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SOME CHEMICAL CHARACTERISTICS OF DIMETHYLSUBERIMIDATE AND ITS EFFECT ON SARCOPLASMIC RETICULUM VESICLES

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SUMMARY

Sarcoplasmic reticulum vesicles treated with dimethylsuberimidate lose the capacity for ATP-promoted Ca^{2+} accumulation and show other properties indicative of leaky vesicles. As an aid to assessing whether this effect was caused by cross-linking or by hydrolysis products, characteristics of dimethylsuberimidate hydrolysis under incubation conditions used were measured. At pH 7.0, 25 °C, dimethylsuberimidate is hydrolyzed with an apparent first order rate constant of 0.016 min^{-1}, to give dimethylsuberate as the principal product. The effect on ATP-promoted Ca^{2+} accumulation was shown to be caused by partially and fully hydrolyzed products of the diimido ester, and not to cross-linking of membrane components.

INTRODUCTION

The bifunctional imidoester dimethylsuberimidate has been employed to cross-link a variety of proteins [1–3], including those of the sarcoplasmic reticulum membrane [4]. The principal reaction involves formation of amidine derivatives from the ε amino groups of lysine residues and release of methanol by displacement. Because various lines of evidence indicate that conformational mobility may be essential to Ca^{2+} accumulation coupled to Ca^{2+}, Mg^{2+}-ATPase activity of sarcoplasmic reticulum vesicles, it was of interest to see if the catalytic capacities of the vesicles could be modified by cross-linking with dimethylsuberimidate. In this report we show that although incubation of the vesicles with dimethylsuberimidate produces an apparent uncoupling effect, which is not reproduced by similar incubation with the monofunctional reagent methylbutyrimidate, the effect observed is probably caused principally by hydrolytic products of the suberimidate.

METHODS

Materials. [γ-^{32}P]ATP was prepared essentially as described by Glynn and Chappell [5]. Methylbutyrimidate was prepared as described by McElvain and Nelson [6], m. p. 77–78 °C. Dimethylsuberimidate was prepared as described by Davies and Stark [2], m. p. 215–215.5 °C.

Sarcoplasmic reticulum vesicles were prepared as described by Kanazawa and Boyer [7], except that Tris was omitted from the buffers containing 0.6 M KCl and 0.1 M KCl, and the resulting vesicles were suspended in 0.1 M KCl, 5 mM maleate, pH 6.5.

Reaction of sarcoplasmic reticulum vesicles with dimethylsuberimidate and methylbutyrimidate. A suspension of sarcoplasmic reticulum vesicles was added to a buffer solution containing freshly dissolved imidoester to give final concentrations of 0.1 M triethanolamine, 40–60 mM suberimidate or 80–120 mM butyrimidate and 5.7 mg protein per ml in a 2 ml total volume. The pH was adjusted periodically to pH 7.0 with small amounts of dilute HCl. After 30–60 min an equal volume of cold 0.1 M maleate buffer, pH 6.1, was added to quench the reaction. The suspension was centrifuged at 54 000 $\times g$ for 30 min. The tube was rinsed with 5 mM maleate buffer, pH 6.5, containing 0.1 M KCl and the pellet was suspended in 2 ml of the same buffer. After another centrifugation, the tube was rinsed and the pellet was suspended in the same buffer.

Ca^{2+} accumulation. This was determined by incubating sarcoplasmic reticulum vesicles (approx. 0.2 mg protein/ml) in 0.05 M Tris/maleate buffer, pH 7.0, containing 5 mM ATP, 5 mM $MgCl_2$. After 2 to 5 min the mixture was applied on a Millipore filter (0.45 μm) previously soaked in 0.05 M Tris/maleate buffer, pH 7.0, containing 0.1 mM unlabeled $CaCl_2$. The radioactivity of the fllter was determined by conventional scintillation counting. The amount of $^{45}Ca^{2+}$ absorbed by the protein was determined from the difference between the radioactivity of the filters to which the sample mixture and the solution containing no sarcoplasmic reticulum had been applied.

Kinetic studies of hydrolysis of dimethylsuberimidate. Dimethylsuberimidate dihydrochloride was added to 0.1 μM triethanolamine buffer, pH 7.0, to a concentration of 5–50 mM, and the pH was quickly readjusted to pH 7.0 with dilute NaOH. Aliquots were withdrawn at various intervals and mixed rapidly with an equal volume of 0.2 M Na_2CO_3 solution and 2 volumes of chloroform. The upper layer was extracted twice more with the same volumes of chloroform. A 5 or 10 μl aliquot of upper layer was added to 3 ml of five-fold diluted Nessler's reagent (Sigma Chemical Co.) and the ammonia was determined at 5 min by absorbance at 425 nm.

Other determinations. Determination of phosphorylated protein and P_i liberation by ATPases was measured essentially as described by Kanazawa et al. [8]. Reaction mixtures contained 10 μM ATP, 5 mM $MgCl_2$, 100 μM $CaCl_2$, 100 mM KCl and 100 mM Tris/maleate buffer, pH 7.0 at 25 °C. In this assay [γ-^{32}P] ATP is used with unmodified vesicles. The measured ATPase activity is greater at higher ATP concentrations or if vesicles are treated with ether, alkali or detergents so as to avoid accumulated internal Ca^{2+}. However, such treatments decrease the $P_i \rightleftharpoons HOH$ exchange activity [7], and thus the assay with unmodified vesicles is preferred.

Protein was estimated by the Lowry procedure [9].

RESULTS AND DISCUSSION

Effect of dimethylsuberimidate on Ca^{2+} accumulation and retention

Table I shows that sarcoplasmic reticulum vesicles treated at pH 7.0 and 25 °C for 30 min with 40 mM dimethylsuberimidate lose most of the capacity for accumu-

TABLE I

EFFECT OF TREATMENT OF SARCOPLASMIC RETICULUM WITH IMIDOESTERS ON Ca^{2+} ACCUMULATION, E-P FORMATION, AND ATP CLEAVAGE

Sarcoplasmic reticulum vesicles (5.7 mg protein/ml) were incubated with the indicated reagents in 0.1 μM triethanolamine buffer, pH 7.0 at 25 °C. The reaction was stopped, the vesicles were separated from excess reagent and assays for Ca^{2+} accumulation, Ca^{2+}-induced ATPase activity (v), and Ca^{2+}-induced phosphoprotein formed from ATP (E-P) were performed as described under Methods. The values for phosphoprotein found in the absence of external Ca^{2+} are generally less than 0.5 nmoles per mg protein and the ATPase activity in absence of Ca^{2+} but presence of Mg^{2+} was less than 0.01 μmoles · mg^{-1} · min^{-1}.

Treatment prior to assay	Ca^{2+} accumulation (nmoles per mg protein)	v (μmoles · mg^{-1} · min^{-1})	E-P (nmoles per mg protein)	$\frac{v}{\text{E-P}}$ (min^{-1})
Experiment 1				
None	102	0.11	2.5	43
Incubated 30 min with:				
No addition	72	0.11	2.2	51
40 mM suberimidate	15	0.28	1.3	209
40 mM butyrimidate	67	0.14	2.7	52
80 mM butyrimidate	55	0.18	2.6	68
Experiment 2				
None	86	0.16	2.6	61
Incubated 60 min with:				
No additions	54	0.10	1.3	78
60 mM suberimidate	7	0.09	0.8	111
120 mM butyrimidate	13	0.34	0.7	484

lation of Ca^{2+}. Concomitant with this loss, the Ca^{2+}-dependent ATPase activity is increased while the phosphoprotein level is decreased. These properties are similar to those of leaky vesicles produced by treatment with phospholipase A and other reagents [10]. Similar incubation with methylbutrimidate at 40 or 80 mM concentration produces only a slight effect on Ca^{2+} accmulation, Ca^{2+}-dependent ATPase activity and the phosphoprotein level. However, more extended treatment with higher concentrations of methylbutyrimidate gives similar results to dimethylsuberimidate.

Vesicles previously loaded with Ca^{2+} readily release this ion upon addition of 30 mM dimethylsuberimidate, while 60 mM methylbutyrimidate causes a slower and less extensive release (Fig. 1). The effect of imidoesters on ion retention is, however, not specific for Ca^{2+}. Vesicles previously incubated with $^{22}Na^{+}$ also lose this ion readily on treatment with dimethylsuberimidate (data not shown).

It was originally anticipated that any effects of dimethylsuberimidate would likely be caused by cross-linking of membrane components, or by modification of the amino groups of proteins and lipids. In experiments not reported in detail here, [^{14}C]dimethylsuberimidate was demonstrated to be covalently attached to protein and lipid components under conditions similar to those used for Table I. Polyacrylamide gel electrophoresis showed evidence of progressive cross-linking of protein components in the membrane. Thus it seemed reasonable that the effects on

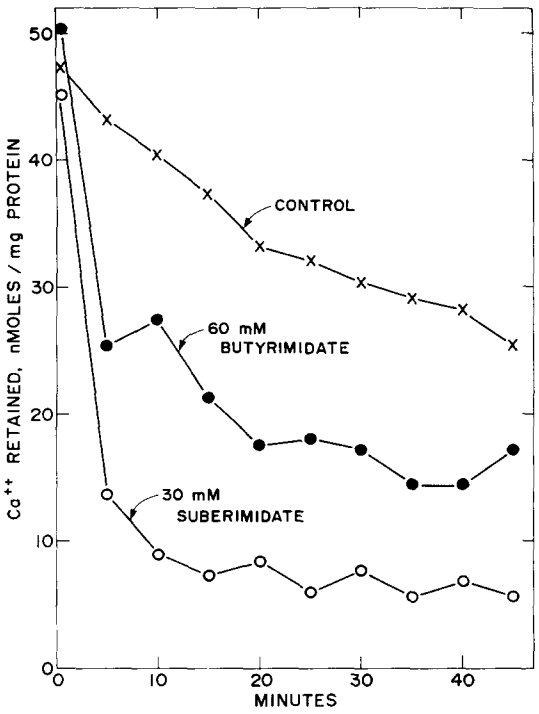

Fig. 1. Effect of imidoesters on Ca^{2+} retention. Vesicles were loaded with $^{45}Ca^{2+}$ and freed from nucleotides and P_i as described by Panet and Selinger [11]. 0.5 ml of the suspension of Ca^{2+}-loaded vesicles (7 mg protein/ml) were added to 9.5 ml of 0.1 M triethanolamine buffer, pH 7.0, containing appropriate amounts of imidoester at 25 °C. At 5 min intervals, 1.0 ml aliquots were withdrawn and $^{45}Ca^{2+}$ remaining in the vesicles was determined as described under Methods. ○, treated with 30 mM dimethylsuberimidate; ●, treated with 60 min butyrimidate; ×, incubated without imidoester.

Ca^{2+} accumulation might be due to cross-linking of membrane components.

However, hydrolysis of added imidoester occurs in competition with reaction with $-NH_2$ groups, and any imidoester that does not react with amino or other groups is subject to eventual hydrolysis. Thus the possbility must be considered that observed effects might be due to partially or completely hydrolyzed products of dimethylsuberimidate. Although the membrane vesicles were washed after treatment, the effects could be exerted by the product formed through covalent attachment to the membrane at one end, and hydrolysis at the other end, through non-covalent but poorly reversible binding of the fully hydrolyzed products, or through irreversible changes produced in the membrane.

Hydrolysis characteristics of dimethylsuberimidate

In order to help distinguish among these possible explanations, better information about the nature and kinetics of hydrolysis of dimethylsuberimidate was essential. Evidence in the literature [12] points to two major hydrolytic pathways for an imidoester, giving either an oxygen ester and ammonia or an amide and an alcohol. We have found that under our experimental conditions at least 84 % of the

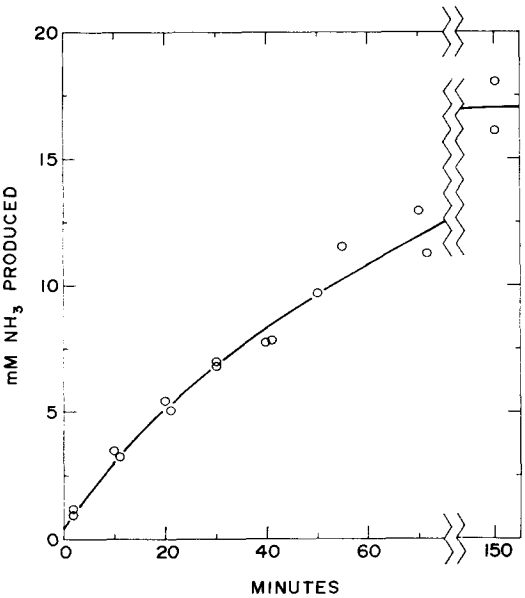

Fig. 2. Time course of hydrolysis of dimethylsuberimidate. Dimethylsuberimidate, 10 mM, was allowed to hydrolyze in 0.1 M triethanolamine buffer, pH 7.0, and the ammonia produced at various times was measured as described in the Methods section.

hydrolysis yields ammonia. Consequently the kinetics of hydrolysis were followed by determination of released ammonium ions with Nessler's reagent. This method is more convenient than the method used by Hunter and Ludwig [13], involving the determination of the imidoester remaining in the course of hydrolysis by acid-base titration.

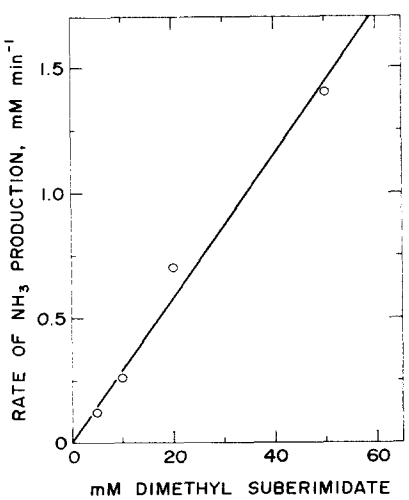

Fig. 3. Rate of ammonia production as a function of dimethylsuberimidate concentration. Conditions and assays were as described with Fig. 2.

Fig. 2 shows a plot of the time course of ammonia liberation on hydrolysis of dimethylsuberimidate at pH 7.0. The data indicate a first-order reaction, as do those in Fig. 3 from initial rate measurements at increasing concentrations of imidoester. The apparent first-order rate constant calculated from these data is 0.016 ± 0.002 min^{-1} corresponding to a half-time of 42 min.

It should be mentioned that our data are not extensive, and do not suffice to show that the hydrolysis is strictly first order. As the monoimidoester is an intermediate in the hydrolysis, slight deviation from first order kinetics would occur if it releases ammonia at a different rate than the diimidoester.

Inhibition of Ca^{2+} accumulation by hydrolysis products

The relative capacity of dimethylsuberimidate and its hydrolytic products to inhibit Ca^{2+} uptake by sarcoplasmic reticulum was assessed by exposing the latter for a short period of time to a solution of dimethylsuberimidate which has been allowed to undergo hydrolysis to various extents. The results given in Fig. 4 show that freshly prepared dimethylsuberimidate has very little effect on Ca^{2+} uptake capacity of sarcoplasmic reticulum vesicles, but that fully hydrolyzed products are inhibitory. This somewhat surprising finding is supported by the results in Fig. 5, showing the

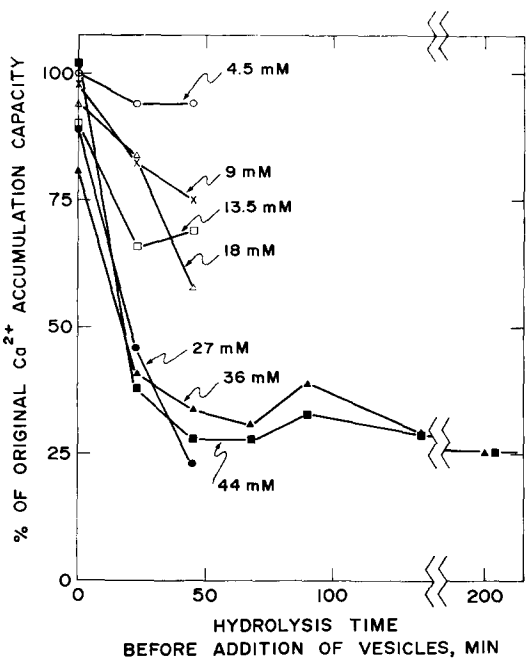

Fig. 4. Effect of fresh and hydrolyzed dimethylsuberimidate on Ca^{2+} uptake. Sarcoplasmic reticulum vesicles (4 mg/ml) were incubated in 0.1 triethanolamine buffer, pH 7.0, with indicated concentrations of dimethylsuberimidate which had been allowed to undergo hydrolysis in 0.11 M triethanolamine buffer, pH 7.0, for various lengths of time. After 4 min, 0.05 ml of this mixture was added to 1.0 ml of the Ca^{2+} uptake mixture and the Ca^{2+} uptake capacity was determined after another 2 min as described in the Methods section. Abscissa values indicate total time before addition to the Ca^{2+} uptake mixture.

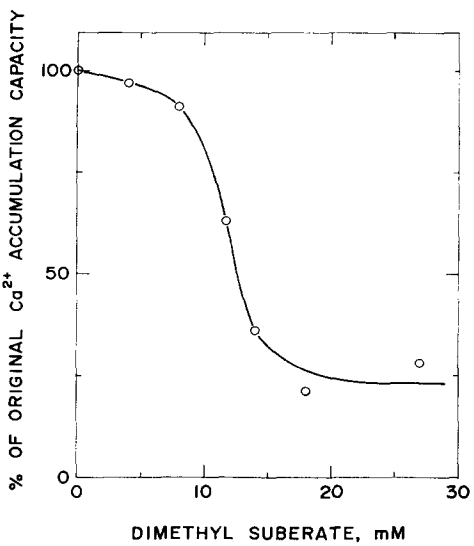

Fig. 5. Inhibition of Ca^{2+} accumulation capacity by dimethylsuberate. Sarcoplasmic reticulum vesicles (4 mg/ml) were incubated with dimethylsuberate for 4 min in 0.1 M triethanolamine buffer, pH 7.0. Then 0.05 ml was added to 1.0 ml of the Ca^{2+} uptake mixture and the Ca^{2+} uptake capacity was determined after another 2 min as described in the Methods section. The saturating concentration of the ester is approximately 18 mM.

TABLE II

INHIBITION OF Ca^{2+} UPTAKE AFTER TREATMENT WITH PARTIALLY HYDROLYZED DIMETHYLSUBERIMIDATE

Vesicles were treated as described in the legend of Fig. 2. The values of observed percentage inhibition are compared with those calculated assuming that they are determined only by the total concentration of ester function produced, and using the inhibition curve in Fig. 3.

Original suberimidate concentration (mM)	Observed inhibition (%)	Inhibition calculated from ester concentration (%)
Time of hydrolysis = 27 min		
9	18	2
13.5	34	3
18	16	4
27	54	9
36	59	27
44	62	33
Time of hydrolysis = 49 min		
9	25	3
13.5	21	6
18	42	11
27	77	45
36	66	71
44	72	72

inhibitory effect of dimethylsuberate, the ester formed as the principal hydrolysis product, on Ca^{2+} uptake. Above the concentration af about 8 mM the ester has an increasing effect up to the saturating concentration of about 18 mM. Methanol at 124 mM shows little effect on the Ca^{2+} uptake.

These results indicate the inhibition of Ca^{2+} uptake observed with fully hydrolyzed products of dimethylsuberimidate can be accounted for largely or wholly by the dimethylsuberate produced. It is interesting to note, as Table II shows, that partially hydrolyzed solution of dimethylsuberimidate shows a greater inhibitory power than would be expected from the total amount of ester function produced, indicating that a half-hydrolyzed molecule with an imidoester function remaining at one end can react with a membrane amino group and exert a greater effect than either dimethylsuberimidate or dimethylsuberate.

The observed effect of hydrolysis products of dimethylsuberimidate may be related to recent findings of Kondo and Kasai on the effect of *n*-alcohols on sarcoplasmic reticulum functions [14]. These workers found that with increasing chain length the alcohols show a greater inhibitory power, and if it is assumed that the effect is caused by partitioning of the alcohols between water and sarcoplasmic reticulum vesicles, the drop in free energy per each methylene group added is similar to that in the transfer from water to *n*-octanol.

Although our results show that effects of dimethylsuberimidate on Ca^{2+} accumulation by sarcoplasmic reticulum are not attributable to cross-linking, it is obviously quite possible that under other conditions effects due to crosslinking may be observed.

Dimethylsuberimidate is gaining rather wide use as a cross-linking agent. Although it may be quite valuable for this purpose, the possible effect of hydrolysis products needs adequate consideration in studies with this reagent.

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