CATALASE DEFICIENCY AS A POSSIBLE CAUSE FOR HEPATOMA AND DISEASE RISK IN STEELHEAD TROUT (O. mykiss) COMPARED TO COHO SALMON (O. kisutch)

Bryant L. Adams, Ph.D.

2343 63rd Ave SE,

Tumwater, WA 98512

ph. 360-412-8008

dahc@healcorp.net

Running title: CATALASE DEFICIENCY AND CANCER

ABSTRACT

Steelhead trout (Oncorhynchus mykiss) were found to be hypocatalasemic compared with coho salmon (Oncorhynchus kisutch) in two types of catalase mediated reactions. One uses H_2O_2 as both substrate and oxidant producing oxygen gas and water. The other uses organic compounds as substrate and requires polyunsturated fatty acids with H_2O_2 through hydroperoxide as oxidant. Correlations are made between differing levels of catalase activity in the two species reported here and in their respective susceptibility to hepatoma (liver cancer) and disease reported by other researchers. Several chemicals including ascorbic acid and tocophrol complemented catalatic metabolism.

Key words:

Ascorbic acid; cancer susceptibility; detoxification; Peroxidase inhibition, Catalase deficiency, hepatoma, afflotoxin polyunsaturated fatty acids.

INTRODUCTION

Steelhead trout (Oncorhynchus mykiss) are more susceptible to hepatoma (7), toxins (2) and viral infections (5), than are coho salmon (Oncorhynchus kisutch) (9). However, these species are sufficiently related genetically that a hybrid of the two species has been studied as a possible disease resistant replacement for trout (22). This genetic relationship, but susceptibility difference suggested that the pair might be of value in determining metabolic factors that were responsible for the variation in resistance to disease, toxins and cancer.

Metabolism of hydrogen peroxide in blood was investigated because it is known to be involved in the cytocidal system of macrophages and leukocytes (22,26). On the other hand excess reactive oxygen species (ROS) including hydrogen peroxide, superoxide and the hydroxyl radical are implicated as causative factors in cellular damage of aging (23), ischemia reperfusion injury (14) and oxidative damaging side effects of certain drugs (13). They may also play a role in regulation of cell growth or division (1).

The results of this investigation of catalase and peroxidase in steelhead trout and coho salmon shows a correlation of activity of these enzymes with known susceptiblity and resistance respectively to cancer and other diseases.

METHODS

Coho salmon, Steelhead trout and Atlantic salmon (Salmo salar) used were reared at the same temperatures, 15^{O} C, in soft water (less than 20 ppm total salts) and the former two species also in hard water (235 ppm). Squafish (Ptychocheilus oregonensis) used were from a natural environment. Blood was let directly from the severed tail into distilled water at 2 drops/ml immediately after killing the individual fish. The lysed sample was mixed and centrifuged to remove the clot. An alliquote of the supernatant was used directly or diluted further so as to equalize the optical density of the hemoglobin peak at 412 nm for different samples. Stock solutions of fatty acids were dissolved in ethanol at 40 mg/ml. For the peroxidase and catalase test reactions, 25 Fl of fatty acid stock solution, 25 Fl solution of lysed dilute blood, and 0.27 Fmoles benzidine dye in 10 Fl ethanol was added to 1.5 ml of 0.05 Molar potassium phosphate buffer, at pH 7.2. The reaction was started by addition of 50 Fl of either 0.3 or 3.0 % H_2O_2 (4.5 or 45 Fmoles) as described in Figures 1 to 3. Peroxidase was measured as the increase in absorbency at 610 nm in a double beam Beckman DB spectrophotometer with a recorder. The above ingredients without H_2O_2 were in the reference cell. Catalase was measured as the volume of gas evolved from a closed hypodermic syringe fitted with a three-way stopcock valve to which was attached a calibrated one mm i.d. plastic tube filled with a colored marker solution. Catalase was also measured by its catalysis of PUFA dependant irreversable oxidation of benzidine to a yellow-brown product.

Pure free fatty acids and their esters were obtained from sigma Chemical Co., St. Louis, MO. Electrophoresis was done in starch gell buffered at pH 7.2 under 100 v. 40 mAmp. for three hours.

RESULTS

1. Distinguishing Features Between Peroxidase and Catalase as Hydroperoxidases.

Catalase exhibits two types of enzymatic activity. One is the disproportionation of H_2O_2 where it is both electron donor and acceptor to form O_2 and H_2O. This reaction predominates increasingly with higher concentrations of H_2O_2. In the second type the electron donor is an organic compound while H_2O_2 is solely an electron acceptor or oxidant (28). Although the second type of reaction may utilize the same substrate as peroxidase, catalase produces a higher redox potential and can produce different end products. This reaction is observed at low H_2O_2 concentrations and is facilitated if not absolutely dependant upon polyunsaturated fatty acids (PUFA) or PUFA-hydroperoxides as described below in this section. Peroxidase does not required the presence of a hydroperoxide. Two other important differences between this catalase mediated oxidation of organic compounds described below and peroxidase is that the latter enzyme has lower oxidation potential and the oxidation mediated by it is reversible for many substrates as in the case with benzidine. Catalase however as reported herein produced a higher oxidation potential and its oxidation of benzidine was irreversible. Consequently the two enzymes produce different end products.

Peroxidase mediates the oxidation of benzidine by hydrogen peroxide to an unstable blue complex called benzidine blue. The complex is thought to consist of one molecule of the unreacted di-amine and one molecule of the di-imine (30,25). Polyunsaturated fatty acids (PUFA) facilitate but are not required for the conversion. The reaction is reversed by reducing compounds. Catalase with PUFA mediates the oxidation of benzidine by hydrogen peroxide to a yellow-brown product which cannot be reversed by reducing compounds nor is benzidine blue an essential intermediate. However the catalase mediated oxidation of benzidine was not observed to occur when PUFA was absent. In a mixed enzyme system with both catalase and peroxidase acting on benzidine all of blue, yellow and brown products are formed. When the oxidation was allowed to continue for some time all color dissapeared. Due to the simultaneous rapid formation and destruction of brownish products of varying absorption maximum in wavelength by catalase, the instrumental quantitation of individual products was impratical for the scope of this paper.

In the absence of any enzyme, titrating benzidine in the presence of permanganate and PUFA while measuring the permanganate equivalents converted to the manganous ion, showed that the blue product formed was the result of one electron oxidation. The high oxidation potential of the half reaction, [$MnO_4^- + 8H^+ + 5e^- = Mn^{+2} + 4H_2O$], made it difficult to stop at a one electron oxidation unless PUFA was present. Permanganate and PUFA could be replaced by ferricyanide to stabilize the blue one-electron oxidation product. When more equivalents of permanganate than dye were added in the presence of PUFA, benzidine was irreversibly oxidized to yellow-brown and then decolorized. The blue intermediate in a low redox media reverts back to colorless benzidine and can be converted back and forth between blue and colorless. When the dye was oxidized beyond the blue to the brownish product however, the reactions were not reversible. By the combination of the above results with those of horse radish proxidase and of bovine liver catalyse described below the color of the product was identified with either a one or a multiple electron oxidation and thereby the product of either peroxidase or catalase respectively.

Horse radish peroxidase mediated the oxidation of benzidine by H_2O_2 to the blue compound. No indication of a brownish benzidine product could be found either with or without linoleic acid present. This was evidence that peroxidase caused one electron oxidation.

Bovine liver catalase in the presence of linoleic acid mediated oxidation of the dye by H_2O_2 irreversibly to a yellow-brown product with no indication of a blue intermediate. This was evidence that catalase produced a multiple electron oxidation with a greater oxidation potential than peroxidase.

At low H_2O_2 concentrations catalase is known to function as a special type of peroxidase in that there is oxidation of organic substrates as with true peroxidase (28). When the H_2O_2 concentration was increased in the current study so did catalase mediated disproportionation of H_2O_2 into water and oxygen thus demonsrating its better known reaction.

The involvement of PUFA was found to be through the fatty acid hydroperoxide since soybean lipoxidase with PUFA could replace hydrogen peroxide in the enzymatic formation of either the blue or the brown benzidine products by proxidase or catalase respectively.

2. Species Difference in Peroxidase Mediated Benzidine Oxidation.

When benzidine was reacted with hydrogen peroxide and PUFA in the presence of buffered lysed blood of steelhead trout or of coho salmon a marked difference in the reaction rates and of the products formed was observed between the two species. Figure 1 shows a typical recording in optical density change at 610 nm due to benzidine blue formation in the reaction with the two types of blood. Click here to see Figure 1

A comparison of peroxidase activity of steelhead trout and of coho salmon blood showed that the capacity of trout to produce benzidine blue was over four times that of coho salmon based on an equal blood concentration in the reaction mixture. The initial addition of H_2O_2 denoted by an arrow to the reaction mixture in Figure 1 demonstrated the presence of a large peroxide sink in coho blood compared with that from trout. Upon the second addition of peroxide to the reaction mixture with coho salmon blood there was more than a two fold increase in optical density compared with the increase after the first peroxide addition. This suggests that over 60% of the peroxide initially added was preferentially absorbed in the coho blood before benzidine oxidation occurred but upon the second addition, since the sink was already saturated or partially filled then H_2O_2 was available for peroxidase. With trout blood the second addition of the same amount of peroxide to the reaction mixture gave a smaller increase than did the first addition. Therefore in trout any metabolism of H_2O_2 other than by true peroxidase was a small percentage of the total hydroperoxidase activity. In other words, the affinity for H_2O_2 by trout blood non-peroxidase enzymes waas low compared to the case with coho. In coho, peroxidase activity ratio to total hydroproxidase was much lower that in trout.

Oxidation by purified (hemoglobin fee) gill microsomes from the above two species showed the same trend in that the preparations from trout gill favored greater formation of the blue intermediate (lower catalase activity) than did coho salmon samples. This data is not shown.

Squawfish blood gave results similar to that of trout whereas Atlantic salmon blood although not shown here reacted very much like that of coho salmon.

3. Species Difference in Catalase Mediated Benzidine Oxidation.

It has long been known that catalase has greater affinity than peroxidase for H_2O_2 (28). The difference in rate of peroxide uptake in Figure 1 could be due to a greater catalase presence and affinity for H_2O_2 in coho than in trout. Further, although trout blood consistently had a higher hemoglobin concentration than coho, decreasing the trout blood concentration to coincide with that in coho (estimated by absorbency at 412 nm) caused an increase in the difference in the two species of their respective conversion rates of benzidine to the

blue compound compared to the reaction in non normalized blood concentrations of the two species. Also decreasing the blood concentration in the reaction mixtures of either species increased the formation rate of the benzidine blue as shown in Figure 2. Click here to see Figure 2

This is difficult to explain purely on the basis of the higher catalase activity in coho than trout since diluting the mixture still leaves the catalase/peroxidase ratio the same. However greater catalase affinity for peroxide or PUFA-hydroperoxide does explain the phenomonon in both Figures 1 and 2. That is, a decrease in the blood concentration would decrease the "hydrogen peroxide sink" with decreased catalase presence and leave more peroxide available to the weaker binding peroxidase in proportion to the dilution.

The end point where excess hydrogen peroxide available to peroxidase was used up was indicated when the increase in benzidine blue formation stopped. However due to the greater affinity for H_2O_2 by catalase the irreversible oxidation of benzidine and benzidine blue to the brownish products continued. This is seen in both Figures 1 and 2 as the dissappearance of the blue intermediate. The formation rate of the yellow-brownish product although not illustrated in this report was much greater in coho than in trout blood. As explained in section 1, horse radish peroxidase would not mimic oxidation to brown products which was mediated by bovine liver catalase.

Figure 2 shows that at all three trout blood concentrations the initial rate of O.D. increase at 610 0m (benzidine blue formation) was the same indicating there was a limiting substrate concentration relative to peroxidase activity. However with coho the higher the blood concentration the lower the initial rate of O.D. increase at 610 0m. This is an apparent consequence of a greater H_2O_2 sink with higher blood concentrations. The inverse ratio of blood concentration to rate of benzidine blue formation shown in Figure 2 demonstrates the effect of the catalase to peroxidase ratio in determining the metabolic outcome of the oxidation products. It underscores the need for proper balance in these two enzymes if indeed not all hydroperoxidases by tissues to accomplish desirable metabolic ends utilizing H_2O_2.

4. Species Difference in Catalase Dissproportionating Activity.

In addition to the higher catalase mediated oxidation of benzidine, coho was also found to posses a higher catalase mediated gas evolution rate from H_2O_2 than trout as shown in Figure 3. The average initial O_2 evolution rate was 28.8 Fl O_2/min/ul blood from coho compared to 9.2 from steelhead. Click here to see Figure 3

The results of other workers with different chemicals suggests that the two reactions demonstrated here, O_2 generation and benzidine destruction are the result of the same catalase enzyme (28). However the same site on a given molecule is not expected to mediate both reactions simultaneously. The catalse molecule has four heme groups and an equilibrium will exists with benzidine and H_2O_2 competing for the same enzyme site to function as a reducing substrate. Equilibrium would shift toward binding H_2O_2 as a substrate at high proxide concentrations and dissproportionation activity would thereby increase. While at low peroxide concentrations organic substrate oxidation would increase.

5. Electrophoretic Separation of Catalase and Peroxidase in the two species

As another means of evaluating species differences for catalase and peroxidase a simple agar gel electrophosis was done of equal concentrations of coho and of Steelhead blood to separate the two enzymes. The result illustrated by Figure 4 showed that the major hemoglobin portion of applied blood moved ahead of the catalase activity. Click here to see Figure 4

As indicated, by runs 6, 7 and 8 steelhead had an additional faster moving hemoglobin band which was

eliminated by pretreatment with H_2O_2. Coho blood however did not exhibit this fast moving moity regardless of substrate presence or absence. The data in Figures 1 & 3 although demonstrating species differences in catalase were not able to define a peroxidase species difference. The electrophoretic pattern shown by Figure 4 did reveal an apparent difference in the hemoglobin associated peroxidase activity for the two species.

6. Co-factor Effects on Peroxidase

Realizing the differences between the several species in the above enzyme activities it was desirable to determine whether the catalase deficiencies in trout could be reversed by any means. Two types of physiologically active agents were investigated, hydrophyllic and hydrophobic antioxidants. For the first type ascorbic acid was added at physiological levels to the same reaction mixture described in Figs. 1 and 2. Figure 5-insert shows that ascorbic acid inhibition (AAI) occurs for benzidine oxidation measured at 610 0M and that it is noncompetitive (i.e. the inhibition of activity or oxidation rate is not dependent upon substrate concentration). Click here to see Figure 5

The control is shown in comparison where no ascorbic acid is present. The inhibition by ascorbic acid was 100% and was not proportional to the ascorbic acid added. However the duration of complete or noncompetitive inhibition was proportional to the ascorbic acid concentration which caused 1.2 seconds inhibition per ug ascorbic acid in the 1.5 ml reaction. After the ascorbic acid was consumed the the optical density at 610 0m (blue intermediate) began to increase. The rate of increase (x/y) at this point was inversely proportional to the duration of inhibition (AAI) shown in Figure 5 insert. This reflects the H_2O_2 decrease caused by catalase during the AAI period. Figure 5 is a plot of the initial peroxidase activity at the end of the AAI period for both steelhead and coho blood when differeing amounts of ascorbic acid was added. It shows the initial rate of O.D. increase per minute at the end of the inhibition period where all ascorbic acid had been consumed on the plot of figure 5-insert. The duration of inhibition is plotted on the horizontal axis of Figure 5 and was directly proportional to the reducing equivalents of ascorbic acid added. A comparison of the reactions in rate of O.D. increase at the zero AAI point (no ascorbic acid added) is seen to be about 0.6 for coho and 1.8 for steelhead or a ratio of 1/3. It is possible if not likely that the lower expression of peroxidase in coho is due to the initial binding of H_2O_2 by the higher catalase in this species (shown earlier with Fig. 1).

Because there was less inhibition by ascorbic acid upon the coho than trout enzyme both species show the same peroxidase activity at the 3 minute point in Figure 5, where 150 Fg of ascorbic acid was consumed. After 100 Fg ascorbic acid was consumed (at 2 minutes) by the steelhead reaction it had the same peroxidase activity as did the coho with no ascorbic acid (0 minutes). Ascorbic acid inhibition was measurable down to about 7 Fg/ml in the reaction mixture. That is within the normal physiological level of 1 Fg/ml in serum and 200 Fg/ml in white blood cells (Wilcox & Grimes 1961; Morse et al. 1956).

Lipophilic agents were investigated and also found to affect the ratio of catalase to peroxidase activity. The agents tested were Turgitol, dimethylsulfoxide, tocopherol and retinol. Only the results with tocopherol and retinol are shown here. Figure 6 shows the peroxidase activity in their presence along with that of ascorbic acid. Click here to see Figure 6

As with figures 1 and 2 this shows the results of peroxidase and the opposing effect of catalase. In contrast to the noncompetitive inhibition caused by ascorbic acid shown in Figure 5 insert and in Figure 6 the lipophyles inhibited peroxidase activity in proportion to their concentration at the outset of addition showing competitive inhibiton. Regardless of the concentration of the lipophylic agent, the change in the rate of peroxidase activity was not observed to revert to the prior rate in contrast to the case with ascorbic acid. This can be explained by the fact that ascorbic acid was consumed through oxidation via peroxidase but the lipophylic agents were not. A plot of the O.D. change at 610 nm for retinol, and tocopherol is shown along with that of ascorbic acid and of the no co-factor control in Figure 6. The concentrations used for both ascorbic acid and tocopherol was in the physiological range while that for retinoic acid used here would be toxic. Although the mechanism of action was different for the lipophyles than for ascorbic acid it was observed that with both types of co-factors

the ratio of catalase to peroxidase activity by trout enzymes could be induced to more closely resemble that seen with coho as was also true for the type of benzidine metabolism occurring.

DISCUSSION

The results reported here reveal a marked deficiency of catalase in steelhead trout compared to coho salmon. This was true for both of two methods of measuring catalase activity - H_2O_2 disproportionation into oxygen plus water and PUFA dependent irreversible oxidation of benzidine. The benzidine test for blood using acetic acid, hydrogen peroxide and benzidine requires that an organic hydroperoxide be formed as the electron acceptor. The reaction is interesting in that with acetic acid a several molar concentration is required. When a longer chain saturated fatty acid such as stearic acid replaces acetic acid no reaction occurs. However if a long chain polyunsaturated fatty acid (PUFA) is employed then only a micro-molar physiological concentration is required. The potential importance of the reaction lies in the fact that a whole class of dyes which are reactive to the test are considered to be carcinogenic and in the fact that the physiological concentration and type of fatty acid present effects the type of reaction that the dyes undergo.

Of further importance is the fact that the ratio of catalase to peroxidase activity could be increased by inhibition of peroxidase activity with ascorbic acid or with several lipophylic agents. The increase in apparent catalase activity could be due both to enhancement of catalase and inhibition of peroxidase. However the inhibitory effect upon peroxidase was the only action studied and reported here.

This report highlights the significance of coho having three fold greater catalase activity than trout as possibly being related to complete resistance to aflatoxin induced hepatoma with the former species in reports by other workers while the latter is highly susceptible. In rainbow trout 96% of animals given 20 ppb dietary aflatoxin B_1 for 20 months developed hepatoma but coho salmon given the same treatment had no tumors (7,9). Although not shown here atlantic salmon showed similar catalase activity as coho and is also apparently aflatoxin resistant. There have been other reports of reduced catalase activity in tissues of cancerous animals (11,19). The PUFA dependedent effect of catalase reported here is a reflection of the work of Canellakis and co-workers (4). Using uric acid as substrate, they reported that catalase plus ethyl hydroperoxide represented a much stronger oxidase system than any of the other enzyme system studied but that H_2O_2 as such and catalase were unreactive toward uric acid oxidation. A review of the work establishing a peroxidative role for catalase has been published (17). Hydrogen peroxide has been shown to be a part of an animal's defense mechanism against bacteria (24,15), virus (3), fungi (16) and murine tumors (31). There is evidence that H_2O_2 is part of the phagocytic activity of leukocytes and that its synthesis is localized in these cells (12). Reduced hydrogen peroxide synthesis was reported in hepatomas and ascites cells (6). The present and others reports suggests that tumor susceptibility upon external challenge to animals may be associated with a deficiency or upset balance of a matrix of factors including peroxide metabolizing enzymes, PUFA concentration, both vitamin and mineral co-factors affecting hydroperoxidases and availability of H_2O_2. Consistent with the above concept are the results reported here demonstrating that physiological levels of ascorbic acid and lipophilic antioxidants increased the catalase to peroxidase ratio. These data may explain one reason why coho salmon have a lower requirement than trout for vitamin C (8) and are less susceptible to certain toxins and viral infections (2,22,29). Lipophilic antioxidants have also been observed to inhibit dimethyl-benz-anthracene induced carcinogenesis in rats (10).

Others workers have shown measurable metabolic changes by increasing catalase activity. This was most effective when combined with other antioxidant enzymes. Transgenic D. malanogaster have been produced with increased superoxide dismutase (SOD) and catalase which increased their lifespan and activity by 30% (23). Transgenic mice with increased catalase and SOD had increased protection against reactive oxygen

species but when only SOD was increased there was less protection (1). An SOD-catalase conjugate given by perfusion protected mice from reperfusion injury of the heart where SOD alone gave no benefit (18). In a like manner a perfusion media supplemented with a mixture of the catalase and SOD as separate enzymes showed reduced injury against reperfusion injury of the heart (14). Transgenic mice having 60 to 100 fold increased catalase were protected against oxidative damage by doxorubicin or free radicals generated thereby (13). However further increase of catalse lost the benefit. Again these studies suggest that the ratio of various hydroperoxidases is critical. Murine L cells enriched for catalase had increased cytotoxic sensitivity to several compounds that promote H_2O_2 production. That work suggests that H_2O_2 is was not the sole or sufficient vector of toxicity generated by their test compounds (27).

Summary

The data presented here demonstrate that Coho salmon blood and tissue compared to steelhead trout have a much higher activity for catalase mediated oxygen evolution and also for a PUFA dependent, irreversible oxidation and destruction of benzidine in the presence of an alkyl hydroperoxide.

! The correlation of catalase deficiency with hepatoma and disease susceptiblity would support the concept that a defined ratio of catalase to other antioxidant enzymes may be a deciding factor in establishing resistance to cancer and other diseases. More work needs to be done to determine how widespread this correlation is.

! The involvement for cis-polyunsaturated fatty acids in expediting catalase mediated irreversible oxidation of an organic substrate underlines the role of essential fatty acids and conceivably the ratio of cis- to trans- or to saturated fatty acids in disease and cancer resistance.

! The inhibitory action on peroxidase by ascorbic acid, retinoic acid and tocopherol wherby catalase is enhanced especially in a hypocatalasemic species or individual raises the importance these co-factors may have in maintaining resistance to cancer and other diseases.

! Since H_2O_2 is essential in the catalatic destruction of toxins, carcinogens or malignant cells it follows that metabolic substrates, enzymes and co-factors which form peroxide would play a role in disease resistance.

! It is likely that all of the above are factors within a matrix of variables which must be properly balanced or regulated to provide the optimum resistance to cancer and disease. Definitions of possible methods to control all these variables as well as where the proper balance lies will be essential for success in understanding and preventing cancer and other recalcitrant diseases.

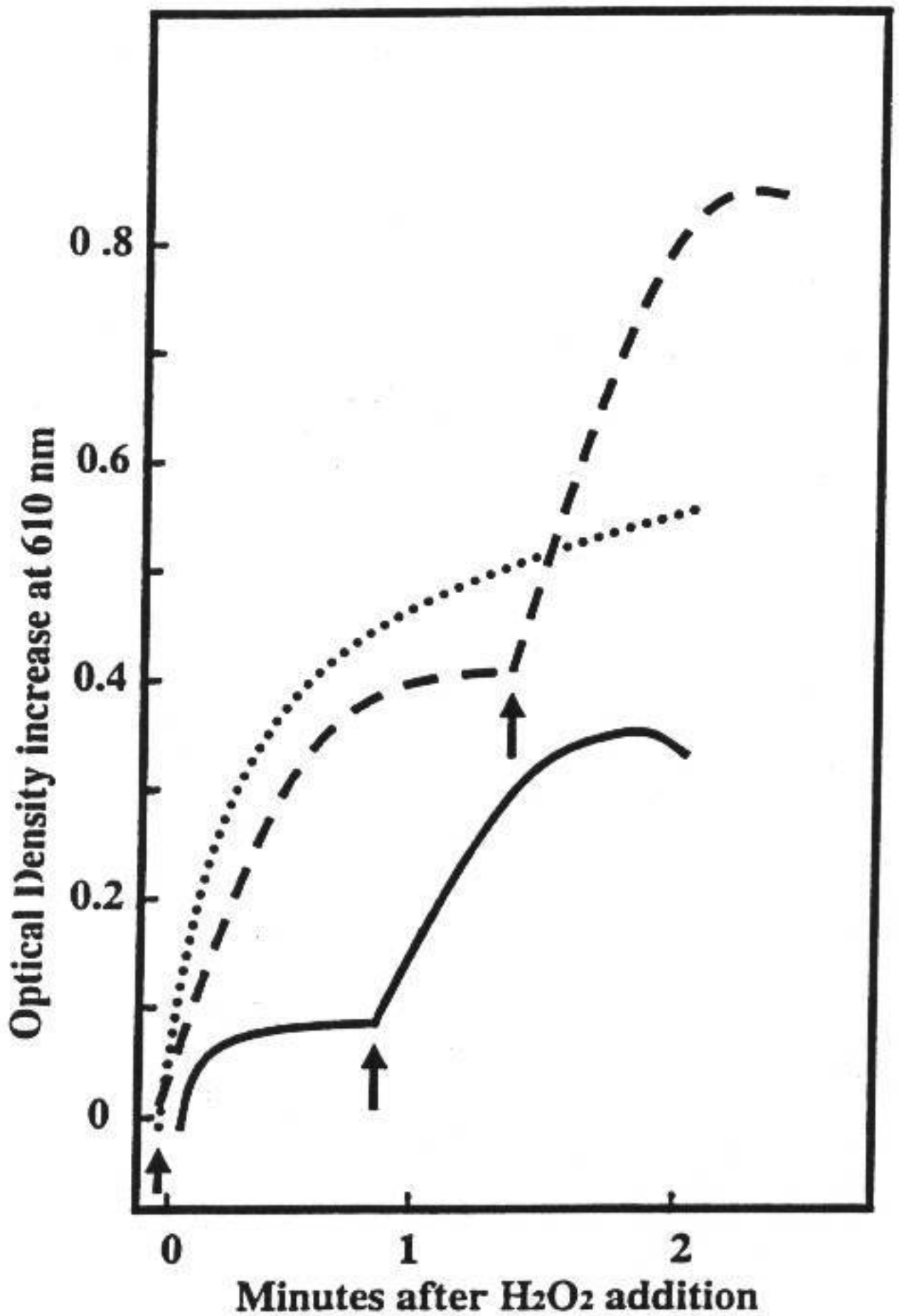

Figure 1. Peroxidase activity using benzidine as the substrate measured as an increase in optical density at 610 nm from blood of steelhead trout (---), coho salmon (___) and squawfish (·····). The initial reaction mixture for each species contained 1.5 ml of 0.05 M phosphate buffer, pH 7.2, 4 μmoles un-esterified essential fatty acids, 50 μl ethanol, 1.25 μl lysed blood supernate, and 0.27 μmoles benzidine. Hydrogen peroxide aliquots at 4.5 μmoles were added where indicated by the arrows.

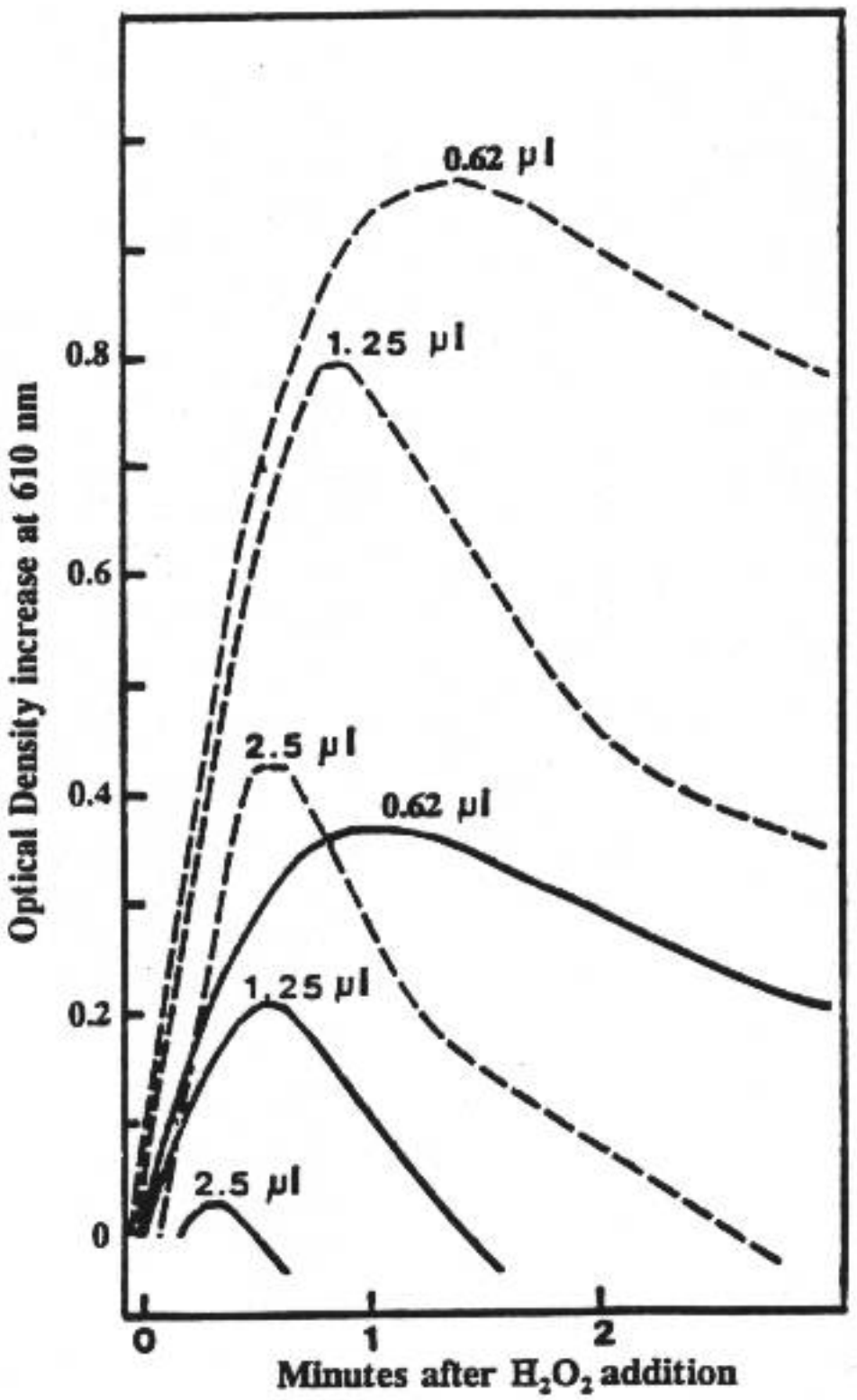

Figure 2. Effect of variation in concentration of lysed blood on peroxidase activity measured as the change in optical density at 610 nm with benzidine as the substrate for Steelhead trout (----) and Coho salmon (___). The amount of lysed whole blood in the reaction mixture is shown in µl. Conditions were the same as in Fig. 1, except, that blood addition varied from 0.65 to 2.5 µl/1.5 ml reaction volume and 9 µmoles of H_2O_2, were added instead of 4.5 to initiate the reaction.

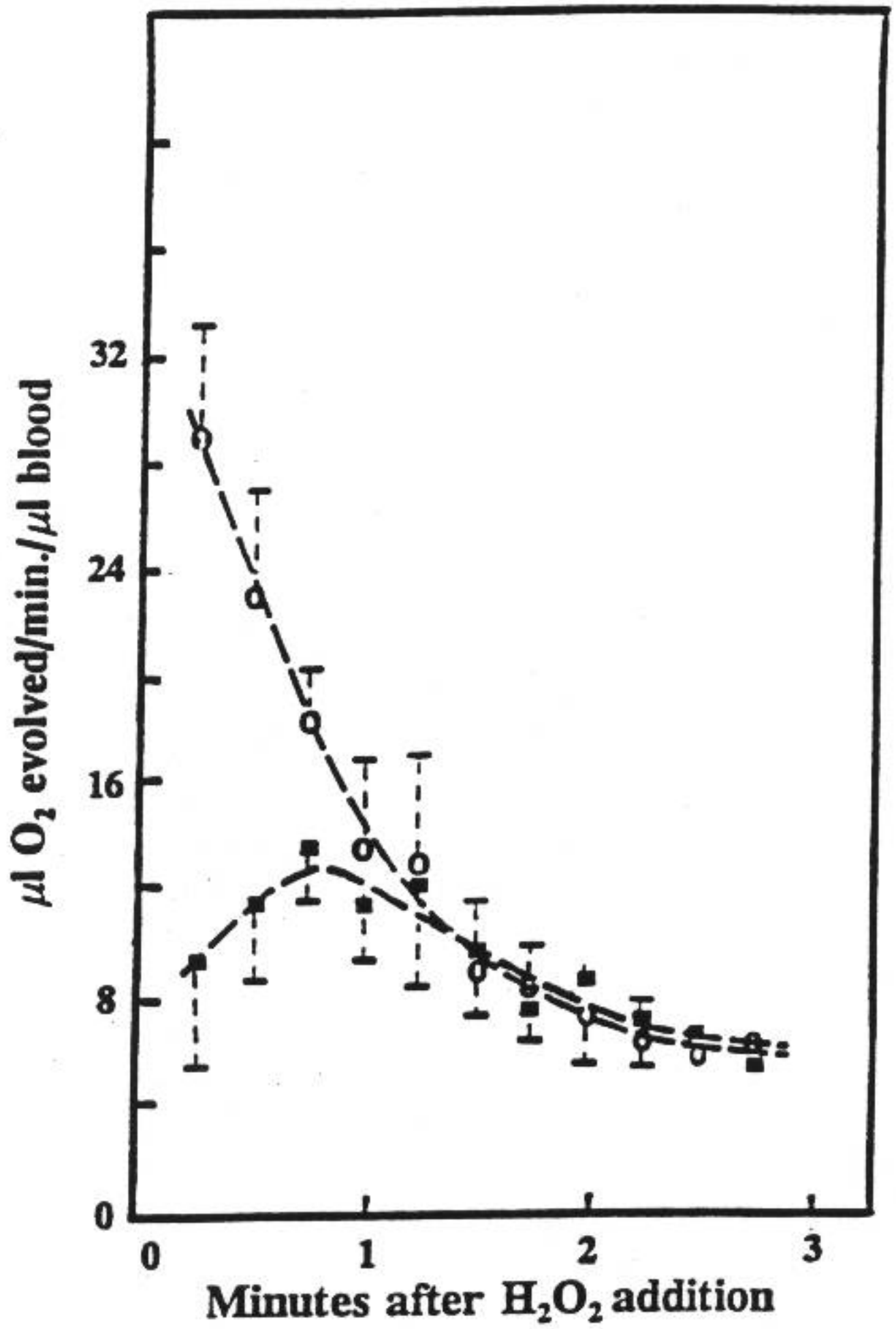

Figure 3. Relative catalase activity in blood of steelhead trout (▪), and coho salmon (o) measured as μl gas evolved per minute per μl whole blood. The closed reaction vessel was connected to calibrated plastic tubing with a marker solution and contained the same ingredients as in fig. 1 except that 45 instead of 4.5 μmoles H_2O_2 were added at time = 0. Each point is the mean of five tests, with standard deviations shown by broken lines.

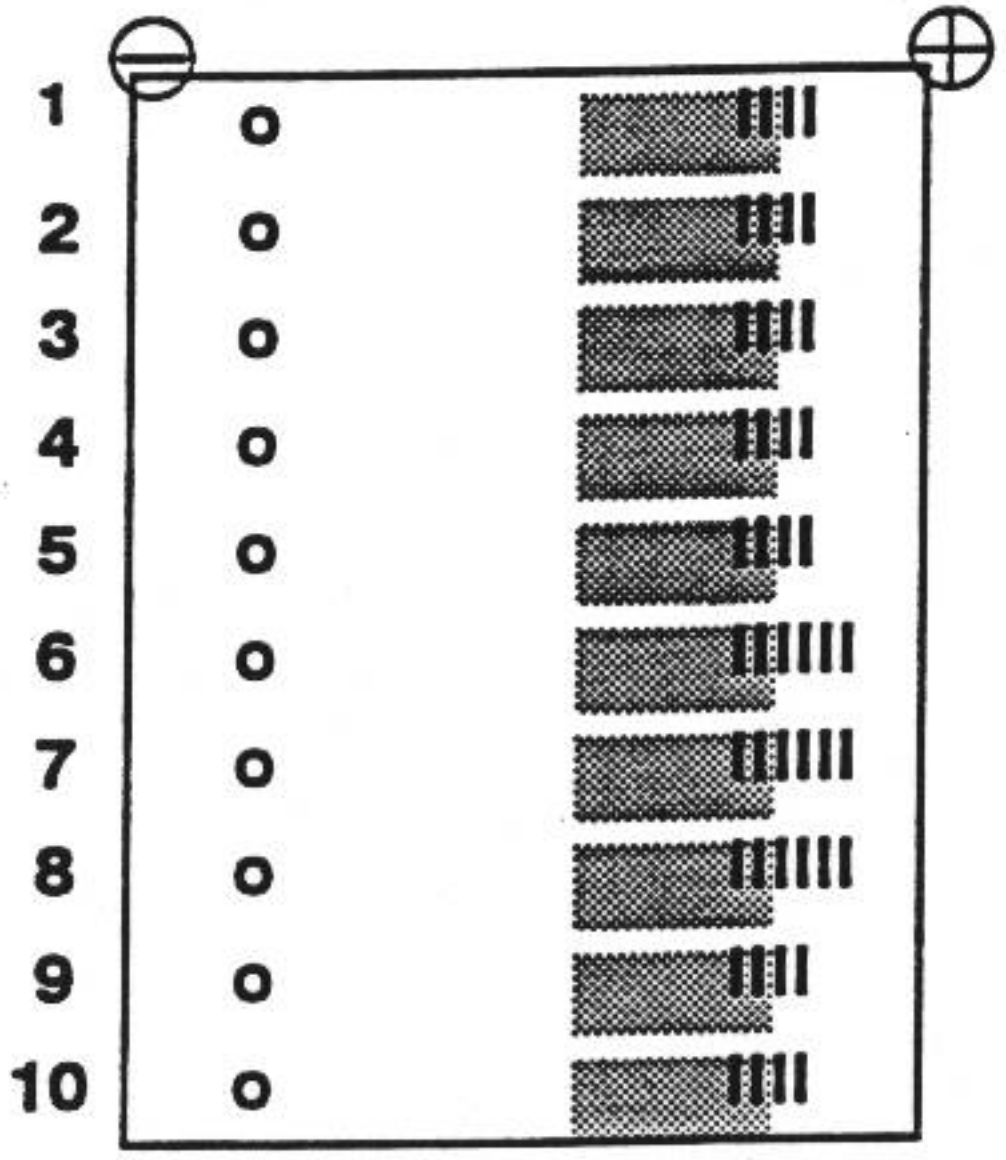

Figure 4. Electrophoretic separation of catalase from peroxidase in lysed blood of coho salmon and steelhead trout. Numbers 1-5, were of coho salmon and 6-10, steelhead trout at 3 μl whole blood each in 25 μl solution. Numbers 1, 2, 9 and 10, had H_2O_2 and in addition numbers 1 and 10 had benzidine. The reagents were added in wells (o) at time 0. Conditions were 2% agar in 0.05 M phosphate buffer pH 7.2, 100 volts, at 40 milliamperes for 3 hours. Oxidative catalase (IIIII), Hemoglobin-peroxidase (▒▒▒▒▒).

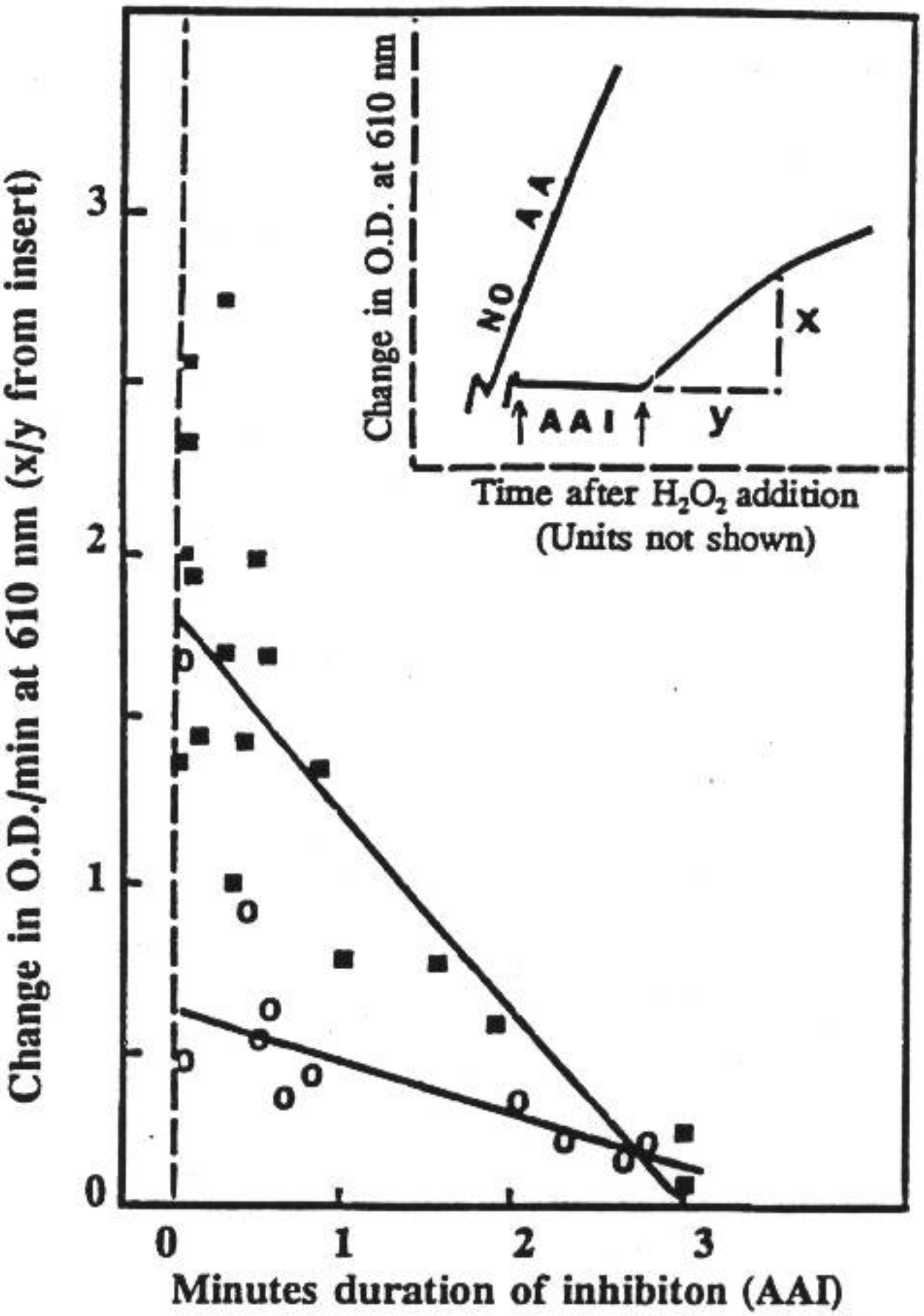

Figure 5. Ascorbic acid induced inhibition (AAI) of peroxidase activity by steelhead trout (▪) and coho salmon (o) blood measured by the increase in O.D. at 610 nm with benzidine as substrate. The reaction mixtures were similar to Fig. 1 except that ascorbic acid was added at 0.06 to 0.90 μmoles per 1.5 ml reaction volume. The H_2O_2 was added last at 9 μmoles where time = 0. The ordinate is the optical density increase per minute (slope of x/y from the insert). The abscissa is the length of time in which ascorbic acid inhibited peroxidase activity (represented by AAI from the insert) which was also proportional to the amount of ascorbic acid added.

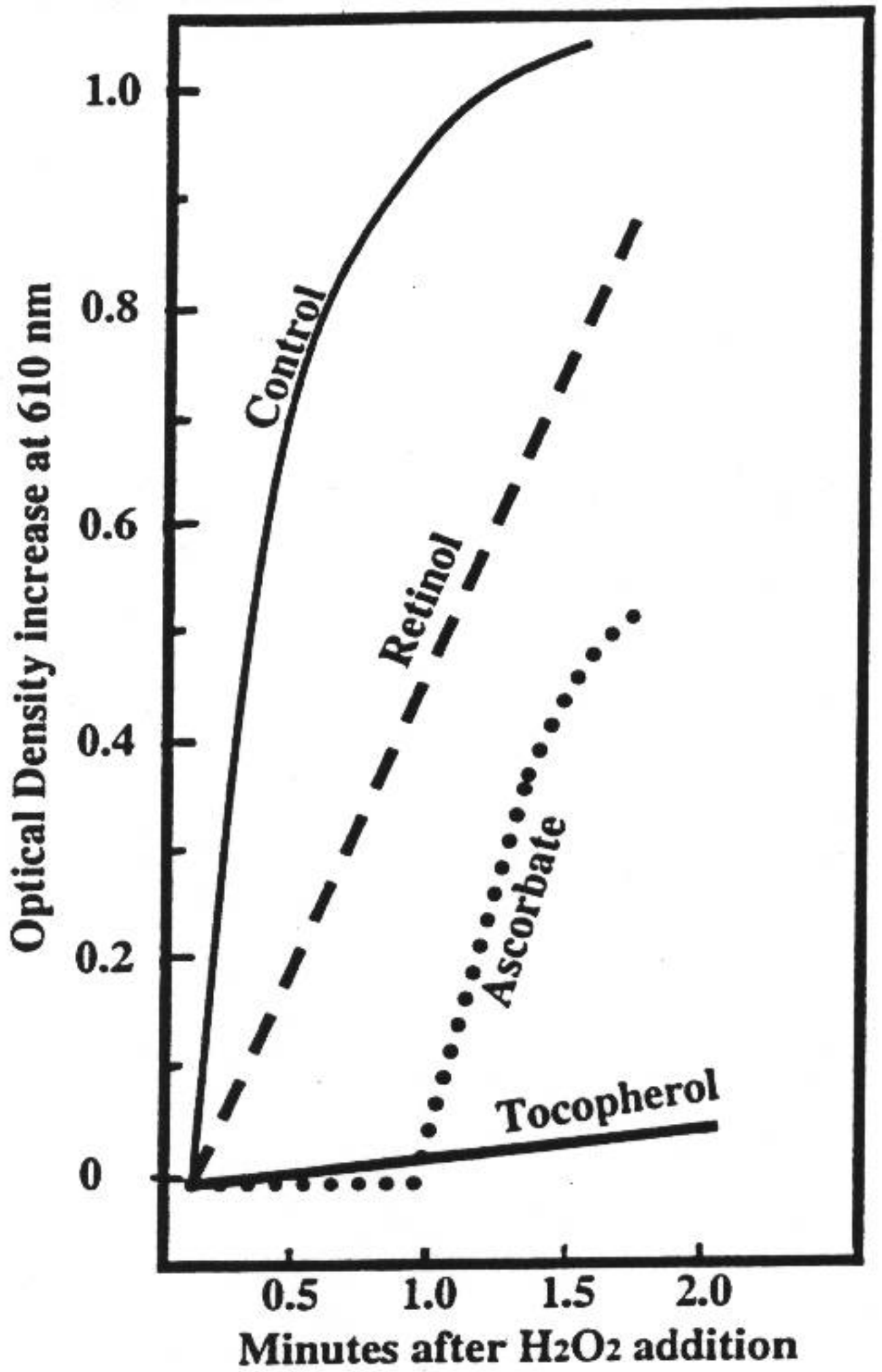

Figure 6. Comparison of the type of co-factor as inhibitors of peroxidase activity in steelhead trout blood. The values are representative of steelhead but are from a particular individual and not overall averages. Conditions were identical to Figure 1 with the following modifications: Control = no co-factors added; ascorbate = 25 μg/ml; retinol acetate = 2880 units of vitamin A activity/ml; tocopherol = 0.13 mg/ml.

REFERENCES

1. Amstad, P.; Peskin, A.; Shah, G.; Mirault, M.; Moret, R.; Zbinden, I.; Cerutti, P. The Balance between Cu,Zn-Superoxide Dismutase and Catalase Affects the Sensitivity of Mouse Epidermal Cells to Oxidative Stress. Biochemistry 30:9305-9313;1991.
2. Ashley, L. M. Nutritional Pathology. In: Fish Nutrition. Halver, J.E. (ed.) New York & London: Academic Press; 1972:439.
3. Belding, M.F.; Klebanoff, S.J. Peroxidase-mediated viricidal systems. Science 167:195-197;1970.
4. Canellakis, E.S.; Tuttle, A.L.; Cohen, P.P. A comparative study of the end-products of uric acid oxidation by peroxidase. J. Biol. chem. 213:397-404;1955.
5. de Kinkelin, P.; LeBerre, M.; Meurillon, A. Demonstration de l'etat refractaire du saumon coho (Oncohynchus kisutch) et de la truite fario (Salmo trutta). Bull. Fr. Piscic. 253:166-176;1974.
6. Dionisi, O.; Galeotti, T.; Terranova, T.; Azzi A. Superoxide radicals and hydrogen peroxide formation in mitochondria from normal and neoplastic tissues. Biochim. Biophys. Acta 403:292-300;1975.
7. Halver, J.E. Aflatoxicosis and trout hepatoma. In: Aflatoxin. Goldblatt, L.A. (ed.) New York, London: Academic Press; 1969:265.
8. Halver, J. E.;Ashley, L. M.; Smith, R. R. Ascorbic acid requirements of coho salmon and rainbow trout. Trans. Amer. Fish. Soc. 98:762-771;1969.
9. Halver, J.E.; Ashley, L.M.; Smith, R.R. Aflatoxicosis in coho salmon. In: Neoplasms and Related Disorders of Invertebrate and Lower Vertebrate Animals. Dawe, C.J.;Harshbarger, J.C. (ed.) Bethesda, MD: National Cancer Institute; 1960:141.
10. Harman, D. Dimethylbenzanthracene induced cancer: Inhibiting effect of dietary vitamin E. Clin. Res. 17:125;1969.
11. Hokama, Y.; Kimura, L.H.; Kobara, T.Y.; Perreira, S.M.; Su, S.W.P.; Torikawa, C.; Oishi, N. Variation in peroxisomal enzyme levels of peripheral leukocytes of cancer, leprosy and tuberculosis patients. Cancer Res. 34:2784-2789;1974.
12. Iyer, G.Y.N.; Islam, M.F.; Quastel,; J.H. (1961) Biochemical aspects of phagocytosis. Nature 192:535-541;1961.
13. Kang, Y.J.; Chen Y.; Epstein P.N. Supression of Doxorubicin Cardiotoxicity by Over exapression of Catalase in the Heart of Transgenic Mice. J. Biol. Chem. 271:12610-12616;1996.
14. Kirshenbaum, L.A.; Singal, P.K. Increase in endogenous antioxidant enzymes protects hearts against reperfusion injury. Am. J. Physiol. 265 (Heart Circ. Physiol. 34):H484-H493;1993.
15. Klebanoff, S.J. (1968) Myeloperoxidase-halide-hydrogen peroxide anti-bacterial

system. J. Bact. 95:2131-2138;1968.

16. Lehrer, R.I. Antifungal effects of peroxidase systems (human). J. Bact 99:361-365;1969.

17. Mannering, G.J.; Van Harken, D.R.; Makar, A.B.; Tephly T.R.; Watkin, W.D.; Goodman, J.T.I. Role of the intracellular distribution of hepatic catalase in the peroxidative oxidation of methanol. Ann. N.Y. Acad. Sci. 168:265-280;1969.

18. Mao, G.D.; Thomas, P.D.; Lopaschukk, G.D.; Poznansky, M.J. Superoxide Dismutase (SOD)-Catalase Conjugates role of hydrogen peroxide and the fenton reactin in sod toxicity. J. Biol. Chem. 268:416-420;1993.

19. Meszaros, I.; Goth, L. Uber die katalaseaktivitat der erythrozyten von krebspatienten. In: Internationales Symposium uber Struktur und Funktion der Erythrozyten. Rapoport, S.; Jung, F. (ed.) Berlin, Acadamie Verlag; 1975:403.

20. Morse, E.H.; Potgieter, M.; Walker, G.R. Ascorbic acid utilization by women. J. Nutr. 58:291-298;1965.

21. Nicholls, P.; Schonbaum, G. R. (1963) Catalases. In: The Enzymes. Boyer, P.D.; Lardy, H.; Myerback, K. (eds.) New York & London: Academic Press; 1963:147.

22. Ord, W.M.; LeBerre, M.; de Kindelin, P. Viral hemorrhagic septicemia: Comparative susceptibility of rainbow trout (Onchorinchus kisutch) and hybrids (S. gairdneri x Oncorhynchus kisutch) to experimental infection. J. Fish. Res. Bd. Can. 33:1205-1208;1976.

23. Orr, W.C.; Sohal, R.S. Extension of Life-Span by Overexpression of Superoxide Dismutase and Catalase in Drosophila malanogaster. Science 263:1128-1130;1994.

24. Paul, B.B.; Strauss, R.R.; Selvaraj, R.J.; Sbarra, A.J. Peroxidase mediated antimicrobial activities of alveolar macrophage granules. Science 181:849-850;1973.

25. Rodd, E.H. (ed.) Chemistry of Carbon Compounds, Vol III Part B. Amsterdam, New York and London: Elsevier Pub. Co. ;1956:1036-1037.

26. Shilotri, P.G. Glycolytic hexose monophosphate shunt and bactericidal activities of leukocytes in ascorbic acid deficient guinea pigs. J. Nutr. 107:1507-1512;1971.

27. Speranza, M.J.; Bagley, A.C.; Lynch, R.E. Cells Enriched for Catalase Are Sensitized to the Toxicities of Bleomycin, Adriamycin, and Paraquat. J. Biol. Chem. 268:19039-19043;1993.

28. Tauber, H. Crystalline Catalase, A Peroxidase. Proc. Soc. Exptl. Biol. Med. 81:237;1952.

29. Wedemeyer G. The stress of formalin treatments in rainbow trout (Salmo gairdneri) and coho salmon (Oncohynchus kisutch). J. Fish. Res. Bd. Can. 28:1899-1904;1971.

30. Welcher, F.J. (ed.) Organic Analytical Reagents, Volume II. Toronto, New York and London: D. Van Nostrand Co. Inc.; 1948;275-277.

31. Weishaupt, K.R.; Gomer, C.J.; Dougherty, T.J. Identification of singlet oxygen as the cytotoxic agent in photo-inactivation of a murine tumor. Cancer Res. 36:2326-2329;1976.

32. Wilcox, E.B.; Grimes, M. Gingivitis-ascorbic acid deficiency in the Navajo I. Ascorbic acid in the white cell-platelet fraction of blood. J. Nutr. 74:352-356;1961.