done by electrophoretic studies. The difficulty in determining this shift by the dye absorption method is that at higher pH levels, the solubility of proteins becomes prohibitive, and it is practically impossible to obtain clear extracts under conditions normally employed. Additionally, at higher pH levels, there is a danger of precipitating the added dyes as well as extracting flavone pigments from the tested materials. Therefore, these studies had been confined to pH values of 2, 3, and 4, respectively. There is a consistent decrease in dye absorption of both basic dyes (the values are given as absorbance of the supernatant) with decreasing pH.

In observing the data for various germ samples stained with a solution at a specified pH level, the higher the damage, the lower the dye absorption. This trend is uniform in any of the pH levels employed and is consistent with findings of lowering of pH due to binding of basic amino groups.

With the acidic dyes, one would expect a higher dye absorption at the lower pH level. This is the case with either of the two acidic dyes employed at both the pH 2 and pH 3 levels, as indicated in Table II. Surprisingly, however, here is no one directional trend in the acid dye absorption of the samples of varying extent of damage tested at a specified pH level.

A shift toward lower pH values, as a result of binding of basic amino groups, should be reflected in higher dye absorption (or lower absorbance of supernatants). No such uniform trend has been recorded. The explanation seems to be that the extent of absorption of acidic dyes depends on two factors acting in opposite directions during storage, shift in pH toward higher acidity and increased acidic dye absorption, concomitant with binding of basic amino groups by reducing sugars and rendering them unavailable for the negative ions of the acidic dye. In Table II, note that though there was no one directional trend for both dyes employed at either pH level, there was a consistent increase in dye binding in the less damaged samples (moisture levels of 15% and below), and only in the highly damaged samples was the staining intensity decreased. This may indicate the relative availability of the basic amino groups in the lightly damaged samples, and decreasing availability of amino groups with progressive damage of the wheat with increasing moisture or length of storage.

Results reported in this investigation are based on studies made on a substrate in which the total protein content was constant. The absorption of acid dyes has been shown (*8*, *10*) to depend on the total number of basic amino groups, and subsequently on the protein level of the tested substrate. Thus, in assessing changes in samples of variable protein content, which undergo a browning reaction, both the protein level and availability of amino groups will affect the amount of bound dye. In testing commercial samples, it might therefore be desirable to express the results on a constant protein basis.

The possible application of the principle of dye binding, as a measure of soundness or storability of grain, and of heat treatment of soy flour to improve its biological value, is being investigated.

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RADIATION STERILIZATION OF FOODS

Comparison of the Radiosensitivities of the Fat-Soluble Vitamins by Gamma Irradiation

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ONE OF THE PROBLEMS in the use of ionizing radiation for sterilizing foods is the destruction of essential micronutrients. Knowledge of radiation lability of vitamins is also important in radiation biology. Although there have been numerous reports on the irradiation of the fat-soluble vitamins A, D, E, and K (*10*), no definitive study has been made of their comparative radiosensitivities under uniform conditions. By irradiating these compounds individually, in pure solution, the complicating factors of interaction with and protection by other solutes may be avoided. By the use of a relatively inert hydrocarbon, iso-octane, as irradiation medium, it was hoped to minimize solvent effects on the vitamins.

Vitamins and Vitamin Assays. Except for α- and β-carotene, all vitamins were obtained from Nutritional Biochemicals Corp. The original concentration of each was the same for both aerobic and anaerobic irradiation.

Vitamin E (*d l* - α - tocopherol), whether irradiated in iso-octane, tributyrin, or lard, was determined by the ferric chloride–1,1′-dipyridyl method (*11*), except that the reaction time was shortened to $1^1/_2$ minutes. To prevent phasic separation, 1 or 2 ml. of acetone were included in each tube.

Initial vitamin A acetate concentrations were determined by the ultraviolet absorption method (*1*) applied directly to the iso-octane solutions. Because irradiation increased the ratio of absorbance at 310 mμ to absorbance at 325 mμ, per cent retentions of vitamin A acetate in irradiated solutions were calculated on the basis of absorbance at 334 mμ.

Mixed α- and β-carotenes were prepared from commercially canned diced carrots (*5*) and assayed by absorbance at 440 mμ.

Vitamin D_3 in iso-octane was determined from its ultraviolet absorbance (*3*). Because the shape of the spectral maximum at 265 mμ was distorted in the more highly irradiated solutions, residual vitamin D was calculated on the basis of absorbance at 274 mμ. Concentrations of vitamin D in salmon oil were determined by the antimony trichloride method (*8*) without saponification.

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In order to compare the radiosensitivities of the fat-soluble vitamins under controlled conditions, they have each been gamma-irradiated in pure solution, aerobically and anaerobically. Vitamin E is by far the most sensitive, followed in order of decreasing sensitivity by carotene and vitamins A, D, and K. Only vitamin E is affected adversely by the presence of oxygen during irradiation; vitamins D and K are considerably stabilized.

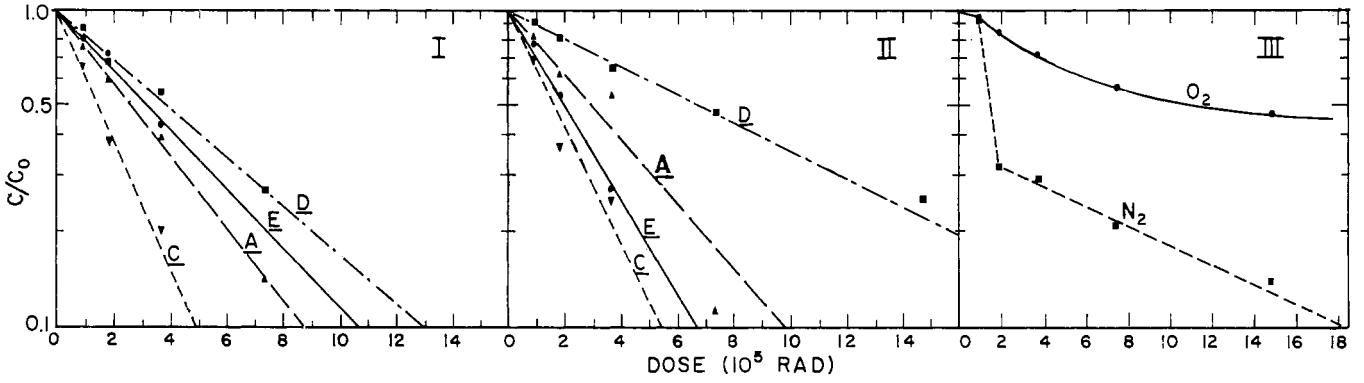

Figure 1. Stabilities of fat-soluble vitamins, γ-irradiated

I. In iso-octane–N_2. *A.* Vitamin A acetate, 8.5×10^{-5}M. *C.* Mixed α- and β-carotenes, 1×10^{-4}M. *D.* Vitamin D, 1.7×10^{-4}M. *E.* Vitamin E, 1×10^{-3}M. II. In isa-octane–O_2. *A.* Vitamin A acetate, 8.5×10^{-5}M. *C.* Mixed α- and β-carotenes, 1×10^{-4}M. *D.* Vitamin D, 1.7×10^{-4}M. *E.* Vitamin E, 1×10^{-3}M. III. Vitamin K_3 in iso-octane, 9×10^{-5}M

Table I. Comparative Stabilities of Fat-Soluble Vitamins Irradiated in Iso-octane

Vitamin	Concn. × 10^5, M	Atmosphere	D_D in 10^6 Rads	D_D/C_0 in 10^9 Rads/Mole/L.
A acetate	8.5	N_2	0.9	10
		Air	0.97	11
Carotene	10	N_2	0.49	4.9
		Air	0.54	5.4
D_3	17	N_2	1.3	7.6
		O_2	2.6	15
E	100	N_2	1.0	1.0
		O_2	0.7	0.7
K_3	9	N_2	18.6	20
		O_2	...	...

Table II. Protection by α-Tocopherol for Carotene and Vitamin A Acetate Irradiated in Iso-octane

Indicator	Atmosphere	Dose, 10^5 Rads: 0.93	1.86	3.72	7.44
		Protective Effect[a]			
Carotene	air	0.6	0.9	0.6	1.1
	N_2	0.7	1.0	0.6	0.9
Vitamin A acetate	air	0.2	0.5	0.7	0.5
	N_2	0.6	0.3	0.8	0.4

[a] Calculated as described by Proctor *et al.* (*6*):

$$\text{Protective effect} = \frac{\text{dose absorbed by protector/concentration of protector}}{\text{dose absorbed by indicator/concentration of indicator}}$$

Because of irregularities in plots of concentration *vs.* dose, protective effect values are based on point-by-point comparisons of vitamin retentions in protected and unprotected solutions rather than on comparisons of over-all destruction rates.

Concentrations: α-tocopherol, $1 \times 10^{-4}M$; carotene, $1 \times 10^{-4}M$; vitamin A acetate, 8.5×10^5M.

Vitamin K_3 (menadione) concentrations in iso-octane were determined colorimetrically as the 2,4-dinitrophenylhydrazone (*9*). Reactions were run for exactly 5 minutes at 100° C. The isoamyl alcoholic extract was clarified with methanol.

Preparation and Irradiation. For anaerobic irradiation, nitrogen-saturated solutions of the vitamins (in iso-octane, tributyrin, stripped lard, or commercial salmon oil) were poured, with bubbling nitrogen, into screw-cap glass vials which were then quickly closed. For aerobic irradiation, a sufficient headspace was gassed with air or oxygen to give a molecular oxygen–vitamin ratio of 11 to 1 for vitamin A and carotene or 100 to 1 for vitamins E, D, and K. The vials were protected by sealing in No. 2 cans and held at −20° C. while at this laboratory. They were packed with solid carbon dioxide and shipped by air to and from the irradiation site, the Materials Testing Reactor, Arco, Idaho. Gamma irradiation at an average energy level of 1 m.e.v. was carried out at 21° C. Samples irradiated under oxygen were regassed with nitrogen upon their return to this laboratory to prevent further oxidation before analysis.

Table III. Stabilities of Vitamin E to α-Irradiation under Various Conditions

Initial Concn. × 10^3, M	Atmosphere	Solvent	Irradiation Dose in 10^6 Rads: 0.5	1	2	4	5	8	10	16	26	D_D in 10^6 Rads	D_D/C_0, 10^6 Rads/Mole/L.
			Per Cent Vitamin E Remaining										
9.8	N_2	Iso-octane	90	82	61	28	..	..	...	..	..	9.5	7.0
7.7	N_2	Tributyrin	..	77	..	..	29	..	3.7	..	..	9.2	1.2
10	N_2	Tributyrin	..	..	73	..	35	..	4	..	..	12	1.2
10	O_2	Tributyrin	..	..	55	..	14	..	1	..	..	6.3	0.63
9.4	N_2	Lard	95	91	85	71	..	54	...	24	10	26	3

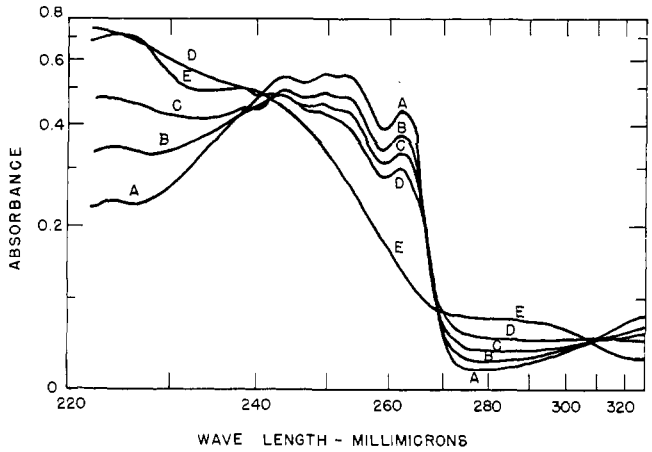

Figure 2. Ultraviolet absorption spectra of vitamin K_3 irradiated in iso-octane

A. Unirradiated; 0.93×10^5 rads, N_2; 0.93×10^5 rads, O_2
B. 3.7×10^5 rads, O_2
C. 7.4×10^5 rads, O_2
D. 14.8×10^5 rads, O_2
E. 1.9×10^5 rads, N_2

Results and Discussion

In Figure 1, part I, per cent retentions of carotene and of vitamins A, D, and E, irradiated anaerobically in iso-octane, have been plotted against the corresponding radiation doses. The data for the irradiation of these vitamins under oxygen are presented graphically in Figure 1, part II. However, as can be seen in Figure 1, part III, vitamin K_3 exhibits broken and curved lines after irradiation under nitrogen and oxygen, respectively.

The slopes of these retention *vs.* dose curves can be defined by the decimal reduction dose, D_D, the dose which reduces the original concentration, C_O, by 90% (*7*). When this semilogarithmic relationship between concentration and dose holds, the percentage destruction per unit of dose varies inversely with solute concentration (*2*). Consequently, the specific decimal reduction dose, D_D/C_O, may be used to compare radiosensitivities in solutions of different concentrations. D_D/C_O values obtained in this study are presented in Table I, allowing a comparison of the radiosensitivities of these vitamins. Since the regression lines for retention of vitamin K are irregular, great significance cannot be attached to the exact value for the specific decimal reduction dose obtained for its anaerobic irradiation. No D_D value can be obtained for the aerobic irradiation of vitamin K. However, under anaerobic conditions the vitamins are radioresistant in the decreasing order: vitamin K_3, vitamin A acetate, vitamin D_3, carotene, and vitamin E. Under oxygen, the order is similar: vitamin K_3, vitamin D_3, vitamin A acetate, carotene, and vitamin E.

Vitamin A acetate was twice as stable to ionizing radiation as was carotene. This is in marked contrast to the results of Kung *et al.* (*4*), who found that carotene was much the more radioresistant, in both whole and evaporated milk. D_D/C_O values for irradiated whole milk were 4×10^{11} roentgens per mole per liter for vitamin A and 4×10^{12} roentgens per mole per liter for the carotenes. These are not only considerably higher, but also reversed in order from the values reported here. There does not appear to be any way to reconcile these differences, because milk is such a complex medium compared to iso-octane. While the presence of unsaturated milk lipides would promote the radiation destruction of other solutes, many of the components of milk, notably vitamin E, would act as protectors against radiation damage. In the present investigation, a limited number of measurements were made of the protective effect of α-tocopherol for vitamin A acetate and carotene irradiated in iso-octane. The resulting data, presented in Table II, show in general that carotene is more stabilized than vitamin A.

Vitamin D in salmon oil is apparently unaffected by ionizing radiation. In the absence of a highly specific assay, the exact magnitude and even the direction of its concentration change are uncertain. It is possible that some of the inactive sterols in the oil may be converted to substances quite like the vitamin, if not identical to it. The vitamin may be protected by tocopherols and other substances in the oil. Any changes of vitamin D concentration in irradiated fish oil, and thus in irradiated fish, would probably be too small to be nutritionally important.

Table III presents additional data for the irradiation of α-tocopherol in iso-octane, tributyrin, and lard. Comparison of the specific decimal reduction doses for vitamin E in the three solvents shows that there is only a slight increase in stability when iso-octane is replaced by tributyrin as solvent. The doubled stability found in lard is probably due, in large part, to the competition for free radicals by various labile constituents of the lard.

The effect on vitamin stability of changing the atmosphere of irradiation from nitrogen to oxygen can be seen from a comparison of the data in Tables I, III, and Figure 1. In view of the well known lability of vitamin E to oxygen, it is not surprising to find it affected adversely by aerobic irradiation. The decrease in stability of 30% is possibly even less than one might predict. But since both solvents are saturated, there is little radiation-initiated chain reaction peroxidation, with consequent damage to the vitamin.

The carotenoids in solution are apparently stabilized to a small extent by the presence of oxygen during irradiation. This is in agreement with the report of Lukton and Mackinney (*5*), who suggest that these compounds are more readily attacked by solvent-free radicals than by the hydroperoxides formed by combination of the latter with oxygen. This oxygen effect in carotenoid irradiation is relatively unimportant in the present investigation.

In the case of vitamins D_3 and K_3, however, the change from nitrogen to oxygen imparts very marked increases in stability. The destruction rate of vitamin D_3 is halved. Because the concentration *vs.* dose plot for aerobic irradiation of menadione is curved rather than linear, it is impossible to quantitate precisely the effect of atmosphere on the radiosensitivity of this vitamin. However, the 50% destruction doses for vitamin K can be estimated from Figure 1, part III, as about 2×10^5 and 1.2×10^6 rads for atmospheres of nitrogen and oxygen, respectively. On this basis the difference in stability of vitamin K is at least sixfold.

This stabilizing influence of oxygen may be ascribed to the lower reactivity of these vitamins toward peroxides than toward alkyl radicals (*5*). A corollary conclusion would be that radiation destruction of these vitamins is chiefly through reductive attack by alkyl radicals and/or hydrogen atoms. This would lead to saturation of double bonds and, in the case of menadione, to reduction or etherification of the benzoquinone oxygen. That menadione is not simply reduced is shown by the inability of its irradiation products to reduce ferric chloride under the conditions of the vitamin E assay.

As Figure 2 shows, menadione irradiated under oxygen retains its characteristic spectrum up to the higher doses. On the other hand, the sharp drop in the plot of concentration *vs.* dose for vitamin K irradiated under nitrogen is reflected in the abrupt change from a spectrum

almost identical with that of the pure substance (*A*, Figure 2) to the relatively featureless spectrum *E* at 1.9×10^5 rads. Some of the molecular species represented by the latter spectrum are evidently changed considerably from the original naphthoquinone structure. It is possible that the changes taking place in menadione, irradiated anaerobically, do not interfere with its reaction with 2,4-dinitrophenylhydrazine as drastically as they apparently reduce its biological potency (*10*).

The curved retention slope, Figure 1, III, given by menadione irradiated under oxygen, might imply that in this system irradiation products are more successful than menadione in competing for free radicals. An alternative explanation is that these products are being reconverted, either to menadione, or to naphtho- or benzoquinones with spectra closely resembling that of the vitamin. More critical chemical methods and/or bioassay would be needed to distinguish between these effects.

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PLANT PROTEIN CONSTITUENTS

Hydroxyproline Content of Seed Meals and Distribution of the Amino Acid in Kernel, Seed Coat, and Pericarp

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Hydroxyproline was found in 63 of 99 samples of solvent-extracted, acid-hydrolyzed seed meals obtained from a wide variety of plants in amounts ranging from 0.1 to 5.7 grams per 16 grams of nitrogen. Eleven seed meals derived from the kernel alone contained no hydroxyproline. Eight samples of only seed coat or pericarp from seven plant families had hydroxyproline contents ranging from 3.1 to 10.0 grams per 16 grams of nitrogen. L-Hydroxyproline was isolated from the seed coat of *Iris germanica* and its identity established by classical methods. Limited solubility studies indicate that the compound is part of the protein in seed coat and pericarp.

THE AMINO ACID content of numerous seed meals is being determined as one phase of a screening program (*17*) to find new crops that could be profitably grown by the farmer. In making such determinations it was observed that most seed meals contained hydroxyproline. This amino acid was frequently present in amounts greater than 1 gram per 16 grams of nitrogen.

Little information was found in the literature concerning hydroxyproline in plants (*7, 9*). Piez, Irreverre, and Wolff (*8*) reported small amounts in the pericarp of dates. The authors reported that soybean seed coat contains 7.6 grams of this amino acid per 16 grams of nitrogen (*10*). Kleinshmidt reported its presence in the poppy plant (*4*).

Hydroxyproline is formed in situ from proline after proline has become part of the peptide chains in collagen formation (*13*). Steward and co-workers (*9, 14, 15*) in a series of papers show that in plant tissue cultures hydroxyproline is formed similarly from proline. Their work indicates it to be part of a stable protein not subject to "carbon turnover." More recently, Dougall and Shimbayashi (*3*) and Lamport and Northcote (*5*) have reported the compound in plant tissue cultures and have used its measurement as a means of following metabolic processes. No reference has been found in the literature to isolation and characterization of the compound from plant sources. Radikrishnan and Giri (*11*) isolated *allo*-hydroxyproline from *Santalum album* in which it was present uncombined. The paucity of information on the presence of the amino acid in proteins from naturally growing plants and its apparently unusual method of formation from proline prompted this further investigation of hydroxyproline in plant materials.

Experimental

Materials. Amino acid analyses were made on solvent-extracted meals derived from seeds, including in most cases seed coat or seed coat and pericarp because these parts could not be easily separated. When it became apparent that hydroxyproline was associated with seed coat and pericarp, selected separate preparations of these tissues were also analyzed. All preparations were ground, extracted with petroleum ether in order to remove oil, and acid-hydrolyzed before analysis as previously described (*16*).

Methods of Analysis. The amount of each amino acid present in the hydrolyzate was determined by the ion exchange, chromatographic automatic analysis method of Spackman, Stein, and Moore (*12*), using the Beckman Spinco, Model No. MS instrument. By operating the long column at 30° C. during the first part of the run, well