and steam distilled. The distillate was then carried through the $HgCl_2$ oxidation as described above. As shown in Table I this HCOOH fraction contains all the carbon derived from C-2 of gluconate.

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Is Acetylcholinesterase a Metallo Enzyme?[1]

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The literature contains numerous examples of the activation of cholinesterases by divalent ions such as Ca^{++}, Mg^{++} and Mn^{++} (see among others).[3,4] The activity of dialyzed preparations is also increased by the presence of salts such as NaCl and KCl. This latter activation is a phenomenon associated with the ionic strength of the medium and is quite distinct from the activating effect of divalent ions which occurs at much lower concentrations and can always be superimposed upon the general salt effect. These observations have been obtained with more or less crude enzyme preparations. Despite the fact that the question of the essentiality of divalent ions in the catalytic process has never been studied in purified preparations, the belief persists that this enzyme is a "metallo enzyme."

Recently a paper[5] appeared which seems to indicate that Mg^{++} ion is essential for catalytic activity and a theory was advanced which incorporated Mg^{++} ion in the elementary processes. The basis of this view was an observed 96% inhibition at *p*H 7 of highly purified acetylcholinesterase preparation from *Electrophorus electricus* caused by the addition of 0.015 *M* citrate to an incubation medium containing 0.1 *M* NaCl, 0.010 *M* $MgCl_2$ and 3.3×10^{-3} *M* acetylcholine chloride. Eighty-six per cent. inhibition was obtained with 0.015 *M* borate. These findings are not in agreement with the experience of this Laboratory (unpublished data of D. Nachmansohn). It therefore appeared necessary to repeat these experiments and also to enquire more carefully into the question of whether some species of divalent ion was essential for activity. The results obtained were not in agreement with those of the previous investigators.[5] Accordingly an exchange of enzymes between the two laboratories was arranged and the experimental basis for the differences was resolved as described in a joint note which follows this paper. The procedure used for determining whether divalent cations are essential was to exclude their salts as completely as was conveniently feasible by using reagent grade analyzed sodium chloride, sodium hydroxide and the disodium salt of ethylenediaminetetraacetic acid[6] and highly purified water. The concentration of polyvalent cations was further reduced by adding the chelating agent ethylenediaminetetraacetic acid (EDTA). It was possible in all cases to calculate the maximum amount of any common polyvalent ion present. Since among the common ions which have an activating effect on this enzyme, Mg^{++} is one of the poorest bound by EDTA and is more or less the most plentiful as an impurity in the various reagents and will, therefore, be present as the free ion in the largest amount, we will present our results in terms of the concentration of this metallic ion.

Methods.—Enzymatic activity was studied by two experimental procedures.

(a) Continual automatic titration with 0.020 *N* NaOH using the Beckman Model K automatic titrator modified slightly so as to increase its rate of response and sensitivity and to decrease the amount delivered to about 3 λ per impulse. The *p*H is adjusted at intervals of less than one second and maintained constant to within less than 0.01 *p*H unit. The medium had a volume of 50–100 ml. and contained the salts indicated under results. The substrate concentration was 0.002 *M* acetylcholine bromide.

(b) Manometric assay[7] in 3.1 ml. of 0.16 *M* NaCl, 0.020 *M* $NaHCO_3$ and 0.1% gelatin medium gassed with 5% CO_2 in N_2 (*p*H 7.4). Additions are indicated with the results. The substrate concentration was 0.004 *M* acetylcholine bromide.

The enzyme preparation used in both procedures had originally been purified[8] to a specific activity (micromoles of acetylcholine hydrolyzed per hour per mg. of protein under optimum conditions) of 6.6×10^4 but in storage had fallen to 4.0×10^4. In addition a cruder preparation of specific activity 2.0×10^4 was used also in the manometric procedure.

The water used in the experiments had been distilled and passed through a resin demineralizer. The resulting water had a conductivity corresponding to 0.1 part per million of NaCl. If we assume 0.1 part per million of Mg^{++} in the water the concentration is about 5×10^{-9} *M*.

Acetylcholine bromide was analyzed for Mg^{++} by flame photometry and found to contain less than 0.05%.

Results

The results obtained by the method of continual titration are summarized in Tables I and II. We find in agreement with earlier observations that the activity is diminished in a medium of low ionic strength (ionic strength of substrate = 0.002 *M*) and can be increased to a maximum of about 20% in a medium of high ionic strength by the addition of 10^{-2} *M* or greater $MgCl_2$. That this Mg^{++} activation is not a general salt effect is shown by the fact that the rate is unaltered by the addition of the amount of NaCl which reproduces the ionic strength. At low ionic strength 10^{-4} *M* $MgCl_2$ is sufficient. The activating effect of Mg^{++} can be offset by the chelating agents citrate ion and EDTA.

Contrary to the results recently reported[5] and referred to above, citrate does not alter the basal activity of this enzyme. In the presence of added Mg^{++} and citrate the rate is somewhat higher than the basal rate and is equal to the rate corresponding to the concentration of free Mg^{++}.

Citrate ion is a relatively poor chelating agent for Mg^{++} ($pK = 3.2$[9]) compared to the tetravalent ion

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(2) Supported by a Grant from the Rockefeller Foundation.

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TABLE I

pH	NaCl, M	Additions, M	Mg^{++}, M	Main source of Mg	Activity, %
7	0.10	None	7×10^{-6}	Impurity in NaCl	100[a]
	.22	None	1×10^{-5}	Impurity in NaCl	99
	.00	None	2×10^{-6}	Acetylcholine bromide	59
	.00	1×10^{-4} $MgCl_2$	1×10^{-4}	Addition	73
	.10	1×10^{-4} $MgCl_2$	1×10^{-4}	Addition	100
	.10	2×10^{-3} $MgCl_2$	2×10^{-3}	Addition	108
	.10	1×10^{-2} $MgCl_2$	1×10^{-2}	Addition	120
	.10	4×10^{-2} $MgCl_2$	4×10^{-2}	Addition	120
	.10	1×10^{-2} $MgCl_2$			
		1×10^{-2} $Na_3Citrate$	2×10^{-3}[b]	Addition	107
	.10	1×10^{-2} Citrate	4×10^{-7}[b]	Impurity in NaCl	99
	.10	1×10^{-2} $MgCl_2$			
		$+1.1 \times 10^{-2}$ EDTA	3×10^{-5}	Addition	90
9	.10	None	7×10^{-6}	Impurity in NaCl	100[a]
	.10	1×10^{-2} EDTA	1×10^{-10}	EDTA	95

[a] 100% activity corresponded to about 5 μmoles acetylcholine hydrolyzed per minute. [b] Calculated from binding constants.

TABLE II

Incubation, 24 hr., 4°	Assay medium, M	Mg^{++}, M	Activity, %
Water, pH 7.0	0.1 NaCl 0.01 EDTA pH 9	1×10^{-10}	100[a]
0.01 M EDTA, pH 7.0	0.1 NaCl 0.01 EDTA pH 9	1×10^{-10}	98
	0.00 NaCl 0.01 EDTA pH 9	1×10^{-10}	102

[a] Has the same meaning as in Table I; enzyme = $2^5 \times 10^{-12}$ M.

of EDTA (pK = 8.7[10,11]). The concentration of the tetravalent ion was calculated from the fourth acidic dissociation constant (pK_4 = 10.3[10,11]). Even this very potent chelating agent does not appreciably lower the basal rate although it readily offsets the activating effect of magnesium ion. An experiment is included at pH 9 to take advantage of a greater concentration of tetravalent EDTA. The slight (5%) decrease in activity in the presence of EDTA in this experiment cannot be attributed to Mg^{++} binding since there is a slightly greater decrease when Mg^{++} is added at pH 7, even though the free Mg^{++} concentration is over 10^5 times greater.

It is apparent that if equilibrium conditions with respect to Mg^{++} binding prevail, the binding of the enzyme for Mg^{++} must be very great indeed if the enzyme is a magnesium enzyme. It is possible, however, that the dissociation of Mg^{++} from the enzyme is a very slow process. To test this possibility the enzyme was incubated in concentrated solution for 24 hours at 4° with 0.01 M EDTA at pH 7, diluted with 0.01 M EDTA and tested in a reaction mixture which contained 0.01 M EDTA and NaCl at pH 9 and compared to a control which was incubated without EDTA, Table II. No inhibition occurred. In one case NaCl was omitted. Mg^{++} ion was introduced in this case mainly as an impurity in the EDTA. It should be noted that 0.01 M EDTA corresponds to an ionic strength in excess of 0.1 M. In this experiment the Mg^{++} concentration is calculated to be on the order of 10^{-10} M.

The enzyme concentration in these experiments can be calculated to be about 3×10^{-12} M from data of Rothenberg and Nachmansohn of their purest enzyme, which had a specific activity of 4.15 $\times 10^5$ and a molecular weight of about 2–3 $\times 10^{-6}$.[8] There is thus far more free Mg^{++} present than enzyme. Since the enzyme is still completely active under these conditions the binding constant would have to be of the order of 10^{11} or larger if this enzyme is a magnesium enzyme. The values so far reported in the literature for magnesium enzymes vary between 10^{+2} and 10^{+4} M.[12] It is interesting to note that if the activating effect of Mg^{++} is interpreted in terms of binding the binding constant is of the order of 10^{+3} M. The activating effect of divalent ions is a phenomenon completely divorced from the question of metallic ion essentiality.

The results obtained by the manometric method with two enzyme preparations of specific activity 2×10^4 and 6.6×10^4 were the same as obtained with the automatic titration method. Citrate (0.015 M) in the presence or absence of Mg^{++} (0.01 M) and independent of the order of mixing in the presence of enzyme did not inhibit. In the presence of EDTA (0.01 M) the rate of CO_2 evolution was decreased by about 10% but in this case at least part of the decline is caused by the buffering action of this compound.

The conditions which the enzyme must satisfy to be a magnesium enzyme are: (a) the binding constant for Mg^{++} must be greater than 10^{11} M or (b) the time required for dissociation must be large compared to 24 hours. These conditions are so severe that it is improbable that this enzyme requires Mg^{++} for function.

While we have presented our data with reference to magnesium binding it is clear that since the concentration of all the common polyvalent ions will have been reduced to levels lower, and in most cases very much lower, than magnesium ion, the implications are quite general. This study shows that there exists at this time no evidence that acetylcholinesterase requires divalent ions or is a metallo

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enzyme. Moreover, it may be concluded that it is very improbable that any species of metallic ion is required for the catalytic function of acetylcholinesterase.

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On the Mg(II) Activation of Acetylcholinesterase[1]

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The activation of the enzyme acetylcholinesterase (AChE) by divalent cations has been studied by various investigators, using enzyme preparations derived from different animal sources. Nachmansohn[3] found that the activity of the enzyme obtained from the electric organ of Torpedo is quite markedly influenced by these cations, with decreasing power of ionic activation in the sequence $Mn^{++} > Mg^{++} > Ca^{++} > Ba^{++}$, and that a 2×10^{-5} M concentration of the most powerful cation Mn^{++} produced enhancement of catalytic activity by a factor of 14. More recently, van der Meer[4] observed that the activity of AChE from the red blood cell or that from the caudate nucleus of the rat is notably enhanced by the presence of Ca^{++} ion.

However, an interesting anomaly has been observed recently in experiments on Mg^{++} activation of purified[5] AChE from the same natural source, namely, the electric organ of the electric eel (*Electrophorus electricus, linnaeus*). Work in the Bethesda laboratories[6] pointed to a large decrease in activity of AChE functioning in a solution 0.01 M in Mg^{++} ion when complexing constituents such as citrate ion were present, at *p*H 7.4. In contrast, Wilson and Cabib[7] at Columbia University found little effect of these constituents on the enzymatic hydrolysis rates, and noted a maximum activating effect of Mg^{++} amounting to about a 20% increase in rate over that with no added Mg^{++} ion. A brief tabulation of the conflicting results for the two enzyme preparations, illustrated with data on both obtained in Bethesda, is presented in Table I. Data are given for the degree of activation produced by Mg^{++} at the 0.016 M level, the effect of added citrate at *p*H 7.4 on the enzymatic rates, and that of added Versene tested as a complexing agent at somewhat higher *p*H values. Enzyme purified at Bethesda is designated as enzyme A; that at Columbia University as enzyme B.

It is seen from Table I that, as noted in the Columbia experiments,[7] enzyme B is relatively insensitive to the presence of citrate ions or Versene, except for a small decrease in activity attributable to the complexing of a fraction of the Mg^{++} present, and is only activated by Mg^{++} at the 0.016 M level to the extent of about 30%. The increase in rate for experiment 4 as compared with experiment 2 can be ascribed at least in part to the increase in activity of AChE with increasing *p*H.[8] Enzyme A, on the other hand, demonstrates a much more marked sensitization by Mg^{++} ion, and desensitization of its catalytic power by agents that are effective in complex formation with this ion. Such behavior was found to be quite reproducible in repeated kinetic experiments made with fresh dilutions of a concentrated stock solution of enzyme A, and the stock solution was found to retain these properties completely on standing at 3–4°. Further, two different stock solutions of AChE prepared at Bethesda over a year's interval showed essentially the same Mg^{++} sensitization properties listed in the enzyme A column of Table I.

TABLE I

COMPARISON OF AChE PREPARATIONS

25.12°, substrate = $3.34 \times 10^{-3} M$, added NaCl = 0.081 M

Expt.	*p*H	Added Mg^{++}, M	Phosphate, M	Citrate, M	Versene, M	Relative activity: Enz. A[a]	Enz. B[a]
1	7.4	0.016	0.012			100	100
2	7.4	0	.012			15	71
3	7.4	.016		0.012		10	92
4	8.2	0	.011		0.014	18	85
5[b]	8.8	.014	.011		.028	8	

[a] Activity in phosphate buffer with 0.016 M Mg^{++} taken as reference value for each enzyme preparation. [b] NaCl concentration, 0.072 M.

The experimental cause for these differences between preparations A and B in Mg^{++} sensitization properties was brought to light by the exchange of diluted solutions of the two enzymes between the two laboratories. A solution of B which reached Bethesda after a day's travel at 3–4° was found to possess the properties in Table I previously noted by the Columbia group. However, a 1:25 dilution of stock enzyme A sent to Columbia, which had also aged at 3–4° for more than a day before being employed in kinetic experiments, was found to have *changed* its Mg^{++}—sensitization properties to those characteristic of enzyme B.

This phenomenon of change in susceptibility to Mg^{++} activation on aging in diluted solution, while the more concentrated protein solution retains its properties indefinitely, was checked in the Bethesda laboratories with the results shown in summary in Table II. Here, the same 1:25 dilution as that sent to Columbia was prepared, stored at 3–4°, and the activity of this dilution checked at approximately 20 and 60 hours after dilution under the conditions shown in Table II.

It is seen from Table II that the relatively high sensitivity to Mg^{++} activation of enzyme A, when tested immediately after dilution from stock, falls progressively on standing in *diluted* solution to the low sensitivity level characteristic[7] of enzyme B.

(1) The opinions in this paper are those of the authors and do not necessarily reflect the views of the Navy Department.

(2) Supported by a Grant from the Rockefeller Foundation.

(3) D. Nachmansohn, *Nature*, **145**, 513 (1940).

(4) C. van der Meer, *ibid.*, **171**, 78 (1953).

(5) Prepared essentially according to the procedures of M. A. Rothenberg and D. Nachmansohn, *J. Biol. Chem.*, **168**, 223 (1947).

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