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Protection of Phagocytic Macrophages from Peroxidative Damage by Selenium and Vitamin E

Sang-Hwan Oh, Myung-Ho Lee and Chang-Jo Chung

Department of Biochemistry, Yonsei University, College of Medicine
Seoul, Korea

The Protective effect of vitamin E and selenium against peroxidative damage in white blood cells was studied. Forty-eight male rats (~100g BW) were divided into four groups and were fed with a torula yeast based diet deficient in Vit. E and Se.

Vit. E (100IU/Kg diet) and Se (0.3ppm) supplementation increased the total peritoneal cell (P.C) population and cell survival rate. Selenium supplementation decreased the hydrogen peroxide generation (half of the control) significantly and Vit. E supplementation reduced the malonaldehyde production during phagocytosis *in vitro*.

However, superoxide generation was not affected by the supplementation of Vit. E or Se. There were no significant differences in catalase activity between groups but glutathione peroxidase activity was increased about twofold by Se supplementation with no effect of Vit. E.

In a separate experiment, activated alveolar macrophages were obtained from BCG infected rabbits fed a diet supplemented with Vit. E (100 IU/Kg diet) or Se (0.3 ppm). Se supplementation increased glutathione peroxidase in cells, and both Vit. E and Se increased the cell survival rate during phagocytosis as compared to the control. Both Vit. E and Se are necessary to protect host cells from peroxidative damage during phagocytosis.

Key Words: Selenium, Vitamin E, Peroxidation, Macrophage, Phagocytosis.

Mammalian phagocytes generate bactericidal agents such as hydrogen peroxide, superoxide (Stossel, 1974; Babior, 1978). They are associated with major changes in oxidative metabolism including increased cyanide insensitive consumption of oxygen (Sbarra & Karnovsky, 1959) and stimulation of glucose oxidation via hexose monophosphate shunt (Stahelin *et al.*, 1957). The primary enzyme of the phagocytic oxidative burst is thought to be a cyanide-insensitive NADPH oxidase that catalyzes the conversion of molecular oxygen to hydrogen peroxide and superoxide (Cagan & Karnovsky, 1964). It was reported that the increased NADPH-dependent glucose oxidation is linked to H_2O_2 metabolism by a glutathione

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system involving glutathione peroxidase and NADPH-dependent glutathione reductase (Reed, 1969; Vogt *et al.*, 1971).

Although metabolic products of the respiratory burst are bactericidal agents, they may cause the host cell destruction if defense mechanisms are insufficient. Several investigators suggested that H_2O_2 is released during phagocytosis and it could affect other cells nearby (Baehner *et al.*, 1971; Sbarra *et al.*, 1972). Hydrogen peroxide is mainly decomposed through systems of catalase and glutathione peroxidase, a selenoenzyme, of which the level is dependent on the dietary selenium intake (Ganther *et al.*, 1976). The essentiality of vitamin E for the protection of tissue from degenerative defects such as fetal resorption, liver necrosis, vascular degeneration, erythrocyte hemolysis was well documented (Mason and Horwitt, 1972). In general, vitamin E is considered as an important antioxidant required for the integration of cell membrane.

A major discovery relative to vitamin E function was the essentiality of selenium and its overlapping in function with that of vitamin E (Schwarz and Foltz, 1957). Then selenium was found to be a component of glutathione peroxidase which destroys H_2O_2 and organic hydroperoxides (Rotruck *et al*, 1973; Oh *et al.*, 1974). Both selenium and vitamin E are recognized as essential but abundant evidence indicated that their biochemical role is independent.

The purpose of this experiment is to investigate the protective effect of selenium and vitamin E on rat peritoneal macrophages and rabbit alveolar macrophages against peroxidative damage during phagocytosis *in vitro*.

MATERIALS AND METHODS

Chemicals: Reduced glutathione, NADPH, cytochrome C (Type V), xanthine, xanthine oxidase, scopoletin, latex beads were purchased from the Sigma Chem. Co., St. Louis, USA. P-nitrophenol phosphate was obtained from Merck, Darmstadt, West Germany and ^{14}C[u] glucose from New England Nuclear, Boston, USA, and Eagles media was obatained from Gibco Lab., NY, U.S.A.

Animals and diet: Forty-eight male Albino rats (~100g BW) were divided into 4 groups and were fed, *ad libitum*, a basal diet supplemented with selenium, vitamin E or both for 4 weeks. The composition of the basal diet is shown in table 1. Torula yeast and Vit. E free lard were purchased from US Biochem. Corporation, Cleveland, USA. DL-α-tocopherol was supplemented to the basal diet (100 IU/Kg) and selenium was supplemented to the diet as sodium selenite (0.3ug Se/Kg diet). Twelve female rabbits (~1 Kg BW) were divided into 3 groups and were fed with bean curd residues. Selenium and vitamin E were supplemented to this diet in the same way as described above.

Hemolysis test: 0.3 ml of heparinized fresh

Table 1. Composition of basal diet

Ingredient	% of the Diet
Torula yeast	30.0
Glucose	55.7
Vitamin E-free Lard	5.0
Cod-liver oil	3.0
Salt mix	5.0
Vitamin mix*	1.0
DL-methionine	0.3
Total	100

* Vitamin mix (per 100g): Glucose, 88.58g; Thiamin-HCl, 40mg; riboflavin, 25mg; Pyridoxin HCl, 20mg; pantothenic acid 200mg; choline chloride, 10g; niacine, 1g; menadione, 10mg; folic acid, 20mg; biotin, 10mg; Vit. B_{12} (0.1% B_{12}) 100mg

blood taken by cardiac puncture was centrifuged and erythrocytes were washed in saline phosphate buffer containing $1.6x10^{-2}$ M glucose. The washed erythrocytes were then suspended in a final volume of 5 ml of isotonic solution. The concentration of added ingredients in the final mixture were $2.2x10^{-3}$ M ascorbic acid, $1x10^{-2}$ M sodium azide, and $1.6x10^{-2}$ M glucose. The ascorbic acid solution was neutralized with sodium carbonate. After the incubation of the final mixture at 37°C for various times, 1.0 ml of the solution was diluted to 5 ml with saline phosphate buffer (0.05 M, pH 7.4). Homolysis was determined by measuring the optical densities of supernates at 540 nm after centrifugation.

Preparation of macrophages: Peritoneal cells were harvested by washing peritoneal cavities of rats with 50 ml portions of heparinized isotonic saline solution twice, and the harvested cells were washed with the same solution and resuspended in the sterilized Eagles media (pH 7.4) at a concentration of $5x10^6$ cells per ml. All rats were injected with 2.0 ml of mineral oil i.p. for the stimulation, 3 days before sacrifice. Rabbit alveolar macrophages were harvested by washing the lung alveoli four times with 25 ml portions of heparinized isotonic saline phosphate solution (pH 7.4). Rabbits were immunized with BCG (Bacillus Calmette-Guerin) by S.C. injection of about 10^7 units of living BCG, one month before the sacrifice. They also received 2 ml of mineral oil i.p. 3 days before sacrifice. The harvested cells were washed and resuspended in Eagles media in the same way and total cell number was counted by a Coulter counter (Model ZBI).

Viability test during phagocytosis in vitro.. Phagocytosis was induced by incubation of macrophages ($5x10^5$ cells/ml) at 37°C after the addition of latex beads (1.0 u diameter) at a bead to cell ratio of about 500. Smears made from the incubation mixture after various incubation periods were microscopically examined by the trypan blue exclusion method and the dead cells were counted by using the hemocytometer.

Enzyme assays: Glutathione peroxidase activity was measured by the method of Paglia and Valentine (1967) and glutathione reductase activity by the method of Racker (1955). Catalase activity was determined by the method described by Bergmeyer *et al* (1974), and superoxide dismutase was measured by the method of McCord and Fridovich (1969). Acid phosphatase activity was measured by the method described by Bergmeyer *et al* (1974).

Measurements of oxidative metabolites during phagocytosis: Hydrogen peroxide generation during phogocytosis was quantitated by measuring the changes in fluorescence of scopoletin added to the incubation mixture. The detailed method is described by Root *et al* (1975). Superoxide (O_2^-) production during phagocytosis was assayed by measuring the O_2^- dependent cytochrome c reduction spectrophotometrically. Malondialdehyde contents in phagocytes were measured after 30 minutes incubation period by the method described by Ernster and Nordenbrand (1967). Total glutathione content was measured by the method of Tietze (1969). $^{14}CO_2$ production from ^{14}C–glucose oxidation was done in a Warburg flask and was measured by the method used by Rabinovitch and Destefano (1973).

RESULTS

The number of peritoneal cells per rat varied between groups and the mortality of rats was higher in the selenium deficient groups (table 2). Se supplementation had an increasing effect on peritoneal cell population, however, the values were not significant between groups.

Table 2. Effect of Vitamin E and selenium on the population of peritoneal leukocytes and mortality of rats.

group	No. animals	Mortality	Cell population (total No. of P.C./rat)
Basal (−E, −Se)	12	2/12*	4,061,126± 2,961,971†
−E, +Se	12	0/12	5,173,293± 2,558,554
+E, −Se	12	3/12	4,043,039± 2,478,127
+E, +Se	12	0/12	7,419,383± 3,258,925

* Numerator is the number of dead and the denominator is the total number of animals.

† All values are mean for alive rats ± standard deviation.

Table 3. Metabolic changes of peritoneal machrophages during phagocytosis by the elicitation

biochemical criteria	control	elicited†
Acid phosphatase (m moles substrate/min/ 10^6 cells)	77.3± 1.0*	190.3± 2.4
$^{14}CO_2$ production** (cpm/hour/10^7 cells)	2,710± 174	3,504± 169

* Values are mean ± standard deviation.

** ^{14}C labelled glucose (0.2 u ci) was added into the incubation mixture.

† Cells were harvested from rat peritoneum 3 days after the injection of 2.0 ml of mineral oil.

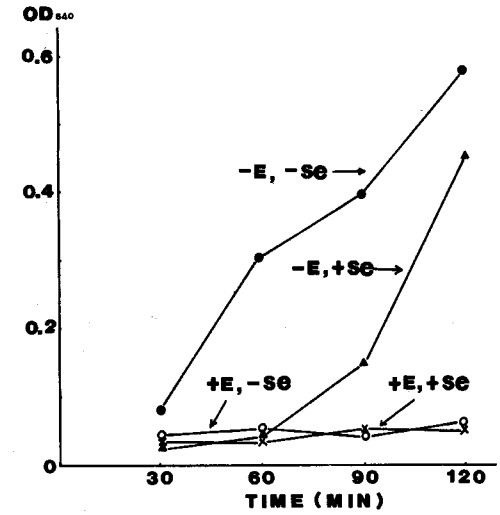


FIG. 1. EFFECT OF SELENIUM & VITAMIN E ON ASCORBATE INDUCED HEMOLYSIS

There was a marked increase in acid phosphatase activity (3X) in peritoneal cells stimulated with paraffin oil compared to the unstimulated normal cells (table 3). Glucose oxidation was higher (150% of control) in stimulated cells than in control normal cells, indicating that stimulated cells were more activated and had more potency for phagocytosis. Status of Vit. E and selenium of rats fed with experimental diets were tested by the degree of hemolysis induced by ascorbate *in vitro*. Vit. E had a protective effect on hemolysis regardless of selenium supplementation in this test. In Vit. E deficient group, selenium had protective effect on hemolysis for the first 60 min of incubation period, but no further protection was observed thereafter (Fig. 1).

There were significant reductions of hydrogen peroxide and malondialdehyde productions by supplementations of both Vit. E and selenium, however, no significant differences in superoxide production were observed between groups (table 4).

Dietary selenium had a reducing effect on hydrogen peroxide production and Vit. E. could reduce malondialdehyde production, though the effects were not statistically significant (table 4).

Selenium supplementation increased glutathione

Table 4. Effect of dietary selenium and vitamin E on the generation of hydrogen peroxide, malondialdehyde and superoxide in rat peritoneal leukocytes during phagocytosis

group†	H_2O_2 (p moles/ min/10^6 cells)	malondialdehyde (n moles/hr/10^6 cells)	O_2^- (n moles/ min/10^6 cells)
Basal (−E, −Se)	229.9±68.3	2.81±0.92	0.58±0.13
−E, +Se	148.7±97.2	2.04±0.78	0.78±0.11
+E, −Se	343.1±171.8	1.55±0.75	0.75± 0.36
+E, +Se	107.2±47.1*	1.38±0.25*	0.52±0.20

† Vitamin E (100 IU/kg diet) was supplemented to the basal diet and Se (0.3 ppm) was supplemented to the diet as sodium selenite.

* Values are significantly different from the basal group ($P<0.05$).

Table 5. Effect of selenium and vitamin E on catalase and glutathione peroxidase in rat peritoneal leukocytes

group	No. animals	Catalase (n moles/min/10^6 cells)	Glutathione peroxidase (n moles/min/10^6 cells)
Basal (−E, −Se)	10	454.5±160.7	26.2±10.0
−E, +Se	12	465.6± 96.7	57.8±14.9*
+E, −Se	9	411.3±141.5	34.4±12.6
+E, +Se	12	388.7±109.5	65.3±16.3*

* Values are significantly different from the basal group.

† All values are mean ± standard deviation.

Table 6. Effect of selenium and vitamin E on glutathione, glutathione peroxidase, glutathione reductase and superoxide dismutase in activated rabbit alveolar macrophages

group	GSH (u moles/ 10^6 cells)	GSH−Rd † (EU/10^6 cells)	GSH−Px (EU/10^6 cells)	SOD (EU/10^6 cells)
control	8.1± 3.3	117.7±11.6	100.4± 3.1	2.4± 0.3
+Se (0.3 ppm)	10.2±4.2	115.0± 4.4	141.5±13.5*	2.8±0.5
+E (100IU/kg)	12.3± 2.1	121.7±14.6	102.4± 8.4	2.5±0.6

† E.U. (glutathione reductase): change in A_{340} of 0.001/min.
E.U. (glutathione peroxidase): m u moles of GSH oxidized/min.
E.U. (superoxide dismutase): inhibiton of cytochrome c reduction by OD550 of 0.0125/min.
Rabbits (−1500g BW) were fed with soybean cake for 30 days and Vit. E (α-tocopherol) and Se (Na_2SeO_3) was added to the diet.

* Value is significantly different from that of other groups (control & + Vit. E).

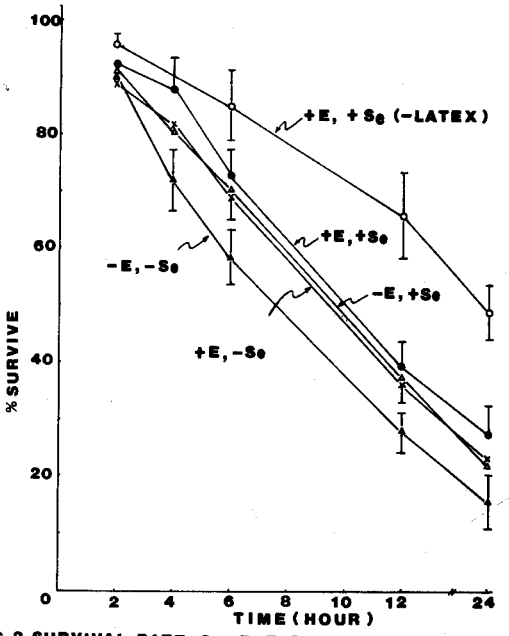
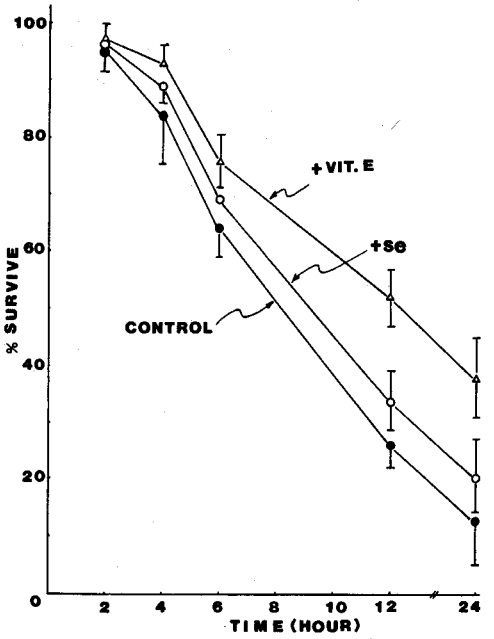


FIG. 2. SURVIVAL RATE OF RAT PERITONEAL PHAGOCYTES



G.3. SURVIVAL RATE OF RABBIT ALVEOLAR MACROPHAGE DURING PHAGOCYTOSIS

peroxidase activities in rat peritoneal cells but Vit. E had effects neither on glutathione peroxidase activity nor catalase activity. There were no significant differences in catalase activity between groups (table 5).

Peritoneal macrophages of rats fed the basal diet had significantly lower survival rate during phagocytosis (Fig. 2), but there were no significant differences in survival rates among groups supplemented with Se, vit E or both. The survival rate of macrophages obtained from the group treated with Vit. E was much higher than that of phagocytes of any other group. Dietary selenium supplementation increased glutathione peroxidase activity in activated rabbit alveolar macrophages but vitamin E has no effect on the enzyme activity. Both vitamine E and selenium had no effect on glutathione concentration, glutathione reductase and superoxide dismutase activities (table 6).

Dietary vitamin E supplementation increased the survival rate of activated macrophage during phagocytosis, however selenium supplementation did not increase the cell survival rate significantly compared to the control group (Fig. 2).

DISCUSSION

Although not accepted by all investigators and in fact, subject to extensive criticism, the antioxidant theory of vitamin E function has remained viable (Witting, 1974). The membrane lipid stabilizing theory of Vit. E has received support from many investigators (Diplock and Lucy, 1973) but the biochemical mechanism of Vit. E function is still obscure. Dietary selenium increased glutathione peroxidase activities in macrophages but dietary vitamin E supplementation had no effect on the enzyme activity in the present study (table 4 & 6). This result is consistent with that previously observed in other tissues (Hoekstra, 1974). Dietary Vit. E supplementation decreased

malondialdehyde production in phagocytes while dietary selenium had little effect on it in this study. The result indicates that Vit. E prevented lipid peroxidation in macrophage membrane probably through the interaction of vitamin E with the lipid membrane of cells but Se functions by destroying H_2O_2 produced during phagocytosis thereby protecting the membrane from oxidative damage. These results imply that the mode of action of selenium is different from that of vitamin E. Phagocytosis by macrophages is accompanied by increases in O_2 consumption, glucose oxidation, and H_2O_2 formation, and they have a killing mechanism which destroys other cells at the expense of active oxygen species produced extensively during the respiratory burst (Klebanoff, and Clark, 1978). Superoxide itself has little microbicidal activity during phagocytosis, but it has an ability to interact with H_2O_2 to generate singlet oxygen and hydroxyl radicals which are considered as strong cytocidal agents. Althouh the overall mechanism of lipid peroxidation is not yet completely understood, the involvement of superoxide, hydroxyl radical and singlet oxygen in the process is well established (Fong *et al.*, 1973). It has been emphasized that hydrogen peroxide in the microbicidal reaction in macrophages is linked to NADP dependent glutathione peroxidative metabolism via the hexose monophosphate shunt and is important for phogocytosis (Vogt *et al.*, 1971). Since hydrogen peroxide as well as other oxygen species may be toxic to host cells, protective mechanisms for the removal of these compounds are implicated. Considering the small effect of superoxide on microbicidal reaction, the dismutation of superoxide will be a protective mechanism for the host cell stability. But vitamin E or selenium had little effect on the release of superoxide in the present study. The relative importance of glutathione and catalase dependent H_2O_2 metabolism is uncertain but the latter was much smaller than glucose oxidation during phagocytosis (Gee *et al.*, 1970). Glutathione peroxidase destroys not only H_2O_2 but also organic hydroperoxides while catalase destroys only H_2O_2. Increased glutathione peroxidase activity in macrophages by dietary selenium supplementation in the present experiment indicates that selenium may potentiate the glutathione metabolism during phagocytosis and can protect host cells from peroxidative damage. The protective effects of Se against peroxide damage were demonstrated in these results; increased cell survival rate and decreased hydrogen peroxide release during phagocytosis. The effect of vitamin E on the protection of host cells from peroxidative damage was relatively greater than that of selenium as judged by host cell survival rates (Fig. 3, 4). Vit. E had a marked effect on the reduction of malondialdehyde production in macrophages during phagocytosis, indicating that Vit. E had prevented the lipid peroxidation maintenance of host cell integrity. The elicitation of macrophages accompanies increases of the respiratory burst, acid phosphatase activity and glucose oxidation in the present study (table 3).

These results are consistent with the previous report by Vogt *et al.* (1971). The activation of rat peritoneal macrophages in the present study has not been attempted because the rats used in this experiment were not guarranted for immunologically similarity. Vit. E treatment in this experiment had no direct effect on glutathione metabolism and the hydrogen peroxide metabolizing enzyme system, but it could prevent lipid peroxidation as judged by the reduced malondialdehyde production and the improved cell viability during phagocytosis. Because lipid membranes of cells are implicated

as the site of vitamin E reaction, the chance of interaction of vitamin E with cidal components in the cytoplasm is small. Therefore, the effect of vitamin E on cidal reaction (phagocytic activity) has to be further clarified.

REFERENCES

Babior BN: *Oxygen-dependent microbial killing by phagocytes. N Engl J Med 298:659-668, 1978*

Baehner RL, Nathan DG, Castle WB: *Oxidant injury of caucasian glucose-6-phosphate dehydrogenase-deficient red blood cells by phagocytosing leucotytes during infection. J Clin Invest 50:2466-2473, 1971*

Bergmeyer HU, Gawehn K & Grassl M: *Catalase and acid phosphatase. Methods in Enzymatic Analysis, Bergmeyer HY ed. vol. I pp438-439 & pp495-496, 1974*

Cagan RH & Karonovsky ML: *Enzymatic basis of the respiratory stimulation during phagocytosis. Nature 204:255-257, 1964*

Diplock AT and Lucy JA: *The biochemical modes of action of vitamin E and Selenium: A hypothesis FEBS Letters 29:205-210, 1973*

Ernster L, Nordenbrand K: *Microsomal lipid peroxidation. Methods in Enzymology, Colowick SP and Kaplan NO eds. Vol. X, pp574-580, 1967*

Fong KL, McCay PB, Poyer JL, Keele BB. and Misra H: *J BC 248:7792-7797, 1973*

Ganther HE, Hafeman DG, Lawrence RA, Serfass RE & Hoekstra WG: *Selenium and glutathione peroxidase in health and disease-A review. Trace Elements in Human Health and Disease. vol II. Academic Press Inc. pp105-234, 1976*

Gee JBL, Vassallo CL, Bell P, Kaskin J, Basford RE and Field JB: *Catalasedependent peroxidative metabolism in the alveolar macrophage during phagocytosis. J. Clin. Invest. 49:1280-1287, 1970*

Hoekstra WG: *Biochemical role of Selenium. In Trace Element Metabolism in Animals 2 (ed Hoekstra, W.G. Suttie J W, Ganther H E and Mertz W) p61 University park press, Baltimore.*

Klebanoff SJ, Clark R.: *The neutrophil: Function and clinical disorders p409, Amsterdam, North Holland.*

Mason KE, and Horwitt MK: *The vitamins: Chemistry, physiology, pathology methods. ed. by Sevrell WH, Jr and Harris RS, Academic press, New York. vol 5 p272, 1972*

McCord JM and Fridovich I: *Superoxide dismutase. An enzymatic function for erythrocuprein. J Biol Chem 244:6049-6055, 1969*

Oh SH, Ganther HE and Hoekstra WG: *Selenium as a component of glutathione peroxidase isolated from ovine erythrocytes. Biochemistry 13:1825-1829, 1974*

Paglia DE, Valentine WN: *Studies on the quantitative characterization of erythrocytes glutathione peroxidase. J Lab Clin Med 70:158-169, 1976*

Rabinovitch M, Destefano NJ: *Macrophage spreading in vitro. I. Inducers of spreading. Exp Cell Res 77:323-328, 1973*

Backer E: *Glutathione reductase. Method in Enzymology, Colowick SP and Kaplan NO eds. vol. II, pp722-725, Academic Press, NY, 1955*

Reed PE: *Glutathione and the hexose monophosphate shunt in phagocytizing and hydrogen peroxide-treated rat leucocytes. J Biol Chem 244:2459-2464, 1969*

Root PK, Metcalf J, Oshino N & Chance B: *H_2O_2 release from human granulocytes during phagocytosis. I. Documentation, quantitation, and some regulating factors. J Clin Invest 55:945-955, 1975*

Rotruck JT, Pope AL, Ganther HE, Swanson AB, Hafeman DG and Hoekstra WG: *Selenium; Biochemical role as a component of glutathione peroxidase. Science 197:588-590, 1973*

Sbarra AJ & Karnovsky ML: *The biochemical basis of phagocytosis. I Metabolic changes during the ingestion of particles by polymorphonuclear leucocytes. J Biol Chem 234:1355-1362, 1959*

Sbarra AJ, Paul BB, Jacobs AA, Strauss RR & Mitchell GW Jr: *Biochemical aspects of phagocytic cells as related to bacterial function. RES J Reticul Soc 11:492-502, 1972*

Schwarz K and Foltz CM: *Selenium as an integral part of factor 3 against dietary necrotic liver degeneration. J Am Chem Soc 79:3292-3293,*

1957

Stossel TP: *Phagocytosis. N Engl J Med 290:717-723, 774-780, 833-839, 1974*

Stahelin H, Karnovsky ML, Farnham AE & Suter E: *Studies on the interaction between phagocytes and tubercle bacilli: III. Some metabolic effects in guinea pigs associated with tubercle bacilli. J Exp Med 105:265-277, 1957*

Tietze F: *Enzymatic method for quantitative determination of nanogram amounts of total and oxidized glutathione: Applications to mammalian blood and other tissues. Anal Biochem 27:502-522, 1969*

Vogt MT, Thomas C, Vassallo CL, Basford RE, Gee JBL: *Glutathione-dependent peroxidative metabolism in the alveolar macrophage. J Clin Invest 50:401-410, 1971*

Witting LA: *Vitamin E-polyunsaturated lipid relationship in diet and tissues. Am J Clin Nutr 27:952-959, 1974*