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Table 1. Stereological parameters studied in CNS of *T. marmorata*. A higher lipofuscin content is observed in the electric lobe

	Forebrain	Optic lobe	Cerebellum	Electric lobe
Average area analyzed per animal ($\mu m^2 \times 10^3$)	2.94	2.94	2.94	2.94
Total average area of lipofuscin granules per animal (μm^2)	12.00	8.53	3.40	48.13
Percent of cytoplasmic area covered by lipofuscin ($\times 10^{-1}$)	4.08	2.90	1.16	16.40

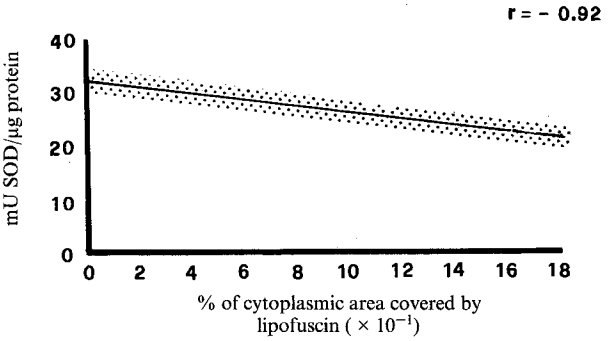
Table 2. Superoxide dismutase activity and percent of cytoplasmic area covered by lipofuscin in the CNS of *T. marmorata*. Note the higher lipofuscin content in the areas with lower SOD activity

	SOD (mU/µg protein)	% of cytoplasmic area covered by lipofuscin ($\times 10^{-1}$)
Cerebellum	34.0	1.16
Optic lobe	28.0	2.90
Forebrain	29.0	4.08
Electric lobe	22.5	16.40

the present work we have assessed more accurately the distribution of lipofuscin in the various areas of the *T. marmorata* brain and in particular we have quantified this by correlation with SOD activity.

Ten specimens of *T. marmorata*, of both sexes and, according to several growth parameters[5] of between 1 and 2 years old, were perfused with Ringer solution until totally bled. The four regions of the CNS were isolated in such a way that half could be processed for electron microscopy using standard techniques and the other half used to determine SOD activity[6]. From the former half, using randomly selected samples, we obtained photographs which were analyzed stereologically to quantify the lipofuscin. For this purpose we utilized an automatic image analyzer system (Ibas I, Zeiss).

The assay of superoxide dismutase was based on its ability to inhibit the autoxidation of epinephrine (0.4 mM) at pH 10.2[7]. In table 1 we show the results of studies of several stereological parameters; the average cytoplasmic area studied per animal, the total average area of lipofuscin per animal and the percentage of the cytoplasmic area occupied by pigment. A higher



Correlation between SOD activity and lipofuscin in the four areas of *T. marmorata*. CNS. y = 31.89–0.59 × ; SD (shaded area) = ±4.5.

lipofuscin content is shown in the electric lobe by the high percentage of the cytoplasmic area covered by the pigment. This parameter is the most interesting in that it gives us on the one hand a relationship between the number and size of the areas of granulation, and on the other hand the cytoplasmic area. Therefore this parameter is used in comparison with the SOD activity of the various regions of the CNS (table 2). To compare these parameters we have calculated the linear regression and the correlation coefficient, which show an inverse proportionality ($r = -0.92$) between high lipofuscin content and low SOD activity (fig.). These results support the theory that lipofuscin is a product of free radicals induced lipoperoxidation[8].

1 Part of the present work was presented at XIIth Int. Pigment Cell Conference, Giessen (FRG), 17–19 September 1983.
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Failure of calcium to stimulate Na, K-ATPase in the presence of EDTA[1]

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Summary. The effect of calcium on Na, K-ATPase activity of rat brain homogenates and its modification by the chelating agent EDTA has been investigated. In the absence of EDTA, free calcium (approximately 10^{-6} mol/l) stimulates Na, K-ATPase activity; in the presence of EDTA the same concentration of free calcium is without effect on the enzyme. In the absence of EDTA the stimulation by calcium of Na, K-ATPase activity is enhanced by the additional presence of calmodulin but in the presence of EDTA, even when calmodulin is added to excess, calcium still fails to stimulate the enzyme. The possibility that EDTA interferes with an interaction between a calcium-calmodulin complex and Na, K-ATPase is discussed.

Key words. Na, K-ATPase; calcium; calmodulin.

In a recently published study from this laboratory it was reported that calcium at low concentration stimulates and at higher concentration inhibits neuronal Na, K-ATPase[3,4]. In addition it was shown that stimulation of the enzyme by calcium is modulated by calmodulin. As far as I am aware our report is unique: of those others who have studied the effects of calcium on Na, K-ATPase, all have demonstrated only an inhibition of the enzyme over the effective concentration range of calcium[5–11]. To explain why others have failed to obtain stimulation of Na, K-ATPase by calcium, we[4] suggested that use of the chelat-

ing agents EDTA or EGTA might be relevant. These compounds were invariably included in the experimental media of the other investigators presumably both to produce a calcium-buffer system and also to remove traces of inhibitory heavy metal contaminants from the preparations thereby to promote a higher basal level of Na, K-ATPase activity suitable for subsequent experimentation. In our experiments we had found that calcium at a concentration which in the absence of EDTA caused stimulation of Na, K-ATPase had no effect in its presence.

The aim of the present experiments was to determine the probable mechanism whereby EDTA prevents calcium: calmodulin mediated stimulation of Na, K-ATPase.

Methods. The methods used to discriminate and quantitate cation-dependent ATP-phosphohydrolase activity in brain homogenates were those reported previously[4]. Briefly, all homogenates were prepared from whole brain of Wistar strain rats of either sex in ice-cold sucrose (0.32 mol/l) either with or without disodium EDTA (1×10^{-3} mol/l).

The experimental media contained Mg (5 mmol/l $MgCl_2$) ± Na (150 mmol/l NaCl) ± K (10 mmol/l KCl) in imidazole: HCl buffer (50 mmol/l, pH 7.4).

When required calcium (as $CaCl_2 \cdot 2\ H_2O$) and calmodulin (Sigma, P2277; one unit stimulates 0.016 activated units of phosphodiesterase, 3′:5′-cyclic nucleotide (Sigma, P0520) to 50% of the maximum activity of the enzyme when saturated with activator) were added (in 0.1-ml aliquots prepared in imidazole: HCl buffer) to the experimental media prior to the addition of the brain homogenates (0.4 ml). The final volume was 2.0 ml and, when present, the EDTA concentration was 2×10^{-4} mol/l. ATP-phosphohydrolase activity was determined as the amount of inorganic phosphate in µmol liberated from vanadium-free Tris-ATP (Sigma A0520; 4 mmol/l) per mg homogenate protein per hour. Na, K-ATPase activity was calculated as the difference between total ATPase activity measured in the presence of Na, K and Mg and that activity determined in the presence of Na and Mg only. Mg-ATPase is given by the value measured in the presence of Mg alone; Na-ATPase activity is given by the difference between this activity and that measured in the presence of Na and Mg.

Except where otherwise stated, all data are expressed as mean values ± SE. Statistical significance of the difference between control and test values was calculated by Student's t-test.

Effects of calcium. All experimental media contain inter alia Mg and ATP. Some contain in addition either Ca or EDTA or both. Since both ATP and EDTA bind both Ca and Mg, the levels of unbound EDTA and Ca in the reaction mixture that are free to affect ATPase activity were calculated from the association constants of the relevant cation-ligand complexes under the prevailing experimental conditions using the algorithm of Perrin and Sayce[12] as modified by Kuchel et al.[13]. The association constants (log K′ apparent) used were for Mg-ATP: 4.20, Ca-ATP: 3.89, Mg-EDTA: 5.82 and Ca-EDTA: 7.72[4,14,15].

In those calculations in which these values were used to estimate the concentrations of cations, ligands and cation-ligand complexes in the reaction mixture, no account was taken of the Mg that would be derived from the homogenate since this would have been small relative to that concentration (5 mmol/l) already present in the experimental medium. However, due account was taken of the Ca deriving from the homogenate and other components of the experimental medium. Estimation of the Ca concentration of the experimental medium using a Ca-selective electrode (Orion 93-20-01) calibrated with the medium containing added $CaCl_2$ (or EGTA $< 10^{-4}$ mol/l to determine the limit of sensitivity to Ca) showed the upper limit of Ca contamination to be 9×10^{-6} mol/l. In medium to which brain homogenate had been added to give the dilution used in the experiments, however, the upper limit of Ca contamination was ca. 2.8×10^{-6} mol/l. It is probable that the lower concentration of Ca in the medium containing homogenate resulted from the ability of the latter to sequester Ca and thereby reduce pCa_{free}.

Results and discussion. Values for ATP-phosphohydrolase activity measured in rat brain homogenates both in the absence and presence of EDTA (2×10^{-4} mol/l) are given in table 1. As with those experiments reported previously[4], EDTA caused a slight stimulation of Mg-ATPase activity, a major stimulation of Na, K-ATPase activity and caused a slight depression of Na-ATPase activity. Only the change in Na, K-ATPase activity is statistically significant.

It is probable that the increase in Na, K-ATPase activity is due to chelation by EDTA of an inhibitory cation derived from the homogenate or reagents used. It is improbable that the effect reflects a direct action of EDTA upon the enzyme.

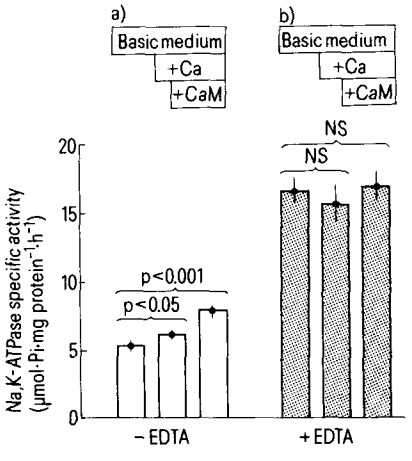
Likewise it is improbable that the changes above, and those changes in Na, K-ATPase activity to be reported below are due to variation in free-Mg concentration occasioned by the different experimental conditions. In this regard, Powis et al.[4] showed that Na, K-ATPase activity remained virtually constant in the face of change in free-Mg concentration between 0.47 and 1.20×10^{-3} mol/l. In the present experiments Mg is calculated to be 1.20×10^{-3} mol/l in the absence of EDTA and 1.02×10^{-3} mol/l in its presence (EDTA 2×10^{-4} mol/l). Calculation shows further that the concentration of Mg_{free} either in the absence or presence of EDTA is unaffected by the presence of Ca $< 10^{-4}$ mol/l.

Effect of calcium on ATP-phosphohydrolase activity. It was shown previously[4] that in the absence of EDTA the stimulatory effect of calcium occurs over the range pCa_{free} 5.5–7.0. In these

Table 1. ATP-Phosphohydrolase basal activity in rat brain homogenates and effect of EDTA (2×10^{-4} mol/l)

	ATP-phosphohydrolase activity (µmol Pi·mg protein^{-1}·h^{-1})		
	Mg-ATPase	Na-ATPase	Na, K-ATPase
− EDTA (n = 12)	7.4 ± 0.69	2.5 ± 0.48	5.4 ± 0.45
+ EDTA (n = 6)	8.5 ± 0.49	1.9 ± 0.58	16.7 ± 0.94
2 p[a]	NS	NS	< 0.001

Values given are mean ± SE. [a]Statistical significance of change in ATPase activity produced by EDTA; NS, not significant.



Na, K-ATPase activity under control conditions and in the presence of either calcium alone or calcium + calmodulin in the absence (a; n = 12) or presence (b; n = 6) of EDTA (2×10^{-4} mol/l).

In part a) $pCa_{free} = 5.93$. [calmodulin] was approx. 40 units; in part b) $pCa_{free} = 6.49$, [calmodulin] was either 40 or 100 units.

Mean values ± SE are shown together with the statistical significance of the differences between relevant mean values.

Table 2. Change in ATP-phosphohydrolase activity (μmol Pi · mg protein^{-1} · h^{-1}) caused by 1) Ca alone, and 2) Ca + calmodulin, in the presence or absence of EDTA (2×10^{-4} mol/l)

		− EDTA (n = 12)	+ EDTA (n = 6)	2P[d] (− EDTA vs + EDTA)
1) Basic medium + Ca[a]	Mg-ATPase	+ 0.7 ± 0.29	+ 0.3 ± 0.29	NS
	p[c]	< 0.05	NS	
	Na-ATPase	− 0.9 ± 0.36	− 0.5 ± 0.26	NS
	p	< 0.025	NS	
	Na, K-ATPase	+ 0.7 ± 0.30	− 0.9 ± 0.72	~ 0.05
	p	< 0.05	NS	
2) Basic medium + Ca[a] + Calmodulin[b]	Mg-ATPase	+ 1.6 ± 0.43	+ 0.5 ± 0.46	NS
	p	< 0.005	NS	
	Na-ATPase	− 1.0 ± 0.62	− 0.8 ± 0.66	NS
	p	NS	NS	
	Na, K-ATPase	+ 2.6 ± 0.51	+ 0.4 ± 0.86	< 0.05
	p	< 0.001	NS	

Values given are mean changes in ATP-phosphohydrolase activity ± SE. Basal values for ATPase activity are given in table 1. [a] In the presence of EDTA $pCa_{free} = 6.49$, in the absence of EDTA $pCa_{free} = 5.93$. [b] Calmodulin approx. 40 units in the absence of EDTA; 40–100 units in the presence of EDTA. [c] Statistical significance of change in ATPase activity caused by calcium or by calcium + calmodulin; NS, not significant. [d] Statistical significance of difference between ATPase activity in absence and presence of EDTA; NS, not significant.

experiments calcium was added to increase the final concentration by 3×10^{-7} mol/l. Together with the Ca deriving from the homogenate (see above) this addition was calculated to result in a pCa_{free} 5.93, (i.e. Ca derived from homogenate and reagents (2.8×10^{-6} mol/l) plus added Ca (3×10^{-7} mol/l) less that Ca chelated by ATP calculated using association constants for CaATP and MgATP (see above). The effects of this concentration of calcium on the activity of the three ATP-phosphohydrolase enzymes are shown in table 2. Specifically Na, K-ATPase activity was stimulated by 13.4% from basal levels ($p < 0.05$, fig.). In the additional presence of calmodulin calcium produced a further stimulation of total ATP-phosphohydrolase activity (table 2). This was due predominantly to stimulation of Na, K-ATPase; its activity in the presence of calcium and calmodulin (40 units) was raised by 47.6% from basal levels ($p < 0.001$, fig.).

In other experiments a range of concentrations of calmodulin up to 500 units was tested; none allowed greater stimulation of Na, K-ATPase by calcium (3×10^{-7} mol/l) than that produced with 40 units calmodulin indicating that this latter concentration is above the saturating level.

It was shown previously[4] that the additional ATP-phosphohydrolase activity produced by the addition of calcium ± calmodulin is ouabain sensitive. This observation confirmed that the enzyme stimulated is Na, K-ATPase and not an independent enzyme recruited by the presence of calcium.

In view of the present and our previous findings it is probable that added calcium is bound by endogenous calmodulin and the complex formed stimulates Na, K-ATPase. Furthermore it appears that in our rat brain homogenate the available store of endogenous calmodulin is small because the stimulatory effect produced by calcium even at very low concentration (10^{-6} mol/l free Ca in these experiments) can be enhanced by the addition of exogenous calmodulin.

In the presence of EDTA (2×10^{-4} mol/l) addition of Ca (3×10^{-6} mol/l) together with that Ca deriving from the homogenate gives a pCa_{free} 6.49. This value is within that range wherein calcium, in the absence of EDTA, has been shown to stimulate Na, K-ATPase[4]. In six experiments such a concentration of Ca caused no significant alteration in ATP-phosphohydrolase activity (table 2). With respect to Na, K-ATPase specifically, Ca altered its activity by − 5.4% (NS) from its basal level (fig.).

Addition of calmodulin (40–100 units) along with calcium still did not produce stimulation of ATP-phosphohydrolase activity: in the presence of EDTA when Ca + calmodulin were added the activities of Na, K-ATPase, Mg-ATPase and Na-ATPase remained at, or near, control levels (fig., table 2).

Table 2 summarizes the effects of calcium on ATP-phosphohydrolase activity in the presence and absence of EDTA. It is clear that the only statistically significant action of EDTA is upon the calcium induced stimulation of Na, K-ATPase: in the presence of EDTA, calcium, both alone and together with added calmodulin at a concentration normally sufficient to saturate the system, failed to increase Na, K-ATPase activity.

It could be argued that since EDTA (2×10^{-4} mol/l) itself increases Na, K-ATPase activity then this leaves no leeway for additional stimulation by calcium. This suggestion has been shown previously to be improbable[4]: at an EDTA concentration of 2×10^{-4} mol/l the activity of Na, K-ATPase is only 50% of that which can be revealed in the presence of this compound.

Only two general mechanisms appear to remain to account for the failure of calcium to stimulate Na, K-ATPase activity in the presence of EDTA: both are based on the assumption that calcium combines with calmodulin to form a complex which stimulates Na, K-ATPase activity (vide infra). Either EDTA inhibits the required combination of calcium with calmodulin to prevent generation of the stimulatory moiety at the outset or EDTA inhibits subsequent combination of the stimulatory moiety with Na, K-ATPase.

In view of the results obtained when calcium and calmodulin were both added to the experimental system together, presumably having combined already to form the active complex prior to coming into contact with EDTA or Na, K-ATPase, the second of the two possibilities appears the more probable.

It is worth noting that other investigators[16,17] have alluded to the possibility that chelating agents somehow interfere with calcium mediation of physiological events.

It is concluded that EDTA does interfere with the events that link calcium and calmodulin to Na, K-ATPase stimulation, possibly by inhibiting the step that involves interaction between calcium: calmodulin and Na, K-ATPase. There are insufficient data to make any further speculation profitable.

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Role of the Δ 8 double bond of agroclavine in lysergic acid amide biosynthesis by *Claviceps purpurea*

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Summary. Agroclavine, given to actively-growing sclerotial tissue of a strain of *Claviceps purpurea* which can not normally elaborate ergot alkaloids, was transformed by this tissue into lysergic acid amide with overall efficiency of approximataly 40%. By contrast, festuclavine (8,9-dihydro-agroclavine) was not transformed, indicating specificity in the mechanism of lysergyl biosynthesis.
Key words. Agroclavine; festuclavine; lysergic acid amide; *Claviceps purpurea;* ergot alkaloids; biosynthesis.

The biosynthetic pathway leading to the ergot alkaloids is fairly well understood though much mechanistic detail remains unclear[1]. A strain of *Claviceps purpurea* which, as a parasite, elaborates trace amounts of dimethylallyltryptophan (**1**), but no ergoline alkaloids, has been shown to metabolize exogenous agroclavine (**2**) or lysergic acid (**6**) to lysergic acid amide (**7**) only[2]. This system is therefore suitable for exploring certain aspects of the biosynthesis of the lysergyl nucleus in parasitic sclerotial tissue.

The most recently proposed speculative scheme for the formation of lysergyl derivatives from clavine alkaloid intermediates[1] involves an activated lysergic acid and excludes free lysergic acid as a direct intermediate[3]. Biosynthesis of the activated lysergic acid from elymoclavine (**3**) is deduced to be via the enol form of Δ 9 lysergaldehyde (**5**) in the formation of which the Δ 8 double bond migrates to the Δ 9 position giving a system conjugated with the aromatic ring. Since in one ergot fungus (*Sphacelia sorghi*) the alkaloids are all dihydrogenated with respect to positions 8,9 and 9,10 of the ergoline nucleus[4], the question arises as to whether, in all of the other ergot fungi producing lysergyl derivates, the Δ 8 is essential in order to stabilize the enol form for activation by coenzyme A.

Sclerotia of the alkaloid-free strain of *C. purpurea* were therefore produced on rye in 1983 and, while actively growing 35 days after inoculation, the distal and proximal 3 mm of each sclerotium was excised and the remaining tissues combined and homogenized briefly in 0.01 M phosphate buffer, pH 6.5. The homogenate was subdivided into three parts and incubated (1 g tissue: 10 ml buffer) with gentle agitation for 24 h at 27 °C as indicated (table). The crystalline substrates, agroclavine and festuclavine (**4**) (8,9 dihydro-agroclavine), were prepared from submerged fermentation of *C. fusiformis*[5] and *Sphacelia sorghi*[6], respectively. After incubation with the substrates the cells were separated from the supernatant by centrifugation, washed and lyophilized. Dry cells were made alkaline with aq. $NaHCO_3$ and exhaustively extracted with diethyl ether[2]. Alkaloids were extracted from the ether with 2% tartaric acid, a portion made alkaline with NH_4OH, extracted exhaustively with chloroform, evaporated to dryness and the alkaloids evaluated spectrophotometrically in methanol at 284 nm and 311 nm, λmax for the clavine alkaloids and lysergic acid amide, respectively. A portion of the extract was examined on silica gel chromatograms developed in chloroform:methanol (4:1) and by HPLC in a Waters Novapak column using acetonitrile:0.01 M ammonium carbonate (4:6) with UV detection at 284 nm. The expected occurrence of lysergic acid amide was confirmed by both chromatographies, as was also the spectrophotometrically-assayed relative abundance of agroclavine and lysergic acid amide.

Lyophilized supernatants from the incubations were also examined by spectrophotometry and chromatography; only given alkaloid was detected.

As expected, tissue incubated with or without agroclavine (table) confirmed previous findings that the tissue was intrinsically alkaloid-free[7]. Additionally, the efficiency of transforming agroclavine into lysergic acid amide may be implied from the calculation that in one day the tissue transformed an amount of alkaloid equivalent to about 10% of that commonly elaborated in ergot sclerotia over several weeks of parasitism. The contrasting failure of such metabolically-active tissue to utilize festuclavine for alkaloid biosynthesis may therefore reflect either a mechanistic requirement for Δ 8 in the biosynthesis of the lysergyl nucleus