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On the Determination of Lipoperoxides in Animal Tissues

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The determination of lipoperoxides in animal tissues was studied through recovery experiments. Quantities of peroxides up to about 0.1 μequiv./g tissue added during the extraction of liver vith chloroform disappeared during the determination. Lung and muscle destroy amounts of peroxides of the same order, whereas with blood and pancreas the recovery is better. Improved recoveries were obtained by adding large amounts of hydrogen peroxide. The principles disturbing the determination of lipoperoxides do not interfere with the thiobarbituric acid reaction.

The occurrence of lipoperoxides has been demonstrated in the adipose tissue of nutritionally deficient experimental animals [1], in subcutaneous tissue of man with peripheral venous diseases [2], and in the atherosclerotic human aorta [3]. Lipoperoxides may also occur as normal constituents of the organism. Gomori [4] has postulated that the presence of lipoperoxides in leucocytes are responsible for the histochemical, so-called stable nadi-reaction; yet, the present authors were able to find, by chemical methods, only small amounts of lipoperoxides in the leucocytes [5].

The determination of lipoperoxides, *e.g.*, in adipose tissue, is simple since only an extraction with a peroxide-free solvent is needed before the usual chemical methods can be carried out. However, the peroxide-group is, chemically, highly reactive. Consequently, a general survey of the presence and function of lipoperoxides in physiology and pathology will have as a necessary prerequisite a study of the applicability of the methods for peroxide determination, especially their use for organs consisting to a major extent of parenchymatous tissue. Such a study was the purpose of this work.

MATERIAL AND METHODS

The organs and other tissues, generally from pigs, were freshly brought from slaughter. Extracts were prepared by grinding 40 g portions of tissue with 120 g anhydrous sodium sulfate and 200 ml chloroform in a Waring blendor. In the recovery studies, known amounts of lipoperoxides in the form of lard were added in the chloroform. The extracts were filtered, and 100 ml evaporated *in vacuo* at low temperature to a small volume which was adjusted to 5 ml with chloroform.

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Table 1. The disappearance of lipoperoxides during the extraction of pig liver. Colorimetric peroxide determinations carried out on liver lipids, the triglyceride and phospholipid fractions of liver lipids, and liver lipids extracted with the addition of lard containing a known amount of peroxides.

Material	Extinction at 530 mμ
Liver extract	0.095
Triglyceride fraction	0.055
Phospholipid fraction	0.040
Liver extract	0.095
Lard	0.170
Liver extract with added lard	0.100

Determinations of lipoperoxides were carried out both by the thiocyanate and the indophenol methods. Generally the latter method was used since, in the presence of high amounts of phospholipids, it gives more quantitative results than the thiocyanate method. Both methods were carried out as described in a previous paper [6]. A Beckman spectrophotometer was used for colorimetry, and corrections made for reagent blanks and for the yellow color of the extracts.

Separate determinations of peroxide groups in the triglyceride and the phospholipid fractions were carried out as described in an earlier paper [7].

The thiobarbituric acid reaction was carried out essentially as described by Wilbur *et al.*[8]: Two g tissue was homogenized with 3 ml 10 % trichloroacetic acid, the mixture centrifuged, and 2 ml of the clear supernatant mixed with 0.67 % thiobarbituric acid. The mixture was heated for 10 min in a boiling water-bath, the tubes cooled, and the optical densities read at 530 mμ.

RESULTS

Attempts to determine lipoperoxides in liver. When the indophenol method for determination of lipoperoxides was carried out on liver, as a rule a distinct, but low value was obtained (Table 1). When triglyceride and phospholipid peroxides were determined separately, small values were usually obtained for both fractions. When, however, a known quantity of lipoperoxide was added

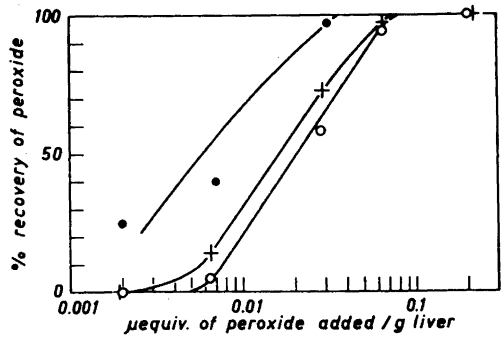

Fig. 1. Recovery of peroxide. Tests carried out on liver with the addition of known amounts of peroxide. Peroxide added before extraction O, after filtration +, and after absorption ●.

with the chloroform during the extraction of liver lipids, sometimes only a very small, sometimes a larger part of the peroxide was recovered.

The disappearance of lipoperoxides was studied in recovery experiments of the type exemplified in Fig. 1. Graded amounts of lipoperoxides were added, (1) in the chloroform, (2) in the filtrate after extraction, and (3) in the solution obtained after absorption on alumina.

These results show that amounts of added lipoperoxide of about 0.01 μequiv./g liver disappeared during the determination, whereas a good recovery was obtained when amounts above 0.1 μequiv./g liver were added. The filtered chloroform extract gave almost as great an inhibition, but only slight losses occurred in the filtrate obtained after absorption of the extract on alumina.

The influence on the destruction of lipoperoxides of varying the experimental conditions, adding enzyme poisons, *etc.*, was studied in further recovery experiments. The peroxide-destroying effect of liver was not affected by heating the liver for half an hour in a boiling water-bath. Likewise, freezing the liver and cooling the chloroform with solid carbon dioxide gave the same result as carrying out the experiment at room temperatures. The use of petrol ether or ethanol instead of chloroform as solvent, or the addition of hydrocyanic acid, ethylenediaminetetraacetic acid, formalin, or sulfosalicylic acid did not influence the peroxide recovery.

An improved recovery was obtained by the addition of large amounts of certain hydroperoxides which probably acted by saturating lipoperoxide-reducing systems. The best results were obtained by adding 20—50 mg perbenzoic acid in the chloroform, and afterwards filtering through alumina which removed the acid. Unfortunately, the instability of perbenzoic acid complicates the use of this substance for routine analysis. Peracetic and monoperphthalic acids destroy the peroxide group. An improved recovery was obtained by the addition of 5—10 ml 30 % hydrogen peroxide. Amounts of lipoperoxide down to 0.01 μequiv./g liver could be recovered, but smaller amounts disappeared.

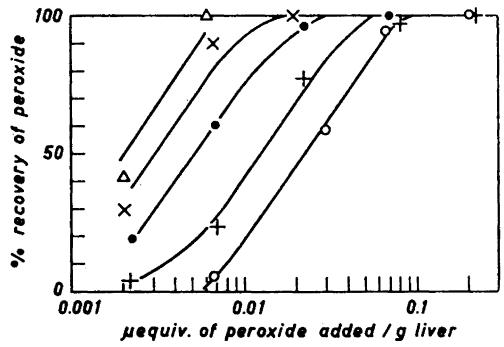

Fig. 2. Recovery of peroxide. Tests carried out on different organs with the addition of known amounts of peroxide. Liver O, muscle +, lung ●, pancreas ×, and blood △.

Thus, the elaboration of a method which gave under all circumstances a quantitative recovery, was not achieved. The best approach resulting from our studies was the following method elaborated for use on chick livers:

Two ml 30 % hydrogen peroxide and 30 g anhydrous sodium sulfate were finely ground in a mortar. 5 g liver were added and ground until the mass was homogeneous. The mixture was extracted with 10 ml chloroform, filtered through a column (12 × 50 mm) of purified alumina [6], the column washed with more chloroform, and the filtrate used for the determination which was carried out as usual.

The use of this method enabled the demonstration of lipoperoxides in livers of chicks receiving a special, vitamin E-deficient diet [9]. Values corresponding to a content of up to 0.1 μequiv. of peroxide per g liver were found while only about 0.01 μequiv. was found in the livers of a control group fed the same diet supplemented with vitamin E.

The lipoperoxide-destroying properties of different organs. Other tissues and organs were examined in the same way as liver, *e.g.*, in recovery experiments with graded amounts of lipoperoxides (Fig. 2). It appears from these results that muscle and lung have peroxide-destroying properties of a potency similar to that of liver, whereas the effects of blood and pancreas are weaker.

The influence of the peroxide-destroying principles on other methods. When recovery experiments were carried out on liver using the thiocyanate method for lipoperoxide determination instead of the indophenol method, similar results were obtained.

A comparison with the thiobarbituric acid reaction was carried out in the following way: Liver was stored in a refrigerator. Samples were taken daily and tested by the thiobarbituric acid reaction. The fresh liver gave no reaction, but an increasingly strong color appeared when the liver was stored for some days. Recovery experiments with the indophenol method were carried out on a sample of liver giving a strongly positive thiobarbituric acid reaction without incubation (optical density 0.6). Peroxide-reducing properties in the stored liver similar to those in fresh liver were found.

DISCUSSION

The peroxide-destroying properties of liver have been profoundly studied by Dubouloz and his co-workers [10]. However, since our procedures differ from theirs, it cannot be settled whether the disappearance of lipoperoxides found here is due to the same peroxide-destroying principles as described by these investigators.

The presence of peroxide-destroying properties in a filtered chloroform extract, and the fact they were influenced neither by hydrocyanic acid, *etc.*, nor by heating, suggest that they are not of enzyme character, but due more likely to some fat-soluble reducing substances. Substances present in the extract might interfere with the peroxide determination in other ways than by reducing the peroxides, or ferric iron, but since the same results were found both with the indophenol and the thiocyanate methods, the reductive effect seems to be the most logical explanation. The nature of the reducing substances is

unknown. Tocopherols, vitamin A, and carotenoids, *etc.*, are oxidized by lipoperoxides and by ferric ions. The reactions are, however, very slow at room temperatures unless catalyzed by heme pigments, and such catalysis seems improbable since hydrocyanic acid does not interfere.

The presence of reducing substances complicates the determination of lipoperoxides which may be present in the same tissue, *e.g.*, deposited in other microscopic structures. In liver amounts less than 0.1 μequiv. per g probably cannot be determined by the usual methods. When hydrogen peroxide is added, to neutralize the reducing substances, the limit concentration which can be determined is probably about 0.01 μequiv. per g liver.

In recent years many studies on the formation of lipoperoxides have been conducted with the thiobarbituric acid reaction of Kohn and Liversedge [11]. A major portion of these studies have been carried out on tissue samples after incubation in the presence of oxygen. Carried out in this way the method gives an expression of the tendency to lipoperoxide formation by the tissue, rather than an estimate of its lipoperoxide content. When the thiobarbituric acid reaction is carried out without incubation, obviously only preformed substances can give the reaction. However, this reaction is not given by the peroxide group proper but by secondary reaction products, and consequently it cannot replace a proper peroxide determination method when quantitative information is required.

It appears from our experiments that the substances interfering with methods for the determination of the peroxide group do not inhibit the thiobarbituric acid reaction. The stability of this reaction towards reducing substances is a definite advantage. Therefore, the thiobarbituric acid reaction, carried out without previous incubation, should be tried when reducing substances impede the use of methods for the determination of lipoperoxides proper.

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