BIO-ASSAYS FOR OXIDATIVE STRESS STATUS (BOSS)

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Bio-Assays for Oxidative Stress Status (BOSS)

INTRODUCTION TO OXIDATIVE STRESS STATUS (OSS)

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The series of chapters included in this volume were originally published as articles in a FORUM, a symposium-in-print that is a feature of the journal *Free Radical Biology & Medicine,* a journal that Dr. Kelvin Davies and I and four outstanding scientists, edit. The FORUM was entitled OSS – OXIDATIVE STRESS STATUS and was the longest and largest FORUM we had yet published – it encompassed 7 issues of *FRBM* and included 31 articles by some of leading authorities in the field. [In 2001, the name FORUM was changed to CONTINUING REVIEWS and, because of the over whelming success of *FRBM*, some modest page-length restrictions were placed on these serial reviews.] Generally, a leading authority is chosen to act as organizer, editor, and facilitator of these serial reviews; for the OSS review, I served in this role.

Many of us in the field of oxidative biology have attempted to design – indeed, have dreamed of the day when it will be routine to use – a method for measuring OSS. This OSS method would give the instantaneous level of oxidative stress in an organism. Why might that be useful? Here are a number of observations relevant to OSS:

- Diseases such as cancer, cataract, and heart disease are known to involve oxidation of a number of types of biomolecules, and therefore detecting the levels of this oxidation could provide an early warning signal.
- Trials of drugs or antioxidant vitamins often take years to develop sufficient case data to allow statistically robust conclusions. If OSS measurements tracked with the decease being studied, it might be possible to use OSS as an early end point.
- Since the oxidized biological molecular products from oxidative stress are generally more stable than the oxidant itself - i.e., oxidized lipids, proteins, and nucleic acids are more stable than the free radicals that effected their oxidation, OSS measurements often turn out to involve determining the level of a Biomarker of Oxidative Stress Status (a BOSS). Thus, one obtains evidence of the activities of an elusive animal by its footprints and usually does not expect to see the creature itself.
- In an article that introduced the first group of OSS FORUM articles, I explained the historical reference for the acronym "OSS" and cited the career of "Wild Bill" Donovan, who founded the Office of Strategic Services (OSS) at the time of World War II. [See *FRBM* 27, 1135-11365(1999); that introduction is also included in this volume].
- We know that all classes of biomolecules are oxidized, but we do not know the relative average rates (or even if there is an "average rate of oxidation of different classes of biopolymer molecules").
- Which oxidation products are best related to a given disease state? For example, are oxidized DNA bases such as 8-oxodeoxyguanosin most closely associated with cancer? Is oxidized LDL most associated with heart disease?
- How much do measures of OSS vary in a group of humans?
- Can a non-invasive, reliable, repeatable measure of OSS be identified?
- Does OSS decrease as a result of life-change factors? From taking vitamin E or other antioxidant vitamins or substances?
- Does OSS increase with age? With disease? With stress? If OSS does increase with age, does that "prove" Denham Harman's theory that free radical oxidation "causes" aging?

We, the editors and publishers of *FRBM* hope that this collection of articles will reveal to the general reader the status of studies of OSS, currently used examples of BOSS, and answers to at least some of the questions posed above.

September 2001

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OXIDATIVE STRESS STATUS: OSS, BOSS, AND "WILD BILL" DONOVAN

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The Forum that makes its first appearance in this issue, *Methods to Measure Oxidative Stress Status*, will be the most extensive yet published by *FRBM*. In this issue we present the first seven articles on this subject; we will publish other groups of papers for this Forum in the coming months, with a total number of contributions near 30.

Almost a decade ago [1,2], Susan Godber and I took upon ourselves the task of searching the literature to discover if many methods for measuring the oxidative stress status (OSS) of individuals had been published, and, if so, if there was a consensus on which methods were the most useful and reliable for measuring what we called OSS.[1] Sad to say, the answers appeared to be "a huge bunch" and "no," respectively. By 1993, the situation had not changed much [4], although the efforts of the Vanderbilt group with isoprostanes, "non-natural" isomers resulting from the nonenzymatic, free radical bicyclization of arachidonate [5], looked very promising [6–12].

This year, the National Institutes of Health (NIH) again visited the question of OSS methods and their reliability. A group of rats were given carbon tetrachloride, a toxin known to act via free radical reaction mechanisms, and the tissue was distributed to a number of groups with the plan that they all would try OSS measurements, meet at the hitching post near the OK Corral at the end of the day, and compare results. Some of those groups, in fact, will report their findings as part of this Forum. The NIH, proving again the Darwinian notion that evolution brings benefits, named the methods "BOSS," for Biomarkers of Oxidative Stress Status. Of course, as readers of *FRBM* are only too aware, radicals generally have only a fleeting existence in vivo, and "footprints" of these elusive species must be discovered—hence, BOSS. Well-recognized examples of BOSS are oxidized lipids (such as aldehydes and isoprostanes), oxidized amino acid residues (such as carbonyl-labeled), or oxidized DNA bases (such as 8-oxodG). The faithful readers of this journal need not be told the names of the many extremely talented groups working in this area and the various methodologies that have been tested and are under development.

The concept that diseases raise the OSS of an individual, or at least of some tissue in that individual, is both obvious and attractive. Many a free radical biologist has dreamed of the day when a simple blood or urine test would divulge the OSS of an individual and the prediction that some organ or tissue might need closer examination. If a marker, a BOSS, could be developed that would predict that an individual has a greater chance of developing disease, or already has a disease, that clearly would be both a confirmation of the concept that oxidative processes can lead to pathologic results and a very useful medical advance. There is some hope, for example, that lower levels of antioxidant nutrients in low-density lipoprotein (LDL) and/or the greater oxidizability of an individuals's LDL might be predictive of cardiovascular disease [13,14].

Originally, our intent in this Forum was to stress BOSS methods that are easily applied techniques that could be used, at least with further development, noninvasively, and thus, could ultimately make a contribution to clinical medicine. However, as authors suggested contributions, and as this Forum developed, the scope of the Forum was expanded. Thus, you will find that all of the contributions to this Forum do not present methods that are now, or perhaps ever could lead to, practical, noninvasive methods for measuring OSS. Indeed, if they did,

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[1]Methods of oxidative stress measures attempt to "spy" out the oxidative status of a subject. By using the acronym "OSS", we remember and honor America's first "big-time" spy, William ("Wild Bill") Donovan [3], the founder of what was called the Office of Strategic Services during World War II (and which later became the CIA—but that is another story).

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there probably would be no need for the Forum! Instead, we hope that by providing a collection of methods, discussed by their developers and protagonists, that readers will both learn the state of the field and, also, get ideas as to how one or more methods could be made more useful, and therefore, a greater contribution to human health.

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NOVEL HPLC ANALYSIS OF TOCOPHEROLS, TOCOTRIENOLS, AND CHOLESTEROL IN TISSUE

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Abstract—Tocopherols and tocotrienols are being increasingly recognized to have an important role in the prevention of atherosclerosis. It has been reported that they protect low-density lipoprotein (LDL) and tissues from oxidative stress and that tocotrienols can reduce plasma cholesterol levels. Two isocratic high-performance liquid chromatography (HPLC) methods for simultaneous analysis of tocopherols, tocotrienols, and cholesterol in muscle tissue were developed. Method A involves basic saponification of the sample, but causes losses of the γ- and δ-homologs of vitamin E. Method B does not involve saponification, thereby protecting the more sensitive homologs. Both permit rapid analysis of multiple samples and neither requires specialized equipment. These methods may provide techniques useful in simultaneous assessment of oxidative stress status (OSS) and cholesterol levels. © 1999 Elsevier Science Inc.

Keywords—Tocopherols, Tocotrienols, Free radical, Cholesterol, HPLC

INTRODUCTION

It is well established that lipid oxidation can have deleterious effects on human health [1–5]. Antioxidants play a major role in controlling lipid oxidation in vivo and in food. The usefulness of supplementation of human diet with antioxidants is still being evaluated [6], but it is generally accepted that increased levels of antioxidants can have beneficial effects on human health by increasing resistance of tissues and lipoproteins to oxidative stress. Therefore, antioxidant quantification provides one method for determining oxidative stress status (OSS).

Vitamin E is one of the most important naturally occurring primary antioxidants and it plays a very important role in protecting LDL from oxidative modification, which has been implicated in the development of atherosclerosis [7].

Vitamin E is a generic term that includes all entities that exhibit the biological activity of α-tocopherol. In nature, eight substances have been found to have vitamin E activity: d-α-, d-β-, d-γ-, and d-δ-tocopherol; and d-α-, d-β-, d-γ-, and d-δ-tocotrienol. Each of these forms of vitamin E has a different biopotency [8]. α-Tocopherol is traditionally reported to have the highest biological activity of all homologs, based on the fetal absorption test or the hemolysis test [9]. However, these are not the most critical tests and their relevance to physiologic importance and health benefits has been questioned [10]. Tocotrienols, on the other hand, exhibit characteristics that would render them more important than originally thought [10–12]. Tocotrienols exhibit a higher recycling efficiency in microsomes and liposomes in the presence of reduced nicotinamide adenine dinucleotide phosphate or ascorbate; they have a more uniform distribution in membrane bilayer; and cause a stronger disordering of membrane lipids, making interaction with lipid radicals

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Paul Addis received his B.S. degree from Washington State University in 1962 and his Ph.D. degree in Food Science from Purdue University in 1967. He joined the University of Minnesota in January of 1967 and was granted a leave for research at the Max Plancx Institute at Marienesee. He has studied at the Unviersity of Washington, University of California at Davis and at San Diego. His research has included muscle protein and enzymes, lipids, lipid oxidation, and more recently, the conversion of cellulose into a soluble fiber.

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more efficient than tocopherols. Tocopherols and tocotrienols not only inhibit radical-induced lipid oxidation, but they can also quench singlet oxygen ($O_2^1\triangle g$) both in vivo and in vitro [13].

Tocotrienols have been known to help lower plasma cholesterol levels by regulating HMG-CoA reductase in the liver [14–17] and, thus, decrease the risk for cardiovascular disease in hypercholesterolemic humans [18]. They have also been shown to reduce the concentration of apolipoprotein B, thromboxane B_2, and platelet factor 4 in pigs with inherited hyperlipidemias [19].

Muscle tissue contains 2–3 ppm of α-tocopherol and traces of α-tocotrienol [20] and, thus, there are not many methods for separating and quantifying tocopherols and tocotrienols in muscle tissues. The recent interest in the use of the other vitamin E homologs as antioxidants and the cholesterol reducing effects of tocotrienols create the need for the development of a rapid method for the analysis of these compounds in muscle. Several HPLC methods for the analysis of α-tocopherol in meat have been developed [21,22]. The method developed by Liu et al. [22] involves a 15-min saponification step and it is a very time-efficient one, allowing for the analysis of many samples per day. There are several HPLC methods for the analysis of all eight homologs of vitamin E for cereal products [23–25], but they are not applicable in muscle tissue. This is mostly due to the fact that muscle contains higher levels of protein, which interferes in the process of extraction (foaming and formation of emulsions).

Cholesterol analysis is usually done by enzymatic [26] or chromatographic methods. The most common chromatographic method is gas chromatography [27,28], although there are some HPLC methods [29]. Most of these chromatographic methods include a laborious saponification-extraction procedure, usually overnight, and a chromatographic run of 15–25 min. Because both vitamin E and cholesterol are nonpolar compounds that absorb in the ultraviolet (UV) range, it is possible that they could be analyzed simultaneously.

Our objective was to develop a rapid method for the efficient extraction, separation, and quantification of vitamin E homologs and cholesterol in muscle tissue.

MATERIALS AND METHODS

Tocopherol and cholesterol standards were purchased from Supelco (Bellefonte, PA, USA). Tocotrienol standards were a gift from Eastman (Kingsport, TN, USA) and the Palm Oil Research Institute of Malaysia (Kuala Lumpur, Malaysia). All solvents were HPLC grade.

Two extraction methods were developed. Method A involved saponification whereas method B did not. The chromatographic conditions were the same for both methods.

Method A

One gram of sample was placed in a screw-cap tube and 0.25-g ascorbic acid and 7.3-ml saponification solution were added. The saponification solution was 55% ethanol in distilled water (v/v) with 11% potassium hydroxide (w/v). The tubes were placed in a shaking water bath for 15 min at 80°C. After the saponification, the tubes were cooled in tap water for 1 min, and 4-ml hexane and 2-ml distilled water were added. The added water increases the polarity of the aqueous phase and improves partitioning of vitamin E and cholesterol into the organic (hexane) phase. Tubes were vortexed and the upper layer (hexane) was collected in a screw-cap vial for analysis by normal phase HPLC.

Method B

Two grams of postmortem bovine muscle were placed in a 100-ml plastic tube, 8-ml of absolute ethanol were added, and the mixture was homogenized for 30 s in a PowerGen 700 (Fisher Scientific, Pittsburgh, PA, USA) homogenizer. Ten milliliters of distilled water were added to the tube and sample was homogenized for 15 s. Finally, 8-ml hexane were added and the sample was homogenized for 15 s. The tubes were capped and centrifuged at 1500 rpm for 10 min. The upper layer (hexane) was collected and analyzed by normal phase HPLC.

Chromatography

The HPLC consisted of a Varian 9010 pump and a Varian 9050 UV detector (Varian Associates, Houston, TX, USA). Twenty microliters were injected into a silica column (Zorbax RX-SIL, 5-μm particle size, 4.6-mm ID $\times$ 25 cm). The mobile phase was hexane-isopropanol (99:1). Flow rate was 1.3 ml/min. The wavelength was programmed at 295 nm for the vitamin E homologs and then it was switched to 202 nm for cholesterol. All eight tocopherols and tocotrienols were eluted within 8 min, the wavelength was changed at 9 min, and cholesterol was eluted at 11 min. A typical run lasted approximately 12 min.

RESULTS AND DISCUSSION

Several extraction procedures and solvents were tested. Methanol is a very good extractant of vitamin E [23,25] in grain and cereal samples, but it also extensively extracts and denatures proteins in muscle, causing foaming, and making volume reduction by rotavaporation impossible. Extraction with methanol:chloroform, 2:1 resulted in poor recovery (~60%). The method of Liu et al. [22] produced the best recoveries.

Method A (which is based on the method of Liu et al. [22]) is useful for the analysis of the α-homologs of vitamin E (α-tocopherol and α-tocotrienol). However, other homologs, and especially the δ-homologs, are rapidly degraded during the saponification process. The recoveries for the homologs after saponification were as follows: α-tocopherol, 95%; α-tocotrienol, 95%; β-tocopherol, 95%; γ-tocopherol, 94%; γ-tocotrienol, 85%; δ-tocopherol, 80%; and δ-tocotrienol, 44%. The recovery of cholesterol was 84%.

It is generally known that vitamin E is less stable in alkaline conditions. There was no reference found in the literature regarding the fact that the δ-homologs, and in particular, δ-tocotrienol, are so much more susceptible to degradation during saponification than the α-homologs. This must be due to the fact that there is only one methyl group on the chromane ring, providing less protection to the hydroxyl group compared with the three methyl groups in the α-homologs. Even if cold saponification was used and α-tocopherol was present at high levels, the δ-homologs were destroyed during saponification. Given the fact that most methods are developed based on α-tocopherol, and that there are no commercially available standards for tocotrienols, it is possible that the contents of the γ- and δ-homologs are being underestimated if the sample is saponified.

It is clear that saponification can be used for α-tocopherol and α-tocotrienol analysis, but not for the other homologs. Method B was developed so that the more sensitive γ- and δ-homologs (especially δ-homologs) could be accurately quantified as all homologs of vitamin E are excellent antioxidants.

For method B, the recovery for vitamin E was approximately 96% for all of the homologs, and for cholesterol was 94%. Cholesterol always exhibits slightly lower recovery than vitamin E because it is more polar and the partitioning coefficient of cholesterol (between the organic and aqueous phase) should be slightly lower than that of vitamin E. The high recovery rates make it clear that virtually no degradation of vitamin E or cholesterol took place during the extraction procedure.

In many of the vitamin E methods published, fluorescence detection was used (excitation at 290 nm and emission at 320 nm). Even though it is far more sensitive than UV detection (at 295 nm), fluorescence detectors are very expensive and in some instances fluorescence quenching by other compounds (i.e., iodine, bromine) can affect the results [9].

In conclusion, method B is a new, rapid method for the extraction, separation, and quantification of all vitamin E homologs and cholesterol in muscle tissue. It is not a laborious method, it does not involve lengthy saponification procedures, and it does not require very specialized equipment, allowing for the analysis of many samples per day with relatively low cost. It is also a useful addition to the current methodology because there are no other good alternatives that combine the foregoing attributes. With further modification, it could be used to analyze for vitamin E and cholesterol content in LDL and other lipoprotein particles in blood.

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ABBREVIATIONS

HMG-CoA—3-hydroxy-3-methylglutaryl coenzyme A
HPLC—high-performance liquid chromatography
LDL—low-density lipoprotein(s)
OSS—oxidative stress status

Bioassays for Oxidative Stress Status (BOSS). Edited by W.A. Pryor

BASELINE DIENE CONJUGATION IN LDL LIPIDS: AN INDICATOR OF CIRCULATING OXIDIZED LDL

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Abstract—The wide acceptance of the diene conjugation-method in monitoring low-density lipoprotein (LDL) oxidation ex vivo has led to development of an assay, which measures the amount of baseline diene conjugation (BDC) in circulating LDL, and is an indicator of oxidized LDL in vivo. The LDL-BDC assay is based on precipitation of serum LDL with buffered heparin, and spectrophotometric determination of baseline level of conjugated dienes in lipids extracted from LDL. Compared to existing methods for oxidized LDL, LDL-BDC is fast and simple to perform. Chemical studies by HPLC and NMR have verified that LDL-BDC is a specific indicator of circulating mildly oxidized LDL. Validity of the assay is further indicated by strong correlation with the titer of autoantibodies against oxidized LDL. Clinical studies have shown that LDL-BDC is closely related to coronary, carotid, and brachial atherosclerosis. Moreover, several independent studies have demonstrated surprisingly strong associations between LDL-BDC and known atherosclerosis risk factors (obesity, physical inactivity, hypertension, diabetes, and arterial functions). Indeed, these studies seem to indicate that as an indicator of the risk of atherosclerosis LDL-BDC clearly exceeds sensitivity and specificity of the common lipid markers of atherosclerosis. It is concluded that LDL-BDC is a promising candidate in search for methods for the evaluation of in vivo LDL oxidation and the risk of atherosclerosis. © 1999 Elsevier Science Inc.

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INTRODUCTION

For several years it has been known that development of atherosclerosis is related to the level of the "noxious" low density lipoproteins (LDL). More recent studies have revealed that ultimate atherogenic agents are in fact the modified, mainly oxidized, forms of LDL [1–7]. Oxidative damage to LDL may range from the slight structural alterations of mildly oxidized LDL to extensive breakdown of lipids and apolipoprotein B of fully oxidized LDL. In vitro studies as well as studies with experimental animals show that the various oxidized forms of LDL contribute to atherogenic processes by multiple mechanisms [1–7].

Despite convincing evidence from the in vitro and animal studies, data concerning the role of oxidized LDL in development of atherosclerosis in humans have remained limited. An obvious reason for the scantiness of human studies has been the lack of methods for direct measurement of oxidized LDL, applicable for the necessary large-scale epidemiologic studies [8]. Thus far, estimation of in vivo LDL oxidation has been based by large on determination of autoantibodies to oxidized LDL [8,9]. This methodology, however, does not seem to be practical for clinical purposes. In particular, repro-

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ducible preparation of antigens (oxidized LDL) is in practice not possible, and this has caused much variability to results, most notably between laboratories [10]. Hence, direct comparison of results from different laboratories is not recommended [9]. Thus, there is "need for a rapid and specific measure of LDL oxidation that could become a part of the laboratory repertoire in the diagnosis and management of atherosclerosis" [7]. In the following, a method will be introduced which measures the amount of baseline diene conjugation in circulating LDL, and is an indicator of oxidized LDL in vivo.

ESTIMATION OF IN VIVO LDL OXIDATION

Due to the heterogeneous nature of the chemistry of LDL oxidation, proper determination of oxidized LDL is problematic. Much of the present knowledge on LDL oxidation comes from studies recording the kinetics of LDL oxidation ex vivo [3]. The estimation of in vivo LDL oxidation has been largely based on immunologic methods. F2-isoprostanes, which seem promising as indicators of whole-body oxidative stress, have also been considered as an alternative analytical means (c.f., [11]). Finally, popularity of the diene conjugation method as an able means to monitor LDL oxidation ex vivo has led to development of an in vivo assay, which measures as an index of oxidized LDL the amount of conjugated dienes present in circulating LDL [12,13].

LDL diene conjugation

Determination of diene conjugation has been for decades one of the basic methods for the measurement of lipid peroxidation. Rearrangement of double bonds in polyunsaturated fatty acids, i.e., the formation of conjugated dienes, is an early event of lipid peroxidation taking place soon after initiation of the chain reaction [14]. The oxidation-induced increase of diene conjugation in LDL lipids is well documented [3], and after introduction of the method by Esterbauer et al. [15], measurement of diene conjugation has become the most popular method to monitor oxidation of LDL ex vivo [8]. For this method of ex vivo oxidizability, LDL is first isolated by ultracentrifugation, then dialyzed, and finally subjected to an oxidizing agent (most commonly copper ions). Proceeding of the LDL oxidation ex vivo can be followed by the various markers of lipid peroxidation [3], but due to its convenience and objectivity, the change of the conjugated diene absorbance at 234 nm has in practice become the most popular means for the monitoring of LDL oxidation. The time course of forced LDL oxidation ex vivo can typically be divided into three consequtive phases: (i) lag phase, (ii) propagation phase, and (iii) decomposition phase. The lag phase (time before onset of lipid peroxidation) has been considered as the most important feature of oxidizability in this assay system [16] although other measures of the assay (e.g., maximum rate of diene formation, total amount of dienes) are also being used. This experimental system has been extensively used to study factors affecting oxidizability of LDL. It has enabled estimation of the contribution of various endogenous antioxidants in protection of LDL from oxidation [3,17,18] and, consequently, the method has become a popular means in testing of novel antioxidant compounds.

Encouraged by usability of the diene conjugation measurement in ex vivo studies, the LDL baseline diene conjugation method (LDL-BDC) was developed to be used as an indicator of LDL oxidation in vivo [12,13]. This method measures the actual amount of conjugated dienes in LDL, but not diene conjugation during or after chemically induced oxidation of LDL ex vivo. Compared with other methods for oxidized LDL in vivo, the LDL-BDC method is fast and easy to perform with instrumentation generally available in clinical laboratories. Since its development and original validation, this method has been successfully utilized in a number of studies on cardiovascular diseases and risk factors (see below).

The LDL-BDC assay is based on (i) precipitation of serum LDL with buffered heparin and (ii) spectrophotometric determination of the baseline level of conjugated dienes (234 nm) in lipids extracted from LDL [12]. Isolation of LDL by heparin does not affect the level of LDL-BDC [13]. Artefactual oxidation of LDL during sample preparation is prevented by addition of ethylenediaminetetraacetate. Chemical studies by HPLC and NMR show that LDL-BDC is almost exclusively ($> 90\%$) due to conjugated dienes in fatty acids, mainly linoleic acid, esterified to cholesterol and triglycerides (Fig. 1). Importantly, HPLC analyses demonstrated that compounds other than LDL constituents are not involved in LDL-BDC. Moreover, NMR spectra clearly indicated a certain degree of peroxidation (diene conjugation) in polyunsaturated fatty acids (Vasankari, T. J.; Ahotupa, M.; Toikka, J.; et al. Oxidized LDL and thickness of carotid intima-media are associated with coronary atherosclerosis in middle-aged men with coronary atherosclerosis: lower levels of oxidized LDL with statin therapy. Submitted for publication.). Together these findings give chemical evidence for specificity and validity of the LDL-BDC assay as an index of LDL lipid peroxidation.

Further evidence on validity of the LDL-BDC assay as an indicator of in vivo LDL oxidation was obtained from comparison with the autoantibody method for oxidized LDL. In a study with healthy middle-aged men, LDL-BDC showed a strong correlation ($r = 0.57$; $p = .001$) with the autoantibody titer [13].