# Existence of Fatty Acid Peroxides in Normal Blood and Tissues of Man and Animals.*† (24858)

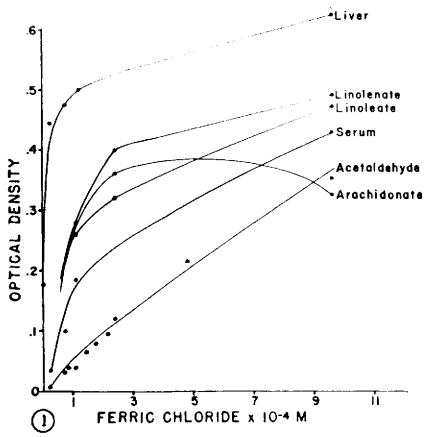
ANDRE C. KIBRICK, LENORE B. SAFIER AND S. J. SKUPP
*Laboratory Service, N. Y. Vet. Admin. Hospital, in affiliation with N. Y. University College of Medicine*

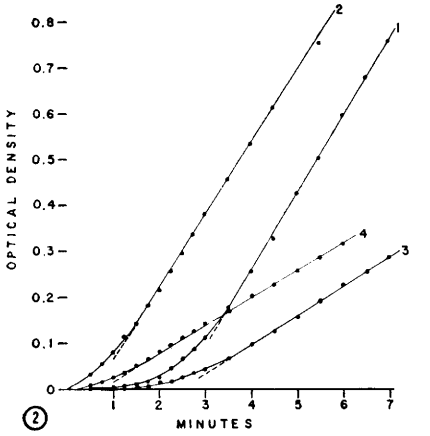
It is well known that fatty acid peroxides are formed in the presence of hemoglobin and other heme compounds(1), and there is evidence suggesting $\alpha$ tocopherol as an *in vivo* inhibitor of unsaturated fatty acid oxidation catalyzed by hematin compounds(2). However, it is not unlikely that fatty acid peroxides may be formed normally in small amounts from unsaturated fatty acids in animal body and may have a role in metabolism. Many methods will detect fatty acid peroxides in pure solution. Of these the thiobarbituric acid method (TBA) of Kohn and Liversedge(3) is positive in extracts of most animal tissues, but negative in blood. The indophenol dye method and thiocyanate method described by Glavind and Hartmann(4) are negative in normal tissues and in blood. It has been claimed that fatty acid peroxides can be detected in blood with methods based on liberation of iodine from potassium iodide (5,6). In spite of many inferences that fatty acid peroxides may be present normally in the animal body, few workers have been willing to conclude that this is so(5,6,7). In this paper we shall present evidence that fatty acid peroxides are present normally in both blood and tissues.

*Methods.* We found that sensitivity of the TBA reaction can be increased markedly by presence of $9.6 \times 10^{-4}$ M ferric chloride. Control experiments with esters of linoleic, linolenic and arachidonic acids showed that autoxidation does not take place under conditions of the reaction. Using a method developed for these conditions, we found much reacting material in blood of man and many species of animals and in most normal and abnormal tissues. However, it was apparent that much of the reacting material was not fatty acid peroxides, but substances which behave like acetaldehyde, which gives the reaction in presence of this concentration of iron. A study was made of the effect of varying concentrations of iron on the TBA reaction. In Fig. 1 it is seen that in alcohol-ether solutions of partially oxidized polyethenoid fatty acids almost maximum color is attained at $1.2 \times 10^{-4}$ M ferric chloride. In acetaldehyde only about 10% of maximum color is attained at this concentration of iron. In alcohol-ether extracts of blood and tissues TBA values obtained at $1.2 \times 10^{-4}$ M are about 43 and 79%, respectively, of those obtained at $9.6 \times 10^{-4}$ M ferric chloride. This indicates presence of acetaldehyde-like material in both blood and tissues which agrees with results of other studies. Blood apparently contains a larger amount of the contaminating substances than tissue. It appeared that 85% of the fatty acid peroxides and only about 10% of the acetaldehyde substances would react at $1.2 \times 10^{-4}$ M iron. To obtain more evidence, especially in the case of blood, that unsaturated fatty acids exist partially in the oxidized peroxide form, the fatty acid fractions of blood and tissues were isolated under anaerobic conditions. Methods used were indophenol dye and thiocyanate methods of Glavind and Hartmann and the unmodified TBA method in absence of iron, since acetaldehyde-like material is not detected under these conditions. Experiments were performed on fresh normal human blood and on liver excised from living rats anaesthetized with ether. Air was excluded at all steps using nitrogen from heated copper coil and the entire procedure was conducted as rapidly as possible. The fatty acid fraction was isolated by the method of Stoddard and Drury(9) in which the acids were finally precipitated from aqueous acid solution in the cold. Peroxides were not detected by any of the 3 methods in fatty

* This investigation was supported in part by a research grant from Nat. Inst. of Arthritis and U. S. P. H. S.

† A preliminary report was presented at Annual Meeting of Am. Chem. Soc., Chicago, Sept. 7, 1958.



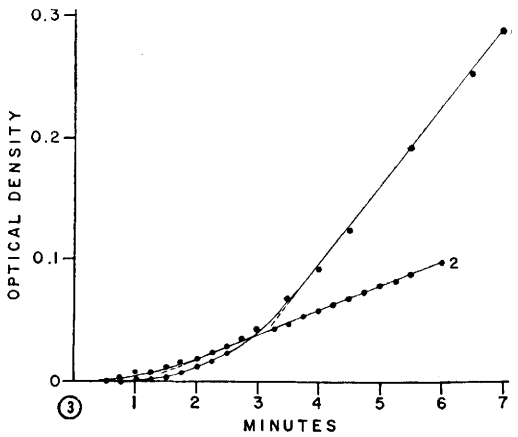




FIG. 1. Effect of varying concentrations of ferric chloride on TBA reaction.

FIG. 2. Effect of linoleate peroxide on lipoxidase-linoleate system. Curve 1, control with saline; 2, same with 0.002 $\mu$M linoleate peroxide; 3, control with 0.05 ml of 4% bovine albumin; 4, bovine albumin plus 0.002 $\mu$M linoleate peroxide. Induction period is indicated at point of origin of dotted line on curves.

FIG. 3. Effect of blood plasma on induction period of lipoxidase-linoleate system. Curve 1, control with bovine albumin; 2, test curve for 0.04 ml plasma instead of albumin.

acids isolated from blood. On the other hand, peroxides were detected in fatty acids isolated from tissues by all 3 methods. However, only about 4-18% of the quantity apparent in original tissue was recovered in the precipitated fatty acids. The results obtained in control experiments with mixtures containing partially oxidized polyethenoid fatty acids were similar, and only about 4% of the reaction was recovered in precipitated acids. Some of the reactive material remained in filtrates from fatty acids of both tissue and in control experiments, but there was still a loss of more than 50% in each case. Stearic acid added to blood and tissues was recovered quantitatively in other control experiments performed similarly except that acids were determined in the usual way by titration with standard alkali.

To show that fatty acid peroxides are present in blood, and that failure of total isolated fatty acids of blood to yield positive reactions with the 3 methods tested was due to low concentration of fatty acid peroxides and to losses encountered during isolation, another method was tried. Haining and Axelrod(8) showed that induction period which occurs during lipoxidase catalyzed oxidation of fresh sodium linoleate, can be abolished by about 5 x $10^{-3}$ $\mu$moles of linoleate peroxide. Since this effect of fatty acid peroxides appeared specific, it seemed a good method to use for demonstration of these substances in blood. Fresh blood was transferred directly from a syringe to ice cold test tube containing oxalate. Nitrogen gas was bubbled through the tube, and plasma prepared in an atmosphere of nitrogen. The procedure of Haining and Axelrod was followed in detail. Fig. 2 shows values of optical density at 234 m$\mu$ in the system of lipoxidase-linoleate. Curves 1 and 2 are saline control and test curve with 0.002 $\mu$moles of linoleate peroxide. Curves 3 and 4 are control with bovine albumin and test curve of albumin plus 0.002 $\mu$M of peroxide. Despite the fact that presence of albumin di-

minishes values of optical density, the induction period in its presence is approximately the same as in its absence. Fig. 3 shows the same system for a bovine albumin control and a test curve containing 0.04 ml of plasma but no added peroxide. It is evident that 0.04 ml of the plasma reduces the induction period approximately by half. This experiment was repeated several times on this plasma with the same result, and similar results were obtained in several other plasmas. From this effect on lipoxidase-linoleate system one can calculate the amount of fatty acid peroxides in blood, since 0.001 $\mu$mole is required to reduce the induction period of the system in half. The value of 2.5 $\mu$moles of fatty acid peroxides/100 ml calculated in this way compares with other values of fatty acid peroxides estimated in 36 human blood serums from normal subjects and patients with a variety of clinical conditions, by the TBA method with $1.2 \times 10^{-4}$ M ferric chloride. Values of TBA reaction calculated in terms of $\mu$moles of linoleate peroxide from the value of 30,000 for the extinction coefficient at 234 m$\mu$ for completely peroxidized linoleic acid reported by Holman (10) yield a range of 8.6 to 35/100 ml. The values calculated from indophenol dye equivalent(4) yield a range of 12.4 to 50.7 $\mu$moles of fatty acid peroxides/100 ml.

Similar calculations of fatty acid peroxides in 52 samples of rat tissues were made from results of the TBA method with $1.2 \times 10^{-4}$ M ferric chloride. Calculation from extinction coefficient at 234 m$\mu$ of oxidized linoleic acid gives a range of 1.86 to 4.86 $\mu$moles of fatty acid peroxides/g of fresh tissue. Calculation of TBA values from analysis of linoleate peroxide with indophenol dye gives a range of 2.68 to 7 $\mu$moles/g. Roughly the same proportion of polyethenoid fatty acids of both tissue and blood exists as fatty acid peroxides.

*Summary.* 1. Evidence is presented for presence of fatty acid peroxides in normal animal tissues and in blood. Total fatty acid fractions isolated from tissues yielded positive values with the thiobarbituric acid method and with indophenol dye and thiocyanate methods. Blood, which was not found to react positively by the 3 methods on isolated fatty acids, contained fatty acid peroxides from their ability to diminish induction period of the lipoxidase-linoleate system. 2. A modified method in presence of $1.2 \times 10^{-4}$ M ferric chloride has been developed for thiobarbituric acid reaction, which apparently yields values for fatty acid peroxides in blood as well as in tissues.

We are indebted to Dr. Joseph L. Haining for suggesting that fatty acid peroxides could be demonstrated by their effect on induction period of lipoxidase-linoleate system.

1. Tappel, A. L., *J. Biol. Chem.,* 1955, v217, 721.

2. ———, *Arch. Biochem. Biophys.,* 1954, v50, 473.

3. Kohn, H. I., Liversedge, M., *J. Pharmacol. Exp. Therap.,* 1944, v82, 292.

4. Glavind, J., Hartmann, S., *Acta Chem. Scand.,* 1955, v9, 497.

5. Shauenstein, E., Pebus, B., *Wien. Klin. Wschr.,* 1956, v68, 376.

6. Shauenstein, E., Wennig, F., *Klin. Wschr.,* 1957, v35, 839.

7. Cole, B. T., PROC. SOC. EXP. BIOL. AND MED., 1956, v93, 290.

8. Haining, J. L., Axelrod, B., *J. Biol. Chem.,* 1958, v232, 193.

9. Stoddard, J. L., Drury, P. E., *ibid.,* 1929, v84, 741.

10. Bergstrom, S., Holman, R. T., in *Advances in Enzymology,* (Nord, F. F., Ed.) v8, pp425-457. Interscience Publishers, N. Y., 1948.

Received February 11, 1959. P.S.E.B.M., 1959, v101.