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INHIBITION OF PROTEIN SYNTHESIS BY PRODUCTS OF LIPID PEROXIDATION

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Key Word Index—*Tortula ruralis*; *Cratoneuron filicinum*; mosses; *Pisum sativum*; Leguminosae; pea; lipid peroxidation; malondialdehyde; protein synthesis.

Abstract—Effects of lipid peroxidation products on *in vivo* and *in vitro* protein synthesis have been studied. Malondialdehyde (MDA), a product, and a routinely used index of lipid peroxidation, inhibits *in vivo* protein synthesis in the two mosses, *Tortula ruralis* and *Cratoneuron filicinum*, and in pea (*Pisum sativum*) leaf discs. When wheat germ supernatant or poly(A)-rich mRNA of *T. ruralis* was incubated with MDA its subsequent activity in a cell-free protein-synthesizing system was reduced. When MDA was added directly to the *in vitro* protein-synthesizing mixture containing moss polyribosomes, the inhibition of amino acid incorporation was small. However, when simultaneous lipid peroxidation was allowed to occur along with *in vitro* protein synthesis there was a strong inhibition of amino acid incorporation and MDA accumulated in the reaction mixture indicating that products of lipid peroxidation other than, and apparently more toxic than, MDA were involved. It was concluded that lipid peroxidation inhibits protein synthesis probably by releasing toxic products which may react with and inactivate some components of the protein-synthesizing complex.

INTRODUCTION

Lipid peroxidation occurs routinely in animal and plant tissues. Thus increased lipid peroxidation has been correlated with the membrane deterioration associated with ageing of animal tissues [1] and foliar senescence in plants [2]. Furthermore, it appears to mediate photoperoxidative destruction of chlorophyll [3] and the drought-induced increase in membrane permeability [4]. Thus, lipid peroxidation appears to modulate important cellular activities. However, its effects on protein synthesis in plant tissues do not appear to have been studied.

Since protein synthesis is an important cellular activity, the present investigation was undertaken as a preliminary study of the effects of lipid peroxidation products on protein synthesis in two mosses, *Tortula ruralis* and *Cratoneuron filicinum*, and pea (*Pisum sativum*) leaf discs. The two mosses were selected since they have been studied earlier and show pronounced differences with respect to drought effects on lipid peroxidation [4] and on protein synthesis [5–8]. The pea tissue was included to determine if lipid peroxidation had similar effects on protein synthesis in a higher plant tissue.

The results obtained from this study show that lipid peroxidation products inhibit *in vivo* and *in vitro* protein synthesis.

RESULTS

Effect of malondialdehyde on in vivo *protein synthesis*

The inhibition of amino acid incorporation into proteins by malondialdehyde (MDA) in the three plant tissues included in this study is shown in Fig. 1. At each concentration the inhibition is much greater in *C. filicinum* and *P. sativum* than that in *T. ruralis*. Thus 0.5 mM MDA causes *ca* 50% inhibition in *C. filicinum* and *P. sativum* but has only a small effect on *T. ruralis*. Likewise, 5 mM MDA inhibits protein synthesis by more than 85% in *C. filicinum* and *P. sativum* and by *ca* 60% in *T. ruralis*.

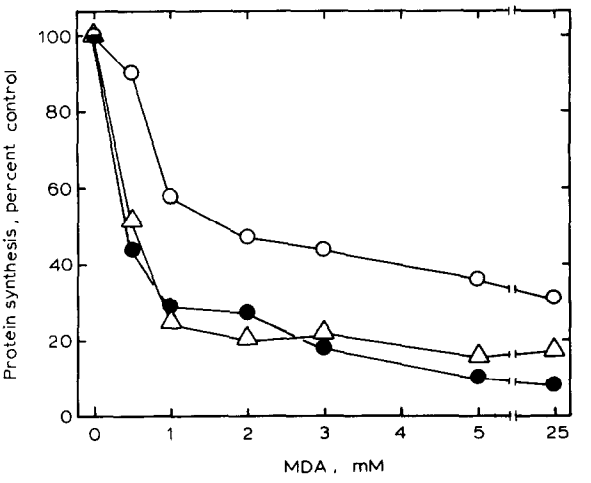

Fig. 1. Effect of various concentrations of malondialdehyde (MDA) on *in vivo* protein synthesis in *T. ruralis* (○), *C. filicinum* (●) and *P. sativum* (△). The results are expressed as percentage of protein synthesis in untreated control tissue. The 100% values for *T. ruralis*, *C. filicinum* and *P. sativum* are 785 cpm/μg protein, 290 cpm/μg protein and 950 cpm/μg protein, respectively. Each value is a mean of three replicates.

The amount of total radioactivity (protein plus soluble) taken up by the tissue was not affected by concentrations

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of MDA up to 1 mM in the case of *C. filicinum* and *P. sativum* and up to 2 mM in the case of *T. ruralis* (data not presented). At higher concentrations of MDA there was a progressive reduction in the total radioactivity but the reduction in the soluble fraction (free amino acid) was never more than 40% of the reduction in the protein radioactivity at corresponding MDA concentration.

Thus it can be concluded that exogenously applied MDA is able to inhibit *in vivo* protein synthesis in *T. ruralis*, *C. filicinum* and *P. sativum*. Furthermore, the possibility is indicated that a small part of the inhibition of protein synthesis caused by higher concentrations of MDA may be due to a decreased uptake of the labelled amino acid (see Discussion).

Effect of malondialdehyde on the activity of mRNA and wheat germ supernatant

The template activity of moss poly(A)-rich mRNA with or without preincubation with MDA is shown in Table 1. Brome mosaic virus RNA, used as control messenger, shows template activity comparable to the one reported earlier [8]. When moss mRNA which has not been pretreated with MDA is used, the incorporation is 7430 cpm. However, when moss mRNA is used after being treated with MDA its template activity is reduced by more than 80%. Another sample of moss mRNA treated in parallel manner with water instead of MDA shows only a small decrease in template activity. It was also noted that when MDA is directly added to the reaction mixture just before the incorporation reaction is started, the inhibition of incorporation is only *ca* 25% (Table 1). Thus, it can be concluded that treatment of poly(A)-rich mRNA with MDA strongly reduces its subsequent template activity in directing amino acid incorporation *in vitro*. The addition of MDA directly to the reaction mixture, however, results in only a small inhibition of incorporation.

Effect of MDA on the activity of wheat germ supernatant during *in vitro* amino acid incorporation by moss polyribosomes is shown in Table 2. It can be seen that the total amino acid incorporation using MDA-treated wheat germ supernatant is only *ca* 30% of that obtained by using untreated supernatant. This is the case with polyribosomes extracted with or without the use of Triton X100.

Effect of simultaneous lipid peroxidation on in vitro *protein synthesis on moss polyribosomes*

In these experiments *in vitro* lipid peroxidation was generated simultaneously during *in vitro* amino acid incorporation. This was done by including in the incorporation mixture, NADPH and the membrane preparation containing NADPH-cytochrome *c* reductase activity. Furthermore, the polyribosomes used in these experiments were extracted from *T. ruralis* in two different ways, one including Triton X100 in the extraction medium and the other without the surfactant.

Table 1. Effect of MDA on the template activity of poly(A)-rich mRNA of *T. ruralis*. The non-specific incorporation in the absence of mRNA and of S-23 was respectively 785 cpm and 125 cpm. For procedures see Experimental section and ref. [8]

Source of template	[U-^{14}C]leucine incorporated (total cpm)
Brome mosaic virus RNA (10 μg)	58900
Untreated moss mRNA (10 μg)	7430
MDA-treated moss mRNA (10 μg)	2140
Untreated moss mRNA (10 μg) + 0.1 mM MDA	5900
Water-treated moss mRNA (10 μg)	6980

Table 2. Effect of MDA on the activity of wheat germ supernatant in an *in vitro* protein-synthesizing system using moss polyribosomes. The non-specific incorporation in the absence of polyribosomes was 438 cpm and in the absence of wheat germ supernatant was 385 cpm. For procedures see the Experimental section

Conditions	[U-^{14}C]leucine incorporation (total cpm)
(a) Polyribosomes extracted without Triton X100	
Complete system with untreated wheat germ supernatant	7790
Complete system with MDA-treated wheat germ supernatant	2640
(b) Polyribosomes extracted with Triton X100	
Complete system with untreated wheat germ supernatant	7860
Complete system with MDA-treated wheat germ supernatant	2560

In the presence of NADPH and the membrane preparation there is production of MDA and a large reduction in total amino acid incorporation (Table 3). This is true with ribosomes extracted with or without the use of Triton X100. In the case of polyribosomes extracted without the use of Triton X100 the addition of NADPH alone (i.e. no membrane preparation added) causes a considerable reduction in total amino acid incorporation and also results in the production of MDA. The addition of membrane preparation alone (i.e. NADPH not added) does not cause much change in total amino acid incorporation or MDA production. When MDA is directly added to the reaction mixture it causes *ca* 15% reduction in total amino acid incorporation.

The time course of amino acid incorporation and MDA accumulation in the reaction mixture in the presence and absence of simultaneous lipid peroxidation is shown in Fig. 2. The kinetics of amino acid incorporation in the absence of lipid peroxidation show that total amino acid incorporation reaches the maximum value in *ca* 45 min. The cumulative increase in the amount of MDA in the reaction mixture continues throughout the reaction time.

It is, therefore, concluded that in the presence of lipid peroxidation there is a large reduction in total amino acid incorporation *in vitro* and that the products of lipid peroxidation, e.g. MDA, are released into the reaction mixture.

DISCUSSION

The present study demonstrates, apparently for the first time, that products of lipid peroxidation can inhibit *in vivo* and *in vitro* protein synthesis in plant tissues.

Exogenously applied MDA inhibits *in vivo* protein synthesis more in *C. filicinum* and *P. sativum* than in *T.*

Table 3. Effect of simultaneous lipid peroxidation on *in vitro* protein synthesis using moss polyribosomes

	Conditions	[U-^{14}C]leucine incorporation (total cpm)	MDA produced (nmol)
(a)	Polyribosomes extracted without Triton X100		
	Complete system	7280	—
	+ 0.1 mM MDA	6030	—
	+ membrane (250 μg protein)	6950	—
	+ NADPH (1 mM)	3880	1.5
	+ NADPH + membrane	1860	5.3
(b)	Polyribosomes extracted with Triton X100		
	Complete system	7690	—
	+ 0.1 mM MDA	6750	—
	+ membrane (250 μg protein)	6830	—
	+ NADPH (1 mM)	6540	0.2
	+ NADPH + membrane	1480	4.6

ruralis. The reasons for this difference are not clear. It appears relevant, however, to point out that *T. ruralis* is able to rapidly control the level of MDA in its tissues [4]. Thus, these tissues may differ in their ability to metabolize MDA and the intracellular concentration of MDA in their tissues may be different. A reduction in the total amount of radioactivity (protein plus soluble) absorbed by the tissue in the presence of higher concentrations of MDA was noted. However, it is not clear from the present study whether this reduced uptake is a cause or a consequence of the reduced incorporation observed. In order to examine the effect of MDA on amino acid uptake, the rate of uptake should be studied in the absence of incorporation. Nevertheless, the present study does not rule out the possibility that a part of the inhibition of amino acid incorporation by higher concentrations of MDA may be due to a reduced uptake of the amino acid.

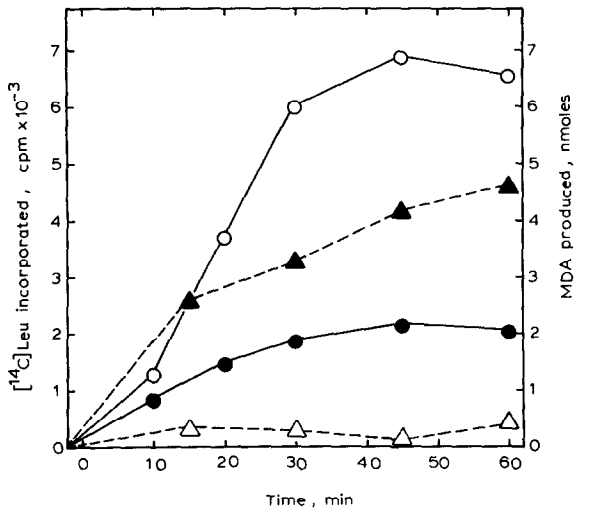

Fig. 2. Time course of *in vitro* amino acid incorporation (circles) and MDA production (triangles) in the presence (solid symbols) and absence (open symbols) of lipid peroxidation. Polyribosomes used in this experiment were extracted from *T. ruralis* without the use of Triton X 100. Each value is a mean of two replicates.

MDA is known to react with and modify the properties of proteins [9] and nucleic acids [10]. Thus, the inhibition of *in vivo* protein synthesis and the *in vitro* inactivation of mRNA and wheat germ supernatant by MDA observed in the present study may be due to such reactions of MDA as mentioned above, with the components of the protein synthesizing complex. It is noteworthy that when MDA is directly added to the *in vitro* protein-synthesizing system it causes only a small inhibition of amino acid incorporation. Probably the components of the protein synthesizing complex are less reactive to MDA when they are in complexed state than when they are in the dissociated form. Alternatively, the reaction with MDA may be relatively slower as compared with the reactions involved in the *in vitro* protein synthesis.

In the present study *in vitro* lipid peroxidation was generated by allowing the reaction between NADPH and a membrane preparation containing NADPH-cytochrome *c* reductase activity [11]. Generation of superoxide radical [12] and lipid peroxidation in liver microsomes [13] catalyzed by NADPH-linked cytochrome *c* reductase has been reported. Lipid peroxidation involves the production of fatty acid hydroperoxides [14] which are extremely reactive compounds [15]. That *in vitro* lipid peroxidation is in fact generated under the conditions used in this study is demonstrated by the production of MDA in the reaction mixture. The inhibition of *in vitro* amino acid incorporation caused by simultaneous lipid peroxidation is greater than the inhibition caused by the direct addition of MDA to the reaction mixture. Thus products of lipid peroxidation other than MDA appear to be involved in causing this inhibition. The nature of these products of lipid peroxidation is not clear at present. Since fatty acid hydroperoxides are known to be early intermediates of lipid peroxidation [14] and are known to be extremely reactive [15] it appears likely that they are involved in causing the inhibition of amino acid incorporation observed in the presence of lipid peroxidation. A similar inhibition of *in vitro* protein synthesis by lipid peroxidation products has been demonstrated in an animal system [16]. In the case of polyribosomes extracted without the use of Triton X100, MDA is produced in the presence of NADPH alone (membrane

preparation not added). A likely reason for this may be that these polyribosomes were complexed with membrane fragments containing some NADPH-linked cytochrome *c* reductase activity.

In conclusion, this study demonstrates that the products of lipid peroxidation can be released from the site of their production on the membrane and inactivate some components of the protein synthesizing complex. Thus, changes in the availability of lipid peroxidation products are likely to produce changes in protein synthesis.

EXPERIMENTAL

Plant material. The two mosses, *T. ruralis* (Hedw.) (Gaertn, Meyer and Scherb) and *C. filicinum* (Hedw.) (Spruce) were collected, stored and prepared for expts as described earlier [5, 6]. The apical 1 cm part of the gametophyte of each moss was used. Pea seedlings (*P. sativum* L. cv Alaska) were grown for 2 weeks in Vermiculite under a 18-hr photoperiod (8 m W/cm^2) and a day/night temp. of 23°/18°. Leaf discs, 1 cm diam., were cut from fully grown but non-senescent leaves.

Preparation of MDA. MDA was prepared from 1,1,3,3-tetraethoxypropane (ICN Pharmaceuticals, Plainview, NY) by the method of ref. [17]. The concn of MDA was determined by the method of ref. [3]. MDA soln was adjusted to a pH of 4.7 and was stored in small aliquots at −20°.

Determination of MDA content. MDA produced during lipid peroxidation in the *in vitro* protein synthesizing reaction mixture was measured by the thiobarbituric acid reaction [3].

Measurement of in vivo *protein synthesis.* Triplicate samples of 250 mg fresh moss or five leaf discs were incubated in 5 ml soln containing 10 μCi of [4,5-^{3}H]leucine (59 Ci/mmol). When the effect of MDA was to be studied, tissue was preincubated in MDA soln of varying concn for 30 min and then the radioactive leucine was added. Incorporation was allowed for 1 hr and then the tissue was washed with an ice-cold soln of 0.2 mg/ml carrier leucine and subjected to protein extraction by the method of ref. [18]. Aliquots of the protein extract were used to determine its protein content by the method of ref. [19] and its radioactivity by liquid scintillation spectrometry.

Preparation of components of in vitro *protein synthesis system.* The prepn of poly(A)-rich mRNA from 2 g fresh *T. ruralis*, and of wheat germ S-23 was as already described [8].

Polyribosomes were extrd from 500 mg fresh *T. ruralis* by the method of ref. [20] with or without Triton X100 in the extraction medium.

The prepn of wheat germ tRNA was according to the method of ref. [21] and the method for preparing wheat germ supernatant (166 500 *g*) is described in ref. [22].

Preparation of membrane fraction. The method employed was that of ref. [23] with modifications. 200 mg of slowly dried *T. ruralis* was used since this moss, when slowly dried, completely loses its polyribosomes [24]. Moss was slowly dried by the method of ref. [7]. Moss samples were homogenized in 7.5 ml of 150 mM tricine buffer, pH 7.5, containing 10 mM KCl, 1 mM $MgCl_2$, 1 mM EDTA (pH 7.5) and 0.4 M sucrose. The homogenate was centrifuged at 1000 *g* for 10 min and a 5-ml portion of the supernatant obtained was layered on top of a nonlinear sucrose gradient. This gradient consisted of a 6-ml cushion of 60%, a 15-ml linear gradient of 35–60%, a 5-ml layer of 28% and a 5-ml layer of 20% sucrose (w/w) contained in a 38.5 ml polyallomer tube. All sucrose solns contained 150 mM tricine, pH 7.5, 10 mM KCl and 1 mM EDTA, pH 7.5. Mg^{2+} was omitted to facilitate the dissociation of any membrane-bound ribosomes. Gradients were centrifuged at 20 000 rpm for 4 hr in a SW-27 rotor in a Beckman L2-65B ultracentrifuge at 4°. All steps of the membrane prepn were carried out at 0–4°. After centrifugation a 4-ml fraction was removed from the gradient corresponding to the 5-ml layer of 28% sucrose. This fraction was found to be rich in NADPH-cytochrome *c* reductase activity and, when incubated with NADPH, carried out lipid peroxidation as determined by MDA production. It should be mentioned here that contamination of this membrane fraction by ribosomes or by membrane fragments of origins other than endoplasmic reticulum would make no difference. The only requirement of the present study is that this membrane prepn should have some NADPH-cytochrome *c* reductase activity and should be able to undergo lipid peroxidation in the presence of NADPH.

Effect of MDA on activity of mRNA and wheat germ supernatant. Determination of the template activity of poly(A)-rich mRNA was according to the method of ref. [8]. The reaction was carried out at 25° for 45 min. In order to determine the effect of MDA on template activity of mRNA, 400 μg of poly(A)-rich mRNA was incubated with 1 mM MDA, total vol. 1 ml, for 90 min at 25°. A control incubation with H_2O was run in parallel. After incubation, RNA was ppted with 2.5 vol. of cold EtOH, pelleted and resuspended in 20 mM Hepes/KOH buffer, pH 7.6

When the effect of MDA on the activity of wheat germ supernatant was studied, wheat germ supernatant prepared by the method of ref. [22] was incubated with 1 mM MDA for 90 min at 25° and then the dialysis step of the prepn was repeated. Aliquots of the treated and redialysed wheat germ supernatant were used in the *in vitro* amino acid incorporation by the moss polyribosomes.

In vitro *protein synthesis using moss polyribosomes.* The reaction mixture, total vol. 250 μl, contained 20 mM Hepes/KOH buffer, pH 7.6, 1 mM ATP, 20 μM GTP, 8 mM creatine phosphate, 8 μg of creatine phosphate kinase, 2.5 mM $Mg(OAc)_2$, 20 mM KCl, 130 mM KOAc, 30 μM total amino acid mixture lacking leucine, 0.5 μCi of [U-^{14}C]leucine (298 mCi/mmol), ribosomes (50 μg as RNA), and wheat germ supernatant (400 μg protein). For introducing simultaneous lipid peroxidation into the reaction mixture, 1 mM NADPH and membrane prepn (250 μg protein) were added. Incorporation was allowed for 45 min. When time course of incorporation and MDA production was to be determined incorporation was stopped at different times. The total radioactivity incorporated was determined by the method of ref. [21] and the amount of MDA produced was determined by that of ref. [3].

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