PA 16

STUDIES OF BIO-CATALIZER α.ρ No. 11 ANTIOXIDANT PROPERTIES

Lucia Marcocci, Rossella D'Anna, Liang-Jun Yan, Nobuya Haramaki, Hirotsugu Kobuchi, Raymond A. Swanson, Lester Packer.*

*Department of Molecular Cell Biology, University of California, Berkeley, CA 94720-3200; *Department of Neurology, VAMC and University of California, San Francisco, CA 94121.*

ABSTRACT

Bio-Catalyzer α.ρ No. 11, Bio-Normalizer (BN), a mixture of products generated by yeast fermentation of several plants has been proposed to possess antioxidant activity. To further define its properties, we analyzed the effect of BN, orally administrated to rats for 6 weeks, against ischemia-reperfusion injury induced in the isolated Langendorff hearts, and against oxidative stress induced in rat tissue homogenates by peroxyl radicals generated *in vitro* from the azo-initiators 2,2'-azobis(2,4-dimethyl-valeronitrile) (AMVN) or 2,2'-azobis(2-amidinopropane)-dihydrochloride (AAPH). We also evaluated the activity of aqueous solutions of BN to protect rat tissue homogenates, isolated human low density lipoprotein (LDL) or phycoerythrin against azo-initiator or copper-derived oxidative damage. Moreover, we studied the efficacy of BN solutions to scavenge the radical cation of (2,2' azinobis(3-ethylbenzothiazoline 6 sulfonate) ($ABTS^+$) (N. Miller, C. Rice Evans collaboration) as well as nitric oxide. Oral BN suppressed the cardiac leakage of lactate dehydrogenase induced by ischemia-reperfusion injury, and protected rat kidney and heart but not liver or brain homogenates against AMVN-dependent oxidation of lipids, proteins and α-tocopherol depletion. BN did not protect homogenates against AAPH-derived damage. Aqueous solutions of BN did not protect rat tissue homogenate or LDL against oxidative damage generated by AMVN, AAPH or by copper, but protected phycoerythrin from AAPH-induced oxidation. BN solutions did not scavenge the $ABST^+$radical cation; however, they decreased the production of sodium nitroprusside-derived nitrite. These findings provide further evidence for an antioxidant activity of BN and point out that it is necessary to use a variety of different metodological systems to evaluate antioxidant activity of complex mixture from natural products.

INTRODUCTION

Bio-Catalyzer α.ρ No.11 (Bio-Normalizer, BN), a food supplement prepared from a yeast fermented mixture of various plants (i.e. *Carica papaya* Linn, *Pennisetum pupureum* Schum, *Sechium edule* Swartz), has been recently proposed as an antioxidant agent due to its ability to scavenge hydroxyl radical *in vitro* and to protect animals against oxidative damage induced in the brain by aging, by iron-treatment or by ischemia-reperfusion injury. However, to delineate antioxidant properties of a substance, it is important to evaluate its efficacy in a wide range of oxidative conditions. Thus, to further define Bio-Normalizer antioxidant character we evaluated:

- The efficacy of BN, administered to rats for 6 weeks in the drinking water, against:
    - ischemia-reperfusion injury induced in isolated hearts in the Langendorff apparatus
    - oxidative stress induced in tissue homogenates by peroxyl radicals generated *in vitro* from the azo-initiators 2,2'-azobis(2,4-dimethyl-valeronitrile) (AMVN) or 2,2'-azobis (2-amidinopropane)-dihydrochloride (AAPH).
- The activity of aqueous solutions of BN to protect against azo-initiator or copper-derived oxidative damage:
    - rat tissue homogenates,
    - isolated human low density lipoprotein (LDL)
    - phycoerythrin
- The interaction of BN with
    - the radical cation of (2,2' azinobis(3-ethylbenzothiazoline 6 sulfonate) ($ABTS^+$),
    - nitric oxide.

MATERIALS AND METHODS

Chemicals: Bio-Normalizer was a kind gift of the Osato Research Institute, Gifu, Japan. AMVN and AAPH were from Polysciences, Inc. (Warrington, PA). All other chemicals were from Sigma (St Louis, MO).

Animals: Male Sprague-Dawley rats (250-300 g) were from Bantin and Kingman (Fremont, CA). All animals were allowed *ad libitum* access to water with or without 0.1 % (w/v) BN for 6 weeks.

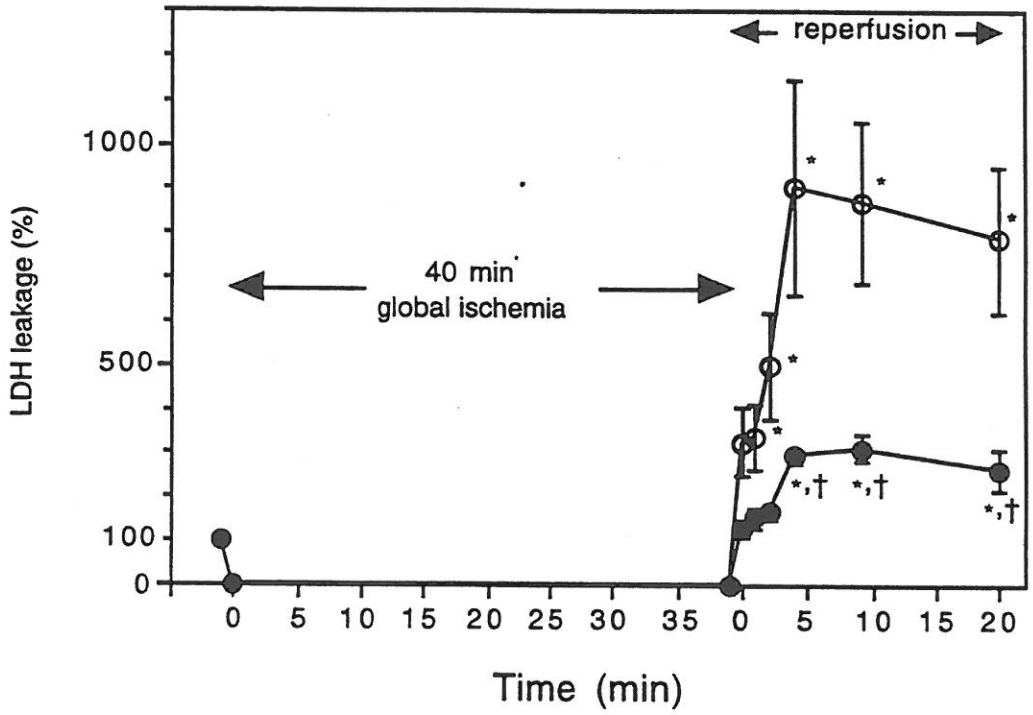
Thiobarbituric reactive substances (TBARS), protein carbonyl, α-tocopherol, cholesterol ester hydroperoxyde(CEOOH) assay: At the end of azo-initiators or copper treatment, butylated hydroxytoluene or EDTA (200 mM or 100 μM, final concentration, respectively) were added to the samples. TBARS and protein carbonyl derivatives were measured spectrophotometrically. 1,1,3,3-tetramethoxypropane (TMP) was used as standard for TBARS. α-Tocopherol and CEOOH were measured by HPLC.

LDH assay. LDH concentration in the coronary effluent from ischemia-reperfusin injured isolate rat hearts was measured by monitoring at 340 nm the oxidation of NADH in the presence of pyruvate.

Nitric oxide assay. Nitric oxide concentration was evaluated spectrophotomentrically as nitrite by the Greiss reagent reaction.

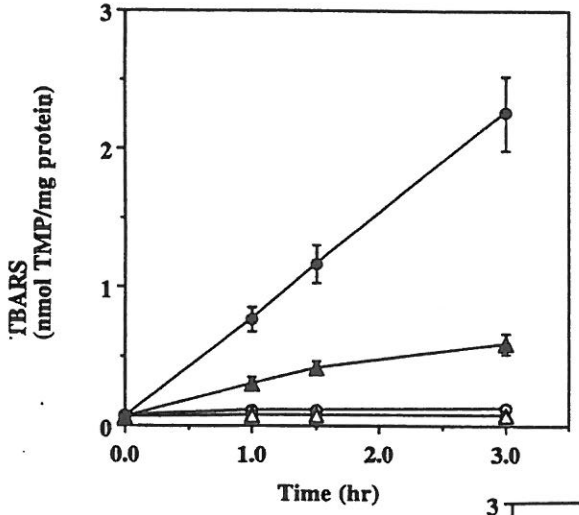
Statistical analysis: Comparisons were carried out using the Student-t test. A difference was considered to be significant when $p<0.05$.

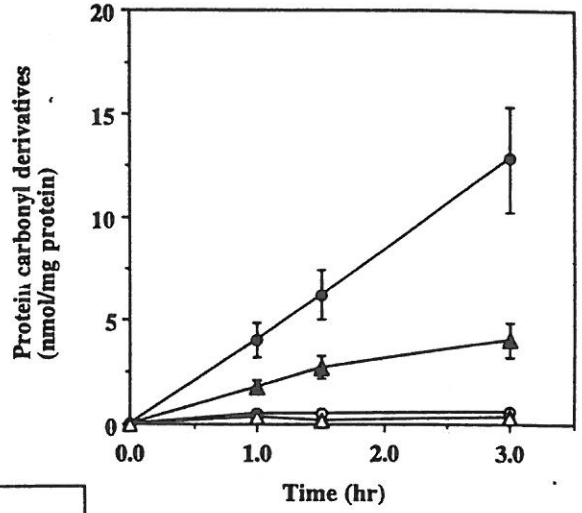
•***Bio-Normalizer supplementation protected isolated rat hearts against leakage of lactate dehydrogenase in the coronary effluent induced by ischemia-reperfusion injury in the Langerdoff apparatus.***

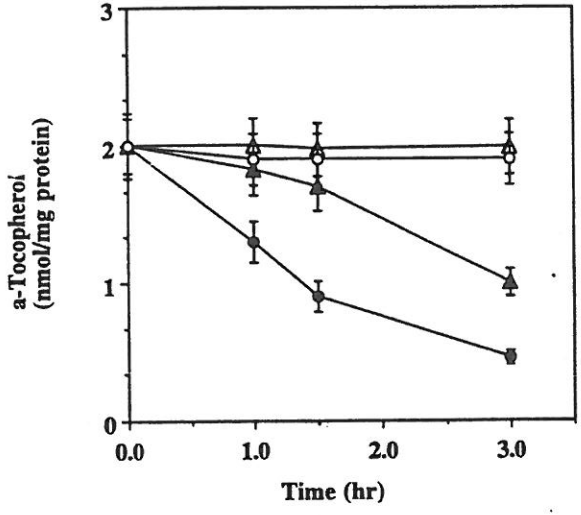


Rat hearts isolated from control (open circles) or from BN supplemented animals (closed circles) was retrogradely perfused at 37°C in a Langerdoff apparatus using a modified Krebs-bicarbonate buffer containing 2.5 mM Ca^{+2} and 10 mM glucose, pH 7.4. Perfusate was gassed with 95% O_2/5% CO_2 and aortic pressure was mantained at 900 mmH_2O. All the sample were pre-equilibrated for 20 min before the beginning of global ischemia. LDH activity during pre-ischemic phase was expressed as 100%. Means±S.E.M of 6 different experiments. *$p<0.01$ compared with pre-ischemic value; † $p<0.01$ compared with the value from control hearts.

•***Bio-Normalizer supplementation protected rat kidney homogenates against AMVN-induced depletion of α-tocopherol, formation of thiobarbituric reactive substances, and of protein carbonyl derivatives.***

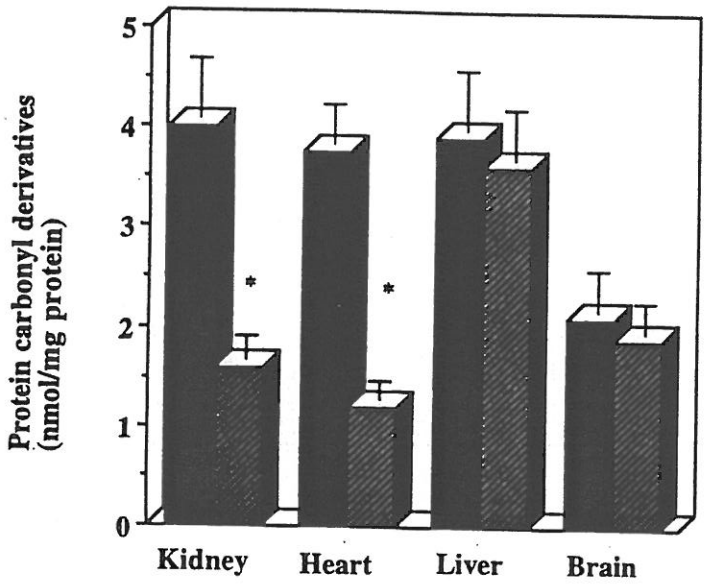


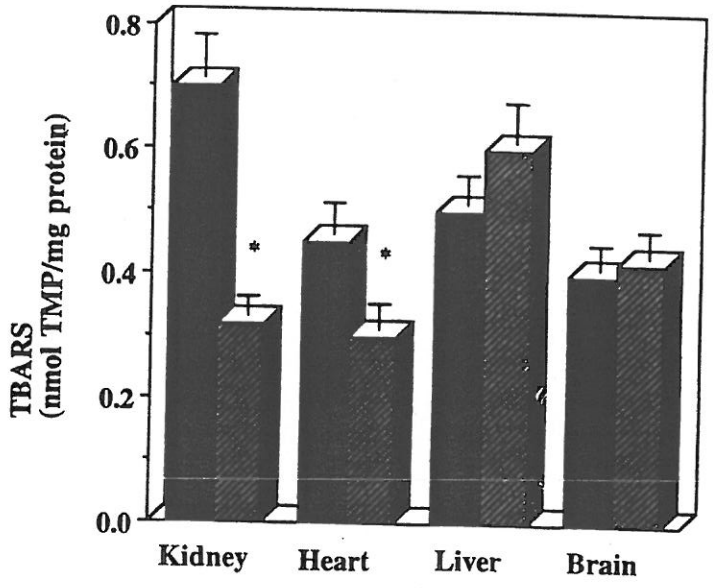




Rat kidney homogenates (10 mg protein/ml) prepared from control (circles) or from BN supplemented animals (triangles), were incubated in 20 mM phosphate buffer pH 7.4 containing 1 mM desferal at 42°C with (closed symbols) or without (open symbols) 5 mM AMVN. Mean ± SD of 3 different samples.

•*The effect of Bio-Normalizer supplementation against AMVN-induced oxidative damage was organ specific.*





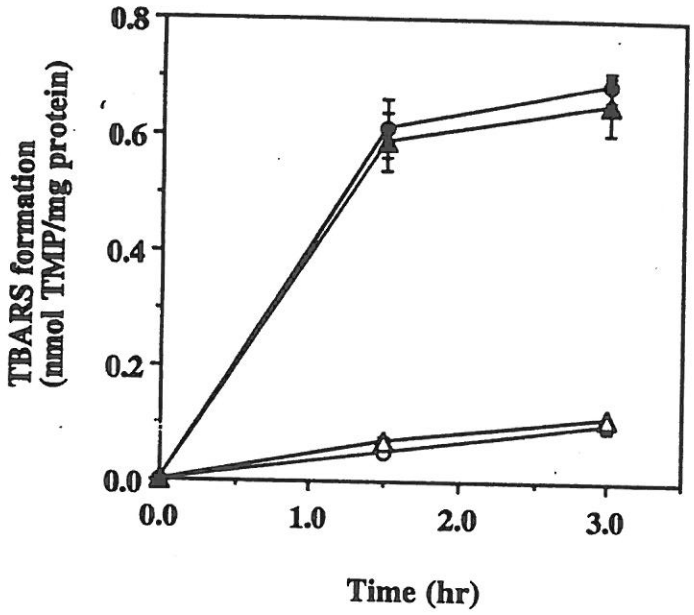
Rat tissues homogenates (10 mg protein/ml) prepared from control (solid bars) or from BN supplemented animals (hatched bars) were incubated in 20 mM phosphate buffer pH 7.4 containing 1 mM desferal at 42°C for 1 hr with 5 mM AMVN. Mean ± SD of 6 different samples. *$p<0.01$ compared with the respective controls.

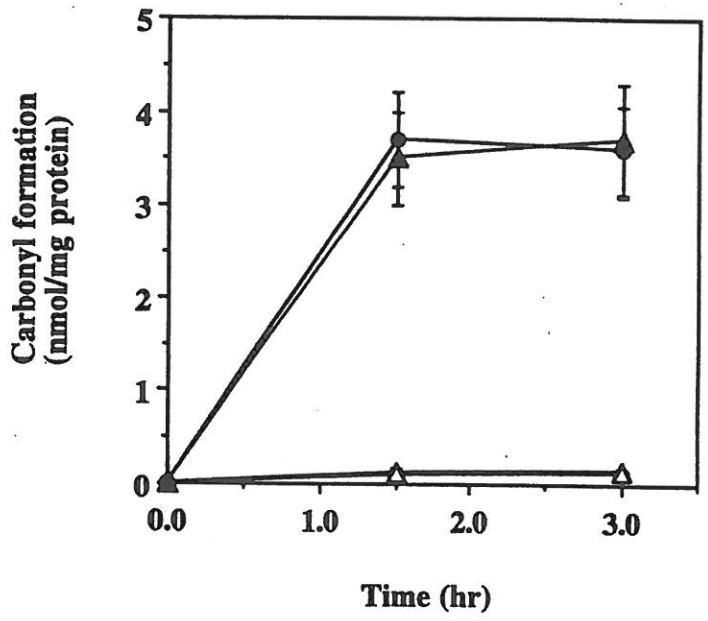
•***Aqueous solution of Bio-Normalizer did not protect tissue homogenates against azo-initiator-induced oxidation.***

Samples	*TBARS formation (nmol TMP/mg protein/hr)*	*Carbonyl formation (nmol/mg protein/hr)*
No BN	**0.76±0.071**	**4.15±0.8**
BN 0.2 mg/ml	**0.80±0.09**	**4.34±0.7**
BN 1 mg/ml	**0.79±0.06**	**4.05±0.5**

Rat kidney homogenates (10 mg protein/ml) were incubated in 20 mM phosphate buffer pH 7.4 containing 1 mM desferal at 42°C with 5 mM AMVN for 3 hours. Mean ± SD of 3 different samples. Similar results were obtained for the heart, liver, and brain homogenates as well as if 5 mM AAPH was used.

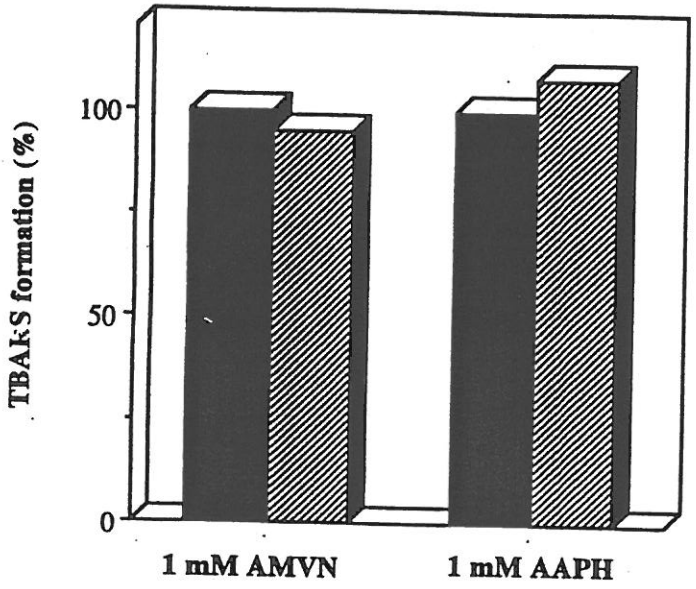
•***Bio-Normalizer supplementation did not protect rat organ homogenates against AAPH-induced oxidative damage.***

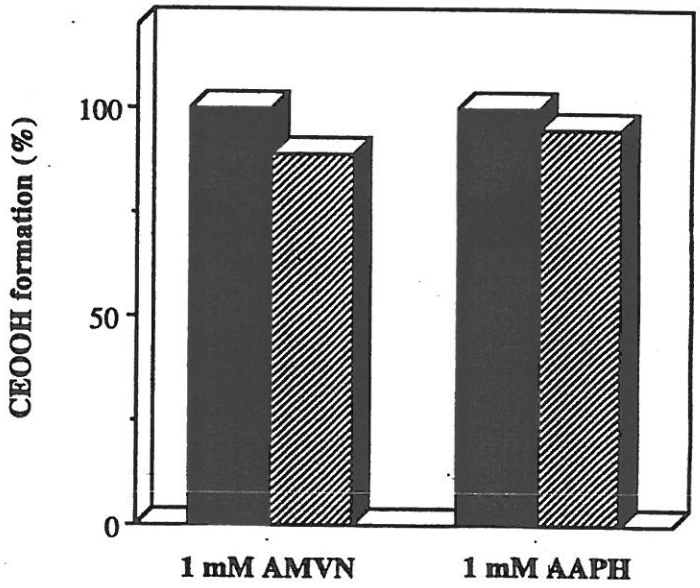




Rat kidney homogenates (10 mg protein/ml) prepared from control (circles) or from BN supplemented animals (triangles), were incubated in 20 mM phosphate buffer pH 7.4 containing 1 mM desferal at 42°C with (closed symbols) or without (open symbols) 5 mM AAPH. Mean ± S.D of 3 different samples. Similar results were obtained with the heart, liver, and brain homogenates.

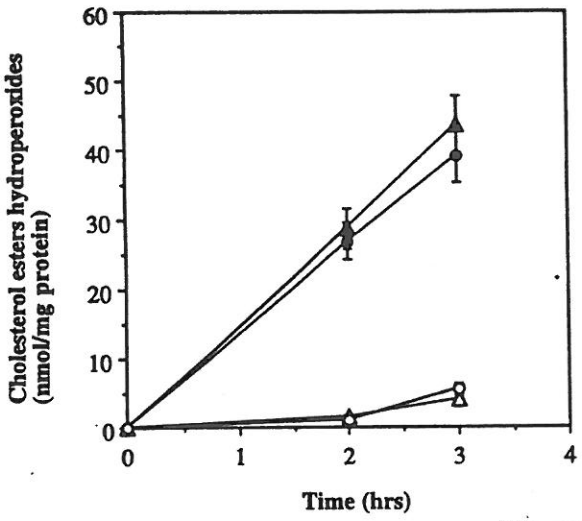
•Bio-Normalizer supplementation did not protect isolated human low density lipoprotein (LDL) against oxidative stress induced by azo-initiators.

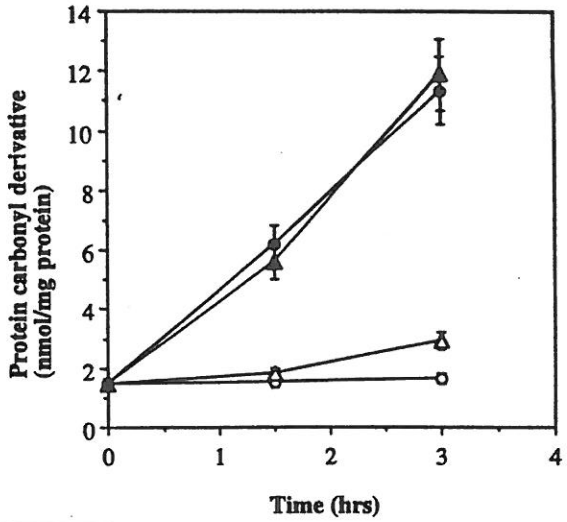


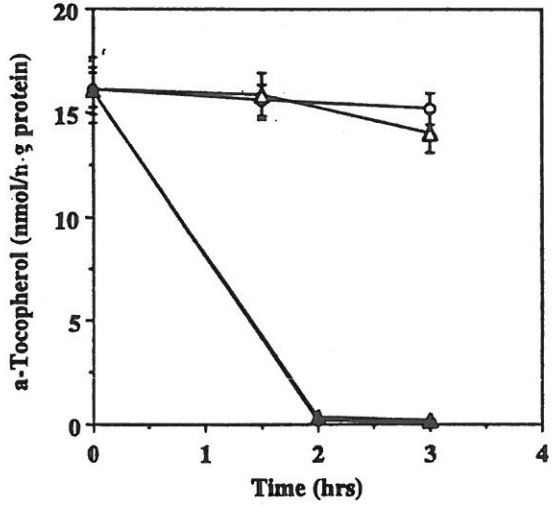


Human LDL (1mg protein/ml) were incubated in 10 mM phosphate buffer pH 7.4 containing 1 mM desferal at 42°C with 1 mM AMVN of 5 mM AAPH up to 6 hours in the presence (hatched bars) or in the absence (solid bars) of 0.2 mg/ml BN. The rate of oxidation in the absence of BN was expressed as 100%. One experiments representative of 3. Similar results were obtained in the presence of 1 mg/ml BN.

•Bio-Normalizer supplementation did not protect isolated human low density lipoprotein (LDL) against Cu (II)-induced oxidative stress.

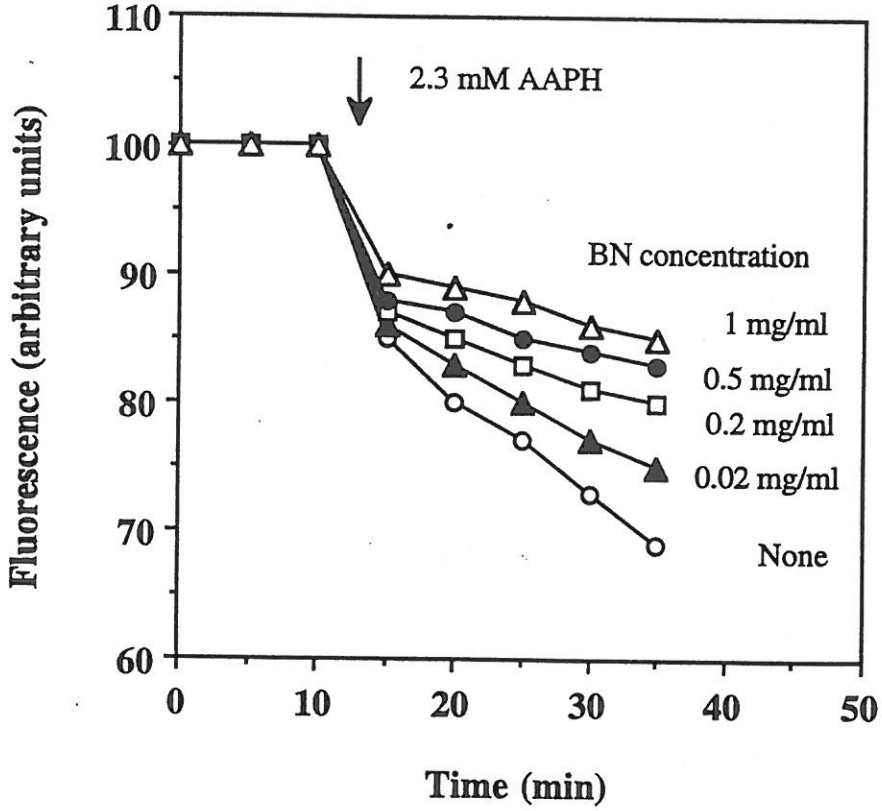






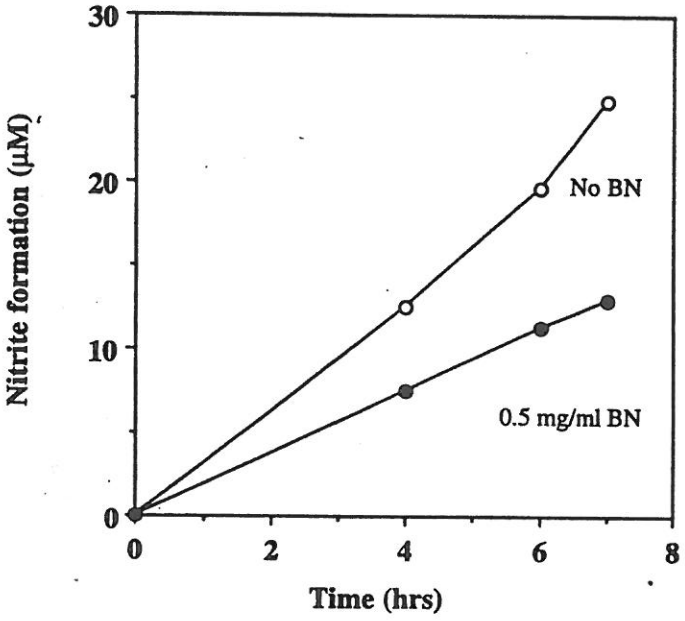
Human LDL (0.5 mg protein/ml) were incubated in 10 mM phosphate buffer pH 7.4 at 37 °C with (closed symbols) or without (open symbols) 10 µM $CuSO_4$ in the presence (triangle) or in the absence (circles) of BN (0. 2 mg/ml, final concentrations). Means ±SD of 3 experiments. Similar results were obtained in the presence of BN 1mg/ml.

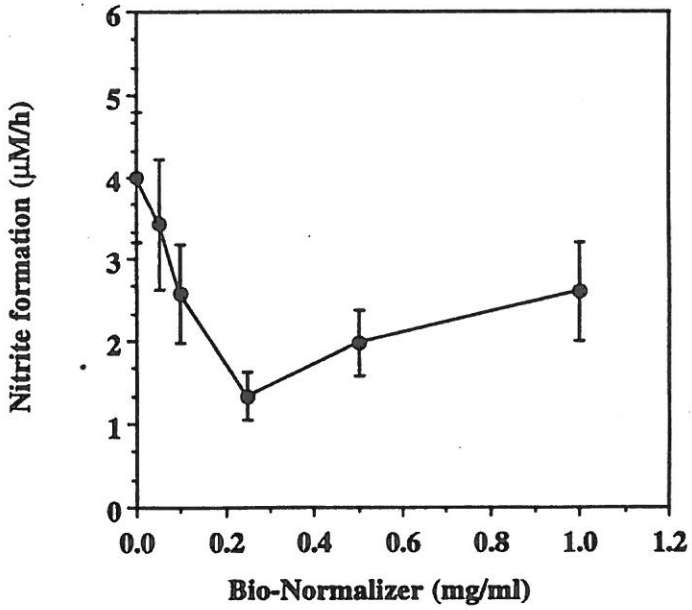
•***Aqueous solution of Bio-Normalizer protected B-phycoerythrin from AAPH-induced oxidative damage.***



B-phycoerythryn (0.02μM, final concentration) was exposed in 20 mM phophate buffer, pH 7.2, to 2.3 mM AAPH at 37°C in the presence of different concentrations of BN. Emission of fluorescence was monitored at 575 nm upon excitation at 540 nm. One experiment representative of 2.

•***Aqueous solution of Bio-Normalizer decreased the production of sodium nitroprusside-derived nitrite.***





Sodium nitroprusside (5 mM, final concentration) in 50 mM phosphate buffer, pH 7.4 was incubated at room temperature with or without BN. Figure on left) One experiment representative of 4. Figure on right) Mean±SD of 4 experiments.

CONCLUSIONS

•Orally administered Bio-Normalizer protected organ homogenates against peroxyl radical induced oxidative stress. Bio-Normalizer action was tissue specific and depended on the site of radical generation. We observed a significant antioxidant effect in heart and kidney homogenates against damage-induced by peroxyl radicals generated in hydrophobic phase from AMVN, but not against damage induced by peroxyl radicals generated in hydrophylic phase from AAPH. In both the cases Bio-Normalizer supplementation did not protect liver or brain homogenates.

•Supplementation of Bio-Normalizer suppressed the leakage of LDH from rat cardiac tissue during ischemia-reperfusion injury. The antioxidant efficacy of Bio-Normalizer supplementation in rat hearts was thus confirmed.

•Controversial data were obtained about the antioxidant effect of aqueous solutions of Bio-Normalizer. Addition of Bio-Normalizer to organ homogenates or to human LDL did not protect against oxidative damage induced by AMVN, by AAPH or by copper treatment. No interaction of Bio-Normalizer with $ABST^{+}$ radicals (data not shown) was also observed. However, Bio-Normalizer protected B-phycoerythryn against oxidation induced by AAPH-generated peroxyl radicals.

•Bio-Normalizer affected the production of nitrite from sodium nitroprusside, thus suggesting an interaction with nitric oxide.

• Our findings provided further evidence for the antioxidant action of Bio-Normalizer. Moreover, they pointed out that it is necessary to use a variety of metodological systems to evaluate antioxidant activity of complex mixture from natural products.