

## On the existence of lipid peroxides in rat tissue

By J. GLAVIND

*Department of Biochemistry and Nutrition, Polytechnic Institute,  
Lyngby, Denmark*

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1. The colorimetric micro-adaptation of the iodometric method and the colorimetric thiocyanate method for the determination of lipoperoxides were compared. Similar results were obtained when methyl linoleate hydroperoxide was tested, but when lipid from rat liver, muscle, kidney and testes was examined, substantial amounts were found by the iodometric, but almost nothing by the thiocyanate method.
2. The main reason for the discrepancy between the methods seems to be that the iodometric micromethod also estimates substances other than true lipoperoxides. The presence of ubiquinone and vitamin A in the organ extracts was shown to interfere in this way in the method.
3. The yellow colour which develops when retinol and its esters are tested by the iodometric micromethod is due not to liberated iodine but to conversion products of retinol.
4. It is concluded that the occurrence of substantial amounts of lipoperoxides *in vivo* has so far been demonstrated only in the adipose tissue, and not in the parenchymatous organs of the rat.

The theory that the mode of action of vitamin E is explained by its function as a biological antioxidant has been criticized in an important publication by Green, Diplock, Bunyan, McHale & Muthy (1967) and a number of subsequent papers in this journal by the same group of investigators. One of the publications (Bunyan, Murrell, Green & Diplock, 1967) claims the existence of significant amounts of lipid peroxides in liver, kidney, testes and leg muscle of the rat. Peroxides were found in normal animals, and the amounts were not influenced by dietary vitamin E.

In our laboratory, all attempts at the determination of lipoperoxides in normal parenchymatous organs have failed (Glavind & Hartmann, 1961). Bunyan *et al.* (1967) conclude that our method for the extraction of lipids has been unsatisfactory, and they also introduce a critical note about our method for the determination of peroxides in the extract. The discrepancy between our negative results and those of Bunyan *et al.* (1967) is, however, so fundamental that it can hardly be explained by an incomplete extraction of lipids in our studies. It was decided, therefore, to reinvestigate the question of the existence of lipoperoxides in rat tissues by means of a comparison between our method and that used by Bunyan and his co-workers.

### EXPERIMENTAL AND RESULTS

#### *Preparation of samples*

Male Wistar rats were given a commercial stock diet (Pelleted Rabbit Diet; Karens-mølle Ltd, Viby, Denmark). They were killed by exposure to nitrogen in a polyethylene bag and dissected. The tissues to be examined were quickly removed and extracted. Great care was taken to prevent any contamination with hair because the

lipids of rat hair contain large amounts of peroxides (Glavind & Faber, 1967). Extraction, rinsing and evaporation of the extracts were carried out as described by Bunyan *et al.* (1967). The dried extract was taken up in chloroform and samples were immediately taken for peroxide determinations.

#### *Analytical methods*

The peroxide determinations were made by the iodometric micromethod used by Bunyan *et al.* (1967) and by our own thiocyanate method (Glavind & Hartmann, 1955). All determinations were made in duplicate. The methods were controlled by recovery experiments in which a known quantity of methyl linoleate hydroperoxide was added to the extract. The hydroperoxide was essentially pure; it produced only one spot when tested by thin-layer chromatography on silica gel G. The solvent system was light petroleum (b.p. 40–60°)–ether–chloroform (6:3:1). The peroxide content determined by the iodometric macromethod (International Union of Pure and Applied Chemistry, 1964) was 7150  $\mu\text{Eq/g}$ .

Iodometric peroxide was determined, following as closely as possible the description of Bunyan *et al.* (1967), but spectrophotometric tubes with gas inlets and outlets were not available. The sample, dissolved in 6.3 ml chloroform–acetic acid (1:2), was placed in a graduated test-tube and de-aerated for 10 min by passing a current of purified nitrogen through the solution. With nitrogen still passing, 0.5 ml de-aerated aqueous KI solution was added. After a further 5 min the gas inlet (a 20  $\mu\text{l}$  measuring pipette) was elevated above the surface of the solution. The stream of nitrogen was continued, and the tube was protected from light. After 1 h the volume of the solution was controlled. A part of it was transferred to a Beckman cuvette, and the extinction at 380 nm was read as quickly as possible. Blanks were made as described by Bunyan *et al.* (1967), and their conversion factor was used for the calculation of the peroxide content.

Peroxide determination by the colorimetric thiocyanate method was carried out as described by Glavind & Hartmann (1955). Atmospheric oxygen was removed from the solutions by nitrogen in the same manner as in the iodometric method.

Attempts to detect chromatographic spots of lipoperoxides were made by thin-layer chromatography on silica gel G. The solvent system was light petroleum (b.p. 40–60°)–ether–chloroform (6:3:1), which is suitable for the separation of hydroperoxides. The acetic acid–KI–starch spray reagent of Bunyan *et al.* (1967), and the leuco-dichlorophenolindophenol–haemin reagent of Glavind & Christensen (1969) for visualization of peroxides were tried.

Unsaponifiable matter from the extracts was prepared by the procedure of Diplock, Green, Edwin & Bunyan (1960).

Further fractionation of liver lipids was carried out by quantitative thin-layer chromatography. All operations were carried out in the dark. The freshly prepared unsaponifiable fraction from 16 g liver was applied in streaks to two 20  $\times$  20 cm plates. Silica gel G (0.25 mm layer) was used as adsorbent, and light petroleum (b.p. 40–60°)–methyl ethyl ketone–acetic acid (94:5:1) was used for development. Zones located by inspection in visible and ultraviolet light were scraped off, eluted with peroxide-free diethyl ether, evaporated in weighed flasks and dissolved in chloroform. Portions

of each eluate were examined by the iodometric micromethod. The most concentrated eluate was rechromatographed on two plates. Development was made twice in light petroleum-methyl ethyl ketone-acetic acid (74:25:1). Otherwise the same procedure was used.

Retinol was estimated by the trifluoroacetic acid method of Neeld & Pearson (1963). Free retinol was used as a standard, and the estimates were expressed as  $\mu\text{g}$  retinol/g tissue.

Ubiquinone was estimated in portions of the unsaponifiable matter dissolved in 2500  $\mu\text{l}$  ethanol. The extinction at 275 nm was measured before and after the addition of 10  $\mu\text{l}$  saturated solution of potassium borohydride in dimethylformamide. The result was calculated on the basis of the decrease in extinction ( $E_{1\text{cm}}^{1\%} = 160$ , mol. wt = 781.2 for ubiquinone 45).

#### *Investigations into methods for determining lipoperoxides in rat tissues*

A comparison of the results of the two methods for determination of peroxide in rat liver is shown in Table 1. The result of the iodometric micromethod for methyl linoleate hydroperoxide was slightly higher than that of the macromethod. The

Table 1. *Comparison of methods for lipoperoxide determination in rat liver*

(Liver (5 g) was extracted as described on p. 20. The extract was evaporated to dryness and redissolved in 5 ml chloroform. The hydroperoxide solution contained 3.24 mg methyl linoleate hydroperoxide concentrate per ml)

| Method ...  | Iodometric                     | Thiocyanate |
|---|--------------------------------|-------------|
| Wavelength (nm)   | 380                            | 500         |
| Volume (ml)   | 6.5                            | 10          |
| Product   | Extinction (blanks subtracted) |             |
| 50 $\mu\text{l}$ liver extract  | 0.188                          | 0.018       |
| 10 $\mu\text{l}$ hydroperoxide solution                                     | 0.310                          | 0.483       |
| 50 $\mu\text{l}$ liver extract +<br>10 $\mu\text{l}$ hydroperoxide solution | 0.487                          | 0.476       |
| Product   | Calculated peroxide content    |             |
| Liver extract ( $\mu\text{Eq/g}$ fresh wt)                                  | 3.1                            | 0.2         |
| Methyl linoleate hydroperoxide ( $\mu\text{Eq/g}$ fresh wt)                 | 7800                           | 8900        |

thiocyanate method gave still slightly higher values, in agreement with other observations on the stoichiometry of this method. However, when a liver extract was tested by the two methods, a substantial amount was found by the iodometric micromethod and almost nothing by the thiocyanate method. When methyl linoleate hydroperoxide was added to the liver extracts, it was reasonably well recovered by both methods.

It was concluded that the reason for the discrepancy between the two methods was the presence in liver and muscle extracts of substances which were estimated only by the iodometric method. Since the discrepancy was especially high for liver, a study of the nature of the substances of rat liver estimated only by the iodometric micromethod was undertaken. The peroxide nature of these substances seemed questionable when it was discovered that they were not destroyed by saponification. They were concentrated from the unsaponifiable matter from liver by thin-layer chromatography. Attempts to locate zones by spray reagents failed. The chromatograms were therefore

divided into zones which were scraped off, eluted and examined by the iodometric micromethod. By repeated chromatography a concentrate was finally obtained which gave a value of about  $5 \mu\text{Eq}$  peroxide/mg. The spectral properties of the concentrate, together with the observation of a blue colour on spraying with 50% (v/v) sulphuric acid, indicated the presence of retinol.

Free retinol was prepared by saponification of the acetate (crystalline, synthetic vitamin A acetate; F. Hoffman - La Roche and Co., A.G., Basle). No peroxide was found when the retinol was tested by the iodometric macromethod but, when graded amounts were tested by the micromethod, values corresponding to a peroxide content of about  $5 \mu\text{Eq}$  peroxide/mg retinol were found.

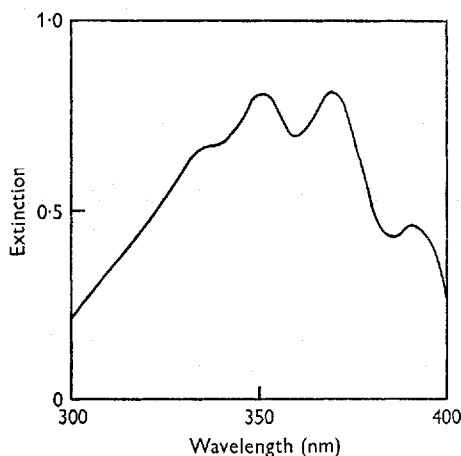


Fig. 1

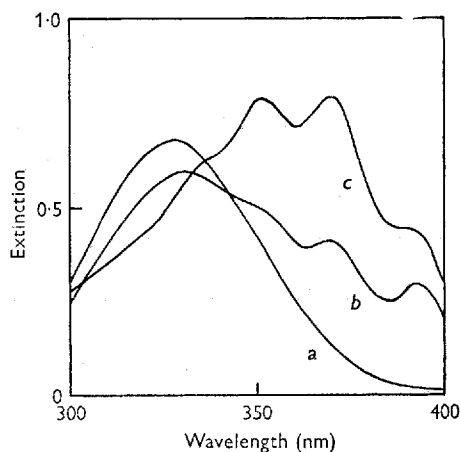


Fig. 2

Fig. 1. Spectrum of yellow material formed by a solution of retinol in acetic acid-chloroform in the presence of aqueous potassium iodide.

Fig. 2. Spectra of retinol and its conversion products in the conditions of the iodometric micromethod. (a) Retinol ( $25 \mu\text{g}$  in 6.5 ml acetic acid-chloroform); (b) 'sample blank' ( $25 \mu\text{g}$  retinol in 6.5 ml acetic acid-chloroform containing 0.5 ml water; spectrum taken after 60 min in the absence of atmospheric oxygen); (c) 'sample' ( $25 \mu\text{g}$  retinol in 6.5 ml acetic acid-chloroform containing 0.5 ml 10% (w/v) KI; spectrum taken after 60 min in the absence of atmospheric oxygen).

In contrast to the macromethod, the iodometric micromethod is a colorimetric method, based on the increment in extinction at 380 nm. The increment in extinction produced when retinol is tested is, however, not due to liberated iodine but to yellow conversion products of retinol. The conversion products were concentrated from a larger amount of retinol treated with chloroform-acetic acid-KI solution as in the conditions of the iodometric micromethod. Water was added, the chloroform phase was separated and washed with water, and the chloroform was evaporated. A yellow oil remained, which was examined in the spectrophotometer. The absorption band at 324 nm had disappeared, and new bands could be observed about 335 (weak), 350, 370 and 390 nm (Fig. 1).

The spectrum was reminiscent of those of anhydroretinol and retroretinyl acetate. Anhydroretinol has absorption maxima at 351, 370 (strongest) and 392 nm and is

formed by treating a solution of retinol in ethanol with dry hydrochloric acid (Edisbury, Gillam, Heilbron & Morton, 1932). Retroretinyl acetate has maxima about 333, 348 (strongest) and 367 nm and is formed by treating retinol acetate in methylene chloride with hydrobromic acid (Beutel, Hinkley & Pollak, 1955). Possibly the conversion product could be a mixture of anhydroretinol and a compound with the retroretinyl chromophore.

The spectra of a 'sample' and the corresponding 'sample blank' of retinol tested by the iodometric micromethod are presented in Fig. 2. Virtually all retinol seems to have been converted into coloured compounds in the 'sample'. The spectrum of the 'sample blank' still shows the presence of retinol, but a partial conversion has taken place.

Yellow material was also formed when retinyl acetate was tested in the conditions of the iodometric micromethod, but the spectrum was different from that of the conversion product formed from free retinol. Absorption maxima were observed at 335 (weak), 350 (strongest) and 370 nm. Probably a formation of retroretinyl ester had taken place. Some conversion had also taken place in the 'sample blank', and the difference between the extinctions at 380 nm of 'sample' and 'sample blank' corresponded to a 'peroxide' content of about 5  $\mu$ Eq/mg retinol acetate, which is of the same order of magnitude as found with free retinol.

A discrepancy between the two methods was, however, also found when tissues other than liver, e.g. muscle, were tested. Since the vitamin A content of rat muscle is negligible, substances other than vitamin A which are estimated only by the iodometric micromethod occur in rat tissues. Clearly the interference of vitamin A in the iodometric method is a special case of a general problem.

Compounds other than peroxides could have the property to liberate iodine when tested by the iodometric method. Among the compounds occurring in lipid extracts of animal tissues in more than negligible quantities, the ubiquinones could be suspected of such a property. A sample of ubiquinone 45 was weighed and dissolved in chloroform and tested by the two methods for peroxide determination. It was found that ubiquinone 45 was quantitatively estimated by the iodometric micromethod (2 Eq/mol) and also gave rise to some colour formation when tested by the thiocyanate method (corresponding to about 0.5 Eq/mol).

The importance of the interference of vitamin A and ubiquinone in the iodometric micromethod was investigated in experiments in which the amounts of the interfering substances in extracts of animal tissues were estimated by specific methods. Typical results of the determination of interfering substances and peroxide estimates in leg muscle, testes, kidney and adipose tissues of rats given the stock diet are presented in Table 2.

Results of the determination of peroxide by the iodometric method and of vitamin A in rat livers are presented in Table 3. The animals were given the basal vitamin E-deficient diet of Bunyan *et al.* (1967), modified by the omission of vitamin A and the replacement of lipid with 10% extra sucrose. They were distributed into three groups which were given no supplement of vitamin A and weekly doses of 300 and 4000 i.u., respectively.

Table 2. *Determination of lipoperoxides in some rat organs*

| Tissue ...  | Muscle |      | Kidney |      | Testes |      | Adipose tissue |     |
|---|--------|------|--------|------|--------|------|----------------|-----|
| Peroxide ( $\mu\text{Eq/g}$ tissue) by:                       |        |      |        |      |        |      |                |     |
| Iodometric micromethod  | 0.25   | 0.40 | 0.26   | 0.20 | 0.07   | 0.06 | 0.04           | 0.7 |
| Thiocyanate method  | 0.01   | 0.02 | 0.02   | 0.03 | 0.02   | 0.02 | 0.02           | 0.7 |
| Content of unsaponifiable matter:                             |        |      |        |      |        |      |                |     |
| Peroxide by iodometric micromethod ( $\mu\text{Eq/g}$ tissue) | 0.08   | 0.05 | 0.22   | 0.15 | 0.05   | 0.04 | 0.02           | 0.1 |
| Ubiquinone ( $\mu\text{mol/g}$ tissue)                        | 0.05   | 0.02 | 0.11   | 0.10 | 0.02   | 0.02 | —              | —   |
| Vitamin A (as $\mu\text{g}$ retinol/g tissue)                 | —      | —    | 2      | 3    | —      | —    | 1              | 5   |

Table 3. *Peroxide determinations by the iodometric micromethod and vitamin A estimates in rat liver extracts*

(Mean values with their standard errors. Male rats, 3 weeks old, were divided into three groups and given a vitamin A-deficient diet. Two of the groups were given 100 i.u. vitamin A three times weekly and 800 i.u. five times weekly, respectively. After 5–6 weeks, one rat of each group was killed on each of 4 successive days, and the livers were removed and examined)

| Weekly supplement of vitamin A (i.u./rat) | Peroxide estimate ( $\mu\text{Eq/g}$ liver) | Vitamin A estimate (as $\mu\text{g}$ retinol/g liver) |
|---|---|---|
| 0   | 0.095 $\pm$ 0.015                           | 8 $\pm$ 0.5   |
| 300                                       | 0.16 $\pm$ 0.02                             | 32 $\pm$ 2  |
| 4000                                      | 1.5 $\pm$ 0.1                               | 375 $\pm$ 20  |

## DISCUSSION

The iodometric micromethod for peroxide determination gave higher estimates than the thiocyanate method for lipid extracts of all organs examined, with the exception of adipose tissue. The discrepancy between the two methods was explained by the presence in the extracts of interfering substances which are estimated only in the iodometric micromethod. Two groups of interfering compounds were identified, retinol and its esters, and ubiquinone and possibly other quinones.

The importance of the interference of vitamin A and ubiquinone in peroxide determinations in normal rat tissues is illustrated in Table 2. As might be expected, since both retinol and ubiquinone will be present in the unsaponifiable fraction of lipids after saponification, the 'peroxide' found by the iodometric micromethod was partly recovered in the unsaponifiable matter. The peroxide-like material in the unsaponifiables from muscle, testes and kidney seems to be predominantly of quinoid nature; vitamin A contributes little in these organs, and the estimates for ubiquinone agree rather closely with the results of the iodometric micromethod (1 mol = 2 Eq).

The extracts, at least those of muscle, also contained, however, significant amounts of substances estimated only by the iodometric micromethod which were not recovered in the unsaponifiable matter. The nature of these substances has not been established. A Sorvall Omni-Mixer was used for disintegration of the muscles; the fluctuating results (0.25–1  $\mu\text{Eq/g}$  muscle) could indicate that artifacts formed during the vigorous mechanical treatment had contributed.



Liver is a special case since, in addition to ubiquinone, it contains the greater part of the body reserves of vitamin A. As illustrated in Table 3, the results of the iodometric micromethod for rat liver will reflect the vitamin A content. The results of the determinations of 'peroxide' and vitamin A were, however, not strictly parallel. The highest 'peroxide' estimates in proportion to vitamin A were found for the livers low in vitamin A, which indicates that substances other than vitamin A, possibly quinones, interfere in the iodometric micromethod in liver.

Bunyan *et al.* (1967) claim that they could confirm the results of their micromethod by a procedure using paper chromatography. A solution containing acetic acid, KI and starch was used as a spray reagent. It is our experience that this reagent, owing to the instability of KI-acetic acid and the necessity of heavy loading with starch, is inconvenient for visualization of weak spots of peroxides. We found a spray reagent containing haemin and leuco-dichlorophenolindophenol (Glavind & Christensen, 1969) more suitable. Our attempts to locate peroxides on thin-layer chromatograms of liver lipids were, however, on the whole negative. Only in some instances a very faint spot could be discerned at the point of application. The spot may be due to artifacts formed by autoxidation of the phospholipids after the extraction. In view of the conclusion that vitamin A is responsible for the larger part of the 'peroxide' estimates of the iodometric micromethod in liver, it seems that the chromatographic methods used by Bunyan *et al.* for the confirmation of their results have been unreliable.

The thiocyanate method has been used in the present studies as a control method for the iodometric micromethod. The thiocyanate method is, however, not specific for peroxides either but will estimate also other oxidizing substances which may be present in extracts of tissues, e.g. ferric ions and porphyrins, and to a certain extent also will be influenced by the presence of quinones. Therefore, it cannot be decided whether or not the minute amounts found by this method represent true lipoperoxides occurring in parenchymatous organs or other oxidizing substances or artifacts formed by autoxidation, especially of the highly unsaturated phospholipids, during the isolation of the lipids, even though protective precautions are taken.

Horgan & Philpot (1964) have extracted mouse organs in an anoxic box and examined the extracts under strictly anaerobic conditions by a colorimetric micromethod for peroxide determination based on the oxidation of leuco brilliant cresyl blue. They found small amounts in a number of organs, in liver and muscle about  $0.1 \mu\text{Eq/g}$ . These results substantiate the idea that normal animal organs contain small amounts of peroxide-like substances, but they give no clue to their final identification, and ubiquinone will also oxidize leuco brilliant cresyl blue. Horgan & Philpot state that the apparent peroxide estimated by their method may be a peroxide but also, for example, a quinone or a ferric compound. It seems reasonable to conclude that this statement holds true for the other methods also.

It must be concluded that the presence of true lipoperoxides in organs which contain significant amounts of interfering substances cannot be established through simple examination of extracts by the iodometric micromethod. For that reason, the evidence provided by Bunyan *et al.* (1967) for the occurrence of lipid peroxides as normal constituents of parenchymatous organs is inadequate. The apparent

constancy of the level of 'peroxides' in most organs is explained more simply as an estimate of metabolites other than peroxides, e.g. ubiquinones, than by the assumption of novel biochemical mechanisms for controlled formation of peroxides independent of vitamin E and unsaturated fatty acids.

On the other hand, although Bunyan *et al.* found significant amounts of peroxides in most tissues by the iodometric micromethod, and only insignificant amounts were found in studies in this laboratory using the thiocyanate method, the results of the two methods agree on one point, that the concentration of lipid peroxides did not increase in tissues pathologically affected by vitamin E deficiency. This is a crucial point of the discussion since, according to the biological antioxidant theory, the primary response to vitamin E deficiency is considered to be peroxidation of unsaturated fatty acids. We can agree with the criticism of Green *et al.* (1967) and Bunyan *et al.* (1967) that direct evidence for increases of peroxide formation in pathologically affected tissues has so far not been given, and that the indirect evidence provided, mostly consisting of so-called lipid peroxide measurements with the malondialdehyde test, is not very convincing.

In contrast to the lipid-soluble substances of parenchymatous organs, adipose lipids contain only negligible amounts of substances known to interfere in the iodometric micromethod. For that reason the iodometric micromethod will give essentially the same results as the macromethod or the thiocyanate method. Bunyan, Green, Murrell, Diplock & Cawthorne (1968) studied the concentration of lipid peroxides in the adipose tissue of rats. Their results agree well with those obtained in our laboratory and elsewhere; no peroxide was found in normal adipose tissue but lipid peroxide could accumulate in vitamin E deficiency when highly unsaturated fatty acids were given.

#### REFERENCES

- Beutel, R. H., Hinkley, D. F. & Pollak, P. I. (1955). *J. Am. Chem. Soc.* **77**, 5166.  
 Bunyan, J., Green, J., Murrell, E. A., Diplock, A. T. & Cawthorne, M. A. (1968). *Br. J. Nutr.* **22**, 97.  
 Bunyan, J., Murrell, E. A., Green, J. & Diplock, A. T. (1967). *Br. J. Nutr.* **21**, 475.  
 Diplock, A. T., Green, J., Edwin, E. E. & Bunyan, J. (1960). *Biochem. J.* **76**, 563.  
 Edisbury, J. R., Gillam, A. E., Heilbron, J. M. & Morton, R. A. (1932). *Biochem. J.* **26**, 1164.  
 Glavind, J. & Christensen, F. (1969). *Acta derm.-vene.* **49**, 536.  
 Glavind, J. & Faber, M. (1967). *Int. J. Radiat. Biol.* **12**, 121.  
 Glavind, J. & Hartmann, S. (1955). *Acta chem. scand.* **9**, 497.  
 Glavind, J. & Hartmann, S. (1961). *Acta chem. scand.* **15**, 1927.  
 Green, J., Diplock, A. T., Bunyan, J., McHale, D. & Muthy, I. R. (1967). *Br. J. Nutr.* **21**, 69.  
 Horgan, J. & Philpot, J. L. S. (1964). *Int. J. Radiat. Biol.* **8**, 165.  
 International Union of Pure and Applied Chemistry (1964). *Standard Methods of The Oil and Fats Division of the I.U.P.A.C.* 5th ed., section II, D.13. London: Butterworths.  
 Neeld, J. B. Jr & Pearson, W. N. (1963). *J. Nutr.* **79**, 454.