Synthesis and Function of 9,12,15-Octadecatrien-6-ynoic Acid in the Moss *Ceratodon purpureus*†

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ABSTRACT: Biosynthesis of *all-cis*-9,12,15-octadecatrien-6-ynoic acid in the moss, *Ceratodon purpureus*, was studied using protonemata cultures with labeled 9,12,15-octadecatrienoic (linolenic) and 6,9,12,15-octadecatetraenoic acids as substrates. Both acids were efficiently converted into the acetylenic and into 5,8,11,14,17-eicosapentaenoic acids. Accordingly, the introduction of a triple bond in position 6 of linolenic acid involves formation of a double bond as a discrete step. Acetylenic acid triglycerides are reserve lipids in the moss. Under suitable growth conditions the acetylenic acids are catabolized and partly reused via acetate for de novo synthesis of fatty acids. They are not used for more direct syntheses of the common polyunsaturated fatty acids.

The occurrence of 5,8,11,14-eicosatetraenoic[1] and 5,8,11,14,17-eicosapentaenoic acids in lipids of cryptogams, in particular of mosses (Schlenk and Gellerman, 1965), indicates that linoleic and linolenic acids can be elongated and desaturated by these plants. Another type of conversion not encountered in higher plants is suggested by the presence of 9,12,15-octadecatrien-6-ynoic, 9,12-octadecadien-6-ynoic, and of 11,14-eicosadien-8-ynoic acids in lipids of some mosses (Åndersson et al., 1974; Anderson et al., 1975). According to their double bond structures, these acetylenic acids may arise from linolenic and linoleic acids by desaturation of the C-6,7 ethane to an acetylene bond. The purpose of the work reported here was to test if such a pathway applies to the biosynthesis of the new acetylenic fatty acids and to explore their further metabolic course.

The moss *Ceratodon purpureus* contains up to 50% 6≡18:3($n-3$) in the total fatty acids (Swanson et al., 1976) and, therefore, was selected for this investigation. By use of axenic cultures, it was found that 18:3($n-3$) and 18:4($n-3$) are precursors in the biosynthesis of 6≡18:3($n-3$) acid.

The acetylenic acids were detected only in triglycerides (Åndersson et al., 1974) which accumulate in the cytoplasm of nutritionally stressed tissue (Swanson et al., 1976). When conditions for normal growth are restored, they are utilized mainly for energy and, to some extent, for fatty acid synthesis via acetate.

Materials and Methods

Materials. Sodium [1-^{14}C]acetate (2 mCi/mmol) and [1-^{14}C]linolenic acid (52 mCi/mmol) were obtained commercially. The radiopurity of the latter was greater than 98% according to thin-layer chromatography and gas–liquid chromatography. [U-^{14}C]-*all-cis*-6,9,12,15-Octadecatetraenoic acid (0.26 mCi/mmol) was prepared biosynthetically in this laboratory (Gellerman and Schlenk, 1965). It contained radioactive contaminations of 5% 18:3($n-3$) and 1% 18:3($n-6$).

Axenic liquid cultures of the moss, *Ceratodon purpureus*, were established by aseptic inoculation of spores into Mueller's modified liquid medium (Mueller, 1973) as previously described (Swanson et al., 1976). The germinated protonemata were grown at 18–20 °C under an 18-h 2700 lux light–6 h dark cycle. After 4 weeks, media were renewed in all cultures. During the subsequent period, fresh media were added at intervals of 11 days to some cultures, while others were kept without addition of new media.

Incubations. Incubations of labeled acids were carried out with cultures in 20 mL of media which had not been renewed for a period of at least 5 weeks. Under such conditions, a culture contained 30–40 mg of dry weight of tissue with 10–14% lipid. Solutions of sodium acetate or ammonium salts of fatty acids (between 1.5 and 2.5 μCi) were prepared in sterile water and added aseptically to the cultures. The weight of 18:3 added was negligible whereas that of 18:4, due to its lower activity, was about 10% in reference to endogenous lipids of the cultures. Incubations for 24 h or less were under continuous light, but the above light–dark cycle was maintained when the periods were longer.

Analyses. Moss tissue was collected by suction on a filter stick and extracted in an Omnimixer with $CHCl_3 + CH_3OH$ (Åndersson et al., 1974). After addition of 0.2 volume of 0.9% aqueous NaCl, the lipids were recovered from the $CHCl_3$ phase by Rotovac at 40 °C. Yields were 3 to 5 mg of lipids per culture. Up to four cultures were used for each incubation experiment.

Distribution of ^{14}C among lipid classes was determined by thin-layer chromatography of aliquots on Silica Gel H using hexane–diethyl ether–acetic acid (70:30:2, v/v) and chloroform–methanol–10 M NH_4OH (65:30:4) as solvents. Spots were localized by exposure to I_2 vapor and were identified by reference to chromatograms of authentic compounds. For extraction and scintillation counting, they were scraped into vials containing 1 mL of methanol to which then was added 15 mL of toluene based scintillation fluid. Radioactivities of the lipid classes and free precursor acids were calculated from results of the two chromatographic fractionations.

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[1] Abbreviation used: UV, ultraviolet. Abbreviations for the common fatty acids are according to IUPAC-IUB Commission (1970), *J. Biol. Chem. 245*, 1511; for example, 20:4($n-6$) = *all-cis*-5,8,11,14-eicosatetraenoic, arachidonic acid. In addition, 6≡18:3($n-3$) = *all-cis*-9,12,15-octadecatrien-6-ynoic acid; 6≡18:2($n-6$) = *cis,cis*-9,12-octadecadien-6-ynoic acid.

TABLE I: Fatty Acid Composition[a] of Lipids from Axenic Cultures of *Ceratodon purpureus*.

Weeks:	0[b]	5	6	8	8
Media Added:		—	—	—	4×
Fatty acids					
16:0	16.8	10.5	5.8	15.0	18.6
16:1	3.5	0.8	1.0	1.4	2.0
16:2	1.9	3.8	3.8	1.2	2.4
16:3	7.2	6.8[c]	5.6[c]	1.6	2.2
18:0	1.0			1.3	1.2
18:1	2.4	2.5	4.1	1.4	1.6
18:2($n-6$)	11.4	19.3	11.2	13.6	16.4
18:3($n-6$)	0.9	0.7	3.4	1.4	1.4
18:3($n-3$)	32.9	25.5	17.7	13.4	19.8
18:4($n-3$)	0.8	0.3	1.0	1.1	1.0
20:3($n-6$)	0.4	1.7[c]	4.2[c]	0.7	0.9
6≡18:2($n-6$)	1.6			7.4	2.2
6≡18:3($n-3$)	2.8	15.5	24.3	27.8	14.9
20:4($n-6$)	10.9	10.3	12.8	10.0	10.3
20:5($n-3$)	1.8	0.6	1.0	2.0	2.0

[a] Area percent of total fatty acid methyl esters in gas chromatogram recordings. [b] The experimental period began 4 weeks after inoculation. At this time new media were given to all cultures. [c] Not resolved in these chromatograms.

Fatty acid methyl esters were obtained from aliquots of total lipids by interesterification with 5% anhydrous HCl in methanol at 90 °C for 1 h. The esters were purified by thin-layer chromatography on Silica Gel H using hexane–diethyl ether–acetic acid (80:20:1) as developing solvent. Samples were applied and plates were dried after chromatography in a nitrogen chamber (Gellerman et al., 1972). The dichlorofluorescein-UV method was used for locating the esters which were then extracted with chloroform. They were analyzed by gas–liquid chromatography at 190 °C over ethylene glycol succinate, 15% on 100–120 mesh Gas Chrom P and over diethylene glycol succinate, 20% on siliconized Chromosorb W. Radioactivities of the esters were determined by collection and scintillation counting (Gellerman and Schlenk, 1965).

Each incubation of the highly unsaturated fatty acids was accompanied by a blank run to check for autoxidation. Aliquots of the NH_4 salts were added to the media and kept under identical conditions, without tissue, for the same time period. The acids were extracted, esterified, and then mixed with methyl esters from nonradioactive cultures. Their analyses were carried out exactly as were those of samples from the radioactive incubations with tissue. Total ^{14}C in autoxidation products and chromatographic artefacts amounted to less than 8%. Some of this radioactivity was in areas of the gas–liquid chromatograms which were pertinent to the conclusions and appropriate corrections were made.

Location of ^{14}C. Radioactivity in the carboxyl group of 9,12,15-octadecatrien-6-ynoic acid was determined by decarboxylation and counting of $^{14}CO_2$ (Schlenk et al., 1969). The acetylenic methyl ester obtained from incubations with [1-^{14}C]linolenic acid was isolated by gas–liquid chromatography, hydrogenated over PtO_2 in methanol, diluted with 20 mg of methyl stearate, and saponified. Total radioactivity of the acid was determined from an aliquot. Decarboxylation by N_3H and absorption of $^{14}CO_2$ in hydroxide of Hyamine 10-X (Packard Instrument Co.) were carried out in duplicate. The radioactivity in Hyamine was counted after mixing with 15 mL of toluene based scintillation fluid. Quench factors were 3–5% as determined by adding [1-^{14}C]palmitic acid after the initial counting. Yields of $^{14}CO_2$ were between 91 and 94% in four experiments using [1-^{14}C]palmitic or stearic acid. The latter was obtained by hydrogenation of an aliquot of the [1-^{14}C]linolenic acid which had been used as substrate in the incubations.

Acetylenic Triglycerides. Triglycerides containing the ^{14}C-labeled acetylenic acids were prepared by incubating [1-^{14}C]acetate (5 mCi) with *C. purpureus* cultures for 1 week. Lipids were recovered as described above with a yield of 20 mg (0.7 mCi). The acetylenic acid triglycerides were isolated by thin-layer chromatography using hexane–diethyl ether–acetic acid (70:30:2) and contained 10 mCi/mmol of acid. Analysis of methyl esters from an aliquot showed 86% of ^{14}C in 6≡18:3($n-3$) and 6% in 6≡18:2($n-6$) acids.

Aliquots of these labeled triglycerides (12.5 μg, 0.4 μCi) in aqueous ethanol were added to cultures of *C. purpureus* from which media had been withheld for 8 weeks. After 24 h, media were withdrawn from one culture, the tissues rinsed thoroughly, and the lipids extracted and counted. The results showed satisfactory incorporation. Media were then withdrawn from all other cultures, the tissues rinsed thoroughly, and fresh media were added for further incubation. These cultures were harvested after 24, 48, and 120 h to follow the metabolism of the acetylenic triglycerides by analytical procedures as described above.

Results

The acids 6≡18:3($n-3$) and 6≡18:2($n-6$) occur in gametophores of the moss *Ceratodon purpureus* in the field at widely different levels depending on the environment (Swanson et al., 1976). The lipid composition can be influenced under controlled conditions in liquid cultures of the protonemata. When media were periodically supplied to the cultures, 6≡18:3($n-3$) increased over a period of 8 weeks from 2.8 to 14.9% of total fatty acids, but without new media, it increased to nearly twice that level (Table I). The same trend of greater accumulation under these conditions was observed with 6≡18:2($n-6$). According to comparisons after different time periods, ≡18:3 became particularly prominent in cultures from which new media had been withheld for longer than 5 weeks.

Some randomization of ^{14}C via acetate was to be expected from incubations with labeled polyunsaturated precursors. Experiments with [1-^{14}C]acetate showed that it was used for synthesis of all acids during this period of growth (Table II). After 12 h incubation, most of the incorporated ^{14}C was in 16:0, 18:1, and 18:2($n-6$), whereas 6≡18:3($n-3$) contained ^{14}C at a much lower level. Accordingly, the polyunsaturated precursors were incubated for 12–24 h to minimize de novo synthesis of the acetylenic acids via acetate.

Between 35 and 50% ^{14}C from both 18:3($n-3$) and 18:4($n-3$) was incorporated into lipids (Table II), although the latter acid had been given in much larger amounts. [1-^{14}C]Linolenic acid yielded ^{14}C levels in 18:4($n-3$), 6≡18:3($n-3$), and 20:5($n-3$) which were much higher than in 16:0, 18:1, and 18:2($n-6$). The effect of the acetate pool was somewhat greater from [U-^{14}C]18:4($n-3$). Nevertheless, ≡18:3 and 20:5, the latter with one exception, were radioactive at higher levels than the de novo synthesized acids. The difference between sequential and de novo acids becomes obvious when the pertinent ratios of ^{14}C labeling are compared. Ratios of ^{14}C in sequential acids (A) to ^{14}C in de novo acids (B) are much larger from labeled 18:3 and 18:4 than from acetate (Table III). It can be concluded that most of the ^{14}C in 6≡18:3($n-$

TABLE II: Incorporation of ^{14}C into Fatty Acids of *Ceratodon purpureus*.[a]

Substrate:	[1-^{14}C]18:3($n-3$)	[U-^{14}C]18:4($n-3$)		[1-^{14}C]Acetate	
Media Withheld (weeks):	12	12	5	5	6
Incubations (h):	18	24	12	12	168
Lipids:	50 (17)[b]	35 (1)[b]	44 (17)[b]	16	28
Fatty acids[c]					
16:0	0.1	0.8	0.3	19.9	16.8
16:1	+	0.1	+	1.6	1.8
16:2	+	0.1	+	1.7	3.4
16:3 / 18:0 [d]	+	0.6	0.2	1.3	2.9
18:1	0.1	0.9	0.3	13.8	2.9
18:2($n-6$)	0.2	2.3	0.4	36.1	22.8
18:3($n-6$)		0.4	0.1	3.9	3.1
18:3($n-3$)	82.6		0.6	4.1	13.1
18:4($n-3$)	6.0[e]	83.6	92.7	1.1	0.9
20:3($n-6$) / 6≡18:2($n-6$) [d]	0.1			5.9	2.8
6≡18:3($n-3$)	8.6[f]	8.3[g]	3.3[h]	4.3	19.6
20:4($n-6$)	0.3			1.5	3.9
20:5($n-3$)	1.0[f]	1.8[i]	2.1[i]	0.6	1.2

[a] Percent of ^{14}C offered. [b] Additional free substrate acid. [c] Percent of total ^{14}C in fatty acids, including free substrate acid. [d] Not resolved. [e] Corrected for trailing of 18:3($n-3$), 0.3%. [f] Corrected for autoxidation products, 0.1%. [g] Corrected for autoxidation products, 0.8%. [h] Corrected for autoxidation products, 1.0%. [i] Corrected for autoxidation products, 0.3%.

TABLE III: Ratios of ^{14}C in Fatty Acids from Different Substrates.

Substrate:	^{14}C in A[a]:^{14}C in B[b]			
	[-^{14}C]-18:3($n-3$)	[U-^{14}C]-18:4($n-3$)		[1-^{14}C]-Acetate
Media Withheld (weeks):	12	12	5	5
Incubation (h):	18	24	12	12
A 6≡18:3($n-3$)				
B 16:0	86	9	10	0.2
B 18:1	86	9	11	0.3
B 18:2($n-6$)	43	3.5	9	0.4
A 20:5($n-3$)				
B 16:0	10	2	6.5	0.03
B 18:1	10	2	7	0.04
B 18:2($n-6$)	5	0.8	6	0.02

[a] A, acids sequential to substrate fatty acids. [b] B, acids synthesized de novo.

3) and 20:5($n-3$) originated from the polyunsaturated precursors without degradation.

This was verified for 6≡18:3($n-3$) from [1-^{14}C]18:3($n-3$) by decarboxylation. The radioactivities obtained in CO_2 were 86.7 and 86.4% of the total activity in the molecule. In reference to the yields of CO_2 from [1-^{14}C]18:3($n-3$), more than 92% of ^{14}C was in the carboxyl group of the acetylenic acid.

Incubation of [1-^{14}C]acetate with *C. purpureus* protonemata for 168 h yielded 20% of ^{14}C in fatty acids as ≡18:3 (Table II). Since the acetylenic acids are accumulated in triglycerides which contain only small amounts of the common fatty acids, these triglycerides can be separated from others by thin-layer chromatography. The acetylenic acid triglycerides from acetate were isolated with a radioactive yield of 2.8%. The radioactive purity of their acetylenic acids was 92% (see first column, Table IV).

When offering the acetylenic acid triglycerides to starved *C. purpureus* protonemata, nearly 40% ^{14}C was incorporated within 24 h. Radioactivity did not appear in phospho- or glycolipids, nor was a significant conversion of ≡18:3 into other acids indicated. After removal of substrate and renewal of media, the level of ^{14}C in tissue lipids decreased to one-third within an additional 24 h period. An appreciable amount of this remaining activity was found in de novo synthesized acids, such as 16:0, 18:1, and 18:2($n-6$). The absolute amount of ^{14}C in these acids increased, while that in acetylenic acids decreased.

Discussion

The introduction of an acetylenic bond into a fatty acid containing three methylene interrupted double bonds is a pathway previously unknown for biosyntheses of acetylenic lipids. In synthesis of 9,12,15-octadecatrien-6-ynoic acid in the moss, the unsaturated system of linolenic acid is extended to form 6,9,12,15-octadecatetraenoic acid. The double bond in position 6 of the intermediate is then further desaturated to a triple bond. The intermediate acid is also precursor for 5,8,11,14,17-eicosapentaenoic acid. Since the tetraenoic acid, added as substrate to moss tissue yields both 6≡18:3($n-3$) and 20:5($n-3$), there is no reason to assume separate enzyme systems for synthesis of the olefinic intermediate to yield one or the other of these products. By implication, one might well assume 6,9,12-octadecatrienoic (γ-linolenic) acid to be the intermediate for synthesis of both 9,12-octadecadien-6-ynoic acid and 5,8,11,14-eicosatetraenoic (arachidonic) acid in *C. purpureus*.

The acetylenic acids in mosses exemplify, like arachidonic and 20:5($n-3$) acids, synthetic capabilities which in the plant kingdom are restricted to cryptogams. Octadeca-6-ynoic (tariric) acid, a constituent of picramnia oil, was the first natural acetylenic fatty acid to be discovered (Arnaud, 1892), but C_{18}-6-ynoic acids with additional unsaturation have not been reported from higher plants (Smith, 1970). 9-Octadecen-12-ynoic (crepenynic) acid became of much greater interest since it is precursor for many acetylenic fatty acids

TABLE IV: Incorporation and Distribution of Labeled 6≡18:3(n − 3) in *Ceratodon purpureus*.

Incubation (h):	0	24		28 + 24[a]	
Lipids[c]:	% ^{14}C[b] (100)[d]	% ^{14}C 39	dpm 3.45×10^5	% ^{14}C 14[e]	dpm 1.2×10^5
Fatty acids					
16:0	1.4	2.8	9 500	14.9	17 400
16:1	0.06	0.09		0.41	
16:2	0.14	0.21		1.1	
16:3 / 18:0 [f]	0.65	1.0		3.8	
18:1	0.52	0.81	2 700	2.8	3 300
18:2(n − 6)	0.49	0.86	2 900	4.1	4 700
18:3(n − 6)	0.14	0.18		0.67	
18:3(n − 3)	0.51	0.72	2 400	2.2	2 600
18:4(n − 3)	0.33	0.34		0.54	
20:3 / 6≡18:2(n − 6) [f]	5.9	6.7	22 600	6.1	7 100
6≡18:3(n − 3)	86.0	82.3	275 700	55.5	65 000
20:4(n − 6)	0.43	0.21		0.85	
20:5(n − 3)	1.0	0.89		0.88	

[a] Time period with new media. [b] Radioactive composition of acids in substrate triglycerides. [c] Percent of ^{14}C offered. [d] 8.6×10^5 dpm was offered. [e] After 48 and 120 h with new media, the values were 9.1 and 5.1%. [f] Not resolved.

(Hitchcock and Nichols, 1971; Bohlmann et al., 1973a). Oleic was found to be an efficient precursor of crepenynic acid (Bu 'Lock and Smith, 1967), but the role of linoleic acid, which would be the intermediate corresponding to 18:4(n − 3) in our investigation, is somewhat in doubt and may be different from system to system. Linoleic acid has been proposed as intermediate in crepenynic acid synthesis (Bohlmann, 1973a; Bohlmann et al., 1973b) but direct experimental evidence is lacking. It served as precursor for cyclic C_{13} acetylene compounds where crepenynic acid was postulated as intermediate (Bohlmann and Schulz, 1968). However, in other experiments, exogenous linoleic acid was not converted into crepenynic acid (Haigh and James, 1967; Haigh et al., 1968). In the latter system, endogenous linoleic acid may remain enzyme bound as a short-lived intermediate.

The demonstration of two discrete desaturation steps for the synthesis of 6≡18:3(n − 3) does not imply the same for the synthesis of crepenynic acid. Likewise, the nonconversion of linolenic into acetylenic acids of higher plants (Bohlmann, 1973b) does not apply to the formation of 6≡18:3(n − 3) acid. In the moss, desaturation to the triple bond takes place in the proximal part of the molecule and, in contrast to many other polyunsaturated acetylenic acids, the polyunsaturated system remains nonconjugated.

The polyenynoic acids of mosses are found only in triglycerides. Also when re-fed to the tissue, their presence in glyco- and phospholipids was not indicated. Thin-layer chromatography of lipids from the 24-h incubation showed only minor amounts of ^{14}C in polar lipids, while incubation for 28 + 24 h yielded approximately 40% of the remaining ^{14}C in polar lipids. The values were rather consistent with ^{14}C in de novo synthesized acids of the two samples (see Table IV).

A corollary to these observations in mosses was encountered with fish. After feeding labeled acetylenic triglycerides to gouramis, 6≡18:3(n − 3) acid was found in neutral, but not in polar lipids of the carcass, liver or roe (unpublished).

Acylation of phospholipids from acetylenic CoA esters in rat liver microsomes has been studied (Tamai et al., 1973). The rate of acyl transfer from octadeca-6-ynoyl-CoA to position 2 was very low whereas to position 1 it was high. Our experiments do not rule out brief transient incorporation of 6≡18:3(n − 3) into polar lipids. The pertinent observation made here is that the triple bond in position 6 greatly alters the properties of linoleic and linolenic acids in regard to incorporation into complex lipids or their turnover as constituents of such.

Triglycerides are often a reserve in plant metabolism and, most likely, this is also the role of the acetylenic triglycerides in moss. The similarity of structures suggested correctly the origin of 6≡18:3(n − 3) from 18:3(n − 3) acid. The similarity of structures may also suggest that the reserve of acetylenic acid triglycerides is a specific source for synthesis of linolenic acid. Our experiments with the triglycerides added to protonemata tissue do not support such a hypothesis. The acetylenic acids were mainly catabolized for energy and any conversion into other fatty acids proceeded via acetate.

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Fluorescence Energy Transfer between Ca^{2+} Transport ATPase Molecules in Artificial Membranes†

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ABSTRACT: The purified ATPase of sarcoplasmic reticulum was covalently labeled with *N*-iodoacetyl-*N'*-(5-sulfo-1-naphthyl)ethylenediamine (1,5-IAEDANS) or with iodoacetamidofluorescein (IAF). In reconstituted vesicles containing both types of ATPase molecules fluorescence energy transfer was observed from the IAEDANS (donor) to the IAF (acceptor) fluorophore as determined by the ratio of donor and acceptor fluorescence intensities, and by nanosecond decay measurements of donor fluorescence in the presence or absence of the acceptor. The observed energy transfer may arise by random collisions between ATPase molecules due to Brownian motion or by formation of complexes containing several ATPase molecules. Experimental distinction between these two models of energy transfer is possible based on predictions derived from mathematical models. Up to tenfold dilution of the lipid phase of reconstituted vesicles with egg lecithin had no measurable effect upon the energy transfer, suggesting that random collision between ATPase molecules in the lipid phase is not the principal cause of the observed effect. Addition of unlabeled ATPase in five- to tenfold molar excess over the labeled molecules abolished energy transfer. These observations together with electron microscopic and chemical cross-linking studies support the existence of ATPase oligomers in the membrane with sufficiently long lifetimes for energy transfer to occur. A hypothetical equilibrium between monomeric and tetrameric forms of the ATPase governed by the membrane potential is proposed as the structural basis of the regulation of Ca uptake and release by sarcoplasmic reticulum membranes during muscle contraction and relaxation.

Sarcoplasmic reticulum membranes operate in two distinct functional states during the contraction-relaxation cycle. (1) The relaxation of muscle is initiated by the ATP-mediated accumulation of Ca^{2+} into the sarcoplasmic reticulum tubules which lowers the cytoplasmic Ca^{2+} concentration below 10^{-7} M (MacLennan and Holland, 1976). (2) During excitation the accumulated Ca^{2+} is rapidly released from sarcoplasmic reticulum. The rate of this process is too great to be explained by a simple reversal of the Ca pump (Martonosi, 1972) and presumably involves the formation of Ca^{2+} channels. The Ca^{2+} release is accompanied by depolarization of the sarcoplasmic reticulum membrane as indicated by changes in birefringence (Baylor and Oetliker, 1975) and membrane potential (Bezanilla and Horowicz, 1975). The only hint that a similar process may occur in vitro is the observed Ca^{2+} release from skeletal muscle microsomes under conditions which are assumed to alter membrane potential (Kasai and Miyamoto, 1976).

The Mg^{2+} + Ca^{2+} activated ATPase of sarcoplasmic reticulum plays a major role in the active accumulation of calcium and in the regulation of the passive Ca^{2+} permeability of the membrane.

Cooperativity in the dependence of Ca^{2+} transport and phosphoprotein formation upon the free Ca^{2+} concentration (Coffey et al., 1975), together with indications of "half of the sites" reactivity (Martonosi et al., 1974), provided the first evidence that interaction between elements of the Ca^{2+} transport complex constitutes an important aspect of active Ca^{2+} uptake. Further indication of interaction between Ca^{2+} transport ATPase molecules in the membrane was the demonstration that the number of 85-Å intramembranous particles revealed in native membranes and in reconstituted ATPase vesicles by freeze-etch electron microscopy was 4–5 times less than the number of 40-Å surface particles seen after negative staining (Jilka et al., 1975). As both sets of particles are as-

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