pletely inhibited by PGI_2 at a dose of 100 ng/ml (Fig. 1). The ED_{50} for PGI_2 inhibition of papain-induced aggregation was 18 ng/ml or roughly equivalent to the ED_{50} for PGI_2 inhibition of B16a-induced aggregation (3×10^6 tumor cells per milliliter) (*20*). Neither cathepsin B activity nor papain activity was directly inhibited by PGI_2 in the fluorometric enzyme assay.

Our results indicate that cathepsin B could be one of the factors responsible for induction of platelet aggregation by tumor cells. We have previously shown that tumor cathepsin B is a property of viable tumor cells and not of host stromal elements, macrophages, or nonviable tumor cells (*8*). There is a positive correlation between cathepsin B activity and metastatic potential (*8*), as well as a positive correlation between the ability of tumor cells to promote platelet aggregation and metastatic potential (*2*). The release of cathepsin B from a variety of human and murine tumors (*6–9*) may facilitate tumor metastasis, and the ability of PGI_2 to act as an antimetastatic agent may result from its ability to inhibit tumor cell cathepsin B–induced platelet aggregation by an as yet unidentified mechanism.

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17. Cathepsin H unlike cathepsins B and L has activity against unblocked amino acid substrates as well as against blocked substrates and thus has been described as an endoaminopeptidase (*10*). Cathepsin L is an endopeptidase with strong activity against natural proteins but little against synthetic substrates (*16*). Up to 50 percent of lysosomal proteolytic activity has been ascribed to cathepsin L (*16*). In an assay of cathepsin H activity with an unblocked substrate, less than 5 percent of the B16a cysteine proteinase activity was cathepsin H when either the L-Arg-4-OMe-β-NA substrate or the L-Arg-AFC substrate, which is about 100 times more sensitive, was used [(*10*); R. Smith, personal communication].
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Inhibition of Steroid Glycoalkaloid Accumulation by Arachidonic and Eicosapentaenoic Acids in Potato

Abstract. *Eicosapentaenoic and arachidonic acids extracted from the fungus* Phytophthora infestans *elicit the accumulation of fungitoxic sesquiterpenoid stress metabolites and inhibit the accumulation of steroid glycoalkaloids in potato tubers. This dual activity, which did not occur with other saturated and unsaturated fatty acids tested, corresponds to the activity of incompatible races of* Phytophthora infestans *and crude elicitor preparations from* Phytophthora infestans *that contain bound forms eicosapentaenoic and arachidonic acids. Arachidonic acid applied to potato slices, which had been aged for various time intervals, elicited the accumulation of sesquiterpenoid stress metabolites and concomitantly inhibited the accumulation of steroid glycoalkaloids.*

The accumulation of steroid glycoalkaloids in potato tuber slices is inhibited when the tissues are inoculated with incompatible races of the late blight fungus *Phytophthora infestans* (Mont.) de Bary or treated with cell-free sonicates of either compatible or incompatible races of the fungus. Inhibition of steroid glycoalkaloid accumulation is associated with the accumulation of sesquiterpenoid stress metabolites (SSM), including rishitin and lubimin (*1–3*). Two polyunsaturated fatty acids in *P. infestans*, *cis*-5,8,11,14,17-eicosapentaenoic and *cis*-5,8,11,14-eicosatetraenoic (arachidonic), are responsible for eliciting SSM accumulation (*4*, *5*). We examined the effect

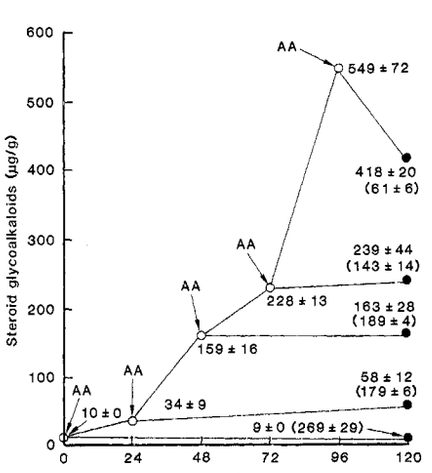

Fig. 1. (●) The effect of arachidonic acid (*AA*) on the accumulation of steroid glycoalkaloids and sesquiterpenoid stress metabolites (in parentheses) in Kennebec potato tuber slices. Values are given as micrograms per gram (fresh weight). Slices were aged for 6 hours, treated with water or with arachidonic acid (100 μg per slice) 0, 24, 48, 72, and 96 hours later, and extracted 120 hours after the initial application (time 0). (○) Steroid glycoalkaloids present in the tissues at the time of arachidonic acid application. Values are the means ± standard error (S.E.) of three replicates containing eight slices per replicate and two determinations per replicate.

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Table 1. The effect of arachidonic and eicosapentaenoic acids on steroid glycoalkaloid and sesquiterpenoid accumulation in Kennebec potato tuber slices. Potato slices were aged for 6 hours, and water or the fatty acids were added to the upper surface of slices at time 0. The top 1 mm of slices was removed for extraction 24, 48, 72, 96, and 120 hours after treatment. Values of steroid glycoalkaloids and sesquiterpenoid stress metabolites are given in units of micrograms per gram (fresh weight); steroid glycoalkaloids are expressed as α-solanine, and sesquiterpenoid stress metabolites are the total accumulations of rishitin and lubimin. Values for eicosapentaenoic acid are in parentheses. The results are the means ± S.E. of three replicates containing eight slices per replicate and two determinations per replicate; N.D., none detected.

Arachidonic acid (μg per slice)	Steroid glycoalkaloids at hours indicated (μg/g)					Sesquiterpenoid stress metabolites at hours indicated (μg/g)				
	24	48	72	96	120	24	48	72	96	120
0	12 ± 1	271 ± 9	463 ± 39	765 ± 51	885 ± 58	N.D.	N.D.	N.D.	N.D.	N.D.
10	9 ± 1	72 ± 9	96 ± 15	229 ± 18	133 ± 9	6 ± 2	33 ± 9	91 ± 9	34 ± 2	41 ± 4
25	N.D.	49 ± 6	101 ± 10	35 ± 2	78 ± 8	7 ± 2	44 ± 5	187 ± 24	108 ± 7	204 ± 19
50	N.D.	44 ± 9	20 ± 4	25 ± 5	30 ± 5	9 ± 3	142 ± 13	256 ± 26	254 ± 9	247 ± 18
100	N.D.	51 ± 4	21 ± 3	22 ± 7	42 ± 8	9 ± 1	156 ± 15	252 ± 13	201 ± 13	236 ± 14
	(8 ± 0)	(13 ± 1)	(15 ± 0)	(23 ± 2)	(31 ± 4)	(3 ± 1)	(120 ± 20)	(152 ± 11)	(371 ± 28)	(362 ± 11)

of these fatty acids on the accumulation of steroid glycoalkaloids.

High concentrations of steroid glycoalkaloids occur in potato foliage, sprouts, and tuber peel, and these glycoalkaloids accumulate in tubers that are cut and aged (*1–3, 6*). The potato cultivar Kennebec, arachidonic acid (Sigma), and eicosapentaenoic acid from *P. infestans* (*7*) were used for our experiments. Potato tuber slices were cut and aged for 6 hours at 20°C in the dark before the application of fatty acids. Stock solutions of fatty acids in chloroform were stored at −20°C and removed shortly before the assay. Chloroform was evaporated under a stream of nitrogen. The acids were then suspended by sonication in sterile distilled water in an atmosphere of nitrogen and applied under aseptic conditions to slices in moist glass petri dishes. Details of the procedures for incubation of slices and assay of SSM have been described (*5, 6, 8*). Since rishitin and lubimin are the predominant SSM (> 90 percent) that accumulated, we considered the sum of the rishitin and lubimin accumulations as representing the elicitor activity of the fatty acids. Total steroid glycoalkaloids were determined by a spectrophotometric assay (*9*). The absorbance of the samples was determined, and the values for steroid glycoalkaloids were calculated from a standard curve for α-solanine (Sigma). The presence of individual steroid glycoalkaloids was verified by thin-layer chromatography (*1, 2*).

Eicosapentaenoic and arachidonic acids markedly inhibited accumulation of steroid glycoalkaloids and elicited the accumulation of SSM (Table 1). Arachidonic acid, applied at 10 and at 100 μg per slice, inhibited steroid glycoalkaloid accumulation approximately 85 and 95 percent, respectively, 120 hours after application. Comparable inhibition was also obtained for eicosapentaenoic acid.

Table 2. The effect of various fatty acids on steroid glycoalkaloid and sesquiterpenoid accumulation in Kennebec potato slices. Potato slices were aged for 6 hours, and water or the fatty acids were added (100 μg per slice) to the upper surface of slices (time 0). The top 1 mm of the slices was removed for extraction 120 hours after treatment. Values of steroid glycoalkaloid and sesquiterpenoids are given in micrograms per gram (fresh weight); steroid glycoalkaloids are expressed as α-solanine and sesquiterpenoids are the total accumulations of rishitin and lubimin. Results are means ± S.E. of three replicates containing eight slices per replicate and two determinations for each replicate; N.D., none detected.

Fatty acid	Steroid glycoalkaloids (μg/g)	Sesquiterpenoids (μg/g)
Arachidonic acid	31 ± 2	209 ± 23
Eicosatrienoic acid	420 ± 30	N.D.
Eicosadienoic acid	597 ± 15	N.D.
Arachidic acid	581 ± 14	N.D.
γ-Linolenic acid	607 ± 33	N.D.
H_2O	588 ± 12	N.D.

Our data are in agreement with those previously reported for the elicitation of SSM by eicosapentaenoic and arachidonic acids (*4, 5*). Four fatty acids tested for this eliciting and inhibiting activity along with arachidonic acid were γ-linolenic (6,9,12-octadecoetrienoic), arachidic (eicosanoic), 11,14-eicosadienoic, and 11,14,17-eicosatrienoic acids (Sigma). Only arachidonic acid inhibited the accumulation of steroid glycoalkaloids with parallel elicitation of SSM accumulation (Table 2).

To examine whether arachidonic acid is capable of inhibiting the accumulation of steroid glycoalkaloids well after the establishment of the steroid glycoalkaloid biosynthetic pathway, we applied arachidonic acid at 100 μg per slice to tuber slices aged for various intervals of time. Further steroid glycoalkaloid accumulation was almost completely inhibited, whether the arachidonic acid was applied shortly after the potatoes were sliced or when steroid glycoalkaloid synthesis was well established (Fig. 1). When arachidonic acid was applied at 100 μg per slice to tuber slices aged for 96 hours, SSM accumulated at concentrations of approximately 60 μg per gram (fresh weight) 24 hours after arachidonic acid application. Less than 10 μg of SSM per gram (fresh weight) was detected in unaged slices 24 hours after inoculation with incompatible races of *P. infestans* or treatment with arachidonic or eicosapentaenoic acids (Table 1) (*5*).

Our data suggest that arachidonic and eicosapentaenoic acids may activate the pathway of SSM synthesis or inhibit that of steroid glycoalkaloid synthesis, or both (by regulation of the presence or activity of key enzymes). If the inhibition of steroid glycoalkaloid synthesis is critical, a site of action could be at the level of squalene synthetase, which converts farnesyl pyrophosphate to squalene (*10*). Inhibition at this site could divert farnesyl pyrophosphate to SSM synthesis.

Arachidonic and eicosapentaenoic acids reproduce all of the characteristic features of the hypersensitive response of potato to incompatible races of *P. infestans*, namely, rapid but restricted cell death, browning, leakage of electrolytes, accumulation of SSM, and inhibition of steroid glycoalkaloid accumulation. These data provide evidence of a mechanism for the action of a chemically characterized elicitor of disease resistance and phytoalexin accumulation in plants, as well as evidence that a fatty acid can inhibit sterol synthesis.

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Radiosensitization of Hypoxic Tumor Cells by Depletion of Intracellular Glutathione

Abstract. *Depletion of glutathione in Chinese hamster ovary cells in vitro by diethyl maleate resulted in enhancement of the effect of x-rays on cell survival under hypoxic conditions but not under oxygenated conditions. Hypoxic EMT6 tumor cells were similarly sensitized in vivo. The action of diethyl maleate is synergistic with the effect of the electron-affinic radiosensitizer misonidazole, suggesting that the effectiveness of misonidazole in cancer radiotherapy may be improved by combining it with drugs that deplete intracellular glutathione.*

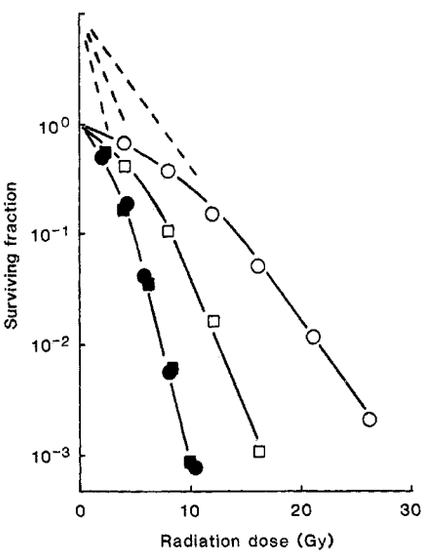
It is well known that hypoxia protects cells from the cytotoxic effects of radiation. Hypoxic cells in solid tumors are therefore considered a problem in the treatment of cancer by radiotherapy, and several methods have been tried to overcome this problem. These include giving radiation in small multiple fractions, treating patients in high-pressure oxygen chambers, using densely ionizing radiations such as neutrons or π^- mesons, and, more recently, administering electron-affinic agents that act like oxygen to enhance radiation-induced damage (*1*). One of these electron-affinic radiosensitizers, the 2-nitroimidazole misonidazole (MIS), is now undergoing extensive clinical trials (*2*).

Another approach to sensitization of hypoxic tumor cells would be to decrease their endogenous radioprotective capacity. Radiation induces the formation of free radicals on DNA either directly by ionization or indirectly by the action of radiolytic products. Within milliseconds of the initial radiolytic event, DNA damage is either restored by hydrogen donation or rendered nonrestorable by reaction with a sensitizer (*3*). Perturbation of this process would be expected to have a dose-modifying effect, that is, to change the slope of the radiation-survival curve. The nonprotein thiol glutathione has been regarded as the main endogenous reducing agent responsible for restoration of radiation-induced lesions by hydrogen donation (*4*).

We used diethyl maleate (DEM) as a reagent to deplete intracellular glutathione in order to examine the hypothesis that depletion of endogenous nonprotein thiol should sensitize hypoxic cells selectively. This possibility is strongly suggested by the observation (*4*) that cells which are genetically deficient in glutathione synthesis have a reduced differential in radiosensitivity between oxygenated and hypoxic conditions.

Diethyl maleate effectively depletes glutathione in Chinese hamster ovary (CHO) cells in vitro without cytotoxicity (no effect on cloning efficiency at $2 \times 10^{-4}M$ DEM for 8 hours at 37°C) and without significant effects on protein thiol content (*5*). When DEM-pretreated cells are irradiated, their sensitivity to radiation is increased in a dose-modifying manner (Fig. 1). The enhancement ratio (ER) of 1.7 ± 0.2—that is, the ratio of the slope of the exponential portion of the survival curve of cells treated with DEM to that of control cells—seen when glutathione is reduced to 1 percent of control levels by $2 \times 10^{-4}M$ DEM is well above that which can be achieved by a clinically tolerable dose of MIS. Oxygenated cells were not radiosensitized by DEM.

In previous work on the importance of endogenous thiols in radioresistance, other reagents such as *N*-ethylmaleimide (*6*) and diamide (*7*) were used; however, these reagents not only decrease intracellular reduced glutathione concentrations but also cause a loss of protein thiol, which could affect enzymatic repair (*5*). The data obtained with *N*-ethylmaleimide and diamide also showed a reduction in the initial shoulder region of



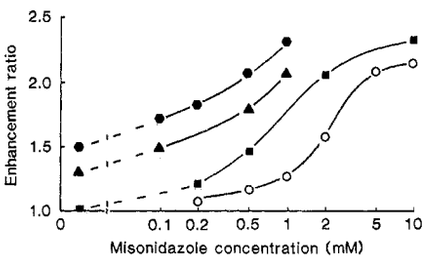


Fig. 1 (left). Effect of glutathione depletion by DEM on the radiosensitivity of CHO cells in vitro. CHO cells were grown in suspension culture (*5*) and preincubated for 1 hour at 37°C with $2 \times 10^{-4}M$ DEM under 5 percent CO_2 in N_2 at 5×10^6 cells per milliliter (□) or 5 percent CO_2 in air at 1×10^6 cells per milliliter (■) in Eagle's minimum essential medium without serum. Cells were irradiated in suspension with a 250-kV x-ray machine. Survival was determined by a clonogenic assay (*8*). The ER for DEM under hypoxia was 1.8 in the experiment shown; the ER for DEM in air (2.8) was identical to that of air alone (●). Survival of controls irradiated under 5 percent CO_2 in N_2 is indicated by open circles. Results are from a representative experiment. One gray (*Gy*) equals 100 rads. Fig. 2 (right). Potentiation of misonidazole radiosensitization of hypoxic CHO cells in vitro by DEM. CHO (HA-1) cells (*5*) were incubated at 25°C for 1 hour with DEM [$2.5 \times 10^{-5}M$ (■), $5 \times 10^{-5}M$ (▲), and $2 \times 10^{-4}M$ (●)] and irradiated in monolayer culture in tissue culture plates under 5 percent CO_2 in N_2 (*8*). MIS was added concurrently with DEM. Controls are indicated with open circles. Glutathione (GSH) was assayed enzymatically (*9*). No effect on the shoulder of the survival curve was seen with any drug combinations. Enhancement ratios for DEM alone were 1.0 for $2.5 \times 10^{-5}M$ at 35 percent of control GSH (no enhancement), 1.3 for $5 \times 10^{-5}M$ at 20 percent of control GSH, 1.5 for $2 \times 10^{-4}M$ at 5 percent of control GSH, and 2.1 for $1 \times 10^{-3}M$ at less than 1 percent of control GSH (data not shown). Data shown are averages from two to four experiments.

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