Tip hairs occurred on the whiplash flagellum of the zoospores of *P. cinnamomi* Rands, *P. megasperma* var. *sojae* Hildebrand, *P. palmivora* and *P. parasitica* (Figure 1, a–d). The length of the hairs varies from 460 to 500 nm on different species. The width as measured on shadow cast specimens is 11–12 nm. The tip hairs were not found on the wiplash flagellum of every zoospore observed as they are apparently quite fragile and probably break off during specimen preparation. Figure 1d, which shows 1 attached hair and 2 detached hairs, suggests that they are fragile and easily lost. Since the number of tip hairs varied within single species, this may be a further indication of their fragility. For example, many flagella of *P. megasperma* var. *sojae* had 7 or 8 tip hairs (Figure 1c) while others had only 1 or 2 (Figure 1a).

Lateral hairs were consistently found on the whiplash flagella of the zoospores of *P. cinnamomi* and *P. megasperma* var. *sojae*. The length and width of these hairs, as measured on shadow cast preparations, were about the same as those reported for *P. palmivora*[5], i.e. ca. 500 nm long and 15 nm wide. Lateral hairs were also observed on the whiplash flagellum of *P. parasitica* (Figure 1b); the latter confirms the finding of REICHLE[6] in his studies of negatively stained flagella of this species. These findings bring the total number of *Phytophthora* species with lateral hairs on the whiplash flagellum to 5, which suggests that their presence is a common phenomenon rather than an unusual one as previously suggested[8,9].

Although the presence of flagellar hairs (mastigonemes) is sometimes difficult to demonstrate because of their fragility, every effort should be made to carefully prepare specimens for electron microscopy before concluding that they are not present. Certainly all types of flagellar hairs are useful in taxonomic studies[10,11].

MANTON[10], in discussing flagellar structure, has recommended that the term 'acronematic' not be used to describe the terminal structure of flagella since such a term suggests a terminal hair similar to lateral hairs on certain flagella. Perhaps the term could be validly used here to describe the tip hairs on *Phytophthora* whiplash flagella since they are indeed similar to the lateral hairs. Nevertheless for the sake of clarity we have simply designated them as tip or terminal hairs.

It has been stated that the lateral hairs on the tinsel flagellum of *P. infestans* arise from the 'axis' (presumably the fibrils) of the flagellum[3,8]. However, this has not as yet been demonstrated in ultrathin sections of flagella[8]. Nothing is known about the point of origin of the hairs on the whiplash flagellum. With respect to the tip hairs described in this report, one might at first wonder if they are extensions of the central fibrils of the flagellum. However, their width (11–12 nm) is less than the diameter of the whole fibril (30–36 nm) and greater than that of the filaments (4–5 nm) described by GRIMSTONE[12] and RINGO[13] as forming the walls of the fibrils. This difference in diameters would seem to suggest that the tip hairs are not extensions of the flagellar fibrils[14].

Résumé. Des mastigonèmes de 460–500 nm de longueur et 11–12 nm de largeur existent à l'extrémité du flagelle «whiplash» des zoospores des *Phytophthora cinnamomi, P. megasperma* var. *sojae, P. palmivora* et *P. parasitica.* Des mastigonèmes latéraux existent aussi sur le flagelle «whiplash» des 4 espèces. C'est la première mention de mastigonèmes latéraux sur le flagelle «whiplash» de *P. cinnamomi* et de *P. megasperma* var. *sojae.*

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COGITATIONES

Divalent Metal Ion Buffers with Low pH-Sensitivity

Introduction. Reaction rate and stability of numerous enzymes are dependent on the presence of certain, mostly divalent metal ions[1–4]. Moreover, metal ions often play an important role in regulation of enzyme synthesis and activity in vivo[5]. It is, however, generally difficult to achieve, and exactly to reproduce, constant levels of free metal ion concentrations of less than about 10 μM in a test medium. Impurities in the reagents often result in an uncontrolled level of divalent metal ions. Metal ions may be chelated by various components of a medium, such as buffer compounds (e.g. histidine), SH-compounds (e.g. cysteine, glutathione), substrates (e.g. ATP) and particularly proteins[6].

The use of metal ion buffers, especially Ca^{2+}-ion buffers, to circumvent these difficulties was proposed by many authors (e.g. Refs.[7] and [8]). It has been recognized[7–10], however, that the application of metal ion buffers poses some other problems, since most of the chelating agents of the polyaminocarboxylate type show considerable protonation.

Addition of a second divalent metal ion, which competes with protons and the primary metal ion Me_1^{2+}, may lessen this pH-dependence. The application of this type of buffer provides an advantage for all investigations which require a constant level of the primary ion over a wide pH- range, e.g. for kinetic studies on the pH-

dependence of K_m- and V_{max}-values of enzymes, especially of 'metal-enzyme-complexes', provided that the second metal ion will not interfere with the enzymatic action.

Results and discussion. Buffer-systems containing one metal ion. In order to convey a better understanding of properties and function of the buffers containing two metal ions, the properties of the buffers containing only one metal ion should be discussed briefly. (For further information see Refs.[7,8] and [10].)

Low metal ion concentrations can be kept constant by using a buffer which consists of a strong chelator and a metal ion. If the metal ion is the divalent cation Me^{2+}, the following equation is valid:

$$[Me^{2+}] = K_{MeL^{(n-2)-}} \cdot \frac{[MeL^{(n-2)-}]}{[L^{n-}]} \quad (1)$$

where L^{n-} is the nonprotonated uncomplexed ligand. Chelators, such as nitrilotriacetate, ethylene diamine-tetra-acetate, etc., which may be used in buffer systems, are protonated to a varying extent (cf. Ref.[9]); e.g. EDTA can occur as L^{4-}, LH^{3-}, LH_2^{2-}, LH_3^-, and LH_4. Thus, the total concentration of a chelator is defined as

$$\overline{\overline{[L]}}_t = [L^{n-}] + [LH^{(n-1)-}] + \cdots + [LH_{n-1}^-] + [LH_n]. \quad (2)$$

Introducing the proton dissociation constants K_1, K_2, $\cdots$ K_{n-1}, and K_n, eq. (2) can be rewritten as

$$\overline{\overline{[L]}}_t = [L^{n-}] \left(1 + \frac{[H^+]}{K_1} + \frac{[H^+]^2}{K_1K_2} + \cdots + \frac{[H^+]^n}{K_1K_2 \ldots K_n}\right) \quad (3)$$

With the definition

$$P = 1 + \sum_{i=1}^{i=n} \frac{[H^+]^i}{\Pi K_i} \quad (4)$$

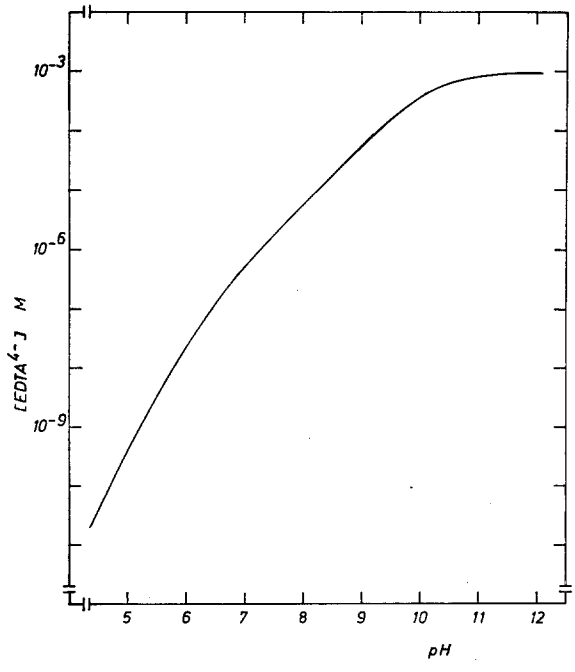

Fig. 1. Calculated pH-dependence of the concentration of nonprotonated $EDTA^{4-}$. $[EDTA]_t = 1$ mM, $\mu = 0.1$. The calculation was performed using eq. (5). (For values of the constants see Table I and Ref.[6].)

the concentration of the unprotonated uncomplexed chelator can be expressed as

$$[L^{4-}] = \frac{\overline{\overline{[L]}}_t}{P}. \quad (5)$$

Figure 1 shows the dependence of the concentration of non-protonated EDTA (L^{4-}) on the pH-value, calculated using equation (5). The values of the dissociation constants K_1–K_4 were taken from Table I (Ref.[6]).

In the presence of a divalent metal ion, the total ligand concentration is given by

$$\overline{[L]}_t \simeq [L^{n-}] \cdot P + [MeL^{(n-2)-}]. \quad (6)$$

This equation is not general but holds for ligands, for which the concentration of the $MeHL^{(n-3)-}$-complexes[10] is negligible in the pH-range of about 5–10, e.g. for EDTA, *t*-CDTA and *o*-PDTA, but not for EGTA, *t*-CPDA and OBDA (Refs. [6] and [10]). Thus, neglecting $[MeHL^{(n-3)-}]$

$$[MeL^{(n-2)-}] \simeq [Me]_t - [Me^{2+}]. \quad (7)$$

Substituting equation (1) (solved for $[L^{n-}]$) and equation (7) in (5), a quadratic equation is derived which can be solved for $[Me^{2+}]$:

$$[Me^{2+}] \simeq -\frac{\overline{[L]}_t - [Me]_t + K_{MeL^{(n-2)-}} \cdot P}{2} + \sqrt{\frac{(\overline{[L]}_t - [Me]_t + K_{MeL^{(n-2)-}} \cdot P)^2}{4} + K_{MeL^{(n-2)-}} \cdot [Me]_t \cdot P} \quad (8)$$

as a function of $\overline{[L]}_t$, $[Me]_t$, $K_{MeL^{(n-2)-}}$, and P.

Figure 2 shows an example for the pH-dependence of the concentration of free Ca^{2+}-ions in an EDTA-buffer. The curves were calculated by use of equation (8) with $n = 4$ and the following concentrations: $[EDTA]_t = 1$ mM, $[Ca]_t = 0.1, 0.2, \ldots, 0.9$ mM, respectively. Figure 2 demonstrates that a distinct Ca^{2+}-concentration can be maintained constant within a certain pH-range only when the composition of the buffer is changed in order to compensate for the effect of ligand protonation. For example, the total Ca-concentration of this buffer system must be varied between 0.1 and 0.9 mM, if the concentration of free Ca^{2+}-ions is to be held constant at 0.01 μM between pH 6.8 and 8.6.

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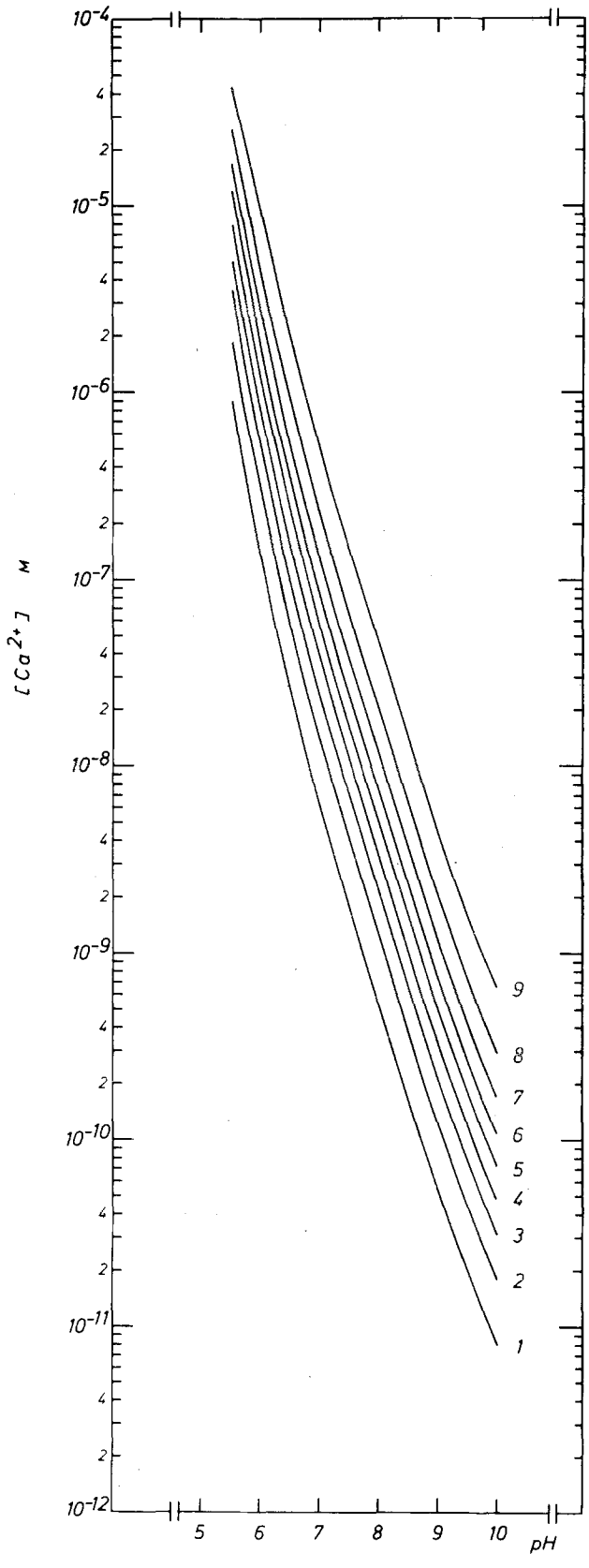

Fig. 2. Behaviour of a 'single-metal-ion buffer system', in which the concentration of complexes of the type MeHL $(^{n-3})-$ can be neglected: $[Ca^{2+}]$ depends markedly on the pH-value for each $[Ca]_t$. $[EDTA]_t$ = 1 mM; $[Ca]_t$: Curve 1: 0.1 mM; 2: 0.2 mM; 3: 0.3 mM; 4: 0.4 mM; 5: 0,5 mM; 6: 0,6 mM; 7: 0.7 mM; 8: 0.8 mM; 9: 0.9 mM. The calculations were performed using eq. (8) and constants of Table I.

Buffer systems containing two metal ions. The pH-dependence of the common metal-ion buffer systems should diminish if the protonation of the ligand is restricted, e.g. if the concentration of free chelator is decreased. This restriction of protonation of the chelating agent is possible if the buffer system consists of a strong chelator and two different metal ions with widely different affinities for the chelating agent, i.e. the metal ion to be buffered ('primary ion') and a second, auxiliary metal ion ('secondary ion').

The metal-buffering efficiency of this type of buffer is maintained under the following conditions:

1. The secondary ion must be bound by the chelator at least 10–100 times weaker than the primary ion[11] in order to achieve sufficiently low concentrations of the primary ion.

2. The total concentration of the primary ion should range between 0.05 and $0.95 \times [L]_t$. Beyond this region, the buffer capacity is decreased too much.

3. The concentration of the secondary metal ion is limited by the condition

$$([Me_1]_t + [Me_2]_t) > [L]_t \,. \tag{9}$$

4. Since there is a strong competition between protons and the divalent metal ions, especially between protons and the secondary metal ion, the free metal ion concentration and the metal buffering efficiency is maintained constant between about pH 6 and 10, if

$$K_{Me_2L^{(n-2)-}} \leq 100\, K_1 \tag{10}$$

and if

$$K_{Me_2L^{(n-2)-}} \leq \sqrt{K_1 K_2} \,. \tag{11}$$

As shown in Table I, both conditions are fulfilled in the case of EDTA, *t*-CPDA, *t*-CDTA and *o*-PDTA with $Me_2^{2+} = Mg^{2+}$, and in the case of all chelators listed in Table I with $Me_2^{2+} = Ca^{2+}$ and heavy metal ions (see Figures 7 and 8).

Besides the well-known complexes of the $MeL^{(n-2)-}$-type, there exist – especially at pH-values < 5 – additional complexes of the $MeHL^{(n-3)-}$-type[17]. The concentration of these complexes has been neglected in the derivation of the eq. (27) on the basis of the following consideration: It can be assumed that the concentration of the free divalent metal ion and the buffer efficiency is practically not changed if less than 1% of the $MeL^{(n-2)-}$-complex is protonated, i.e. if

$$\frac{[MeL^{(n-2)-}]}{[MeHL^{(n-3)-}]} \geq 100 \,. \tag{12}$$

[11] This condition can be verified, e.g., if Ca^{2+} or heavy metal ions as the primary ions are combined with Mg^{2+} as the secondary ion, EDTA being the ligand. This combination is very useful for the study of some enzymes (e.g. Ca^{2+}-dependent ATPase[12–14], alkaline phosphatase[15,16]), which are only active in the presence of Mg^{2+} and a second divalent metal ion (Ca^{2+}, Zn^{2+}, respectively), if the second divalent metal ion is present in enzyme-saturating concentrations or if its free ion concentration can be determined at any moment or under any condition.

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With

$$\frac{[MeL^{(n-2)-}]}{[MeHL^{(n-3)-}]} = \frac{K_{MeHL}{}^{(n-3)-}}{[H^+]} \quad (13)$$

we find that the concentration of the $MeHL^{(n-3)-}$-complexes can be neglected if

$$[H^+] \leq \frac{K_{MeHL}{}^{(n-3)-}}{100} . \quad (14)$$

By means of condition (14), a lower pH-limit for the validity of eq. (27) can be calculated for every chelator. These values are listed in Table III for $Me_1^{2+} = Ca^{2+}$ and $Me_2^{2+} = Mg^{2+}$. Moreover, they have been taken into consideration in the figures.

Neglecting the concentration of the $MeHL^{(n-3)-}$-complexes, the equation for a two-metal-ion buffer system is derived as follows: The total ligand concentration is given by

$$[L]_t \simeq [L^{n-}] \cdot P + [Me_1L^{(n-2)-}] + [Me_2L^{(n-2)-}] \quad (15)$$

while the total concentrations of the divalent metal ions are expressed approximatively by

$$[Me_1]_t \simeq [Me_1^{2+}] + [Me_1\,L^{(n-2)-}] \quad (16)$$

and

$$[Me_2]_t \simeq [Me_2^{2+}] + [Me_2\,L^{(n-2)-}] . \quad (17)$$

With equation (16) and (17), eq. (15) may be rewritten as

$$[L]_t \simeq [L^{n-}] \cdot P + [Me_1]_t - [Me_1^{2+}] + [Me_2]_t - [Me_2^{2+}] . \quad (18)$$

The concentration ratios [free divalent metal ion]/[complex] are expressed as

$$\frac{[Me_1^{2+}]}{[Me_1\,L^{(n-2)-}]} = \frac{K_{Me_1L}{}^{(n-2)-}}{[L^{n-}]} \quad (19)$$

and

$$\frac{[Me_2^{2+}]}{[Me_2\,L^{(n-2)-}]} = \frac{K_{Me_2L}{}^{(n-2)-}}{[L^{n-}]} . \quad (20)$$

In order to eliminate $[L^{n-}]$ (19) and (20) may be combined to form

$$[Me_2^{2+}] = [Me_1^{2+}] \frac{K_{Me_2L}{}^{(n-2)-}}{K_{Me_1L}{}^{(n-2)-}} \frac{[Me_2\,L^{(n-2)-}]}{[Me_1\,L^{(n-2)-}]} \quad (21)$$

which has to be inserted into (18) resulting in

$$[L]_t \simeq [L^{n-}]\,P + [Me_1]_t + [Me_2]_t - \\ - [Me_1^{2+}] \left(1 + \frac{K_{Me_2L}{}^{(n-2)-}}{K_{Me_1L}{}^{(n-2)-}} \frac{[Me_2\,L^{(n-2)-}]}{[Me_1\,L^{(n-2)-}]}\right) . \quad (22)$$

Combining eq. (16) and (19), $[L^{n-}]$ can be expressed as

$$[L^{n-}] = K_{Me_1L}{}^{(n-2)-} \frac{[Me_1\,L^{(n-2)-}]}{[Me_1^{2+}]}$$

$$\simeq K_{Me_1L}{}^{(n-2)-} \frac{[Me_1]_t - [Me_1^{2+}]}{[Me_1^{2+}]} . \quad (23)$$

Table I. Values[6] of the dissociation constants K_1, K_2, etc., K_n, and $K_{MeL}(n\text{-}2)$- of the chelators *t*-CDTA, *t*-CPDA, EDTA and EGTA (T = 20 °C, μ = 0.1)

		t-CDTA	*t*-CPDA	EDTA	EGTA
K_1	*M*	2.0×10^{-12}	5.5×10^{-11}	5.5×10^{-11}	3.47×10^{-10}
K_2	*M*	7.6×10^{-7}	3.63×10^{-8}	6.9×10^{-7}	1.41×10^{-9}
K_3	*M*	3.02×10^{-4}	2.34×10^{-3}	2.14×10^{-3}	2.09×10^{-3}
K_4	*M*	3.72×10^{-3}	1.78×10^{-2}	1.03×10^{-2}	1.0×10^{-2}
$K_{MgL}(n\text{-}2)$-	*M*	4.8×10^{-11}	8.5×10^{-10}	2.04×10^{-9}	4.0×10^{-6}
$K_{CaL}(n\text{-}2)$-	*M*	8.4×10^{-13}	4.8×10^{-12}	2.57×10^{-11}	7.77×10^{-11} [a]
$K_{MnL}(n\text{-}2)$-	*M*	1.7×10^{-17}			
$K_{ZnL}(n\text{-}2)$-	*M*			3.1×10^{-17}	

[a] Ref.[20].

Table II. Values[6] of the dissociation constants K_1, K_2, etc., K_n, and $K_{MeL}(n\text{-}2)$- of the chelators HEDTA, OBDA, and *o*-PDTA (T = 20 °C, μ = 0.1)

		HEDTA	OBDA	*o*-PDTA
K_1	*M*	1.9×10^{-10}	3.4×10^{-10}	2.0×10^{-7}
K_2	*M*	5.62×10^{-6}	1.45×10^{-9}	1.58×10^{-5}
K_3	*M*	9.12×10^{-3}	1.74×10^{-3}	2.0×10^{-4}
K_4	*M*	—	1.78×10^{-2}	1.26×10^{-3}
$K_{MgL}(n\text{-}2)$-	*M*	1.66×10^{-6}	4.9×10^{-9}	8.0×10^{-8}
$K_{CaL}(n\text{-}2)$-	*M*	7.25×10^{-9}	8.9×10^{-11}	1.0×10^{-9}
$K_{CoL}(n\text{-}2)$-	*M*		2.0×10^{-15}	
$K_{ZnL}(n\text{-}2)$-	*M*	3.16×10^{-15}		

Table III. Suitability of chelators for use in two-metal-ion buffers

Chelator	pH-range of validity of eq. (27)	Suitability in the pH-range of validity of eq. (27) $Me_2^{2+} = Mg^{2+}$	$Me_2^{2+} = Ca^{2+}$
o-PDTA	4.5–10	++	++
t-CDTA	5 –10	+	++
EDTA	5.5–10	+	++
HEDTA	6.0–10	–	++
t-CPDA	6.5–10	+	++
OBDA	6.5–10	±	++
EGTA	7.5–10	–	++

Inserting eq. (23) into (22), rewriting and solving the square equation for $[Me^{2+}]$, and using the abbreviations

$$\Delta = [Me_1]_t + [Me_2]_t - [L]_t \tag{24}$$

$$R = \frac{K_{Me_2L}{}^{(n-2)-}}{K_{Me_1L}{}^{(n-2)-}} \frac{[Me_2L^{(n-2)-}]}{[Me_1L^{(n-2)-}]}{}^{18} \tag{25}$$

leads to the final equation, which describes the dependence of the concentration of the free primary metal ion on the pH-value, the total concentrations of the primary and the secondary metal ion and the chelator:

$$[Me_1^{2+}] \simeq \frac{\Delta - K_{Me_1L}{}^{(n-2)-} \cdot P}{2(1+R)} + \sqrt{\left(\frac{\Delta - K_{Me_1L}{}^{(n-2)-} \cdot P}{2(1+R)}\right)^2 + \frac{[Me_1]_t K_{Me_1L}{}^{(n-2)-} \cdot P}{1+R}} \tag{27}$$

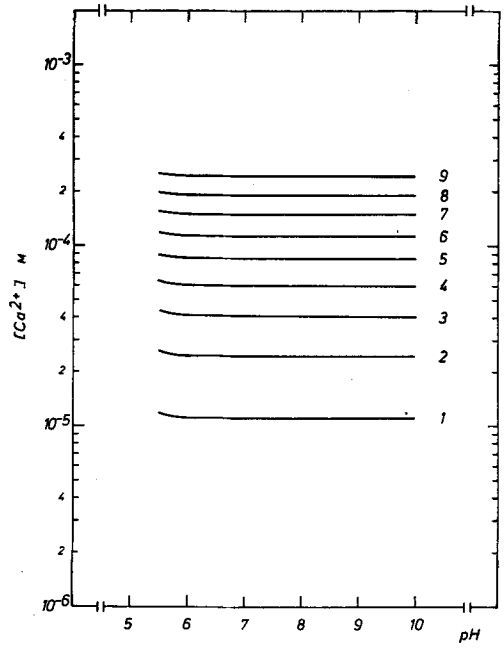

Fig. 3. Behaviour of a 'two-metal-ion buffer system' with Ca^{2+} as primary, Mg^{2+} as secondary ion and L as chelator: $[Ca^{2+}]$ depends only slightly on the pH-value within the range of pH 5.5–6.0. $[EDTA]_t$ = 1 mM; $[Ca]_t$: Curve 1: 0.1 mM; 2: 0.2 mM; 3: 0.3 mM; 4: 0.4 mM; 5: 0.5 mM; 6: 0.6 mM; 7: 0.7 mM; 8: 0.8 mM; and 9: 0.9 mM. $[Mg]_t$ = 10 mM. For calculations eq. (27) and constants of Table I were used.

Eq. (27) shows that the concentration of any divalent cation can be changed in three different ways:

1. The concentration of the free primary ion can be varied by changing the total concentration of the primary ion. Figures 3, 4 and 5 give three examples for this $[Me_1^{2+}]$-variation with Ca^{2+} as primary, Mg^{2+} as secondary ion and EDTA as chelator, and Zn^{2+} as primary, Ca^{2+} as secondary ion and HEDTA as chelator.

2. The second possibility of achieving a change in the concentration of the free primary ion consists in the variation of the total concentration of the secondary ion. Figure 6 shows the dependence of the free Ca^{2+}-concentration on the total Mg^{2+}-concentration within the pH-range

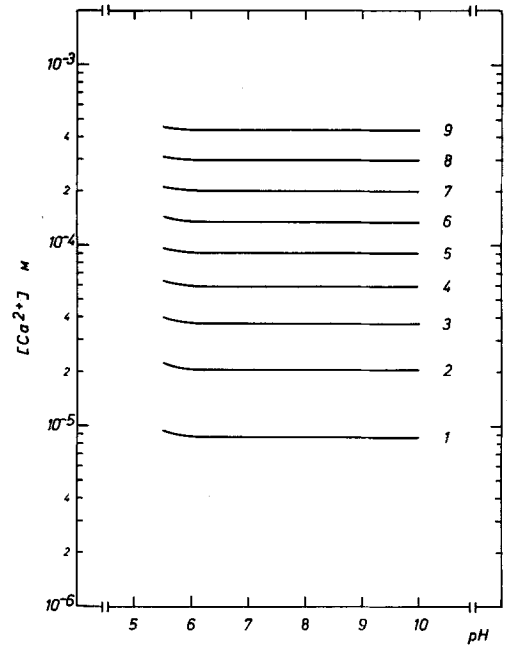

Fig. 4. Dependence of $[Ca^{2+}]$ on the pH-value and $[Ca]_t$ with $[EDTA]_t$ = 4 mM; $[Ca]_t$: Curve 1: 0.4 mM; 2: 0.8 mM; 3: 1.2 mM; 4: 1.6 mM; 5: 2.0 mM; 6: 2.4 mM; 7: 2.8 mM; 8: 3.2 mM; 9: 3.6 mM. $[Mg]_t$ = 10 mM. For calculations eq. (27) and constants of Table I were used.

[18] For practical use, the ratio $[Me_2L^{(n-2)-}]/[Me_1L^{(n-2)-}]$ is expressed in terms of $[L]_t$ and $[Me_1]_t$ by means of eq. (15), (16) and (19) as

$$\frac{[Me_2L^{(n-2)-}]}{[Me_1L^{(n-2)-}]} \simeq \frac{[L]_t}{[Me_1]_t - [Me_1^{2+}]} - \frac{K_{Me_1L}{}^{(n-2)-}}{[Me_1^{2+}]} \cdot P - 1. \tag{26}$$

$[Me^{2+}]$ is than calculated by a simple iteration procedure.

of 5.5–10 with L = EDTA. It is quite remarkable that the increase in the total Mg^{2+}-concentration not only causes an increase in the Ca^{2+}-concentration but also an extension of the pH-range, within which the Ca^{2+}-concentration is practically constant.

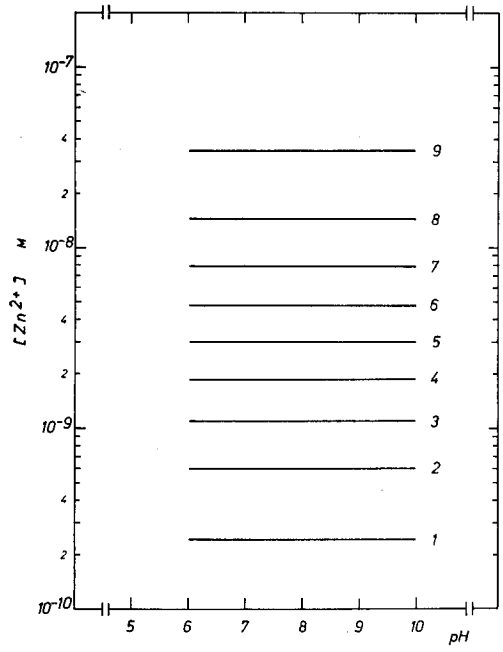

Fig. 5. Dependence of $[Zn^{2+}]$ on the pH-value and on $[Zn]_t$ in a buffer system with Zn^{2+} as primary, Ca^{2+} as secondary metal ion and HEDTA as chelator. $[HEDTA]_t$ = 1 mM, $[Ca]_t$ = 2 mM, $[Zn]_t$: Curve 1: 0.1 mM; 2: 0.2 mM; 3: 0.3 mM; 4: 0.4 mM; 5: 0.5 mM; 6: 0.6 mM; 7: 0.7 mM; 8: 0.8 mM; 9: 0.9 mM. For calculations eq. (27) and constants of Table II were used.

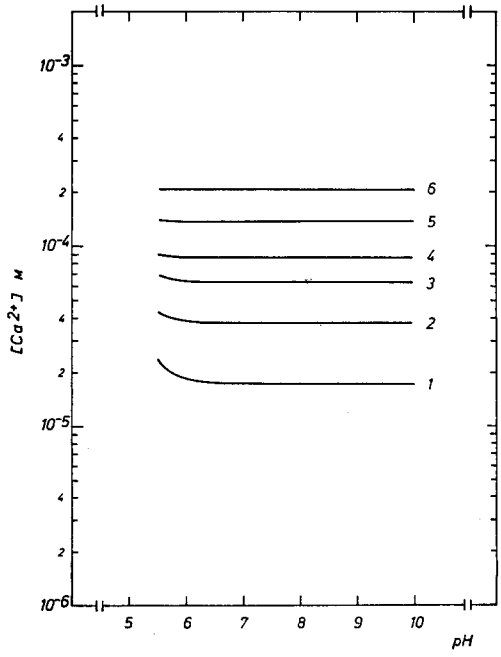

Fig. 6. Dependence of $[Ca^{2+}]$ on the pH-value and on $[Mg]_t$ as secondary metal ion. $[EDTA]_t$ = 1 mM, $[Ca]_t$ = 0.5 mM, $[Mg]_t$: Curve 1: 2 mM; 2: 4 mM; 3: 7 mM; 4: 10 mM; 5: 20 mM; and 6: 40 mM. For calculations eq. (27) and constants of Table I were used.

3. The third way of $[Me_1^{2+}]$-variation results from the change of the ratio $K_{Me_2L}{}^{(n-2)-}/K_{Me_1L}{}^{(n-2)-}$, i.e. the choice of an appropriate chelator. Figure 7 shows the results of calculations of a series of different chelators with $[Mg]_t$ = 2.0 mM and $[Ca]_t$ = 0.5 mM. The values of the constants $K_{MgL}{}^{(n-2)-}$, $K_{CaL}{}^{(n-2)-}$, K_1, K_2, etc., K_n are given in the Tables I and II (Ref.[6]). The curves differ in two respects: a) The concentration of free Ca^{2+}-ions varies with the affinity-ratio. b) The pH-range, within which $[Ca^{2+}]$ remains constant, varies with the type of ligand.

The reasons for the second observation are the different metal ion and proton dissociation constants of each ligand. Thus, both constants are important for the suitability of a chelator in a buffer system. This becomes obvious when chelators of nearly identical values of the ratio $K_{MgL}{}^{(n-2)-}/K_{CaL}{}^{(n-2)-}$ are compared, eg. the chelators EDTA and *o*-PDTA or *t*-CDTA and OBDA. They differ markedly with respect to their proton dissociation constants (Tables I and II). This causes a ligand-specific pH-dependence of the Ca^{2+}-level.

The Ca^{2+}-level is independent of the pH-value within ΔpH = 3.5–5.0 in the case of those ligands which meet the conditions given by eq. (10) and (11) (*o*-PDTA, EDTA, *t*-CPDA and *t*-CDTA), and only within ΔpH = 1.5 in the case of those ligands which do not meet these conditions (HEDTA, EGTA). OBDA holds an intermediate position with ΔpH = 3.0: condition (10) is met, but not (11).

The two-metal-ion buffers are able to buffer the Me_1^{2+}-concentration over a wide range; e.g., it is possible to buffer the Ca^{2+}-concentration between 0.007 and 200 μM, when EDTA and EGTA are used as chelators. This range can be extended in the cases of Co^{2+}- and Zn^{2+}-ions, which are known to affect stability and reaction rate of some enzymes, e.g. alkaline phosphatase[15]. The concentration

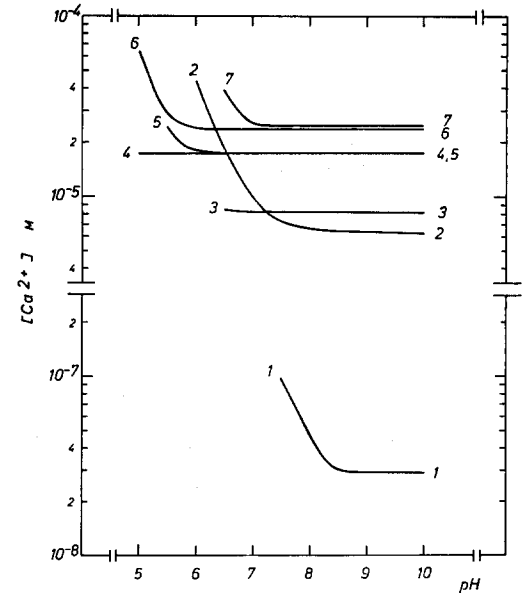

Fig. 7. Comparison of $[Ca^{2+}]$ in buffer systems with different chelators. The chelators differ with respect to their ratio $K_{MgL}{}^{(n-2)-}/K_{CaL}{}^{(n-2)-}$, which causes variable $[Ca^{2+}]$-values. In addition, they differ with respect to their proton dissociation constants, which causes a differing pH-dependence of the Ca^{2+}-level. $[L]_t$ = 1 mM, $[Ca]_t$ = 0.5 mM, $[Mg]_t$ = 2 mM. Curve 1: L = EGTA, $K_{MgL}{}^{(n-2)-}/K_{CaL}{}^{(n-2)-} = 5.15 \times 10^4$; 2: HEDTA, 229; 3: *t*-CPDA, 177; 4: *o*-PDTA, 80; 5: EDTA, 79,5; 6: *t*-CDTA, 57; 7: OBDA, 55. For calculation eq. (27) and constants of Tables I and II were used.

of Co^{2+}-ions can be buffered in the range of 10^{-13}–$10^{-8}M$ by application of OBDA, EDTA, and *t*-CDTA, Zn^{2+}-ions in the same range by these ligands and EGTA. These chelators have been shown to be quite suitable for the investigation of stability and reaction rate dependence of alkaline phosphatase on Zn^{2+}-concentrations[15,16,19].

Finally, not only the primary metal ion concentration, but also the buffer capacity $d[Me_1]_t/d(-\log[Me^{2+}])$ of the two-metal-ion buffers is practically independent of the pH-value. In order to prove this independence, calculations were performed for a variety of buffer systems consisting of different primary and secondary ions and chelators of various concentrations. The results shown in Figures 8, 9 and 10 indicate that.

1. The buffer capacity is slightly dependent on the pH-value in some cases, i.e. in buffer systems with $Me_2^{2+} = Mg^{2+}$, especially in the presence of low Mg^{2+}-concentrations (curve 1 in Figure 8), or with L = HEDTA, which does not meet the conditions (10) and (11) (curve 12 in Figure 9).

2. The buffer capacity is constant within the validity-range of eq. (27) in systems with $Me_2^{2+} = Ca^{2+}$, irrespective of the nature of Me_1^{2+} and L, and the concentrations of Me_1^{2+}, Me_2^{2+} and L.

As outlined before, in a single-metal-ion buffer the total concentration of the metal ion has to be changed in order to maintain a constant level of the free metal ion concentration within a certain pH-range (e.g. 0.01 μM at pH 6.8–8.6; see Figure 2). As shown in Figure 11, this change in the composition results in a marked pH-dependence of the buffer capacity, compared with a two-metal-ion buffer system.

In practice, the design of any buffer system with a constant concentration of the free primary metal ion can be accomplished even in a pH-range, in which protonation of the chelator occurs (e.g. pH 5.5–6.5 in the case of EDTA with $Me_1^{2+} = Ca^{2+}$ and $Me_2^{2+} = Mg^{2+}$; see Figure 7). Since often both concentrations $[Me_1^{2+}]$ and $[Me_2^{2+}]$ have to be maintained constant, in the following equations $[Me_1]_t$ and $[Me_2]_t$ are expressed as functions of $[L]_t$, $[Me_1^{2+}]$, $[Me_2^{2+}]$, $[H^+]$, $K_{Me_1L}{}^{(n-2)-}$, $K_{Me_2L}{}^{(n-2)-}$, K_1, K_2,

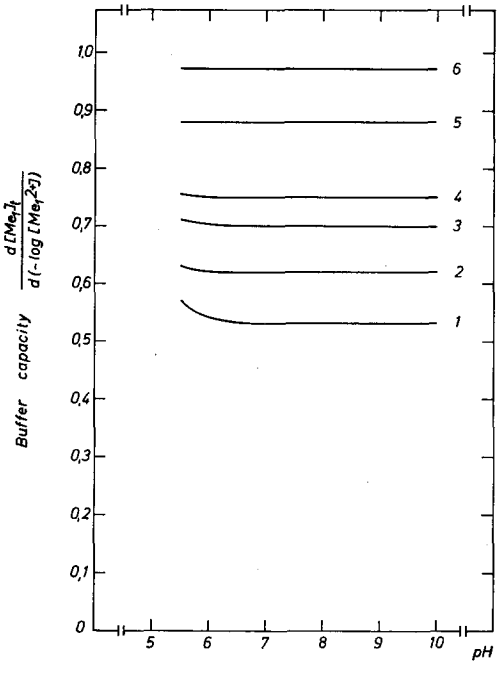

Fig. 8. Dependence of the capacity of various two-metal ion buffer systems on the pH-value. The buffers are composed as follows:

Curve No.	L	$[L]_t$ (mM)	Me_1^{2+}	$[Me_1^{2+}]_t$ [mM]	Me_2^{2+}	$[Me_2^{2+}]_t$ (mM)
1	EDTA	1	Ca^{2+}	0.5	Mg^{2+}	2
2	EDTA	1	Ca^{2+}	0.5	Mg^{2+}	4
3	EDTA	1	Ca^{2+}	0.5	Mg^{2+}	7
4	EDTA	1	Ca^{2+}	0.5	Mg^{2+}	10
5	EDTA	1	Ca^{2+}	0.5	Mg^{2+}	20
6	EDTA	1	Ca^{2+}	0.5	Mg^{2+}	40

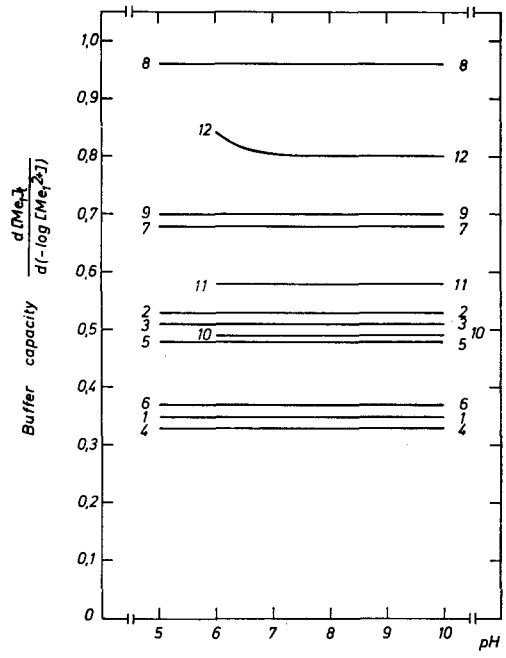

Fig. 9. Dependence of the capacity of various two-metal-ion buffer systems on the pH-value. The buffers are composed as follows:

Curve No.	L	$[L]_t$ (mM)	Me_1^{2+}	$[Me_1^{2+}]_t$ (mM)	Me_2^{2+}	$[Me_2^{2+}]_t$ (mM)
1	*o*-PDTA	1	Ca^{2+}	0.2	Mg^{2+}	1
2	*o*-PDTA	1	Ca^{2+}	0.5	Mg^{2+}	1
3	*o*-PDTA	1	Ca^{2+}	0.8	Mg^{2+}	1
4	*t*-CDTA	1	Mn^{2+}	0.2	Ca^{2+}	2
5	*t*-CDTA	1	Mn^{2+}	0.5	Ca^{2+}	2
6	*t*-CDTA	1	Mn^{2+}	0.8	Ca^{2+}	2
7	OBDA	2	Co^{2+}	0.2	Ca^{2+}	4
8	OBDA	2	Co^{2+}	0.5	Ca^{2+}	4
9	OBDA	2	Co^{2+}	0.8	Ca^{2+}	4
10	HEDTA	1	Zn^{2+}	0.5	Ca^{2+}	2
10	EDTA	1	Zn^{2+}	0.5	Ca^{2+}	2
11	HEDTA	1	Zn^{2+}	0.5	Ca^{2+}	10
11	EDTA	1	Zn^{2+}	0.5	Ca^{2+}	10
12	HEDTA	1	Ca^{2+}	0.5	Mg^{2+}	40

[19] R. Cohen and J. B. Wilson, Biochemistry *5*, 904 (1966).
[20] Y. Ogawa, J. Biochem., Tokyo *64*, 255 (1968).

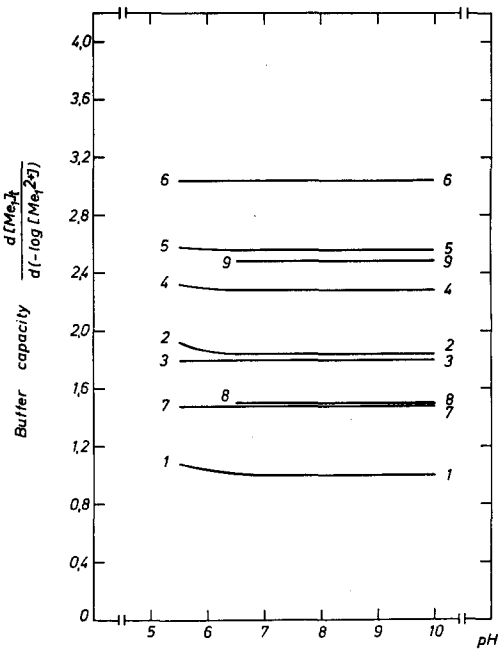

Fig. 10. Dependence of the capacity of various two-metal-ion buffer systems on the pH-value. The buffers are composed as follows:

Curve No.	L	$[L]_t$ (mM)	Me_1^{2+}	$[Me_1]_t$ (mM)	Me_2^{2+}	$[Me_2]_t$ (mM)
1	EDTA	4	Ca^{2+}	0.8	Mg^{2+}	5
2	EDTA	4	Ca^{2+}	2.0	Mg^{2+}	5
3	EDTA	4	Ca^{2+}	3.2	Mg^{2+}	5
4	EDTA	4	Ca^{2+}	2.0	Mg^{2+}	10
5	EDTA	4	Ca^{2+}	2.0	Mg^{2+}	20
6	EDTA	4	Ca^{2+}	2.0	Mg^{2+}	40
7	EDTA	4	Ca^{2+}	0.8	Mg^{2+}	40
8	*t*-CPDA	4	Ca^{2+}	0.8	Mg^{2+}	20
9	*t*-CPDA	4	Ca^{2+}	2.0	Mg^{2+}	20

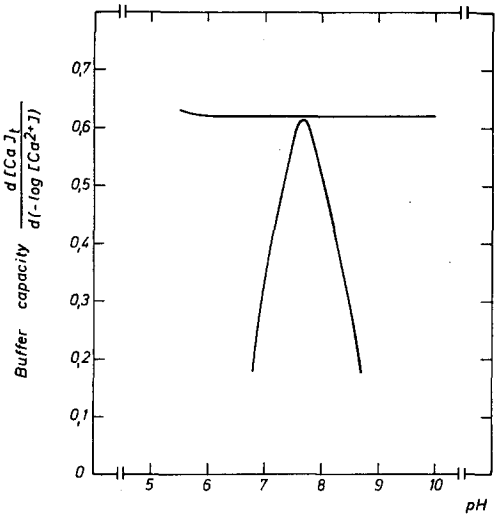

Fig. 11. Comparison of different buffer systems with respect to the pH-dependence of their buffer capacities. The lower curve represents the pH-dependence of a single-metal-ion buffer consisting of $[EDTA]_t$ = 1 mM and $[Ca]_t$ = 0.1–0.9 mM. This buffer yields a constant Ca^{2+}-level of 0.01 μM within the pH-range of 6.8–8.6 (the values were calculated from Figure 2). The upper curve represents a two-metal-ion buffer system with $[EDTA]_t$ = 1 mM, $[Ca]_t$ = 0.5 mM, and $[Mg]_t$ = 4 mM (curve 2 of Figure 8).

K_3, ..., and K_n. These equations are derived as follows:

Inserting (19), solved for $[L^{n-}]$, and (21), solved for $[Me_2L^{(n-2)-}]$, into (15) yields

$$[L]_t \simeq K_{Me_1L}{}^{(n-2)-} \frac{[Me_1L^{(n-)2-}]}{[Me_1^{2+}]} P + [Me_1L^{(n-2)-}] \left(1 + \frac{[Me_2^{2+}]}{[Me_1^{2+}]} \frac{K_{Me_1L}{}^{(n-2)-}}{K_{Me_2L}{}^{(n-2)-}}\right). \quad (28)$$

Solving for $[Me_1L^{(n-2)-}]$ and substituting into (16) leads to

$$[Me_1]_t \simeq \frac{[L]_t}{1 + \frac{K_{Me_1L}{}^{(n-2)-}}{[Me_1^{2+}]} \left(P + \frac{[Me_2^{2+}]}{K_{Me_2L}{}^{(n-2)-}}\right)} + [Me_1^{2+}] \quad (29)$$

The total concentration of the secondary metal ion can be calculated using the equation

$$[Me_2]_t \simeq \frac{[L]_t}{1 + \frac{K_{Me_2L}{}^{(n-2)-}}{[Me_2^{2+}]} \left(P + \frac{[Me_1^{2+}]}{K_{Me_1L}{}^{(n-2)-}}\right)} + [Me_2^{2+}] \quad (30)$$

which is obtained by a derivation similar to that of eq. (29).

In Figure 12, eq. (29) was used to calculate the total Zn^{2+}-concentration in a Zn^{2+}, Mg^{2+}-EDTA-buffer system as a function of the pH-value within the range of pH 5.5–10. It is noteworthy that the total Zn^{2+}-concentration necessary to maintain a constant concentration of free Zn^{2+}-ions is decreased between pH 5.5 and 6.5, where a significant protonation of EDTA occurs.

A limitation of the applicability of the metal ion buffers is given by the metal ion hydroxide formation at high pH-values. In the example discussed here, the solu-

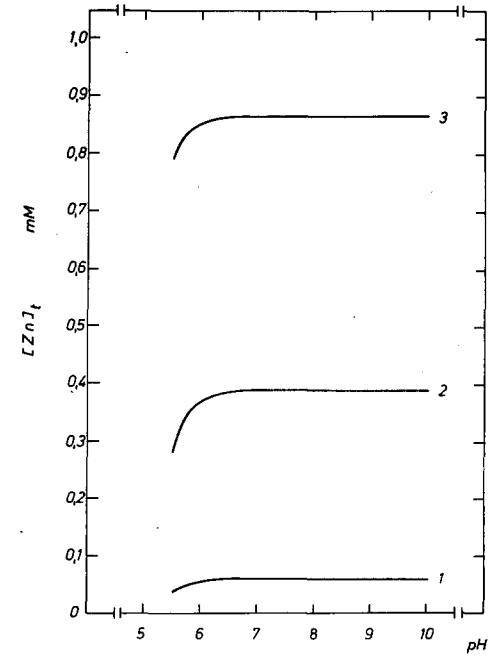

Fig. 12. pH-dependence of $[Zn]_t$ necessary to maintain a constant concentration of free Zn^{2+}-ions. $[EDTA]_t$ = 1 mM; $[Zn^{2+}]$: Curve 1: $10^{-12}M$; 2: $10^{-11}M$; 3: $10^{-10}M$. The caluclation was performed using eq. (29). For values of constants see Table I. The $[Mg]_t$-value, which has to be calculated by means of eq. (30), is not shown in this Figure.

bility product of the respective $Me(OH)_2$-compounds is not exceeded under the conditions chosen here. However, in some cases (e.g. at $[Mg]_t > 10$ mM and pH > 10 (Refs. [6] and [10]) a correction of the $[Me]_t$-value with respect to the formation of $Me(OH)^+$ and $Me(OH)_2$ may become necessary.

In order to give hints for the practical use of two-metal-ion buffers, the pH-range of the validity of eq. (27) and the suitability for use in a two-metal-ion buffer are listed in Table III for each chelator discussed in this paper. The item on the suitability is based on the pH-independence of the Me_1^{2+}-level and the buffer capacity within the pH-range covered by eq. (27).

Zusammenfassung. Es werden Puffersysteme für divalente Metallionen beschrieben, die im Bereich von etwa pH 6–10 gegenüber den herkömmlichen Metallpuffern eine bemerkenswert niedrige pH-Abhängigkeit der Metallionenkonzentration und der Pufferkapazität zeigen.

Die Puffersysteme bestehen aus einem starken Komplexbildner (vorzugsweise einer Polyaminocarbonsäure) und zwei verschiedenen, divalenten Metallionen, die bezüglich der Bindung durch den Komplexbildner in Konkurrenz stehen. Dasjenige Metallion, dessen Konzentration gepuffert werden soll, wird als Primärion bezeichnet. Das zweite Ion, das Sekundärion genannt wird, soll vom Komplexbildner mindestens um den Faktor 10–100 schwächer gebunden werden.

Die Gesamtkonzentration des Primärions ist geringer als diejenige des Komplexbildners, während die Summe der Gesamtkonzentrationen der beiden divalenten Metallionen höher ist als diejenige des Komplexbildners.

Die Konzentration des freien Primärions kann in den beschriebenen Puffersystemen auf drei verschiedene Arten variiert werden: 1. durch Änderung der Gesamtkonzentration des Primärions, 2. durch Änderung der Gesamtkonzentration des Sekundärions, und 3. durch die Wahl eines passenden Komplexbildners mit entsprechenden Metallionen- und Protonendissoziationskonstanten.

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[21] Acknowledgments. The author wishes to thank Miss U. Stechert for the design of the graphs. This work was supported by a grant of the Deutsche Forschungsgemeinschaft.

Abbreviations and symbols

t-CDTA, *trans*-Cyclohexane-1,2-diamine-tetraacetate.

t-CPDA, *trans*-1,2-Cyclopentylenedi-[iminodi(acetate)].

EDTA, Ethylenediamine-N, N, N', N'-tetraacetate.

EGTA, 2,2'-Ethylenedioxybis[ethyliminodi(acetate)].

HEDTA, N'-(2-Hydroxyethyl)-ethylenediamine-N, N, N'-triacetate.

OBDA, 2,2'-Oxybis[ethyliminodi(acetate)].

o-PDTA, *o*-Phenylenediamine-N, N, N', N'-tetraacetate.

Me^{2+}, divalent metal ion.

Me_1^{2+}, 'primary' metal ion.

Me_2^{2+}, 'secondary' metal ion.

$[Me^{2+}]$, concentration of the free metal ion (not bound by a chelator).

L, chelator.

n, number of dissociable protons of L.

K, dissociation constant.

$K_{MeL}{}^{(n-2)-}$, dissociation constant of the complex $MeL^{(n-2)-}$.

$K_1, K_2 .. K_n$ } proton dissociation constants (definitions see text).

$K_{MeHL}{}^{(n-3)-}$, proton dissociation constant of the complex $MeHL^{(n-3)-}$.

i, total index.

Definitions.

$$\overline{\overline{[L]}}_t = [L^{n-}] + [LH^{(n-1)-}] + \cdots + [LH_{n-1}^-] + [LH_n] .$$

$$\overline{[L]}_t = \overline{\overline{[L]}}_t + [MeL^{(n-2)-}] .$$

$$[L]_t = \overline{\overline{[L]}}_t + [Me_1L^{(n-2)-}] + [Me_2L^{(n-2)-}] .$$

$$P = 1 + \sum_{i=1}^{i=n} \frac{[H^+]^i}{\Pi K_i} .$$

$$R = \frac{K_{Me_2L}{}^{(n-2)-}}{K_{Me_1L}{}^{(n-2)-}} \frac{[Me_2L^{(n-2)-}]}{[Me_1L^{(n-2)-}]} .$$

$$\Delta = [Me_1]_t + [Me_2]_t - [L]_t .$$

Lipid Mobility and Function in Biological Membranes

Recent advances in bioenergetics[1–7] and immunology[8] show that the fluidity of membrane lipids is of prime physiological significance. The recent 'fluid mosaic' model of cell membranes envisages membrane proteins as floating like icebergs in fluid lipid[9] and lipid-protein interactions can increase substantially the collapse pressure of such film components. For example, rhodopsin can rotate freely in the retina membrane which has a viscosity of about 2 poise[10]. It seems that the above properties are linked with conformational changes in the membrane[4,5].

Any membrane reaction between protein 'icebergs' and an external reactant which expands the protein would tend to compress the lipid film since the protein would function like the moveable barrier in a surface balance. Energy would thereby tend to transfer from the reaction to the compressed lipid and from there to another process. Alternatively, lipid compression could be relieved by expansion of the membrane. Hence the compression would normally be transient. Lipid therefore provides a mechanism for the storage and transmission of energy. The mechanism proposed is more generally applicable than my earlier concept of 'lipid rubbers'[4] but the two concepts are not mutually exclusive.

If there were much cholesterol, or ceramides, in a lipid film, as in some nerve and plasma membranes, the film would inherently be substantially compressed[11] and its viscosity is high (5–20 poise[10]). A small expansion of a protein 'iceberg', or penetration of the film by a lipo-