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## Chemical Nature of the Thiobarbituric Acid Test for the Oxidation of Unsaturated Fatty Acids

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Thiobarbituric acid was used by Kohn and Liversedge<sup>1</sup> as a reagent for a compound formed by aerobic incubation of tissue suspensions. It was found by Bernheim *et al.*<sup>2</sup> that the colors obtained upon addition of thiobarbituric acid to incubated tissues are due to a product of oxidation of unsaturated fatty acids, especially linolenic acid. Subsequently, Wilbur *et al.*<sup>3</sup> studied further thiobarbituric acid as a reagent for oxidized fatty acids. They found that the strongest colors were produced with oxidized linolenic acid, weaker with oxidized linoleic and arachidonic acids. Furthermore, while oxidized fatty acids gave orange-red colors, various sugars and aliphatic aldehydes gave yellow colors, and glyoxylic acid a pink color.

As stated by Wilbur *et al.* the chemical reaction underlying the thiobarbituric acid reaction has not been completely elucidated. However, a comparison of

thiobarbituric acid with phloroglucinol with respect to chemical properties will probably contribute to its explanation. The two compounds show some similarity with respect to chemical structure, including an analogous tautomerism and both are used as sensitive reagents for the quantitative determination of aldehydes, especially furfural. Furthermore, phloroglucinol is used as a reagent for the detection of oxidative changes of fats in the well-known Kreis-test<sup>4</sup>. The question therefore arises whether thiobarbituric acid is a reagent for the presence of the same chemical structures.

In order to investigate this question we have compared the two reactions using a number of fatty acids and other products of various origin. The thiobarbituric acid reaction was carried out as described by Wilbur *et al.* In order to facilitate the comparison of the results, the Kreis-test was carried out in a one phase-system using the modification of Pool *et al.*<sup>5</sup>. Finally, the peroxide values of the substances were determined by Hartmann and Glavind's colorimetric method<sup>6</sup>.

Some representative results are given in Table 1. The colors were measured on the Beckman spectrophotometer at the respective wave-lengths. For presentation in the table they are calculated as the extinctions obtained in the three methods by the same amount of substance in the same final volume of 10 ml using a 1 cm cuvette.

It can be seen from the table that a minute amount of purified methyl oleate hydroperoxide gave a strong color in the thiobarbituric acid reaction. Such a strong color cannot be explained by the presence in the oleate of small amounts of linolenate. This observation does not agree with the statement by Wilbur *et al.* that thiobarbituric acid is a reagent for oxidized linolenic acid alone.

A study of the table further shows that the thiobarbituric acid reaction and the Kreis-test follow each other rather closely,

Table 1. Intensity of colors produced by different methods for assay of oxidative changes in fats.

Method	Thio-barbituric acid reaction	Kreis-test	Peroxide method of Hartmann and Glavind
Wave length	532 m $\mu$	550 m $\mu$	520 m $\mu$
Substance	Extinction		
Lard, 400 mg	0.175	0.325	0.73
Fatty acids from cod liver oil, 1.3 mg*	0.165	0.35	1.65
Methyl oleate hydroperoxide**, 2.5 mg	0.10	0.36	7.3
Epihydrin aldehyde***	0.64	0.60	0.89
Benzoyl peroxide, mg	0.00	0.00	2.5

\* Highly unsaturated fraction precipitated at  $-75^{\circ}\text{C}$  from acetone solution after more saturated acid had been removed at higher temperatures. Stored for 7 years at room temperature.

\*\* Prepared from purified oleic acid by the method of Swift *et al.*<sup>7</sup>. Peroxide value 5 000 milliequivalents per 1 000 gramms.

\*\*\* To 330 mg freshly distilled acrolein dissolved in saturated KCl was added 1 ml 30%  $\text{H}_2\text{O}_2$ . Since all reactions were to be carried out in a non-aqueous medium, the mixture was extracted with 40 ml  $\text{CHCl}_3$ , and 2.5 ml of the extracts was used in each trial.

while there is no such parallelism with Hartmann and Glavind's method for the determination of peroxides. Benzoyl peroxide reacts quantitatively with leucodichlorophenolindophenol in the latter reaction but produces no color in the Kreis- or thiobarbituric acid tests. Purified methyl oleate hydroperoxide produces rather weak colors in the thiobarbituric acid as well as the Kreis-test as compared with the colors produced with leucodichlorophenolindophenol. On the other hand, strong colors are produced in the two tests in comparison with Hartmann and Glavind's method by such substances as oxidized fatty acids and lard. Especially intense colors are produced with epihydrin aldehyde which, according to Powick<sup>8</sup>, is the carrier of the Kreis-test.

These observations seem to justify the conclusion that while Hartmann and Glavind's method is a specific reaction for peroxide groups, the thiobarbituric reaction like the Kreis-test is indicative of more complex structures formed in the course of the peroxidation process, and which, in accordance with Powick's explanation, are split off in the form of epihydrin aldehyde by the treatment with strong acids used in both methods.

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## Paper Chromatography on Borate-Impregnated Paper

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Paper impregnated with phosphate buffers has been employed with advantage for the separation of organic acids<sup>1,2</sup>. As emphasized by Levi in a review<sup>3</sup> it should also be possible to utilize paper treated in various other ways, and in connection with work on lichen acids capable of forming complexes with boric acid it seemed desirable to investigate the possible use of borate buffers.

A number of phenols, phenolic aldehydes and phenolic acids as well as some sugars were studied. With every substance or group of isomeric substances parallel