Results. Data reported in figure 1 show that calcium dobesilate produced a dose-dependent inhibition of PG synthesis in microsomes of pregnant human myometrium. Figure 2 shows the inhibition of PG synthesis by indomethacin. As with calcium dobesilate, the greater the concentration of indomethacin the greater was the inhibition of PG synthesis. In our assay system, the potency of indomethacin was greater than that of calcium dobesilate, as shown in the table.

Discussion. The above results indicate that calcium dobesilate exerts a potent inhibitory effect on the human pregnant myometrial PG synthesis in vitro. The results of this experiment are more or less comparable to those with etamsylate[1]. Quantitatively the inhibitory potency, expressed in I_{50}-values, for calcium dobesilate (mM) is less than that of indomethacin (μM) (table).

The inhibitory potency of aspirin on PG synthesis tested in vitro on bovine seminal vesicles[8] was of the same order as that of calcium dobesilate. In our experiments the inhibition of PG synthesis, as evaluated by the I_{50}-values for the different arachidonate metabolites, was more marked with calcium dobesilate than with etamsylate.

The findings of the present study are of particular interest in determining the possible therapeutic role of PG synthetase inhibitors like calcium dobesilate, in certain pathological conditions related to various hematological disturbances or microcirculatory disorders.

Inhibition by calcium dobesilate and indomethacin of PG synthesis in microsomes of pregnant human myometrium

PG products	Ca dobesilate I_{50} (mM)	Indomethacin I_{50} (μM)
6-oxo-$PGF_{1\alpha}$	0.87	0.85
$PGF_{2\alpha}$	0.92	0.81
PGE_2	1.01	1.00
TXB_2	0.74	0.91

PG synthesis was measured as described in the text. I_{50} = concentration (mM or μM in final dilution) producing 50% inhibition. I_{50} values were calculated from 10 points of a concentration curve, using regression analysis when the transformations were ln [y/(100-y)] = b lgx + const. for calcium dobesilate, and ln (100-y) = b lgx + const. for indomethacin.

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0014-4754/84/020190-02$1.50 + 0.20/0

Free radicals and aflatoxin biosynthesis*

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Summary. The addition of some halogenated alkanes (bromotrichloromethane, carbontetrachloride and chloroform) to cultures of *A. parasiticus* and *A. flavus* have shown a high stimulating effect on aflatoxin biosynthesis. When the production of aflatoxin increases during the stimulating effect the peroxidase activity is inhibited.

We have shown previously that compounds with an epoxide ring (cerulenin, tetrahydrocerulenin, methyl 9, 10 epoxystearate and methyl 9,10:12,13 diepoxystearate)[1-3] and lipoperoxides[4,5] promoted aflatoxin production to a remarkable extent when added to cultures of *Aspergillus parasiticus* or *A. flavus.* In addition, on the basis of the results of the aflatoxins analyzed in various non-aged and aged seeds inoculated with *A. parasiticus* and *A. flavus* we thought it probable that the products of the oxidation of unsaturated fatty acids and lipids (lipoperoxides and their breakdown products) played a leading role in the production of aflatoxins.

Recently, the presence of compounds with polysubstrate monoxigenase (PSMO) activity, viz. cytochrome P-450 reductase, has been established in the microsomes of *A. parasiticus*[6]. In this connection we decided to investigate the effects of the addition of some halogenated alkanes, viz. bromotrichloromethane (CCl_3Br), carbontetrachloride (CCl_4) and chloroform ($CHCl_3$) on cultures of *A. parasiticus.* It has been postulated, with good evidence, that the basis of the hepatotoxicity of CCl_3Br and CCl_4 is the peroxidation of lipids of endoplasmic reticulum by the highly reactive trichloromethyl radicals (CCl_3) formed by the interaction with NADPH-cytochrome P-450 system[7,8]. In addition, the amount of peroxidase activity in the mycelia of *A. parasiticus* was studied.

Materials and methods. Aspergillus parasiticus (strain NRRL 2999) was used in this study. In some experiments we also used 2 toxigenic strains of *A. flavus:* strain ATCC 22548 and CF 1 isolated in our Institute from wheat seeds (*Triticum vulgare* var. Manitoba). Stock cultures were maintained on Czapek Dox agar (Difco) supplemented with 5 mg/l $ZnSO_4 \cdot 7\ H_2O$ and 1 mg/l $NA_4MoO_4 \cdot 2\ H_2O$ at 4 °C.

The fungal strains were normally grown on Czapek Dox Broth medium + 5 mg/l $ZnSO_4 \cdot 7\ H_2O$ and 1 mg/l $Na_2MoO_4 \cdot 2\ H_2O$ in 100 ml conical flasks at 30 °C. The inoculum was 10^6 15-day-old conidia from culture grown

Table 1. Growth and aflatoxin production by *A. parasiticus* grown at 30 °C on synthetic medium (SM) supplemented with dimethyl sulfoxide (DMSO), bromotrichloromethan ($BrCCl_3$), carbontetrachloride (CCl_4) and chloroform ($CHCl_3$). Each result represents the average ±SEM of 5 determinations

	Days of incubation 6		10	
	Dry weight (mg)	Total aflatoxins (μg/50 ml)	Dry weight (mg)	Total aflatoxins (μg/50 ml)
Control	289.2 ± 53.3	2.5 ± 1.2	273.8 ± 46.3	2.2 ± 0.8
SM + DMSO (0.2% v/v)	206.8 ± 41.5	12.3 ± 0.6	244.5 ± 43.5	8.7 ± 1.2
SM + $BrCCl_3$ (0.2% v/v)	130.5 ± 60.4	80.5 ± 27.4	287.0 ± 39.6	870.5 ± 88.9
SM + CCl_4 (0.8% v/v)	144.6 ± 53.7	50.3 ± 10.5	301.6 ± 74.2	673.2 ± 75.4
SM + $CHCl_3$ (1% v/v)	175.8 ± 27.4	1.6 ± 0.9	274.1 ± 53.3	270.8 ± 47.1

In all cases the most quantitatively important aflatoxins are B_1 and G_1. The 2 strains of *A. flavus* (a = strain ATCC 22548; b = strain CF1) after 10 days of incubation at 30 °C on SM supplemented with $BrCCl_3$ (0.2% v/v) produced respectively a: 705.8 ± 177.8; b = 788.9 ± 155.4 μg of total aflatoxins/50 ml).

on Czapek Dox agar medium. With the inoculum, bromotrichloromethane ($BrCCl_3$), carbontetrachloride (CCl_4), chloroform ($CHCl_3$) or dimethyl sulfoxide ($(CH_3)_2SO$) were added to the culture medium separately and at different concentrations.

Dimethyl sulfoxide (DMSO) was added as solvent control. The concentrations of all the assayed compounds were chosen according to the reasons previously described[1-5], and they differed because the inhibiting capacity on spore germination of each compound was different. The halogenated methanes, having a higher density than water, formed a layer below the medium in the flasks. So, unlike DMSO, they were not in direct contact with growing cells.

The fungal growth was estimated by mycelium dry weight as previously described[9]. The aflatoxins were extracted with $CHCl_3$: CH_3OH (2:1 v/v) and analyzed by TLC and HPLC as described[2,3].

Extraction and assay of peroxidase enzymes: a known amount of mycelium was chilled and ground with cold 0.05 M Tris-HCl buffer (pH 7.4) + 0.1% Sodium deoxycholate (Merck) using acid-washed sea sand, in a precooled mortar for 10 min and centrifuged at 15,000 rev/min in a Heraeus (Christ) Cryofuge 20-2 refrigerated centrifuge. The enzymes were estimated in the supernatant. Peroxidase activity was determined using 4-aminoantipyrine as hydrogen donor and hydrogen peroxide as an oxidant as previously described[10], with slight modifications: 1 mM NaN_3 (sodium azide) was added to inhibit the catalase activity that interfered with the measurements of peroxidase activity. All data are presented as mean ± SEM.

Results and discussion. Halogenated methanes highly enhance the biosynthesis of aflatoxin when added to cultures of *A. parasiticus* and *A. flavus*, in the order $BrCCl_3 > CCl_4 > CHCl_3$ (table 1). The order is parallel to the hepatotoxic potency of the 3 halogenated methanes and it might be directly correlated with the bond-dissociation energies (49,68 and 90 kcal/mole respectively for $BrCCl_3$, CCl_4 and $CHCl_3$)[8,11]. A low bond-dissociation energy implies a greater tendency to homolytic cleavage ($CCl_3-X \rightarrow CCl_3 + \cdot X$). In the case of $BrCCl_3$ and CCl_4 the most likely mechanism of aflatoxin induction may be considered to be the interaction of these drugs, as in the liver, with the NADPH-cytochrome P.450 system of *Aspergillus* leading to the highly reactive CCl_3^- radical. According to Slater[12], a pioneer in the study of liver injury produced by CCl_4, the CCl_3^- radical, which has a very short half-life, attacks neighboring components in the endoplasmic reticulum causing covalent binding to proteins and lipids and lipoperoxidation of unsaturated lipids in the environment. In the liver, all these reactions may lead to death, above all if protective antioxidants are lacking. In the case of *A. parasiticus*, the aflatoxin biosynthesis may be considered to be a system of detoxification from lipoperoxides and probably form many other toxic products following breakdown of lipoperoxides.

From this point of view, lipid peroxidation may be considered to be a basic step inducing aflatoxin production. In fact, lipoperoxides and probably some of their breakdown products, either in vitro (synthetic lipoperoxides added to cultures of *Aspergillus*) or in vivo (aged seeds whose extracted oils showed a high number of peroxides) remarkably stimulated the biosynthesis of aflatoxins[4,5].

More complicated is the attempt to give an explanation for the induction of aflatoxin biosynthesis by chloroform. The activation of $CHCl_3$ to a CCl_3^- radical is not as favorable because of the strong bond energy (96 kcal/mole) of C-H bond[11]. In vitro studies[13] have shown that chloroform undergoes a bioactivation by cytochrome P-450-dependent oxidation and not by a reduction as for CCl_4. According to these authors, $CHCl_3$ is oxidized to highly unstable CCl_3OH, which further decomposes to toxic phosgene ($COCl_2$), which is responsible for the depletion of the hepatic content of glutathione. According to Brown et al.[14], in phenobarbital-pretreated rats exposed to hepatotoxic doses of $CHCl_3$ the increased levels of lipid conjugated dienes may reflect an enhanced activation of $CHCl_3$ to a free radical species. In conclusion, the formation of an unstable intermediate (CCl_3OH) with the subsequent depletion of glutathione, a known radical scavenger, may be responsible for the effect of chloroform.

DMSO would not give rise to reactive radicals and was found to have no remarkable effect on aflatoxin production.

Another aspect deserves to be taken into consideration; the function of peroxidase and lipoxidase. Doyle and Marth[15] showed that the enzyme may be involved in the degradation of aflatoxins by *A. parasiticus:* 'when peroxidase activity was great, the rate of aflatoxin degradation was substantially greater than when peroxidase activity was minimal'. From our results (table 2) it is evident that a total inhibition of peroxidase corresponds to a high production of aflatox-

Table 2. Amounts of peroxidase activity present in the supernatant fluid from homogenates of 6- and 10-day-old mycelia of *A. parasiticus* grown at 30 °C on synthetic medium (SM) supplemented with bromotrichloromethane, carbontetrachloride and chloroform. Each result represents the average ±SEM of 5 determinations

	Days of incubation 6 (units/g)	10 (units/g)
Control	2.4 ± 0.8	28.9 ± 3.4
SM + $BrCCl_3$ (0.2% v/v)	–*	–*
SM + CCl_4 (0.8% v/v)	0.4 ± 0.1	–*
SM + CCl_4 (0.5% v/v)	0.7 ± 0.2	–*

* Less than 0.1 unit/g.

ins. Homolytic cleavage of the CCl_3–Cl bond evidently produces such a shower of CCl_3 radicals that any protection effort by peroxidase is overwhelmed. Rao et al.[16] also reported that lower activities of lipid peroxidase and nucleotide oxidase might be favorable for optimum aflatoxin biosynthesis, but they started from presuppositions quite different from ours. According to them, in fact, lipid peroxidation was increased within the mycelium under conditions of reduced aflatoxin production; under these conditions lipid synthesis and growth are enhanced and greater availability of lipids would elevate lipid peroxidase activity.

* Acknowledgments. Supported by 'Cassa di Risparmio di Roma', funds from Ministry of Education and Faculty of Science of 'La Sapienza' Rome University.

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0014-4754/84/020191-03$1.50 + 0.20/0

Physiological state of submitochondrial particles and their susceptibility to Triton X-100[1]

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Summary. The solubilizing effect of Triton X-100 on beef heart submitochondrial particles (ETP_H) has been studied under various physiological conditions. Coupled, uncoupled and azide-inhibited ETP_H particles have been studied. Quantitative and qualitative differences are found in the proteins solubilized by the detergent from ETP_H particles under the various conditions tested.

There have been various reports of differences in susceptibility of membranous systems to the solubilizing action of detergents, according to the physiological state of the membranes[1-4]. Some of these studies have been carried out on microorganisms, or microbial membranes, generally by inducing inhibited states through cyanide or sodium azide. Detergent action was usually assessed by the release of cytoplasmic contents.

Studies on the effect of detergents on mitochondria and mitochondrial membranes have also been carried out, in this and in other laboratories[5-7]. It was found that Triton X-100 was very useful in this respect, because of its selective solubilizing action and mild effect on enzyme activities at low concentrations, together with a high solubilizing power at higher detergent/membrane ratios.

The well-known conformational changes accompanying variations in the mitochondrial physiological state[8], together with the selectivity of Triton X-100 action[6,9] and indications that the accessibility of membrane lipids and proteins to externally added reagents varies with energization/deenergization processes[4,8,10-12] suggest that mitochondria in different physiological states may behave differently towards detergents. However, this hypothesis was difficult to test with the usual lengthy methods (centrifugation, dialysis, etc.) of separating the solubilized from the non-solubilized membrane fraction, because of the problems of maintaining mitochondria in a defined functional state for such long time intervals. In this paper, we describe a fast and efficient filtration method to achieve that aim, and we also record the quantitative and qualitative differences found in Triton X-100 solubilized protein from mitochondria in different physiological states.

Materials and methods. Beef heart submitochondrial particles (ETP_H) were prepared according to[13]. They were washed and resuspended in a few ml of 0.25 M sucrose, 0.01 M Mg^{2+} and 10 mM Tris-HCl, pH 7.2.

The physiological state of ETP_H was tested by means of a Clark-type oxygen electrode (Rank Bros., Bottisham, U.K.) using an assay medium containing 2 mM K_2HPO_4, 75 mM KCl, 0.5 mM EGTA, 1 μM rotenone and 10 mM Tris-HCl (pH 7.0). With succinate-maintained respiration, respiratory control ratios were 1.2-1.4 at 38 °C. The ETP_H suspension was diluted to a final concentration of 1 mg protein/ml in the assay medium indicated above. 'Respiring' submitochondrial particles (1 mg protein/ml) were assayed in the presence of 20 mM succinate and 0.6 mM ADP; 8 μM carbonyl cyanide m-chlorophenyl hydrazone (CCCP) was added in order to obtain 'uncoupled' ETP_H; finally, 'inhibited' submitochondrial preparations contained 20 mM succinate, 0.6 mM ADP and 20 mM sodium azide. Polarographic observations showed that the submitochondrial preparations remained in their respective functional states for longer than 1 min.

The different ETP_H suspensions were treated with the required amounts of Triton X-100 in order to obtain final detergent concentrations ranging from 10^{-5} to 10^{-3} M, and