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EFFECT OF pH ON IN VITRO OXYGEN CONSUMPTION AND MUCUS PRODUCTION OF GILL TISSUE OF RAINBOW TROUT (SALMO GAIDNERI)

presented by

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Major professor

Date August 26, 1982

# EFFECT OF pH ON *IN VITRO* OXYGEN CONSUMPTION AND MUCUS PRODUCTION OF GILL TISSUE OF RAINBOW TROUT (*SALMO GAIRDNERI*)

By

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ABSTRACT

EFFECT OF pH ON *IN VITRO* OXYGEN CONSUMPTION AND MUCUS PRODUCTION OF GILL TISSUE OF RAINBOW TROUT (*SALMO GAIRDNERI*)

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The mechanism causing fish death after exposure to low pH remains undefined. Anoxia, as evidenced by decreased oxygen consumption, has been suggested as a causative factor. The transfer capacity of fish gills is influenced by their oxygen consumption and by the diffusion distance across the gill. This investigation was conducted to determine the effect of pH on both of these parameters. Trout were exposed to acid water (pH 6.0, 5.0 and 4.0) for 2 h and oxygen consumption and mucus content of the gill tissue were determined. Gill tissues from exposed fish showed higher oxygen consumption and greater mucus production than controls (pH 7.4). Histologically, exposed tissues revealed more mucus cells and exhibited greater damage. It is suggested that increased mucus production influences the oxygen transfer capacity of gills three ways: (a) by increasing diffusion distance, (b) by preventing proper ventilation and (c) by increasing gill metabolism.

DEDICATION

To my beloved parents

Hj. Mohd. Noor Hj. Daud and Hjh. Asma' Hj. Saad

From a grateful son

# ACKNOWLEDGEMENTS

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# INTRODUCTION AND LITERATURE REVIEW

## A. Water Pollution and Its Consequence

Pollution of our surface waters has been a problem worldwide for many years. Many fish and other aquatic animals which are directly affected by pollution, not only serve as an important protein source for a lot of people, but also provide various medically and industrially important chemicals. Therefore, anything which is a threat to the life of these animals is undesirable. Currently acidification of fresh water lakes and rivers is perhaps the most widespread serious problem of water pollution.

Acidification of our environment is indirectly due to the steady increase in our demand for energy. Acid loading of lakes arises either from dilute acid in precipitation or from dry deposition of oxides of sulfur or nitrogen which, on contact with water, form their respective strong acids (Conroy et al., 1976; Dillon et al., 1978; Likens et al., 1979). Galloway, Likens and Egerton (1976) concluded that sulfuric and nitric acids are the primary sources of excess $H^+$ in acid precipitation. Petroleum used for power generation and in internal combustion engines is a prime source of oxides of nitrogen whereas combustion of coal in electric power plants, smelters and steel mills leads to production of oxides of sulfur.

Some lakes are more susceptible to acidification than others because of a lower buffering capacity of the watershed (Likens et al., 1979). For example, the pH of George Lake in Ontario, a poorly buffered lake, declined at an estimated rate of 0.13 pH units per year from 1961 to 1973 (Beamish et al., 1975).

Research on the biological effects of water pollutants during the past 30 to 40 years has been primarily concerned with the determination of concentration of pollutants which kill test animals. The European Inland Fisheries Advisory Commission (1969) pointed out that the safe range of environmental pH for fish survival appears to be 5-9 and for maximum productivity the pH value should be between 6.5 and 8.5. These criteria have been generally accepted.

Freshwater fish are vulnerable to lowered environmental pH because the buffer capacity of their body fluids is low and consequently is easily overwhelmed if acid enters the fish or if metabolic acid is prevented from being excreted. Since the plasma $P_{CO_2}$ of fish is low (2-3 mmHg), the bicarbonate content must be correspondingly low in order to maintain the pH in the physiological range. In addition, blood protein and hemoglobin in fish are low compared to mammals; thus the non-bicarbonate buffer capacity is also low. Furthermore, fish do not have the luxury of manipulating the bicarbonate/carbonic acid buffer system by changing respiratory ventilation, viz., respiratory ventilation is dedicated to providing adequate oxygen to the fish.

B. Effect of Acid Stress on Growth and Reproduction of Fish

Data from field studies on the effect of acid stress on growth of fish are difficult to assess due to a variable food supply. Environmental acidifiction is often accompanied by an increase in heavy

metal ion concentration and it is very difficult to separate the effects of these ions and that of pH on growth (Beamish, 1974). There are conflicting results from well controlled hatchery-laboratory experiments where there was no increase in heavy metal ion concentration with increased acidification. Leivestad et al. (1976) showed that brook trout fed ad libitum grew two times faster at pH 6.0 than those at pH 4.6. Jacobsen (1977) found that brown trout yearlings fed a daily ration of 2.9% of initial body weight had similar growth rates at pH 6.26, 5.44 and 5.00 over a 48 day test period. Menendez (1976) reported that in a 5-month experiment adult brook trout exposed to pH 4.5 and 5.0 grew slower than controls (pH 7.0) during the first 3 months but there was no difference during the next 2 months.

Acid stress significantly affects the reproduction and development of fish. Beamish (1976) observed that most females of a particular species taken from acidified lakes did not release their ova prior to death and this failure to spawn was associated with the inability of the females to maintain normal serum calcium levels. Under laboratory conditions Mount (1973) reported that egg production and egg hatchability of fathead minnows exposed to pH 5.9 and below were reduced and that all eggs were abnormal. Most researchers agree that the no effect level of pH for successful reproduction of fishes is around 6.5 (Craig & Baski, 1977; Menendez, 1976; Mount, 1973).

C. Physiological Studies on the Effect of Acid Stress on Fish

In addition to field studies documenting fish loss, many physiological studies have been performed in an attempt to clinically describe the cause of death of fish due to acid stress (reviewed by Fromm, 1980). The gills of teleost fish are in direct contact with

water, and continuous exposure of fish to acid stress can cause alteration of gill membranes and/or coagulation of gill mucus. Damage to the gill epithelium may also cause disturbances in gill functions such as ionoregulation (Packer and Dunson, 1970; Lloyd and Jordan, 1964), acid-base regulation (Reeves,1977) and oxygen uptake (Packer and Dunson,1972). These changes may actually represent the primary cause of death of fish exposed to severe acid stress. The actual mechanism of these processes, however, is not fully understood.

1. Failure of Ionoregulation

Since freshwater fish are hyperosmotic to their environment, there is an osmotic influx of water and diffusive loss of ions across the gills. The excess water is excreted via the kidneys with a concurrent loss of ions in the copius volume of dilute urine. The ions lost may be recovered from the diet or absorbed directly from the water via gill $Na^+/H^+$ or $Na^+/NH_4^+$ and $Cl^-/HCO_3^-$ exchanges or other active transport pumps. In addition to aiding the maintainance of the NaCl balance of the freshwater fish, gill ion exchange is important because the excretion of $HCO_3^-$ provides for a net loss of metabolic $CO_2$ and excretion of $H^+$ provides for the net loss of metabolic acid. Excretion of $NH_4^+$ represents a net loss of both acid and nitrogenous waste.

Total body sodium and chloride of acid exposed fish decrease with decreased pH of the environmental water (Packer & Dunson, 1970; Leivestad et al., 1976; McDonald & Wood, 1981). At low pH the influx of $Na^+$ and $Cl^-$ is blocked, but when fish are transferred to clean water, influx returns to normal (Packer & Dunson, 1970). McWilliams and Potts (1978) measured $Na^+$ influx and efflux across brown trout gills at an ambient pH ranging from 7.0 to 4.0 ($H_2SO_4$) and found that at pH below

6.0 a reduced influx and increased efflux probably contributed equally to salt loss. Based on their measurements of gill electrical potentials they also concluded that $Na^+$ effluxes were due to simple diffusion and that the permeability of the gill to $Na^+$ and to $H^+$ increased as pH decreased.

Water-borne calcium ion also affects the rate of sodium loss from freshwater fish (McWilliams and Potts, 1978). If calcium is high, diffusional salt losses are relatively slow and acid-base disturbance is the predominant symptom noted in acid stressed fish. At low calcium, failure of ionoregulation is the predominant symptom. The increased gill permeability with decreasing water-borne calcium and decreasing pH would account for the observed loss of $Na^+$ and $Cl^-$ down their electrochemical gradients.

In summary, failure of ionoregulation in acid exposed fish may be due to:

a) $H^+$ interfering with $Na^+$ uptake directly by competing with $Na^+$ for carrier sites

b) $H^+$ altering ion transport mechanisms

c) $H^+$ depressing the metabolic rate of transporting cell

d) $H^+$ displacing $Ca^{++}$ in the gill thus producing an overall increase in permeability.

Ionic imbalance due to loss of NaCl from the body may be one of the causes of death of fish subjected to severe acid stress.

2. Failure of Acid Base Regulation

Failure of acid-base regulation in fish as a result of exposure to acid stress will cause variations in blood pH, which in turn affect functions as diverse as blood oxygen transport (Riggs, 1970), cardiac

contractility (Poupa & Johansen, 1975) and plasma ionoregulation (DeRenzis & Maetz, 1975). More importantly, protein function will be affected if blood pH is changed. Reeves (1977) has pointed out that the ultimate effect of acid-base regulation is to preserve the net charge of protein. Severe fluctuations in blood pH when acid-base regulation fails may therefore cause death of fish by alterations in cellular metabolism.

Decreases in blood pH can be due to respiratory acid (i.e., $CO_2$), metabolic acids, e.g., lactate which could be metabolized, or "fixed" acid which must be excreted. The latter can arise directly from the influx of environmental $H^+$ across the gill.

How much is arterial blood changed in response to a decrease in environmental pH? Neville (1979b) found that there was a gradual change in arterial blood pH from 7.92 to 7.47 over 5 days in rainbow trout exposed to pH 4.00 (HCl). Packer (1979) showed that the arterial blood pH of brook trout exposed to pH 3.15 - 3.50 decreased from 7.8 to 7.2. Dively et al. (1977) exposed brook trout to pH 4.2 for 5 days and found that the arterial blood pH decreased by 1.5%, i.e., from 7.8 to 7.6. McDonald and Wood (1980) have shown that blood acidosis seems relatively minor during acid exposure in soft water as opposed to hard water. A decrease in blood pH and blood total carbonate has been observed in fish exposed to acidic hard water (Lloyd & Jordan, 1964; Neville, 1979a; Packer, 1979; McDonald and Wood, 1980; 1981). At sublethal pH, there is little change in blood $P_{CO_2}$ in salmonids (Neville, 1979a; Packer, 1979; McDonald and Wood, 1980; 1981) but there is an increase in arterial $P_{CO_2}$ and a reduction in $HCO_3^-$ in the white sucker, indicating a compound metabolic and respiratory acidosis in the latter. Measurements of blood

lactate show that the blood metabolic acid load is not derived from lactic acid (Neville, 1979a; McDonald and Wood, 1980). Therefore, the $H^+$ responsible for the metabolic acidosis observed in acid stressed fish almost certainly comes from the environment and is not endogenous in origin.

Decreases in blood pH can also be caused by environmental hypercapnia (increased $P_{CO_2}$). Cameron and Randall (1972) found that there was a slight decrease in arterial pH when rainbow trout were exposed to increased ambient $CO_2$. Lloyd and Jordan (1964) have demonstrated the joint toxic action of environmental hypercapnia and low pH by exposing rainbow trout to pH 4.5 (HCl) combined with elevated water $P_{CO_2}$, (about 19 mmHg) for one week. They found that this condition caused a much greater reduction in blood pH than when the fish were exposed to acid alone. This observation was supported by Neville (1979b) who found that fish exposed to hypercapnic and acid stress experienced a significantly more rapid decrease in $pH_a$ than those exposed to acid stress alone. Hypercapnia also increased ventilation rate, whereas acid exposure alone did not.

An important mechanism for pH regulation in mammals (Pitts, 1974) and in fish is intracellular buffering of $H^+$. Since this is only an internal redistribution of $H^+$, the excess $H^+$ must eventually be excreted to return the animal to normal status (Wood & Caldwell, 1978). Eddy (1976) infused approximately 4 mEq $H^+$/ml of blood and calculated that 20% of this load was buffered in the blood, the rest being buffered by interstitial fluid and intracellular fluid (ICF).

Ion losses can occur as a result of acid-base disturbance because the $H^+$ entering across the gill is not completely removed by the kidney

(McDonald and Wood, 1981). The $H^+$ then progressively penetrates the ICF at the expense of other cations which must come out of the cell under the constraint of electroneutrality. This considerable ionic disturbance could have complex physiological effects on the fish.

3. Failure of Oxygen Uptake

The respiratory process basically consists of 1) ventilation; 2) diffusion of oxygen from water to blood across the gill; 3) transport of oxygen via the blood to the tissues; and 4) consumption of oxygen at the tissues. Any interruption of the process can cause a reduction in the general metabolism or oxygen consumption. Wiebe et al. (1934) showed that fish had progressively greater difficulty extracting oxygen from test solutions down to pH 3, below which no data were reported. Packer and Dunson (1972) noted variable but eventual decreases in oxygen consumption to death over a period of 3 to 6 hours in brook trout exposed to a range of pH from 2.0 to 3.25, and they suggested that oxygen deprivation is the primary cause of death at low pH. Ultsch (1978) has shown that there is a decrease in oxygen consumption measured over a pH range of 7.0 to 3.5 in channel catfish, but bluegills and goldfish were unaffected in these acute experiments.

Blood $P_{O_2}$ is governed by the uptake of oxygen at the gill surface and by removal of $O_2$ from the plasma for tissue metabolism. In fish, as opposed to mammals, blood oxygen level, not blood $CO_2$, controls respiratory ventilation (Wood and Jackson, 1980). Janssen and Randall (1975) found that there was a gradual increase in the ventilation volume when rainbow trout were exposed to pH 5.0 which they suggested might have been due to hypoxemia arising from gradual accumulation of mucus on

the gills. An increase in ventilation is one of the compensatory mechanisms for hypoxemia caused by low ambient pH. Vaala (1972) supported this observation with his report that blood $P_{O_2}$ was decreased in acid stressed fish but observations by others indicate that arterial $P_{O_2}$ is unaffected by acid exposure, or that there is only a transient decrease in this blood parameter. Eddy (1976) and Neville (1979b) both reported that arterial $P_{O_2}$ was little changed in fish exposed to acid stress.

Data obtained on oxygen consumption and ventilation rates indicate that the hypoxia associated with acid exposure may be the primary cause of death only at very low ambient pH (probably pH 3 or below).

How is hypoxic stress induced by acid exposure? A number of studies have been done to determine if this is due to decreased diffusion of oxygen across the gill or reduced transport by the blood. The volume of oxygen transferred across the gills ($\dot{V}_{O_2}$) is inversely related to the thickness (t) of the barrier separating water from blood as expressed in the following equation:

$$\dot{V}_{O_2} = (KA \cdot \Delta P_{O_2}).t^{-1}$$

where:

K = Krogh permeation coefficient ($\mu l\ O_2\ h^{-1} mmHg^{-1} cm^{-1}$)

A = area of respiratory surface ($cm^2$)

t = thickness of barrier separating water from blood (cm)

Alterations in fish gills induced by water pollutants including $H^+$ are usually associated with hypersecretion of mucus. The mucus may increase the thickness of the barrier separating water from blood and thereby decrease the diffusion of oxygen across the gill.

Ultsch and Gros (1979) found that the diffusion rate through body mucus isolated from acid exposed carp was 70% of that through water. They suggested that the major effect of mucus was to reduce or prevent water circulation between secondary gill lamellae rather than acting as a diffusion barrier. The net result would be reduced oxygen flux from water to blood.

Wiebe et al. (1934) showed that fish are less able to extract oxygen when the pH of the ambient water is decreased, but they did not indicate that this was due to gill mucus coagulation. Ellis (1937) stated that impairment of gills at low pH was due initially to precipitation and coagulation of mucus on the gills and then by coagulation of gill membranes themselves. In their review of the literature Doudoroff and Katz (1950) concluded that the death of fishes at low pH caused by inorganic acids was due to mucus coagulation.

Plonka and Neff (1969) found bits of cellular debris embedded in mucus on the gills of brook trout exposed to acutely toxic acid pH. They suggested that mucus adhering to cellular debris from the respiratory epithelial cells is involved in the mechanism of coagulum formation which decreases ventilation of secondary lamellae. Janssen and Randall (1975) observed that there was a build-up of mucus on the gills of rainbow trout subjected to hypercapnic stress. They suggested that this build-up could have interfered with oxygen transfer and that the subsequent rise in ventilation volume may have been related to a slowly developing hypoxemia. Daye and Garside (1976) observed that the mucus cells of gills and the integument of brook trout char exhibited progresive degrees of hypertrophy and excessive secretion of mucus with increased pH stress.

## RESEARCH RATIONALE

Studies on the effects of various toxicants such as $H^+$ on the transfer capacity of fish gills for oxygen could provide valuable information on possible subtle effects that environmental acidification has on a fundamental or basic process common to all freshwater aquatic communities, namely oxygen uptake.

If death by anoxia does occur, it may be by a decrease in the rate of transfer of oxygen across the gills resulting from an increase in the oxygen consumption of the gill tissue and/or increased diffusion distance (due to mucus deposition on the gill). Anoxia may also be caused by an inhibition of the oxygen transport capacity of the blood via a combined Root and Bohr shift, which would simultaneously lower both the oxygen capacity and the oxygen affinity of the blood.

Isolated-perfused gill preparations have been employed to study various aspects of the physiology of fish gills. Girard & Payan (1976), Smith (1977) and Wood (1974; 1975) studied vascular resistance; Bergman, Olson & Fromm (1974) and Haywood, Isaia & Maetz (1977) worked on urea influx as an index of branchial surface area; Sorenson and Fromm (1976) found that heat exchange could be used as an index of branchial vascular geometry; Payan et al. (1975) studied various ionic exchanges and Jackson and Fromm (1980) worked on the physical and biological factors affecting the movement of water across the gill using the isolated-perfused gill preparation. However, the fundamental parameter

isolated-perfused gill preparation. However, the fundamental parameter on which the physiology of fish gills is based, viz., respiratory gas exchange, has received no such experimental attention. Wood, McMahon & McDonald (1978) used a totally perfused whole trout preparation to examine the effects of variations in internal and external perfusion flow rates, adrenergic stimulation and blood pressures on oxygen exchange and vascular resistance in the branchial and systemic vascular beds. They stated that the oxygen used by gill tissue i.e., oxygen taken up from the external ventilatory flow by the gills but not transferred to the internal perfusate, was significant and must be considered in calculating the amount of oxygen exchanged across the gill.

The effect of ambient pH on the transfer of oxygen across the gill could be studied using the isolated-perfused gill preparation. However, before the preparation is used for this purpose a few questions regarding the basic characteristics of the gill tissue itself need to be answered. They are:

1. What is the oxygen consumption of the gill tissue?

The isolated perfused gill technique measures the difference in $P_{O_2}$ of the fluid going into the gill (afferent vessel) and the fluid going out of the gill (efferent vessel) in calculating the transfer capacity of the gill for oxygen. Therefore, the oxygen consumption of the gill tissue itself has to be considered in making the calculation.

2. Is the oxygen consumption of the gill tissue affected by $P_{O_2}$ of the bath solution and the time taken to make the measurement?
3. Is the oxygen consumption of the gill tissue affected by the pH of the bath solution?

4. Is there any difference in oxygen consumption of the gill tissue if the fish are exposed to acid water prior to measuring the oxygen consumption of the gill tissue?
5. Mucus may interfere with diffusion across the gill and has been reported frequently but has not been quantified. How much mucus is produced by the gill of the fish exposed to various pH?

Experiments were done in an attempt to answer the above questions.

# MATERIALS AND METHODS

## Experimental Animals

Rainbow trout (Salmo gairdneri) weighing between 150-350 g were used as experimental animals. The fish were obtained from a local hatchery (Midwest Fish Farm Enterprises, Inc., Harrison, Michigan) and maintained at Michigan State University in large fiberglass tanks supplied with flowing tapwater (pH 7.4 $\pm$ 0.2) from which chlorine and excess minerals had been removed. The temperature of the room in which the fish were kept was maintained at 10$\pm$2C. Animals were fed twice weekly and starved at least 5 days prior to experimental use.

Fish to be exposed to various pH levels were acclimatized in small tanks (38x33x7cm) in a cold room (10$\pm$1C) for two days prior to exposure. In other tanks of similar size, concentrated $H_2SO_4$ was used to adjust the pH to the desired level. Fish were then transferred carefully from the acclimatization tanks to the exposure tanks. Exposure time was two hours.

## Gill Tissue Preparation

The fish were immobilized by severing the spinal cord immediately posterior to the opercula and placed ventral side in a V-shaped trough. A rubber tube inserted in the mouth directed flowing tapwater over gills. A three-inch incision was made ventro-caudally from the jaw to expose the heart. The ventricle was cannulated with PE 90 tubing which

was attached via a pump to a perfusion fluid reservoir. The tubing was secured in place with ligature around the bulbus arteriosus. The gills were pump-perfused (for perfusion fluid composition see Appendix I) until cleared of blood (5-10 minutes).

Of the four gill arches, the second arch of the right side was removed and used for oxygen consumption study, the left second arch was used for histology and the first, third and fourth arches were used for sialic acid determination.

Oxygen Consumption of Gill Tissue

A sample of gill filaments (60-90 mg) was cut from the middle of the arch. Excess fluid was blotted off and the samples were weighed and suspended in 3 ml of citric acid/sodium phosphate buffered incubation medium (Appendix II) having an osmotic concentration of 270-300 mOsm/kg. The incubation medium was aerated with room air for 10 minutes prior to use and the oxygen content was determined using the Winkler procedure. The oxygen consumption was then measured polarographically with a Y.S.I. model 53 biological oxygen monitor (Yellow Springs Instrument Co., Yellow Spring, OH), and recorded with a two channel strip chart recorder (Linear Instruments Model 486, Linear Instrments Corp., Irvine, CA). The temperature of the samples in the incubation vials was maintained at $10 \pm 0.5$C with a constant temperature water bath (Haake, Inc., Model DI, Temperature Control Equipment Div., Saddle Brook, NJ).

The recorded tracings for each experiment were digitized at one minute intervals. The oxygen consumption at each digitized point (time t) was calculated based on the difference between the initial oxygen content of the incubation medium (100% saturation) and the content at

time t. The formula for calculating the oxygen consumption is as follows:

$$\dot{Q}_{O_2} = (\Delta P_{O_2} \cdot a) t^{-1} w^{-1}$$

where:

$\dot{Q}_{O_2}$ = oxygen consumption in µl/h/mg dry wt.

$\Delta P_{O_2}$ = Δ% saturation x barometric pressure x 0.21 (mmHg)

t = time in hours

w = dry weight in mg

a = initial oxygen content (µl) at 100% saturation.

The data used to compare treatment effects were based on the oxygen consumption during the first 20 minutes statistically extrapolated to time zero utilizing an exponential curve fit routine.

Sialic Acid Content of Gill Tissue

The first, third and fourth gill arches of both sides were used. The filaments were trimmed from the cartilagenous arch and blotted free of excess fluid. The tissue was then weighed and frozen in distilled water after it was sonified for 5 minutes using a sonifier cell disruptor (Heat Systems Co., Melville, NY). A procedure described by Warren (1963) was used to determine the amount of sialic acid present. (Appendix III).

Histological Observation of Gill Tissue

The second arch of the left side was removed from the fish and fixed in Detrick fixative. The tissues were dehydrated in tetrahydrafuran and embedded in paraffin. Tissue sections of 8 µm thickness were stained in haematoxylin and eosin and observed for any histological changes. Gill

tissue sections for histochemical identification of mucous cells were stained using Periodic Acid Schiff technique (Pearse, 1968).

Statistical Treatment of Data

Data for oxygen consumption and sialic acid content were subjected to a one-way analysis of variance and the Student-Newman-Keul test was used to compare treatment means. Fiducial limits were set at 0.05.

# RESULTS

## A. Oxygen Consumption of Gill Tissues

1. Experiment 1. What is the effect of pH of the incubation media on the oxygen consumption of gill tissues?

To answer this question samples of gill tissue were taken from fish which had been exposed to a non-stressful ambient pH of 7.4. The samples were placed in incubation media having a pH of 7.4, 6.0, 5.0 and 4.0 and oxygen consumption was measured. The results, displayed in Figure 1, show that as pH of the incubation media decreased there was a decline in oxygen consumption. The means for oxygen consumption at pH 5.0 and 4.0 were both significantly lower than that for controls (pH 7.4).

2. Experiment 2. What is the effect of exposing the gill tissues for one hour in the incubation media prior to measuring their oxygen consumptions?

Gill tissue samples were obtained from fish as noted above and divided into two groups. One group was placed in aerated (room air) incubation media having a pH of 7.4 and the other at pH 6.0. One hour was allowed to elapse prior to determining the oxygen consumption of these tissues. The data obtained were compared to appropriate control (i.e., same pH) values from Experiment 1 and are displayed in Figure 2. Results show that the one hour exposure of gill tissue to *in vitro* conditions had no effect on oxygen consumption.

Figure 1. Effect of pH of the incubation media on oxygen consumption of gill tissues. Mean values ± S.E. are plotted and the number in each column indicates the N values. Means (µl/h/mg dry wt.) not underlined by the same line are significantly different ($P<0.05$).

| pH 7.4 | pH 6.0 | pH 5.0 | pH 4.0 |
|---|---|---|---|
| 2.51±0.20(9) | 1.98±0.17(6) | 1.65±0.22(4) | 0.76±0.18(4) |

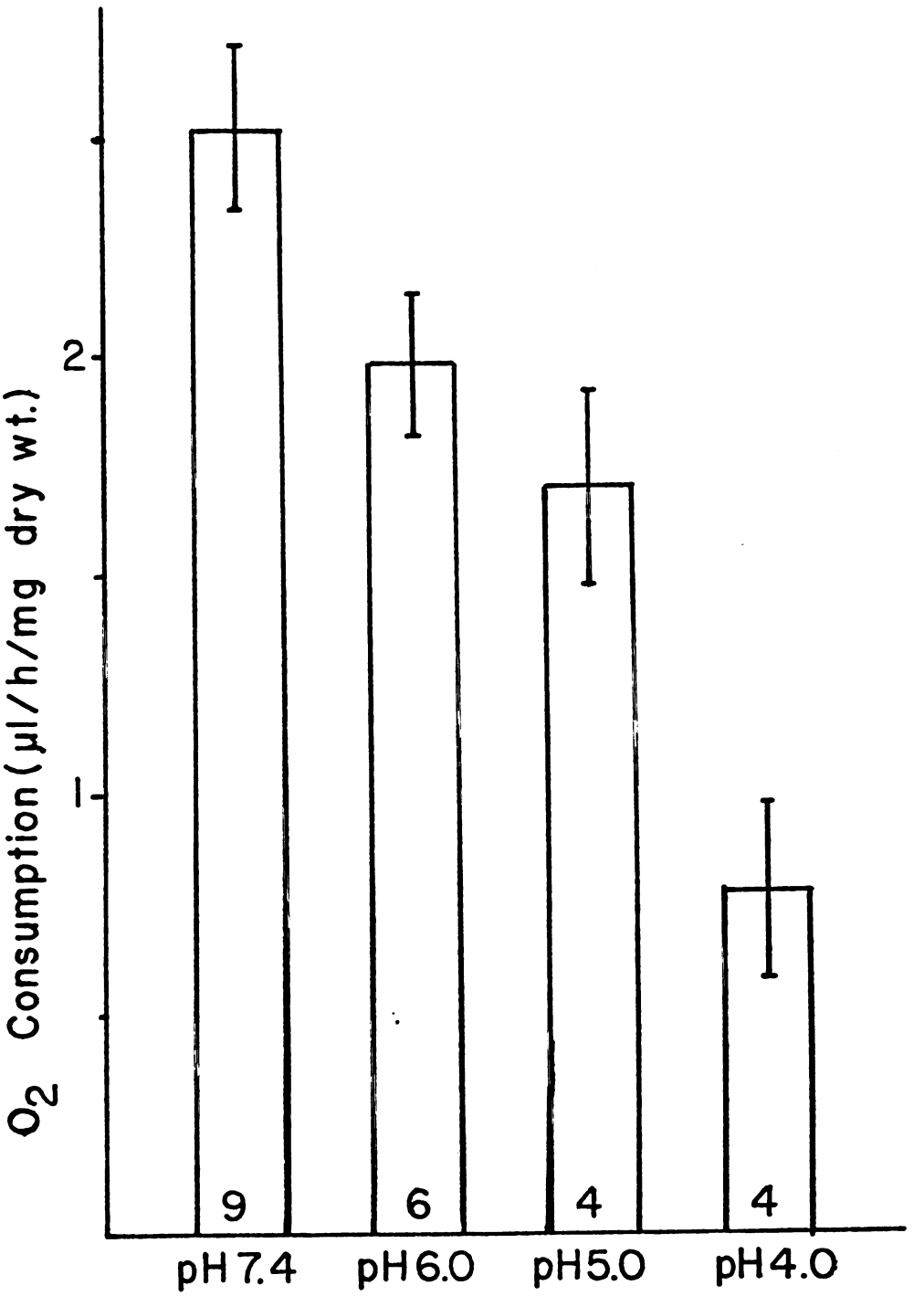



Figure 1

Figure 2. Effect of exposing gill tissues for one hour in the incubation media prior to measuring oxygen consumption. Mean values ± S.E. are plotted and the number in each column indicates the N value. Means (µl/h/mg dry wt.) not underlined by the same line are significantly different ($P<0.05$).

| pH 7.4 | | pH 6.0 | |
|---|---|---|---|
| No exposure | 1-h exposure | No exposure | 1-h exposure |
| 2.51±0.20(9) | 2.34±0.06(4) | 1.98±0.17(6) | 1.84±0.05(4) |

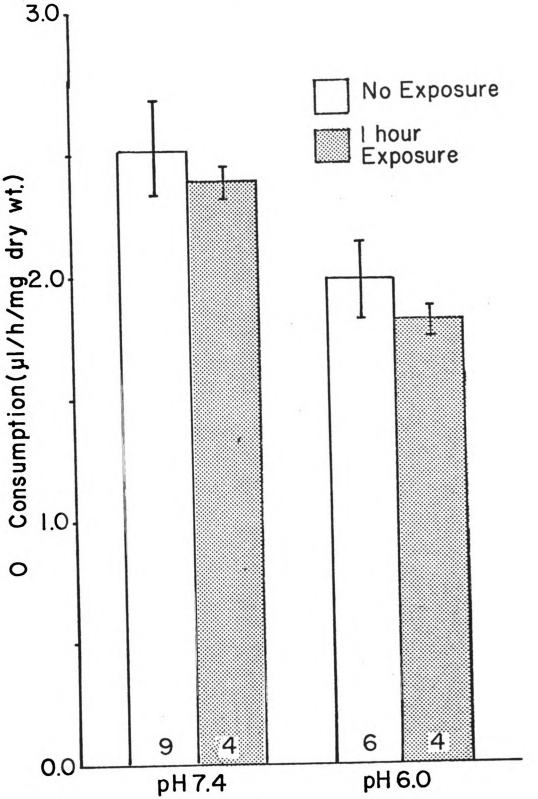

3.0
2.0
1.0
0.0
O Consumption(µl/h/mg dry wt.)
No Exposure
1 hour Exposure
9
4
6
4
pH 7.4
pH 6.0

Figure 2

3. Experiment 3. What is the effect of $P_{O_2}$ of the incubation media on oxygen consumption of gill tissues?

Oxygen consumption data for tissues measured at pH 7.4 in Experiment 1 were used to compare the effect of $P_{O_2}$ on the oxygen consumption of gill tissue. Since the Y.S.I. model 53 biological oxygen monitor used to make the measurements is a closed system, the $P_{O_2}$ of the incubation medium drops as oxygen is consumed. The average rate of oxygen consumed for each 20 mm Hg drop in $P_{O_2}$ was calculated for gill tissues measured at pH 7.4. The results are shown in Figure 3. During the time required to reduce the $P_{O_2}$ from 130 mm Hg or above to 90 mm Hg (20 minutes or less) there was a slight but statistically insignificant decline in oxygen consumption. As $P_{O_2}$ decreased further there was an accompanying decrease in oxygen consumption and at some $P_{O_2}$ between 10 and 30 mm Hg the decline in oxygen consumption appeared to become greater.

4. Experiment 4. What is the effect of exposure of fish to various pH levels prior to obtaining gill samples and measuring the oxygen consumption at the pH of exposure?

Gill tissue samples were taken from fish which had been exposed for two hours at pH 7.4, 6.0, 5.0 and 4.0. The samples were then placed in the incubation chamber containing incubation media at a pH similar to that of exposure. Results of this experiment are shown in Figure 4. The mean oxygen consumption of gill tissue showed a uniform and statistically significant decrease as pH of the exposure water was decreased.

Figure 3. Effect of $P_{O_2}$ on oxygen consumption of gill tissues. Plotted are mean ± S.E. Means (µl/h/mg dry weight) not underlined by the same line are significantly different ($P<0.05$). N = 6 for each mean.

$P_{O_2}$ (mmHg)

| >130 | 110-130 | 90-110 | 70-90 | 50-70 | 30-50 | 10-30 | <10 |
|---|---|---|---|---|---|---|---|
| 2.85 ±0.04 | 2.54 ±0.30 | 2.26 ±0.31 | 2.04 ±0.27 | 1.76 ±0.20 | 1.57 ±0.15 | 1.28 ±0.12 | 0.74 ±0.05 |

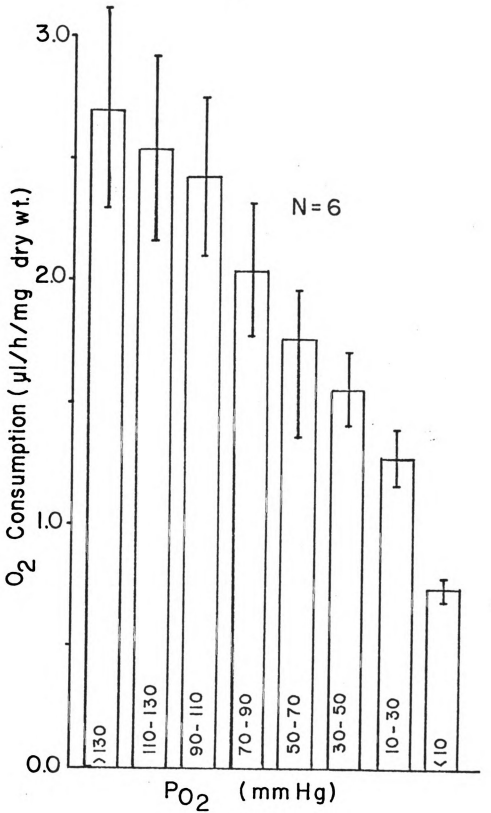

3.0
2.0
1.0
0.0
N=6
O2 Consumption (µl/h/mg dry wt.)
>130
110-130
90-110
70-90
50-70
30-50
10-30
<10
PO2 (mm Hg)

Figure 3

Figure 4. Effect of exposing the fish for two hours at various pH levels on *in vitro* oxygen consumption of gill tissues. Measurements of oxygen consumption were made at pH of exposure. Plotted are mean values ± S.E. and the numbers in each column indicate N. Mean values (µl/h/mg dry wt.) are as follows:

| pH 7.4 | pH 6.0 | pH 5.0 | pH 4.0 |
|---|---|---|---|
| 2.54±0.21(8) | 2.06±0.18(8) | 2.00±0.17(4) | 1.75±0.30(7) |

The oxygen consumption at pH 4.0 is significantly lower than at pH 7.4 ($P<0.05$, Student t-test).

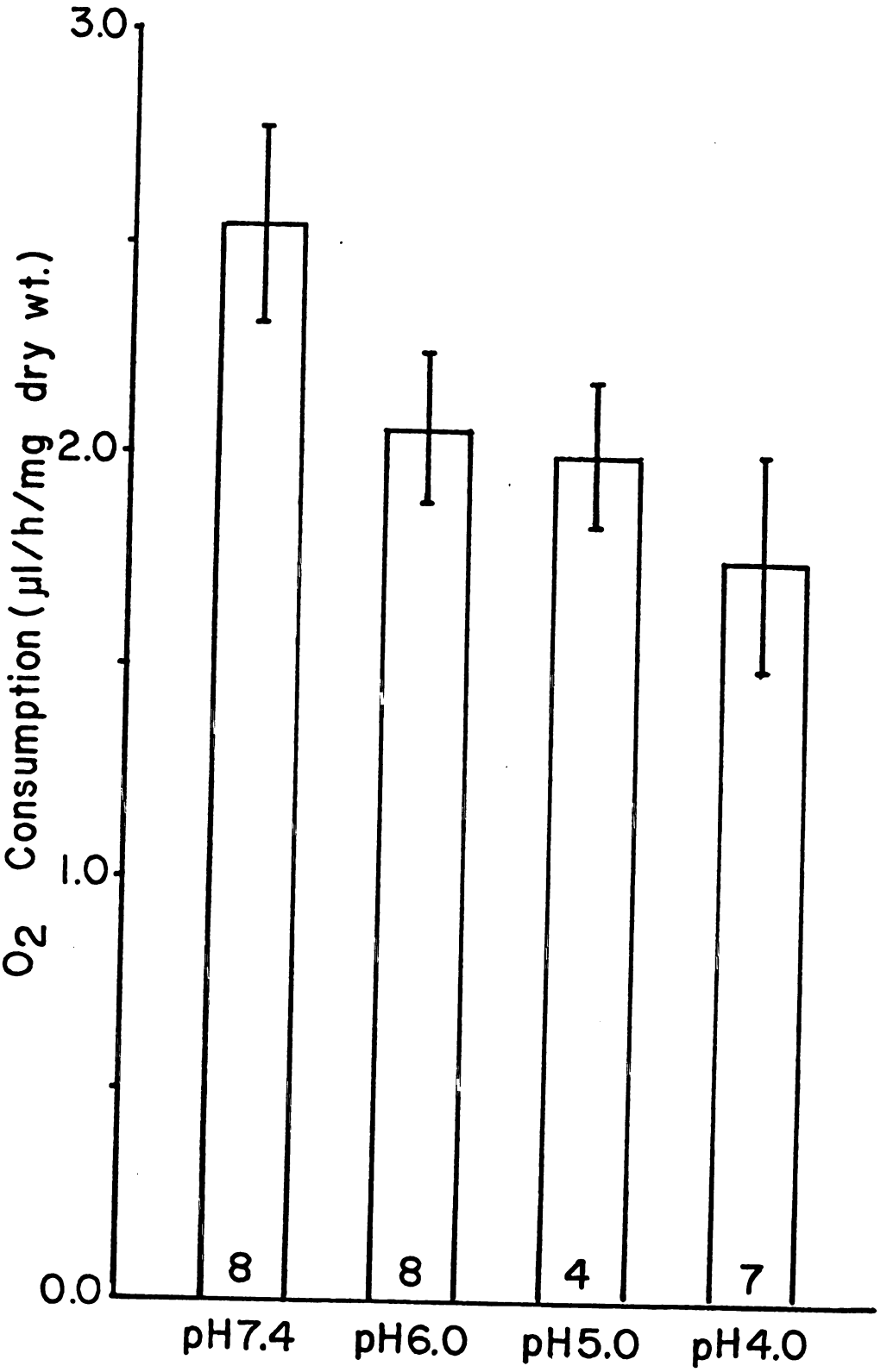

3.0
2.0
1.0
0.0
O2 Consumption (µl/h/mg dry wt.)
8
8
4
7
pH7.4
pH6.0
pH5.0
pH4.0

Figure 4

Figure 5. Effect of prior exposure of fish for two hours at various pH levels on *in vitro* oxygen consumption of gill tissue. All measurements of oxygen consumption were made at pH 7.4. Plotted are mean values ± S.E. and the numbers in each column indicates N. Mean values (µl/h/mg dry wt.) are as follows:

| pH 7.4 | pH 6.0 | pH 5.0 | pH 4.0 |
|---|---|---|---|
| 2.54±0.21(8) | 2.19±0.07(3) | 2.73±0.34(3) | 2.83±0.71(3) |

Statistically ($P<0.05$) there were no differences between any of the means.

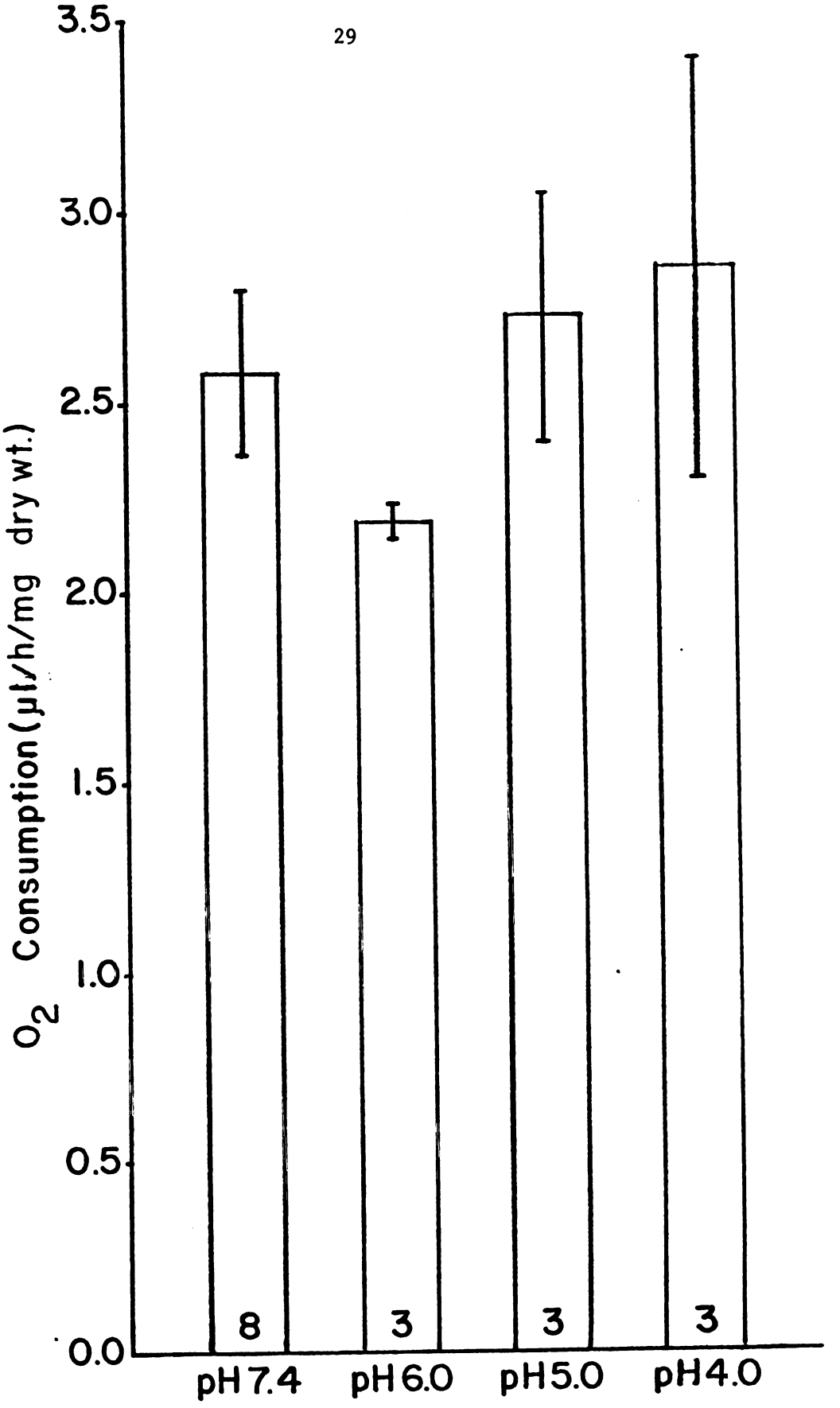

3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
$O_2$ Consumption (µl/h/mg dry wt.)
8
3
3
3
pH 7.4
pH 6.0
pH 5.0
pH 4.0

Figure 5

5. Experiment 5. What is the effect of exposing the fish for two hours at various pH levels prior to measuring the oxygen consumption at pH 7.4?

The fish and gill tissue samples were treated as in Experiment 4 except that all oxygen consumption measurements were made in incubation media at pH 7.4. Results from this experiment are shown in Figure 5. The oxygen consumption of gill tissue samples from fish previously exposed to different pH levels were not significantly different from controls and no uniform trend in the data was evident.

B. Sialic Acid Content Of Gill Tissues

Since sialic acid is an important component of mucus, an assay for this acid was used to estimate the mucus content of gill tissues of experimental animals. Intact fish were transferred from a holding tank and acclimated for two days in small plastic tanks. After equilibration they were exposed for two hours to water at pH 7.4, 6.0, 5.0 and 4.0.

Table 1. Effect of exposing the fish for 2 h at various pH levels on sialic acid content of the gill tissue. Amount of sialic acid is in μMoles/g wet weight. The amount of sialic acid in gill tissues of fish exposed to pH 4.0 is significantly more than the amont in controls. Data are presented as Means±S.E.(N).

| Control | Exposed to | | | |
|---|---|---|---|---|
| (no exposure) | pH 7.4 | pH 6.0 | pH 5.0 | pH 4.0 |
| 1.07±0.04(4) | 1.10±0.09(4) | 1.25±0.06(4) | 1.30±0.06(4) | 1.42±0.04(4) |

The sialic acid content of gill tissue from fish exposed to pH 7.4 was compared to that of controls, i.e., gill tissue from fish obtained from the holding tank, to test for the effect of transfer from the holding tank to the small exposure tanks. Data for fish exposed to pH

6.0, 5.0 and 4.0 were compared to data for fish at pH 7.4. The data are presented in Table 1. Transfer of fish from the holding tank to the small exposure tanks and 48 hours of acclimation had no effect on gill sialic acid content. The short, two-hour exposure to acidic conditions caused a significant increase in the sialic acid (mucus) content of gill tissue.

C. Histological Observations

Figures 6, 7, 8 and 9 indicate that as the stress of lowered pH is increased there is a progressive increase in the tissue damage, accompanied by an increase in the number of mucous cells. A more detailed description is given in the legends for each photomicrograph.

Figure 6. Photomicrograph of longitudinal section of gill filaments from rainbow trout exposed to pH 7.4 for two hours. Mucus cells which are stained dark (m) are very few in number at the junctions of the gill lamellae (l) and gill filaments (f), and very few in the bodies and tips of of the lamellae. Practically no mucus can be seen between the lamellae. Arrows indicate the few areas of separation between the epithelial cells (e) and the pilaster cells (p). No separation is seen in the areas around the tips of the lamellae.

Figure 7. Photomicrograph of longitudinal section of gill filaments from rainbow trout exposed to pH 6.0 for two hours. Mucus cells are few in number at the junctions of gill lamellae and gill filaments and in the bodies and tips of the lamellae. Practically no mucus can be seen between the lamellae. Arrows indicate the areas of separation between the epithelial cells and pilaster cells. The separation is seen in almost all lamellae especially at their bases. No separation is seen in areas around the tips of the lamellae.

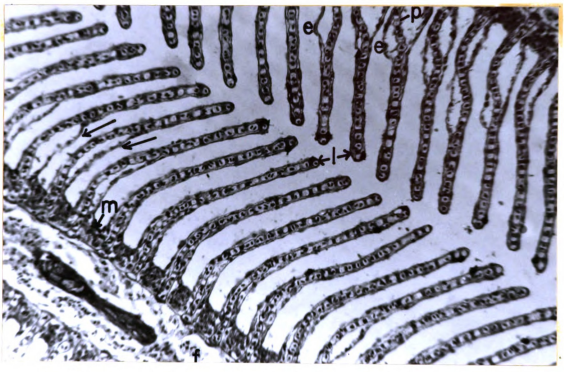



FIGURE 6

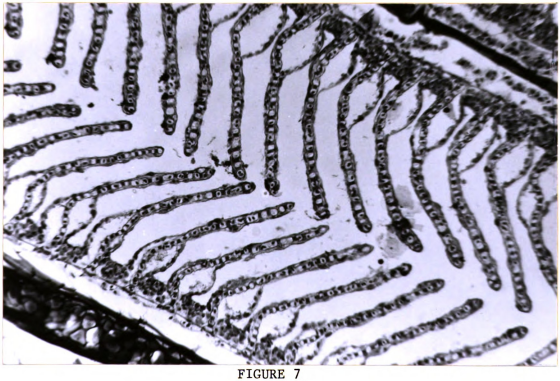


FIGURE 7

Figure 8. Photomicrograph of longitudinal section of gill filaments from rainbow trout exposed to pH 5.0 for two hours. More mucus cells are seen at the junctions of the gill lamellae and gill filaments and also at the tips of the lamellae. Dark areas between the lamellae represent secreted mucus. The areas of separation betwen the epithelial cells and pilaster cells are more pronounced.

Figure 9. Photomicrograph of longitudinal section of gill filaments from rainbow trout exposed to pH 4.0 for two hours. Mucus cells fill the junctions of the gill lamellae and gill filaments and more are present at the tips of the lamellae. Mucus can be seen filling the spaces between the lamellae. The areas of separation between the epithelial cells and pilaster cells are very conspicuous.

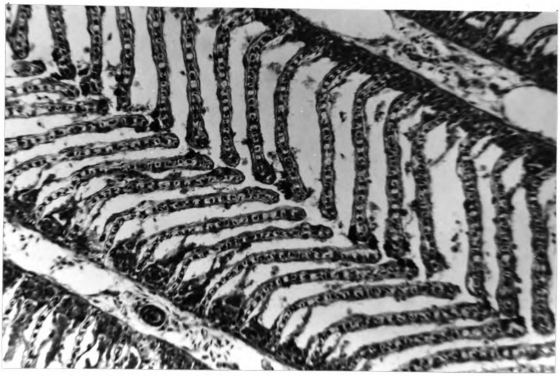


FIGURE 8

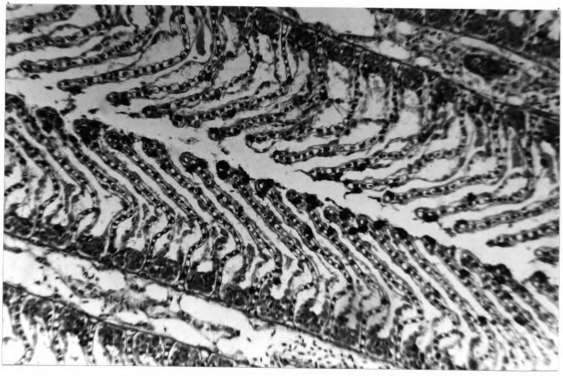


FIGURE 9

# DISCUSSION

The purpose of this study was to measure the rate of oxygen consumption of fish gill tissue and to determine whether oxygen consumption and mucus production of gill tissue are affected by lowered pH.

In the procedure used to measure oxygen consumption three factors (pH, $P_{O_2}$ and time) may interactively affect the data obtained. Three series of experiments were done to separate out the effects of these variables. Data from Experiment 2 show that after removal of gill tissue from a fish and storage in incubation media aerated with room air for 1 hour, the oxygen consumption of this tissue was not different from the oxygen consumption of gill tissue which was measured immediately upon removal. After the start of the measurement period the rate of oxygen consumption showed the same decrease with time in both of the aforementioned cases. Therefore, in the experiments reported here, the time period from removal of gill tissue from fish until the end of the oxygen consumption determination can be eliminted as a factor influencing oxygen consumption. Thus any change in oxygen consumption must be due to other variables, viz., $P_{O_2}$ and/or pH.

Data from Experiment 3 show that the $P_{O_2}$ of the incubation media does not affect oxygen consumption of the gill tissue until the $P_{O_2}$ drops below 90 mm Hg. The time required to reduce the $P_{O_2}$ to around 90 mm Hg averaged 25 minutes. Data used to study the effect of pH

(Experiment 1) were obtained during the first 20 minutes of the measurement period. The data show that gill tissue oxygen consumption at pH 5.0 and 4.0 are significantly lower than that for controls (pH 7.4). Therefore, any change in oxygen consumption during the first 20 minutes of the measurement period is due to pH alone.

The pH of the incubation media causes a decrease in the oxygen consumption of the gill tissue possibly by causing physical damage to the tissue membrane. This in turn could lead to edema, ionic imbalance and possible intracellular pH change. This pH change could affect the activity of the respiratory enzymes.

The next experiments were done to determine if exposure of fish to various pH levels prior to obtaining gill samples and measurement of oxygen consumption had any effect on the rate of oxygen consumed by the gill tissue. Data from Experiment 4 show that there is a uniform but not significant decrease in oxygen consumption as the pH of the exposure water is decreased. When these data were compared to the appropriate data in Experiment 1, the oxygen consumption of the gill tissue from the exposed fish were consistently higher than those taken from the unexposed fish but only those exposed to pH 4.0 showed a statistically significant difference. The oxygen consumption of the gill tissue from fish exposed to pH 4.0 showed a significantly higher oxygen consumption than those taken from unexposed fish. (See Figure 10).

The above data suggest that when intact fish are exposed to acid water for 2 hours the detrimental effect of pH on metabolism of the gill tissue is overridden by an opposite effect (increased metabolism) which is also caused by low ambient pH. When fish are exposd to low pH increased rate of metabolism of gill tissue may simply reflect the

increased energy requirement caused by the increase in mucus production. In order to verify the two opposite effects of pH on the metabolism of the gill tissue, Experiment 5 was done. When the fish were exposed to pH 7.4, 6.0, 5.0 and 4.0 for two hours in small tanks and all the oxygen consumption of the gill samples was measured at pH 7.4, there were no significant differences in the oxygen consumption and no uniform trend was evident. When the data were compared to those of Experiment 1 (measured at pH 7.4 only), again there were no significant differences. Therefore the pH of the water in which the fish had been exposed to does not significantly affect the oxygen consumption of the gill tissue if the oxygen consumption measurement is made at pH 7.4. However, when the data are compared to the appropriate data in Experiment 1, the oxygen consumption of the gill tissue of fish exposed to pH 4.0 and measured at pH 4.0 is significantly lower than the oxygen consumption of the gill tissue from fish exposed to pH 4.0 and measured at pH 7.4. (See Figure 10).

In Salmonidae, the major epithelial mucin component appears to be sialic acid (Harris , Watson and Hunt, 1973). Lemoine and Olivereau (1971) used sialic acid assay to determine the degree of skin mucification in Anguilla anguilla. The assay of sialic acid may therefore provide evidence of the presence of an environmental stressor, producing alterations in the secretion of gill mucus.

Miller and Mackay (1982) studied the relationship of secreted mucus to copper and acid toxicity in rainbow trout. They found that the amount of mucus secreted into the water by fish exposed to pH 4.0 for eight hours was significantly greater than that secreted by fish exposed to pH 7.3. The data cannot be used to explain a decrease in oxygen

Figure 10. Effect of exposing the fish for 2 h at various pH levels on *in vitro* oxygen consumption of gill tissue. Experiment 1: Controls i.e., fish not exposed; $O_2$ consumption measurements made at pH 7.4, 6.0, 5.0, and 4.0. Experiment 4: Fish exposed to pH 7.4, 6.0, 5.0 and 4.0, and the oxygen consumption of the gill tissues were measured at the pH of exposure. Experiment 5: Fish exposed as above and the tissues measured at pH 7.4. Plotted are mean values (µl/h/mg dry wt.)±S.E. The numbers in each column indicate N values. The oxygen consumption of gill tissue from fish exposed to pH 4.0 and mesured at either pH 4.0 or pH 7.4 is significantly greater than that of controls.

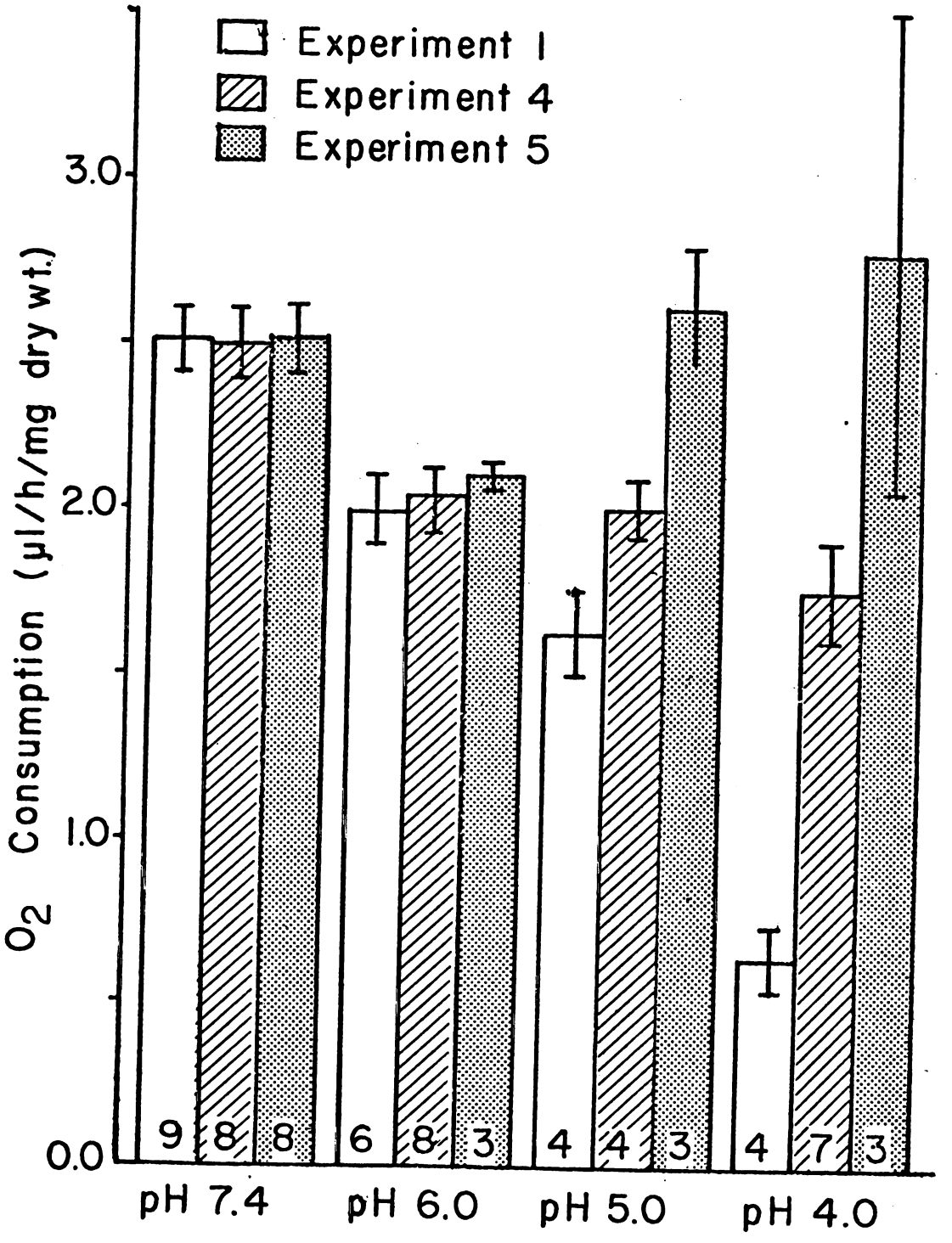
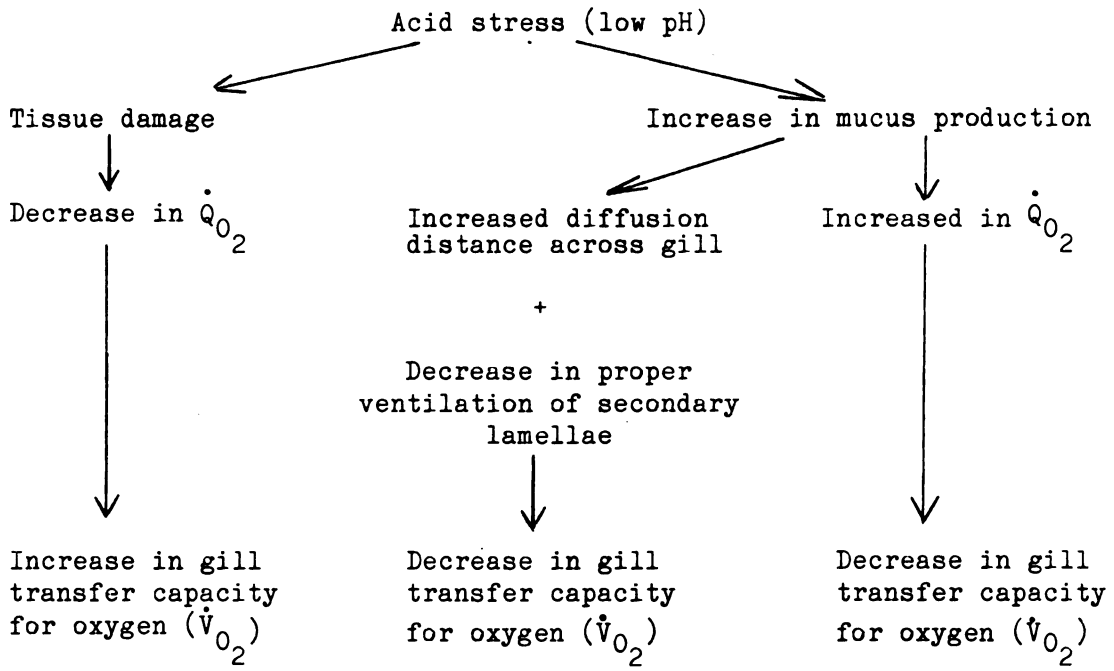

Experiment 1
Experiment 4
Experiment 5
$O_2$ Consumption (µl/h/mg dry wt.)
3.0
2.0
1.0
0.0
9
8
8
6
8
3
4
4
3
4
7
3
pH 7.4
pH 6.0
pH 5.0
pH 4.0

Figure 10

transfer across the gill because mucus secreted into the water does not act as a diffusion barrier. Furthermore, mucus secreted into the water does not indicate gill mucus alone; mucous cells are widely distributed over the general body surface of fishes. It is believed that an assay for the amount of mucus synthesized and/or secreted by the gill tissue itself is the only valid method that can be used to explain the decrease in oxygen transfer across the gill.

A series of experiments was done to determine mucus production of the gill tissue of fish exposed to pH 7.4, 6.0, 5.0 and 4.0. The data represent the mucus secreted on the gill tissue as well as the mucus produced by the mucous cells but not yet extruded. The data show that the amount of mucus steadily increased as the ambient pH was decreased. The gill tissue taken from fish exposed to pH 4.0 had a significantly greater amount of mucus than those taken from the controls (pH 7.4). A histological study was done on acid stressed gill tissues to illustrate the response of this tissue to low ambient pH. The gills exhibited two types of reaction to pH stress. Hypertrophy of mucous cells was observed at the base of the secondary lamellae with exposure to increased hydrogen ion concentration. The accumulation of mucus on the gill is shown in the photomicrographs and increased mucus production is also indicated by the data on the sialic acid content. In addition, as pH stress increased more tissue damage, especially near the tips of the lamellae, was apparent. A more pronounced separation of epithelial cells from the underlined cell layers is more evident with increased pH stress.




The data on gill oxygen consumption and mucus production presented above indicate that exposure of this tissue to acidic conditions may result in multiple effects. It is postulated that pH stress, in one case, causes tissue damage which leads to a decrease in gill oxygen consumption. Other things being equal, this would result in an increase in the transfer capacity of gills for oxygen. On the other hand, pH stress stimulates mucus production which in turn could produce variable effects. As shown in the diagram above an increase in mucus production could increase oxygen consumption i.e., energy must be provided for the synthetic process. Secondly, increased mucus production could result in a decrease in the proper ventilation of secondary lamellae and also increase the diffusion distance for oxygen across the gill from the ventilate to the vascular space. Both of these effects should result in a decrease in the transfer capacity of the gills for oxygen.

# CONCLUSIONS

1. The *in vitro* oxygen consumption of gill tissue from rainbow trout not previously exposed to acid water decreased as the pH of the incubation media was decreased.
2. The *in vitro* oxygen consumption of gill tissue from rainbow trout exposed to lowered pH decreased as the pH of the incubation media (similar to exposure pH) was decreased.
3. The *in vitro* oxygen consumption (measured at pH 4.0) of gill tissue from rainbow trout exposed to pH 4.0 was higher than of none exposed fish.
4. There is a progressive increase in sialic acid content as the pH of exposure decreases. However, the only significant difference noted is the sialic acid content at pH 7.4 and pH 4.0.
5. Histologically gill tissue of the more severely acid-stressed fish had more mucous cells and exhibited a greater degree of filamental damage.

# APPENDICES

# APPENDIX I

## Composition of Perfusion Fluid

| | |
|---|---|
| NaCl | 7.31 g |
| $CaCl_2$ | 0.11 g |
| KCl | 0.38 g |
| $NaH_2PO4\ H_2O$ | 0.41 g |
| $NaHCO_3$ | 1.00 g |
| $MgSO_4\ 7H_2O$ | 0.12 g |
| $MgCl_2\ 6H_2O$ | 0.10 g |
| Glucose | 1.00 g |
| Albumin | 30.00 g |
| Distilled water | to 1 liter |

## APPENDIX II

### Composition of Citric Acid/Sodium Phosphate Buffer at Different pH Levels.

Procedure:

1. Prepare 0.2 M $Na_2HPO_4$ in 1% Cortland saline*.
2. Prepare 0.1 M Citric Acid in 1% Cortland saline.
3. To make the buffer at different pH levels, take different volumes of 0.2 M $Na_2HPO_4$ and 0.1 M Citric acid as indicated in the table below.
4. Adjust the osmolarity of the buffer to 270-300 mOsm/Kg either by adding NaCl or distilled water.

| pH | 0.2M $Na_2HPO_4$, ml | 0.1M Citric Acid, ml |
|---|---|---|
| 4.0 | 8.0 | 12.0 |
| 5.0 | 10.0 | 10.0 |
| 6.0 | 12.0 | 8.0 |
| 7.4 | 17.0 | 3.0 |

* Composition of Cortland saline:

| | |
|---|---|
| NaCl | 7.25 g |
| $CaCl_2$ $2H_2O$ | 0.23 g |
| KCl | 0.38 g |
| $NaH_2PO_4$ $H_2O$ | 0.41 g |
| $NaHCO_3$ | 1.00 g |
| $MgSO_4$ $7H_2O$ | 0.23 g |
| Glucose | 1.00 g |
| Distilled water | to 1 liter |

## APPENDIX III

Thiobarbituric Assay for Sialic Acid - for Use in Estimating Mucus.

Reagents:

0.1N $H_2SO_4$

Sodium metaperiodate (0.2M) in 9M phosphoric acid (5%)

Sodium arsenite (10%) in 0.5M sodium sulfate solution

2-Thiobarbituric acid (0.6%) in 0.5M sodium sulfate solution

Cyclohexanone

Procedure:

Trim filaments from gill cartilage and place 1 g in 5.5 ml sulfuric acid.

Sonify for 5 minutes.

Incubate at 80 C for one hour.

Centrifuge at 10,000 rpm for 10 minutes.

Put 0.2 ml supernatant in test tube.

Add 0.1 ml sodium periodate.

Mix and allow to stand at room temperature for 20 minutes.

Add 1.0 sodium arsenite solution. Shake vigorously.

Add 3.0 ml 2-thiobarbituric acid solution. Mix by inversion.

Place in boiling water bath exactly 15 minutes.

Cool in tap water for 5 minutes.

Add 4.0 ml cyclohexanone. Shake vigorously.

Centrifuge briefly.

Transfer top,clear cyclohexanone phase to cuvette of 1 cm light path.

Determine optical densities at 532 nm and 549 nm.

$$\text{Micromoles of sialic acid} = (0.084 \times OD_{549}) - (0.031 \times OD_{532})$$

NOTE: The derivation of this equation is dependent upon the molecular extinction coefficients at 532 nm and 549 nm of sialic acid and the interfering material (considered 2-deoxypentose) (Warren, 1963).

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