

cated that the C-C bond in hexaphenylethane is weaker than a normal bond by about 35 kcal., which we attributed to steric hindrance. If one assumes that the C-C bond in hexaphenylethane is a normal bond then there is no way of explaining a bond in diphenyl di-biphenylene ethane which is 20 kcal. stronger. The inference from these data is that steric hindrance is much less when the benzene rings are tied together as they are in this compound, in agreement with the appearance of the model of the molecule.

The surprising result from these experiments is that the decrease in steric hindrance is so large in this compound. If this were the only factor involved in the dissociation of the ethane it would be impossible to explain the fact that the difference in the free energy of dissociation of this compound and hexaphenylethane is only three or four kcal. A solution to the problem is possible if one assumes that phenyl fluoryl has a much larger resonance energy than triphenylmethyl, thereby canceling to some extent the increased strength of the bond in diphenyl di-biphenylene ethane. This is just the opposite of the conclusion reached by Pauling and Wheland in their discussion of this compound.

The above conclusions are based on data in-

volving materials in solution or in the solid state. A much more satisfactory treatment would be based on reactions in the vapor phase. We hope to have some such data to report in the near future but since this work is being interrupted for the present it seems desirable to report the progress which has been made to this time.

In comparing tetraphenyl di- α -naphthyl ethane with hexaphenylethane we are dealing with a difference in heats of oxidation which is not as large as in the case just discussed and therefore the interpretation is more in doubt. Since we have no data on the heat of solution of the peroxide it will not be possible to study this reaction in the vapor phase by the method which we are using with hexaphenylethane.

Summary

1. The heat of oxidation of diphenyl di-biphenylene ethane is found to be about 20 kcal. less than that of hexaphenylethane.

2. This datum indicates that steric hindrance is less than in the case of hexaphenylethane and that the free radical formed by dissociation, phenyl fluoryl, has more resonance energy than triphenylmethyl.

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[CONTRIBUTION FROM THE BIOCHEMICAL LABORATORY, STATE UNIVERSITY OF IOWA]

Antioxidants and the Autoxidation of Fats. VI. Inhibitors¹

By H. S. OLCOTT AND H. A. MATTILL

Previous work from this Laboratory^{2,3,4} has demonstrated that the unsaponifiable lipid fractions of vegetables and vegetable oils contain compounds which are active antioxidants toward lard. It is proposed to call these compounds as a class "inhibitors," a name which indicates their function as inhibitors and also the invariable occurrence of hydroxyl groups, upon which their inhibiting action depends. Although concentrates

have been prepared from various sources,⁵ only the lettuce inhibitor has been crystallized.³ The present paper contains a description of the preparation and properties of the inhibitor concentrates from wheat germ, cottonseed and palm oils.

The method used for obtaining the most active concentrates from wheat germ or cottonseed oil is exactly the same as that described for obtaining vitamin E concentrates.⁶ Indeed, the physical and chemical properties of vitamin E

(1) Presented at the Kansas City meeting of the American Chemical Society, April, 1936.

(2) H. A. Mattill and B. Crawford, *Ind. Eng. Chem.*, **22**, 341 (1930).

(3) H. S. Olcott and H. A. Mattill, *J. Biol. Chem.*, **93**, 59, 65 (1931).

(4) E. M. Bradway and H. A. Mattill, *THIS JOURNAL*, **56**, 2405 (1934).

(5) Inhibitors are present in lettuce, tomatoes, carrots, alfalfa, spinach; in wheat germ, cottonseed, corn, sesame, palm, soy bean and peanut oils; and probably in many other vegetable substances. No demonstrable amounts of inhibitors are present in yeast, lard; or in olive (trace), cod-liver, palm kernel or castor oils.

(6) H. S. Olcott and H. A. Mattill, *J. Biol. Chem.*, **104**, 423 (1934).

and the inhibitols from these oils are so similar that it has thus far been impossible to separate the two. The demonstration that the vitamin E and inhibitols of lettuce and tomatoes could be separated by preferential solubilities,^{3,4} thus providing a vitamin E concentrate free of antioxygenic activity, has been the principal basis for the assumption that the two properties are not inherent in the same molecule.

Experimental

Preparation.—Wheat germ or cottonseed oil is saponified with alcoholic potash,⁷ and extracted with ether (previously shaken with 10–20% aqueous sodium hydroxide to remove peroxides). The ether extracts are evaporated to dryness and the residue dissolved in petroleum ether, from which most of the sterols separate on cooling. The sterols are removed by filtration, and the residue obtained after removal of the solvent is extracted several times with hot methanol. The methanol-soluble fractions are combined and cooled. If more sterols crystallize they are removed. The solvent is evaporated under reduced pressure and the residue is then carefully distilled in a high vacuum. The inhibitol distils at 190–210° (0.1 mm.).

The palm oil inhibitol was concentrated as follows. The unsaponifiable fraction was dissolved in petroleum ether, diluted with an equal volume of methyl alcohol and allowed to stand cold for several weeks. Some of the carotene crystallized and was removed. The petroleum ether was then evaporated from the filtrate and an insoluble oil separated; the methanol solution was poured off, the oil was washed twice with methanol and the extracts combined and hydrogenated with platinum as the catalyst. The purpose of this step was to destroy the remaining pro-oxygenic carotenoids.⁸ The catalyst

(7) For the complete saponification of large or small amounts of fat with a minimum of time, effort and opportunity for oxidation, the following proportions of reagents have been used in this Laboratory: 26 g. of potassium hydroxide is dissolved with stirring in 22 cc. of water and poured while hot into 89 cc. of 95% ethanol. This mixture is poured directly into a flask containing 100 g. of the oil or melted fat. The flask is swirled vigorously until the mixture becomes homogeneous; usually less than a minute is required. For the extraction of unsaponifiable constituents, the soaps are allowed to cool to 40–50° and poured into 450 cc. of water; 450 cc. of ethyl ether is added and the mixture is shaken. The ether layer separates immediately. The water-alcohol solution of the soaps may be extracted repeatedly with ether without emulsification. This procedure was developed by Dr. R. B. French, who also devised a method for large scale saponification and extraction which will be described in another place.

(8) Newton [*J. Oil and Soap*, **9**, 247 (1932)] found that fats containing carotene were more stable than colorless ones, and, further, that lards could be protected from oxidation by the addition of palm oil which contains carotene. However, we had previously demonstrated that purified crystalline carotene acted as a pro-oxidant in lard-cod-liver oil mixtures [H. S. Olcott and H. A. Mattill, *J. Biol. Chem.*, **91**, 105 (1931); H. S. Olcott, *THIS JOURNAL*, **56**, 2492 (1934)]. It now seems clear that the fats containing carotene also contained inhibitols, which, in fact, ensured the presence of carotene by protecting it from oxidation. The palm oil inhibitols were responsible for the protection afforded by the addition of palm oil to lard. The method of incorporation described was such as to destroy the pro-oxygenic carotene by heat treatment and thus stabilize the fat still further.

was removed by filtration and the filtrate evaporated to dryness. A white inactive powder separated from a petroleum ether solution of the residue and was filtered off. The limpid oil remaining after evaporation of the filtrate amounted to 0.1% by weight of the original oil. It was carefully fractionated in high vacuum (0.03 to 0.04 mm.) and the antioxidant obtained most highly concentrated in the fraction distilling within the 165–180° range. Several of a number of runs with palm oils have yielded concentrates of high vitamin E potency. With palm oil as with wheat germ and cottonseed oils, it was not possible to separate the inhibitols from vitamin E.

Attempts further to concentrate the inhibitols from these sources or to obtain a crystalline product have not been promising. Physical methods have included crystallization from solvents at low temperatures (–80°), careful fractional distillation in high vacuum (0.002 mm.), and adsorption on silica, alumina and magnesia. No notable concentration has been effected by any of these methods. In each case all of the fractions were antioxygenic to lard. Separation of the material into soluble and insoluble fractions from methanol at 0° consistently yielded somewhat more active fractions in the soluble portion. Diphasic separation between 92% methanol and petroleum ether removed a small amount of an inactive oil in the 92% methanol layer. The inhibitols were preferentially soluble in petroleum ether.

Physical and Chemical Properties.—Inhibitols concentrates are light yellow transparent oils of medium viscosity, which do not crystallize on long standing. They are stable under ordinary laboratory conditions for years. Some physical constants and chemical analyses are given in Table I. The concentrates are soluble in the following organic solvents: ether, petroleum ether, methyl and ethyl alcohols, pyridine, glacial acetic acid, chloroform, benzene, dioxane, etc.

TABLE I
COMPOSITION OF INHIBITOL CONCENTRATES

Source	Wheat germ oil		Cottonseed oil	Palm oil
Concentrate no.	W5-10	W4-12-2	C44	P17-8-1
C ^a	82.3	82.5	81.6	81.6
H ^a	11.7	11.4	11.5	11.1
M. w. ^a	317	379	279
I no.	106 ^{b,c}	120 ^c	90 ^b	170 ^c
Refr. index (20°)	1.5161	1.5254	1.5090	1.5193

^a Dr. Ing. A. Schoeller. ^b Ralls' method.¹¹ ^c Rosenmund-Kuhnnehn method.¹⁰ We have found it difficult to obtain consistent iodine numbers of unsaponifiable lipid fractions.

The effects of various reagents on the activity of inhibitols concentrates have been used to characterize the structure of the inhibitols. Table II includes representative results of such experiments. The inhibitols are always inactivated by reagents which combine with a free hydroxyl group, including acetyl chloride, acetic anhydride in pyridine, benzoyl chloride, methyl iodide with silver oxide, dimethyl sulfate, diethyl sulfate, phenyl isocyanate, *p*-nitrophenyl isocyanate and many others. None of these derivatives yielded crystalline products. Those which contained ester linkages, including the substituted ure-

thans, were hydrolyzed with alcoholic sodium hydroxide. The recovered material was again antioxygenic although in most cases some of the activity was lost. Diazomethane did not affect the antioxygenic action.

TABLE II
EFFECT OF REAGENTS ON INHIBITOL ACTIVITY

Concentrate ^a	Fat	Treatment	Induction period (75°C.)	
			With inhibitor (0.02% hrs.)	Without inhibitor, hrs.
C5-9	Lard	None	34	8
		Chlorine	7	8
		Chlorine + Zn + HCl	34	8
C65	Lard-cod-liver oil	None	158	16
		Ozone	11	11
W5-10	Lard	None	62	15
		Methyl iodide	13	12
		Dimethyl sulfate	13	12
		Ethyl iodide	17	15
W5-1572	Lard	None	53	4
		Acetic anhydride	4	4
		Phenyl isocyanate	4	4
		Phenyl isocyanate + NaOH	50	6
W3-18	Lard-cod-liver oil	None	23	7
		Acetic anhydride	5	5
		Acetic anhydride + NaOH	12	4

^a C indicates cottonseed oil inhibitol, W that from wheat germ oil.

The inhibitols are destroyed by direct bromination or chlorination, presumably by addition to a double bond. The activity can be regenerated by boiling with zinc and hydrochloric acid in methanol. Hydrogenation has uniformly failed to inactivate the inhibitols. One sample was subjected to hydrogen at 250° and 230–280 atmospheres for two hours without loss of activity.⁹ The recovered material was still unsaturated, although the iodine number^{10,11} had been reduced from 105 to 70. Perbenzoic acid in the cold destroyed the inhibitols. Ozone was also destructive. These facts have been interpreted to mean that inhibitol contains a difficultly hydrogenated double bond which is essential to its activity.

The inhibitols are not destroyed by dry hydrogen bromide in methanol, nor by phosphorous tribromide, at room temperature. Potassium permanganate in pyridine, chromic acid in glacial acetic acid, potassium amide in liquid ammonia, potassium ethylate in ethyl alcohol and lead tetraacetate in glacial acetic acid destroyed the activity.

The methods used for determining the effect of some of these reagents upon concentrates have been described briefly elsewhere.^{12,13}

(9) We are indebted to Professor Adkins of the University of Wisconsin for several hydrogenation experiments.

(10) K. W. Rosenmund and W. Z. Kuhnemann, *Untersuch. Nahrungs-u. Genussmittel*, **46**, 154 (1923); M. Yasuda, *J. Biol. Chem.* **94**, 401 (1931).

(11) J. O. Ralls, *THIS JOURNAL*, **56**, 121 (1934).

(12) H. S. Olcott, *J. Biol. Chem.*, **107**, 471 (1934).

(13) H. S. Olcott, *ibid.*, **110**, 695 (1935).

The ultraviolet absorption spectra of inhibitol concentrates show a distinct broad band with maximum at 2940 Å., the height of which is roughly proportional to the antioxygenic activity. Acetylation causes a loss in height and migration of the peak to 2810 Å. Several authors have suggested that this band is a property of vitamin E.^{14,15} In our experience, the height of the band has been more nearly proportional to the antioxygenic than to the physiological activity. Most striking of such experiments is a comparison of the properties of concentrate, P16-9, from palm oil with those of concentrates from wheat germ or cottonseed oil. Although P16-9 contained less than one-fifth as much vitamin E as did the others, the band in the ultraviolet was equally strong (Table III). Concentrates W5-156 and W5-157 were consecutive fractions from a fractional vacuum distillation, equally effective as antioxidants, but not containing equal amounts of vitamin E.

TABLE III
THE RELATION OF THE INHIBITOL CONCENTRATION AND THE VITAMIN E CONTENT TO THE ABSORPTION BAND AT 2940 Å.

Concentrate	Antioxidant index ^a	E ₁ % at 2940 Å.	Minimum dose, ^b mg.
C65	4-7	70	4
P16-9	5-8	90	>30
W5-10	5-8	100	3
W5-156	5-8	65	>10
W5-157	5-8	70	2

^a Antioxidant index is the ratio of the induction period of the protected sample to that of the control. The figures are approximations because of the natural variability in duplicate assays (Table VI). ^b The minimum amount required to ensure the birth of a litter in a vitamin E-deficient animal.

Assay.—All fractions obtained during these studies were assayed for antioxygenic activity by the procedure

TABLE IV
COMPARISON OF THE ANTIOXYGENIC ACTIVITY OF SEVERAL TYPES OF INHIBITORS ON LARD

Inhibitor	Concentration %	Antioxidant index
Hydroquinone ¹⁷	0.01	29
<i>a</i> -Naphthol	.01	6-10
Inhibitol concentrate W5-10	.02	5-8
Inhibitol concentrate C65	.02	5-8
Inhibitol concentrate P17-8-1	.02	5-8
Commercial lecithin ^a	.10	1.2-1.5
Maleic acid ^b	.10	1.2-1.5
Phosphoric acid ^c	.02	1.2-1.5
Carotene ^d	.02	0.5

^a H. Bollman, U. S. Patent 1,464,557; *C. A.* **17**, 3234 (1923). ^b G. R. Greenbank and G. D. Holm, *Ind. Eng. Chem.*, **26**, 243 (1934). ^c E. W. Eckey, U. S. Patents 1,982,907; 1,993,152; A. S. Richardson, F. C. Vibrans and J. T. R. Andrews, U. S. Patent 1,993,181, *C. A.*, **29**, 518, 2770 (1935). ^d See Newton, reference 8.

(14) J. C. Drummond, E. Singer and R. J. MacWalter, *Biochem. J.*, **29**, 456, 2510 (1935).

(15) H. M. Evans, O. H. Emerson and G. A. Emerson, *J. Biol. Chem.*, **113**, 319 (1936).

previously described,^{16,17} which measures the induction period of lard or lard-cod-liver mixtures by the oxygen absorption method. The flasks containing the samples were placed in constant temperature baths at 75°, and the time of the beginning of active oxygen absorption was recorded automatically. The most active inhibitol concentrates protected lard to a degree approaching that afforded by the phenolic inhibitors (Table IV) and were much more effective than other compounds which have been suggested for use in edible fats.

Inhibitol concentrates are effective antioxidants for purified fatty acids and esters (Table V). Preliminary observations suggest that they also protect other easily oxidized organic compounds. They are, however, ineffective as antioxidants for the vegetable fats and oils from which they are prepared.

TABLE V
EFFECT OF AN INHIBITOL CONCENTRATE ON VARIOUS SUBSTRATES

Substrate fat	Inhibitol concentrate	Induction period at 75°	
	W5-10 %	With inhibitor, hrs.	Without inhibitor, hrs.
Ethyl ricinoleate	0.10	56	16
Ethyl linolate (58°)	.10	8	0.5
Methyl oleate	.03	10	4
	.10	40	4
Oleic acid	.02	30	4
β -Ionone	.10	4	0.5
9,10-Octadecene	.13	84	13

The assays are subject to interference by many factors, some unknown. Attention has been called elsewhere¹⁷ to the variability in fats and among samples of the same fat, not only in keeping quality, but in their reactions to added inhibitors. Table VI indicates the results which

TABLE VI
EFFECT OF INHIBITOL CONCENTRATES ON DIFFERENT SAMPLES OF LARD

+0.02% C65,			+0.02% P17-8-1,			+0.02% W5-10,		
Blank	Blank	A. I. ^a	Blank	Blank	A. I. ^a	Blank	Blank	A. I. ^a
hrs.	hrs.		hrs.	hrs.		hrs.	hrs.	
82	13	6.3	84	13	6.5	47	13	3.6
59	6	10.0	67	15	4.5	77	15	5.1
46	15	3.1	84	12	7.0	84	12	7.0
55	12	4.6	53	10	5.3	34	10	3.4
65	13	5.0	49	5	9.8	50	5	10.0
52	10	5.2	50	10	5.0	40	10	4.0
2	5	6.4						
51	9	5.3						

^a Antioxidant index.

(16) H. A. Mattill, *J. Biol. Chem.*, **90**, 141 (1931).

(17) R. B. French, H. S. Olcott and H. A. Mattill, *Ind. Eng. Chem.*, **27**, 724 (1935).

may be expected in assays of inhibitol concentrates on different lots of the same kind of fat.

Furthermore, the mere fact that an inhibitor is effective in one fat at an elevated temperature does not mean that it will be effective in other fats or at other temperatures. Preliminary experiments do show, however, that inhibitols protect lard at room temperature.

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Summary

The unsaponifiable lipid fractions of many vegetables and vegetable oils contain compounds which are active antioxidants to lard and which are here named inhibitols. The inhibitols from wheat germ and cottonseed oils may be concentrated by processes of crystallization and distillation similar to those used for obtaining vitamin E concentrates from which the inhibitols have not been separated. The preparation of inhibitol concentrates from palm oil is aided by the destruction by hydrogenation of the accompanying pro-oxygenic carotenoids.

Inhibitol concentrates are transparent oils which have resisted crystallization. Some chemical and physical properties are outlined. The inhibitols are destroyed by reagents which attack a hydroxyl group or saturate a double bond. Inactive esters may be hydrolyzed to regenerate the activity. They are resistant to hydrogenation. Chlorine or bromine addition products can be reactivated with zinc and hydrochloric acid. The concentrates have a strong absorption band at 2940 Å. roughly proportional to their activity.

The inhibitol concentrates have been assayed by an oxygen absorption method. They are shown to be much more effective antioxidants in lard than any edible compounds which have been suggested for use as commercial antioxidants. The inhibitols protect purified fatty acids and esters but do not protect the vegetable oils from which they are obtained.

IOWA CITY, IOWA

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