^{3}H-polyrC. All polymers were from Miles. PolyrI was dissolved in 0.02 *M Tris* buffer (pH 7.8) containing 0.1 *M* NaCl and extracted twice with buffer saturated phenol. The aqueous phase was then dialyzed in the same buffer to eliminate minimal traces of phenol. ^{3}H-polyrC was diluted with 0.02 *M Tris* buffer and dialyzed to eliminate ethanol from the commercial preparation. While in initial experiments phenol extraction was performed, this was found unnecessary in further trials. The formation of the bihelical homopolymer duplex ^{3}H-polyrC:polyrI was evaluated by the hypochromic effect at 233 nm. The temperature of the product was found between 60 °C and 70 °C in 0.1 *M* NaCl. The duplex was also tested for stability to digestion by RNase B (Worthington) for 30 min at 37 °C. For concentrations of the enzyme up to 1 µg/ml, no effect was observed on the amount of acid precipitable counts of the duplex in most preparations of ^{3}H-polyrC:polyrI, whereas the single stranded ^{3}H-polyrC was made completely acid soluble. The reaction mixture contained an amount of ^{3}H-polyrC:polyrI equivalent to about 0.5 nmoles of P and variable amounts of nuclear extracts in a final volume of 1 ml of buffer (1 *M* or 0.14 *M* NaCl, 0.01 *M* MgAcet. and 0.02 *M Tris* · HCl). The reaction was stopped after 30 min by addition of 2 ml of 10% cold TCA. 50 µg of albumin were added as carrier, and the precipitate was filtered through Millipore filters (HAWP25). The filters were dried and counted in a Packard Tricarb scintillation spectrometer. Assays were always performed in duplicate, and average values are presented.

Results. All the nuclear extracts assayed in our experiments displayed a significant digesting activity on the double-stranded substrate. Results obtained with reaction mixtures in 0.14 *M* NaCl buffer are shown in the Table. The proportion of substrate radioactivity digested in buffer of this molarity was always greater than that observed in 1 *M* NaCl buffer, even though control experiments showed that a significant amount of activity was still bound to the precipitate formed after dialysis. The rate of hydrolysis of the labelled duplex was irregular and proportionality to the amount of nuclear extract added to the reaction mixture could not be demonstrated. All the results shown in the Table were obtained with 200 µl of extract, corresponding to about 3×10^6 nuclei. The activity in this amount of nuclei from unstimulated lymphocytes of donor No. 2 brought to hydrolysis of 0.08 nmoles of double-stranded substrate, whereas the activity in the same number of nuclei, after 24 h of PHA stimulation, brought to hydrolysis of 0.17 nmoles of substrate. Single-stranded ribonuclease activity was present in each of the samples where double-stranded ribonuclease activity was detected. However, in all donors examined, double-stranded ribonuclease activity in nuclei of PHA-stimulated lymphocytes was about twice that before stimulation. On the contrary, only a small average increase in single stranded ribonuclease activity was observed in lymphocyte nuclei after PHA stimulation. Furthermore, hydrolysis of ^{3}H-polyrC was markedly stimulated by EDTA, which had, on the contrary, a slight inhibitory action on double-stranded ribonuclease activity. These results suggest that single-stranded and double-stranded ribonuclease activities are associated with different nuclear proteins. Experiments are now in progress in the attempt to separate these proteins.

Dissociation-Constants of Metal-Ion-Complexes with Alkaline Phosphatase from Pig Kidney

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Summary. Using metal-ion buffers it was possible to remove Zn^{2+}, Mg^{2+} and Mn^{2+} ions of pig kidney alkaline phosphatase reversibly. The dissociation constants obtained are K_{EMg}: $4 \cdot 10^{-7}$ *M*, K_{EMn}: $4 \cdot 10^{-8}$ *M* and K_{EZn}: $8 \cdot 10^{-13}$ *M* (22 °C, pH: 9.6, µ: 0.07).

Alkaline phosphatase (EC 3.1.3.1) is a metal-containing enzyme. It requires Zn^{2+} ions both for preservation of its structure and for its enzymic activity (e.g. ref.[3]). Zinc may be replaced by cobalt, copper, cadmium, nickel or manganese[4-7]. In addition to these metal ions, the alkaline phosphatase from mammalian tissue needs Mg^{2+} ions for activity[8,9]. Other divalent cations, especially Mn^{2+}, Co^{2+}, Ni^{2+}[9] and Ca^{2+} have been reported to be capable of replacing Mg^{2+} as activator. Most of these experiments have been performed in the absence of complexing agents. Therefore, due to contamination of the reagents and of the enzyme preparation, there are various metal ions in the assay. Furthermore, the concentration of metal ions were varied in only a few cases.

We determined the dissociation constants of several metal ion complexes with alkaline phosphatase in the presence of a suitable complexing agent under conditions which guarantee that the removal of metal ions is reversible and that an equilibrium is achieved. Furthermore, we solved the problems arising from a system consisting of more than one sort of metal ion, complexing agent and protein by means of a FORTRAN IV program or an approximate formula.

Experimental. All chemicals were obtained from E. Merck, Darmstadt, Germany. Pig kidneys were used as source for the alkaline phosphatase.

Methods. The preparation of pig kidney alkaline phosphatase and the determination of enzyme activity was described recently[8,10]. The test medium for kinetic mea-

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surements contained 50 m*M* sodium carbonate buffer, pH 9.6, 10 m*M* β-glycerophosphate and different amounts of enzyme, $MeCl_2$, $ZnCl_2$ and NTA as reported in 'Results'. The temperature was 30 °C. The enzyme was preincubated under conditions given in 'Results' until equilibrium was reached. All results are averages of 5 experiments.

The calculations of free metal ions, free complexing agent etc. are performed as reported in the 'Appendix'. The dissociation constants of the metal-ion-ligand complexes are from SILLÈN and MARTELL[11].

Symbols and abbreviations: NTA, nitrilotriacetate; L, ligand; Me, metal ion; K_{MeL}, dissociation constant of the metal-ligand complex; $[Me]_t$, total concentration of the metal ion. All other symbols and abbreviations have been defined recently[8,10].

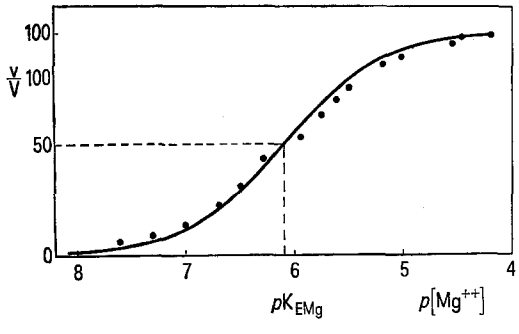
Results and discussion. The dissociation constant of the E-Mg complex. We used NTA as complexing agent, because with it the concentration of free Zn^{2+}-ions could be adjusted, preventing dissociation of the Zn^{2+}-E-complex. Furthermore, the concentration of free Mg^{2+} ions can be adjusted to concentrations where the Zn^{2+}-E-Mg^{2+} complex dissociated nearly completely into Zn^{2+}-E and Mg^{2+}.

To control the reversibility of the dissociation of this complex under the test conditions, we used the following method: The alkaline phosphatase was incubated for 24 h with 2 m*M* NTA and 2 m*M* ZnNTA to remove nearly all Mg^{2+} ions from the enzyme. Then the enzyme was incubated for 1 h with definite concentrations of free Mg^{2+} ions and afterwards the activity was determined by adding 10 m*M* β-glycerophosphate. The reversibility of the reaction was controlled simultaneously by comparing the activity with a reference assay, incubated for the same time without complexing agent.

Dissociation constants K_{MeE} of metal-ion enzyme complexes.

Complex	Temperature (°C)	K_{MeE} (*M*)
EMg	30	$8 \cdot 10^{-7}$
EMg	22	$4 \cdot 10^{-7}$
EMn	22	$4 \cdot 10^{-8}$
EZn	22	$8 \cdot 10^{-13}$

The results were taken from dissociation curves, e.g. Figure. Calculations were performed as described in the 'Appendix', pH: 9.6, μ = 0.07.



Plot of the relative activity versus the negative logarithm of the concentration of free Mg^{2+} ions. T = 30 °C, pH: 9.6 (50 m*M* carbonate-buffer). To obtain the various concentrations of free Mg^{2+} ions a NTA buffer was used. O, Measured values; solid line, theoretical dissociation curve.

In the Figure the relative activity is plotted versus $-\log[Mg^{2+}]$. We obtained typical dissociation curves, indicating that the removal of Mg^{2+} ions was reversible. The values fit best with a theroetical dissociation curve of a Me_1L_1 complex with a dissociation constant given in the Table. This value is in good agreement with K_{Mg-E} = 0.98 μM obtained from plots of 1/v versus $1/[Mg^{2+}]$ for different substrate concentrations[8,10]. To estimate the influence of temperature on the dissociation constant, the experiments reported above were repeated at 22 °C. The *pK* value obtained is shown in the Table. From this it can be seen that the temperature has a significant influence on the dissociation constant and that Mg^{2+} ions are bound better at a lower temperature.

Influence of Mn^{2+}, Ca^{2+}, Fe^{2+}, Zn^{2+} and Cu^{2+}. Using Mn^{2+} instead of Mg^{2+} we also obtained a typical dissociation curve with a dissociation constant shown in the Table. These results confirm the assumption that Mg^{2+}-ions can be replaced by Mn^{2+} ions, resulting in an active enzyme. However, the maximum activity is only 74% of the activity obtained with Mg^{2+} under the same conditions, although Mn^{2+} ions are bound much more strongly. With Ca^{2+} ions we did not get any dissociation curve.

When the alkaline phosphatase is preincubated with 10 μM Fe^{2+}, Zn^{2+}, Mn^{2+} or Ca^{2+} and afterwards different concentrations of Mg^{2+} are added in the absence of NTA, we obtained curves similar to those shown in the Figure but with much higher apparent pK_{Mg}-values, indicating that these ions compete with Mg^{2+} for the active site and must be more strongly bound than Mg^{2+}. 10 μM Cu^{2+} leads to irreversible inactivation (not shown). These results show that estimating the dissociation constant for the E-Mg complex in the absence of complexing agents necessarily leads to higher values.

Determination of K_{Zn-E}. To determine the dissociation constant of the E–Zn^{2+} complex, one has to ensure that a reversible dissociation occurs and that the Mg^{2+} concentration keeps constant while varying $[Zn^{2+}]$ (see Appendix). Furthermore, to achieve complete reactivation, the concentration of Zn^{2+} has to be so low that no inhibition by reaction with the Mg^{2+} binding site occurs.

Reversibility of the reaction was achieved by the following conditions[12]. The alkaline phosphatase was preincubated for 24 h at pH 9.6 with 2 m*M* EDTA and different amounts of $ZnCl_2$ and $MgCl_2$ to achieve a concentration of 0.1 m*M* Mg^{2+} at varying $[Zn^{2+}]$. Then the activity in one aliquot was determined directly and another aliquot was incubated with 1.9 m*M* $ZnCl_2$, leading to $4 \cdot 10^{-11}$ *M* to $4 \cdot 10^{-10}$ *M* free Zn^{2+}. Afterwards the activity was determined.

We obtained a typical curve for reversible dissociation of a Me_1L_1 complex with a dissociation constant shown in the Table.

Appendix. 1. Dependence of one equilibrium on another. A system consisting of 2 complexing agents, e.g. enzyme and NTA, and 1 metal ion, which can be bound by both complexing agents, e.g. Mg^{2+}, will be examined. The concentration of one complexing agent is greater than the other: e.g. $[NTA]_t \gg [E]_t$. We have to calculate $[Mg^{2+}]$, [NTA], [MgNTA] as well as $[E]/[E]_t$ and $[EMg]/[E]_t$, as the absolute concentration of E is not known.

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As $[NTA]_t \gg [E]_t$, the problem can be divided into two parts: 1. an independent MgNTA/NTA system, which can be calculated according to

$$(1)\quad [Me] = \frac{[L]_t - [Me]_t + K_{MeL}}{2} + \sqrt{\frac{([L]_t - [Me]_t + K_{MeL})^2}{4} + \frac{}{K_{MeL}[Me]_t}}$$

$$(2)\quad [MeL] = [Me]_t - [Me]$$

$$(3)\quad [L] = [L]_t - [MeL]$$

and 2. a system EMg/E, which is a function of the first system. From

$$(4)\quad K_{EMg} = \frac{[E] \cdot [Mg^{2+}]}{[EMg]}$$

and

$$(5)\quad [E] = [E]_t - [EMg]$$

we get

$$(6)\quad \frac{[EMg]}{[E]_t} = \frac{[Mg]}{[Mg] + K_{EMg}}$$

As the substrate concentration is constant,

$$(7)\quad v = k_2\,[EMg]$$

Introducing (7) and $V = k_2\,[E]_t$ into (6) we get

$$(8)\quad \frac{v}{V} = \frac{[Mg]}{[Mg] + K_{EMg}}$$

From equations (1) and (8) the theoretical curves, e.g. Figure, have been calculated.

2. Calculation of $[Zn^{2+}]$ as a function of $[Zn^{2+}]_t$, $[Mg^{2+}]_t$ and $[L]_t$ in the system ZnL/MgL/Mg^{2+}. To calculate the dissociation constant of the Zn-enzyme complex, we had to obtain definite concentrations of Zn^{2+} in the region of 10^{-10} *M* to 10^{-14} *M* at 0.1 m*M* Mg^{2+}. Because of the extreme differences in concentrations, this problem could only be solved by combination of a Zn^{2+} buffer system with unbuffered Mg^{2+}: ZnEDTA/MgEDTA/Mg^{2+}.

Wolf[13] has evaluated the equations necessary to solve this problem. Ackermann[14] has calculated the concentrations of free metal ions in this system by means of a FORTRAN IV programm.

When the following conditions are fullfilled

$$(9)\quad [Zn]_t < [L]_t$$

and

$$(10)\quad K_{ZnL} \ll K_{MgL}$$

then

$$(11)\quad [ZnL] \approx [Zn]_t$$

and

$$(12)\quad [L]_t \approx [L]_t - [ZnL] \approx [L]_t - [Zn]_t$$

These approximations enable us to calculate the concentration of free Zn^{2+} ions more easily. From

$$(13)\quad K_{ZnL} = \frac{[Zn]\,[L]}{[ZnL]}$$

and

$$(14)\quad K_{MgL} = \frac{[Mg]\,[L]}{[MgL]}$$

we obtain

$$(15)\quad [Zn] = \frac{K_{ZnL} \cdot [ZnL]}{K_{MgL} \cdot [MgL]} \cdot [Mg].$$

Substituting equation 11 we get

$$(16)\quad [Zn] = \frac{K_{ZnL} \cdot [Zn]_t}{K_{MgL}} \cdot \frac{[Mg]}{[MgL]}.$$

$[Mg^{2+}]$ we calculate from equation (1) as a function of $[Mg]_t$ and $[L]_t$ and MgL from equation (2).

The results obtained by this approximation are in complete agreement with those calculated from the exact equation for all measurements taken.

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Acetylcholinesterase in Erythrocytes and Lymphocytes: its Contribution to Cell Membrane Structure and Function

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Summary. Based on biochemical, histochemical and immunofluorescent studies on erythrocytes and lymphocytes, we propose an active function for acetylcholinesterase in membrane structure.

There is considerable recent interest and research in the structure and function of plasma membranes in the dynamic phenomena involved in their participation in physiologic processes. There are several existing hypotheses of the physicochemical structure of membranes[2–4]. Acetylcholinesterase (AchE) is known to be a membrane component on the basis of biochemical[5] and histochemical data[6]. We present immunofluorescent localization of AchE in human lymphocytes, particularly the thymus-dependent (T) cells which form spontaneous rosettes with sheep red cells, and propose that AchE forms the bond between lecithin and protein in structural membranes and contributes to their stability and function.

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