PERMEABILITY OF SANDDOLLAR OVA AND OF *MODIOLUS* AND *MYTILUS* GILLS TO VARIOUS ACIDS AND THEIR SALTS

By LYLE V. BECK and ROBERT CHAMBERS

Lilly Research Laboratories, Marine Biological Laboratory, Wood's Hole, Mass., and New York University, Washington Square College, New York, N.Y.

(*Received* 28 *November* 1936)

INTRODUCTION

Several investigators have indicated that certain intracellular effects of readily penetrating acids are lessened by the presence of their salts in the medium. This effect has been assumed to be produced either by the actual penetration of the salt together with its acid, or by the penetration only of the acid followed by a more or less extensive exchange of ions between the cells and the medium.

Among the papers dealing with this question are the following: Smith & Clowes (1924), and Smith (1925, 1926) noted a physiological antagonism between penetrating acids and their salts (carbonates and various fatty acids) in their effects on the segmentation of echinoderm ova and on the heart beat of the turtle. R. S. Lillie (1926) found that the capacity of 0·001 *M* acetic acid to induce artificial parthenogenesis in starfish eggs is diminished when the salts of the acid are also present in concentrations of 0·008 and 0·016 *M*. More recently, E. Howard (1931) showed that the lower fatty acids decrease the internal viscosity of *Arbacia* eggs and that this effect is counteracted when the salts of the acids are also present in relatively high concentration in the medium. Beck (1935) found that the inhibitory effects of butyric acid on cytochrome reduction are counteracted by the presence of the corresponding sodium salt in the medium.

In all the above-mentioned work the criteria used to demonstrate acid penetration are somewhat indirect, since such processes as segmentation, heart beat, changes in viscosity, etc. can be affected by factors other than changes in intracellular hydrogen-ion concentration. The experiments described in this paper are based on a colorimetric method (by vital staining) for detecting changes in intracellular reaction.

Methyl red was used, since it has a pK value (5·1) which approaches closely those of the acid-salt systems being tested. In colorimetric studies on intracellular pH it has been found (Chambers, 1928) that this indicator is a vital stain from media of pH 5·0–5·5. The stained cells take on the yellow colour of the alkaline range of the indicator, the dye being rapidly segregated in cytoplasmic vacuoles

where the buffer capacity is so low that the dye readily changes to the red colour of its acid range when the cells are exposed to penetrating acids.

The experiments described in this paper deal with the effects of immersing the eggs of the sanddollar (*Echinarachnius parma*) and the gill filaments of two molluscs (*Modiolus* and *Mytilus*), stained with methyl red, in sea water containing various concentrations of hydrochloric, benzoic and several of the lower fatty acids in the presence and absence of their salts. The criterion for acid penetration was the change of the methyl red in the cells from the colour of its alkaline to that of its acid range, while that for a possible counteracting effect of the salt was a diminution or absence of a colour shift.

MATERIALS AND METHODS

The value of a vitally staining *p*H indicator to detect the counteracting effect of salts on the intracellular acidification by their acids depends upon the *pK* values of the indicator and of the penetrating acid. Neutral red, for example, having a *p*H colour range of 6·8–8·2, cannot be used to detect the counteracting effect of the salts of the lower fatty acids, since the buffer range of the fatty acid-salt systems extends only between *p*H 3·0 and 6·0. An illustration of this is the increased acid colour of neutral red in starfish eggs immersed in alkaline solutions of the salts of various fatty acids. On the other hand, cells stained with methyl red, which has a *p*H range of 4·4–6·0, should show the counteracting effect of the salt if the salt penetrates.

These considerations may explain the results reported by Harvey (1915) who found that the natural indicator in the cells of *Stichopus* changes colour more slowly with the lower fatty acids than with mineral acids, each acid being tested in 10^{-2} *M* concentration. He concluded that the mineral acids penetrated more rapidly than the lower fatty acids. It is justifiable to assume, however, that the natural indicator in these cells has a low *pK* value and, therefore, that the lower fatty acids, while penetrating more rapidly, could not shift the colour of the indicator, whereas the stronger, more slowly penetrating mineral acids would still be capable of causing a colour shift. For example, the acid *p*H range of thymol blue is 1·2–2·8. Consequently this indicator will not show an acid colour in 10^{-2} *M* concentration of any of the lower fatty acids, since solutions of these acids at this concentration have a *p*H of 3·2 or more, but will show an acid colour with 10^{-2} *M* HCl, which has a *p*H of 2·0. In case the fatty acid is neutralized in part within the cells during its penetration the *p*H, in the regions where the indicator is present will, in all probability, be appreciably greater than 3·2.

In our experiments the minimum concentration of fatty acid in the medium to induce a shift of methyl red to the acid colour has been found to be about 10^{-3} *M*, while neutral red shows such a shift when the concentration of the acid is only 10^{-6} *M*.

Methyl red penetrates living cells only from media of *p*H less than 6·0. The *p*H of the staining medium used for our experiments was 5·3–5·4. At this *p*H the cells are, of course, not under normal conditions, but no more so than in the usual experimental procedures for testing acid and acid-salt penetration.

The *p*H at which loss of methyl red from the cells may interfere with experimental work varies with the composition of the medium. Stained eggs or gill strips may be left in artificial sea water, brought to *p*H 6·0 with phosphate buffer, for upwards of half an hour without appreciable diminution in colour. On the other hand, in fatty acid-salt solutions at a *p*H of 5·5–5·6 the loss of dye begins to be appreciable after 10 min., while in media containing benzoic acid and benzoate the rate of loss is more rapid, even at a *p*H as low as 5·0. Because of this danger of leakage, and the risk to viability from too long a sojourn in the more acid-salt solutions, the period of experimentation with fatty acid and acid-salt solutions was limited to 4–5 min. Within this period the colour comparisons were quite satisfactory.

Owing to the abnormal acidic conditions of the medium in permeability experiments with fatty acid and salt mixtures, some of the experiments were modified so as to secure indications regarding the penetrability of organic salts from media having *p*H values within physiological limits, viz. that of sea water.

Artificial unbuffered sea water. This was prepared according to the following formula from Howard (1931) to correspond with the sea water at Wood's Hole:

M/1 NaCl	...	...	420 c.c.
M/2 KCl	...	...	18 c.c.
M/2 $MgSO_4$	...	...	51 c.c.
M/2 $MgCl_2$	...	...	46·7 c.c.
M/2 $CaCl_2$	...	...	18·7 c.c.
Water ...	...	...	to 1000 c.c.

Acid sea water. A *p*H of 5·3–5·4 was obtained by adding 1 c.c. of a mixture of 9 parts *M*/2 KH_2PO_4 and 1 part *M*/2 Na_2HPO_4 to 99 c.c. of the artificial sea water.

Methyl red staining solution. The methyl red, which at *p*H 5·3 is orange in colour, was obtained from Eimer and Amend (New York) as a powder of the water-insoluble acid. A solution which colours cellular tissue within 5–10 min. was secured by heating a suspension of the powder in acid sea water to 50° C. for a few minutes. The solution, filtered while hot, was prepared fresh daily.

Organic acid and salt solutions. Stock 0·1 *M* solutions of acetic, propionic, butyric, valeric, and benzoic acids were prepared in appropriate NaCl solutions to render them isotonic with Wood's Hole sea water. Stock 0·5 *M* solutions of the corresponding sodium salts were prepared in distilled water. These are approximately isotonic with sea water. The various experimental acid and acid-salt solutions were prepared by mixing the stock solutions and artificial sea water in the required proportions.

The materials used were short strips of the ciliated gills of *Modiolus* and of *Mytilus*[1] and the fertilized eggs[2] of the sanddollar (*Echinarachnius parma*). Strips

[1] The gills of *Modiolus* and *Mytilus* during the summer months are orange in colour because of the accumulation of diatoms. In the late autumn and winter the *Mytilus* obtained in New York City had colourless gills which were better suited for colorimetric experiments.

[2] In order to test the continued viability of the eggs by observing their subsequent cleavage it is necessary to fertilize the eggs prior to the acid treatment since it has been found (cf. Clowes & Smith, 1924) that a high percentage of sanddollar eggs cannot be fertilized after even moderate acid treatment.

of the gills, showing active ciliary movement, were placed in acid sea water containing methyl red where they became coloured a vivid yellow, the colour on the alkaline side of the range of methyl red. Tests for viability were made by noting whether or not the ciliary beat returned after acid treatment. The relatively transparent fertilized eggs of *E. parma* were passed through silk bolting cloth to remove the surrounding jelly and fertilization membranes in order to avoid as far as possible the carrying over of one solution to another during the experiments. With methyl red, in acid sea water, the eggs stain a uniform yellow colour. As a test for viability, the eggs were returned to normal sea water after the various experimental procedures and examined for extent of cleavage and development.

EXPERIMENTS WITH PIECES OF *MODIOLUS* AND *MYTILUS* GILL STAINED WITH METHYL RED

A. *Colour changes on immersion in acids*.

Table I gives the results obtained with hydrochloric acid, with the fatty acids (acetic to valeric), and with benzoic acid, all at a concentration of 3×10^{-3} *M* in artificial sea water. All induce a change of the yellow colour of the methyl red in

Table I. *Colour changes induced in methyl red stained* Modiolus *gills by various acids in* 3×10^{-3} *M concentration*

Acid employed	*p*H ±0·2	Time at which colour change was noted	Time to secure return to original colour in artificial acid sea water min.	Minimum time required to stop ciliary beat min.	Time required to secure return of ciliary beat in sea water min.
Hydrochloric	2·5	2–4 min.	10–20	3–5	20–30
Acetic	3·5	*ca.* 15 sec.	*ca.* 1	½–1	*ca.* 5
Propionic	3·5	*ca.* 10 sec.	*ca.* 1	½–1	*ca.* 5
Butyric	3·5	5–10 sec.	*ca.* 1	½–1	*ca.* 5
Valeric	3·5	5 sec. or less	*ca.* 1	½–1	*ca.* 5
Benzoic	3·3	5 sec. or less	*ca.* 1	½–1	*ca.* 5

*p*H values given were calculated and checked to ±0·2 *p*H units by colorimetric methods.

the cells to the red colour of its acid range without causing irreversible injury, as shown by the return of ciliary beat when the pieces are returned to normal sea water. The difference in time required by the various acids to induce a colour change conforms with the well-known findings regarding the difference in penetration rate of lipoid-soluble and lipoid-insoluble acids. It is significant (cf. column 3 in the table) that there is a similar difference with regard to their exit from the cells. The effect on ciliary beat was noted only in a general way, the times given in the last two columns being taken for the cessation and return of beat of the majority of the cilia. *Mytilus* gills were found to react in the same way as *Modiolus* gills.

B. *Comparison between immersion in the fatty acid and in mixtures of the fatty acid and its salts in unbuffered artificial sea water*

For each acid and its salt, four solutions were prepared of the artificial sea water, in each of which the concentration of added fatty acid was the same, i.e. 3×10^{-3} *M*, while that of the salt was varied. To the first solution the acid alone was added; in the second the ratio of acid and salt added was 1 : 1; in the third 1 : 3; and in the fourth 1 : 9.

The degree of dissociation of the acid in each of these mixtures was calculated for the strongest and the weakest of the four acids, namely acetic and propionic, on the basis of *pK* values of 4·48 for acetic and 4·59 for propionic acid. The *pK* values for the fatty acids are taken from Drucker (1905) and corrected by the addition of −0·25 unit, found necessary by Michaelis & Kruger (1921), for acetic acid and its salts in a solution containing 0·5 *M* NaCl and 0·02 *M* $CaCl_2$. This solution, which is isotonic with sea water, gives the closest approximation of the *pK* value of acetic acid in sea water thus far available. The calculated dissociation values for the two acids are as follows: for solutions to which acid alone was added they were 10·5 and 9·5 per cent respectively; for solutions having an acid/salt ratio of 1 : 1, they were 3·2 and 2·9 per cent; for solutions having an acid/salt ratio of 1·3, they were 1·1 and 0·9 per cent and, for solutions having an acid/salt ratio of 1 : 9, they were 0·36 and 0·29 per cent.

In accordance with the generally accepted assumption that weak electrolytes penetrate more readily in the undissociated than in the dissociated form, one would expect a greater penetration of acid when the salt is present. However, the solutions containing the lowest concentrations of undissociated acid were found to be more effective in inducing intracellular acidification than the various mixtures of the acid and the salt in which the dissociation of the acid should be less. The purpose of this paper was to study the counteracting intracellular effect of the salt to its acid. Therefore, the possible source of error arising from the differences in concentration of undissociated acid in the several solutions was disregarded since the differences, if appreciable, would count against our observed results.

The effects of these various fatty acid-salt solutions on the colour of stained strips of *Modiolus* gill are given in Table II. The yellow stained gills, when placed in the solutions with the acid/salt ratios of 1 : 0 and 1 : 1, began to shift immediately to the pink colour of the acid range of methyl red, reaching a maximum within 3–4 min., after which the colour remained unchanged. In the solutions with an acid/salt ratio of 1 : 3 the colour shifted only to a pink-orange indicating a less intense, although appreciable, intracellular acidification. In the solution with an acid/salt ratio of 1 : 9 the colour shift for the acetic, propionic, and butyric systems was also slight, although distinct, while for the valeric system no change was observed. These findings show that the intracellular acidification decreased with the increase in concentration of the salts in the medium.

At the end of each experiment the reversibility of the colour reactions was tested by transferring the gill strips to artificial acid sea water. When this was done

Table II. *Change in colour of methyl red stained* Modiolus *gills on transference to solutions of fatty acids and their salts in artificial sea water. (Methyl red colour: at* pH 6·0, *yellow;* pH 5·5, *orange; at* pH 5·0, *pink-orange; at* pH 4·5, *red; at* pH 4·0, *violet-red)*

Acid/salt ratio and *p*H of solutions	Acetic systems		Propionic and butyric systems		Valeric systems	
	At 15–20 sec.	At 3–4 min.	At 10–15 sec.	At 1 and 3–4 min.	At 10 sec.	At 1 and 3–4 min.
(*a*) 1 : 0 *p*H *ca.* 3·5	Pink-orange	Red	Pink-orange	Red	Pink-orange	Red
(*b*) 1 : 1 *p*H *ca.* 4·5	Pink-orange	Red	Pink-orange	Red	Pink-orange	Red
(*c*) 1 : 3 *p*H *ca.* 5·0	Pink-orange	Pink-orange	Pink-orange	Pink-orange	Pink-orange	Pink-orange
(*d*) 1 : 9 *p*H *ca.* 5·5	Yellow-orange	Orange	Yellow-orange	Orange	Yellow-orange	Yellow-orange

*p*H values given were calculated and checked within ±0·2 *p*H units by colorimetric methods.

after a sojourn of 5 min. in any one of the experimental solutions, there was a complete return of the original yellow coloration. Ciliary motion was re-established when the strips were then returned to normal sea water.

The decrease in degree of colour shift caused by the presence of the salts might be from leakage of dye although there was no appreciable diminution in colour intensity of the gill strips during the experimental period of 4–5 min. That some leakage must be occurring was indicated by the distinct decrease in colour intensity after the gill strips had been left for 10 min. in the 1 : 9 acid/salt solutions. However, the actual alkaline colour tint persisted, and, on transfer to 0·01 or 0·03 *M* solution of a fatty acid, the gill strips invariably showed an immediate change to a pronounced red colour.

Gill strips, which had changed to a pink or pink-orange in solutions having acid/salt ratios of 1 : 0, 1 : 1 and 1 : 3, showed a return to the original yellow-orange colour when transferred to artificial sea water and were then capable of again showing a change to a pink colour when returned to the fatty acid solutions.

C. *Effects of salts of organic acids on intracellular alkalinity in the absence of acid; relative alkalinizing effects of* Na *and* K *salts*

These experiments were designed as a test for a possible penetration of salts of organic acids from media of approximately physiological *p*H and in absence of added acid.[1] *Mytilus* gills were used. The calculated *p*H of the fatty acid salts in unbuffered sea water was 8·6, that of the benzoate, 8·4. These *p*H values approximate that of Wood's Hole sea water which has a *p*H of 8·2 (glass electrode).

After the tissues had been in the several salt solutions for a given time the possibility of the given salt having penetrated was tested by staining the tissue with

[1] The concentration of free acid formed by dissociation of the salts is of a very low order, by calculation *ca.* $1{\cdot}6 \times 10^{-6}$ *M* for 0·05 *M* benzoate and $1{\cdot}1 \times 10^{-6}$ *M* for 0·05 *M* acetate and butyrate solutions.

methyl red and immersing it in a solution of the corresponding acid. While in this acid solution the colour was compared with that of a methyl red stained and acid-treated tissue which had not been exposed to the fatty acid salt. A diminution or lack of colour shift in the tissue, having had a prior treatment with the salt solution, would indicate that the effect of the entering fatty acid was more or less neutralized by the salt already having accumulated in the cells.

An illustration of the procedure is given in the following experiment with acetic acid (see Table III). Pieces of gill were placed in three dishes: one, containing ordinary sea water (*p*H 8·4); another, 9 parts artificial, unbuffered sea water plus

Table III. *Colour of* Mytilus *gill strips in solutions of various acids after previous exposure for* 20 *min. to solutions of the corresponding salts in artificial sea water at p*H 8·4–8·6. (*See text for the procedure of staining with methyl red*)

Acid employed	Salt solution used during the preliminary 20-min. exposure period		Control
	(*a*) Potassium salt solution, 0·5 *M*, 1 part; artificial sea water, 9 parts	(*b*) Sodium salt solution, 0·5 *M*, 1 part, artificial sea water, 9 parts	Normal sea water, *p*H 8·4
	Each gill strip was now stained by immersing for 5 min. in methyl red in artificial sea water buffered to *p*H 5·3 with phosphate, and then exposed to the corresponding acid in artificial sea water. Change of colour from yellow shown below		
Acetic	Orange at 30 sec.	Orange at 15 sec.	Red at 15 sec.
Butyric	Orange at 20 sec.	Orange at 20 sec.	Red at 10 sec.
Benzoic	Orange at 30 sec.	Red at 10 sec.	Red at 10 sec.

1 part 0·5 *M* Na acetate; and a third, 9 parts artificial unbuffered sea water and 1 part 0·5 *M* K acetate. Twenty minutes later the gill pieces in each dish were transferred to a solution of methyl red in acid sea water (*p*H 5·3), left there for 5 min. to be stained, and then immersed in 3×10^{-3} *M* solution of the acetic acid in artificial sea water (*p*H 3·5). The same procedure was employed with the salts of butyric and benzoic acids.

The results, given in Table III, indicate that there is some penetration of the salts of acetic, butyric, and benzoic acids from a medium with a *p*H approximating that of the normal environment, in amounts sufficient to counteract the effect of the subsequently penetrating acids. It is to be noted that this penetration took place from a medium in which the concentration of free acid was of a very low order, viz. *ca.* 10^{-6} *M*.

It is significant that the reaction could be obtained only when the gill tissue had been exposed for at least 20 min. to the salt solutions. With a 10-min. period the effect was barely noticeable and, with a 5-min. period, was not observed. This result, compared with those obtained when the acid was present with the salt, indicates a much slower penetration of salts in the absence of their acids.

A third point of interest relates to potassium. Tissues, previously exposed to the solution made up with potassium acetate or butyrate (1 part of 0·5 *M*) and

artificial sea water (9 parts), showed a lesser colour shift with the acid than when the tissues were exposed to the solution prepared with the sodium salt. This indicates a more rapid penetration of the potassium than of the sodium salt.

EXPERIMENTS ON STAINED FERTILIZED SANDDOLLAR EGGS

A. *Colour changes upon immersion in hydrochloric acid*

Eggs, stained yellow with methyl red and then immersed in 10^{-3} and 3×10^{-4} *M* HCl in artificial sea water, changed gradually to an orange colour which reached a maximum after 1 min. in the stronger and after 3–4 min. in the weaker solution. Among the eggs a certain number (up to 30 per cent in the stronger solution) became opaque and irreversibly injured. These eggs could be distinguished at once by the fact that their colour had changed to a decided pink.

On transfer from the HCl solution to artificial acid sea water (*p*H 5·3–5·4) the healthy, orange-coloured eggs showed a slow return to the yellow colour characteristic of the eggs before they had been exposed to the HCl solutions. The dead, red eggs changed rapidly to the orange-pink characteristic of methyl red at the *p*H of the artificial acid sea water. The eggs were then removed to normal sea water in which the colour rapidly washed out. Those which had been red in the acid solution remained unsegmented while those which had changed only to orange underwent segmentation and developed into apparently normal gastrulae (top-swimmers).

B. *Comparison between butyric acid and mixtures of butyric acid and butyrate* (Na, K, *etc. of artificial sea water*)

Solutions of butyric acid and its sodium salt were prepared in artificial sea water in the same way as for the experiments already described with gill filaments. The acid was used in 10^{-3} *M* concentration, and the acid/salt ratios in the four solutions were 1 : 0, 1 : 1, 1 : 3 and 1 : 9.

All the eggs were fertilized, freed of their jelly and fertilization membranes and stained with methyl red. The tests were made as follows: stained eggs, washed free of all extraneous dye by rinsing several times in fresh changes of artificial acid sea water, were collected in a heap on the bottom of a watch-glass by gentle rotation of the glass. With a medicine dropper, a drop of concentrated suspension of the eggs was deposited on the bottom of each of two dry watch-glasses and as much fluid as possible withdrawn with a capillary pipette. 3 c.c. of the solution containing butyric acid without its salt were added to one dish and, simultaneously, 3 c.c. of one of the acid-salt mixtures to the other. The eggs, scattered by this procedure, were collected as soon as possible by twirling the dishes and an ocular comparison made of the colour of the mass of eggs in each dish. About $\frac{1}{2}$ min. was required

[1] On the basis of a *pK* value of 4·53 for butyric acid (cf. Drucker, 1905 and Michaelis & Drucker, 1931) this acid, by calculation, is about 84 per cent undissociated, while the amount of undissociated acid in the presence of 9×10^{-3} *M* concentration of its salts approaches 100 per cent. While there is an appreciable difference in concentration of undissociated acid in the two solutions, the experimental findings indicated greater acidification of the eggs in the absence than in the presence of the salts.

to get the eggs sufficiently collected to make this possible. The comparisons were facilitated by using coloured backgrounds consisting of two large, covered Petri dishes, each one being placed on a white sheet of paper and filled with a solution of methyl red, one at *p*H 5·6 and the other at *p*H 4·8. Red and pink-orange eggs stand out sharply against the former (orange) background while orange and yellow eggs do so against the latter (red) background.

Eggs, exposed to solutions having acid/salt ratios of 1 : 0 and 1 : 1, changed their colour within a few seconds from yellow to a pink-orange and remained so over a period of 5–10 min., beyond which no observations were made.

In the solution with an acid/salt ratio of 1 : 3 the shift in colour from yellow also was definite but the extent of shift was less, the colour which persisted during the exposure being distinctly less pink than in the two aforementioned solutions. In the solution with an acid/salt ratio of 1 : 9 there was also a shift to the acid colour but to a still lesser extent than in the other solutions. When the eggs in the four solutions were compared after 5–10 min. of exposure there was apparent a progressive gradation in intracellular colour, from a decided pink-orange in the more acid solutions to a decided yellow-orange in the less acid solutions. It is significant that in none of the solutions did the intracellular colour attain that characteristic of the *p*H of the external solution.

There was no observable loss of dye from the eggs in any of the solutions. However, eggs which had been several minutes longer in the most alkaline solutions were transferred to the strongest acid solution as an additional test for continued presence of the dye. In such eggs, the orange colour immediately shifted to the pink-orange colour characteristic of eggs which had been placed directly in that solution.

In all cases reversal to the original coloration was obtained on transfer of the eggs to artificial acid sea water and normal cleavage and development of top-swimmers appeared on transfer to normal sea water.

DISCUSSION

The methyl red staining method for detecting intracellular buffer effects of the salts of the rapidly penetrating fatty acids has served to demonstrate the occurrence of such effects within a shorter period of time than is possible with any of the indirect methods which have been used previously. Howard (1931) noted a salt antagonizing effect at the end of 4 min., the shortest time tested, and Beck (1935), at the end of the first determination which required about 2 min. In our experiments, colour differences could be noted within 10–20 sec. between cells in the presence of the acid alone and in the presence of the acid and its salt in a ratio of 1 : 9, the concentration of total added acid being the same in the two solutions.

The observed colour changes of the methyl red cannot be attributed to colour changes of adsorbed dye at the cell surfaces for the following reasons:

(1) In both gill strips and echinoderm eggs (sanddollar and starfish) the colour changes are dependent, not on the *p*H of the medium, but on the nature of the chemical agent employed. Fatty acid-salt solutions, having an acid/salt ratio of

1 : 1 and consequently a *p*H of about 4·5–4·7, induce a rapid and intense acid colour shift which is rapidly reversed if the cells are transferred to 0·5 *M* NH_4Cl (calculated *p*H 4·5). On the other hand, artificial sea water, acidified to *p*H 4·5 or even to *p*H 3·5 with HCl or KH_2PO_4, does not induce an acid coloration.

(2) In centrifuged sanddollar and starfish eggs in which the stained granules become sharply localized in the heavier or lighter ends of the cells the colour changes could be observed to occur in the granular zone.

Exposing the cells to organic salts in artificial sea water has given evidence for penetration of the fatty acid salts and of benzoate in the almost complete absence of their acids. This feature was brought out by one of the experiments of Howard (1931) but not emphasized by her. However, our experiments and those of Howard show that the rate of penetration of these salts is markedly increased when the corresponding acid is also present in the medium. The acetate or butyrate salt at 0·027 *M* concentration will counteract the effect of 3×10^{-3} *M* of the acid within 10–20 sec. (cf. Table II). To get this effect with the acid after a prior exposure to the salt alone it is necessary to expose the cells to 0·05 *M* concentration of the salt for at least 20 min. (cf. Table III). The time-concentration difference in action of the salts of these two acids is roughly 120 to 240 times greater in the presence than in the absence of appreciable amounts of the acid.

Another relation (cf. Tables I and II) which suggests a dependence of the rate of salt penetration on the presence and rate of penetration of the acid is the more rapid counteracting effect of the higher than of the lower fatty acid salts. This parallels the increase in rate of penetration of the higher over that of the lower fatty acids, and is in line with the observations by Smith and by Howard that the counteracting effect of a particular salt is greater, the greater the original effect of the corresponding acid.

From the remarkable parallel relation observed between acid penetration and apparent salt penetration, both Smith and Howard concluded it unlikely that the anions or salts in question actually migrate through the cell membrane. This conclusion was based on the fact that there is no evidence that the physical properties of these salts are related to the physical properties of the corresponding acid molecules to an extent sufficient to account for the observed apparent similarities in permeability behaviour. It should be emphasized that these relations between acid and salt action have been observed only under conditions where both acid and salt are present in the medium, and that when only the salt is present, its permeability is of a relatively low order.

The two authors have advanced independent hypotheses as to the manner in which the presence of a penetrating acid in the medium might be responsible for accumulation of salts of the corresponding acid within the cell. Smith suggested that H^+ and Cl^- as ion pairs, may pass out of the cell, following penetration of the fatty acid into the cell. On the basis of this hypothesis, it is necessary to assume that these ion pairs, formed as a result of interaction of the penetrating organic acid with intracellular constituents, will pass out of the cell because of the relatively high concentration of the salt of the penetrating acid in the medium.

Howard suggested the entrance into the cell of the organic acid in the undissociated form followed by a cation exchange, the external base being exchanged for the hydrogen ion derived from the intracellular dissociation of the acid. This is supported by her findings that the rate of effectiveness of the salt varies with different cations, viz. Rb > K > Na. It is noteworthy that she was able to observe this variation by adding such small amounts of the specific cation as 0·001–0·01 *M*. She attributed these effects to the different mobilities of Na, K and Rb, which, in water and in collodion membranes, are in the order Rb > K > Na. If we assume that a similar order of mobilities applies to the cell membrane, the observed ionic effects are to be expected on Howard's hypothesis. Smith's hypothesis does not explain these specific ionic effects, since such slight variations in the ionic medium as were employed by Howard can hardly be thought of as being capable of appreciably altering the rate of loss of an acid from the cells. This objection naturally applies to such acids as carbonic, lactic, etc., as well as HCl.

However, a serious objection may be raised regarding Howard's hypothesis, which is based on the assumption that intracellular hydrogen ions can exchange for extracellular cations. The phenomenon of intracellular salt effects which the hypothesis was designed to explain is a rapid one. There is required an additional and rather improbable assumption, viz. that there is a greater rate of exchange across the cell membrane of internal hydrogen ions for external cations than of external hydrogen ions for internal cations. If the membrane were equally permeable in both directions the acidification with mineral acids should be even more rapid than the action of the fatty acid salts, since (1) the chief cation within the cell is the highly mobile K ion whereas the chief extracellular cation in salt penetration studies is the less mobile Na ion, and (2) the concentration of HCl in the external medium in our HCl experiments was sufficiently great to give an external hydrogen-ion concentration hundreds of times higher than can conceivably be produced within the cell by a penetrating weak acid, and yet the rate of intracellular acidification was very slow.

Future experimental work should give consideration to other hypotheses regarding the mechanism involved in the rapid intracellular buffering effects exerted by the salts of fatty acids when such acids are also present in the medium. A possibility which has received no consideration is that the nature of the cell membrane may be so altered by acidic conditions in the medium or by intramembrane effects as to make the cells more permeable to such salts. This altered state need not be thought of necessarily as a destructive one. For example, it may be postulated that the fatty acids are retained within the lipoid cell membrane in amount sufficient to increase the solubility of their salts in the membrane, and in this manner favour their passage through.

SUMMARY

1. The *p*H indicator, methyl red, as a vital stain (from a medium at *p*H 5·3) and having a relatively low *pK* value, was used for testing the penetration of acids and of salts of organic acids into cells. The cells were those of gill strips of

Modiolus and *Mytilus* and the fertilized eggs of the sanddollar, *Echinarachnius parma.*

2. The penetration of various acids into cells was studied by immersing the cells in artificial, unbuffered sea water acidified with the given acid at 3×10^{-3} *M* concentration in experiments with gill strips and at 10^{-3} *M* concentration in experiments with sanddollar eggs. The colour change indicating intracellular acidification was almost instantaneous with benzoic acid (5 sec. or less), slightly less rapid (5–20 sec.) with the fatty acids, valeric to acetic, and slow ($\frac{1}{2}$–2 min.) with HCl. The rate of the return of the methyl red to the original alkaline colour upon the transfer of the cells to artificial acid sea water (*p*H 5·3) was in the same order for the various acids.

3. Intracellular acidification with a fatty acid was diminished when salts of the acid were present in the medium. Since the counteracting effect of the salts does not appear until the concentration of these salts exceeds that of the fatty acid by at least three times we may conclude that when both acid and salt are penetrating, the acid does so more rapidly than its salts.

4. The penetration of salts of acetic, butyric and benzoic acids also occurred in the absence of added acid and at an alkaline *p*H approximating that of normal sea water. However, the rate of penetration was much slower than in the presence of added acid. Evidence for penetration of the salts in absence of added acid was obtained by exposing the cells to the salts prior to treatment with the corresponding acid.

5. Effects obtained by prior treatment with the salts alone were enhanced by increasing the concentration of potassium in the medium.

REFERENCES

BECK, L. V. (1935). *Biochem. J.* **29**, 2424.
CHAMBERS, R. (1928). *Biol. Bull. Wood's Hole*, **55**, 369.
CLOWES, G. H. A. & SMITH, H. W. (1924). *Amer. J. Physiol.* **64**, 144.
DRUCKER, K. (1905). *Z. phys. Chem.* **52**, 641.
HARVEY, E. N. (1915). *Publ. Carneg. Instn*, No. 212, p. 143.
HOWARD, E. (1931). *Biol. Bull. Wood's Hole*, **60**, 132.
LILLIE, R. S. (1926). *J. gen. Physiol.* **8**, 339.
MICHAELIS, L. & KRUGER, R. (1921). *Biochem. Z.* **119**, 307.
SMITH, H. W. & CLOWES, G. H. A. (1924). *Amer. J. Physiol.* **68**, 183.
SMITH, H. W. (1925). *Amer. J. Physiol.* **72**, 347.
— (1926). *Amer. J. Physiol.* **76**, 411.