Color Tests for the Detection of Alpha-Dicarbonyl Compounds Formed in the Autoxidation of Fats[1]

EDWARD A. PRILL[2]
McArdle Memorial Laboratory, University of Wisconsin, Madison

The supposition that α-diketo compounds, as well as their probable intermediate precursors, the α-hydroxyketo compounds, may occur among the autoxidation products of fats has been discussed in recent publications (11, 19). The tests developed by the author provide definite evidence for the occurrence in autoxidized unsaturated fats of compounds containing either the α-diketo (C-CO-CO-C) or the α-ketoaldehyde (C-CO-CO-H) group. These compounds apparently have fairly long carbon chains.

The tests are based upon the formation of the colored nickelous (7, 16), cupric (4, 16) and bis-pyridine-ferrous (16) derivatives of the dioximes of the α-dicarbonyl compounds. The procedures are, in part, similar to those used in certain methods for the determination of diacetyl, methylglyoxal and other homologous α-dicarbonyl compounds in which the compounds are converted to the corresponding nickelous (1, 12, 13, 18) and diammino-ferrous (13, 14) derivatives of their dioximes. Each of the new tests involve, (a) treatment of the fatty material with a reagent containing hydroxylamine, (b) fixation of the excess hydroxylamine with acetone, (c) addition of the proper heavy metal ion together with certain auxiliary reagents, and (d) extraction of the metallic derivative of the dioximes into a clear organic solvent phase. Metallic derivatives of the dioximes of α-dicarbonyl compounds which contain fairly large alkyl groups are generally quite soluble in benzene and other organic solvents. Dilute solutions of the nickelous, cupric and bis-pyridine-ferrous derivatives of such dioximes in organic solvents are colored, respectively, yellow, yellowish olive (or orange in some cases) and red. Solutions of nickelous derivatives of homologous dioximes have been reported to exhibit similar absorption spectra (17).

Autoxidized unsaturated fatty materials were found to give definite tests for the presence of α-dicarbonyl compounds when tested directly or when tested after preliminary treatment with ferrous chloride to destroy preformed peroxides. The fresh fatty materials did not respond to any of the tests for the presence of α-dicarbonyl compounds.

The application of the proposed tests may provide useful information in regard to autoxidation of fats and related phenomena. In view of the recognized reactivity of α-dicarbonyl compounds, it would not be surprising if such compounds exerted activities, possibly including physiological activity, in addition to their suggested rôle (8, 11, 19) of imparting yellow color to autoxidized oils. Since it has been reported (10, 15) that diacetyl added to butter fat promoted autoxidation, it might be expected that α-dicarbonyl compounds formed during autoxidation might act similarly to autocatalyze the oxidation.

Procedure

About 0.5 g. of fatty material was weighed into a test tube for each individual test. Two methods of oximation, designated as the "pyridine method" and the "acetate method," were employed for the conversion of any fatty α-dicarbonyl compounds to their dioximes. For the "pyridine method," 2 ml. of a freshly prepared 3 per cent solution of hydroxylamine hydrochloride in pyridine was added to each test tube containing a sample to be tested. The tubes were then heated for two hours in a bath maintained at 80° C., and after cooling, 0.3 ml. of acetone was added to each tube and the mixture allowed to stand for five minutes before proceeding with the subsequent steps of the tests. For the "acetate method," 2 ml. of a 3 per cent solution of hydroxylamine hydrochloride in absolute ethanol and 1 ml. of a 10 per cent solution of potassium acetate in absolute ethanol were added in place of the pyridine solution of hydroxylamine hydrochloride, otherwise the treatment was the same as outlined above.

For the purpose of determining whether formation of hydroxamic acids (R-CO-NHOH), particularly the higher members, accompanied the above oximation reactions, the ferric chloride test (5) for hydroxamic acids was modified in the respect that the red colored ferric derivative formed from higher hydroxamic acids was extracted from the reaction mixture (the mixture being slightly acidic to Congo red) into a benzene phase. This procedure was found applicable in trials with reference hydroxamic acids prepared (5) from ethyl laurate and ethyl stearate. By use of this modified test appreciable amounts of higher hydroxamic acids were found in the products obtained when autoxidized fats were treated by either of the two oximation methods, but little or none was found in the products from similarly treated fresh fats. Obviously, it was necessary to include measures in the following steps of the tests for α-dicarbonyl compounds to prevent interference by metallic derivatives of hydroxamic acids. Addition of tartrate and an alkali served this purpose and also prevented interference by extractable colored metallic salts of free fatty acids as well.

The reagents used in the various tests, both on the oximated fatty materials and on known reference dioximes, are shown in Table I. When a given reagent was used in a test it was added in the amount indicated in the table and the mixture shaken thoroughly. When a metallic derivative was extracted into a benzene phase the addition of a few drops of *n*-butanol served to break any emulsion which formed. Blank tests omitting the heavy metal salts, as well as others omitting the oximation, were performed on each material tested.

In order to compare the tests obtained with the oximation products of the unidentified α-dicarbonyl compounds present in autoxidized fats, similar tests

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[2] Present address: The Borden Company Research Division, Bainbridge, N. Y.

were made with several known dioximes. For this purpose the dioximes of di-*n*-butyryl (13), acetylcaproyl and *n*-amylglyoxal (13) were used. Solutions were prepared by dissolving one millimole of a reference dioxime in 100 ml. of ethanol and diluting to one liter with water.

TABLE I
Test Reagents[1]

Reagent	Concentration g. compound in 100 ml. of water	Quantity used, ml.
Tartrate (Rochelle Salt)	60	1
Ammonium Hydroxide	Conc.	2
Nickel Chloride	5	1
Copper Sulfate	5	1
Ferrous Sulfate	5	1
Sodium Acetate	20	1
Sodium Bicarbonate	8	3
Potassium Hydroxide	100	4
Pyridine (pure)	......	2
Ethanol	......	3
Benzene	......	2
Water (for dilution)	......	5-10

[1] The reagents for the oximation reactions are given in the text.

Nickelous Derivative of Dioximes. Addition of nickel chloride and sodium acetate to small amounts of each of the reference dioxime solutions produced the corresponding nickelous derivatives. On dilution with water and shaking with benzene, yellow colored solutions of the nickelous derivatives in the benzene phases were obtained. When tartrate, ammonium hydroxide, or pyridine, or any combination of these was added the intensity of the colors of the benzene extracts was unaffected.

Nickel chloride, tartrate and ammonium hydroxide were added to fatty material that had been oximated by one or the other method, after which the mixture was diluted with water and shaken with benzene. This test for the presence of α-dicarbonyl compounds was considered positive if the benzene phase showed a yellow color similar to that observed in the case of the reference dioximes.

Cupric Derivative of Dioximes. Addition of copper sulfate and either sodium acetate or pyridine to each of the reference dioximes, dilution with water and extraction with benzene yielded benzene layers that were colored in each case by the extracted cupric derivative. Di-*n*-butyryl dioxime gave rise to a yellowish olive colored extract while the other two dioximes gave rise to orange colored extracts. Addition of tartrate and ammonium hydroxide to each of these specimens and shaking of the mixtures (a measure which would often be necessary in tests on fats in order to remove the blue copper derivatives of hydroxamic acids (5) from the benzene layers) caused some reduction in the intensity of the colors of the benzene phases owing to the relative instability (4, 16) of the cupric derivatives of these fairly low molecular weight dioximes. It appears that in a series of dioximes, increase in molecular weight may be a factor in causing increased stability of the various metallic derivatives.

Pyridine was added to each sample of fatty material oximated by the "acetate method" and ethanol was added to each sample oximated by the "pyridine method." Copper sulfate was then added to each specimen followed by the addition of sodium bicarbonate, after which the mixtures were shaken with benzene and diluted with water. In some of the positive tests the benzene phases immediately showed a yellowish olive color similar to that obtained with di-*n*-butyryl dioxime. However, in many cases the benzene phases were colored bluish or greenish, and in these latter cases addition of tartrate and ammonium hydroxide and shaking of the mixtures usually removed the blue or green compounds from the benzene phases leaving, in the positive tests, the characteristic yellowish olive color in the benzene phase.

Bis-pyridine-ferrous Derivative of Dioximes. Addition of pyridine, tartrate and ferrous sulfate to each of the reference dioximes, followed by the addition of sodium bicarbonate to neutralize any acid, produced intensely red colored derivatives which were easily extracted by benzene.

Addition of an excess of saturated potassium hydroxide to a mixture consisting of the dioxime of a reference α-diketone, pyridine, tartrate and ferrous sulfate caused the salting out of a pyridine layer containing the red dioxime derivative which was formed. When applied to fats this latter procedure avoids interference by the similarly colored ferric derivatives of hydroxamic acids (presence of ferric iron is unavoidable), which are not completely decomposed by treatment with any less alkaline reagents.

Tartrate and ferrous sulfate were added to fatty materials oximated by the "pyridine method" followed by the addition of saturated potassium hydroxide which was added slowly and with shaking. In the positive tests the pyridine layers developed red colors similar to those obtained with the similarly treated reference dioximes.

This procedure provides a very sensitive test for the presence of α-dicarbonyl compounds in fats. However, when applied to materials oximated by the "acetate method" and followed by later addition of pyridine, the presence of too much ethanol makes the salting out of a pyridine layer unsatisfactory.

Results of the Tests on Some Autoxidized and Fresh Fatty Materials

Table II summarizes the results of the several tests for the presence of α-dicarbonyl compounds in fatty materials. In all cases the blank tests showed no color or only the slight color attributable to the fat itself. All of the unsaturated materials which had become autoxidized through heating and aeration, or through long storage, gave evidence of containing α-dicarbonyl compounds through the formation of the three types of metallic derivatives. The corresponding fresh materials did not respond to any of the tests. The relative intensities of the tests are indicated. Comparisons of the colors obtained with fats and those obtained with known amounts of di-*n*-butyryl dioxime makes possible estimates of the molecular concentration of the α-dicarbonyl compounds, if it is assumed (a) that the oximation leads to the quantitative production of *anti*-dioximes (6, 7), and (b) that solutions of the same molarity with respect to a given type of metallic derivative of the *anti*-dioximes of different α-dicarbonyl compounds exhibit approximately the same intensity of color. On the basis of these assumptions most of the autoxidized materials appeared to contain the equivalent of 20 or more millimoles of an α-dicarbonyl compound per kilogram.

Because the final reaction mixtures were alkaline, those compounds which contain an acidic group in addition to the α-dicarbonyl group were not able to form extractable metallic derivatives. Besides the α-dicarbonyl compounds, there are a few other types of compounds (3, 6), which after oximation can also form certain metallic derivatives, but the possibility that these latter compounds were responsible for the colors produced can be reasonably excluded.

TABLE II

Results of the Several Tests for α-Dicarbonyl Compounds Obtained With Some Fatty Materials[1]

Fat or ester	Condition or treatment[2]	Oximated by "Pyridine method"			Oximated by "Acetate method"	
		Metallic derivatives				
		Ni	Cu	$Fe^{\cdot\cdot}$ 2 Py[3]	Ni	Cu
Ethyl Stearate	Fresh	0	0	0	0	0
	I	0	0	0	0	0
Ethyl Linoleate	Fresh	0	0	0	0	0
	I	+++	+++	+++	+++	+++
Corn Oil (Refined)	Fresh	0	0	0	0	0
	I	++	++	++	++	++
	II	++	++	++	++	++
	III	+++	++	—[4]	+++	?
Cottonseed Oil (Refined)	Fresh	0	0	0	0	0
	I	++	++	++	++	?
	II	++	++	++	++	++
Olive Oil (Pure)	Fresh	0	0	0	0	0
	I	++	++	++	++	?
	II	+	+	+	+	+
Lard	Fresh	0	0	0	0	0
	IV	++	++	++	++	++

[1] On the basis of the comparisons with the colors produced with a reference dioxime, the positive results are expressed in terms of the apparent number of millimoles per kilogram as follows:
+ = about 10 to 20; ++ = 20 to 30; +++ = 30 to 40.

[2] The treatments were as follows:
I—Heated at 100° C. for 20 hours with constant aeration.
II—Aged at ordinary room conditions for a year or more.
III—Aged oil further treated as in I.
IV—Held at 37° C. for two months.

[3] Fe · 2Py = Bis-pyridine-ferrous.

[4] In this case the test was definitely positive but the red colored derivative did not dissolve in the pyridine phase but collected at the interface. Since the oil was very viscous, it is possible that the α-dicarbonyl compound was present in the form of a polymerized substance.

Tests on Fatty Materials with Previous Destruction of Peroxides

It was considered important to determine whether the peroxides present in autoxidized fats might oxidize hydroxylamine to nitrous acid or to the nitrosyl radical (2), which in turn might react with components other than α-dicarbonyl compounds to bring about the formation of a dioxime capable of responding to the tests. Ferrous chloride and other ferrous salts are known to decompose true peroxides (20, 21). For the destruction of peroxides, 50 ml. of absolute ethanol, 2 ml. of glacial acetic acid and 2 g. of ferrous chloride were added to a 2 g. sample of fatty material and the mixture was refluxed for an hour. After dilution with 300 ml. of water and extraction with peroxide-free ether, the ether extract was fitered and washed with 5 per cent sulfuric acid, then with 5 per cent phosphoric acid, and finally with water. When autoxidized materials which had been thus treated were tested for peroxides by the method of French *et al.* (9), the tests were found to be negative. Aliquots of the ether solutions were evaporated to remove the solvent and the various tests for α-dicarbonyl compounds performed. The intensities of the colors produced were approximately the same as those obtained with the corresponding materials from which peroxides had not been removed. It was assumed therefore, that the action of peroxides was not responsible for the observed color formation, and it may be concluded that the method actually is a test for the presence of α-dicarbonyl compounds in autoxidized fats.

Summary

Tests have been devised for detecting α-dicarbonyl compounds in autoxidized fats. The tests are based upon the conversion of the α-dicarbonyl compounds to their dioximes, formation of the nickelous, cupric and bis-pyridine-ferrous derivatives of the dioximes, and extraction of these colored metallic dioxime derivatives into organic solvent phases.

The presence of α-dicarbonyl compounds in a number of autoxidized fatty materials has been demonstrated by these tests.

Peroxides in autoxidized fatty materials could be destroyed by treatment with ferrous chloride without destruction of α-dicarbonyl compounds.

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