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From the Department of Food Hygiene, College of Veterinary Medicine, Helsinki, Finland.

THE EFFECT OF pH AND POTASSIUM PHOSPHATE BUFFER ON THE TOXICITY OF CADMIUM FOR BACTERIA

By

H. Korkeala and *T. J. Pekkanen*

KORKEALA, H. and T. J. PEKKANEN: *The effect of pH and potassium phosphate buffer on the toxicity of cadmium for bacteria.* Acta vet. scand. 1978 *19*, 93—101. — An increase in the pH of Plate Count Agar led to increased toxicity of cadmium (Cd) for Micrococcus luteus ATCC 9341, Staphylococcus aureus ATCC 12600, Clostridium perfringens ATCC 8798, Escherichia coli ATCC 11775 and Pseudomonas aeruginosa ATCC 10145. The effect of the pH on toxicity was not clearly observed with Streptococcus bovis ATCC 9809 and was absent with Bacillus subtilis ATCC 8473.

When potassium phosphate buffer was added to the growth medium, the Cd toxicity for M. luteus and B. subtilis was enhanced. The toxicity of Cd for S. bovis decreased when potassium phosphate buffer was added to the medium. When the pH increased at differing phosphate concentrations, the sensitivity of M. luteus to Cd decreased.

In the authors' opinion, the effect of pH on Cd toxicity for bacteria is due to a continuously increasing negative charge towards an alkaline value by most bacteria which increases the affinity of cations towards the cell wall.

cadmium; pH; phosphate; bacteria.

Cadmium (Cd) is a potentially hazardous pollutant in the environment. Much attention has been given to Cd toxicity studies on animals (e. g. *Friberg et al.* 1974), but less attention to the effects of Cd on plants and microorganisms.

Some studies have shown that the pH and the phosphorus content of the soil influence the Cd uptake by plants. *Miller et al.* (1976) and *Williams & David* (1976) observed that extensive Cd accumulation and growth inhibition of plants were especially apparent at a low pH. Increasing available soil phosphorus was related to increased Cd accumulation (*Miller et al.*).

In the studies of *Abelson & Aldous* (1950) the toxic effects of Cd on the metabolism of bacteria were apparent. Later *Bernheim* (1973) described the effect of pH on the actions of Cd salts on Pseudomonas aeruginosa. *Babich & Stotzky* (1977) found that for bacteria, in contrast to plants, Cd toxicity was generally potentiated at a high pH.

The present study was undertaken to investigate the sensitivity of certain bacteria to Cd by the minimum inhibitory concentration technique and the effects of the pH and a phosphate buffer on that sensitivity.

MATERIAL AND METHODS

Organisms

The microbial strains used in the study were Micrococcus luteus ATCC 9341, Staphylococcus aureus ATCC 12600, Bacillus subtilis ATCC 8473, Streptococcus bovis ATCC 9809, Clostridium perfringens ATCC 8798, Escherichia coli ATCC 11775 and Pseudomonas aeruginosa ATCC 10145.

Chemicals and water

KH_2PO_4, K_2HPO_4 and $CdCl_2$ 21/2 $\times$ H_2O were of pro analysis grade and obtained from J. T. Baker (Chemical Co. Deventer, Holland and Phillipsburg, N. J., USA). The water used was double distilled and deionized. Before the experiments the Cd content of the water was determined by flameless atomic absorption spectrophotometry and found to be below the lowest limit of detection (0.2 ng). The apparatus used was a Perkin-Elmer 303 atomic absorption spectrophotometer equipped with a graphite furnace and graphite cell power supply HGA 72.

Media and culture conditions

The basic medium used throughout the experiments was Plate Count Agar (Difco Laboratories, Detroit, Michigan, USA). The incubation time was 48 hrs. and the incubation temperature for M. luteus and B. subtilis 30°C and for the other bacteria 35°C. All the experiments were conducted on cells from overnight cultures in Nutrient Broth (Difco). The Cd content of the Plate Count Agar and the Nutrient Broth was determined before the experiments, as above. The greatest concentrations found were 0.0006 mg of Cd/l substrate and 0.0057 mg of Cd/l substrate, re-

spectively. The studies on C. perfringens were carried out in anaerobic conditions (BBL GasPak jars equipped with GasPak H + CO_2 generator envelopes, BBL, Cockeysville, Maryland, USA) and on the other bacteria in aerobic conditions.

Sensitivity testing

Minimum inhibitory concentration (M. I. C.) for Cd (used as $CdCl_2$) was determined by a plate dilution test (*Barber & Waterworth* 1964) as the lowest concentration of Cd which inhibits growth completely. A barely visible haze or a single colony was disregarded. Sterile 10 ml volumes of $CdCl_2$ solutions of certain Cd concentrations were added to 90 ml of the autoclaved Plate Count Agar to give the desired Cd concentrations and the recommended amounts of water in the media. The final concentrations of Cd were 0, 0.001, 0.002, 0.004, 0.006, 0.008, 0.01, 0.02, 0.04, 0.06, 0.08, 0.1, 0.2, 0.4, 0.6, 0.8, 1, 2, 4, 6, 8, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250, 300, 350 and 400 mg of Cd/l medium. Three replicate plates were employed for each concentration of Cd. The inoculum from the diluted overnight cultures was spread on the agar surface with a standard 1-mm loop. The volume of one loop was used for each test. The cultures in the Nutrient Broth were first diluted with sterile isotonic saline so as to contain 170 $\times$ 10^4 colony forming units (CFU)/ml of M. luteus, 60 $\times$ 10^4 CFU/ml of S. aureus, 75 $\times$ 10^4 CFU/ml of B. subtilis, 366 $\times$ 10^4 CFU/ml of S. bovis, 395 $\times$ 10^4 CFU/ ml of C. perfringens, 63 $\times$ 10^4 CFU/ml of E. coli or 70 $\times$ 10^4 CFU/ml of P. aeruginosa.

The effect of pH of the growth medium on Cd toxicity

The experiments were carried out by determining the M.I.C. values of Cd as described above. The pH of the Plate Count Agar was adjusted to 6.0 and 6.5 with 4 N-HCl and 7.5 and 8.0 with 4 N-NaOH. Experiments were also made at the normal pH of Plate Count Agar, which is pH 7.0.

The effect of phosphate buffer at different pH values on Cd toxicity

The experiments were carried out by determining the M.I.C. values of Cd as above. Plate Count Agar was buffered with potassium phosphate buffer to pH 6.0, 7.0 and 8.0. Potassium phos-

phate was selected instead of sodium phosphate because it is known that some heavy metal compounds (mercurials) increase the swelling rate of Pseudomonas aeruginosa to a lesser extent in potassium phosphate than in sodium phosphate (*Bernheim* 1970). The phosphate concentrations used at each pH value were 0.05 M, 0.1 M and 0.2 M. The phosphate concentration of the Plate Count Agar was found to be 0.001 M, as determined by the method of *Neraal & Hamm* (1972). This is not taken into account when the final phosphate concentrations of the media are given. Sterile $CdCl_2$ solution was added to the media after sterilization in order to avoid the possible precipitation of Cd with phosphate on autoclaving (*Donald et al.* 1952).

Because a precipitate was observed when $CdCl_2$ was mixed with phosphate buffer to give Cd concentrations high enough to inhibit the growth of the strains of S. aureus, C. perfringens, E. coli and P. aeruginosa used in the sensitivity testing without added phosphate, the effect of phosphate buffer at different pH values on Cd toxicity was studied only on M. luteus, B. subtilis and S. bovis.

RESULTS

The effect of the pH on the toxicity of Cd with M. luteus, B. subtilis and S. bovis is presented in Table 1 and with S. aureus, C. perfringens, E. coli and P. aeruginosa in Fig. 1. The results of the studies on the effects of potassium phosphate buffer and pH on the toxicity of Cd for M. luteus, B. subtilis and S. bovis are given in Table 1. The three replicate plates for each Cd concentration always gave the same M.I.C. value with all used media.

DISCUSSION

When the results presented in Table 1 and Fig. 1 are examined, it is obvious that the C. perfringens ATCC 8798, S. aureus ATCC 12600, E. coli ATCC 11775 and P. aeruginosa ATCC 10145 strains used in the experiments are more resistant to the toxic effects of Cd at most pH values and phosphate concentrations than M. luteus ATCC 9341, B. subtilis ATCC 8473 or S. bovis ATCC 9809, but because it is well known that within a bacterial species there exist more or less Cd resistant strains, no general conclusions concerning interspecies sensitivity can be made from the results (*Novick & Roth* 1968, *Kondo et al.* 1974, *Tokuyama &*

Table 1. The effect of pH and potassium phosphate buffer at different pH values on the toxicity of Cd for Micrococcus luteus ATCC 9341, Bacillus subtilis ATCC 8473 and Streptococcus bovis ATCC 9809.

Medium	M. luteus	B. subtilis	S. bovis
Plate Count Agar. No added phosphate			
pH 6.0	2[1]	4	4
pH 6.5	4	4	4
pH 7.0	2	4	4
pH 7.5	0.6	4	4
pH 8.0	0.6	4	2
Plate Count Agar prepared in 0.05 M phosphate buffer solution			
pH 6.0	0.08	2	8
pH 7.0	0.4	2	8
pH 8.0	2	1	4
Plate Count Agar prepared in 0.1 M phosphate buffer solution			
pH 6.0	0.008	0.8	8
pH 7.0	0.2	2	8
pH 8.0	0.8	1	6
Plate Count Agar prepared in 0.2 M phosphate buffer solution			
pH 6.0	—[2]	0.6	8
pH 7.0	0.1	2	10
pH 8.0	0.2	1	—

[1] The minimum concentrations of Cd (mg/l) in the test medium which inhibited the growth of the test microbe (M.I.C. value).
[2] No visible growth without added Cd in the medium.

Asano 1974, *Tynecka et al.* 1975, *Nakahara et al.* 1977). It can further be seen from the results that most of the bacteria used in the experiments are generally more sensitive to Cd at a high than at a low pH when phosphate is not added to the substrate. This is in accordance with the results obtained by *Babich & Stotzky* (1977), who showed the toxicity of Cd for eubacteria, actinomycetes and fungi to be similarly pH-dependent. On the other hand this is in contradiction to the observations made on plants, where Cd is most toxic at low pH values of the soil (*Miller et al.* 1976). According to *Miller et al.* this is due to the increased solubility of Cd compounds, e.g. phosphates, at low pH values. *Babich & Stotzky* explained the pH effect of Cd referring to

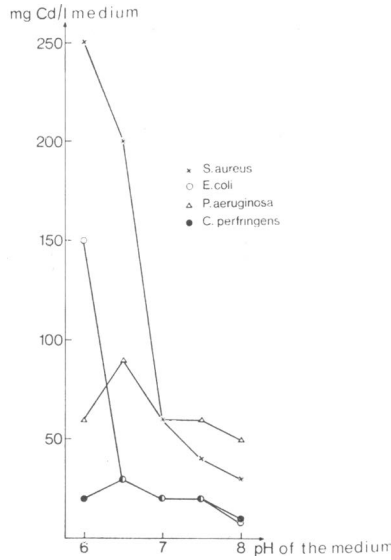

Figure 1. The effect of the pH of the growth medium on the toxicity of Cd for Staphylococcus aureus ATCC 12600, Clostridium perfringens ATCC 8798, Escherichia coli ATCC 11775 and Pseudomonas aeruginosa ATCC 10145. The pH of the Plate Count Agar which contained 1, 2, 4, 6, 8, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250, 300, 350 and 400 mg of Cd/l medium was adjusted to pH 6.0, 6.5, 7.5 and 8.0 with 4 N-HCl or 4 N-NaOH. The normal pH of the Plate Count Agar is pH 7.0. Each point represents the lowest concentration of Cd which inhibits the growth of the test microbe in the respective medium (M.I.C. value).

Hahne & Kroontje (1973), who observed that in an aqueous system most Cd at pH values under 8 is in an ionic Cd^{2+} state. According to *Babich & Stotzky,* Cd^{2+} should readily react with the constituents of the growth medium and thus be less available to the microbe than the $CdOH^{+}$ ion which begins to form at pH values higher than 7.5. On the other hand, *Ramamoorthy & Kushner* (1975) showed that the constituents of bacterial growth mediums do not bind large amounts of the Cd^{2+}. According to the present authors the greater toxicity of Cd for bacteria in an alkaline medium than in an acid medium might be more due to a continuously increasing negative charge towards an alkaline value by most bacteria (*Lamanna et al.* 1973), which increases the affinity of cations towards the cell wall, than to other factors.

The effect of pH on the toxicity of Cd was not clearly observed with S. bovis and was absent with B. subtilis. The reason for the lack of the effect remains obscure. A reversal of or difference in the negative charge on the bacteria might explain the difference.

When potassium phosphate buffer was added to the growth medium the toxic effects of Cd on M. luteus were enhanced at pH 6.0 and 7.0 of the medium. A similar increase in toxicity was observed for B. subtilis at the pH values studied, but not for S. bovis, for which the toxicity of Cd decreased when potassium phosphate buffer was added to the medium. It was also observed that when the pH increased at differing phosphate concentrations the sensitivity of M. luteus to Cd decreased. Obviously the stress of the low water activity at an acid pH increases the sensitivity of the microbe to Cd. Finally, when the phosphate concentration is high enough, i.e. 0.2 M at pH 6.0, the growth of M. luteus is inhibited even without added Cd in the substrate (Table 1). M. luteus is relatively resistant to high solute concentrations in the growth medium (*Baird-Parker* 1974) as is also B. subtilis (*Gibson & Gordon* 1974). Differences in the structure and composition of the cell wall, e.g. the absence of teichoic acids in M. luteus (*Baird-Parker*) but their presence in B. subtilis and also in S. bovis (*Heptinstall et al.* 1970, *Lamanna et al.*) might explain the differences observed regarding the sensitivity of the microbes to high phosphate concentrations at varying pH values. It is also well known that there are fundamental differences in the intake of inorganic phosphates between bacteria (*Mitchell* 1953).

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SAMMANFATTNING

Inverkan av pH och kaliumfosfatbuffer på toxisiteten av kadmium för bakterier.

Toxisiteten av kadmium (Cd) för Micrococcus luteus ATCC 9341, Staphylococcus aureus ATCC 12600, Clostridium perfringens ATCC 8798, Escherichia coli ATCC 11775 och Pseudomonas aeruginosa ATCC 10145 ökade vid stigande pH i bakterieräkningsmediet. Inverkan av pH på toxisiteten var mindre utpräglad för Streptococcus bovis ATCC 9809 och utblev för Bacillus subtilis ATCC 8473. När kaliumfosfatbuffer tillsattes i odlingsmediet tilltog toxisiteten av Cd för M. luteus och B. subtilis men minskade för S. bovis. När pH-värdet i mediet steg vid olika fosfatkonsentrationer minskade känsligheten av M. luteus för Cd. Författarna är av den åsikten att effekten av pH på toxisiteten av Cd är för de flesta mikrober förorsakad av den ökade negativa laddningen vid tilltagande alkalisitet, vilket ökar cellväggens affinitet för kationer.

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Reprints may be requested from: the Department of Food Hygiene, College of Veterinary Medicine, Hämeentie, 57, 00550 Helsinki 55, Finland.