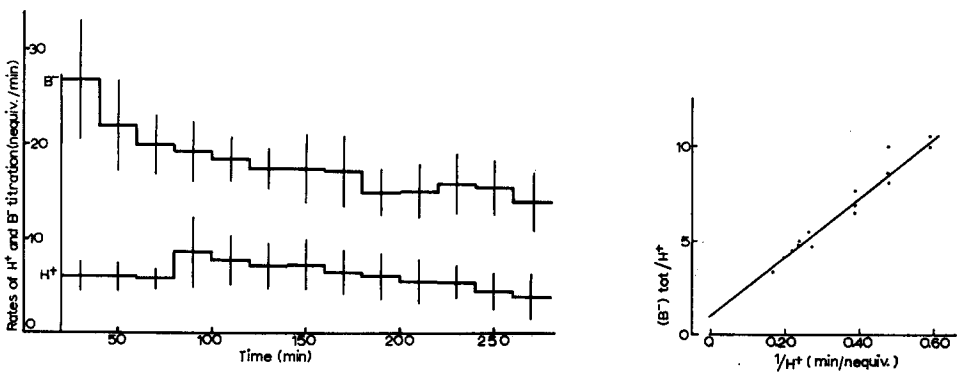

Fig. 3. H^+ and B^- titration rates at pH 7.0. Mean values obtained from 11 experiments. The vertical bars represent ± S.D.

Fig. 4. Relation between the values calculated in one experiment for the ratios $(B^-)_{tot}/(H^+)$ and $1/(H^+)$. Regression curve calculated by the least mean squares method: $(B^-)_{tot}/(H^+) = 1.00 + 15.7$ nequiv/min $\times 1/(H^+)$.

amounts of total B^- and H^+ titrated were plotted against time, and the respective average rates were calculated at 5-min intervals; then the values of $(B^-)_{tot}/(H^+)$ were determined and plotted against $1/(H^+)$. The plot could be fitted by a straight line, as shown in Fig. 4; the slope of this line represents the fraction $(B^-)_{ind}$, and its interception with the y axis the stoichiometric factor A. This supports the assumptions that a part of the total base is stoichiometrically related to the acid and that another part is "independent" and constant. Regression curves were calculated for these experiments, and the respective values for A and (B^-) are tabulated in Table III. The mean value of the stoichiometric factor in this group of experiments was 1.00, with a standard deviation of $\pm$ 0.24; for the independent B^- fraction, a value of 12.6 $\pm$ 3.4 nequiv/min was observed. This last value compares well with the mean differences between total B^- and H^+ rates obtained in the former group of experiments after the first 80 min (Fig. 3).

TABLE III

RELATION BETWEEN H^+ AND B^- TITRATION RATES

In all experiments, the stoichiometric factor, A, and the independent B^- fraction, $(B^-)_{ind}$, were obtained from individual regression curves. Values of $(B^-)_{ind}$ are expressed as nequiv/min per 7.1 cm^2.

Expt. No.	A	$(B^-)_{ind}$
1	1.31	10.9
2	1.22	7.0
3	0.83	6.9
4	0.79	15.7
5	0.94	17.5
6	0.65	13.8
7	1.00	15.7
8	0.77	15.0
9	0.90	11.0
10	0.70	10.2
11	1.23	15.3
12	1.22	9.2
13	1.38	15.9
Mean $\pm$ S.D.	1.00 $\pm$ 0.24	12.6 $\pm$ 3.4

An attempt was made to characterize the cause of the rapid pH increase in the initial phase of the free-run experiments and the high rates of base titration registered in the first periods of the titration experiments. This was done by beginning the titration procedure at pH 7.0 immediately after the setting up of the preparation. The titrant consumption was measured at 1-min intervals, so that the corresponding quantities of base could be plotted against time in a sufficiently smooth curve. This protocol was utilized in three experiments, and the cumulative plots had the aspect shown in the typical example of Fig. 5 (Curve A). The exponential decrease of the rates of base titrated during the initial phase suggested the washout of some basic substance from an accessible, finite compartment, possibly the intercellular space, into an infinite one. In this event, one might predict that the cumulative increase of base would be described by an equation of the type

$$B = B_0(1 - e^{-kt})$$

with B_0 equal to the total amount of base washed out by this process and B equal to the amount of base washed out until time t. The parameters of this empirical function were calculated from the base excretion values determined for each experiment during the first 60 min. In the example shown in Fig. 5, B_0 had a value of 1.84 μequiv, and k of $3.6 \cdot 10^{-2}$ min^{-1}, good agreement being found between the theoretical curve (Curve D) and the experimental values. Similar results were obtained in the other experiments in which the same protocol was followed; the calculated values of B_0 and k were, respectively, 1.6 and 2.5 μequiv, and 4.3 and $3.6 \cdot 10^{-2}$ min^{-1}.

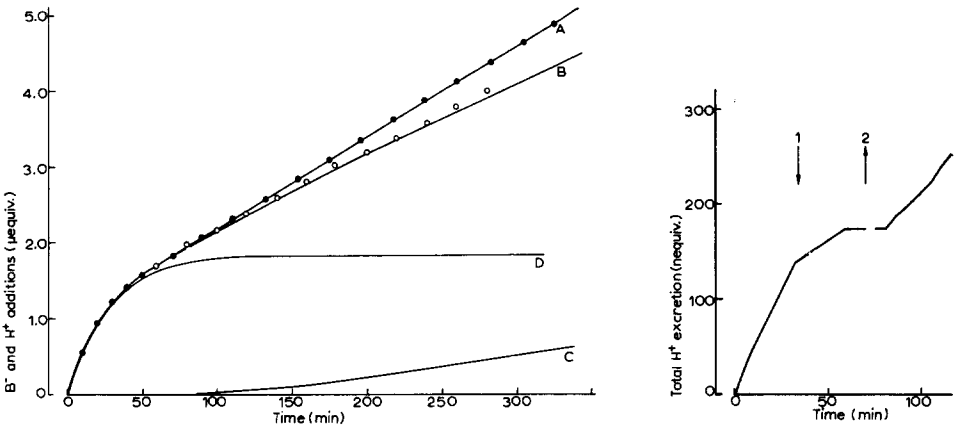

Fig. 5. Cumulative plot of the total base (Curve A) and H^+ (Curve C) titrated at pH 7.0 in one experiment. Curve B is the independent B^- fraction, obtained by subtracting C from A. The white dots represent the mean cumulative independent base calculated from the experiments in Fig. 3. Curve D represents the empirical function $B = B_0 (1 - e^{-kt})$; $B_0 = 1.84$ μequiv, $k = 3.6 \cdot 10^{-2}$/min.

Fig. 6. Effect of acetazolamide on H^+ cumulative titration at pH 7.0. At arrow 1, the solution in the corium half-chamber was substituted by 0.25 mM acetazolamide in Ringer. At arrow 2, the chambers were rinsed and refilled with normal Ringer.

In this group of preparations, H^+ extrusion towards the epidermal side began after an appreciable delay and proceeded at lower rates than in former experiments. This is evident in the example of Fig. 5 by the cumulative plot of titrated H^+ (Curve C); nevertheless the differences between simultaneous B^- and H^+ titration rates were again fairly constant. The plot of these differences closely approaches a straight line with a slope of 9 nequiv/min (Curve B), corresponding to the "independent" base fraction.

Effect of acetazolamide. A group of experiments was conducted to test whether the use of a carbonic anhydrase inhibitor would affect the acid–base changes observed in our preparations. The compound utilized, acetazolamide, was added to the Ringer's solution in final concentrations of 0.25 mM and 0.50 mM, and the pH value was adjusted to 7.0 with HCl. The pH-stat technique was followed in these experiments; as soon as steady values for the titration rates of H^+ and B^- were obtained, the Ringer's solution containing acetazolamide was introduced into the corium or the epidermal half-chamber for a period of 30–60 min. At the end of this period, both

sides were thoroughly rinsed and refilled with fresh Ringer, so that final control values could be obtained.

A decrease of the acid titration rates at the epidermal side was evident after the application of the drug to either surface of the skin, gradually tending to zero. This effect was immediate when acetazolamide was used externally and was preceded by a time lag of 10–20 min when the drug was applied to the corium side Some degree of recovery of the acidification process was always observed after the chamber had been rinsed, though there was a considerable delay when the drug had been used on the corium side. A cumulative plot of H^+ titration against time in one of these experiments is shown in Fig. 6.

The effect on the alkalinization process was not so clearly defined; the titration rates seemed unaffected when the Ringer containing the inhibitor was introduced into the epidermal chamber, and the same rates were maintained during the final control period. When it was introduced into the corium chamber, the rates of titration often had an increase, which may perhaps be attributed to the impairment of the sensitivity of the measurement procedure due to the high buffer capacity of the acetazolamide solution; sometimes a decrease of alkalinization did occur, parallel with the decrease of acidification (Fig. 7). During the final control period the rates of titration were often smaller than during the experimental period. Table IV summarizes the results obtained in this group of skins.

Effect of anaerobiosis. In a group of six experiments the effect of suppressing the O_2 supply to the preparation was tested. A pH level of 7.0 was maintained on both sides of the chamber until stable values were obtained for H^+ and B^- titration rates; the solutions in the chambers were then substituted by fresh Ringer that had been bubbled with N_2 for 2 or 3 h and whose pH had been adjusted to the original value; stirring of the fluids in the chambers was obtained by bubbling with N_2 during the experimental period of about 30 min. The initial conditions were then reset, that is, oxygenated Ringer was introduced into the chambers and bubbled with CO_2-free air as usual. In every test acidification was completely inhibited by the exclusion of oxygen. A reduction in the B^- titration rates was also found, so that the difference

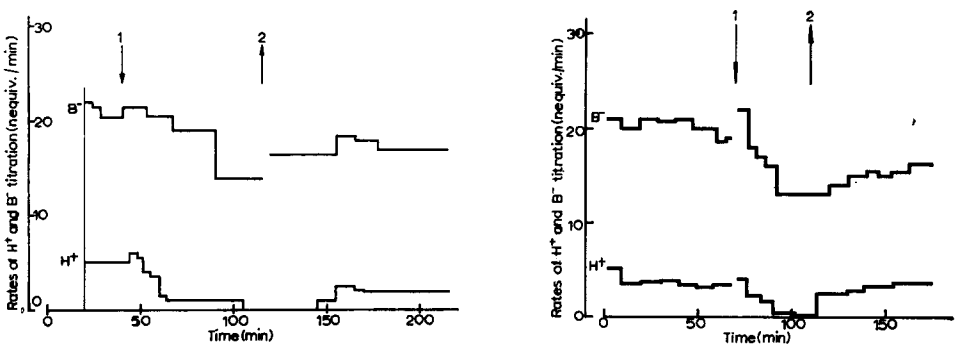

Fig. 7. Effect of acetazolamide on H^+ and B^- titration rates at pH 7.0. At arrow 1, the solution at the corium half-chamber was substituted by 0.25 mM acetazolamide in Ringer. At arrow 2, the chambers were rinsed and refilled with normal Ringer.

Fig. 8. Effect of anaerobiosis on H^+ and B^- titration rates at pH 7.0. At arrow 1 anaerobic conditions were introduced. At arrow 2 the O_2 supply was reset.

TABLE IV

EFFECT OF ACETAZOLAMIDE ON H^+ AND B^- TITRATION RATES

Acetazolamide was applied to the corium side of the skins (Expts. 1–5) and to the epidermal side (Expts. 6–10) in the concentrations of 0.5 mM in Expts. 1 and 2 and 0.25 mM in all the others. A and C, control periods before and after using the drug; B, experimental period. The values of the potential difference (PD) and short circuit current (s.c.c.) were recorded at the end of each period.

Expt. No.	*Period*	(H^+)	(B^-)	*PD*	*s.c.c.*	*Expt. No.*	*Period*	(H^+)	(B^-)	*PD*	*s.c.c.*
		(nequiv/min)		*(mV)*	*(μA)*			*(nequiv/min)*		*(mV)*	*(μA)*
1	A	8	24	32	360	6	A	4	25	22	200
	B	0	27	15	210		B	0	15	18	172
	C	—	20	30	260		C	2.5	14	18	150
2	A	11	20	39	380	7	A	7	17	16	150
	B	0	23	18	205		B	2.5	17	11	110
	C	6	15	25	175		C	3	15	22	115
3	A	5	16	27	175	8	A	5	17	30	260
	B	0	16	28	170		B	1	17	22	240
	C	3	13	27	140		C	2	16	25	200
4	A	4	20	18	175	9	A	4	20	24	210
	B	0	20	18	170		B	2	15	15	160
	C	2	17	16	140		C	0	15	20	160
5	A	5	25	39	280	10	A	9.5	26	20	100
	B	0	27	29	230		B	0	21	19	80
	C	2	19	37	180		C	4	21	14	70

between H^+ and B^- simultaneous titration rates was again constant. After the O_2 supply had been restored the capacity of extruding H^+ was partially recovered and a corresponding increase in B^- titration was also noticed. These results are shown in Table V, as well as the values of the overall potential difference and the short circuit current measured in the three experimental periods. Fig. 8 represents the graphic plot of the H^+ and B^- titration rates in a typical experiment.

DISCUSSION

Our results show that the isolated skin of *Rana ridibunda* Pallas can produce large changes in the pH conditions of the liquids in contact with its surfaces. The pH gradients observed are larger than those reported in previous studies, obtained in similar conditions but in a different species of frog, *Rana pipiens*[2]. The inversion of the characteristics of the two surfaces of the skin in relation to acid–base changes, suggesting an ability to keep the environmental pH near the neutrality point[3], was never observed in our experiments, at least within the limits which could be spontaneously reached at the two surfaces.

The cause of the time lag preceding the gradual H^+ addition to the epidermal half chamber could be the disturbance of intracellular metabolic processes, due either to the handling of the tissues or to the different ambient conditions. This has been suggested to explain a similar finding in the isolated bull-frog gastric mucosa, where there are signs of low metabolic activity in the first 30–60 min of an experiment[10].

TABLE V

EFFECT OF ANAEROBIOSIS ON H^+ AND B^- TITRATION RATES

O_2-free Ringer was introduced into the chambers for periods of 30 min approximately (periods B), and N_2 was used for stirring. A and C, control periods before and after the withdrawal of O_2. The values of the potential difference (PD) and short circuit current (s.c.c.) were recorded at the end of each period.

Expt. No.	*Period*	(H^+) *(nequiv/min)*	(B^-)	*PD (mV)*	*s.c.c. (μA)*
1	A	5	22	33	200
	B	0	17	35	85
	C	4	20	40	190
2	A	4	11	27	90
	B	0	6	11	35
	C	1	7	33	80
3	A	3	20	35	170
	B	1	17	33	62
	C	2.5	18.5	37	120
4	A	3	18	42	220
	B	0	13	36	37
	C	3	15	38	150
5	A	2.5	19	53	260
	B	0	12	38	72
	C	2.5	*	64	190
6	A	7	16	33	130
	B	0	—	18	35
	C	2	12	35	100

* Free-run period.

However, in our preparations the potential difference and the short circuit current measured immediately after the chambers had been set up had values similar to those maintained throughout the experiment, indicating that other transport properties requiring metabolic activity were present. Alternatively, this delay can be explained by the necessity of creating a pool of H^+ within the cells before the triggering of an extrusion mechanism.

The delay at the beginning of acidification contrasts with the immediate and strong alkalinization of the liquid bathing the corium, but after the initial period the spontaneous pH changes seem to run in a symmetrical way in time, until a state of apparent equilibrium is reached in both sides of the chamber. This symmetry is not confirmed by direct quantitative measurement at a fixed pH 7.0 level, the amount of base added to the corium half-chamber always being higher than the amount of acid added to the epidermal one. This is particularly evident during the first hour of each experiment. It seems reasonable to presume that a large part of this base is HCO_3^-, as its presence in the corium fluids is confirmed by microtitration and its total amount has the same order of magnitude as the HCO_3^- stored in frog skin, according to existing data[6]. This would also explain the "washout-like" aspect of the cumulative plot of base titrated during the first hour, as titration at pH 7.0 liberates

as CO_2 practically all the HCO_3^- added to 6 ml of Ringer's solution at rates similar to the alkali rates prevailing in the skin preparations.

Apart from the initial phase, and in spite of the asymmetry noted in the rates of alkali and acid titration, a relation between the two phenomena is suggested by their parallel evolution with time, a parallelism that is maintained in anaerobic conditions. The mathematical correlation of these two variables permitted the partition of the total amount of base titrated in the corium half-chamber into two fractions —one that is stoichiometrically related to the H^+ titrated in the epidermal chamber, and another that is titrated at a nearly constant rate. No formal explanation can be offered at the moment for this base excess. Characterization and quantification of different substances contributing to the neutralization of the acid added to the corium solutions are now under study. There is some evidence that a fraction of the titrant H^+ is exchanged for K^+ from the corium structures of the skin. Preliminary K^+ determinations revealed an excess of this ion in the liquids taken from the corium side of titrated chambers, representing an amount of 5 nequiv/min, approximately. As for the remaining base, it seems probable that its major part is comprised of HCO_3^- and lactate ions. Studies on the lactate production by the frog skin have shown that this ion appears asymmetrically in the bathing solutions, the amounts appearing at the corium side being three to five times larger than at the epidermal side. The amount of lactate determined by LEAF AND RENSHAW[11] in aerobiosis (0.2 μmole per 100 mg wet skin per h) roughly corresponds to 5 nmoles/7 cm^2 per min, which is $^1/_3$–$^1/_4$ of the mean rates of steady base titration in our experiments. This value agrees well with data recently obtained in this laboratory. The results of the electrolyte and lactate determinations mentioned will be fully reported in a following paper.

FRIEDMAN *et al.*[6], using results obtained from intact frogs and from experiments with skin bags, attributed the acidification of the epidermal fluid to the release of respiratory CO_2. According to their results, good agreement was found between O_2 consumption (equal to CO_2 production, assuming a R.Q. of 1.0) and the amount of alkaline titrant necessary to keep the pH of the epidermal bathing fluid at pH 7.0. However, no CO_2 determinations were done in the bathing fluids, so these experiments do not exclude the possibility that the hydration of metabolic CO_2 might be performed intracellularly. The mean rates of H^+ titration at steady conditions (pH 7.0) reported by these authors are within the range found in our experiments (0.07 μequiv/cm^2 per h, corresponding to 8 nequiv/7 cm^2 per min).

The hypothesis that the fall in pH might be due to the reabsorption of HCO_3^- from the bathing media has been put forward by SCHILB AND BRODSKY[12] to interpret a similar phenomenon of acidification in isolated bags of turtle bladder; these authors were using a CO_2-rich medium for their experiments and based their theory on the finding of a decrease of the p_{CO_2} together with a decrease in pH at the mucosal side of their preparations. This hypothesis does not seem plausible in our conditions, since no extrinsic HCO_3^- was available and the metabolic CO_2 which might be eliminated by the outer surface of the skin was efficiently removed from the bulk of the bathing fluids. A last possibility remains that a considerable amount of HCO_3^- may be present in the epidermal unstirred layer, formed by the hydration of that CO_2. This was considered unlikely by STEINMETZ[13] who studied the same epithelium in conditions that were closer to our own. In a later work, GREEN, STEINMETZ AND FRAZIER[14] could not confirm the decrease in p_{CO_2} at the mucosal side described by SCHILB AND

BRODSKY[12]. Their results supported the hypothesis that acidification by the isolated turtle bladder is actually due to the active transport of H^+ into the mucosal medium.

Although the spontaneous potential difference across the skin favours the observed pH gradient, the values of this gradient are much larger than could be expected from the effect of the potential difference alone; when the rates of acid titration were measured at constant pH, short circuiting the skin had but a small effect on these rates, which could only be demonstrated when alternate short circuit and open circuit periods were run in the same experiment. In the absence of an overall potential difference large enough to account for the pH gradient, this could be determined by an intracellular potential positive in relation to the epidermal surface of the skin by 150 mV, approximately. Data obtained from microelectrode studies do not indicate the existence of such a potential; typical intracellular potential values reported in the literature for skins bathed in chloride Ringer are between 40 and 60% of the total potential across the skin, under open circuit conditions[15]. Under short circuit conditions, negative intracellular potentials are generally found; in neither condition could the observed H^+ gradient be determined by these electrical potentials. Another possible cause for the pH decrease of the outer medium would be the existence of a H^+ concentration gradient, that is, the interior of the cells, or at least some intracellular compartment close to the outer barrier, would have a pH of less than 5. Severe theoretical and technical difficulties are involved in the methods of intracellular pH determination[16], and no direct information is available on the pH of the frog skin. Overall values obtained in other transporting epithelia, for instance, the toad bladder, are in the slightly alkaline range[17]. This gives no support to the hypothesis of a concentration gradient contributing to the acidification of the outer medium, though the possibility remains that some intracellular compartment near the outer barrier may have a very low pH.

Assuming that the frog skin is not very different from other epithelia with respect to the pH characteristics, the transfer of H^+ cannot be explained on the basis of an electrochemical gradient. The observation that the exclusion of O_2 from the preparation prevents acidification indicates a dependence of this process on the oxidative metabolism. On the other hand, acidification is interrupted in the presence of a carbonic anhydrase inhibitor, acetazolamide, which suggests that it is also dependent on the catalyzed hydration of CO_2. In our preparations, the clear effect of acetazolamide on stopping the acidification and the recovery observed after the withdrawal of that compound strongly argues for the presence of carbonic anhydrase. So far, this enzyme has not been found in frog skin[18], though it is known to be widely distributed in other tissues associated with transport phenomena, as well as in the red blood cells of this animal; it cannot be excluded that the observed effect is due to contamination by blood. On the other hand, though it is generally accepted that this type of drug has no effect on other enzymic systems, a depression of chloride transport by methazolamide in the absence of carbonic anhydrase has been described by KITAHARA, FOX AND HOGBEN[19] in the frog cornea, and the authors suggest that another enzyme is being inhibited here. Determinations of carbonic anhydrase activity and its histochemical localization will be of interest to further enlighten the mechanism of action of acetazolamide in the frog skin preparations. Recently, it has been possible to confirm the preferential localization of carbonic anhydrase near extracellular membrane-bordered spaces in a variety of transporting epithelia[20], and it has also

been suggested that it may facilitate the penetration of CO_2 through biological membranes[21]. This would justify the presence of the enzyme in the frog skin, since this organ acts as a path of elimination of CO_2 in the intact animal. In our working conditions, however, the role of the enzyme might be different, as no extrinsic CO_2 is present. One may predict that it favours the hydration of metabolic CO_2, thus furnishing a source of H^+ which are extruded towards the outside, and HCO_3^- eliminated towards the corium side; the paradoxical effect of enhancement of the base titration observed in some experiments, when acetazolamide is applied to the corium, remains unexplained unless two different sites of enzymic action are admitted; the inhibition of the hydration of CO_2 at the corium structures would then prevent the utilization of OH^-, which would be titrated in larger amounts than under the usual conditions. This effect is not detectable when the drug is applied to the epidermal side of the chamber.

The extrusion of H^+ generated in the cells may be directly dependent on an energy requiring mechanism or coupled with the transport of some other ions, but no data are now available to distinguish these processes. A suggestion that H^+ transport is independent of Na^+ transport is provided by the observation that no simple relation is found between the short circuit current and the capacity of the skins to acidify the outer medium. On the other hand, no effects were noted on the short circuit current which could be attributed to the spontaneous pH changes, within the limits previously mentioned. Namely, the skins tolerated a pH 7.0 solution at the corium side throughout the experiments. This condition has been reported to cause a 50% fall of the short circuit current[22], but the effect might be due to the simultaneous use of a high phosphate concentration in the medium utilized for the experiments[23].

In summary, our results suggest that the acid–base changes across the isolated frog skin can be explained by the following mechanisms: immediately after the setting up of the preparation, a process of washout of HCO_3^- from the extracellular compartments of the skin causes the rapid alkalinization of the inner fluid. In the meanwhile, H^+ accumulates in the cells as the result of intermediary metabolic reactions, including the hydration of CO_2; after a short delay, a gradual addition of acid to the epidermal chamber can be observed, which may be attributed to the triggering of an extrusion mechanism for H^+. HCO_3^- and other organic anions of metabolic origin, or formed in some structures of the skin such as the glands, accumulate preferentially in the solution bathing the corium. The cause of the apparent base excess that is found when the pH in the chamber is kept at 7.0 is not known at the moment; there is reason to suppose that a fraction of the acid titrant is exchanged for K^+ from the corium structures.

The inhibition of acidification that follows the removal of the O_2 supply or the addition of acetazolamide supports the hypothesis that the hydration reaction of metabolic CO_2 is the source of the H^+ responsible for the acidification; the effect of acetazolamide on frog skin preparations cannot yet be fully understood, as there is no direct evidence of the presence of carbonic anhydrase in this tissue.

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