Water-soluble inhibitor(s) of tumor respiration formed from ultraviolet-induced oxidation of linoleic and linolenic acids

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ABSTRACT Inhibition of Ehrlich ascites carcinoma respiration by aqueous extracts of oxidized linoleic or linolenic acid (aqueous emulsions UV-irradiated, 90 min) was associated entirely with relatively involatile compounds which were both thiobarbituric acid (TBA)-reactive and peroxidase-reactive. Inhibitory compounds were heat stable and migrated in thin-layer chromatography with aldehydes, "hydroperoxides," and TBA-reactive compounds. Peroxidase-catalyzed reduction of the "hydroperoxide" diminished the inhibition. At 4.7×10^{-5} M "hydroperoxide" concentration, the residues from both linoleic and linolenic acid inhibited tumor oxygen consumption to a similar degree. However, at this concentration of "hydroperoxide" only the dried extract from linolenic acid was able to produce inhibition (100%) of aerobic glucose utilization by tumor cells. No glycolytic inhibition by the dried residue of oxidized linoleic acid was observed. At least 12 compounds (approximate chain length, 7C–13C) containing α,β-unsaturated carbonyl groups were isolated by gas–liquid chromatography (GLC) of dried extracts of oxidized linolenic acid. No single fraction inhibited tumor respiration, but the recombined mixture of all compounds caused complete respiratory inhibition of ascites tumor cells. Less material was required to inhibit oxygen consumption before than after GLC presumably because the more highly inhibitory components of the extract (along with "hydroperoxides" and TBA-reactive compounds) were lost during GLC. Extracts from oxidized linolenic acid were found to produce in all tumor cells cytoplasmic evaginations which were readily detected by phase microscopy.

KEY WORDS polyunsaturated fatty acids · autoxidation · tumor respiration · water-soluble metabolic inhibitors · thiobarbituric acid reactivity · hydroperoxides

Abbreviations: TBA, thiobarbituric acid; TLC, thin-layer chromatography; GLC, gas–liquid chromatography.

ATTEMPTS TO EXPLORE the correlation between thiobarbituric acid (TBA) reactivity of aqueous extracts of autoxidized lipids and inhibition of tumor respiration, first described by Shuster (1), have been hampered for several reasons. First, concentration of the extracts resulted in loss of most of the TBA-reactivity (2). Second, most of the TBA-reactive material appeared to be malonaldehyde (2), which did not inhibit tumor respiration (3). Third, inhibition could be correlated with peroxidase reactivity as well as TBA reactivity (3). Despite extensive efforts to separate inhibitory TBA-reactive and peroxidase-reactive compounds from each other, our attempts have failed. However, we have found a mild procedure for separating inhibitory from noninhibitory TBA-reactive and peroxidase-reactive compounds which should prove helpful in further attempts to isolate metabolic inhibitors from the early oxidation products of polyunsaturated fatty acids. This procedure is described here and in the previous paper (2). Our earlier work, not published in detail heretofore, correlating peroxidase reactivity as well as TBA reactivity with inhibition of tumor respiration is also presented.

METHODS

Linoleic acid and linolenic acid were emulsified in water and exposed for 90 min to UV-irradiation as previously described (2). The emulsions were extracted with petroleum ether and the aqueous residue was assayed for peroxidase and TBA reactivity (2).

The water extracts were separated into volatile and relatively involatile components by sublimation in a Thunberg tube (2). Conditions for the removal of H_2O_2 by catalase and of the "hydroperoxide" function by re-

action with peroxidase and *o*-anisidine were also the same as those used in the preceding paper (2). Reactions were always stopped by placing samples in a boiling water bath for 5 min.

TLC was performed as in the previous study (2, 4). GLC of 14 combined, dried extracts (2) was performed at 175°C on a 180 × 0.5 cm I.D. column, 20% silicone fluid 96 on 60–80 mesh Johns-Manville Chromosorb, flow rate of argon 40 ml/min. A Barber Coleman Model 10 chromatograph equipped with ionization detector with tritium as an ionizing source was used. The metal effluent tube was heated with electrical tape to approximately 190°C and connected tightly to the tip of a 17 gauge needle by a 24 cm loop of flexible plastic tubing (Pennsylvania Fluorocarbon Co., Inc., Clifton Heights, Pa., No. 16 gauge) which was heated in a water bath at 70–80°C. The needle supported a vertically oriented, 2 ml syringe barrel (85 mm × 6 mm I.D.) containing 50–200 μl of absolute ethanol (distilled from NaOH pellets and stored over Linde molecular sieve). The lower half of the syringe was submerged in the hot water bath but the upper half extended into the air. A rubber septum, previously extracted with alcohol, and metal outlet were inserted in the top of the syringe to allow exit of carrier gas.

Ehrlich ascites tumor cells were harvested after 7–14 days of intraperitoneal growth in mice. They were washed twice in Krebs-Henseleit phosphate buffer (5) and centrifuged (20 × *g* for 10 min), to remove differentially most of the red blood cells. The suspension was diluted and counted microscopically to determine the tumor cell concentration. Samples of 6.4 × 10^7 cells in 0.50 ml of Krebs phosphate buffer were added to Warburg flasks containing the test substance dissolved in 2.5 ml of Krebs phosphate buffer. In assaying GLC eluates for inhibition of tumor oxygen consumption, 70 μl of absolute ethanol was included in the incubation mixture. No effect of purified ethanol itself was noted. Tumor cells were kept at 4°C during washing and until they were added to the Warburg flasks. The time of addition was controlled so that, immediately after exposure of cells to inhibitor, each flask was placed in the 37°C bath for temperature equilibration. Approximately 30 min later measurements of oxygen consumption were begun.

Glucose utilization ("glycolysis") was measured at 37°C by adding 3.6 μmoles of glucose in 0.20 ml of the phosphate buffer to each Warburg flask and removing aliquots for glucose assay 1 and 10 min later. Glycolysis was stopped and glucose determined by adding 0.10 ml of the tumor cell suspension to 1.0 ml of ethanolic glucostat reagent (4). Disappearance of glucose during the interval 1–10 min was proportional to cell concentration when 2–4 × 10^7 cells were present in each flask. Only those glycolysis experiments in which 3.2 × 10^7 cells per flask were used are presented here, although all-or-none inhibition was readily detectable and frequently tested by using 6.4 × 10^7 cells per flask (the concentration used for oxygen consumption studies).

In several instances cells were examined by phase microscopy after completion of the respiration study to determine whether morphological evidence of cell damage was present.

RESULTS

Inhibition of Tumor Oxygen Consumption by the Involatile Compound(s)

After removal, by flash evaporation, of most of the volatile components from water extracts of UV-irradiated, oxidized linoleic or linolenic acid, a water-soluble inhibitor(s) of tumor respiration remained in the residue, as shown in Table 1. Although flash evaporation, which was employed as the means of removing volatile components, does not remove H_2O_2 completely, the latter may be removed by catalase treatment (2). Removal of hydrogen peroxide was without significant effect on the inhibition of tumor respiration (Table 1). Separate experiments also established that neither H_2O_2 nor malonaldehyde, which are known to be present in the initial extracts (2), inhibited tumor oxygen consumption significantly (Table 2).

Extracts of linolenic acid were dried in Thunberg tubes (2) so that volatile and relatively involatile fractions could be tested for their ability to inhibit tumor respiration separately and in combination with each

TABLE 1 INHIBITION OF ENDOGENOUS TUMOR RESPIRATION BEFORE AND AFTER REMOVAL OF RESIDUAL H_2O_2 BY CATALASE TREATMENT OF DRIED WATER EXTRACTS OF UV-IRRADIATED UNSATURATED FATTY ACIDS

Irradiated Fatty Acid	ROOH* (M × 10^5)	Percentage Inhibition of Oxygen Consumption by Water Extract, Dried by Flash Evaporation†	
		No Catalase	Plus Catalase‡
Linolenic	1.8	19 (7–24)	33 (17–49)
	3.0	40 (39–41)	59 (41–76)
	3.7	68 (45–94)	74 (56–88)
	4.7	89 (80–97)	84 (62–96)
Linoleic	1.8	11	17
	2.7	29	32
	4.7	72	62

* Peroxidase-reactive material, excluding H_2O_2.

† Each experiment using linolenic acid is the mean (range in parentheses) of results obtained with 2–3 different water extracts of UV-irradiated linolenic acid. The experiment using linoleic acid is based upon a single preparation. Inhibition by extracts of nonirradiated unsaturated fatty acids, ranging from 0 to 7%, was not significant.

‡ No correction has been applied for an observed 10% stimulation which resulted from catalase treatment alone, in the absence of the water extract of irradiated fatty acid.

TABLE 2 EFFECT OF MALONALDEHYDE OR HYDROGEN PEROXIDE ON OXYGEN CONSUMPTION OF EHRLICH ASCITES TUMOR CELLS

Experiment	Addition	Concentration (M × 10^5)	Percentage Inhibition
1	H_2O_2	5.5	0
2		16.4	16
3		16.4	0
4	Malonaldehyde	3.1	10
5		3.1	0
6		3.1	4

TABLE 3 INHIBITION OF TUMOR RESPIRATION BY RELATIVELY INVOLATILE COMPONENT OF WATER-SOLUBLE EXTRACT OF UV-IRRADIATED OXIDIZED LINOLENIC ACID

Additions	ROOH* (M × 10^5)	Equivalent Malonaldehyde† (M × 10^5)	Q_{O_2}‡	Percentage Inhibition
(i) None	0	0	247	0
(ii) Concd water extract of nonirradiated linolenic acid	0	0	224	7
(iii) Concd water extract of UV-irradiated linolenic acid	3.6	1.6	21	92
(iv) Residue of (iii) after drying by sublimation in vacuo	3.6	1.4	29	88
(v) Sublimate of (iii)	0	0.2	262	0
(vi) Concd water extract of UV-irradiated linolenic acid, dried by sublimation, then reconstituted by sublimation in the reverse direction [equivalent to (iv) plus (v)]	3.6	1.6	31	87

* Peroxidase-reactive material excluding H_2O_2. The latter's concentration was 0.6 × 10^{-5} M before sublimation in the concentrated water extract of UV-irradiated linolenic acid.

† Assayed by thiobarbituric acid (TBA). The residual TBA-reactive material after sublimation is not malonaldehyde since malonaldehyde is quantitatively recovered in the volatile fraction under the present conditions.

‡ μl O_2/120 min per 10^8 cells. Each value is the mean of triplicate determinations.

other. The results of these experiments, shown in Table 3, established that all of the inhibitory compounds are relatively involatile and that addition of the volatile fraction does not influence significantly the inhibitory properties of the involatile residue.

Influence of the "Hydroperoxide" Moiety

The results of experiments shown in Table 4 indicate that when the "hydroperoxide" moiety in the water extract of UV-irradiated linolenic acid was completely reduced by peroxidase and *o*-dianisidine a major part of the inhibitory properties of the extract remained. However, the preparation was less inhibitory than if the hydroperoxide groups were not reduced prior to the respiration study.

Control experiments established the absence of appreciable inhibitors of tumor respiration in extracts of linolenic acid that had not been irradiated (Tables 3 and 4).

Heat Stability of Inhibitors of Tumor Respiration

After being treated with either catalase (Table 1) or peroxidase (Table 4) the reaction mixtures were exposed to high temperature (100°C for 5 min); nevertheless, their inhibitory properties remained unimpaired.

Recovery of Inhibitor of Tumor Respiration after TLC

The inhibitor(s) of tumor respiration migrated, at least in part, to $R_f = 0.75$ in the solvent system used in previous studies (2, 4), as shown in Table 5. Aldehydic, TBA-reactive, peroxidase-reactive, yellow, UV-absorbing components of the dried residue also moved to this position, as shown previously (2). An eluate of Silica Gel G from a plate to which no extract of oxidized fatty acid had been applied was inhibitory. In experiments 1–3 and 7 (Table 5), the appropriate controls were run, but the controls for experiments 4–6 (Table 5) were not included in the experiment. Nevertheless, eluates from regions immediately above and below that corresponding to R_f = 0.75 were less inhibitory in each case than the eluate

TABLE 4 EFFECT OF REDUCTION OF THE HYDROPEROXIDE GROUP BY PEROXIDASE AND DYE ON INHIBITION OF ENDOGENOUS TUMOR RESPIRATION BY WATER-SOLUBLE OXIDATION PRODUCT OF UV-IRRADIATED LINOLENIC ACID

Addition	Q_{O_2}* Expt. 1	Q_{O_2}* Expt. 2
(a) None	618	503
(b) Water extract† of nonirradiated linolenic acid	618 (0)	540 (−7)
(c) Water extract† of irradiated linolenic acid (ROOH = 3.6 × 10^{-5} M)	73 (88)	111 (78)
(d) Same as (c) but ROOH reduced by peroxidase–dye	270 (56)	195 (61)
(e) H_2O_2 (3.6 × 10^{-5} M) but reduced by peroxidase–dye	(0)‡	490 (3)

* μl O_2/120 min per 10^8 cells. Each value is the mean of triplicate determinations. (Percentage inhibition is shown in parentheses).

† Concentrated by flash evaporation to approximately 25% of the initial volume. ROOH is peroxidase-reactive material, excluding H_2O_2.

‡ Prior to experiment 1, the Q_{O_2} of a separate population of tumor cells was shown to be unaffected by peroxidase plus H_2O_2-oxidized dye (*o*-dianisidine).

TABLE 5 INHIBITION OF ENDOGENOUS TUMOR RESPIRATION BY TLC ELUATES OF DRIED WATER EXTRACTS OF UV-IRRADIATED, OXIDIZED UNSATURATED FATTY ACIDS

Expt.	Irradiated Fatty Acid	ROOH (M × 10^6)	Percentage Inhibition of Oxygen Consumption: Uncorrected*	Percentage Inhibition of Oxygen Consumption: Corrected
1	Linolenic	2.4	82	70
		silica gel blank	12	
2	"	3.7	100	77
		silica gel blank	23	
3	"	5.0	88	76
		silica gel blank	12	
4	Linoleic	1.8	39	10
		"blank"†	29	
5	"	2.6	100	32
		"blank"†	68	
6	"	2.7	65	25
		"blank"†	40	
7	"	3.3	46	37
		silica gel blank	9	

* Uncorrected for inhibition from silica gel blank. Inhibitor was eluted from a 1 cm band at R_f 0.75 (2).

† Corresponding blank region not tested. Value is average per cent inhibition obtained with silicia gel eluate from regionsi mmediately above and below band corresponding to maximum ROOH.

which included most of the peroxidase reactivity. The corrected values for percentage inhibition (last column, Table 5) represent minimal values in experiments 4–6, since the "blank" which was subtracted (Table 5) probably included some inhibitory compounds in addition to those in the appropriate blank. This is substantiated by the previously reported finding that approximately 33% of the ROOH material in the extract of oxidized linoleic acid was found in the regions immediately above and below the major ROOH-containing region (Fig. 4, reference 2), and by the data in the next to last column of Table 5, which suggest that the inhibition produced by eluates of silica gel ranged from 9 to 23% whereas that of the "blanks" in experiments 4–6 ranged from 29 to 68%.

Aerobic glucose utilization was inhibited completely by dried extracts of oxidized linolenic acid, which contained 4.5×10^{-5} M ROOH (Fig. 1), as it was by chromatographed and nonchromatographed samples of the dried extract under the assay conditions (6.4×10^7 cells per flask) used to study oxygen consumption. On the other hand, the extract of oxidized linoleic acid did not inhibit glucose utilization at this concentration of ROOH, as shown in Fig. 1 and in separate experiments using higher cell concentrations (6.4×10^7 cells per flask). The method used to measure glycolysis is not highly accurate, although it was reproducible as a means of demonstrating complete inhibition when it occurred. Interference in the glucose assay due to the presence of a substance, soluble in 60% ethanol, derived from the tumor cells was usually observed, but could be corrected for by taking a 1 min sample instead of a zero-time control. The method also gave spurious results occasionally, if tumor cells were not carefully dispersed. The one high value for inhibition by the extract of oxidized linoleic acid at 1.2×10^{-5} M ROOH (Fig. 1) could not be confirmed in other experiments and is considered atypical.

GLC of the Dried Extract

GLC of the sublimated extract established the presence of at least eight major peaks (Fig. 2A, upper tracing). Continuous collection of the effluent in ethanol and reinjection of a portion of the effluent immediately thereafter showed that all eight peaks had been recovered proportionately and that there was little or no breakdown of these eight components during chromatography (Fig. 2A, lower tracing). Eight individual fractions enriched with respect to each of the eight components were accumulated from five repeated injections of the master preparation (approximately 3.6 mg each passage). The composition of each fraction collected was checked by GLC. Tracings of the latter records are shown in Figs. 2B and C. Fractions 1, 2, 3, and 4 were found to consist of two definite components each; peaks 7 and 8 each had a shoulder. There was also some contamination of each of fractions 2–5 by components of the mixture which had earlier retention times. Fractions 7 and 8 each showed an early peak (Fig. 2C, X_7 and X_8) which may have resulted from decomposition during the 1 day of storage of the isolated fraction prior to its reinjection. No decomposi-

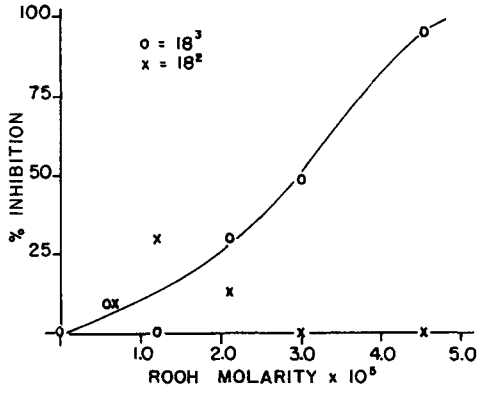

FIG. 1. Inhibition of tumor glucose utilization by dried water extracts of UV-irradiated, oxidized linoleic (18^2) or linolenic (18^3) acid. Assays were performed in duplicate. Of five determinations using extract of oxidized linoleic acid, only at 1.2×10^{-5} M ROOH concentration was significant inhibition observed, and this was considered to be spurious (see text). The extracts used here had been flash evaporated to dryness, reconstituted in 95% ethanol, dried by sublimation in vacuo, and redissolved in phosphate buffer. They were free from both H_2O_2 and malonaldehyde.

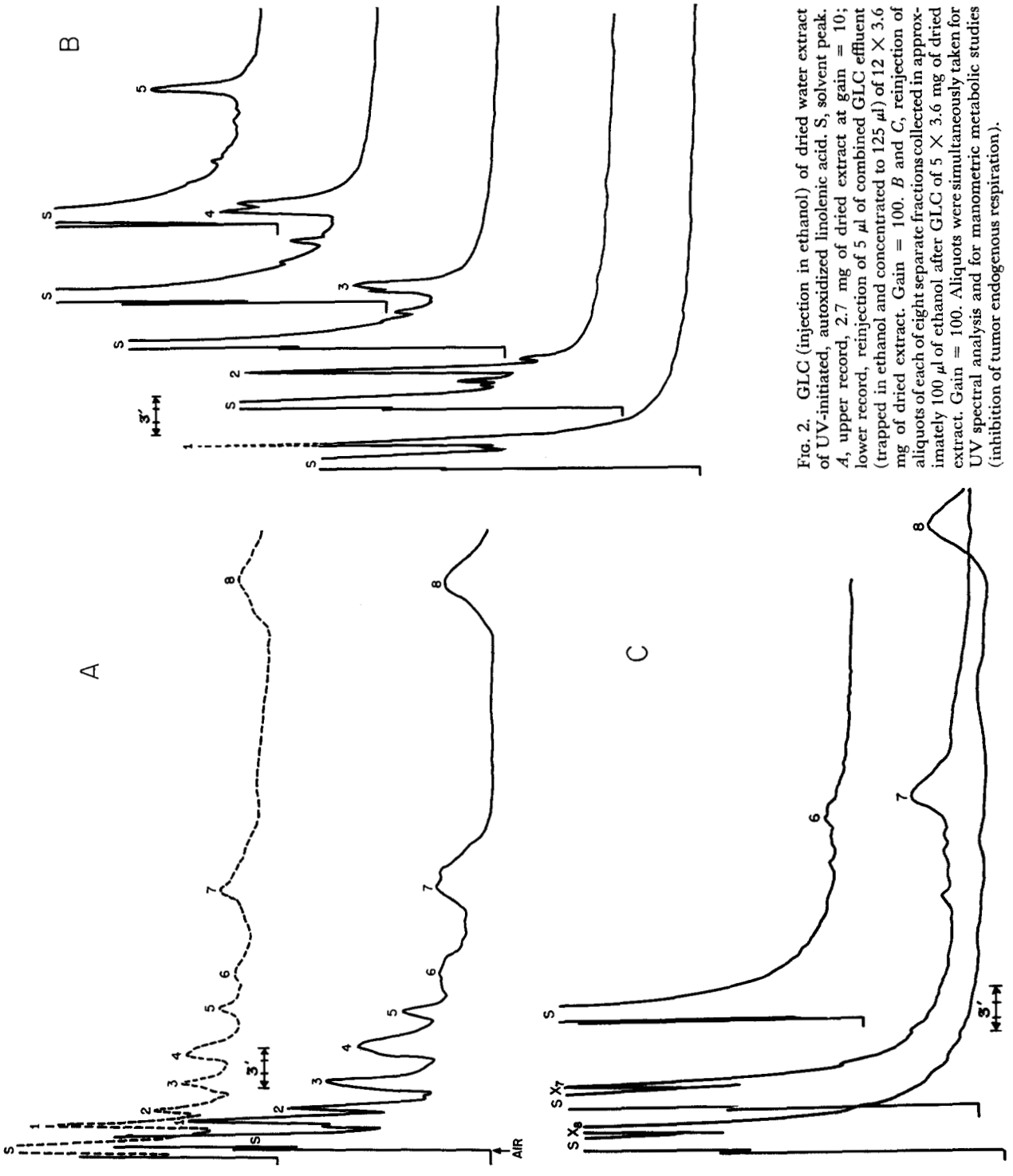

FIG. 2. GLC (injection in ethanol) of dried water extract of UV-initiated, autoxidized linolenic acid. S, solvent peak. *A*, upper record, 2.7 mg of dried extract at gain = 10; lower record, reinjection of 5 μl of combined GLC effluent (trapped in ethanol and concentrated to 125 μl) of 12 × 3.6 mg of dried extract. Gain = 100. *B* and *C*, reinjection of aliquots of each of eight separate fractions collected in approximately 100 μl of ethanol after GLC of 5 × 3.6 mg of dried extract. Gain = 100. Aliquots were simultaneously taken for UV spectral analysis and for manometric metabolic studies (inhibition of tumor endogenous respiration).

tion of this sort during GLC has been observed by reinjection of either fractions 7 or 8 immediately after their collection in other experiments.

The retention times of the eight major peaks were those expected of two homologous series of compounds having chain lengths of approximately 7–13 carbon atoms. The two major components (fractions 7 and 8) had retention times corresponding approximately to C_{11} and C_{13} aliphatic acids or to C_9 and C_{11} semialdehydes. Methylation (5% HCl in anhydrous methanol, 1 hr, 60°C) caused prolongation of the retention time of each of the eight components in the mixture as though conversion of aldehydes to acetals had occurred. However, it may be misleading to infer chain length from relative retention times because homologous series of unsaturated aliphatic acids, saturated acids, semialdehydes, aldehydes, and methyl esters show different relationships between retention time and chain length.

All eight fractions of Fig. 2 had similar UV spectra (illustrated by those of fractions 5 and 8 in Fig. 3; fractions 1–7 resembled fraction 5 more than they did fraction 8). The spectra suggest that each fraction contained α,β-unsaturated carbonyl compounds. Although initially we were able to collect some TBA-reactive compound(s) from our extract after GLC, none of the peaks shown in Fig. 2 had significant TBA or peroxidase reactivity. Reference α,β-unsaturated aldehydes which are TBA-reactive, such as cinnamaldehyde, were recovered in excellent yield, unaltered, under the present conditions.

Effect of Fractions Separated by GLC on Tumor Oxygen Consumption

After a year's storage, the pooled extract of UV-irradiated, oxidized linolenic acid was still able to inhibit oxygen consumption of tumor cells and had approximately the same composition, as judged by GLC analysis, peroxidase reactivity, and TBA assay, as when it was first prepared. The 50% inhibiting concentration was 1.2 mg/3 ml. If any single compound detected and recovered from GLC were responsible for this inhibition then we could estimate the weight of this substance which would produce 50% inhibition from its peak area in GLC. These estimates, for each of the eight fractions collected, are given in Table 6. For example, component 3 made up about 10% of the total GLC effluent; therefore, if this component were responsible for producing 50% inhibition of oxygen consumption, a maximum[1] of 10% of 1.2 mg, 0.12 mg, should suffice to produce 50% inhibition. However, as shown in Table 6, none of the eight fractions collected caused any detectable inhibition of tumor oxygen consumption even in concentrations far in excess of those present in a 50% inhibiting concentration of extract. Moreover, when all eight fractions were collected and combined after GLC (lower record in Fig. 2A), the concentration of material required to inhibit tumor respiration was greater than that of the extract before GLC (Table 6). This observation is consistent with other evidence which correlates hydroperoxides and (or) TBA-reactive materials, which were lost during GLC, with inhibition of tumor oxygen consumption. The compounds isolated by GLC are probably of little importance as far as inhibition of tumor respiration is concerned. For this reason, no further characterization of these compounds has been carried out.

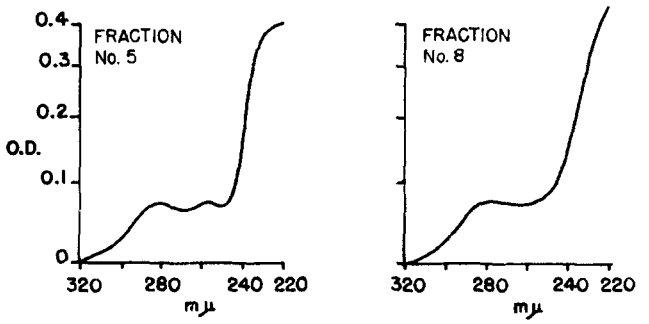

FIG. 3. Typical UV absorption spectra of individual fraction collected after GLC. Fractions 5 and 8 refer to corresponding peaks in Figs. 2B and 2C.

TABLE 6 EFFECT OF FRACTIONS ISOLATED BY GLC ON ENDOGENOUS OXYGEN CONSUMPTION OF EHRLICH ASCITES TUMOR CELLS

	Before GLC	After GLC	
Fraction*	50% Inhibiting Concentration (mg/3 ml)†	Concentration‡ Tested (mg/3 ml)†	Percentage Inhibition of Tumor, QO_2
1	0.16	1.0	0
2	0.07	0.6	0
3	0.12	0.6	0
4	0.17	1.0	0
5	0.02	0.4	0
6	0.04	0.2	0
7	0.25	0.6	0
8	0.37	1.5	0
1–8 inclusive	1.2	1.1	0
"	1.2	4.2	100
"	1.2	5.9	100

* The fractions tested here represent aliquots of the same solutions as those used to obtain the GLC records shown in Figs. 2A–C.
† Estimated from areas of peaks on GLC records.
‡ In the case of individual fractions only the weight of the major component (estimated from GLC record peak areas) is given; in addition other compounds were also present (see Fig. 2).

[1] Estimates are maximal since they assume that the percentage of each component which is detected after GLC is the same as the percentage of that component prior to GLC. This results in overestimation of the mass of each component in the original mixture since some compounds may be preferentially retained on the column and others may pass through the column and cell undetected.

Phase microscopic examination of tumor cells incubated with the extract of oxidized linolenic acid (5×10^{-5} M ROOH) showed that every cell examined contained numerous protrusions attached to the main cytoplasm by short, narrow stalks. The cytoplasm was still surrounded by a plasma membrane. Only rarely were protrusions seen in control cells incubated with aqueous extracts of nonirradiated linolenic acid.

DISCUSSION

Shuster first showed that water-soluble oxidation products of UV-irradiated methyl linoleate and methyl linolenate inhibited both glycolysis and oxygen consumption of Ehrlich ascites tumor cells (1). He correlated inhibition with TBA reactivity of the extract. TBA reactivity (λ_{max} 532–535 mμ) is generally considered to be a (nonspecific) measure of malonaldehyde (e.g., 6–10). However, relatively involatile TBA-reactive substances other than malonaldehyde have now been found as autoxidation products of linoleate and linolenate by workers in at least two different laboratories, under considerably different conditions of autoxidation (2, 11).[2]

We have also shown that after a relatively brief exposure of linoleic or linolenic acid to UV light, water-soluble peroxidase-reactive compounds form (2). It is apparent from these findings and from the work of Schauenstein and coworkers (12–17), who have separated large numbers of oxidation products from autoxidized methyl linoleate, that in order to explore Shuster's correlation between TBA reactivity and inhibition of tumor respiration, it is necessary to isolate the TBA-reactive compounds to see whether they are inhibitory or not. Schauenstein et al. (14, 15) have identified, in a highly complex mixture of autoxidation products of methyl linoleate, two inhibitors of tumor respiration which would be expected to be TBA-reactive since they are both α,β-unsaturated aldehydes (18). However, the method of preparation employed by Schauenstein and coworkers involves long-term exposure to oxygen (4–10 days in water emulsion), which might give rise to extensive secondary oxidation of initial oxidation products. Moreover, their method of fractionation eliminates both the most polar and the relatively volatile oxidation products.

We have employed a different method of fractionation which separates volatile from relatively involatile water-soluble components by sublimation in a closed system (2). This method has been used here to demonstrate that only the relatively involatile compounds inhibit oxygen consumption of tumor cells. Removal of H_2O_2 by catalase or by sublimation did not influence the results. However, treatment with peroxidase caused a marked diminution in the inhibitory properties of the involatile fraction obtained from UV-irradiated linolenic acid. Significant inhibition of oxygen consumption still occurred after reduction (by peroxidase and dye) of the "hydroperoxide" group. The inhibitor(s) was highly stable during exposure to heat and prolonged storage.

A large part of the material responsible for inhibiting tumor respiration was recovered after TLC. The inhibitor(s) had an R_f value previously shown (2) to be characteristic of highly polar compounds containing acid, aldehyde, conjugated aldehyde (TBA-reactive), and "hydroperoxide" groups.

Our results indicate that the aqueous extract of UV-irradiated linolenic acid contains a group of relatively involatile, medium-chain polar compounds with a total mass over a thousand times greater than that predicted on the basis of the TBA assay with malonaldehyde as a reference compound. The bulk of the material has been separated from relatively small amounts of "hydroperoxide" and TBA-reactive materials and resolved by GLC into individual components, none of which inhibited tumor respiration in the concentrations tested. Although the combination of all of these components recovered after GLC inhibited tumor oxygen consumption, its activity was reduced 2- or 3-fold by GLC. On a weight basis, we may estimate that the hydroperoxide and (or) TBA-reactive components of the mixture, which are lost on GLC, are probably at least 100 times more inhibitory than the combination of compounds isolated by GLC.

The GLC system employed was chosen because it allowed isolation of medium-chain length, α,β-unsaturated aldehydes in high yield and without loss of TBA reactivity. Since almost all TBA reactivity (and peroxidase reactivity) of our extract was lost during GLC, we conclude that these particular TBA-reactive compounds are unusually labile or possess some functional groups which result in their being retained on the column. Yet, the bulk of the material in the extract was recovered after GLC. This evidence substantiates further the correlation between TBA and peroxidase reactivities and inhibition of tumor respiration.

The partial separation of products of autoxidation which has been attained thus far has helped to clarify an earlier observation by Shuster (1). He reported that UV-irradiated methyl linoleate gave rise to a water-soluble product which inhibited tumor oxygen consumption to a greater degree than did the extract of irradiated methyl linolenate when expressed per unit of TBA reactivity (1). We have shown (2, see also 19) that relative to linoleate, linolenate gives rise to a larger proportion of malonalde-

[2] Saslaw, Anderson, and Waravdekar (11) exposed methyl esters to UV light in the absence of water for 18 hr prior to extraction with water. We (2) irradiated the free acids, emulsified in water, with UV light for only 90 min.

hyde, which is TBA-reactive but not inhibitory. If malonaldehyde is removed before testing, the degree of inhibition per unit of TBA reactivity becomes more nearly similar for the oxidation products of the two unsaturated lipids. Moreover, if the correlation is made between peroxidase reactivity and inhibition of oxygen consumption, the dried aqueous extracts of irradiated linolenate and linoleate behave similarly.

Evidence was obtained that "hydroperoxide" was not correlated with inhibition of tumor cell glycolysis, since glycolysis was completely inhibited in the presence of 5×10^{-5} M ROOH from linolenate but entirely uninhibited in the same concentration of ROOH derived from linoleate. Either the "hydroperoxides" in each extract are different or the "hydroperoxide" alone is not responsible for the inhibitory properties of the extract. Another difference between the dried residues of oxidized linoleate and linolenate was reported previously (2). Twice as much ROOH per unit of TBA reactivity was obtained from linoleate than from linolenate.

Further work is required to determine whether the widely different conditions of autoxidation used by various workers (1, 2, 11–17) all yield common, inhibitory, water-soluble products. Although extraction of oxidation products with water has been a common initial step, we have found that the vast bulk of material in our aqueous extracts, when dried, is relatively insoluble in water. In fact, most compounds can be almost completely extracted from water with a common lipid solvent (chloroform–methanol). Schauenstein and coworkers have focused their attention on those late oxidation products of methyl linoleate which may be extracted from alkaline solutions with chloroform. Our work has been centered on early oxidation products of linolenic acid, which may be expected (because of its greater unsaturation) to yield shorter-chain, more polar compounds than linoleate. The present study shows that all of the inhibitors of tumor respiration in aqueous extracts of UV-initiated, autoxidized linolenic acid may be dried by sublimation in a closed system. Attempts to isolate and to identify the TBA-reactive and hydroperoxide moieties of the complex mixture are in progress.

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