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Antioxidants in Animal Tissue

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Tentative methods for the determination of antioxidants in biological materials, based on the reaction with the stable free radical α,α-diphenyl-β-picrylhydrazyl, are described. The methods are used in a study on the occurrence of antioxidants in mammalian blood and livers. Three groups of substances giving the reaction are present: Fat-soluble antioxidants, water-soluble antioxidants, and proteins containing, *e.g.*, thiol-groups. The amounts of the three groups of substances are determined, and the results discussed in relation to data compiled from the literature on the occurrence of such substances.

A method for antioxidant determination by the use of the stable free radical α,α-diphenyl-β-picrylhydrazyl (DPPH) was proposed by Blois [1]. He found that the substance reacted quantitatively with cysteine, glutathione, ascorbic acid, tocopherol, polyhydroxy aromatic compounds, and certain aromatic amines. The SH groups of proteins were oxidized, whereas glucose, purines, pyrimidines or aromatic compounds with only a single hydroxyl group, *e.g.*, tyrosine, were not oxidized. A preliminary survey, carried out by Blois' method, on the occurrence of antioxidants in mammalian blood and liver has been carried out in this laboratory.

EXPERIMENTAL

Reagent solution. The solution is prepared by dissolving a few milligrams of DPPH in an amount of chloroform adjusted so as to give blanks of a suitable optical density (about 0.5).

Determination of antioxidants. Principle. The decrease in optical density at 517 mμ of a solution of DPPH, caused by the addition of a solution containing antioxidants, is a measure of the amount of antioxidants present. The strong absorption band at 517 mμ disappears by the addition of an antioxidant; instead of a deep violet color, a yellow color appears which gives only slight absorption at 517 mμ. Correction for turbidity or interfering colored substances can therefore be made by adding a surplus of an antioxidant, *e.g.*, pyrogallol, and taking a new reading. Interfering substances can also be eliminated by extracting the free radical and the yellow hydrazine compound with xylene from aqueous methanol or ethanol.

Procedure. Fat-soluble antioxidants. The antioxidant solution is pipetted into a test-tube, the volume adjusted to 3 ml with chloroform or ethanol, and 1 ml of the reagent

added. After 5 min the mixture is transferred to a 1 cm cuvette, and the optical density at 517 mμ is read in a Beckman spectrophotometer against a cuvette containing chloroform/ethanol as a blank. One drop of a concentrated solution of pyrogallol in ethanol is added and distributed in the cuvette with a small spatula. A new reading is taken after a few seconds, when constant, and subtracted from the first reading before the addition of pyrogallol. The difference between the two readings is called G_s.

The optical density of the radical solution is measured in the same way: One ml of the reagent is mixed with 3 ml solvent and the mixture treated exactly as when antioxidant is present. The difference between the readings at 517 mμ, before and after decolorization with a drop of pyrogallol solution is called G_r.

The reaction may be carried out in organic solvents other than chloroform and ethanol. If so, it must be taken into consideration that the light absorption of the free radical at 517 mμ depends on the solvent. It is higher, *e.g.*, in hexane than in ethanol, and the same solvents must be used for constructing the standard curve as for carrying-out the reaction.

Water-soluble antioxidants. The aqueous tissue extract is pipetted into a centrifuge tube, brought to 1.5 ml with water, and mixed with 3 ml of a solution of the reagent in methanol. After 5 min 4 ml of xylene is added, the contents of the tube mixed by shaking, and separated again by brief centrifuging. The upper xylene-layer is transferred to a Beckman cuvette, and the extinction, G_s, is read. At the same time a blank determination is carried out in the same way but using 1.5 ml distilled water instead of the tissue extract. The optical density of the blank is called G_r.

Calculation. $G_r - G_s$ is a measure of the amount of antioxidant. The amount is expressed as microequivalents. Calculations are made from a standard curve constructed with the use of pure ascorbic acid, *d,l-α*-tocopherol, or hydroquinone; 0.1 μequiv. of either of the substances will produce a decrease in optical density of about 0.22. Stoichiometry: Tocopherol, ubichromenol, hydroquinone, ascorbic acid: 1 μmole = 2 μequiv. Glutathione, ergothioneine or other substances containing 1 thiol-group per molecule: 1 μmole = 1 μequiv.

Extraction and fractioning of antioxidants. The antioxidants of animal tissues may be fat-soluble or water-soluble, and in addition the free radical may react with, *e.g.*, thiol-groups of non-dissolved proteins.

Antioxidants in blood were studied in heparinized blood from calves or rats. Plasma and corpuscles were separated by centrifuging. The non-dialyzable fraction of both was prepared by dialysis for 24 h against distilled water. Protein-free fractions were made by precipitation with tungstic acid[2], filtration, and adjusting the filtrates to pH 5.6 with sodium acetate. Ferricyanide-treated corpuscles were prepared by adding 0.5 ml of a 10 % potassium ferricyanide solution to 50 ml dialyzed corpuscles followed by renewed dialysis. When whole blood or corpuscles were diluted with water for the determination, time was allowed for hemolysis to take place before the methanolic reagent solution was added.

Suspensions of pig or calf liver were prepared with distilled water in a tissue homogenizer. Fractions of the homogenates were prepared with tungstic acid precipitation and in other simple ways in the same manner as in the case of blood.

Fat-soluble antioxidants were studied in extracts prepared by grinding liver in a Waring-blendor with chloroform in the presence of anhydrous sodium sulphate. The filtrate was evaporated *in vacuo* to a small volume and tested as described above.

The unsaponifiable fraction of the lipids was prepared in the following way: About 1 g chloroform extract was treated with 25 ml absolute ethanol by gentle heating. A minor amount of the extract was left as undissolved while the solution was transferred to a 250 × 33 mm test tube fitted with a reflux condensor and placed in a boiling water bath. After the ethanol had been boiling for 1 min, the reflux condensor was removed for a moment while 1 ml of a freshly boiled 50 % aqueous solution of potassium hydroxide was added. Boiling was continued for 10 min. The test tube was then taken out of the water bath, the reflux condensor removed, and a piece of dry-ice thrown into the ethanol to obtain rapid cooling without the access of atmospheric oxygen. The content of the tube was rapidly transferred to a separatory funnel containing 50 ml hexane which had been deaerated for 5 min in the funnel with a stream of nitrogen. After mixing, 25 ml water, freshly boiled and cooled with a piece of dry-ice, was added, and the contents of the funnel moderately shaken. The upper phase was washed five times with water, emulsions being

discarded. The hexane solution was dried over anhydrous sodium sulphate, filtered, and antioxidant determination carried out on an aliquot part.

The unsaponifiable fraction was further fractioned by chromatography on a column of alumina (anionotropic, Woelm). The fraction was brought on the column dissolved in hexane-benzene, and fractions collected using successively stronger eluents: ether, acetone, and chloroform-methanol. Each fraction was evaporated, dissolved in ethanol, and determinations carried out on aliquot amounts.

Ferric chloride-bipyridyl reaction. Determinations of ferric chloride-reducing substances were carried out on the lipid extract and its fractions in the following way: An aliquot part was dissolved in 3 ml ethanol, 0.2 ml 0.5 % α,α'-bipyridyl in ethanol was added, followed by 0.2 ml 0.2 % ferric chloride (hexahydrate) in ethanol. The mixture was rapidly transferred to a 1 cm Beckman cuvette, and the density read at 520 mμ, 30 sec and 3 min after the addition of ferric chloride. The results were calculated from a standard curve employing pure α-tocopherol and expressed as μequiv. ferric chloride-reducing substances.

RESULTS

Typical results from the determination of antioxidants in mammalian blood and livers and their fractions, carried out by the methods given above, are presented in Tables 1, 2, and 3.

Table 1. Antioxidants in fractions of blood.

Nature of fraction	Antioxidant μequiv./ml blood
Whole blood	19.6
Plasma	0.30
Corpuscles	19.2
Lipid extract of whole blood	0.01
Plasma	0.30
Dialyzed plasma	0.18
Tungstic acid filtrate of plasma	0.11
Corpuscles	19.2
Dialyzed corpuscles	17.6
Ferricyanide treated corpuscles	7.7
Tungstic acid filtrate of corpuscles	2.8

Table 2. Antioxidants in fractions of liver.

Nature of fraction	Antioxidant μequiv./g liver
Homogenate of liver, 1 part with 15 parts of water	27
Supernatant from gentle centrifuging of homogenate	25
Non-dialyzable fraction of supernatant	12
Tungstic acid filtrate from supernatant	12
Fraction of supernatant soluble in 90 % ethanol	11
Chloroform extract of liver	0.31

Table 3. Antioxidants in fractions of a chloroform extract of liver.

Nature of fraction	Antioxidant by DPPH-method	Ferric chloride-reducing substances, colorimetry after	
		30 sec	3 min
	μequiv./g liver		
Chloroform extract	0.31	0.37	0.67
Unsaponifiable	0.27	0.29	0.49
Chromatographic fraction eluted with			
Hexane-benzene	0.02	0.02	0.02
Ether	0.11	0.11	0.13
Acetone	0.12	0.07	0.17
Chloroform-methanol	0.01	0.00	0.00

DISCUSSION

It will be understood that the most prominent substances of antioxidative properties, detected by the DPPH-method, that are known to occur in animal tissues, can be divided in the following groups: 1. Glutathione, ergothioneine, ascorbic acid and other dialyzable, water-soluble substances. 2. Thiol- and other reactive groups of proteins. 3. Tocopherols and other fat-soluble antioxidants.

It appears clearly from Table 1 that the bulk of the antioxidant activity of blood is found in the corpuscles and is caused mainly by non-dialyzable substances. A larger part of the activity of dialyzed blood cells (9.9 μequiv./ml blood) disappears by treatment with potassium ferricyanide and is probably due to the ferrous iron of hemoglobin, while the remaining activity (7.7 μequiv./ml) may be attributed to the thiol-groups which, according to Mirsky and Anson,[3] are oxidized to only a small extent at neutral pH. The hemoglobin molecule has 4 iron atoms, and ox hemoglobin was found by Snow [4] to contain 2 active thiol-groups per molecule. The hemoglobin content of the blood was estimated at 15.5 %. Assuming a molecular weight of 68 000, it can be calculated from the figures that the iron and the thiol-groups of the hemoglobin should contribute about 9.1 and 5.0 μequiv., respectively, to the antioxidant activity of the blood, in reasonable agreement with the findings just reported.

The principal dialyzable antioxidants of mammalian erythrocytes are glutathione, ergothioneine, and ascorbic acid. Estimates of the concentrations of the substances in the erythrocytes, compiled from the literature,[5] are: Glutathione 0.5, ergothioneine 0.1, ascorbic acid 0.01 mg/ml whole blood, corresponding to about 1.6, 0.4, and 0.1 μequiv./ml or a total of about 2 μequiv./ml blood. This figure again agrees fairly well with the results reported in Table 1: 2.8 μequiv./ml blood was found in the tungstic acid filtrate from the corpuscles, and 1.6 μequiv./ml can be calculated, probably as a less exact estimate, as the difference between non-dialyzed and dialyzed corpuscles.

An activity of only about 0.3 μequiv./ml blood which decreased by dialysis to 0.18 μequiv./ml was found in plasma. The decrease agreed well with the value 0.11 μequiv./ml blood found in plasma tungstic acid filtrate, and corresponds to an ascorbic acid content in plasma of 0.01 mg/ml blood. The results, for both nondialyzable and dialyzable antioxidants are of the same order as

the figures from the literature,[5] ascorbic acid about 0.01 mg/ml plasma, thiol-groups in serum-proteins 0.54 μmole/ml plasma.

An amount of fat-soluble antioxidants in blood of 0.01 μequiv./ml was found. The only fat-soluble antioxidants known to occur in blood are tocopherols and ubichromenols. Edwin *et al.*[6] found a content of 0.014—0.015 mg ubichromenol and 0.012—0.015 mg α-tocopherol per g blood clot and about 0.005 mg α-tocopherol per g serum of rats on an adequate stock diet. From the figures a content of about 0.07 μequiv. antioxidants per ml blood can be calculated. A probable explanation of the much lower figure reported in Table 1 would be that the method of extraction, the same as was used for liver, is inadequate for blood.

When the figures of Table 2 are studied, it can be calculated that the soluble, but non-dialyzable substances contributed about 12 μequiv./g to the antioxidant properties of liver, while only 2 μequiv./g liver could be attributed to the precipitate removed by centrifuging. However, the method should probably be modified to be considered appropriate for the use on non-dissolved substances.

With respect to the dialyzable antioxidants, Register [7] reported for rat liver a content of glutathione 8.18, ergothioneine 0.70, total non-protein thiol-groups 9.32 μequiv./g. When the ascorbic acid content is also taken into consideration (about 0.2—0.3 mg/g liver,[5] corresponding to about 3 μequiv./g), the total amount of dialyzable antioxidants can be calculated to 12—13 μequiv./g liver. The figure agrees well with the results reported in Table 2, 12 μequiv./g liver in the tungstic acid filtrate, 13 μequiv./g when the difference between non-dialyzed and dialyzed supernatant is calculated.

An amount of fat-soluble antioxidants of 0.31 μequiv./g liver was found, *i.e.*, almost two orders of magnitude less than that of the water-soluble antioxidants.

The fat-soluble antioxidants in liver were studied in more detail in the experiments reported in Table 3. After saponification the activity could be recovered in the unsaponifiable compounds. Further attempts to concentrate the active compounds were made by chromatography. All fractions were tested both by the DPPH-method for antioxidants and by the ferric chloride-bipyridyl reaction. The latter test has been widely used for the determination of tocopherols. It is, however, not specific but is also given by ubichromenol, and, furthermore, by, *e.g.*, vitamin A and carotenoids. Vitamin A and carotenoids do not react with DPPH, and the reaction with ferric chloride-bipyridyl is slower than in the case of α-tocopherol and ubichromenol. When the ferric chloride-bipyridyl reactions were carried out, the optical densities were read after 30 sec and after 3 min. By the first reading carotenoids etc. will have only partially reacted.

When inspecting Table 3 it is seen that the antioxidant activity of the different chromatographic fractions follows rather closely the content of ferric chloride-reducing substances estimated from the reaction after 30 sec. The results indicate that the substances accounting for the antioxidant activity found in liver lipids are probably tocopherols and ubichromenols. Obviously it cannot be finally settled whether other fat-soluble antioxidants are present since no proper identification was made. From a quantitative view-point the

value 0.31 μequiv./g corresponds fairly well with the results of Edwin *et al.*[6], who found about 0.05 mg ubichromenol and 0.02 mg α-tocopherol per g liver of rats on an adequate stock diet, corresponding to a total of about 0.21 μequiv. antioxidants per g.

As a conclusion it can be stated that the DPPH-method seems very promising for the study of the function of biological antioxidants. It should be very appropriate in further studies to decide whether antioxidants other than those already known occur in the organism. The method, due to the unusual character of its specificity, probably also will appear valuable for special purposes, *e.g.*, the determination of tocopherols and ubichromenols, or of thiol-groups of proteins. It must be remarked, in this connection, that the inclusion of thiol-containing proteins among the antioxidants is a matter of definition: The substances giving the DPPH-reaction were divided into three groups, fat-soluble, dialyzable, and non-dialyzable. The first group is represented by tocopherol whose action as a biological antioxidant is well established, and by ubichromenol which shows some protection against certain symptoms of vitamin E-deficiency in animals. Certain water-soluble, dialyzable substances like ascorbic acid and cysteine have effects similar to those of ubichromenol, so that the action as biological antioxidants is known for this group also. It is less current to consider the thiol-groups of proteins as functional antioxidants. They will probably react with free radicals like the true antioxidants do, but in certain cases the result might not be that of a protective effect but rather the attack on a vulnerable part of the protein-molecule.

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