Compounds Producing the Kreis Color Reaction With Particular Reference to Oxidized Milk Fat[1]

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MUCH of the literature on fats and oils states, without reservation, that the compounds responsible for the Kreis color reaction (7) of oxidized fats are epihydrin aldehyde or its acetals. Most of these statements are based on the findings of Powick (12), who made a rather thorough study of the Kreis reaction. That epihydrin aldehyde or its acetals are responsible for the positive Kreis test of oxidized fat may be open to question for a number of reasons. Epihydrin aldehyde as such is known only as a very volatile, unstable compound, melting considerably below 0°C.(14). Its acetal derivatives which have been prepared (12, 14, 15, 16) have not been subjected to rigorous proof of structure. Moreover it has been assumed that hydrolysis of these acetals gives rise to free epihydrin aldehyde, which may or may not be the case. In order to satisfy thoroughly the theories concerning epihydrin aldehyde, it would seem desirable to isolate the pure compound, or a derivative thereof, from oxidized fat. This does not appear to have been done.

The impetus for the present study was the preparation of 2,4-dinitrophenylhydrazones from oxidized milk fat by Keeney and Doan (5). These workers isolated a derivative from which the formula $C_3H_{4\text{-}6}O_2$ was indicated for the parent compound. One of the oxygens was shown to be non-carbonyl, and theoretical considerations suggested the derivative to be that from β-hydroxy propanal. The similarity of this compound to epihydrin aldehyde led to a re-examination of the role of oxygenated three-carbon compounds in the Kreis test.

Results from this investigation have indicated that the basic three-carbon compound which is responsible for the Kreis color from oxidized milk fat is malonic dialdehyde. In Powick's investigation malonic dialdehyde was eliminated as a possibility in the Kreis test by somewhat indirect means. Powick noted that reaction mixtures of acrolein and H_2O_2 give an intense red color when treated with the Kreis reagents, phloroglucinol and concentrated HCl. By photometric study he established that the color from this system is identical with that obtained in the Kreis test of oxidized fat. The literature (1, 2) points out that malonic dialdehyde in aqueous solution gives an intense red coloration with ferric chloride. Powick was unable to observe a ferric chloride reaction in his acrolein-H_2O_2 systems and therefore discarded malonic dialdehyde as a possibility. It is interesting to note that Powick's study did not include submission of malonic dialdehyde to the Kreis test or examination of oxidized fat fractions with regard to the ferric chloride test. The present study has revealed that malonic dialdehyde does give a Kreis test and that the spectral characteristics of the color are identical with that obtained in the Kreis test of oxidized milk fat. Moreover it has been shown that certain fractions isolated from oxidized milk fat give a ferric chloride color (red) which closely resembles the color given by malonic dialdehyde in this test. Reaction mixtures of acrolein and H_2O_2 have been submitted to the ferric chloride test and the consistent formation of a red-brown color noted. Finally the chemical properties of certain Kreis positive fractions from oxidized milk fat were observed to be at variance with the expected properties of epihydrin aldehyde but closely consistent with those of malonic dialdehyde.

Experimental

Compounds giving a positive Kreis test. Further to elucidate the nature of the Kreis color reaction of oxidized fats a rather large number of compounds and reaction mixtures were submitted to the test. For the most part these were in addition to those previously investigated (12). The test was conducted as follows: To 5-10 mg. of the compound or mixture in a 3-inch test tube were added with agitation 1 ml. of concentrated HCl and then 1 ml. of saturated ethereal phloroglucinol. Since oxidized fat gives a typical red color rather quickly, tests were considered positive when a red color appeared in the HCl layer within 15 minutes of performance.

The following compounds or reaction mixtures were submitted to the test: lactic, pyruvic, propionic, acrylic, β-methoxyacrylic (prepared as by Owen and Somade [11]), β-hydroxybutyric, and levulinic acids, diacetyl, acetol, maltol, glyoxal, methyl glyoxal, crotonaldehyde, malonic dialdehyde (10% aqueous solution prepared[3] from the diacetal as directed by Rehm [13]), malonic dialdehyde diacetal (Union Carbide and Carbon Corporation), diethoxytetrahydrofuran (Union Carbide and Carbon Corporation), diethyl malonate, diacetone alcohol, acetyl acetone, acetonyl acetone, aldol, epichlorohydrin, glycidol (epihydrin alcohol) and its oxidation (H_2O_2) products, trimethylene glycol and its oxidation (both by H_2O_2 and $KMnO_4$) products, acrolein and its oxidation (H_2O_2) products, β-hydroxy propanol (prepared as by Nef [9]), and lactic aldehyde (prepared in a manner similar to that of Goto [3]).

Reactions resulting in various colors, some appearing immediately and others after a considerable period of time, were noted in the case of many of the compounds. However rapid appearance of the characteristic red color was limited to the following compounds and reaction mixtures: malonic dialdehyde, malonic dialdehyde diacetal, the oxidation (either by H_2O_2 or $KMnO_4$) products of trimethylene glycol, and the oxidation (H_2O_2) products of acrolein. The fact that malonic dialdehyde gave a positive test would explain adequately positive results with its diacetal and with oxidized trimethylene glycol. The diacetal would hydrolyze readily under the acid conditions of the test to yield the free dialdehyde. Oxidation of trimethylene glycol also should produce small quantities of malonic dialdehyde. The positive results with the acrolein-H_2O_2 system has not been resolved entirely

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[3] Essentially, this consisted of refluxing the diacetal with water in the presence of a trace of mineral acid.

in view of Powick's conclusions. However acrolein-H_2O_2 systems have been observed repeatedly to give positive ferric chloride tests. The color varies from brown to reddish brown and indeed is not unlike the color given with malonic dialdehyde in this test. Thus it is conceivable that even the positive Kreis tests which result with acrolein-H_2O_2 systems are attributable to malonic dialdehyde.

Spectral characteristic of the Kreis color from oxidized milk fat and from malonic dialdehyde. The significance of malonic dialdehyde, as a compound related to oxidized fat with regard to the Kreis test, would depend in a large measure on similarity of the colors obtained with the two materials in the test. Accordingly absorption spectra of Kreis colors from malonic dialdehyde and oxidized milk fat were determined with the aid of a Beckman model DU spectrophotometer. The sample of dry milk fat had been aged in the laboratory for 6 months in a stoppered clear glass bottle. Kreis color was developed by adding to 1 g. of the melted fat in a test tube, 2 ml. of concentrated HCl, mixing thoroughly and then adding 2 ml. of saturated ethereal phloroglucinol. The mixture was agitated periodically throughout a 5-minute holding period after which 10 ml. of water were added to give a suitable color density. The contents of the tube were mixed, the aqueous layer containing the color, removed and rendered as clear as possible by centrifuging.

The malonic dialdehyde sample was prepared in the same manner; use of a dilute solution of the compound (approximately 50 p.p.m.) was necessary in order to give color density roughly equivalent to that from the fat sample. Absorption curves for the two colors are presented in Figure 1. The somewhat greater basic level of absorption in the color spectrum from the fat sample was no doubt due to slight turbidity which could be plainly observed. Even so, the great similarity between the two spectra is quite evident, with shoulders in the vicinity of 480-500 mμ and maxima at 543 mμ. These curves also bear a close resemblance to those obtained by Powick in a similar manner from acrolein-H_2O_2, epihydrin aldehyde diethylacetal and rancid lard.

Observations on Kreis positive fractions from oxidized milk fat. Various fractions from oxidized milk fat, which had been prepared by Keeney and Doan were subjected to the Kreis and ferric chloride tests. The details of fractionation and some other properties of the fractions have been reported elsewhere (5). The fractions were prepared and tested essentially as follows: Milk fat (2.5 Kilo) was held at 22-25°C. in a clear glass container exposed to diffuse sunlight. After seven months' storage under these conditions the fat gave a strongly positive Kreis test. It was then vacuum distilled, with a mantle temperature of 140-150°C. at a pressure of approximately 0.5 mm. of Hg., to yield 10 g. of volatile material which reacted positively (red color) to both the Kreis and ferric chloride tests. This material was fractionated by selective solvent extractions to yield a Kreis positive material having the following characteristics: water soluble from which solution it could not be readily extracted with ether, volatile with steam, strongly acidic as evidenced by its complete resistance to steam distillation or ether extraction when in $NaHCO_3$ solution, stable in hot solutions of $NaHCO_3$ or dilute HCl, enolic as indicated by a red-brown

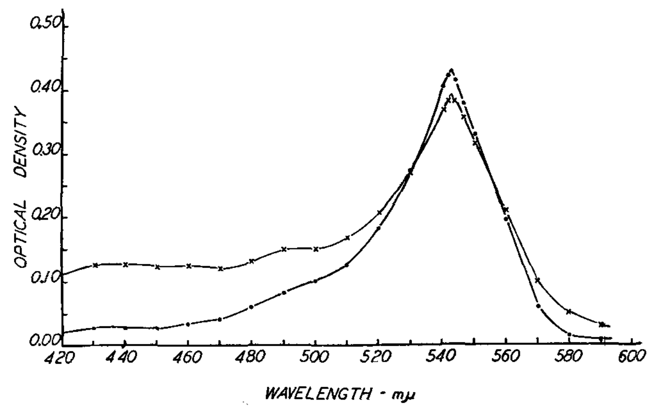

FIG. 1. The absorption spectra of Kreis colors from oxidized milk fat (x) and from malonic dialdehyde (o).

ferric chloride reaction that was invariably associated with it, and carbonyl in nature as evidenced by its loss of Kreis test activity after reaction with carbonyl reagents. This water soluble component represented only part of the Kreis positive substances from the oxidized fat. However, as isolated, it should have constituted the low molecular weight compound(s) concerned in the Kreis test. Its properties are closely consistent with those reported in the literature (1) and, as observed in this laboratory, for malonic dialdehyde. Indeed it is difficult to designate any other compound which might have the above mentioned properties. Considering the proposed structure of epihydrin aldehyde, it seems quite unlikely that it is a strong acid, that it would give a red color reaction with ferric chloride, or that it would be relatively stable to hot dilute solutions of acid or base.

Discussion

Epihydrin aldehyde and malonic dialdehyde are isomeric ($C_3H_4O_2$). Both are obscure compounds, and neither is known in pure form. Possibly the two compounds undergo a number of chemical reactions in common, and it is conceivable that both give the Kreis test and even form the same pigment in the reaction. Lea (8) has suggested that epihydrin aldehyde may be formed by rearrangement of malonic dialdehyde in the oxidation of certain unsaturated fatty acids. Thus it appears to be largely an academic question as to which one is involved in the Kreis test of oxidized fats. This question is somewhat beside the point since a number of compounds or, more likely, a characteristic structure appear responsible for the Kreis reaction of oxidized fat. As noted in this study some volatile, Kreis positive compounds from oxidized fat can be extracted from ether solution, some cannot. Of those extracted from ether solution by sodium bicarbonate, some appear mainly water-soluble, others ether-soluble. In addition, it has been observed in connection with this study that no matter what procedures are used to remove the Kreis positive substances from oxidized fat, a substantial proportion of them always remain with the fat and thus are perhaps an integral part or structure in the triglyceride molecule.

However if malonic dialdehyde is partially responsible for the Kreis reaction of oxidized fat, which at least is strongly indicated in this investigation of milk fat, the fact may have significant bearing on theories concerning fat oxidation. Kobert (6) concluded from his studies that only compounds containing an allyl group or a substituted allyl group are capable of giving positive (red) Kreis tests. Since the ferric chloride test given by malonic dialdehyde shows it to be partially in the enol form, it would satisfy Kobert's observation. In a general sense any unsaturated fatty acid would conform to this requirement. According to Holm and Greenbank (4), autoxidized oleic, ricinoleic, linoleic, and linolenic acids give positive Kreis tests.

Patton and Kurtz (10) have shown that oxidized milk fat when heated with thiobarbituric acid gives a red color. The absorption spectrum of this color (maximum at 532 mμ) is very similar to the Kreis color (maximum at 543 mμ) of oxidized milk fat. In a similar manner these workers demonstrated that malonic dialdehyde gives a red color with thiobarbituric acid reagents and that this color is spectrally identical with that obtained from oxidized milk fat. Further it may be noted that the enol form of 2-thiobarbituric acid bears a close structural relationship to phloroglucinol. With respect to measurement of milk fat oxidation the thiobarbituric acid test appears more sensitive than the Kreis test. The chemical mechanisms of the two seem similar.

The present paper constitutes a preliminary report. The question concerning the roles of malonic dialdehyde and epihydrin aldehyde in fat oxidation would be considerably clarified by conclusive demonstration of their presence or absence in oxidized fat. Research efforts toward this end are under way in this laboratory.

Summary

Evidence is presented which suggests that epihydrin aldehyde and its derivatives are not necessarily solely responsible for the Kreis color reaction of oxidized fats. Malonic dialdehyde has been shown to give a positive reaction in the Kreis test and the resulting color demonstrated to be spectrally similar to the Kreis colors obtained with epihydrin aldehyde diethyl acetal, acrolein treated with H_2O_2 rancid lard, and oxidized milk fat. Characteristics of a water-soluble, low molecular weight, Kreis positive, carbonyl compound from oxidized milk fat were observed to be very similar to those reported for malonic dialdehyde, i.e., strongly acidic, enolic as indicated by the ferric chloride test, and relatively stable to heating with dilute mineral acids. These properties would not be expected of epihydrin aldehyde. Three highly sensitive colorimetric reactions, involving reactions with ferric chloride, 2-thiobarbituric acid or the Kreis reagents, might well serve as the basis for quantitative measurement of malonic dialdehyde.

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The Centrifugal Acetone Foots Test Applied to Crude Soybean Oil. I. Rapid Estimation of Phosphatide Content[1]

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ALTHOUGH most crude edible vegetable oils are graded according to the refining loss as determined by the official A.O.C.S. cup method, there are many occasions when it is desired to know the content of the phosphatides and related constituents, for example, as estimated by the acetone insoluble content. The HCl heat break (Gardner break) content of soybean oil indeed continues to be used to some extent despite its displacement by refining loss methods as the primary means of evaluating crude soybean oil for refining purposes.

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In the early days of the rapid growth of the American soybean industry, a committee (1) conducting cooperative analyses intended to lead to the adoption of a suitable method for grading soybean oil in trade, investigated the acetone foots test which had been in use for some time in grading raw linseed oil. This committee found that test to be far less reproducible when applied to soybean oil as compared with its performance with raw linseed oil (on which its application has been the subject of much criticism). Considering this test therefore altogether unsuitable for the purpose, the committee investigated the HCl heat break content and found the method to