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**ETHIDIUM BROMIDE EFFLUX BY SALMONELLA: Modulation by
metabolic energy, pH, ions and phenothiazines.**

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Abstract

The main efflux pump of Salmonella Enteritidis serovar typhimurium that obtains its energy for the extrusion of a noxious agent from the proton motive force (PMF) has been studied with the aid of the Ethidium Bromide semi-automated method under conditions which define the role that metabolic energy, ions and pH play in the extrusion of the universal substrate ethidium bromide (EB). The results obtained in this study indicate that in minimal medium containing sodium, whereas at pH 5, efflux of EB is independent of glucose, at pH 8, metabolic energy is an absolute requirement for the maintenance of efflux. In deionised water pH 5.5, metabolic energy is required for maintenance of efflux. The inhibitory effect of the ionophore carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) on efflux is shown to be minimised by low pH, and at high pH, by metabolic energy. Similarly, thioridazine, an inhibitor of metabolic enzymes inhibits efflux of EB only at pH 8 and the degree of inhibition lessened by the presence of metabolic energy.

Keywords: proton motive force; hydronium ions, Salmonella Enteritidis, efflux pump; pH; metabolic energy; ions, CCCP, thioridazine.

1. Introduction

Infections caused by food-borne salmonella continue to manifest themselves even in well developed western countries where control over the preparation of food is unquestionably of high quality (1). Moreover, because many of these foods are meats that have been obtained from animals that have been treated for prolonged periods with tetracycline, and prolonged exposure of Gram-negative bacteria to this antibiotic causes the development of multi-drug resistant phenotypes (2-4), there is the distinct possibility that meats may not only be contaminated with salmonella or other coliform bacteria, but with MDR phenotypes of these bacteria (5).

The MDR phenotype of Gram-negative bacteria is mainly due to over-expressed efflux pumps which extrude members from two or more antibiotic classes before the antibiotics can reach their intended targets (6). The AcrAB-TolC efflux pump is the main MDR efflux pump of salmonella (6) and belongs to the genetically defined class resistance nodular division family (RND). RND efflux pumps derive the energy for activity from the proton motive force (PMF) which can be collapsed by exposure of the bacterium to cyanide m-chlorophenylhydrazine (CCCP) (7).

The components that make up the AcrAB-TolC efflux pump of salmonella are: i) the trimeric transporter protein AcrB that is attached to the inner membrane (plasma membrane) and recognizes and binds the substrate that is present in the periplasm, or within outer leaflet of the plasma membrane, or in the cytoplasm (8), and rapidly transfers the substrate to ii) the TolC component which is a conduit to the surface of the cell. The third component of the AcrAB-TolC efflux pump are two fusion proteins AcrA which bridge the joining of the AcrB to TolC and

which have been suggested to assist the extrusion process by a “peristaltic” type mechanism (9,10).

Ingested salmonella must survive the acid rich environment of the duodenum which receives bile salts from the liver that are toxic to the organism (11). When the organism reaches the colon, the pH is near neutral but the presence of bile salts still presents a toxic situation which must be dealt with for the entire duration of its colonic presence. The means by which the organism escapes injury from bile salts is by the activity of its AcrAB-TolC efflux pump which extrudes the noxious compounds prior to their reaching their intended targets (11). One may suppose that as long as the organism resides in the colon, the activity of the AcrAB-TolC efflux pump also protects it from antibiotics, and therefore, this would be the major reason as to why therapy of salmonellosis is problematic.

Recent studies have shown that the dissociation constant (K_d) for a substrate bound to the AcrB transporter is markedly affected by pH (12). Whereas at low pH of 5 the K_d is highest, with increasing pH the K_d decreases to a point where dissociation is almost absent (pH 8.5). If the AcrB transporter is to function, the dissociation of the substrate must be high, and if not, the transport function would slow down, thereby compromising the existence of the organism when present in an environment containing a noxious agent (bile salts, antibiotics). Because the AcrAB-TolC efflux pump is in reality a “tube” whose open terminal end (TolC channel) provides exposure of the transporter to the pH of the milieu, one would expect that when the salmonella organism is in the duodenum where the pH is lower than pH 5, dissociation of the bile salt or antibiotic from the site of transporter to which it was initially bound, would be rapid. At pH 8 the

dissociation of the substrate (bile salt or antibiotic) from the AcrB transporter would be slow.

Because the organism survives in the colon (*ca.* pH 7) where the concentration of bile salts is high, the dissociation of the substrate from the transporter must be rapid if the efflux pump is to protect the organism. How does the organism accomplish this in a pH milieu where dissociation of the substrate from the transporter is slow?.

The study to be described evaluates the *in vitro* effect of varying pH on the efflux of the universal efflux pump substrate-ethidium bromide (EB), a fluorochrome that is used for the assessment of efflux via an automated method (13), the modulation of efflux by metabolic energy, and the role of ions in efflux.

2. Materials & Methods.

2.1 *Bacteria Salmonella Enteritidis* NCTC 13349.

2.2 *Materials*

Media employed in this study for maintaining *Salmonella Enteritidis* NCTC 13349: Mueller-Hinton (MH) in powder form purchased from Difco for the preparation of broth and agar; ethidium bromide (EB), phosphate buffered sodium chloride (PBS), sodium chloride, glucose, sodium pyruvate, ethanol, ATP, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) thioridazine (TZ) and orthovanalate (OV) purchased from Sigma-Aldrich Química SA (Madrid Spain). Sodium Chloride purchased from Aldrich.

2.3 *Minimum inhibitory concentrations (MICs)* of EB, CCCP, TZ and OV performed in MH as per CLSI guidelines (14). MICs were determined for each of these agents in order to select concentrations that had no effect on the viability of the bacterium.

2.4 *Real-time accumulation of EB*

was performed using our previously developed semi-automated method (11, 12). Briefly, the study strain was cultured in MH broth reaching an optical density (OD₅₄₅) of 0.6, the tubes centrifuged at 13,000 rpm for 3 minutes and the supernates discarded. The recovered pellets re-suspended in phosphate buffered saline (PBS) pH 7.4, and centrifuged as before. The final washed pellet was then re-suspended in tubes containing PBS buffered to different pH ranging from 5 through 8 or deionised water. The final OD₅₄₅ was adjusted to 0.6.

To 100 μ l tubes a volume of 50 μ l containing PBS at varying pH or deionised water to match that of the initial cell stock with and without glucose to yield a final concentration per ml of 0.6 % or other sources of metabolic energy, and 1 mg/L of EB. 50 μ l of the cell suspension maintained in varying pH of PBS or deionised water was added to the phosphate-saline pH matching tubes that lacked or contained metabolic energy or deionised water that lacked or contained metabolic energy. Similarly, agents such as CCCP, TZ and OV in pH matched PBS or deionised water were added. Tubes were placed into the Corbett 3000 PCR Thermocycler that had been programmed for temperature of 37°C, length of cycle in minutes, total number of cycles and for the identity of each tube with respect to its contents. The amount of relative fluorescence calculated by the Corbett 3000 instrument is a measure of accumulated EB within the bacterial population (15). Accumulation assays were repeated by three different co-authors and the qualitative results obtained were similar.

2.5 Assessment of real-time efflux of EB

In order that efflux is assessed, sufficient accumulation of EB must first be established. Therefore, conditions which maximize accumulation of EB (identified in the text and its figures) were employed and accumulation of EB at 37°C allowed for a specific period of time after which the instrument was stopped, various reagents that would allow definition of their effects on efflux were then added in a volume of 10 μ l (controls received 10 μ l of matching pH), the instrument re-started and any change in fluorescence recorded during the remainder of the run. These efflux assays were repeated by three of the co-authors of this study and the results obtained did not vary qualitatively.

3. Results

The MICs for EB, CCCP, TZ and OV against Salmonella Enteritidis NCTC 13349 are presented by Table 1. The concentrations of each of these reagents employed in the following sections were well below $\frac{1}{2}$ their MICs and had no effect on the viability of the bacterium.

The accumulation and efflux of EB by bacteria has been shown to be modulated by pH (16) and independent of metabolic energy at pH 5 during the assay (15, 16). The effect of pH and energy source on the accumulation of EB by the NCTC strain maintained in PBS is depicted by Figure 1. As noted by Figure 1A accumulation of EB in the absence of glucose is markedly affected by pH. In the presence of glucose during the course of the assay, as noted by Figure 1B, no accumulation takes place at pH 5; a little accumulation of EB takes place at pH 7.4 and slightly more at pH 8. These results suggest that accumulation of EB by the NCTC strain, as is the case with other Gram-negative bacteria (15, 16), is affected by pH and that at pH of 7.4 or greater, glucose plays a major role in reducing the amount of EB accumulated. That indeed metabolic energy plays a role in the control of EB accumulation, replacement of glucose with ethanol or pyruvate provides the needed energy to maintain accumulation at a lower level as opposed to the absence of metabolic energy alone (data not shown). However, as is the case for glucose, the need for metabolic energy is greatest with increase of pH. At pH 5, metabolic energy is not needed during the assay for maintaining a low level of EB accumulation and no additional decrease of EB accumulation was observed in the presence of pyruvate, glucose or ethanol (data not shown). At pH 7.4 and pH 8 replacement of glucose with ethanol or pyruvate reduces accumulation.

Conditions which promote the accumulation of the substrate are considered to be those that modulate efflux (2, 6, 13, 15-17). Nevertheless, in order that such conditions are identified, an efflux assay that describes extrusion of the substrate must be conducted. As shown by Figure 2, in PBS of pH 8, efflux by the salmonella strain takes place only when metabolic energy (glucose, ethanol, pyruvate) is present in the medium during the duration of the assay. At pH 5, efflux takes place readily in the absence of any metabolic energy (data not shown).

CCCP, a proton ionophore, causes the collapse of the proton motive force (PMF) and hence, deny protons needed for the activity of an efflux pump system dependent upon the PMF (6, 18). If low pH is sufficient to maintain efflux, the possibility that at low pH CCCP would have a much smaller effect on efflux than at high pH, was investigated. As shown by Figure 3, at a pH of 5 the effect of CCCP on accumulation of EB is much less than at pH 8 when glucose is present. Similarly, the effect of CCCP on the efflux of EB is much less at pH 5 than at pH 8 (data not shown). Whereas the lowest concentration of CCCP that promoted accumulation and inhibited efflux at pH 5 was 2.5 mg/L at pH 8 concentrations of CCCP as low as 1 mg/L produced significant accumulation and inhibition of efflux at pH 8 (data not shown).

Phenothiazines such as thioridazine (TZ) are known to inhibit enzymes involved in metabolism (19). Because metabolic energy is required for efflux of EB as shown in this study, the effect of TZ on accumulation and efflux was studied, and the results depicted by Figure 4. Briefly, TZ inhibits efflux at pH 8 despite the presence of glucose in the system and has less of an effect at pH 5. Similar results have been obtained with *Escherichia coli* (20).

OV is known to affect the activity of metabolic enzymes that furnish energy (ATP) for the activity of ABC transporters (21). Because ABC transporters hydrolyse ATP subsequent to their binding the substrate, reduction of ATP levels reduces efflux of the substrate. At pH 8 the hydrolysis of ATP by ATP synthase is favoured (22-24). In order to determine whether the marked inhibition of efflux promoted by TZ at pH 8 is due to an inhibition of an ABC transporter, the efflux assay was conducted in glucose-saline pH 8 with increasing concentrations of OV. Concentrations of OV as high as 200 mg/L had no effect on efflux of EB (data not shown) thereby indicating that the effects of TZ on EB efflux at pH 8 did not involve an ABC transporter.

As shown by the studies so far described, efflux at pH 5 does not require metabolic energy for the duration of the assay. In our laboratory, deionised water has a consistent pH of 5.5 and therefore this pH is close to that of the PBS of pH 5. Accumulation and efflux were therefore conducted in deionised water pH 5.5 with and without energy source. As shown by Figure 5A and Figure 5B, metabolic energy is needed to prevent or reduce accumulation of EB as well as to maintain efflux of EB. Therefore, pH alone does not maintain conditions needed for efflux of the substrate EB. The addition of NaCl to the deionised water to yield a final concentration of 0.95% and a pH of 5.5 produced accumulation and efflux data similar to that of PBS of pH 5 and no glucose (data not shown).

The ionophore CCCP is used for the demonstration of a PMF dependent efflux system. Interestingly, we have not been able to find a single study that shows where CCCP has its effects on the bacterial PMF. The brilliant papers by Mulkidjanian and colleagues (25-27) present strong

evidence that protons generated by metabolism are transported as hydronium ions via channels to the surface of the cell where they are distributed and bound to reactive groups of LPS. These hydronium ions are then transported via other channels (e.g. porins) to the periplasm of the Gram-negative bacterium where they are used by the pump of the efflux system for rapid extrusion of the substrate. KM Pos and colleagues recently published a series of studies and reviews demonstrating the path of the “proton” through the AcrB component of the efflux pump (28, 29).

If we substitute the “proton” with the hydronium ion, the passage of the hydronium ion would establish the needed condition for the dissociation of the substrate from the transporter as predicted by Nikaido’s group (30). Because the latter group were able to show that the dissociation of the substrate is very slow at high pH (7 or greater) and very high at low pH (5.5), the passage of the hydronium ion would create the needed acid pH for dissociation of substrate from the transporter. Because CCCP would be expected to reduce the availability of hydronium ions to the periplasm, the substrate would remain tightly bound to the transporter and its extrusion would be slowed down or not at all. Employing varying CCCP concentrations in the deionised water efflux assay (pH 5.5), it would be possible to determine the effects on accumulation. As shown by Figure 6A the presence of CCCP at varying concentrations produces rapid and huge increases in the accumulation of EB when there is no glucose in the system. The amount of EB accumulated is significantly greater than that taking place in glucose-free deionised water. When glucose is present, the same concentrations of CCCP are less effective. The effect of the higher concentration of CCCP on efflux and the modulation of this effect by glucose is depicted by Figure 6B. It is evident that the inhibition of efflux by CCCP alone can be

246 eliminated by metabolic energy in deionised water of pH 5.5. These results suggest that a
247 sufficient metabolic source of energy can overcome the effects of CCCP even when no ions are
248 present in the system.

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4. Discussion

The activity of the main efflux pump of a Gram-negative bacterium has been previously shown to be modulated by pH and by metabolic energy (6, 9, 16, 17). At the low pH of 5, efflux activity is greater than at the high pH of 8. Whereas at high pH efflux is dependent upon metabolic energy, at low pH, efflux does not depend on metabolic energy, at least for the duration of the assays employed. In the current study, the efflux system of Salmonella Enteritidis NCTC 13349 is more effective at the low pH of 5 than at the high pH of 8; and at low pH it is less dependent of metabolic energy (glucose, ETOH, pyruvate). However, when ions are omitted from the medium, efflux is dependent upon metabolic energy even though the pH of the ion free milieu (deionised water) is low (pH 5.5). Sodium therefore appears to serve as an anti-port for efflux of the universal substrate EB. Or [Na] contributes to the chemical potential necessary for energising efflux.

The phenothiazine thioridazine is known to promote accumulation of EB by inhibiting efflux of this substrate (19). In the current study, the inhibition of efflux by thioridazine has also been noted. However, the inhibition of efflux takes place at the high pH of 8 and not at the low pH of 5. Thioridazine is known to inhibit enzymes involved in metabolism (19). Because the presence of glucose does not reduce the inhibition of efflux produced by thioridazine, the inhibition of efflux at pH 8 is assumed to be the result of an inhibition of the enzyme system involved in metabolism.

Summarising the results obtained in the current study, we can say that efflux is supported by ions and metabolic energy and that the degree of support provided by ions and energy is modulated by

pH. But how do these results relate to the mechanism by which AcrAB, the main efflux pump of Salmonella, functions? Crucial to understanding the mechanism of efflux lies in studies that demonstrate two main characteristics of the AcrAB transporter, namely, that the binding of the substrate (example EB) that takes place at neutral pH is stable (low dissociation constant), and, the dissociation of the substrate requires a pH lower than 6 (12, 28). Association and dissociation does not require metabolic energy as is the case for an ABC transporter (31, 32). Because dissociation of the substrate from the transporter must take place for the efflux pump to continue to extrude the noxious agent to the exterior of the cell, the internal pH of the transporter must be reduced to about 6 or lower for the dissociation of the substrate and its eventual release through the TolC conduit. It is the opinion of the authors that the reduction of internal pH of the transported takes place from the passage of hydronium ions that either come directly from metabolism when the pH of the milieu is high, or from the surface of the cell when the pH of the milieu is low (when the pH of the milieu is high, the dissociation of protons (hydronium ions) from the surface of the cell into the bulk medium is encouraged (25-27)). Results obtained in the current study suggest that the activity of the proton ionophore CCCP on efflux is modulated by pH. Because at high pH, CCCP has its greatest inhibition on the efflux of EB that is obviated by the availability of metabolic energy, we may assume that at high pH it is metabolism that provides the protons in the form of hydronium ions that as they pass through the internal cavity of the transporter, the pH is reduced to the level needed for the dissociation of the substrate. It is further suggested that the AcrAB-TolC provides the means by which molecules is extruded from the cell as a consequence of peristalsis provided by the movement of the AcrA fusion proteins as described by Eicher *et al.* (8).

When the pH of the milieu is low, the source of hydronium ions needed for passage through the transporter is assured due to the high concentration of surface bound protons (hydronium ions) (25-27). The difference between this high concentration of protons and that of the cytoplasm creates an electrochemical gradient (through the inner membrane) that manifests itself into the proton motive force (PMF). The movement of these protons is propagated by events within the periplasm and cytoplasm of the cell such as export functions. It is postulated that routinely, with export functions taking place, the need for hydronium ions is present and the translocation of these via the PMF, takes place through “aquaporins” (33). The result of the current study that shows that efflux at pH 5 does not require metabolic energy as long as ions are present in the system, is interpreted to reflect the availability of the required protons for passage through the transporter and subsequent reduction of pH resulting in the release of the substrate and subsequent extrusion provided by the flow of water to the surface of the cell. Lastly, extrapolation of the results of this current study to the in vivo passage of the Gram-negative bacterium that eventually colonises the colon, we may assume that during its passage through the stomach where the pH may be as low as 2, the bacterium is relatively immune to any noxious agent due to automatic efflux possible at this low pH. When it reaches the duodenum, the pH is higher but still quite acid (pH 4-5) and the bile salts present can easily be extruded automatically since the low pH of the environment will maintain a source of hydronium ions bound to the surface of cell, and hence, maintain the PMF required for movement of the hydronium ion to the periplasm and passage through the efflux pump thereby promoting release of the bile salt from the efflux pump and extrusion via the movement of water through the pump-possible assisted by the fusion protein promoted peristaltic movement. When the organism reaches the colon, the pH is near neutral. Here the organism faces toxicity presented by bile salts, which are recognised

when they are present in the periplasm and bound tightly to the transporter. If these toxins are to be released at the sites of the transporter where they are bound, the pH within the transporter must be reduced. It is here where metabolism of glucose and intermediates of metabolism furnish the needed hydronium ions for passage through the efflux pump, subsequent release of the substrate, and its expulsion into the medium.

Declarations

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Ethical Approval: Not applicable

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Table 1. Minimum Inhibitory Concentration (MIC) of compounds used in this study.

MIC (mg/L)	
Compound	<i>Salmonella Enteritidis</i> NCTC 13349
EB	256 ug/ml
TZ	128 ug/ml
OV	> 5000 ug/ml
CCCP	25,6 ug/ml

EB, ethidium bromide; **TZ**, thioridazine; **OV**, orthovanadate; **CCCP**, carbonyl cyanide m-chlorophenylhydrazone.

Title and Legends of Figures

Figure 1A. Accumulation of EB at pH 5, 7.4 and 8 in absence of glucose

Legend. Bacteria suspended in glucose free-saline buffered pH 5, 7.4 and 8 containing 1 mg/L of EB incubated at 37C and accumulation of EB followed for 50 minutes. Note that at pH 5 no accumulation of EB takes place from 1 through 50 minutes of assay. At pH 7.4 and 8 accumulation is essentially the same and increases for duration of assay.

Figure 1B. Accumulation of EB at pH 5, 7.4 and 8 in presence of glucose

Legend. Bacteria suspended in glucose-saline buffered pH 5, 7.4 and 8 containing 1 mg/L of EB incubated at 37C and accumulation of EB followed for 50 minutes. Note that at pH 5 and 7.4 no accumulation of EB takes place from 1 through 50 minutes of assay. At pH 8 EB begins to accumulate after 30 or so minutes. Comparison of accumulation at pH 8 in absence (Figure 1A) and presence of glucose (Figure 1B) indicates that the presence of metabolic energy reduces significantly accumulation of EB.

Figure 2. The effect of metabolic energy on efflux of EB at pH 8

Legend. Accumulation of EB in glucose free-PBS pH 8 conducted for 30 minutes; the instrument is paused and glucose free-PBS, PBS with glucose, pyruvate or ethanol is added; instrument re-started and efflux of EB followed for an additional 30 minutes. Note that whereas accumulation of EB continues with the addition of glucose free-PBS, addition of glucose promotes efflux. Addition of Pyruvate or ethanol also promote efflux.

Figure 3. The effect of CCCP on accumulation of EB at pH 5 and 8 in medium that contains glucose

Legend. Accumulation of EB in glucose-saline pH 5 and 8 with and without 5 mg/L of CCCP.

Note: The effects of CCCP are concentration dependent with minimal effects with 2.5 mg/L (data not shown).

Figure 4. The effect of TZ on the efflux of EB by salmonella in glucose saline pH 5 and 8

Legend. Accumulation of EB at pH 5 and 8 in glucose-free saline; instrument paused and 10 mg/L of TZ with glucose-saline of pH 5 and 8 added, and the instrument re-started.

Note: Effect of TZ on efflux is greatest at pH 8 than at pH 5.

Figure 5A. Accumulation of EB by salmonella in deionised water pH 5.5 and modulation by glucose

Figure 5B. Efflux of EB by salmonella in deionised water pH 5.5 and modulation by glucose

Figure 6A. Effect of CCCP on accumulation of EB in deionised water pH 5.5

Note. Presence of CCCP in glucose-free deionised water produces accumulation of EB in a concentration dependent manner. The amount of EB accumulated is significantly greater than that taking place in glucose-free water alone. The presence of glucose significantly reduces the amount of EB accumulated produced by CCCP. The glucose reduced level of CCCP accumulated is lower than that present in glucose-free deionised water.

552 **Figure 6B. Effect of additions of CCCP on efflux of EB in deionised water pH 5.5**

553 **Note.** Despite the addition of a high concentration of CCCP, efflux of EB takes place when the
554 addition contains glucose.

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Figure 1A. Accumulation of EB at pH 5, 7.4 and 8 in absence of glucose.

Figure 1B. Accumulation of EB at pH 5, 7.4 and 8 in presence of glucose.

Figure 2. The effect of metabolic energy on efflux of EB at pH 8.

Figure 3A. The effect of CCCP on accumulation of EB at pH 5 and 8 in medium that contain glucose.

Figure 4. The effect of TZ on the efflux of EB by salmonella in glucose saline pH 5 and 8.

Figure 5A. Accumulation of EB by salmonella in deionised water pH 5.5 and modulation by glucose.

Figure 5B. Efflux of EB by salmonella in deionised water pH 5.5 and modulation by glucose.

Figure 6A. Effect of CCCP on accumulation of EB in deionised water pH 5.5.

Figure 6B. Effect of additions of CCCP on efflux of EB in deionised water pH 5.5.