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EXTRACTION AND PARTIAL CHARACTERIZATION OF DIALYSABLE PRODUCTS ORIGINATING FROM THE PEROXIDATION OF LIVER MICROSOMAL LIPIDS AND INHIBITING MICROSOMAL GLUCOSE 6-PHOSPHATASE ACTIVITY*

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Abstract—The dialysate obtained from a system containing actively peroxidizing liver microsomes shows inhibitory effects on glucose 6-phosphatase activity of freshly prepared liver microsomes (test system). The inhibitory factors were recovered in extracts obtained from the dialysate with ethyl ether or ethyl acetate. The extraction procedure completely removed the inhibitory activity from the dialysate. A partial separation of the products present in the dialysate extract was obtained by thin-layer chromatography. When the lipid materials eluted from individual bands or groups of bands were tested for inhibitory activity, it was found that various chromatographic bands contained various degrees of inhibitory activity, and that the highest inhibitory activity occurs in a well resolved band that is stained yellow by a *N, N'*-dimethyl-*p*-phenylene-diamine reagent. Additional studies indicated that this band contains most of the carbonyl functional groups detectable in the unfractionated dialysate extract, while it contains peroxide functional groups in trace amounts only. The peroxide functional groups present in the unfractionated dialysate extract were found to occur in various chromatographic bands without a well defined relationship with the toxicological activity. It is concluded that lipoperoxidation products highly active in inhibiting microsomal glucose 6-phosphatase activity are fatty aldehydes or other carbonyl compounds, probably provided with a relatively long carbon chain.

Peroxidation of unsaturated lipids in the biological membranes is known to produce deleterious effects on the membrane structure and function [1–5]. Also, lipoperoxidation is believed to play an important role in many conditions of cellular damage [6–10] including CCl_4-induced liver injury [11–13]. The mechanism by which lipoperoxidation acts in producing its effects has been the object of several investigations. Molecular alterations of membrane fatty acids may be important in this respect. However, studies carried out in order to elucidate the mechanism by which CCl_4 causes the inactivation of membrane-bound enzymes, indicated that the enzyme impairment could be due to the evolution of products originating from the peroxidation of membrane lipids [14, 15]. The concept therefore developed according to which factors derived from lipoperoxidation can produce damaging effects on biological structures. Supportive evidence for this concept, which implies that the toxic products diffuse from the original locus of peroxidation and act at distant loci, was found in the results of earlier experiments [16, 17] in which erythrocytes, used as a revealing system, were added to liver microsomes peroxidizing in the NADPH-dependent system. It was in fact observed that such an addition results in an almost complete hemolysis. According to Pfeifer and McCay [17] the lysis is due to free radicals originating from the microsomal oxidation of NADPH. According to Roders *et al.* [18, 19], on the other hand, the lysis is caused by hemolytic factors originating from the peroxidation of microsomal lipids. Previous studies from our laboratory [20–22] greatly reinforced the view that during the course of the peroxidation of liver microsomal lipids, products are formed which have the capacity of inducing cytopathological effects at distant loci. In these studies, in fact, the effects were shown to occur in revealing or target systems, which were separated from the peroxidizing system (liver microsomes, plus NADPH) by a dialysis membrane. The cytotoxic products are therefore dialysable. They have the capacity of damaging cellular membranes, as shown by the lytic effects on erythrocytes (when the target system consists of blood red cells), and of impairing the activity of enzymatic molecular complexes bound to the membranes, as shown by the partial inactivation of microsomal glucose 6-phosphatase (G-6-Pase)† and cytochrome P_{450} (when the target system consists of freshly prepared liver microsomes). The cytotoxic products were shown to have a relatively long life span [22]. In fact, they can display their effects not only when the dialysis tube containing the target system is introduced into the flask containing the system destined for lipoperoxidation at the beginning of the incubation, but even when the target system is introduced into the flask after a preincubation step during which the microsomes destined for lipoperoxidation have been allowed to peroxidize. The effects of the cytotoxic products are moreover evident when the target system (in the dialysis tube) is challenged with the supernatant fraction obtained by centrifuging the preincubation mixture at the end of the preincubation. All the above studies indicate therefore that the products with cyto-

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† Abbreviations: G-6-Pase, glucose 6-phosphatase; MDA, malonic dialdehyde; EDTA, ethylene diaminetetraacetate; t.l.c., thin-layer chromatography; g.l.c., gas–liquid chromatography; u.v., ultra-violet.

pathological activity are not of a free radical nature.

The next step in the problem is that concerned with separation and characterization of these dialysable products originating from lipoperoxidation. To this end, experiments were designed in which the incubation system was similar to the one mentioned above and previously reported [20–22], except that only a buffer, without the target system, was contained in the dialysis tube. It is obvious that if the cytotoxic products that enter the dialysis tube do not interact with the target system, they should be more easily separable from the content of the dialysis tube. A successful approach to the separation by extraction procedures of the products that inhibits microsomal G-6-Pase is reported in this communication. Studies concerned with the chemical characterization of these products are also reported.

MATERIALS AND METHODS

Male Sprague–Dawley rats (200–250 g) maintained on a pellet diet (Nossan, Correzzana, Milano, Italy) of preservative compounds were used. The animals were starved overnight before sacrifice.

Preparation of liver microsomes destined for lipoperoxidation. Liver microsomes destined for lipoperoxidation were prepared from 10% (w/v) liver homogenates in ice-cold 0.15M-KCl buffered with 0.05M-Tris–maleate, pH 6.6. The homogenate was centrifuged at 2700 *g* for 10 min. The supernatant fraction was centrifuged at 100,000 *g* for 30 min. The resulting microsomal pellet was appropriately resuspended with the homogenization buffer.

Incubation procedures. The composition of the incubation mixture was, for the experimental sample (plus microsomes, plus NADPH), microsomes derived from 50 mg liver/ml, 0.15M-KCl, 0.05M-Tris–maleate buffer, pH 6.6, 0.025mM-$FeSO_4$, and an NADPH-generating system [0.4 mM-NADP, 5 mM-$MgCl_2$, 2.5 mM-nicotinamide, 20 mM-DL-isocitrate and 0.1 μM units/ml isocitric dehydrogenase (Boehringer)]. $FeSO_4$ was added to enhance lipid peroxidation. The final volume was 40 ml or 200 ml in the experiments in which ethyl ether or ethyl acetate were used for the extraction of the inhibitory products, respectively. The incubation was carried out aerobically, for 60 min, at 37°, in appropriate Erlenmeyer flasks. The concentration of malonic dialdehyde (MDA) formed in the experimental sample was quite similar with either volume of incubation mixture. At the beginning of the incubation a section of a dialysis tube (for the characteristic of the dialysis tube, see [20]) containing the KCl–tris–maleate buffer used for the incubation, was introduced into the Erlenmeyer flask. The tube was bent into a U shape, with the upper limbs pressed between the cork and the neck of the flask and the U-shaped lower portion (containing the buffer) immersed in the incubation mixture. The amount of the KCl–Tris–maleate buffer contained in the dialysis tube was 16 ml or 100 ml according to whether ethyl ether or ethyl acetate was used for the extraction of the products provided with inhibitory activity. In the former case lower volumes were employed in order to avoid large amounts of ethyl ether for the extraction (see below). In the latter case larger volumes of dialysate were desired to obtain sufficient material to carry out, together with the test of the inhibitory activity, chromatographic or other studies in the same experiments.

Control samples were identical to the experimental sample except that no microsomes [plus NADPH-generating system–$FeSO_4$ (indicated as NADPH), minus microsomes] or no NADPH-generating system–$FeSO_4$ (plus microsomes, minus NADPH) was added, or neither (no additions) were added.

Extraction and test of the toxicological activity. At the end of the incubation, the content of the dialysis tube of each sample was recovered. After the addition of EDTA (6 mM) to prevent lipid peroxidation and consequent loss of the enzymatic activity in the following microsomal test of G-6-Pase*, an aliquot (1.5 ml) was directly used to test the inhibitory activity of the 'whole dialysate'. Another aliquot was extracted, after acidification to pH 3 with HCl. The acidification was performed in order to extract compounds of peroxidic nature [23], eventually present in the dialysate. It was subsequently observed, however, that the inhibitory products could be extracted even without previous acidification.

In a first group of experiments the extraction of the inhibitory products was performed with ethyl ether and subsequently, for the reasons that will be discussed below, with ethyl acetate. In the former case, 10 ml of the content of the dialysis tube were extracted three times with ethyl ether rectified twice on metallic sodium and free of peroxides, as tested by the method of Dugan [24] as modified by Pesh-Imam *et al.* [25]. The upper ether phases of each extraction were collected, washed with water and dried down under an oxygen-free nitrogen stream. The residue was resuspended with 1.5 ml of 0.15 M KCl, 6 mM EDTA, 0.05 M Tris–maleate buffer, pH 6.6, containing 0.001% (w/v) Tween 80. The suspension was then tested for inhibitory activity of the 'dialysate extract'. The lower, aqueous phase after the extraction was brought back to pH 6.6 with NaOH, after removal of the residual ether content with a nitrogen stream. An aliquot (1.5 ml) of this phase was then tested for inhibitory activity of the 'dialysate-after-the-extraction'.

When the extraction of the inhibitory products was performed with ethyl acetate, approximately 95 ml of the content of the dialysis tube were acidified as above and then extracted three times with birectified ethyl acetate. The upper phases of each extraction were collected and washed several times with H_2O. Since some water remains in the upper phase, the separation of the water content from the ethyl acetate extract was achieved by cooling the extract at −20° and by removing the ice crystals by means of rapid filtration through a sintered glass funnel. The ethyl acetate extract derived from 20 ml† of dialysate was dried down in a rotatory evaporator; the residue was resuspended as above and the suspension was tested for toxicological activity of the dialysate extract. In these experiments too, the

* NADPH can in fact enter the dialysis tube; thus lipoperoxidation can be promoted in the microsomes of the test system when the whole dialysate, or the dialysate-after-the-extraction (see below), is tested for toxicological activity, if EDTA is not added to the content of the dialysis tube.

† The recovery of the ethyl acetate in the upper phase of each wash was not complete, as part of the ethyl acetate remained in the lower aqueous phase. For this reason the extract derived from a double amount of dialysate, with respect to that (10 ml) used in the previous experiments, was employed.

toxicological activity of the whole dialysate and that of the dialysate-after-the-extraction were tested.

The inhibitory activity of the various dialysate preparations was tested on G-6-Pase activity of freshly prepared liver microsomes. The microsomes were prepared from 10% (w/v) liver homogenates in 0.25 M-sucrose, 6 mM-EDTA, pH 7.4, according to de Duve *et al.* [26]. The microsomal pellet was resuspended with 0.15M-KCl, 6mM-EDTA, 0.05M-Tris–maleate buffer, pH 6.6. An aliquot of 0.1 ml (containing the microsomes equivalent to 50 mg liver) of this suspension was incubated aerobically with 1.5 ml of the various dialysate preparations for 60 min at 37°. The G-6-Pase activity of the microsomal suspension was then assayed as reported by Glende *et al.* [14] with minor modifications. Microsomal protein was determined according to Lowry *et al.* [27], using bovine serum albumin as a standard.

Aliquots of the mixture destined for lipoperoxidation, of the content of the dialysis tube, and of the systems used to test the toxicological activities of the various dialysate preparations were drawn off for MDA determination, at the end of the respective incubations. MDA was measured as previously reported [28], using a MDA standard produced by the acid hydrolysis of 1,1,3,3-tetraethoxypropane.

Absorption spectrum of the chromogen produced by the dialysate extract in the Dugan's assay for peroxides. For these, as well as for the subsequent experiments, the extraction of the lipoperoxidation products from the content of the dialysis tube was performed by using ethyl acetate. An aliquot of the dialysate extract was dried down, redissolved in chloroform and allowed to react with *N,N'*-dimethyl-*p*-phenylenediamine according to Dugan's method [24] for peroxide determination, as modified by Pesh-Imam *et al.* [25]. The absorption spectrum was then recorded from 400 to 600 nm, after maximum color development (30 min).

In some experiments, at the end of the incubation, lipoperoxidation was stopped by the addition of EDTA (6 mM). The incubation mixture was then centrifuged at 100,000 *g* for 60 min and the microsomal sediment and the supernatant fraction were recovered. The microsomal sediment was then resuspended in the incubation buffer. Both the suspension of peroxidized microsomes and the supernatant fraction (incubation medium) were acidified to pH 3 and extracted with ethyl acetate. The upper phases (separated by centrifugation) were washed with H_2O and aliquots were dried down, redissolved in chloroform and processed as above according to the modified Dugan's method.

Thin-layer chromatographic analysis of the dialysate extract. A sample of the dialysate extract was dried down and the residue, dissolved in a small volume of ethyl acetate, was applied to a 20 × 20 cm chromatoplate coated with a 0.25 mm layer of Anasyl H (Analabs Inc., New Haven, Conn., 06473). Thin-layer chromatography (t.l.c.) was performed by using a solvent system of n-heptane–ethyl acetate–acetic acid (75:25:1, by vol). The bands were detected by spraying the chromatoplate with a solution of *N,N'*-dimethyl-*p*-phenylenediamine [29], used as a reagent to detect organic peroxides.

Test of the various t.l.c. bands for the inhibitory activity. In the experiments in which the various t.l.c. bands were eluted from the chromatoplate and tested for inhibitory activity, a larger (six fold) amount of dialysate extract (i.e. the extract derived from 120 ml of dialysate) than that used to test the inhibitory activity of the unfractionated sample, was applied to the central part of the edge of the chromatoplate in a line sample. Additional samples were applied at both sides. After developing the chromatogram, the area corresponding to individual bands or groups of bands in the central part of the chromatoplate, which was protected from the spray by a glass sheet, was scraped off. The lipid material was eluted first with the solvent system, second with 0.5% (v/v) acetic acid in ethyl acetate, and third with n-heptane–ethyl acetate–acetic acid (50:50:0.5, by vol.). After evaporation of the solvent, the residue was resuspended with 1 ml of 0.15 M-KCl, 6 mM-EDTA, 0.05 M-Tris–maleate buffer, pH 6.6, containing 0.001% (w/v) Tween 80. The liver microsomes of the test system were prepared in the same way as those obtained to test the inhibitory activity of the various dialysate preparations (see above). The microsomal sediment was resuspended with 0.15 M-KCl, 6 mM-EDTA, 0.05 M-Tris–maleate buffer, pH 6.6. An aliquot of 0.1 ml (containing the microsomes equivalent to 33.3 mg liver) of this suspension was added to each sample. After a 60 min incubation at 37°, the G-6-Pase activity was assayed as above. Silica gel blanks (obtained by eluting corresponding areas of a chromatoplate to which no material was applied) did not affect microsomal G-6-Pase activity.

Other analytical procedures. The amount of peroxide functional groups contained in the dialysate extract and the lipid materials eluted from the various t.l.c. bands was determined by iodimetric titration, according to Wheeler's method [30] as reported by Swern [31].

The amount of aldehyde (carbonyl) functional groups contained in the dialysate extract and the lipid materials eluted from the various t.l.c. bands was determined by converting aldehydes (or carbonyl compounds) to their *p*-nitrophenylhydrazone derivatives, according to the method of Wittenberg *et al.* [32]. This method, which has been used for the estimation of higher aldehydes present in tissue lipids, satisfactorily detects aldehydes (or carbonyl compounds) of only 14 or more carbon atoms, although C_8 and C_{10} aldehydes are partially determined. The *p*-nitrophenylhydrazones of aldehydes of shorter chain length have distribution coefficients in favor of water in the water–petroleum ether system used in this method for the extraction of the *p*-nitrophenylhydrazone derivatives.

The determination of free fatty acids in the dialysate extract was performed either by Duncombe's method [33] or by gas–liquid chromatography (g.l.c.). In the former case, a modified Duncombe's method adapted for microdetermination was used. In the latter case, methyl esters from free fatty acids were prepared by heating an aliquot of the dialysate extract (dried under a nitrogen stream) with the boron trifluoride–methanol reagent (Analabs) at 75° for 2 min. Fatty acid methyl esters were extracted and analyzed by g.l.c. as previously reported [34]. In some experiments, fatty acid methyl esters were purified by t.l.c. as previously described [35]. The weight of the fatty acids was determined by using a known amount of internal standard

Table 1. Effect of various preparations of a dialysate derived from a peroxidizing microsome system on the glucose 6-phosphatase activity of freshly prepared liver microsomes (test system)

	Whole dialysate		Dialysate extract		Dialysate-after-the-extraction	
	Glucose 6-phosphatase activity*	Percentage variation	Glucose 6-phosphatase activity*	Percentage variation	Glucose 6-phosphatase activity*	Percentage variation
No addition	9.52 ± 0.23	—	8.73 ± 0.23	—	9.29 ± 0.22	—
+NADPH −microsomes	8.95 ± 0.28	−6.0	8.69 ± 0.24	−0.5	8.81 ± 0.18	−5.2
+Microsomes −NADPH	9.91 ± 0.29	+4.1	8.53 ± 0.26	−2.3	9.20 ± 0.30	−1.0
+Microsomes +NADPH	7.67 ± 0.26†	−19.4	1.84 ± 0.13‡	−78.9	8.85 ± 0.19	−4.7

The dialysate from the peroxidizing microsome system [plus microsomes, plus NADPH-generating system–$FeSO_4$ (indicated as NADPH)] or from the control systems (no additions; plus NADPH, minus microsomes; plus microsomes, minus NADPH) was prepared as described in Materials and Methods. Freshly prepared liver microsomes (test system) were incubated aerobically with the whole dialysate, the dialysate extract and the dialysate-after-the-extraction, for 60 min at 37°. At the end of the incubation the glucose 6-phosphatase activity was assayed. The extraction of the inhibitory activity from the dialysate was performed with ethyl acetate.

Values are the means ± S.E.M. of 4 experiments.

* Glucose 6-phosphatase activity is expressed as μmoles of inorganic phosphorus produced in 20 min per milligram of microsomal protein.

† Significantly different from each control ($P < 0.01$).

‡ Significantly different from each control ($P < 0.001$).

(heptadecanoic acid, Fluka AG, Buchs, Switzerland).

The determinations of lipid phosphorus and glycerides in the dialysate extract were performed according to Shin [36] and Van Handel and Zilversmit [37], respectively.

RESULTS

Loss of G-6-Pase activity of liver microsomes exposed to products extracted from the dialysate derived from a peroxidizing microsome system. As previously mentioned, the inhibitory activity of the whole dialysate (i.e. the content of the dialysis tube before the extraction), of the dialysate extract (i.e. the substances extracted from the content of the dialysis tube) and of the dialysate-after-the-extraction was evaluated by using the G-6-Pase activity of freshly prepared liver microsomes as a test system.

As shown in Table 1, when the whole dialysate was used, a slight (20–25%) but significant decrease was observed in the experimental sample (obtained by using the dialysate derived from the sample in which lipid peroxidation occurred) as compared to the control samples (obtained by using the dialysates from the samples in which, because of the absence of microsomes, or NADPH, or both, lipoperoxidation either did not occur or occurred to a very slight extent, as in the case of the control sample in which only microsomes were present). This result therefore indicates that the dialysate recovered at the end of the incubation contains the inhibitory products. An approach to the extraction of the inhibitory products, therefore, can be confidently made.

Table 2. Amount of malonic dialdehyde produced during the peroxidation of liver microsomal lipids and recovered in the dialysate and in the systems used to test the toxicological activity of the whole dialysate, the dialysate extract and the dialysate-after-the-extraction

	Malonic dialdehyde concentration*				
	In the peroxidizing system (outside dialysis tube)	In the dialysate	In the test system for the whole dialysate	In the test system for the dialysate extract	In the test system for the dialysate after the extraction
Experiment 1					
+Microsomes +NADPH	7.96 ± 0.68†	7.65 ± 0.64	6.92 ± 0.63	0.32 ± 0.15	5.64 ± 0.51
+Microsomes −NADPH	0.57 ± 0.17	0.42 ± 0.03	0.36 ± 0.02	0.01 ± 0.003	0.31 ± 0.02
Experiment 2					
+Microsomes +NADPH	6.61 ± 0.15	5.89 ± 0.15	5.39 ± 0.24	0.28 ± 0.03	2.96 ± 0.16
+Microsomes −NADPH	0.71 ± 0.04	0.30 ± 0.01	0.31 ± 0.01	0.03 ± 0.01	0.22 ± 0.01

Conditions were as described in Table 1. The malonic dialdehyde concentration was measured in the various incubation systems at the end of the respective incubations. The extraction of the inhibitory activity was performed with ethyl ether in experiment 1 and with ethyl acetate in experiment 2.

* Malonic dialdehyde is expressed as μg per ml.

† Values are the means ± S.E.M. of 3 experiments for experiment 1 and of 4 experiments for experiment 2.

Table 1 shows that when the dialysate extract was tested for inhibitory activity with the same system, an almost complete inactivation of microsomal G-6-Pase was seen in the experimental sample. Table 1 shows the results obtained in the experiments in which the extraction of the inhibitory products from the dialysate was performed with ethyl acetate. Identical results were also obtained when ethyl ether was used for the extraction of the inhibitory factors. It appears therefore that the products provided with inhibitory activity can be extracted equally well from the dialysate with either solvent.

On the other hand, when the dialysate-after-the-extraction was tested for inhibitory activity with the same system, no loss of microsomal G-6-Pase was seen (Table 1). It appears, therefore, that the inhibitory products derived from peroxidation of liver microsomal lipids, and present in the content of the dialysis tube, have been completely extracted from the dialysate with the above reported technical procedures. The higher degree of enzyme inactivation found by using the dialysate extract as opposed to the whole dialysate, is most probably due to the different amounts of dialysate used in either case (10 ml or an approximately equivalent amount for the extraction and 1.5 ml for the test of the whole dialysate).

Table 2 shows that during the incubation of liver microsomes in the NADPH-dependent system a vigorous production of MDA occurs (outside the dialysis tube). The MDA concentration outside and inside the dialysis tube nearly reached the equilibrium. MDA was found in approximately the same concentration in the test system in which the whole dialysate was used. On the contrary, almost no MDA was present in the system in which the dialysate extract was tested for the inhibitory activity, either before (not shown in Table 2) or after the incubation (Table 2). The concentration of MDA in the system in which the dialysate-after-the-extraction was tested was only slightly lower than that found in the test system in which the whole dialysate was used, in the experiments in which the extraction was performed with ethyl ether (Table 2, experiment 1); it was clearly lower in the experiments in which ethyl acetate was used for the extraction (Table 2, experiment 2). This could be due to the repartition of MDA (which is water soluble) in the water that remains in the upper phase (see Materials and Methods) after the extraction of the dialysate with ethyl acetate. Even in this case, however, no MDA was found in the test system in which the dialysate extract was used.

The reason why ethyl ether was replaced by ethyl acetate as extraction solvent was that, in some experi-

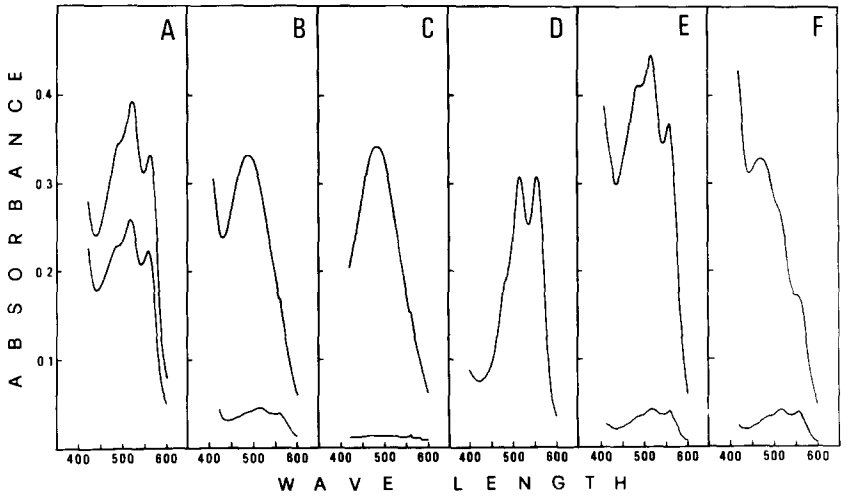

Fig. 1. Absorption spectra of the chromogens produced in Dugan's assay for peroxide. Panel A, upper trace: extract derived from sedimented microsomes incubated in presence of NADPH. Panel A, lower trace: extract derived from sedimented microsomes incubated in absence of NADPH. Panel B, upper trace: extract derived from the incubation medium (i.e. the supernatant fraction) obtained by centrifuging the incubated microsome–NADPH mixture. Panel B, lower trace: extract derived from the incubation medium (i.e. the supernatant fraction) obtained by centrifuging the incubated control microsomes (no NADPH). Panel C, upper trace: extract of dialysate derived from the incubated microsome–NADPH mixture. Panel C, lower trace: extract of dialysate derived from the incubated control microsomes (no NADPH). Panel D: H_2O_2. Panel E: arachidonic acid irradiated with U.V. for 3 hr (upper trace) and not irradiated (lower trace). Panel F: arachidonic acid exposed to a stream of air for 48 hr at room temperature (upper trace) or not exposed to a stream of air (lower trace). Amounts used in the assay were: the lipids extracted from the microsomal sediment recovered from 8 ml of incubation mixture (see Materials and Methods) (A); the lipids extracted from the supernatant fraction (incubation medium) recovered from the same amount of incubation mixture (B); the lipids extracted from 8 ml of dialysate (C); 0.05 μmoles of H_2O_2 (D); 0.66 μmoles of arachidonic acid (peroxide content after the irradiation, 0.25 μmoles/mg, as determined by iodimetric titration) (E); 0.82 μmoles of arachidonic acid (peroxide content after the exposure to air, 2.4 μmoles/mg, as determined by iodimetric titration) (F). Lipids dissolved in 1 ml of chloroform were allowed to react with 1 ml of the *N,N'*-dimethyl-*p*-phenylenediamine reagent. Hydrogen peroxide standard was prepared as described by Pesh-Imam *et al.* [25] and allowed to react as above for 90 min.

ments in which the inhibitory activity was tested after storing the dialysate extract at −20° overnight, some inactivation of microsomal G-6-Pase was seen even in the control samples. This could be due to some inhibitory factors formed in ethyl ether during the storage. In some experiments, in fact, ethyl ether extracts from the control samples, although free of peroxides immediately after the extraction, showed the presence of peroxides (which can easily form in ethyl ether), when tested by Dugan's method after a 24-hr storage. Since the ethyl acetate extracts from the control samples remained free of peroxides and did not inhibit G-6-Pase activity even after a 48-hr storage, in the subsequent experiments ethyl ether was not used.

Absorption spectrum of the chromogen produced by the dialysate extract in the Dugan's assay for peroxides. The dialysate extract from the experimental sample exhibited a marked reactivity with the *N,N'*-dimethyl-*p*-phenylenediamine reagent for peroxides (see Materials and Methods). However, the absorption spectrum of the chromogen (Fig. 1C, upper trace) was different from that of the chromogen obtained with hydrogen peroxide (Fig. 1D) or with arachidonic acid irradiated with u.v. for 3 hr (Fig. 1E, upper trace). The latter spectra showed two peaks at 515 and 560 nm and a shoulder at 485 nm, while the spectrum of the chromogen obtained with the dialysate extract (Fig. 1C, upper trace) showed an apparently single peak with maximum at 480–485 nm and a little shoulder at 560 nm. The origin of the substances responsible for the spectrum obtained with the dialysate extract was studied in additional experiments in which the extract obtained from the peroxidized microsomes and that obtained from the incubation medium (supernatant fraction obtained by recentrifuging the incubation mixture at the end of the incubation, see Materials and Methods), as well as the dialysate extract, were allowed to react with the *N,N'*-dimethyl-*p*-phenylenediamine reagent. It was found that the chromogen (frankly pink) obtained with the extract from the peroxidized microsomes showed a spectrum (Fig. 1A, upper trace) similar to that observed with H_2O_2 or with peroxidized arachidonic acid, while the chromogen obtained with the extract from the incubation medium exhibited a spectrum (Fig. 1B, upper trace) that was almost identical to that observed with the dialysate extract. In the two latter spectra, the 485 nm component of the spectrum seen in the case of peroxidized microsomes is by far the major component and this explains the change of the overall color to a yellowish orange. A somewhat similar configuration was found in the spectrum of arachidonic acid peroxidized in air for 48 hr (Fig. 1F, upper trace). The peroxide concentration (determined by iodimetric titration) in the latter (2.4 μmoles/mg) was higher than that (0.25 μmoles/mg) found in arachidonic acid irradiated with u.v. for 3 hr (Fig. 1E, upper trace). These results

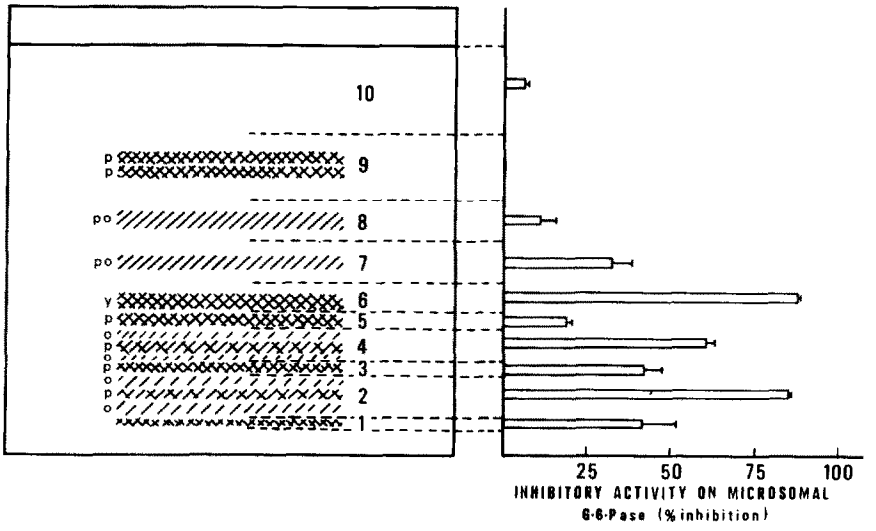

Fig. 2. Thin-layer chromatography of the lipid material extracted from a dialysate derived from a peroxidizing microsome system and inhibitory activity of the various chromatographic bands on microsomal glucose 6-phosphatase. The dialysate from the peroxidizing system was prepared and extracted as described in Materials and Methods. T.l.c. was performed by using a solvent system of n-heptane–ethyl acetate–acetic acid (75:25:1, by vol.). The bands were detected by spraying the chromatoplate with a solution of *N,N'*-dimethyl-*p*-phenylenediamine [29]. When the various chromatographic bands were tested for the inhibitory activity, the extract derived from 120 ml of dialysate was applied to the chromatoplate in the central part, which was protected from the spray, as described in Materials and Methods. The chromatoplate was divided into 10 parts corresponding to individual bands or groups of bands, as depicted in the figure. The various areas of the chromatoplate were scraped off and the lipid materials were eluted, resuspended and tested for inhibitory activity on the G-6-Pase activity of freshly prepared liver microsomes (test system) as described in Materials and Methods. The inhibitory activity of the various areas of the chromatoplate was expressed as percentage of the glucose 6-phosphatase activity of the microsomes of the test system incubated in the absence of the materials eluted from the chromatoplate. Results are the means ± S.E.M. of five to eight experiments. Colours of the various chromatographic bands after detection with the *N,N'*dimethyl-*p*-Phenylenediamine solution: 0 = orange; p = pink; y = yellow; po = pale orange.

Table 3. Content in peroxide and carbonyl functional groups in the various bands obtained in the thin-layer chromatography of the extract from a dialysate derived from a peroxidizing microsome system

	Peroxide functional groups (nmoles/extract from 100 ml dialysate)	Carbonyl functional groups (nmoles/extract from 100 ml dialysate)
Unfractionated sample	267 ± 17.0	687
Area no. 1	24 ± 0.9	7
Area no. 2	90 ± 16.8	28
Area no. 3	19	14
Area no. 4	24 ± 8.2	23
Area no. 5	22 ± 3.2	26
Area no. 6	14 ± 4.4	318
Area no. 7	19	35
Area no. 8	23	5
Area no. 9	4	2
Area no. 10	0	3
Per cent recovery	89.5	67.1

The thin-layer chromatographic analysis was performed as described in Fig. 2. The chromatoplate was divided into 10 parts as reported in Fig. 2. The lipid materials were eluted from the various areas of the chromatoplate and assayed for the content in peroxide and aldehyde functional groups as described in Materials and Methods. Results are the means of either three experiments (shown with standard errors) or of two experiments.

seem to indicate that during the incubation peroxides are formed in the microsomal lipids and that products related to or derived from these peroxides are released into the incubation medium and can be recovered in the dialysate. A similar spectrum of the chromogen produced in the reaction of peroxidized microsomal lipids with *N,N'*-dimethyl-*p*-phenylenediamine has also been reported by Pesh-Imam *et al.* [25]. On the other hand, when liver microsomes were incubated in the absence of NADPH generating system–$FeSO_4$ (control sample), although some peroxides were formed in the microsomal lipids, as shown by the spectrum of the chromogen produced in the reaction with the *N,N'*-dimethyl-*p*-phenylenediamine reagent (Fig. 1A, lower trace), almost no chromogen was formed when the extracts from either the incubation medium (Fig. 1B, lower trace) or the content of the dialysis tube (Fig. 1C, lower trace) were allowed to react with the same reagent. It seems therefore that the Dugan-positive materials (Fig. 1A, lower trace) which appear in the extract of sedimented control microsomes (i.e. no added NADPH) are not released into the medium (Fig. 1B, lower trace).

Thin-layer chromatographic analysis of the dialysate extract. An attempt at the separation of the products present in the dialysate extract derived from the experimental sample was performed by using t.l.c. Figure 2 shows the typical pattern of bands obtained with the solvent system described in Materials and Methods, after detection with a spray of a *N,N'*-dimethyl-*p*-phenylenediamine solution [29]. As can be seen, a number of pink and orange bands were observed; some of them were poorly resolved. Moreover, a yellow band with an R_f value of 0.33 was clearly visible. A similar pattern of bands, although with a lower resolution, was seen when we used a solvent system of n-heptane–ethyl ether–acetic acid (60:40:1, by vol.) as used by McCay *et al.* [38] to separate metabolites derived from linoleic acid hydroperoxide. Detection of the bands with a spray of an aqueous solution of sodium fluorescein (0.025%, w/v) showed similar distribution of bands.

No detectable bands were seen when the extract of the dialysate derived from the control sample (plus microsomes, minus NADPH) was analyzed by t.l.c. as above.

Test of the various t.l.c. bands for the inhibitory activity on microsomal G-6-Pase. The chromatoplate obtained from the experimental sample was divided into 10 parts corresponding to individual bands or group of bands, as depicted in Fig. 2. The lipid materials eluted from the various areas of the chromatoplate were tested for their capacity to inhibit the G-6-Pase activity of freshly prepared liver microsomes (test system). As reported in Fig. 2, the highest inhibitory activity was found in the chromatographic band that was stained yellow by the *N,N'*-dimethyl-*p*-phenylenediamine spray (area no. 6). The products eluted from areas no. 2, 4, 3 and 1 (origin) also exhibited a marked inactivating capacity, while the products eluted from the areas no. 8, 9 and 10 showed minor effects. Some inhibitory activity was also found in the areas no. 7 and 5.

No inhibition of microsomal G-6-Pase activity was found in the control samples, obtained by scraping the silica gel from the corresponding areas of the control (plus microsomes, minus NADPH; minus microsomes, plus NADPH; no additions) chromatoplate and run under the same conditions as those used for the experimental sample.

Analysis of the dialysate extract. Studies were carried out in order to investigate the nature of the products inhibiting microsomal G-6-Pase activity. The unfractionated dialysate extract from the experimental sample as well as the lipid material eluted from the various t.l.c. bands were analyzed for the content of both peroxide and carbonyl functional groups (Table

3). The peroxide content of the unfractionated extract derived from 100 ml of dialysate was somewhat higher (335 ± 21) when determined by the *N,N'*-dimethyl-*p*-phenylenediamine reagent against H_2O_2 as a standard, than determined by iodimetric titration (see Table 3). The peroxide content in the various areas of the chromatoplate (according to the scheme depicted in Fig. 2) was determined by the iodimetric titration only, since the *N,N'*-dimethyl-*p*-phenylenediamine method, used in some experiments, seemed to give unreliable results*. As shown in Table 3, the highest peroxide content was found in the lipids eluted from the area no. 2. The lipids eluted from the areas no. 4, 1 (origin), 8 and 5 also contained some peroxidic functions, while the band in the area no. 6, as well as the remaining areas of the chromatoplate, contained only minor amounts. No peroxides were found either in the unfractionated dialysate extract or in any of the corresponding areas of the chromatoplate derived from the control sample (plus microsomes, minus NADPH).

The amount of carbonyl functional groups contained in the unfractionated dialysate extract was much higher than that of peroxide functional groups (687 vs 267). When the amount of carbonyl functional groups was determined in the various t.l.c. bands, it was found (Table 3) that the major part of carbonyl functional groups was contained in the band that is stained yellow by the *N,N'*-dimethyl-*p*-phenylenediamine spray (area no. 6). The other areas of the chromatoplate, on the contrary, contained minor amounts of carbonyl functions. Therefore, most of the aldehydes or carbonyl compounds that can be detected by the method [32] used in the present experiments (long chain fatty aldehydes, see Materials and Methods) can be separated in an area of the chromatoplate which contains the highest inhibitory activity. Probably aldehydes or ketones of short chain length, which cannot be extracted by petroleum ether with the method [32] used for aldehyde determination, are contained in some other areas of the chromatoplate, especially in area no. 2. This was deduced by the intense yellow colour appearing in the respective samples after the addition of nitrophenylhydrazine and before the extraction with petroleum ether. No carbonyl compounds were found either in the unfractionated dialysate extract or in any of the corresponding areas of the chromatoplate derived from the control sample (plus microsomes, minus NADPH).

The dialysate extract, whether from the experimental or the control (plus microsomes, minus NADPH) sample, does not contain lipid phosphorus, thus excluding phosphatides as the inhibitory factors. Also, no glycerides were found in the dialysate extracts. The possibility that the inhibitory factors were free fatty acids released from the microsomal phospholipids by phospholipases (see Niehaus and Samuelsson [39]) was taken into consideration. Studies on the phospholipid requirement of G-6-Pase [40] suggested, in fact, that free fatty acids (released by phospholipase action on microsomal lipids) and lysophosphatides could inhibit G-6-Pase activity. When Duncombe's method was used for the determination of free fatty acids, it was found that the apparent content in free fatty acids of the dialysate extract from the experimental sample was 120 μg in the extract derived from 100 ml of dialysate. The apparent fatty acid content of the dialysate extract from the control sample (plus microsomes, minus NADPH) was found to be minor. When, on the other hand, the fatty acid content of the dialysate extracts was determined by g.l.c. it was found that similar amounts of free fatty acids occur in the dialysate extracts from either the experimental or the control sample (10.0 and 15.9 μg in the extract derived from 100 ml of dialysate, respectively). Since a similar amount of free fatty acids was also found by g.l.c. in the dialysate extract derived from the control samples in which no microsomes were added (no additions: plus NADPH, minus microsomes), it seemed likely that the amount of fatty acids detected by g.l.c. was present in all samples as contaminants derived from the solvents or the buffer used. Since g.l.c. detects fatty acid molecules, while Duncombe's method measures organic acid functions, it appeared that most of the products detected by Duncombe's method in the dialysate extract from the experimental sample are modified fatty acids bearing additional functional groups, such as hydroxy, peroxy or carbonyl groups and probably formed during lipoperoxidation. This view was supported by the fact that the acid functions determined by Duncombe's method were found in almost all areas of chromatoplate after t.l.c. of the dialysate extract from the experimental sample, while standard fatty acids were seen to move to the level of area no. 7 (with a minor portion in area no. 8) in the chromatographic system used. G.l.c. analysis of the lipid materials eluted from the various areas of the chromatoplate derived from the experimental and control (no addition; plus NADPH, minus microsomes) samples revealed the presence of fatty acids in areas no. 7 and 8 only; again, similar amounts of fatty acids were found in the experimental and the control samples. A similar pattern of fatty acids ($C_{16:0}$, 46%; $C_{16:1}$, 12%; $C_{18:0}$, 15%; $C_{18:1}$, 19%; $C_{18:2}$, 8%; no detectable $C_{20:4}$) was also seen in all the samples. These observations confirmed the occurrence of fatty acid contaminants in our samples. Studies carried out to investigate the effects of fatty acids on microsomes G-6-Pase, showed that the addition of stearic acid to microsomes, under the condition used to test the various t.l.c. bands for the inhibitory activity, has no effect even in a relatively high amount (0.879 mM). When a mixture of palmitic, stearic, linoleic and arachidonic acid (1:1:1:1, by wt) was used, some inhibitory effect was observed, but only with an amount of 0.356 mM of the mixture. The latter inhibition could well be due to the peroxidation of arachidonic acid during the incubation.

DISCUSSION

The present paper shows that dialysable products originating from the peroxidation of liver microsomal lipids and inhibiting microsomal G-6-Pase activity, can be separated from the dialysate by means of extraction procedures. Both ethyl ether and ethyl acetate are equally effective in extracting the inhibitory products. The latter solvent was preferred throughout the present work because of its chemical stability even after prolonged periods of storage. Other solvents (n-heptane, chloroform, dichloromethane) appeared to be less ef-

* The yellow colour obtained with the eluate from area no. 6, for instance, rapidly faded away and a pink colour, increasing with time, appeared.

fective in extracting the inhibitory factors. The fact that the inhibitory products can survive the extraction procedure indicated, as previously suggested [20–22], that the factors responsible for the impairment of microsomal G-6-Pase activity are chemicals of a stable nature. Since the extraction procedure completely removes the inhibitory activity from the dialysate, the inhibitory factors appear to be substances of a hydrophobic nature.

A partial separation of the inhibitory factors from other substances present in the dialysate extract was achieved by t.l.c. After t.l.c. of the dialysate extract, in fact, the inhibitory activity was concentrated in a few chromatographic bands. Among these bands, the band that is stained yellow by the *N,N'*-dimethyl-*p*-phenylenediamine spray reagent (area no. 6), contains the highest inhibitory activity on microsomal G-6-Pase. Preliminary studies from our laboratory have shown that the products eluted from this band also exhibit a very powerful hemolytic activity, when a test system consisting of blood red cells was used. It is therefore conceivable that this t.l.c. band contains the main products responsible for the cytopathological effects observed in the target systems contained in the dialysis tube, as shown in our previous studies [20–22].

The present work offers some information on the nature of the lipoperoxidation products that inhibit microsomal G-6-Pase activity. The studies in which the extracts from the peroxidized microsomes, the incubation medium, and the dialysate were allowed to react with the *N,N'*-dimethyl-*p*-phenylenediamine reagent, seem to indicate that products related to or derived from peroxides formed in the microsomal lipids, occur in the dialysate extract. However, peroxides do not appear to be the primary inhibitory products, since very low amounts of peroxide functional groups were found in the band (area no. 6) that shows the highest inhibitory activity. Even if both the highest level of peroxides and a very high inhibitory activity were found in area no. 2, it must be considered that this area contains a number of unresolved bands which are differently stained by *N,N'*-dimethyl-*p*-phenylenediamine. Thus, products of different natures may occur in this area, and therefore no clear indications can be deduced from this result.

The study of the distribution of carbonyl functional groups in the various t.l.c. bands indicate that the carbonyl functions are mainly concentrated in the chromatographic band (area no. 6) that exhibits the highest inhibitory activity. Since as previously stated, almost no peroxidic functions can be found in this band, it can be concluded that highly active inhibitory products originating from the peroxidation of liver microsomal lipids, are compounds bearing carbonyl functional groups, as aldehydes and/or ketones. Since, as stated above, the method [32] used for aldehyde determination satisfactorily detects aldehydes (or carbonyl compounds) of only 14 or more carbon atoms, it should be concluded that the lipoperoxidation products that are highly active in inhibiting microsomal G-6-Pase activity and that also exhibit a marked hemolytic effect (see above), are fatty aldehydes or other carbonyl compounds, provided with a relatively long carbon chain. In addition, part of the inhibitory factors may be short chain aldehydes or ketones, which can be partially extracted from the dialysate by the relatively polar solvents used, and which probably are contained in some areas of the chromatoplate (see above). However, it can be stated that MDA, which in the present work was taken as an index of lipoperoxidation, is not the inhibitory factor. In fact, MDA does not occur in the test system in which there is inhibition of G-6-Pase activity, while it is present in the test system in which G-6-Pase is not inhibited (see Table 2).

The results of the experiments in which the effects of fatty acids on the microsomal G-6-Pase activity were studied (see the Results section) seem also to exclude the possibility that free fatty acid molecules are the primary inhibitory factors.

In conclusion, even if part of the inhibitory factors may be compounds of peroxidic nature, the present results indicate that during the course of the peroxidation of liver microsomal lipids, products bearing carbonyl functional groups and having the capacity of inducing pathological effects are formed. Products of aldehydic nature are well known to originate from the breakdown of lipid peroxides. The inhibitory activity of aldehydes on enzymatic systems of primary biological interest has been known for a long time [41–44]. The possibility that aldehydes covalently bind to proteins and to other biological substances is also well known [41, 45, 46]. In this respect, it can be mentioned that previous work from our laboratory [47] showed that during the peroxidation of liver microsomal lipids (which, in these experiments, contained labeled arachidonic acid) products are formed which have the capacity of binding to microsomal protein. It was also observed [22] that these products can pass the dialysis membrane (in an experimental device similar to that used in the present work) and bind to the non lipid constituents of the microsomes of the target system (contained within the dialysis tube).

The recognition that cytopathological products originate from the peroxidation of liver microsomal lipids offers a new approach to the understanding of the mechanisms by which halogenated hydrocarbons such as CCl_4 and CCl_3Br or other chemical or physical agents that promote the peroxidation of membrane lipids, may act in inducing their pathological effects.

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