tion of the vitamin are probably environmental rather than hereditary. The failure of cobalt bullets in animals located at Tifton and Reidsville to result in increased production of vitamin B_{12} suggests that the supply of cobalt is not the primary factor limiting secretion of the vitamin in milk elaborated by animals situated at these two locations.

In this study and in the majority of reports in the literature of vitamin B_{12} content of cows' milk, major portions of the variation observed are associated with differences in milk produced by one animal at different times and with differences in milk produced by various animals maintained under the same environmental conditions. For example, Gregory, Ford, and Kon (*4*) observed that day-to-day and animal-to-animal variations in concentrations in milk were greater for vitamin B_{12} and biotin than for thiamine, riboflavin, pantothenic acid, or vitamin B_6. This extreme variation indicates that secretion of the vitamin into milk is ultimately influenced to a great extent by conditions within the animal which can change markedly in a short period of time or which would vary among different animals maintained under the same environmental conditions.

Little is known of the actual mechanisms by which vitamin B_{12} is synthesized in the rumen or of the factors which control its secretion into milk. Investigations of types of microorganisms which synthesize vitamin B_{12} and of conditions in the rumen which would support growth of these organisms, of substrates and cofactors required in the biosynthetic pathways, and of factors involved in transportation of the vitamin from rumen to udder will probably be necessary before it would be possible to predict the vitamin B_{12} content of milk accurately or to produce milk of uniformly high vitamin B_{12} content.

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IRRADIATION OF FATS

Effect of Ionizing Radiations on Antioxidants in Fats

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The effect of high energy radiations on several antioxidants dissolved in methyl myristate or methyl linoleate has been studied. When used at a concentration of 0.01% in methyl myristate and irradiated under vacuum, 27% of butylated hydroxyanisole, 50% of propyl gallate, and all of the tocopherol were destroyed with a dose of 5 megarads. In oxygen the same dose almost completely destroyed all antioxidants. Citric acid did not protect propyl gallate from destruction. No further changes occurred during storage of vacuum-irradiated samples. Destruction was greater in methyl myristate than in methyl linoleate.

IRRADIATION of fats produces free radicals which, in the presence of oxygen, form hydroperoxides. With unsaturated fats chain oxidation reactions are initiated and autoxidation proceeds rapidly. Antioxidants have been shown to have no effect on the formation of peroxides during irradiation (*5*) and are much less effective in preventing accumulation of peroxides during storage of irradiated materials than in simple autoxidation. This can be attributed either to the large number of chain reactions initiated by irradiation or to the destruction of antioxidant during irradiation.

Several investigators have reported that tocopherols are readily destroyed as a result of irradiation (*3, 8–12, 14*), but it has been suggested that other antioxidants such as propyl gallate and butylated hydroxyanisole can be added to fats prior to irradiation to prevent or minimize loss of stability (*1, 9*). However, little information was available on the effect of irradiation on common antioxidants. This paper reports the destruction of propyl gallate, butylated hydroxyanisole, and tocopherol when irradiated and stored under different conditions.

Experimental

Materials Used. The samples of irradiated methyl myristate and methyl linoleate containing the antioxidants studied were the same as those described earlier (*5*).

Determination of Antioxidants. The analytical procedure was a modification

of the methods described by Mahon and Chapman (*13*) and Austin (*2*), using the ferric chloride–bipyridine reagents of Emmerie and Engel (*7*). Color was developed and measured in the same solution, using absolute ethanol to dissolve the reagents and the sample.

REAGENTS. Ethanol was purified by refluxing for several hours over granulated aluminum (8-mesh) and potassium hydroxide followed by distillation through a 6-bulb Snyder column.

The color reagents were 0.1% 2,2′-bipyridine (Eastman Organic Chemicals) in purified absolute ethanol and 0.0832% $FeCl_3.6H_2O$ (Mallinckrodt, AR) also in purified ethanol.

PROCEDURE. Methyl Myristate. An aliquot of the methyl ester containing the antioxidant (0.2 ml. for propyl gallate, 0.3 ml. for butylated hydroxyanisole, and 0.8 ml. for α-tocopherol) was weighed into a 5-ml. volumetric flask. The sample was dissolved in 2 ml. of bipyridine reagent, an equal amount of the ferric chloride solution was added, and the mixture was made up to the 5-ml. volume with purified absolute ethanol. After mixing, the color was allowed to develop at room temperature in the dark for 40 minutes and the absorbance of the solution was measured at 522 mμ in a Beckman spectrophotometer against distilled water. The absorbance of the reagents was determined also using an equal amount of pure methyl myristate, free of antioxidant, and this value was applied as a correction to the absorbance of the samples. Light is known to influence the rate of chromogenesis during this reaction and, to minimize this effect, the addition of the reagents, mixing of the reaction mixture, and transfer from the volumetric flask to the spectrophotometer cuvettes were performed in an amber light of less than 1 foot-candle intensity.

Methyl Linoleate. This compound was slightly oxidized and it reacted with the Emmerie and Engel reagents to give blanks that were too high when 0.2 ml. of ester was used. For this reason, antioxidant measurements in irradiated methyl linoleate have been limited to one sample containing 0.1% propyl gallate. This high concentration of antioxidant allowed the use of only 0.02 ml. of sample and this amount of methyl linoleate gave satisfactory blanks.

Standard curves were obtained for each antioxidant by performing the analyses of known concentrations of the substances dissolved in pure unirradiated methyl myristate.

Results and Discussion

Antioxidant Measurements. Procedures for measuring antioxidants in fats usually require a preliminary extraction of the antioxidant from the fat with an aqueous-alcoholic solvent. The solubility of methyl esters of fatty acids in aqueous methanol precluded the use of such techniques for these studies. Furthermore, because of the large number of samples to be analyzed, a rapid method capable of measuring antioxidants directly in the substrate was desired. The ferric chloride–bipyridine procedure first introduced by Emmerie and Engel (*7*) was selected for this purpose because it is sensitive, simple, and applicable to all antioxidants that were to be studied. Its nonselectivity was not a problem, since no combination of antioxidants was to be used. Citric acid, which was used as a synergist with propyl gallate, has no effect on the determination of the latter and in these mixtures only propyl gallate is measured.

Figure 1 shows the standard curves obtained for the three phenolic antioxidants studied. All three compounds obey Beer's law but differ considerably in their response to the analytical procedure. There appears to be no simple relationship between the intensity of color given by an antioxidant and its molecular weight or the number of phenolic groups present in the molecule. However, the slopes of the curves agree with the generally accepted antioxidant activity of the three compounds, with butylated hydroxyanisole slightly lower than propyl gallate and α-tocopherol much weaker than the other two.

As already reported (*5*), irradiation of methyl esters free of antioxidant produces small amounts of compounds able to reduce ferric to ferrous ions and thus give apparent antioxidant values. Although the nature of these reducing substances is unknown, they can be calculated in terms of any of the antioxidants employed by using the corresponding standard curve. When expressed as propyl gallate, the color developed was equivalent to 6, 11, and 14 mg. per kg. of ester after irradiation with 2, 5, and 8 megarads, respectively. When calculated as α-tocopherol, however, these values were much higher and became 30, 56, and 71 mg. per kg. for the same doses. The level of these materials remained the same whether irradiation was performed in vacuum or in an atmosphere of nitrogen, and it was not affected by storage.

It has been assumed that these chromogenic substances developed to the same extent in the samples containing the antioxidants as in the irradiated antioxidant-free controls. In Tables I to III, the antioxidant contents of the samples have been corrected by subtracting from the measured values the antioxidant equivalents calculated from the color given by the irradiated controls. In the case of tocopherol this correction often resulted in negative values which have been recorded as zero. This suggests that a lower amount of these reducing substances accumulated in the samples containing tocopherol than in the controls. Although this may be true also for propyl gallate and butylated hydroxyanisole, it is not apparent from the available data. The antioxidant contents shown in the tables, therefore, may be slightly too low, but the error for propyl gallate and butylated hydroxyanisole should be insignificant because the corrections for these antioxidants are small.

Destructon of Antioxidants in Methyl Myristate. Table I shows the effect of irradiation and storage under vacuum at different temperatures on the antioxidant content of methyl myristate.

All three antioxidants studied show some destruction as a result of irradiation under vacuum. Tocopherol is most sensitive and only one tenth of the amount added remained after irradiation with 2 megarads, and higher doses resulted in complete destruction. Propyl gallate is considerably more resistant to destruction than tocopherol, only half of the amount used being destroyed by a dose of 5 megarads. Citric acid had no protective effect on the destruction of propyl gallate during irradiation and subsequent storage under vacuum. Of the three antioxidants studied, butylated hydroxyanisole is most resistant to irradiation under vacuum and only about 40% of the amount present was destroyed after a dose of 8 megarads.

In all cases, antioxidant destruction was noted when the samples were first examined as soon after irradiation as

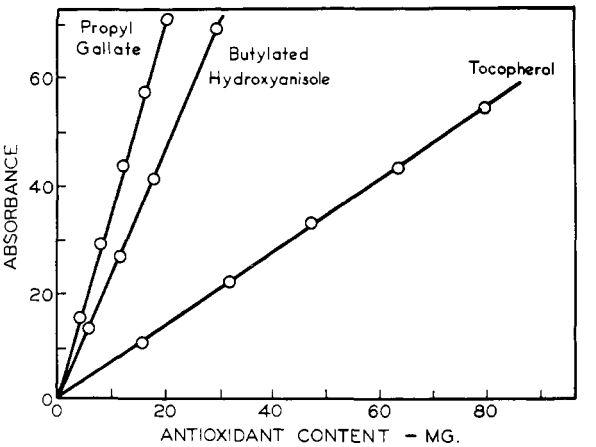

Figure 1. Standard curves for determination of antioxidants

Table I. Antioxidant Content of Methyl Myristate Irradiated and Stored under Vacuum

Antioxidant	Dose, Megarads	Antioxidant Content (Meq./Kg.) after Storage under Specified Conditions									
		25° C.				2° C.			−20° C.		
		0 week	2 weeks	8 weeks	20 weeks	2 weeks	8 weeks	20 weeks	2 weeks	8 weeks	20 weeks
0.01% α-Tocopherol	0	112	..	...	...	...	...	116	..	...	...
	2	11	..	...	...	47	44	2	..	...	...
	5	0	0	0	0	0	0	0	0	0	0
	8	0	..	...	...	0	0	0	..	...	...
0.01% Propyl gallate	0	113	..	113	115	...	117	119	..	116	123
	2	95	85	100	98	92	88	95	97	95	100
	5	58	56	55	61	55	60	71	61	59	60
	8	41	38	62	35	60	49	62	51	53	48
0.01% Propyl gallate + 0.01% citric acid	0	120	..	...	...	...	119	125	..	...	...
	2	95	..	...	...	99	102	90	..	...	...
	5	57	53	59	55	60	56	54	53	57	59
	8	40	..	...	...	55	59	31	..	...	...
0.01% Butylated hydroxyanisole	0	122	..	...	...	...	123	121	..	...	...
	2	112	..	...	...	112	115	114	..	...	...
	5	88	89	38	88	92	96	92	91	94	90
	8	72	..	...	...	76	72	73	..	...	...

possible. Although some of the values obtained on stored samples differ appreciably from the concentrations measured before storage, there is no general trend to indicate either further destruction or regeneration of antioxidant during storage. The occasional large differences in the antioxidant content of similar stored samples are beyond the limits of experimental error of the analytical method employed and, therefore, they represent real variations in antioxidant concentration. They may reflect, in part, variations in the actual dose received by individual samples, defective seals which permitted contamination of the atmosphere of the sample with oxygen, or other unknown and uncontrolled factors.

When based on the over-all total doses received by the samples, the G values for destruction of antioxidants are very low (between 0.04 and 0.11), but this is still 100 to 500 times higher than the values expected from chance ionization of the antioxidant molecules, calculated from the mole concentration of the antioxidants in the substrate. The destruction of these antioxidants, therefore, appears to be caused to only a slight extent by the direct action of ionizing radiations on the antioxidant molecules and largely through secondary reactions between activated substrate molecules and the antioxidant.

On a mole basis, the destruction of propyl gallate is approximately 25% greater than that of BHA. BHA is also more resistant than propyl gallate to destruction by heat, as evidenced by the carry-through antioxidant activity of BHA in baked and cooked products (*6*).

Table II shows that the antioxidants are destroyed much more rapidly in an atmosphere of oxygen than in vacuum. A dose of 2 megarads destroyed completely all tocopherol and about 80% of propyl gallate. Again, citric acid had no effect on propyl gallate destruction. In an atmosphere of oxygen, butylated hydroxyanisole was slightly more sensitive to irradiation than propyl gallate, at least 90% being destroyed by 2 megarads. In most cases, storage at the higher temperature resulted in further slight losses. The pronounced destructive effect of oxygen cannot be due to autoxidative chain reactions because, although free radicals are formed (*4*), no chain propagation is initiated in saturated materials such as the methyl myristate employed in this study (*5*). This greater destruction is more likely to be due to reaction of activated antioxidant molecules with oxygen or with the highly oxidizing hydroperoxide radicals which have been shown to result when the myristate alkyl free radicals originally formed react with oxygen (*4*).

Destruction of Antioxidants in Methyl Linoleate. Table III shows the destruction of propyl gallate added to methyl linoleate at a concentration of 0.1% and irradiated under vacuum or in an atmosphere of oxygen. With such a high antioxidant concentration, irradiation under vacuum resulted in the loss of less than 12% of propyl gallate at the highest irradiation dose used. In contrast, irradiation under oxygen with the same dose destroys nearly 90% of the

Table II. Antioxidant Content of Methyl Myristate Irradiated and Stored under Oxygen

Antioxidant	Dose, Megarads	Antioxidant Content (Meq./Kg.) after Storage under Specified Conditions									
		25° C.				2° C.			−20° C.		
		0 week	2 weeks	8 weeks	20 weeks	2 weeks	8 weeks	20 weeks	2 weeks	8 weeks	20 weeks
0.01% α-Tocopherol	0	110	...	...	...	113	109	112	...	...	...
	2	0	...	...	...	0	0	0	...	...	...
	5	0	...	0	0	0	0	0	0	0	0
	8	0	...	...	...	0	0	0	...	...	...
0.01% Propyl gallate	0	112	110	103	112	114	113	116	113	115	115
	2	22	15	14	10	11	10	7	18	15	13
	5	14	5	4	0	6	4	3	15	12	11
	8	14	8	2	3	7	5	4	16	13	10
0.01% Propyl gallate 0.01% Citric acid	0	113	...	...	...	117	118	124	...	...	...
	2	19	...	...	...	14	8	8	...	...	...
	5	14	8	5	9	7	5	5	13	13	15
	8	13	...	...	...	7	7	9	...	...	...
0.01% Butylated hydroxyanisole	0	121	...	...	...	123	123	117	...	...	...
	2	6	...	...	...	2	2	4	...	...	...
	5	10	4	3	8	6	4	12	6	8	16
	8	12	...	...	...	6	7	12	...	...	...

Table III. Propyl Gallate Content of Irradiated Methyl Linoleate

Irradiation Atmosphere	Dose, Megarads	Propyl Gallate Content (Meq./Kg.) after Storage under Specified Conditions: 25° C. 0 week	25° C. 2 weeks	25° C. 8 weeks	25° C. 20 weeks	2° C. 2 weeks	2° C. 8 weeks	2° C. 20 weeks	−20° C. 2 weeks	−20° C. 8 weeks	−20° C. 20 weeks
Vacuum	0	1015	...	...	...	...	1000	1013	...	...	...
	2	959	...	...	...	986	984	970	...	...	...
	5	939	903	870	924	915	912	963	939	907	946
	8	897	...	...	...	940	938	910	...	...	...
Oxygen	0	1023	...	...	...	1063	985	952	...	...	...
	2	571	...	...	...	517	562	394	...	...	...
	5	151	55	77	75	134	116	60	157	159	145
	8	115	...	...	...	121	54	43	...	...	...

antioxidant. There is no significant change in the antioxidant content of the samples irradiated under vacuum during postirradiation storage.

In general, the antioxidant concentration in the samples irradiated and stored under oxygen continues to decrease during storage at the higher temperatures. This further destruction of antioxidant is probably due to the continued autoxidation of these samples during storage (*5*).

The *G* value for the destruction of propyl gallate in methyl linoleate during irradiation in vacuum is again low (0.07 to 0.13), but in spite of the higher concentration of antioxidant, the *G* value is still 15 to 25 times greater than what would be expected on the chance ionization of the antioxidant molecules during irradiation. As in the case of methyl myristate, this indicates also that the antioxidant is destroyed mostly by secondary reactions, although, in this case, this effect appears to be less than with methyl myristate. This may suggest that the linoleate free radicals are destroyed more rapidly through recombination and polymerization than through reaction with antioxidant molecules. Rose *et al.* (*14*) arrived at a similar conclusion from their observations that α-tocopherol was more sensitive to radiations when dissolved in methyl myristate than in methyl linoleate.

To detect possible differences in the effectiveness of antioxidants in radiation-induced oxidation and simple autoxidation, identical amounts (0.005%) of butylated hydroxyanisole were added to two samples of methyl linoleate, one irradiated in oxygen to a peroxide value of 240 meq. per kg. and the other autoxidized at room temperature to a similar peroxide value (200 meq. per kg.). Figure 2 shows that the same amount of antioxidant was considerably more effective in preventing further oxidation of the autoxidized sample than of the irradiation-oxidized sample, even if a correction is applied for the difference in the original peroxide content of both samples. Such a correction can be effected approximately by considering only the portion

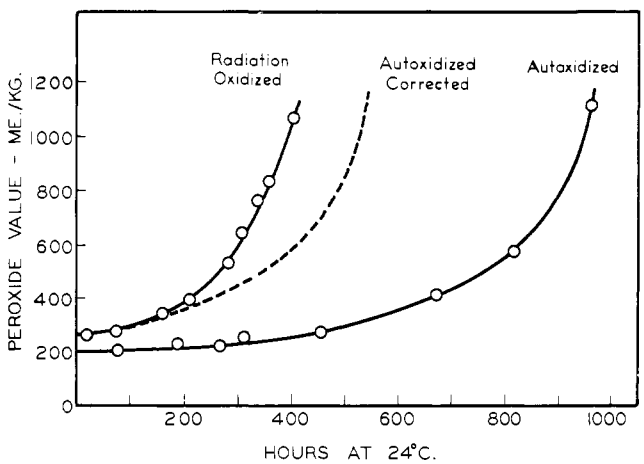

Figure 2. Comparison of efficiency of 0.005% BHA added to radiation-oxidized and autoxidized linoleate

of the autoxidized curve above a peroxide value of 240. This is shown as a broken line in Figure 2. Such a procedure gives a slight overcorrection because, while the autoxidized sample oxidized from its original peroxide value to a peroxide content of 240, some of the antioxidant originally added would have been destroyed. Comparison of the autoxidation of both samples above the peroxide value of 240, therefore, means that the autoxidized material has a slightly lower antioxidant content than the irradiated sample. The lower effectiveness of BHA in the irradiated sample may be explained by the large number of short-chain reactions that were initiated during irradiation as compared to the smaller number of longer chains prevailing in the autoxidized material. Since antioxidant action is due to interruption of chain reactions, these compounds would be expected to be more effective under the latter conditions.

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