gluten. Additional work is needed on storage stability, nutritional value, and processing costs.

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WORLD-WIDE RESEARCH

Studies on the Chemistry and Biological Effects of Cyclopropenoid Compounds

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"Pink white" and other disorders in eggs arise from the ingestion by hens of leaves and/or seeds of many plants in the order Malvales. The active principles are two cyclopropenoid acids, malvalic (C_{18}) and sterculic (C_{19}), which were isolated and characterized in the fraction giving a positive Halphen color test. When more than 2 mg. of these active acids are fed daily to hens, characteristic disorders develop in the eggs, and are probably caused by greatly increased permeability of membranes within the yolk. The cyclopropene ring structure is essential for biological activity. To aid further biological studies, methods are being investigated for the preparation of cyclopropenoids specifically labeled with C^{14} in the methylene group. Quantitative methods for the estimation of each acid by gas-liquid chromatography have been devised.

STUDIES on the cyclopropenoid compounds form part of the work of the Animal Products Section of the C.S.I.R.O. Division of Food Preservation. The section's work on eggs includes not only disorders induced by diets but also the changes occurring in the whites and yolks of normal eggs during storage. The division's Food Chemistry Section has given considerable help, particularly in those aspects concerned with the physical chemistry of egg proteins and lipids. The division's Plant Physiology Unit has also helped on some aspects of the synthesis of cyclopropenoid compounds in plant cells. This paper comprises a survey of C.S.I.R.O. work on these compounds but is not intended to be a comprehensive review of studies in this field.

Pink White Disorder in Eggs

It has been known for over 40 years that whites and yolks of hens' eggs will discolor during storage if the hens are fed diets containing crude cottonseed products. Sherwood (*34, 35*) showed that they gave rise to two different disorders—a bronze discoloration of the yolk induced by gossypol, and pink whites caused by an unknown factor. Lorenz and coworkers (*19*) showed that seeds of another plant in the family *Malvaceae*, *Malva parviflora*, also induced the pink white condition.

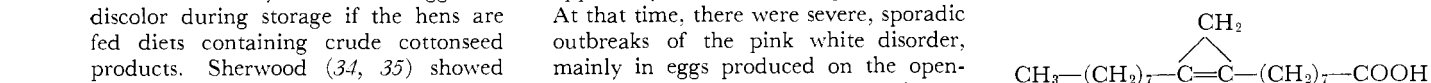
Lorenz (*18*) stated that only those oils giving a positive Halphen color reaction produced pink whites in eggs. The Halphen test (*11*) has been used for many years as an empirical method of detecting the adulteration of more expensive oils by cottonseed oil. This test involves adding to the oil one volume of carbon disulfide containing 1% sulfur, and one volume of pentanol. The carbon disulfide is distilled slowly from an open tube which is heated to 110° C. A pink or red color indicates a positive reaction.

Nature of Active Principle

About 12 years ago, a series of investigations was started on disorders in eggs apparently associated with poultry diets. At that time, there were severe, sporadic outbreaks of the pink white disorder, mainly in eggs produced on the open-range system, and, therefore, a study was commenced on the nature of the active principle causing pink whites.

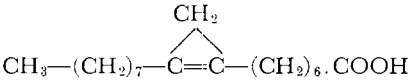
Guided by Lorenz's observation (*18*) on the association between the Halphen color test and the pink white disorder, the oils from three species of *Malvaceae* were examined—cottonseed (*Gossypium hirsutum*) and the leaves and seeds of two mallows, *Malva parviflora* and *M. verticillata*. In all five oils, a positive Halphen test was always associated with the unsaturated fatty acid fraction. Further fractionation to eliminate oleic, linoleic, and linolenic acids, by methods to be described later, gave an apparently pure substance, with a melting point of 10.3° C. and a molecular weight of 283, and a Halphen color reaction detectable at concentrations down to 10 p.p.m. When fed to laying hens at the rate of about 50 mg. per day, this Halphen-reacting acid produced the typical symptoms of the pink white disorder in stored eggs (*31*).

This biologically active fraction had some of the properties of sterculic acid, the C_{19} cyclopropenoid whose structure, given by Nunn (*25*), is as follows:



After Faure (*9*) reported that sterculic acid gave a positive Halphen test, as-

cribed to the presence of the cyclopropene ring, the authors showed that the predominating compound in their biologically active fraction was a homologous C_{18} cyclopropenoid to which they gave the trivial name malvalic acid (*27*). Malvalic acid has the following structure:



$$CH_3—(CH_2)_7—C\overset{CH_2}{=}C—(CH_2)_6.COOH$$

In oils from cottonseed and the mallow species, small amounts of sterculic acid were found, but malvalic acid always predominated in the cyclopropenoid acid fractions.

Occurrence of Cyclopropenoid Acids

Oils obtained from the seeds or some leaves of about 45 species in the plant order Malvales and one species in the order Ebenales have been reported to give a positive Halphen test. Since the two cyclopropenoid acids are the only naturally occurring compounds known to give a true Halphen reaction, these plant species are presumed to contain one or both acids.

The amounts of cyclopropenoid acids occurring in the oils of 15 species have been determined by nine groups of investigators using a variety of methods, including the Durbetaki hydrogen bromide titration, the Halphen color reaction, and gas-liquid chromatography of hydrogenated oils. By far the richest source is in the seed oil of the tropical tree, *Sterculia foetida*, where the cyclopropenoid acids constitute 50 to 70% of the total acids. It is one of the few oils so far examined where the sterculic acid content greatly predominates, the ratio of sterculic to malvalic acid being about 10:1. In the oils of most other species, malvalic acid usually comprises about 80% of the total cyclopropenoids.

For the determination of the amounts of malvalic and sterculic acids in the leaves and seeds of six plant species, a semiquantitative colorimetric method based on the Halphen reaction (*32*) was used. In crude cottonseed oils from various sources, some variability was found in the total cyclopropenoid acids and in the ratio of malvalic to sterculic acid. The totals varied from about 1.0 to 2.5% and the ratio from 3:1 to 5:1. Up to 30% of cyclopropenoids was found in the leaf oils of *Malva parviflora*, of which about two thirds was malvalic acid; corresponding figures for the seed oils of *Brachychiton* spp. were about 12% total, of which over 80% was malvalic acid.

The modified hydrogen bromide titration method recently described by Harris and coworkers (*12*) is to be preferred in assays for total cyclopropenoid acid content when samples exceeding several grams are available.

Isolation of Cyclopropenoid Acids

Both cyclopropenoid acids are extremely labile, undergoing rapid thermal polymerization, isomerization, and oxidation at room temperature. Procedures adopted in isolating these compounds are, therefore, selected to avoid exposure to elevated temperatures or to oxygen.

The oil from seeds of *Sterculia foetida*, where sterculic is the predominant acid, permits simpler isolation procedures than those which would be used with oils having a low concentration of cyclopropenoids—e.g., cottonseed. Nunn (*25*) obtained sterculic acid from *Sterculia foetida* oil by submitting the mixed fatty acids to fractional adduction with urea and removing the noncyclopropenoids in the first two crystalline adducts. Crystallization of the fatty acids isolated from the subsequent adducts in acetone at −50° C. gave the purified acid (m.p., 18.2° C.).

For oils having low cyclopropenoid contents, various combinations of the techniques of low temperature crystallization from solvents and urea adduction of the acids failed to isolate the cyclopropenoids, owing to losses incurred in unresolvable mixtures. This failure occurred not only because of the low concentration of cyclopropenoids, but also because the malvalic-sterculic acid ratio did not favor one or the other compound. Moreover, the other fatty acids in oils from seeds of cotton, *Malva* spp., and *Brachychiton* spp. contain a high proportion of oleic and linoleic acids; *Malva* leaf oil, in addition, contains substantial amounts of linolenic acid. Other unsuccessful methods of isolation included vacuum distillation of methyl esters, use of molecular sieves, and those based on surface-active properties.

A successful method for isolation was developed by using low temperature crystallization of the mixed acids to remove saturated acids and a portion of the oleic acid, followed by reversed-phase, liquid-liquid partition column chromatography (*13*). This chromatographic technique resolved malvalic–linoleic and sterculic–oleic acids into two distinct fractions. After this critical separation, the cyclopropenoids could be obtained in sufficient concentrations, sometimes after re-application to the columns, to allow malvalic or sterculic acid to be crystallized from solvent solution and purified.

The oils used for separation were extracted from seeds or dried leaf with petroleum solvent (b.p. 60° to 70° C.) and the acids were obtained after saponification for 2.5 hours at 40° C. in an ethanol solution of KOH having a concentration just sufficient for complete reaction. The use of acetone to extract cottonseed subsequently gave acid mixtures which could not be resolved.

The total fatty acids were separated from unsaponifiable material and then crystallized from petroleum solvent using a concentration and a temperature suitable to the composition of the acid mixture. It was not possible to obtain a concentration of more than about 50% cyclopropenoids in the filtrate acids. Efforts to increase this value by crystallizing more acids (higher solute concentration and lower crystallizing temperature) resulted in excessive losses of cyclopropenoids from the filtrates.

Cottonseed acids provide an example of the special case where the original cyclopropenoid concentration is very low (1 to 2%), and conditions are chosen to remove portions of the oleic acid and also some linoleic acid to achieve the required concentration of cyclopropenoids. The mixed acids were dissolved in petroleum solvent at a concentration of 6.5 grams per 100 ml. and crystallized at −75° C. The filtrate obtained after separation from the crystals on a glass filter maintained at −75° C. contained a high proportion of the original cyclopropenoid acids at a concentration of about 20%. Further recovery of the cyclopropenoids from the crystals was obtained by recrystallization.

Brachychiton acerifolium and *B. populneum* oils yielded acid mixtures with a concentration of 10 to 12% cyclopropenoids with oleic and linoleic as the other unsaturated acids. These mixtures were crystallized from solutions of 10 grams per 100 ml. of petroleum solvent at −25° to −30° C. At lower crystallization temperatures, excessive amounts of cyclopropenoids were separated with the crystals.

Malva leaf-oil acids, which contained up to 30% cyclopropenoids, were crystallized at a concentration of 10 grams per 100 ml. of petroleum solvent at a temperature of −60° C. The presence of linolenic acid in these mixed acids increases the solubility of all other acids, and these low-temperature conditions are needed to produce concentrates of cyclopropenoids.

Some modifications were made to the original methods of Howard and Martin (*13*) for the preparation and running of the reversed-phase partition columns. The vapor-phase transfer of dichlorodimethyl silane for the deposition of a silicone surface layer on the diatomaceous earth column support was unsatisfactory. Stable columns were made by mixing 1 kg. of Hyflo Super-Cel with 2.5 liters of a 5% solution of Dow-Corning 200-type silicone oil (350 or 500 centistokes) in petroleum solvent. The solvent was evaporated, and the mixture was heated at 300° C. for one hour. Dichlorodimethyl silane (10% solution) may be used instead of silicone oil, but the mixture with Super-Cel was heated to 120° C. for one hour. The stationary phase, paraffin oil (Shell Ondina 17), was incorporated by stirring the oil with the

support in the ratio 1 to 1.12 v./w. The mobile phase used was 85% methanol in water, equilibrated with paraffin oil.

The glass columns were packed by suspending the oil-treated support in the mobile phase and tamping with a perforated metal plunger to produce an evenly packed column. Alternatively, it was equally effective to sieve the oil-treated support into the columns and to add the mobile phase subsequently. The columns were kept at a constant temperature of 30° C. during chromatographic runs.

For separation of quantities of acids up to 100 mg., columns 2 cm. in diameter and 80 to 114 cm. long were used. The eluate was collected in measured fractions (approximately 10 ml.), and each was titrated with alkali in order to follow the elution of the acids. Larger quantities, up to 5 grams, were separated on a column 10 cm. in diameter and 140 cm. long. With the larger columns, 26-ml. fractions were collected, and every tenth tube was titrated. The 2-cm. diameter columns were operated at a methanol flow rate of 75 to 90 ml. per hour over a period of about 6 hours, and the 10-cm. diameter column was run for about 50 hours at a flow rate of 350 to 450 ml. per hour.

Individual tubes were grouped into fractions, from which the dissolved paraffin oil was removed by making the solutions alkaline and extracting with diethyl ether. The fatty acids in each fraction were extracted with diethyl ether after acidification. These acids were submitted to crystallization from solvent to yield pure acids, or were reapplied to chromatographic columns to gain a higher degree of separation in more difficult mixtures, before crystallization was used.

An example of the chromatographic separation of the cyclopropenoids from cottonseed oil is shown in Figure 1, which illustrates the initial separation with a "load" of 5 grams of the filtrate acids placed on a column 10 cm. in diameter. Malvalic acid (fraction III) occurs in the tail of the largest peak, linoleic acid; and sterculic acid (fraction V) in the tail of the oleic acid peak.

To obtain malvalic acid, the acids from fraction III were again subjected to reversed-phase chromatography on a column 2 cm. in diameter, a typical run being shown in Figure 2. The acids in the major peak (hatched area, Figure 2) were crystallized five times from acetone solutions at −30° C., and malvalic acid of constant melting point (10.3° C.) was obtained.

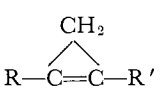
A similar procedure was adopted for the isolation of sterculic acid from fraction V (Figure 1).

In oils from *Malva* species, there is evidence of the occurrence of a shorter chain cyclopropenoid acid (possibly C_{16} or C_{17}) which, so far, has not been isolated in sufficient amounts for characterization.

Characterization of Cyclopropenoid Acids

The structural constitution has been clearly established by many workers for sterculic acid (*2, 10, 25*) and malvalic acid (*3, 21*). The infrared spectra of these acids show a strong band at 1008 cm.$^{-1}$ and a weaker band at 1870 cm.$^{-1}$ attributed to vibrations of the cyclopropene ring, which is their unique structural feature.

The preparations of derivatives of these acids (*24, 33*) have indicated that the molecular configuration essential for a positive Halphen test is a substituted cyclopropene, containing the cyclopropenylene radical, such as:



The cyclopropanoid derivative of malvalic acid, dihydromalvalic acid, for instance, gives a negative Halphen test. Masson (*22*) has shown that, when heated above 200° C., methyl sterculate undergoes isomerization and fails to give a Halphen color test.

Biological Effects

Pink White Disorder. Shenstone and Vickery (*31*) working with malvalic acid and Masson and coworkers (*23*) with sterculic acid, were the first to show that ingestion of cyclopropenoid acids, fed to hens at the rate of about 20 to 25 mg. per day, caused the onset of pink whites in stored eggs. At this feeding rate, about 25% of the cyclopropenoid compounds are deposited in the egg yolks (*33*).

The work of Kemmerer and coworkers (*15*) shows that the minimum daily dose of cyclopropenoid acids necessary to produce the disorder is about 1.5 to 2 mg. It is of some interest to examine the effects of a hen's ration containing 10% cottonseed meal which may contain 3.5% residual oil. If the oil contains about 1.5% cyclopropenoids and if the hen consumes 11 grams of the meal per day, then the daily intake of cyclopropenoid compounds will be about 5 mg. This amount is ample for the production of typical disorders in the eggs.

The minimum duration of feeding needed to produce the disorder varies with the daily dosage, but it can be as low as four days (*18*).

When feeding of the cyclopropenoids ceases, they continue to be deposited in the yolks for a period of up to 10 days, presumably by mobilization from the adipose tissues of the body (*33*).

While substantial amounts of the cyclopropenoids may accumulate in the yolk lipids a few days after the start of feeding, the symptoms in the eggs are

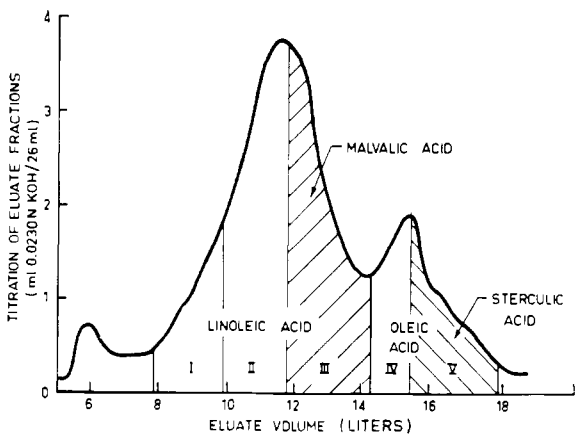


Figure 1. Liquid-liquid partition chromatogram of fatty acids from cottonseed oil on a reversed-phase column

Five grams of the filtrate from low temperature crystallization of the total oil acids applied to a 10- × 140-cm. column and run in 85% methanol

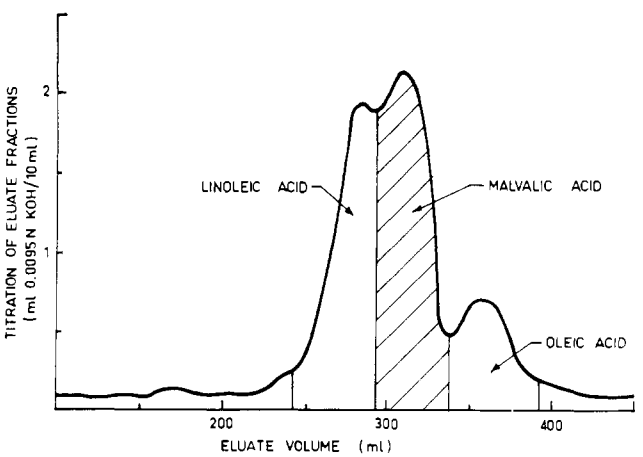


Figure 2. Liquid-liquid partition chromatogram of fatty acids from cottonseed oil after repeat chromatography on a reversed-phase column

Five-hundredths gram of fraction III (malvalic acid cut from the separation shown in Figure 1) applied to a 2- × 88-cm. column

first apparent only after a period of storage whose duration depends upon the temperature. At 20° C., for instance, a faint pink color in the whites appeared after about 8 to 10 days and at 1° C. after 8 weeks. Other symptoms may appear still earlier.

While the most obvious effect on eggs owing to ingestion of cyclopropenoid compounds by the hen is the onset of pink whites, this is only one of several effects, some of which will be briefly described.

pH Changes. In normal eggs, the pH values of the yolk and white after storage for a few days are about 6.4 and 9.0, respectively. In affected eggs, however, the pH values converge rather rapidly, approaching about 7.4 in the yolks and 7.6 in the whites (*33*).

Water Transfer. A considerable transfer of water from the white to the yolk takes place. In normal yolks, the water content remains about 50%, but in eggs from hens fed with cyclopropenoid compounds, it may rise to 57% (*33*).

Protein Transfer. There was a considerable migration of proteins of the white to the yolk. Evans and coworkers (*7*) have confirmed this, but they have shown that this migration of proteins is limited to ovalbumin, conalbumin, and lyzozyme. They found, too, that the cyclopropenoids induced a migration of the protein livetin from yolk to white.

Nonprotein Nitrogen Transfer. The cyclopropenoids also cause a major transfer of nonprotein nitrogen compounds—chiefly amino acids—from the yolk to the white. For instance, the glutamic acid concentration was eight times greater in egg whites from hens fed on both cyclopropenoid acids than in whites from hens fed the control ration (*33*).

Texture of Yolks. The onset of a pasty condition in the yolks of eggs containing cyclopropenoid compounds, and held at temperatures below about 10° C., is another striking effect. The work of Evans and his colleagues (*4, 6, 8*) has demonstrated that the ingestion of the cyclopropenoid acids causes changes in the hen's metabolism of fatty acids which give rise to increased proportions of stearic acid and reduced proportions of oleic acid in the lipids of the yolk and the body organs. Therefore, the pasty texture in affected yolks is probably due to synthesis of higher melting-point glycerides.

Effects on Egg Production. The authors found (*33*) that a daily dose of 250 mg. of sterculic acid fed to laying hens quickly caused cessation of laying, but that 25 mg. per day had no effect. Schneider and coworkers (*28*), who fed about 120 mg. of mixed cyclopropenoid acids from *Sterculia foetida* oil daily to pullets, found considerable retardation of ovary and oviduct development.

Hatchability. As has been recognized for many years, the ingestion of cottonseed products by hens may inhibit hatchability. Schneider and coworkers (*29*), who fed the oil of *Sterculia foetida* seeds to hens, found that when the daily dose exceeded 10 mg. there was an increasingly severe effect on hatchability. When the dose reached 50 mg. (about 30 mg. cyclopropenoids), 97% of the embryos were dead at the 22nd day of incubation. Increasing doses resulted in 100% mortality at decreasing times of incubation. Experiments conducted by McDonald and Shenstone (*20*) using pure sterculic acid gave similar results. The fertility of mice, however, was not affected by daily doses of sterculic acid as high as 200 mg. per kg. of body weight (*1*). Sheehan and Vavich (*30*) have recently found, however, that the feeding of *Sterculia foetida* oil to female rats decreased the frequency of estrous cycles.

Mechanism of Action

Schaible and Bandemer (*27*) showed that pink whites are caused by iron diffusing from the yolk and chelating with conalbumin in the white. The authors found (*33*) that ingestion of cyclopropenoids caused the iron concentration in the white to rise as high as 7 μg. per gram as compared with only 0.2 μg. in the control whites.

Schaible and Bandemer suggested that the active principle causing the pink-white disorder had a specific effect in increasing the permeability of the vitelline membrane, and that this increased permeability could account for other symptoms such as uptake of water by the yolk and convergence of the pH values of the white and yolk. The cyclopropenoid acids appear to have strong surface-active properties (*31*).

While one cannot exclude a specific action on the permeability of the vitelline membrane, it seems more likely that the cyclopropenoids exert their effects through changes in the microstructures in egg yolk and at boundary layers within the yolk. The authors have been unable, for instance, to demonstrate any combination of cyclopropenoids with the constituents of the vitelline membrane, whereas, of course, they are combined in measurable quantities in the yolk lipids.

The mode of action of the cyclopropenoids in increasing membrane permeability is unknown as is the mechanism by which they increase the deposition of stearic acid at the expense of oleic acid. Recently, however, Kircher (*17*) prepared stable compounds of methyl mercaptan and β-mercaptopropionic acid with cyclopropenoids where the sulfhydryl added to the double bond of the cyclopropene ring. Kircher suggested that a possible mechanism to account for the diverse biological effects is the reaction of the cyclopropene ring with sulfhydryl groups in physiologically active proteins.

The cyclopropenoids exert their biological effects in very low concentrations. Rather less than one part in 3000 of yolk lipids is needed in the egg. This suggests that they are active at highly specific structural sites and in specific enzyme systems.

In studying the inactivation of cyclopropenoid compounds, the authors found (*33*) that hydrogenation of malvalic and sterculic acids to dihydromalvalic and dihydrosterculic acids destroyed the biological activity of the cyclopropenoids. Polymerization of sterculic acid by heating (*24*) and substitution of one hydrogen attached to the cyclopropene ring (*16*) also had similar effects.

Generally, chemical changes which result in a negative Halphen test abolish biological activity. One exception was reported by Evans and coworkers (*5*) who heated crude cottonseed oil for one hour at 200° C. to give a negative Halphen test without destroying the ability of the oil to induce pink white disorder.

Some Current Work

Reliable methods of estimating small amounts of each cyclopropenoid acid are needed in biochemical studies, and this can best be done by gas-liquid chromatography. Since destruction of the cyclopropenoid ring occurs during gas-liquid chromatography, it is necessary first to hydrogenate the methyl esters to the cyclopropanoid compounds. Hydrogenation, however, causes some ring opening, and depending on conditions used, variable amounts of straight-chain derivatives and branched-chain isomers are produced. This is important because stearate produced from malvalate is admixed with stearate present or derived from unsaturated straight-chain C_{18} esters in the original oil. A study has, therefore, been made of methods of hydrogenation which will minimize formation of the straight-chain derivative (*14*). Hydrogenation of the methyl esters with palladium catalyst (10 to 1 w./w.) in methanol at room temperature and atmospheric pressure for 30 minutes gave negligible amounts of straight-chain compounds. For example, when pure methyl sterculate was hydrogenated with 10% palladium on calcium carbonate in methanol, the products were 79% dihydrosterculate, 2% C_{19}, and 19% branched C_{19}. The corresponding figures for platinum oxide in methanol were 70% dihydrosterculate, 22% C_{19}, and 8% branched C_{19}.

The authors are endeavoring, biologically, to label the cyclopropenoid acids with C^{14} in the methylene group (*26*). This work has the dual purpose of giving information on the mechanism of biosynthesis of the cyclopropenoids and providing labeled acids for the biochemical studies on the mechanism of their biological activity.

Incubation of slices of immature *Malva parviflora* seeds with C^{14}-labeled one-carbon donors, such as methionine, formate, acetate, and malonate, leads mainly to the labeling of dihydrosterculic acid and only minor amounts of labeled dihydromalvalic, malvalic, or sterculic acid, despite the fact that in mature seeds malvalic acid greatly predominates. The methyl group of methionine was the most efficient source of the ring-methylene carbon. Whether dihydrosterculate is an intermediate in the synthesis of cyclopropenoid acids is not yet known.

In view of the disappointing yields of biologically labeled cyclopropenoid acids, an attempt is being made to obtain labeled acids by chemical synthesis via stearolic acid and labeled methylene iodide.

There are a number of interesting matters on which further knowledge is urgently needed. These include: the mechanism of synthesis of cyclopropenoids in plant cells, whether the compounds are deposited unchanged in the lipids of eggs and body organs, the molecular species into which they are incorporated, the mechanism of their action on cell membranes, the way in which they react with enzymes or coenzymes or substrates to change fatty acid metabolism, and why malvalic acid appears to have only about one half the biological activity of sterculic acid.

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WORLD-WIDE RESEARCH

Proteolytic Activity of Crystalline Rennin and Caseins Associations

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THE CLOTTING of milk by rennin is one of the key steps in cheese making. The cow does not always give the same milk, and the variations which are observed in milk composition will, in turn, affect the properties of the coagulum formed when rennet is added to milk.

These properties depend to a large extent upon the mineral substances present in the milk, particularly the calcium in its various forms (ionized, etc.). The proteins themselves do play a role which, very likely, is far from negligible. This is especially true when one considers the varieties of cooked cheese—such as Swiss cheese—in which the water content is relatively low. In these varieties, the physical properties of the curd during the cheese-making process and those of the cheese during the curing process are important because of their influence on the quality of the finished cheese.

The present cheese-making techniques and the use of automation show, better than past methods, the interest in and the need for a better knowledge of the asso-