THE RELATIONSHIP OF THE HALPHEN REACTION AND PINK EGG DISCOLORATION

by

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INTRODUCTION

Pink discoloration of stored eggs was first reported by Sherwood (28) in 1928 and was attributed to the presence of cottonseed products in the diet of laying hens. The eggs became discolored after several months of cold storage. Typical of the discoloration were pink whites and enlarged, watery, salmon colored yolks, with increased fragility of the vitellin membrane.

Extensive investigation of the problem by Schiable, Bandimer and Davidson (21,22,23,24,25) showed that the pink color produced in the white was due to a combination of conalbumin, a protein constituent of the white, with ferrous ion, an egg yolk component. The yolk and white are normally separated by the vitellin membrane, but in the process of discoloration, migration of the protein from the white to the yolk takes place. In the yolk, the conalbumin-ferrous ion complex blends with the natural yellow and gives the apricot appearance. Reverse migration of the complex, or migration of the ferrous ion alone gives the pink color to the white. Examination of eggs in the earlier stages of discoloration shows the color to be most intense in the area immediately adjacent to the vitellin membrane. Older eggs show an even distribution of the color throughout the white and yolk. The pH of the white was found to be about the same as that of the yolk, whereas in normal eggs, the yolk is much more acidic. This convergence of pH was apparently caused by diffusion. These authors postulated that some component of the cottonseed meal or oil must cause increased transport of egg white components through the vitellin membrane.

Lorenz and Almquist (16,17) found that the ether soluble fat fraction from the yolks of discolored eggs gave a positive Halphen reaction. The Halphen test is made by dissolving the fat in a solution of sulfur in carbon disulfide and isoamyl alcohol and heating to a temperature of 100° C. or more for at least an hour (33). A red color indicates the presence of cottonseed oil in the fat. No other oils commonly marketed in the United States give this reaction. However, other plant oils are known to give the Halphen reaction. Kapok seed oil, the oil of Malva parviflora seed and the oil of Sterculia foetida are three examples of oils reported to give the reaction (4,8,16,20,27). Kapok seed oil, and the oil of Malva parviflora have been shown to cause pink egg discoloration.(16,27). No evidence is available as to whether or not the oil of Sterculia foetida will cause pink discoloration.

The fatty acid composition of Sterculia foetida has been investigated (5,6,19,30,31). It contains 13 per cent oleic acid, 15 per cent myristic and palmitic acids and more than 70 per cent of an unusual acid called sterculic (9). Nunn, Faure, and Smith (5,6,19) have proposed the following structure for sterculic acid:

$$CH_3-(CH_2)_7-\overset{CH_2}{C=C}-(CH_2)_7-CO_2H$$

This structural assignment has been based mainly on the production, by means of a reductive ozonolysis, of 9,11-dioxononadecanoic acid, which in turn has been degraded to yield methyl-n-octyl ketone, 9-oxodecanoic acid, and azelaic acid. Nonanoic acid, another expected product, was not found.

Varma, Nath, and Aggerwal (30,31) disagreed with this structural assignment. On the basis of a permanganate oxidation which yielded among

other products, methyl-n-hexyl ketone, they believed the structure to be:

$$CH_3-(CH_2)_5-\overset{CH_2}{\underset{\diagdown}{\diagup}}CH-CH-CH=CH-(CH_2)_7-CO_2H$$

This does not seem reasonable because such a system would be expected to show an ultra-violet absorption maximum and Nunn (19) has showed that sterculic acid does not. Also, the infra-red spectra of the acid is unlike that of other cyclopropane conjugated systems. Infra-red absorption of freshly prepared samples of sterculic acid shows a maximum absorption at 9.92 microns (6) which appears to be characteristic of the cyclopropene ring. Dijkstra and Duin (4) reported that whenever the cyclopropene ring is altered by chemical reactions, the Halphen test is negative.

They believe there is a similar but not an identical acid in kapok seed oil. Its exact structure has not been elucidated. A similar fatty acid containing a cyclopropane ring has been found in the bacteria Lactobaccillus arabinosis and Lactobaccillus casii (10,11,12,13,14,15). It has been shown to have the structure:

$$CH_3-(CH_2)_x-\overset{CH_2}{\underset{\diagdown}{\diagup}}CH-CH-(CH_2)_y-CO_2H \qquad \text{where } x+y=14$$

The infra-red absorption of this acid exhibits a maximum at 9.8 microns which is characteristic of cyclopropane derivatives. It has been found to be isomeric with, but not identical to, the product obtained by partial hydrogenation of sterculic acid.

As first proposed by Lorenz and Almquist (16,17) and later studied by Shenstone and Vickery (27), the component in cottonseed responsible for the Halphen reaction and the one causing pink egg discoloration are either identical or very closely related. Recently Faure (5) has shown that pure sterculic acid is capable of causing the Halphen reaction.

Shenstone and Vickery proposed that the acid responsible contains a cyclopropane ring but they were unable to isolate it because of its instability. Fatty acids containing the cyclopropane ring are not known to be labile (10). Only under stringent conditions is the ring structure broken. Shenstone and Vickery (27) also stated that an infra-red absorption maximum was obtained at 1008 cm^{-1} (9.92 microns). This is not consistant with the cyclopropane structure. All monosubstituted cyclopropane derivatives on which absorption maxima have been determined accurately have their peaks at 9.79 ± 0.04 microns or slightly lower when more than one hydrogen has been displaced (29).

Recently, Gunstone (7) proposed that the structure responsible for the Halphen reaction in cottonseed oil is a fatty acid containing the cyclopropene ring, but he offers no evidence.

The purpose of this investigation is to determine the relationship between the Halphen reaction and pink egg discoloration.

EXPERIMENTAL

Isolation of Sterculia foetida Oil

Seeds from the tropical Java Olive tree, Sterculia foetida, were hulled and the kernels ground and extracted with Skellysolve B for eight hours in a soxhlet apparatus. The residue was reground and re-extracted to obtain a small additional yield. The extract was dried over magnesium sulfate and the solvent was evaporated by vacuum distillation. Yield was about 50 per cent of the kernel weight.

Preparation of Fatty Acids of Cottonseed Oil

Crude cottonseed oil was saponified by treating a 200 ml. sample of the oil with an equal volume of 10 per cent alcoholic potassium hydroxide. The mixture was kept at 40° C. for two days, then diluted with 400 ml. of water and extracted with 400 ml. ethyl ether three times to remove unsaponified fat and the unsaponifiable matter. The water solution was then acidified with HCl and re-extracted with ethyl ether. This latter ethyl ether extract was evaporated under water pump vacuum to concentrate the free fatty acids. The same procedure was used for Sterculia foetida oil.

Separation of Fatty Acids as Urea Adducts

Straight chain saturated hydrocarbons, fatty acids, and esters can be separated from the branched chain, and unsaturated ones by formation of slightly bound addition products with urea (26). Straight chain compounds form these adducts with relative ease, while unsaturated or branched chain compounds form adducts less readily or not at all. A sample

of fatty acids was added to an equal weight of methanol saturated with urea at the boiling point of the methanol solution. The solution was cooled to room temperature and the crystals collected on a Buchner funnel and washed twice with cool solution of methanol saturated with urea. Enough fresh urea was added to the filtrate to make a saturated solution at the boiling point (about one gram urea to three ml. methanol) and the solution was reheated to boiling and again cooled. Sometimes it was necessary to concentrate the filtrate rather than add urea in order to precipitate the branched acids. Each adduct fraction was freed from the urea by addition of acidified water, and the free fatty acids were separated by ethyl ether extraction. In the separation of crude sterculic acid for preparation of methyl sterculate, the third urea complex and the filtrate remaining after complex three were combined, diluted with acidified water and extracted with ethyl ether. The ethyl ether extract was dried over magnesium sulfate overnight and used directly in the diazomethane esterification process. For separation of the cottonseed oil fatty acids, each ether extract was concentrated under water pump vacuum.

Diazomethane Esterification Procedure

Diazomethane is one of the mildest esterifying agents known, and is preferred for use on labile or expensive acids where high yield and mild esterification conditions are essential (32). Its extreme toxicity and explosiveness require that adequate safeguards be maintained. All reactions should be carried on in a well ventilated hood, and a safety glass or screen should be placed in front of the operator. The intermediate, N-nitroso-N-methyl-p-toluenesulfonamide, used in the esterification was prepared according to the directions of DeBoer and Backer (3). The

esterification procedure was essentially that of Miramon (18). The esterification apparatus is shown in Fig. 1. About 5 to 15 grams of the dry sterculic acid obtained from the urea complex separation were dissolved in anhydrous ethyl ether (about 5 to 10 ml. per gram of acid) and placed in the reaction flask. The N-nitroso-N-methyl-p-toluenesulfonamide (about 30 per cent excess of theoretical) was dissolved in a minimum amount of ethyl ether and placed in the distilling flask. Several grams of benzoic acid were dissolved in 150 to 200 ml. of absolute methanol and placed in the gas absorption bottle. Potassium hydroxide in 1:1 methanol-water was put in the dropping funnel (1.5 grams KOH per gram fatty acid to be esterified). Both the reaction flask and the distilling flask were cooled to 0° C. with an ice-salt mixture and the system was then connected. The potassium hydroxide solution was then added to the N-nitroso-N-methyl-p-toluenesulfonamide slowly to keep the temperature low. When the addition was completed, the ice-salt bath was removed and replaced by a heating mantle. Heat was applied slowly until the ether began to distill over at a steady rate as evidenced by bubbles forming in the reaction flask. After about 15 minutes of bubbling, the magnetic stirrer was started and run during the remainder of the reaction. When essentially all the ether had distilled over, the joint between the reaction flask and the distilling flask was quickly disconnected and replaced by a plug in the reaction flask to prevent the solutions from sucking back due to condensation of the ether. The stirrer was allowed to run 15 to 30 minutes longer and then the ice-salt bath was removed. Stirring was continued for several minutes to rid the solution of any excess diazomethane. The esters were separated from any remaining fatty acids by

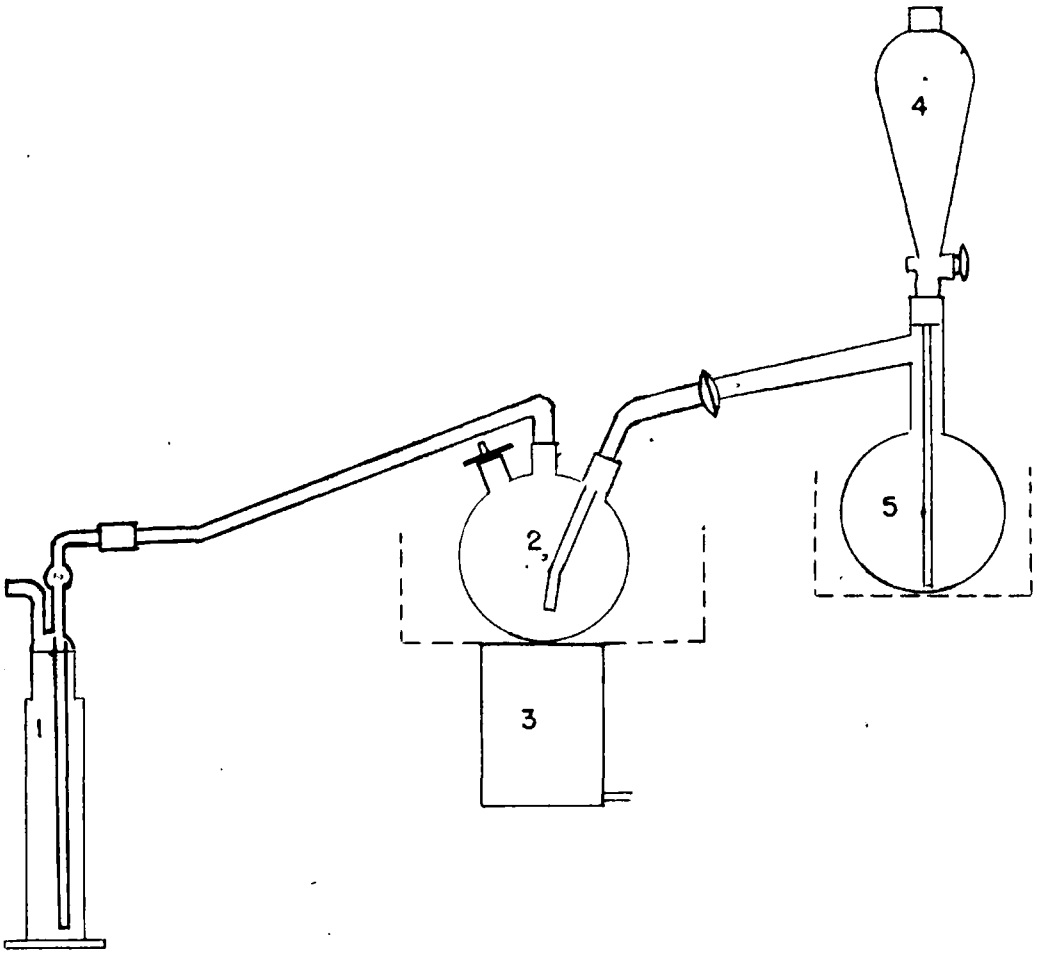

Fig. 1 Diazomethane Esterification Apparatus

1. Gas washing bottle
2. Reaction flask
3. Magnetic stirrer
4. Dropping funnel
5. Distilling flask

washing the ether solution with sodium bicarbonate solution. The ether solution was then dried over sodium sulfate and the ether evaporated under reduced pressure.

Hydrogenation Procedure

Preparation of Catalyst

The method of Busch and Stove (2) for preparation of the 2 per cent palladium on calcium carbonate catalyst was employed. Fifty grams of calcium carbonate, freshly prepared from a solution of calcium chloride by addition of a solution of sodium carbonate was heated, filtered, washed and resuspended in several hundred ml. of water. A solution containing 1 gram of palladium chloride in 10 ml. of water was added and the suspension was heated until all of the palladium was precipitated on the carbonate as indicated by the supernatant becoming clear. The water was then decanted, the precipitated product was washed with distilled water, filtered and rewashed until the filtrate was chloride free. Excessive washing with water was avoided because of the solubility of the palladium hydroxide. The washed product was dried in a desiccator and stored in a tight bottle.

Hydrogenation Apparatus

Two hydrogenation procedures were used. In one, a Parr Hydrogenation Apparatus* was employed with the use of pressure from 1 to 2 atmospheres. This was not too satisfactory as measurement of hydrogen uptake was not possible. For quantitative determinations the apparatus shown in Fig. 2 was found more useful.

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*Parr Instrument Co., Moline, Illinois

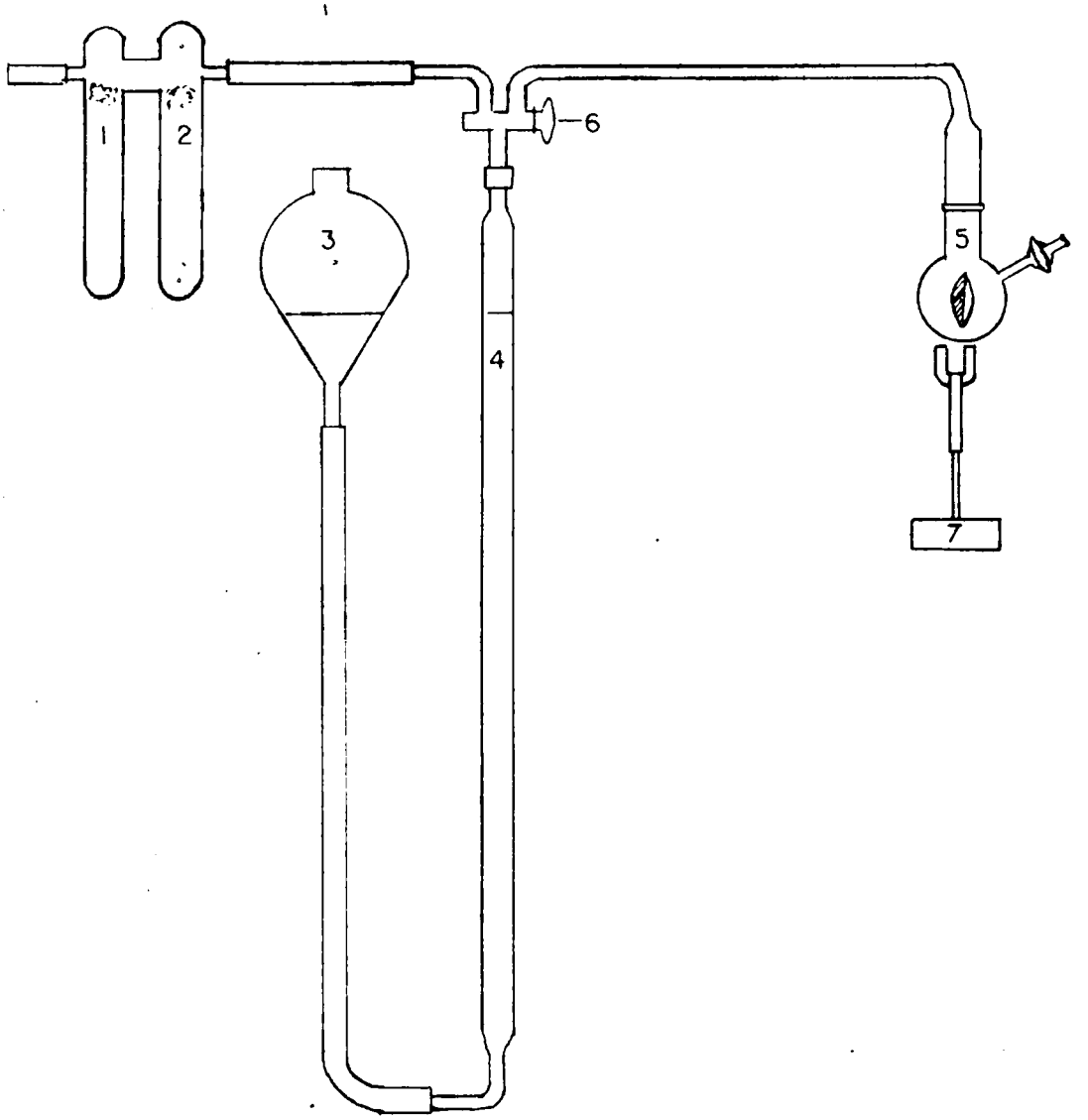

Fig. 2 Micro Hydrogenation Apparatus

1. Drierite tube
2. KOH tube
3. Balancing bulb
4. Graduated gas burette
5. Hydrogenation flask
6. Two way stopcock
7. Magnetic stirrer

In quantitative runs the catalyst was first reduced in ethanol, and the oil was added by medicine dropper. The weight of oil added was determined by difference. When the Parr apparatus was used, both sample and catalyst were added at the same time. About 10 ml. of solvent was used per gram of reactant. When using fats which were insoluble in cold ethanol, hexane or benzene was used as the solvent. Equal weights of palladium catalyst and fat or fatty acid were used. The reduced fat or fatty acids were separated from the catalyst by filtration and the solvent was evaporated by vacuum distillation.

Halphen Reaction on Egg Yolk Fat

The method of Lorenz and Almquist (16) for the extraction of egg yolk fat for the Halphen reaction was tried. The yolk was separated from the white and the broken yolk was allowed to stand in several volumes of ethyl ether for 24 hours. The ether extract was then decanted and the ether evaporated at room temperature. The fat residue was then redissolved in a solution of 5 ml. of one per cent sulfur in carbon disulfide and five ml. of isoamyl alcohol. The test tubes containing these mixtures were heated in a saturated salt solution on a hotplate slowly until the carbon disulfide and residual ether was boiled off, and then vigorously for at least an hour. This method proved to be unreliable since the Halphen reaction was occasionally negative for discolored eggs or for eggs that would eventually discolor. Several factors may have been responsible for this variation. The most critical variable appears to be the efficiency of extraction of the egg yolk fat. Several variations of the extraction procedure were tried in an effort to improve the reproducibility of the test. Hard boiling the eggs before the extraction appeared to give about the same results as using unboiled eggs. Hard boiling, followed by oven

drying and Soxhlet extraction of the yolk with Skellysolve B resulted in a solution containing so much dark pigmentation that it was difficult to observe production of a red color during the Halphen test. The most effective procedure was to mix about 20 grams of anhydrous sodium sulfate with the yolk when the ether was added. The mixture was kept at room temperature in stoppered 125 ml. erlenmeyer flasks for 24-36 hours. The extract was then filtered and washed with more ether. This modification gave more consistant results.

Infra-red Procedure

All infra-red data were taken on a Beckman model IR-2 Infra-Red Spectrophotometer with a rock salt prism. Spanning time for the 15 to 1 micron range was 43 minutes.

RESULTS

Halphen Reaction on Egg Yolk Fat

Two groups of four laying Leghorn hens each were used in the experiment. One group was fed a diet containing 6 per cent crude cottonseed oil, the other 6 per cent refined cottonseed oil*. Eggs were collected and stored after 5 days of feeding. The refined cottonseed oil was tested and found to give a weak Halphen reaction in 1:5 dilution. Halphen tests were run when the eggs were about 4 days old. The yolks were extracted by means of the improved procedure described previously. In an attempt to compare the results more precisely than visual observation would permit, the filtered solutions were diluted with isoamyl alcohol and the absorption at various wavelengths determined with a Beckman model DU Quartz Spectrophotometer. For ease in comparing spectra, actual values are multiplied by a proportionality constant so that all values in a particular set converge at one wavelength. Then the similarity or divergence of the values obtained for other wavelengths can be easily observed. This method was found to give results which confirmed visual observations and in addition were more specific and precise. The spectra of those samples which gave red coloration were characterized by a small decrease in absorption from 450 to 460 millimicrons and a rather sharp increase from 460 to 475 millimicrons followed by a medium decline from 475 to 490 millimicrons.

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*Wesson oil

Spectra of samples appearing yellow or only slightly orange showed a greater decline from 450 to 460 millimicrons and little or no increase from 460 to 475 millimicrons. The most characteristic difference however appeared to be the much greater decline from 475 to 490 millimicrons.

All Halphen reaction products from crude cottonseed oil yolks appeared red. Those on the refined cottonseed oil gave orange while the controls were yellow.

Individual corrected values are shown in Table 1. Comparison of the group averages is made in Fig. 3.

The Halphen Reaction of Cottonseed Oil Fatty Acids

The fatty acids of crude cottonseed oil, prepared by cold saponification were found to give a positive Halphen test. When the saturated fatty acids were separated from the unsaturated by urea complex formation, the unsaturated fatty acids were found to give a positive Halphen test, whereas the saturated did not. Mild hydrogenation (Parr apparatus) of the unsaturated acids destroyed the ability to give a positive Halphen reaction. No diminution in the intensity of the Halphen reaction after three months of standing at room temperature in a closed container was noted.

Esterification of the free fatty acids of cottonseed oil with methanol and sulfuric acid did not eliminate the Halphen test. Treatment of refined cottonseed oil with bromine destroyed the test completely.

Table 1

Comparison of Visible Spectra of Halphen Reaction on Egg Yolk Fat from Hens Fed Various Diets

Diet	Density Wave Length – Millimicrons					
	450	460	470	475	480	490
Crude C.S. oil	.500	.446	.471	.486	.467	.340
" " "	.500	.447	.464	.477	.460	.328
" " "	.500	.447	.469	.487	.467	.346
" " "	.500	.439	.449	.464	.442	.312
" " "	.500	.442	.462	.479	.460	.343
Refined C.S. oil	.500	.423	.423	.430	.398	.245
" " "	.500	.414	.414	.418	.387	.240
" " "	.500	.422	.423	.431	.398	.253
" " "	.500	.420	.419	.427	.397	.248
" " "	.500	.421	.421	.428	.395	.248
Control	.500	.419	.413	.420	.386	.225
"	.500	.408	.389	.395	.362	.222
"	.500	.413	.409	.413	.381	.226
"	.500	.415	.408	.417	.386	.218
"	.500	.419	.406	.414	.387	.228

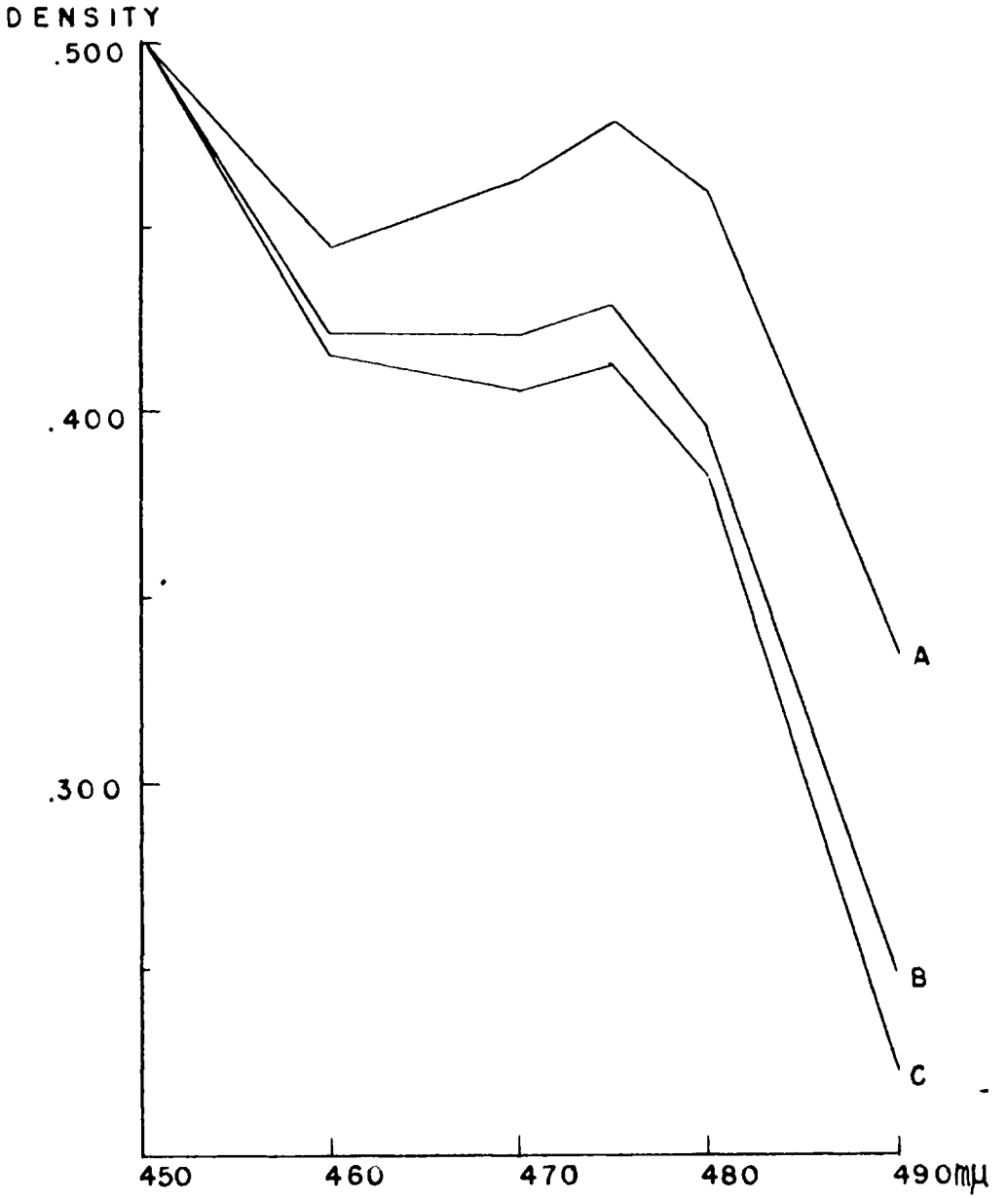

Fig. 3 Comparison of Halphen Reaction Color Products of Egg Yolk Fat from Hens on Various Diets (5 egg averages)
A. Hens fed diet containing 6 per cent crude cottonseed oil
B. Hens fed diet containing 6 per cent Wesson oil
C. Hens fed normal diet

The Halphen Reaction of *Sterculia foetida* Oil

The oil of the tropical Java Olive tree, *Sterculia foetida* was extracted and isolated as previously described. Infra-red spectra showed a strong absorption at 9.92 microns (Fig. 4), a frequency previously assigned to the cyclopropene ring (4). This oil gave a strongly positive Halphen reaction in dilutions of 1:60. The Halphen color produced appeared to be orange-red in contrast to the red obtained by Halphen reaction of cottonseed oil. The visible spectra results bore out this observation (Fig. 5,6). The absorption maxima with refined cottonseed oil was found to be at 500 millimicrons while with *Sterculia foetida* was at 490 millimicrons.

Cold saponification of the oil, followed by acidification and urea complex separation of the free fatty acids gave several fractions of which the last to crystallize consisted mainly of sterculic acid. The fatty acids in these fractions were converted to their methyl esters by the diazomethane procedure previously described. The methyl sterculate thus produced also gave a strongly positive Halphen test.

Nunn (19) has previously shown that hydrogenation under mild conditions will hydrogenate double bonds but will not cleave the cyclopropane ring structure. A sample of *Sterculia foetida* oil (.8168 grams) hydrogenated at room temperature with 0.8 grams of 2 per cent palladium on calcium carbonate catalyst absorbed 62.0 ml. of hydrogen (at 700 mm. and 25° C.). Comparison of the infra-red spectra of the hydrogenated product with the original sample is made in Fig. 4. The absorption band has shifted from 9.92 microns in the original oil to 9.8 microns in the hydrogenated product and the band has decreased about 50 per cent in intensity. Cyclopropane derivatives are reported to have absorption at

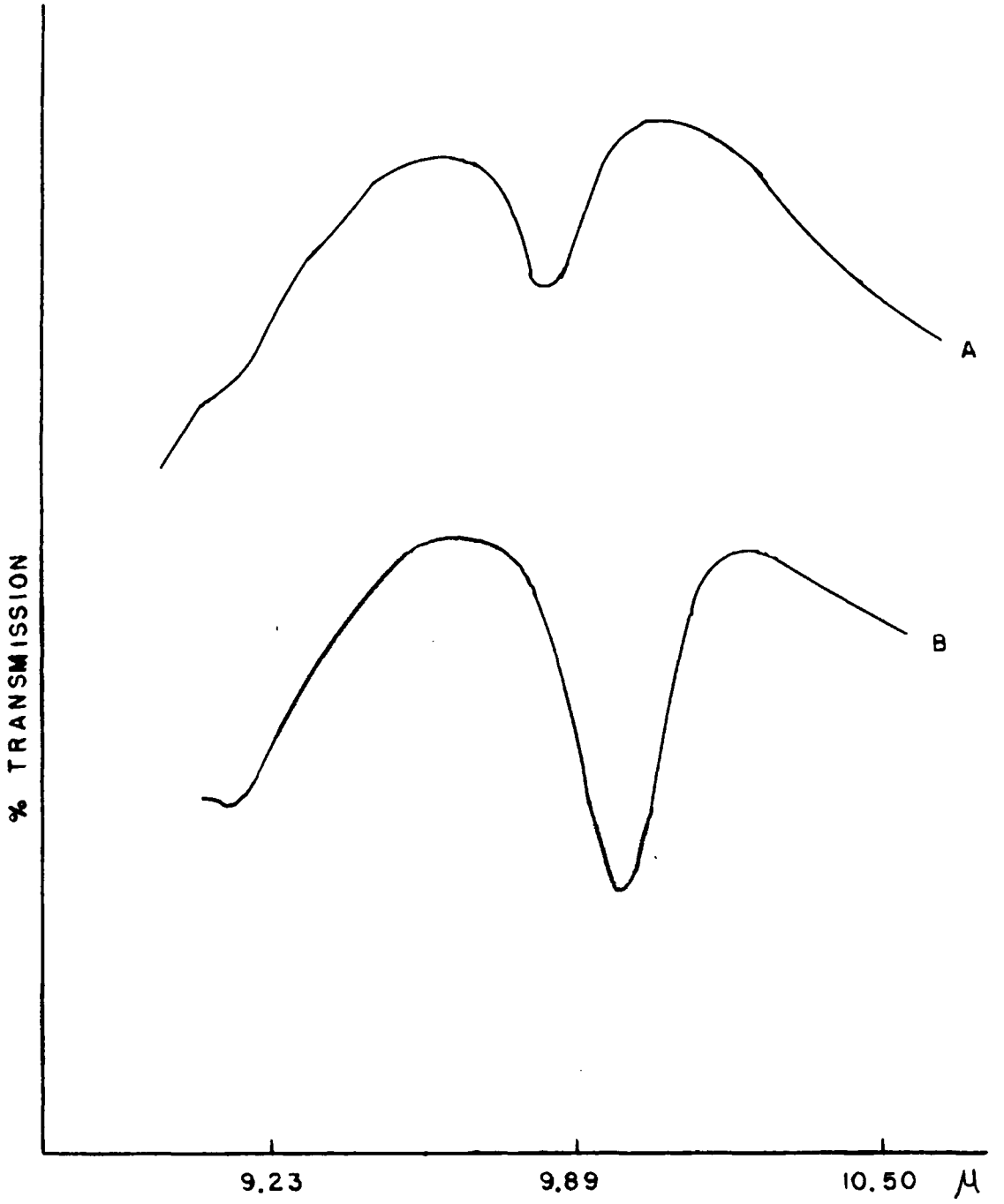

Fig. 4 Comparison of Infra-red Spectra of Crude and Hydrogenated Sterculia foetida oil
A. Hydrogenated (Pd on $CaCO_3$ in ethanol) oil
B. Crude oil

9.79± .04 microns (29). The hydrogenated product gave no evidence of Halphen reaction.

Faure has suggested (5) that only the carbon disulfide is active in the Halphen test, and its action is addition across the cyclopropene ring structure in sterculic acid. A sample of sterculic acid left in carbon disulfide for 5 hours turned orange-red. When the excess carbon disulfide was evaporated and the deep red residue taken up in isoamyl alcohol, the resulting spectra, shown in Fig. 5, had the same maximum (590 microns) as the Sterculia foetida oil when the Halphen reaction was run with sulfur in carbon disulfide and the tube heated. Samples of refined cottonseed oil let stand in carbon disulfide produced no color.

To evaluate the necessity of sulfur in the Halphen reaction samples were heated with carbon disulfide alone. Sterculia foetida oil gave only a very pale orange tinge, although its maximum absorption was the same as those treated normally.

Refined cottonseed oil however turned violet when heated with carbon disulfide alone. Comparison of the resultant spectra with that given in a normal Halphen test is shown in Fig. 6.

When heated with sulfur in benzene refined cottonseed oil did not give a color test. Sterculia foetida oil, run similarly, turned yellow upon standing.

Feeding of Cottonseed Oil Fractions

In order to determine the fraction of the cottonseed oil responsible for the pink discoloration, 6 groups of laying hens (3 to 5 hens per group) were fed the following fractions:

Group 1 hexane extracted unsaponifiable

Group 2 hexane extracted saponifiable, acidified

Group 3 ethyl ether extracted unsaponifiable

Group 4 filtrate from heating with 1 per cent alkali

Group 5 precipitate from heating with 1 per cent alkali

Group 6 crude cottonseed oil

All were fed at levels equal to 6 per cent crude cottonseed oil.

After four months of storage, all eggs in groups 2,3,4 and 6 showed typical pink discoloration, whereas none in groups 1 and 5 did.

To further identify the fraction, another trial was run in which the hexane extracted saponifiable fraction was re-extracted with ethyl ether. Only the hexane saponifiable extract caused pink discoloration. It also showed a strongly positive Halphen reaction.

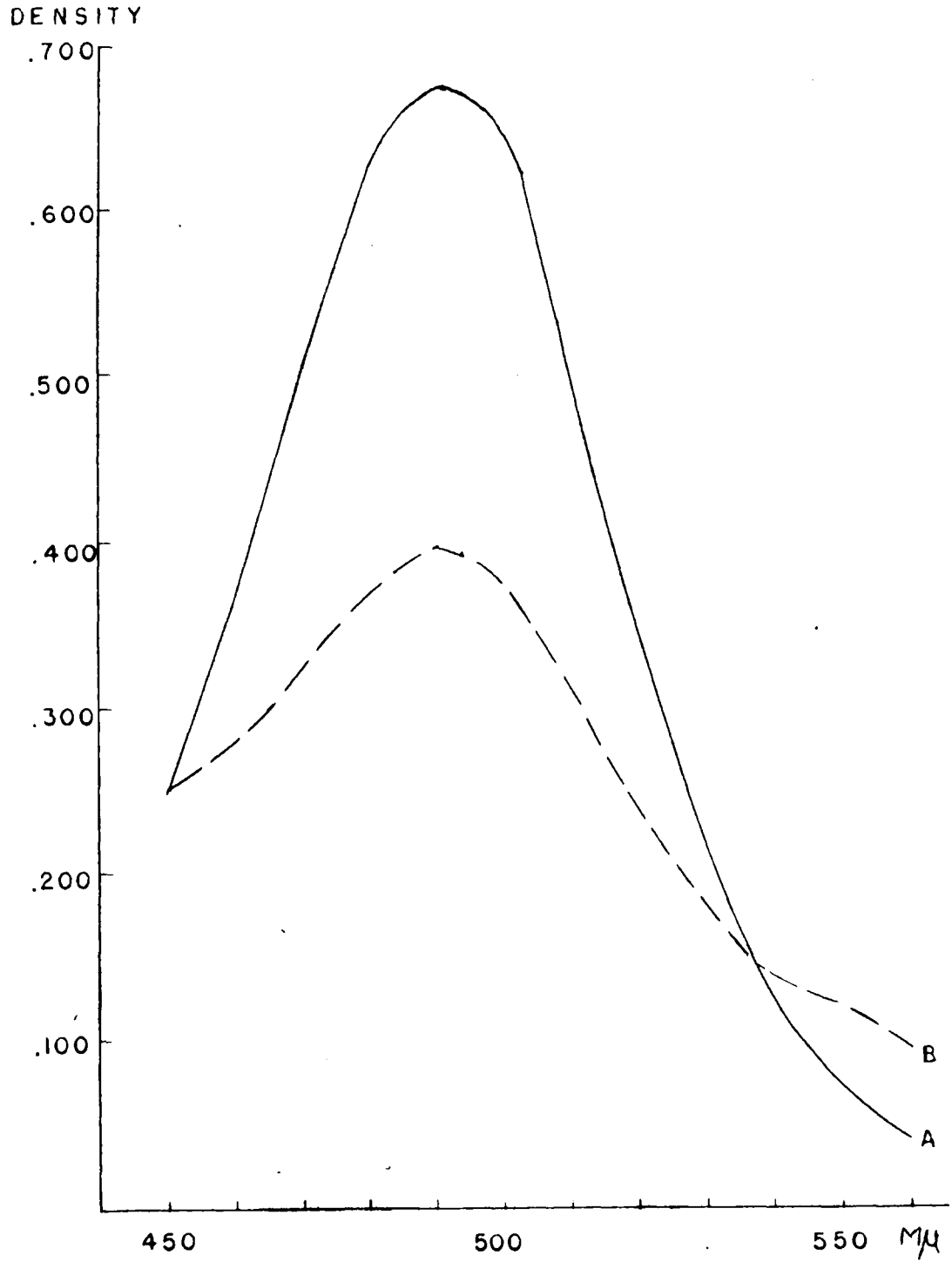

Fig. 5 Comparison of Halphen Reaction Color Products of Sterculia foetida oil in Isoamyl Alcohol

A. Crude sterculic acid dissolved in cold CS_2 (then CS_2 evaporated)

B. Sterculia foetida oil heated with S in CS_2

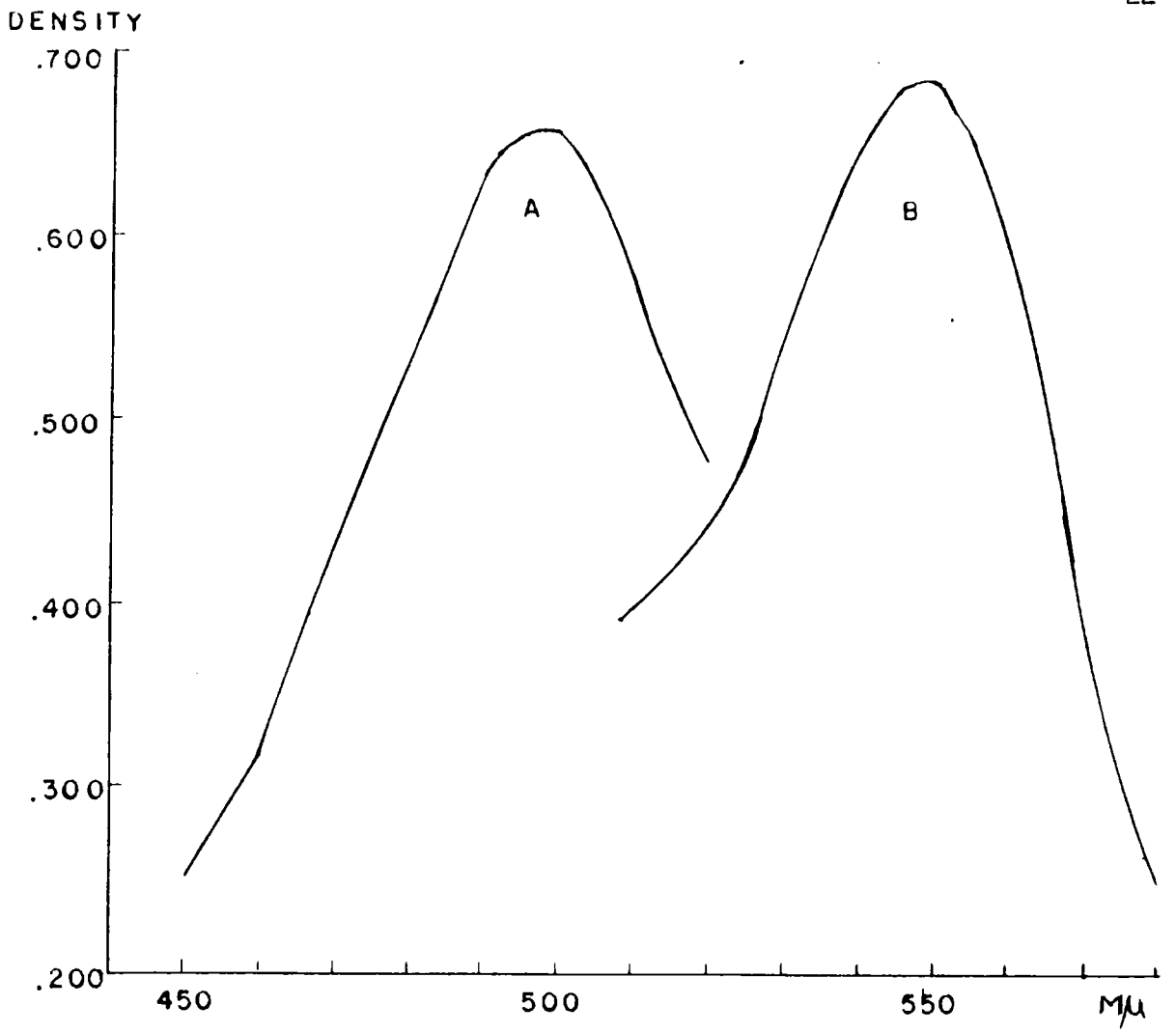

Fig. 6 Comparison of Halphen Reaction Color Products of Refined Cottonseed Oil with and without Sulfur
A. Refined cottonseed oil heated with sulfur in carbon disulfide
B. Refined cottonseed oil heated in carbon disulfide alone

DISCUSSION AND CONCLUSIONS

Additional evidence was found in hen feeding experiments to corroborate Shenstone and Vickery's (27) conclusion that a particular fatty acid was responsible for the Halphen reaction which also caused pink egg discoloration. These experiments showed that the component responsible for the discoloration was in the saponifiable fraction of the oil.

By use of an improved method of egg yolk fat extraction utilizing anhydrous sodium sulfate, consistent results were obtained for the Halphen reaction of egg yolk fat. Table 1 shows how closely the spectrophotometric values for the eggs from hens on a particular ration converged. The spectrophotometric estimation method developed is based on the premise that the ratio of yellow pigmentation to total fat is about the same for all eggs. When samples for the Halphen reaction are diluted the yellow and red components should dilute equally. If the values found at a wavelength where strong yellow absorption takes place (450 millimicrons) are multiplied by a factor to make them equal to some arbitrary value, and all other values found for the sample multiplied by the same factor, then the relative amount of red absorption can be estimated. Crude cottonseed oil gave consistantly higher values than did the refined oil when run under identical conditions, indicating the likelihood that some of the constituent responsible for the test is destroyed or modified during the refining process.

Confirmation of Faure's (5) observation that sterculic acid gives the Halphen reaction was obtained. Methyl sterculate was prepared by the reaction of the free acid with diazomethane and was also found to give a positive Halphen reaction. Methyl sterculate was found to be stable at room temperature. As already shown by other workers (4,6,29) Sterculia foetida oil has a prominent absorption band, due to a cyclopropene or conjugated cyclopropane ring, at 9.92 microns. When hydrogenated with palladium on calcium carbonate in ethanol, the band shifted to 9.80 microns, characteristic of the cyclopropane ring (10,11,12,14,29). The hydrogenated product does not give a Halphen reaction. Refined cottonseed oil reacts similarly to hydrogenation.

Some differences in the Halphen reaction of sterculic acid and that given by cottonseed oil were found. Sterculic acid and Sterculia foetida oil gave the Halphen reaction upon standing in carbon disulfide at room temperature. Refined cottonseed oil did not. This however may be explained on the basis of the wide difference in the concentration of the constituent causing the test. Some difference exists in the color produced by the heating of sulfur in carbon disulfide with Sterculia foetida oil and with refined cottonseed oil. The product of Sterculia foetida oil heated in carbon disulfide alone gives a weak test, but is orange-red in color. Refined cottonseed oil when treated in this manner gives a violet color. These differences seem to indicate that the sulfur acts in the role of a catalyst in the Halphen reaction with Sterculia foetida oil or with the free sterculic acid or its methyl ester, but that it may play a more important role in the reaction with cottonseed oil.

Further indication of the possibility of differences in the Halphen

reaction of Sterculia foetida oil and that of cottonseed oil is indicated by the rapid polymerization of the free fatty acids of Sterculia foetida oil (5) so that within a short time, the resultant oil gives no Halphen reaction. Free cottonseed oil fatty acids left stand for several months, were found to give a strong Halphen test. Nunn (19) has postulated that the polymerization reaction of sterculic acid is that of ester formation between the cyclopropene ring and the free carboxyl group. Evidence in favor of this mechanism is the increasing equivalent weight, the disappearance of the infra-red absorption band characteristic of the cyclopropene ring, and the hydrolysis product with the same equivalent weight as the original sterculic acid. Added evidence is the relative stability of the methyl and glyceryl esters which contain no free carboxyl grouping. If the same structure were present in cottonseed oil, it would be reasonable to expect a similar polymerization. Since the exact identity of the grouping in sterculic acid is still in doubt, the exact nature of the difference is impossible to predict.

The fatty acids responsible for pink egg discoloration by cottonseed oil, and by kapok seed oil have been postulated as 18 carbon acids. Sterculic acid contains 19 carbons, and typically, fatty acids with an odd carbon chain length have low intestinal absorption. It will be interesting to see if feeding of Sterculia foetida oil causes pink egg discoloration.

SUMMARY

The relationship between the component of cottonseed oil responsible for the Halphen reaction, and that causing pink egg discoloration has been explored further. Evidence supporting previous workers' conclusions that both the Halphen test and pink egg discoloration are caused by the same agent has been obtained. Feeding tests were used to determine which fraction of the crude oil contained the constituent causing the discoloration. In line with the findings of other workers the component is found to be a fatty acid, presumably one with a cyclopropene ring or some similar structure.

By means of an improved extraction procedure, a definite difference was found in the intensity of the Halphen reaction of yolk fat given by eggs from hens fed various rations. Those hens on rations containing crude cottonseed oil laid eggs that gave a more intense Halphen test than did eggs from hens fed the same level of refined cottonseed oil.

The exact nature of the Halphen reaction has been studied. Tests seem to indicate that a difference may exist in the Halphen reaction given by Sterculia foetida oil, and that from cottonseed oil. The mixed glyceryl esters of Sterculia foetida oil, free sterculic acid, and methyl sterculate have all been found to give a positive and apparently identical Halphen reaction. The Halphen reaction of refined cottonseed oil apparently differs depending whether or not sulfur is included in the test reagent.

Partial hydrogenation of either Sterculia foetida oil or refined cottonseed oil was found to eliminate the Halphen reaction. This change

was attended, in the case of the Sterculia foetida oil, with a shift in the infra-red absorption band from that indicating a cyclopropene ring (or a cyclopropane ring conjugated with a double bond) to that frequency assigned the cyclopropane ring structure.

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