Sherman (1) has shown that carotene is destroyed in the gastrointestinal tract when fed with linolate or linolenate to vitamin A-deficient rats. Carotene-free refined vegetable oils or their non-saponifiable fractions prevented this destruction of carotene. It was shown later (2) that $\alpha$-tocopherol was highly effective in preserving carotene under such conditions. This fact was noted independently by Quackenbush, Cox, and Steenbock (3). These results have been confirmed by Hickman and coworkers (4–6), and extended in detail to show that $\beta$- or $\gamma$-tocopherols or fat-soluble derivatives of ascorbic acid and hydroquinone are equally as effective as $\alpha$-tocopherol.

The antioxidant property of gossypol for the protection of lard, in vitro, was first demonstrated by Mattill (7). Gossypol is effective in protecting carotene, in vitro, against preformed fat peroxides (8). Therefore, it seemed logical to determine whether gossypol could act as a carotene-protecting antioxidant, in vivo, and whether cottonseed products containing gossypol could inhibit carotene destruction and rancidity development, in vitro.

**EXPERIMENTAL**

*Carotene Protection, in Vivo*—The procedure used by previous workers has been to deplete rats on the U. S. P. vitamin A-low diet, or modifications thereof. When body weights have plateaued, small amounts of carotene or other vitamin A-active materials were fed daily along with the antioxidant to be tested. The resultant growth rate is the criterion for effectiveness of the antioxidant.

*Ration*—The U. S. P. vitamin A-low basal diet contains 8 per cent of dry yeast as the source of water-soluble vitamins. It was found in this laboratory (8) that the 1.3 per cent of crude fat in the dry yeast is rich in some fat-soluble antioxidant. At the 8 per cent level, therefore, the yeast would contribute 0.1 gm. of crude fat and 30 $\gamma$ of antioxidant, expressed as tocopherol, to 100 gm. of the basal diet. Because of the presence of these materials, yeast was omitted from the ration for the experiments.

* Published with the approval of the Director of the Alabama Agricultural Experiment Station.
reported in this paper and the water-soluble vitamins were supplied as the synthetic compounds. The diet, indicated as Diet 60, was made up as follows: extracted casein 18, sucrose 77.5, Salts 186 (9) 4.5, thiamine, riboflavin, and pyridoxine 10 \( \gamma \) per gm. of ration, calcium pantothenate 25 \( \gamma \) per gm. of ration, \( i \)-inositol 100 \( \gamma \) per gm. of ration, choline hydrochloride, 1 mg. per gm. of ration, and calciferol 0.25 \( \gamma \) per gm. of ration.

The extracted casein was prepared by refluxing 500 gm. batches of commercial casein with 2 liters of 95 per cent alcohol, and filtering while hot on a Büchner funnel. This was repeated five times. The first reflux was carried out with 10 ml. of concentrated sulfuric acid added to the alcohol.

Besides being low in vitamin A activity, Diet 60 is low in fat and all fat soluble factors except vitamin D.

Animals—Black and white hooded rats were used in this work. Litters were obtained from the stock colony at an average weight of 35 gm. and placed on the basal diet. A plateau in the weight curve began in 5 weeks at 90 to 130 gm. Within any one litter, the weights deviated by about \( \pm 7 \) gm. from the mean. Daily weights were taken until a plateau was assured, at which time the litter was started on experiment. Only one or two litters of six to eight animals each were started on depletion at a time. For the work reported here, litters were started at intervals over a 6 month period. Each litter was made into a complete and self-contained experiment, with usually only one rat on each supplemental variation. Thus, the final results represent the average of several small scale experiments. By the adoption of this procedure, assurance was given that the results obtained were not artifacts caused by variation between litters or other conditions of the experiment, such as deterioration of materials.

Supplements—Some workers (3, 4) have preferred to feed daily supplements by calibrated dropper. However, Sherman (1) has had success in dispersing the appropriate quantities of supplement solutions on a small quantity of basal ration, mixing, and feeding. He has shown conclusively that no less of carotene occurs prior to consumption by the rat. This procedure was continued, but with some modifications. Since iron and copper salts are well known prooxidants, a special supplement diet has been used, identical with Diet 60, except that the salt mixture was omitted. Exactly 1 \( \pm 0.1 \) gm. of this special diet was placed in the supplement cups.

The carotene and antioxidant solutions were pipetted onto this and were allowed to disperse for 20 to 30 minutes. The fat or oil, if used, was then measured in and the contents of the jar mixed with a clean glass rod. 2 hours previously the regular feed jars had been removed from the cages. The supplement cups were offered to the rats, and the supplement was practically always consumed in less than 15 minutes. 2 hours later the
regular feed jars were replaced. By this procedure the possible complicity of inorganic salts in the destruction of carotene, either before or after consumption by the rat, was minimized. To compensate for the absence of salt mixture in this 1 gm. of feed, the salt content of the basal diet was increased from 4.0 to 4.5 per cent.

Crystalline carotene (Smaco, 90 per cent β-, 10 per cent α-) was dissolved in Skellysolve B. The concentration of this solution was checked every 10 days. A new solution was made up every 6 weeks. Merck's synthetic α-tocopherol was dissolved in Skellysolve B. Gossypol and dianilinogossypol were prepared as previously indicated (8). Gossypol was used in a peroxide-free ether solution in a concentration of 10 mg. per ml. Dianilinogossypol was used as the solution in reagent grade chloroform at a concentration of 2 mg. per ml.

Methyl linolate was prepared and stored as previously described (1). Its peroxide number was less than 2 milliequivalents per kilo at the end of the experiment. Swift's Silverleaf brand lard was used, and its peroxide number at the end of the experiment was less than 1.5 milliequivalents per kilo.

All rations and supplements were kept in a refrigerator room at all times. The gossypol was shown to be free of tocopherols by application of the Parker-McFarlane treatment and iron-bipyridine reagent as previously described (10).

Results

The data in Fig. 1 show the average growth rates of male and female depleted rats receiving 2 γ of carotene per day, alone, or with the addition of 0.1 gm. of lard, 0.1 gm. of hydrogenated coconut oil, 0.8 mg. of α-tocopherol, or one of the fats plus the tocopherol.

Rats receiving carotene alone failed to grow appreciably, declined in weight, and died. Altogether, seventeen rats received this supplement with 100 per cent mortality by the end of 12 weeks. This differs from Sherman's results (1) in that his rats, receiving 2 γ of carotene only, grew steadily throughout the experiment, although very slowly. This may be explained by the presence of the small amount of fat and antioxidant present in the dry yeast used in his basal diet. The initial xerophthalmia of the control rats, whose growth record is shown in Fig. 1, was cured in a week or two after the 2 γ of carotene per day were received. However, the fat acid deficiency symptoms persisted and became progressively more acute. The skin around the eyes and nose became dry, caked, and scaly. The dry flakiness around the paws gradually changed to a moist appearing swollen dermatitis. There was dandruff on the coat which was rough, with many loose hairs.
Feeding of lard plus carotene to depleted rats had little beneficial effect on the growth response. Altogether, fourteen rats received this supplement and only two survived to 12 weeks. Fatty acid deficiency symptoms were absent, but the rats developed a recurrence of xerophthalmia and a severe incoordination, with extreme leg weakness in those cases in which growth was evident. Thus, this lard appeared to have the same adverse effect as does pure methyl linolate, as shown by Sherman (1).

Feeding of carotene plus tocopherol (but no fat) resulted in some
growth, but an early plateau. No deaths occurred in this group. All vitamin A deficiency symptoms disappeared, but scaliness around the paws and nose persisted. The feeding of hydrogenated coconut oil plus carotene resulted in growth about equal to that obtained on the tocopherol plus carotene supplement, with similar persistence of fatty acid deficiency symptoms. Since this coconut oil gave negative tests for tocopherol (10) or antioxidants (8), the possibility of a mechanical protection of carotene in its passage through the tract must be considered. Lard plus tocopherol plus carotene supported good growth. The coconut oil plus tocopherol and carotene resulted in better growth than was evident in the group receiving the coconut oil and carotene (with no tocopherol), but it was not as great as on lard plus carotene and tocopherol.

In a preliminary experiment to determine the effectiveness of gossypol as an internal antioxidant, Diet 60 was altered by including 10 per cent of lard and increasing the casein to 20 per cent. Rats were depleted on this ration and started on experiment. Of six rats receiving 1.6 γ of carotene per day only, all were dead by the end of 3 weeks with weight losses ranging up to 36 gm. Six rats receiving 0.3 mg. of pure gossypol plus the carotene showed an average weight gain of 28 ± 6 gm. in 4 weeks. Four rats receiving 0.3 mg. of α-tocopherol in addition to the carotene showed an average weight gain of 44 ± 12 gm. by the end of 4 weeks. This experiment looked promising and was repeated in greater detail.

The data in Fig. 2 show the average growth records of rats that received 1 mg. daily doses of gossypol, dianilinogossypol, α-tocopherol, or α-tocopherol plus gossypol, in addition to 0.1 gm. of lard or 25 mg. of ethyl linolate. Diet 60 was used unaltered in this work.

From the results in Fig. 2, it is apparent that both gossypol and dianilinogossypol are quite effective internal antioxidants. Altogether, fifteen rats have received gossypol in addition to carotene plus a fatty material and satisfactory responses have been observed in each case. Ten rats have been on the dianilinogossypol supplement with good responses in all cases. The rats receiving linolate did not grow as well as those on the lard supplement. This may be due to the different quantities of these fatty materials used.

Carotene Protection, in Vitro, and Inhibition of Rancidity Development by Cottonseed Products—Crystalline carotene (Smaco, 90 per cent β-) was dissolved in pure ethyl oleate and was added to several commonly used feedstuffs in such quantities that the final mixtures contained 80 γ of carotene per gm., and 30 per cent of ethyl oleate. The feedstuffs had been previously ground to pass a 24 mesh screen. The wheat products were fresh and all milled from the same batch of whole wheat.1

1 The wheat milling fractions were obtained from the Pillsbury Flour Milling Company, through the courtesy of Mr. C. G. Harrel.
Fig. 2. The growth responses of vitamin A-deficient rats, whose weights reached a plateau, to daily supplements of 2 γ of carotene (X); to the carotene plus either 0.1 gm. of lard or 25 mg. of methyl linolate; to the carotene with one of the fatty materials plus 1 mg. of an antioxidant as indicated. (TOCO) α-tocopherol, (GOSS) pure gossypol, (DAG) pure dianilinogossypol. The numbers in parentheses indicate the number of animals. Broken lines indicate one or more deaths.
oil meal and other products were obtained from the stock-feed barn of the Experiment Station. Carotene determinations were made by the method of the Association of Official Agricultural Chemists. Total tocopherol determinations were carried out on the 24 hour Skellysolve B extracts of the products by the iron-bipyridine method, as previously described (10). The samples were stored at 68°C in open dishes. 2 gm. aliquots were removed at 6, 20, and 31 days for carotene determinations. The percentage losses of carotene were calculated on the basis of the original determinations before storage.

**Table I**

*Stability of Carotene Added at a Level of 80 γ per Gm. to Feedstuffs Mixed with Ethyl Oleate at a 30 Per Cent Level and Stored Open at 68°C*

<table>
<thead>
<tr>
<th>Product</th>
<th>Original tocopherol content</th>
<th>Carotene loss in</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg. per gm.</td>
<td>6 days</td>
</tr>
<tr>
<td>Wheat germ</td>
<td>0.28</td>
<td>16.5</td>
</tr>
<tr>
<td>Cottonseed oil meal</td>
<td>0.05</td>
<td>20.0</td>
</tr>
<tr>
<td>Red dog flour</td>
<td>0.08</td>
<td>25.5</td>
</tr>
<tr>
<td>Soy bean oil meal</td>
<td>0.07</td>
<td>34.0</td>
</tr>
<tr>
<td>Wheat bran</td>
<td>0.07</td>
<td>34.0</td>
</tr>
<tr>
<td>2nd clear flour</td>
<td>0.022</td>
<td>40.5</td>
</tr>
<tr>
<td>1st &quot; &quot;</td>
<td>0.016</td>
<td>50.5</td>
</tr>
<tr>
<td>Peanut oil meal</td>
<td>0.016</td>
<td>60.0</td>
</tr>
<tr>
<td>Patent flour, wheat</td>
<td>0.011</td>
<td>68.0</td>
</tr>
<tr>
<td>Whole wheat</td>
<td>0.03</td>
<td>71.0</td>
</tr>
<tr>
<td>Skim milk powder</td>
<td>0</td>
<td>97.2</td>
</tr>
<tr>
<td>Corn-starch</td>
<td>0</td>
<td>78.9</td>
</tr>
</tbody>
</table>

The results are given in Table I. It is apparent that cottonseed oil meal provides good protection to carotene under these conditions, in spite of the fact that its tocopherol content is lower than several of the other products tested.

Some experiments were also carried out to determine the relative stability of cottonseed, soy bean, and peanut oil meals. The ground products were stored in half full jars at 60°C, and the development of rancidity followed organoleptically. The results are shown in Table II. These can only be considered as rough estimates because of the type of experiment, but they do have practical implications. The age of the products studied varied from less than 2 months for peanut oil meal Sample III to more than a year, under practical conditions, for peanut oil meal Sample I and the cottonseed oil meal. The defatted ground cotton seeds were fresh. They had been extracted with Skellysolve until no determinable fat or tocopherols re-
CAROTENE PROTECTION BY GOSSYPOL

When this product was added to peanut oil meals at a level of 10 per cent, the keeping quality of these meals was increased 4 to 5 times. This defatted, ground cotton seed contained 0.8 per cent free gossypol by isolation. The fat contents of the commercial oil meals used in this study varied from 4.8 to 5.7 per cent.

### Table II

<table>
<thead>
<tr>
<th>Oil meals</th>
<th>Admixture at a 10 per cent level</th>
<th>Keeping time at 60°</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cottonseed</td>
<td></td>
<td>70</td>
</tr>
<tr>
<td>Soy bean</td>
<td></td>
<td>56</td>
</tr>
<tr>
<td>Peanut, Sample I</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&quot; &quot; &quot; II</td>
<td>Cottonseed oil meal</td>
<td>47</td>
</tr>
<tr>
<td>&quot; &quot; &quot; &quot;</td>
<td>Soy bean oil meal</td>
<td>40</td>
</tr>
<tr>
<td>&quot; &quot; &quot; &quot;</td>
<td>Ground, defatted cotton seeds*</td>
<td>107</td>
</tr>
<tr>
<td>&quot; &quot; &quot; III</td>
<td>Cottonseed oil meal</td>
<td>38</td>
</tr>
<tr>
<td>&quot; &quot; &quot; &quot;</td>
<td>Soy bean oil meal</td>
<td>09</td>
</tr>
<tr>
<td>&quot; &quot; &quot; &quot;</td>
<td>Ground, defatted cotton seeds*</td>
<td>202</td>
</tr>
</tbody>
</table>

* Fresh, ground, cottonseed meats thoroughly extracted with Skellysolve B.

### DISCUSSION

The data show that pure gossypol and dianilinogossypol are about equally effective as carotene-preserving antioxidants when fed to rats, and are only slightly inferior to \( \alpha \)-tocopherol in this respect.

Defatted cotton seeds, containing gossypol, extended the keeping quality of peanut oil meals, in vitro, by 4 or 5 times, when mixed at a 10 per cent level into these meals. Commercial cottonseed oil meal proved to be equal to wheat germ in stabilizing a carotene solution in ethyl oleate. The tocopherol contents of these cottonseed products were much too low to explain their stabilizing action. Possibly this action is related solely to the gossypol or gossypol derivatives present.

Gossypol, or cottonseed products containing this factor, are known to be toxic to many animals. The minimum toxic dose for rats is said to be about 6 mg. per day, orally (11). Other animals such as the hog or chicken are much more sensitive (12), while cattle or goats are, like the rat, quite resistant. Raw cotton seeds contain about 1 per cent of free gossypol. Commercial cottonseed oil meals contain from 0.05 to 0.20 per cent of free gossypol, with an average of about 0.10 per cent. Thus, the 1 mg. per day level fed to rats in these experiments corresponds roughly to 10 per cent.
cottonseed oil meal in the ration. This is a safe level to feed to most farm animals. In the pressing of cotton seeds, a fair amount of gossypol goes into the oil. This is eliminated during the alkali refining process.

Apparently, a large part of the toxicity of gossypol is related to its two carbonyl groups. Apogossypol, which is gossypol with the two carbonyl groups eliminated by strong alkali, is much less toxic than the original product (13). In dianilinogossypol, the free carbonyl groups of gossypol are tied up by the anilido complex. It has never been determined whether this compound has lost the high toxicity that is characteristic of the free gossypol. The data presented in this paper show that the dianilinogossypol has not lost the antioxidant activity characteristic of the free compound.

The demonstration of a carotene-preserving action of gossypol and cottonseed products in no way changes the situation relative to the toxic properties of these. However, it does suggest that a balance can be struck between the good and bad effects. A more efficient utilization and better preservation of carotene are especially significant during the present crisis in supplies of vitamin A-active materials for stock feeding. If the toxicity of gossypol can be lessened or destroyed while its antioxidant properties are maintained, a much more important and far reaching benefit can be derived from cottonseed products.

SUMMARY

1. Small amounts of lard fed with small doses of carotene to rats that were deficient in vitamin A and essential fat acids resulted in growth failure and death. This result was similar to the previously demonstrated effect of methyl linolate on carotene fed to such rats.

2. Pure gossypol and dianilinogossypol were effective antioxidants, at daily doses of 1 mg., for the preservation of carotene fed with lard or methyl linolate to depleted rats. These compounds were only slightly inferior to \( \alpha \)-tocopherol in this respect.

3. Cottonseed oil meal was equal to wheat germ in stabilizing a carotene solution in ethyl oleate, \textit{in vitro}, and much superior in this respect to several other common feedstuffs. Defatted cotton seeds added to peanut oil meals at a 10 per cent level extended the keeping quality of the meals by 4 to 5 times.

4. The significance of these findings is discussed in relationship to commercial cottonseed products.

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GOSSYPOL AS A CAROTENE-PROTECTING ANTIOXIDANT, IN VIVO AND IN VITRO
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