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ABSTRACT

AEROBIC AND ANAEROBIC GLYCOLYSIS IN THE RETINA OF A TELEOST (*Salmo gairdneri*)

by Dennis A. Baeyens

Carbohydrate metabolism was investigated in the rainbow trout retina by measuring glucose utilization and lactic acid production under aerobic and anaerobic conditions and varying glucose concentrations. The retinas were incubated in various culture media for varying time periods at 13C.

The teleost retina was found to be capable of producing lactic acid in the presence of high oxygen tensions, thereby confirming the occurrence of aerobic glycolysis. More glucose was utilized (41.5%) and more lactic acid was produced (33%) under an anaerobic environment than an aerobic environment. The inhibition of glycolysis by oxygen (Pasteur effect) was thus substantiated in the teleost retina.

The inhibition of the tricarboxylic acid (TCA) cycle by high levels of glucose (Crabtree effect) was verified in the teleost retina by varying the glucose concentration of the incubation media. Under physiological levels of glucose in the media all carbohydrate metabolism

could be accounted for by glycolysis. At increasing levels of glucose in the media, up to 100 mg%, the TCA cycle became predominant in carbohydrate metabolism. At levels in excess of 150 mg% glucose an inhibition of TCA cycle activity was demonstrated.

Glycolysis in the teleost retina was affected by the type of incubation media employed. In modified Medium 199 (pH 7.0) there was more glucose utilized and lactic acid produced than in modified Mammalian Krebs Saline Medium (pH 7.6). It was further demonstrated that glycolysis is dependent upon the integrity of the retinal cells, being almost completely abolished by cellular disruption. Finally, it was shown that an exogenous source of lactic acid could not be utilized by the teleost retina as a substrate for the TCA cycle.

The Crabtree effect is only evident under glucose concentrations in excess of those normally encountered <u>in vivo</u> and probably does not have a great deal of physiological significance in the teleost retina. The inability of the retina to utilize lactic acid as a substrate coupled with the aerobic glycolysis occurring in the retina would result in the accumulation of acid metabolites. The acid metabolites would shift the oxygen dissociation curve to the left causing the release of oxygen from hemoglobin in the choroid region. This release of O_2 might explain in part

the high P_{O_2} encountered in the vicinity of the retina. The Pasteur effect may function as a possible component of a negative feedback control loop functioning to control the set point for the oxygen concentrations generated in the teleost eye.

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By

Dennis A. Baeyens

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Dedicated to my parents, Hector and Marion Baeyens, whose moral and financial support made this study possible.

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INTRODUCTION

Warburg (1927) showed that the mammalian retina had the highest rate of respiration (Q_{O_2}) and anaerobic glycolysis, as measured by lactate production, of any tissue he studied. It is now known that no other tissue in the body, with the possible exception of the brain and the gonads, has a higher rate of anaerobic glycolysis than the retina. Since the discovery of the high rate of glycolysis by the retina, considerable interest has been attached to this as a possible source of energy for the photochemical visual processes. Moreover the retina has a unique capacity for aerobic glycolysis with the production of lactic acid in the presence of oxygen that is only equaled by neoplastic tissue.

It is well known that the retina, which is primarily nervous tissue, utilizes glucose as the predominant substrate for its energy metabolism. Glucose is catabolized in the retina by two major pathways. The predominant metabolic pathway employed is the Embden-Meyerhof-Parnas (EMP) scheme. Under anaerobic conditions the glycolytic pathway (EMP) results in the breakdown of glucose to lactate as the end product. Under aerobic conditions, those normally occurring <u>in vivo</u>, glucose can be completely oxidized by the second major pathway, the tricarboxylic acid cycle (TCA), with the formation of carbon dioxide and water and

the liberation of substantially more energy than via the EMP pathway. A third minor metabolic scheme employed by the retina to break down glucose is the hexose monophosphate shunt (HMP). The reactions of the HMP shunt result in the formation of CO_2, reduced pyridine nucleotides (NADPH), various intermediates of the EMP pathway, and pentose sugars.

Oxygen tension (Pasteur effect) and glucose availability (Crabtree effect) in the immediate environment of a tissue are known to determine its predominant carbohydrate metabolic pathways. Within the past fifteen years considerable interest has been generated in the study of these two factors.

Due to its high metabolic rate the avascular teleost retina is an ideal tissue for the in vitro study of nervous tissue metabolism. Even at the reduced temperatures normally encountered by this poikilothermic organism the high metabolic activity of the retina allows one to easily quantitate glucose metabolism. It can be readily removed from the eye with minimal injury to the cells and is thin enough to allow for adequate diffusion of metabolites and therefore negates the necessity of slicing and injury resulting therefrom.

It is known that the mammalian retina is capable of aerobic glycolysis, but few attempts have been made to demonstrate aerobic glycolysis in the retina of poikilotherms.

Oxygen tensions have been measured and were found to be high in the choroid region of the teleost eye (Fairbanks, Hoffert, and Fromm, 1969). It was hypothesized that these high oxygen tensions were due to a shift of the oxygen dissociation curve to the left which caused a release of oxygen from hemoglobin coupled with a counter current diffusion multiplying system. A shift in the oxygen dissociation curve to the left could be accounted for by the formation of acid metabolites. These workers further speculated that lactic acid derived from glycolysis and CO_2 from the TCA cycle were the acid metabolites causing this shift. To help explain these phenomena a high rate of aerobic glycolysis which would provide the needed lactic acid was assumed to be present in the teleost retina.

In only one study, in the abundance of work on retinal metabolism, has the measurement of aerobic glycolysis in the teleost retina been attempted. In this study de Vincentiis (1951) measured lactic acid production in a marine teleost and found that aerobic glycolysis was 14% less than anaerobic glycolysis. Due to the paucity of data presented in this study it is impossible to draw any definite conclusions as to the statistical significance of the magnitude of either aerobic or anaerobic glycolysis. In an erroneous interpretation of de Vincentiis' data Davson (1962) concluded that there was no aerobic glycolysis

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occurring in the teleost retina. The above facts justify a study of both aerobic and anaerobic glycolysis in the trout retina.

The objectives of this study were to investigate the following questions concerning the carbohydrate metabolism of the teleost retina:

1. What effects do gas mixtures of different oxygen concentrations have on glucose utilization and lactic acid production by the teleost retina?

2. Does the teleost retina demonstrate a Pasteur effect?

3. What is the rate of glucose utilization and latic acid production by the teleost retina as affected by duration of incubation and tissue disruption?

4. Does the teleost retina demonstrate a Crabtree effect?

5. Is the teleost retina in the absence of glucose capable of utilizing lactate?

LITERATURE REVIEW

Anatomy of the Retina and Related Structures

In no other vertebrate class does the retina vary more in thickness than in the teleost (Walls, 1942). The nervous elements of the retina including cell bodies, dendrites, and axons which are usually mixed together throughout the CNS, have been sorted into discrete layers. Much of the variation in thickness of the teleost retina is caused by variation in the number of conductive elements per number of visual cells. The horizontal cells of the teleost retina have small bodies and are slender. Where cones predominate, as in the case in trout, conductive and integrative elements pile up resulting in a thick inner nuclear layer and a compact ganglion cell layer. In almost all teleosts there are three types of visual cells: rods, single cones, and twin cones. The twin cones are peculiar to the teleosts and appear to function on exposure to bright light.

The teleost retina in contrast to the mammalian retina is poorly vascularized. However, due to the high metabolic rate of the retina, it seems obvious that this nervous tissue would be sensitive to any interference with its supplies of raw metabolic materials and oxygen. These come in the most part from the direction of the choroid which is devoted to the nutrition of the visual and neural cells. The choroid,

in addition to its pigmented vascular layers, characteristically contains the choroid gland. The choroid gland is horseshoe shaped and surrounds the optic nerve. It only exists when the remnant of the first gill arch, the pseudobranch, is present. Blood flows into the pseudobranch, is directed into an efferent artery (opthalmic artery) which passes through the sclera, along with the optic nerve, and breaks up into a set of capillaries (rete mirabile) in the choroid gland (Barnett, 1951). From these capillaries the blood enters the chorio-capillaris circulation, returning to the rete mirabile and leaving the eye by way of the opthalmic vein.

The falciform process is a ridge, formed of pigmented and vascular choroidal tissue, which projects upward into the vitreous cavity from the floor of the eyeball. The arterial blood supply of the falciform process comes from the lentiform body. The blood supply to these structures is derived from the retinal artery and does not pass through the pseudobranch (Barnett, 1951). A possible function of the falciform process is to provide the inner layer of the retina with nutrients. Glucose passes out of the falciform process, diffuses in all directions through the vitreous, to be absorbed by the retina.

Chemical Environment of the Retina

In vivo P_{O_2} measurements were made in the eye of rainbow trout (Fairbanks, Hoffert and Fromm, 1969) and oxygen tensions behind the retina were found that were 20x higher than arterial blood and 3.5x those of the environmental water. This ability to concentrate oxygen in the eye is associated with the development of the choroidal rete mirabile (Wittenburg and Wittenburg, 1962). Fairbanks et al. (1969) suggest the participation of the choroid gland, erythrocyte and retinal carbonic anhydrase as the major components in the oxygen concentrating mechanism of the teleost eye.

Adler (1931) found that the sugar content of the normal vitreous was always considerably lower than that of the aqueous or blood serum in cats. All but the anterior portion of the vitreous is bounded by the retina. The fact that the retina has such high glycolytic activity explained the low concentration of sugar found in the vitreous. The major sources of glucose to the mammalian retina are derived from the choroidal and retinal circulation, whereas in the teleost the falciform process functions in place of the retinal circulation.

Adler in the same study demonstrated that degenerative changes took place in the retina, and the aqueous and vitreous glucose concentrations were higher than normal after the optic

nerve had been severed. The glycolytic activity of the atrophied retinas was considerably less than that of the normal. These facts support the hypotheses previously made that the low concentration of glucose in the vitreous is due to the high rate of glycolysis of the normal retina.

Futterman and Kinoshita (1959) found that whole cattle retinas could use lactic acid in the presence of iodoacetate for cellular respiration *in vitro*. There are two exogenous sources of lactic acid which the teleost retina may use *in vivo*. One source is from the blood. Blood levels of lactic acid have been measured in unexercised two year old sockeye salmon and were found to average 19.5 mg% (Black, 1957). The value goes as high as 200 mg% in exercised fish. Adler (1959) found that changes in the chemical environment of the blood are reflected, after a time lag, in the vitreous body. Therefore the retina has a supply of lactic acid from the blood. A second source of lactic acid for the retina comes from the aqueous humor. Kronfeld and Bothman (1928) observed that anaerobic glycolysis occurs in the mammalian lens. Anaerobic glycolysis was also found to occur in the trout lens (Hoffert and Fromm, 1970). It can therefore be assumed that lactic acid is present in the aqueous humor of the trout. Adler (1959) cites data which indicates that substances which are manufactured by the tissues of the eye freely pass into and out

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of the anterior and posterior chambers. This indicates that any lactic acid in the aqueous may pass into the vitreous for utilization by the retina. The two exogenous sources of lactic acid would only be available if the concentration of lactic acid in the retina is lower than that found in the vitreous or plasma, thus setting up a diffusion gradient into the retina.

Glycogen was first detected in the retina of the frog by Ehrlich in 1833, and has now been found in the retinas of all vertebrate phyla. A comprehensive attempt was made to compare the content and distribution of glycogen in the retinas of a variety of species by Kuwabara and Cogan in 1961. They found that the variations in glycogen content in different species depended on the blood supply, and the amount of glial tissue in the retina. Highest glycogen contents were found in those retinas which had a high tissue to capillary ratio. These retinas accumulated glycogen during periods of low glucose utilization to be used subsequently when they were unable to meet their substrate requirements by diffusion of glucose from the blood stream.

In the same study Kuwabara and Cogan, arranging animals in increasing retinal tissue to capillary ratios, found abundant glycogen in the guinea pig and rabbit; moderate glycogen in the human, cat, and fish (unknown species); little glycogen in the rat; and none in the mouse. Glycogen

was more abundant in the peripheral than central portions of the vascular retinas. This result is attributed to the poorer vascular supply in the periphery of the retina. It was found that the glycogen in the fish retina was associated with Muller's cells and fibers, and was also present in the horizontal cells. It was further shown that fish retinas were capable of synthesizing glycogen when incubated in vitro in a media high in glucose and potassium. The synthesis of glycogen occurred predominantly in Muller's cells and fibers and in the horizontal cells. The problem still remains to discover if glycogen can be used as an endogenous substrate for glucose formation in the teleost retina.

General Aspects of Retinal Metabolism

Many measurements have been made of the RQ of the retina, and the values found closely approximate 1. An RQ of 1 implies the oxidation of carbohydrates. The major scheme for glucose degradation in the retina, both aerobically and anaerobically, is glycolysis. Under anaerobic conditions glucose is converted to lactic acid via the EMP pathway with little production of energy. Aerobically glucose may be oxidized to carbon dioxide and water via the TCA cycle with a much higher energy output. A third pathway for glucose catabolism, which is probably of minor significance in the retina, is the HMP shunt. The HMP shunt diverges

from glycolysis at the level of glucose-6-phosphate (Fig. 1).

A great deal of work has gone into the determination of the contribution of the visual cells to the overall metabolism of the retina. Noell (1952) used retinas in which the visual cells had been inhibited by iodoacetate and found that half of the in vitro Q_{O_2} and half the lactic acid production of the adult rabbit's retina was contributed by the visual cells, whereas anaerobically about two thirds of the lactic acid comes from the inner layers. Noell used these observations to explain why the inner layers are relatively high in glycolytic enzymes and relatively low in enzymes of the citric acid cycle. Fonner, Hoffert and Fromm (1969) used histochemical evidence to show that the EMP and TCA pathways are concentrated in the photoreceptor ellipsoids of the rainbow trout. They suggest that the HMP shunt is in the inner layers of the retina and not the ellipsoids.

The Contribution of the HMP Pathway to Retinal Metabolism

There has been some controversy concerning the importance of the HMP pathway in retinal metabolism. A few workers have attributed as much as 25% of the metabolism of the retina to the HMP shunt, while others have found it operating to a negligible extent. The method used to test the occurrence of this metabolic pathway consists of comparing the rate of appearance of label in the respired CO_2

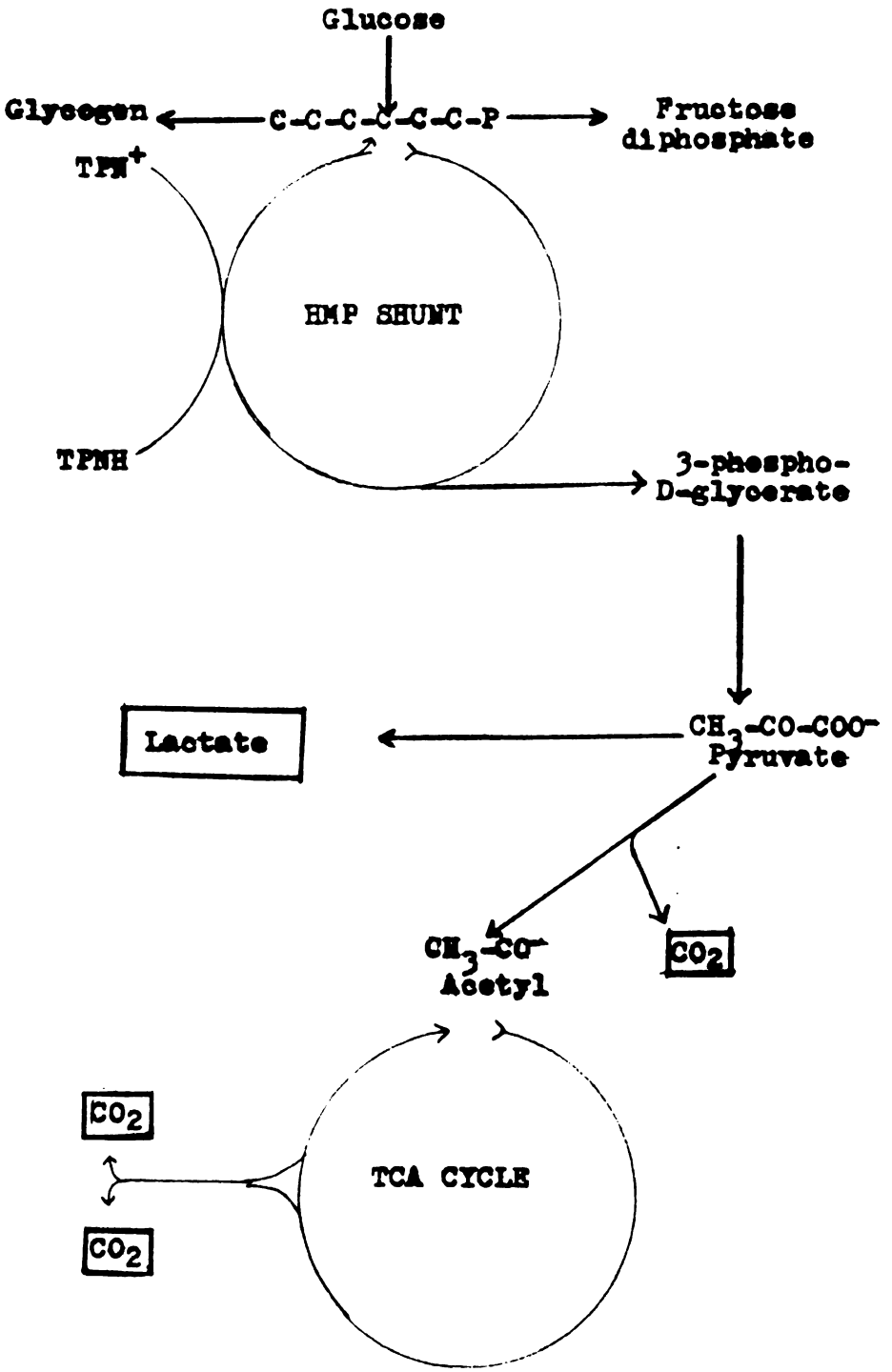

FIGURE 1.--Scheme demonstrating the three metabolic pathways occurring in the teleost retina.

derived from 1-^{14}C glucose with labeled CO_2 produced from other positions. The molecules of CO_2 produced from the HMP shunt pathway all have their origin in C-1 of glucose. The rate of glucose 6-^{14}C oxidation is used as a standard for comparison with glucose 1-^{14}C because the 1-C and 6-C carbons, when only the TCA cycle is operational, appear in CO_2 at equal rates. Any deviation of the ratio 1-C/6-C above unity indicates some participation of the non-glycolytic pathway (HMP) (Hoar and Randall, 1969).

To determine the significance of the HMP pathway in the aerobic metabolism of glucose by the retina, intact cattle retinas were incubated with glucose-1-^{14}C or glucose-6-^{14}C. No evidence of any significant preferential oxidation of the carbon 1 atom of glucose was observed by Futterman and Kinoshita (1959). These results suggest that the HMP pathway does not play a significant role in the utilization of glucose by the retina. In a study by Kerley and Rahman (1961) it is shown that the ratio of $^{14}CO_2$ formed during incubation with glucose labeled at 1-^{14}C to that formed with 6-^{14}C was near unity in ox retinas. However, these workers found that the difference in the isotope content of the lactate produced from the two glucose samples was greater than that found by Futterman and Kinoshita (1959). This, together with low values found for the specific activity of CO_2, has led Kerley and Rahman to conclude that the HMP

shunt may be of more importance for glucose metabolism in the retina than has been previously reported. Although the HMP shunt does not hold the importance it has in corneal epithelium or in the lens, some glucose is metabolised by this route, and under aerobic conditions it is not suppressed completely as has been suggested by Futterman and Kinoshita (1959). Kerley and Rahman conclude that one glucose molecule in four is metabolized by the HMP shunt pathway in the ox retina.

The capacity for retinal oxidation of glucose by the HMP shunt was also investigated by Cohen and Noell (1960). The HMP shunt path operated in both adult and young rabbit retinas under in vitro conditions but was severly restricted by the availability of electron acceptors. The conclusion was drawn that at both ages the capacity for glucose oxidation by the HMP shunt pathway was small. Von Holt et al. (1959) used bovine retinas to see if there was a difference in the oxidation time for 1-^{14}C glucose and 6-^{14}C glucose. It was found that the retina shows only a small preponderance of C-1 oxidation which again indicates that the HMP shunt pathway holds little significance in retinal metabolism.

Hoffert and Fromm (1970), using both diseased and normal retinas, studied retinal metabolism in rainbow and lake trout. Their results indicate that slightly more glucose was metabolized by the EMP pathway than the TCA cycle. In

both the diseased and normal trout retinas the HMP shunt accounted for less than 5.5 % of the total glucose metabolized.

Factors Affecting Retinal Metabolism

Kornblueth, Yardini-Yaron and Wertheimer (1953), using the retinas of white rats found that glucose utilization is dependent on the integrity of the cells. It was discovered that after the retina was cut into 20 pieces or more, the utilization of glucose was not appreciably different from that found in intact retina. When the tissue was homogenized, however, the glucose utilization fell to zero.

In the study of retinal metabolism, when one removes the retina by severing the optic nerve, the ganglion cell bodies themselves are not directly injured because only the axone is cut. This injury to the retinal ganglion cell axone occurs at some distance from the cell body, and does not cause severe trauma to the neural elements of the retina.

Retinal metabolism is known to vary *in vitro* with the type of incubation media used. As early as 1936, Laser used rat retinas and found that respiration is about twice as high in bicarbonate buffered Ringer as it is in phosphate buffered Ringer. In a later study, Craig and Beecher (1943)

used the retinas of mongrel white rats to study lactic acid production in a medium containing phosphate. They found that retinal Q_{O_2} in a phosphate medium were sensitive to P_{O_2}. Craig and Beecher in the same study experimented with retinal metabolism in a bicarbonate medium subjected to varying oxygen tensions and found no significant change in respiration but glycolysis increased to nearly the anaerobic level when the oxygen tension was lowered from 95 % to 5 %. Kornblueth et al. (1953) found that serum and Krebs-Ringer bicarbonate were the best media for glucose utilization by the retina of white rats. They surmised that bicarbonate was necessary for the synthesis of the Krebs cycle intermediates.

Differing gaseous environments may affect retinal metabolism. In 1936, Laser measured the respiration of rat retinas under varying gas concentrations. In 95 % nitrogen with 5 % oxygen, he found the respiration to be normal. It was also demonstrated that 95 % carbon monoxide with 5 % oxygen leads to no inhibition of retinal respiration. In a later study Craig and Beecher (1943) discovered oxygen uptake of the nearly intact rat retina in the presence of glucose was doubled by raising the carbon dioxide tension from 1 % to 5 %. This result led the investigators to speculate that carbon dioxide may be important at an intermediate stage in metabolism for the synthesis of dicarboxylic acids.

The effect of light on retinal metabolism was studied by Lindeman in 1940. The Warburg manometric method was used to measure oxygen consumption of frog retinas. The results indicated that there was no consistant difference in the rate of oxygen consumption in the dark as compared with the light when the retinas were observed under alternating periods of darkness and light.

Iodoacetate is a metabolic inhibitor which is known to block the EMP pathway. The site of action is the inhibition of glyceraldehyde-P-dehydrogenase (Mahler and Cordes, 1966). Lenti (1940) used minced calf retina suspended in Ringer solution to study the effects of iodoacetate on retinal metabolism. A 0.02M solution of iodoacetate was found to completely inhibit glycolysis. The fact that iodoacetate inhibits glycolysis has proved to be an important analytical tool in the study of retinal metabolism. Noell (1952) used iodoacetate in an attempt to demonstrate that aerobic glycolysis predominates over the TCA cycle in yielding the energy for certain functions of the retina. His findings suggest that in the higher vertebrates (rabbit and cat) glycolysis does in fact provide the predominate support of retinal functions. In the lower vertebrates (frog), however, aerobic glycolysis is of minor importance compared with the TCA cycle in maintaining retinal functions. To demonstrate experimentally that glucose or glycogen was

not the principle endogenous substrate of bovine retinas, a study of the effect of iodoacetate was undertaken (Futterman and Kinoshita, 1959). These workers discovered that during the first hour of incubation Q_{O_2} could be supported by endogenous substrate even in the absence of an active glycolysis. These experiments suggested that lactic acid was a major endogenous substrate contributing to respiration in the presence of iodoacetate.

Various chemical substances have pronounced effects on retinal metabolism. Using the Summerson manometric technique, Robbie and Leinfelder (1948) did simultaneous determinations of Q_{O_2} and glycolysis in white rat retinas. They attempted to simulate an anaerobic environment through the action of cyanide which is an inhibitor of the cytochrome oxidase system. They found that when a concentration of 10^{-3}M cyanide was added to the media there was an inhibition of the Pasteur effect and an increased rate of anaerobic glycolysis. When a high concentration of cyanide was added (10^{-2}M) there was a decrease in anaerobic glycolysis as measured indirectly by CO_2 production.

Lenti (1940) found that phlorizin (0.1 %) did not affect glycolysis in calf retinas <u>in vitro</u>. In the same study it was found that although glyceraldehyde was converted to lactic acid it had an overall inhibitory effect on glycolysis when added to the medium. Von Holt et al.

(1959) experimented with the effect of insulin on bovine retinal metabolism and found that insulin caused a significant increase in glucose oxidation.

Pasteur Effect

In all cells that are capable of degrading glucose both in the presence and absence of oxygen, the sugar will disappear, and the lactic acid will be formed more rapidly under anaerobic than under aerobic conditions. This inhibition of glycolysis by oxygen was first recognized by Pasteur and later confirmed by Meyerhof and Warburg, and is known as the Pasteur effect. Aerobiosis will remove inorganic orthophosphate (P_i) and ADP, a great deal more effectively than will anaerobiosis. A decrease in the availability of P_i and ADP leads to a diminution in the rate of glycolysis and an increase in the rate of gluconeogenisis (Mahler and Cordes, 1966).

The presence of a Pasteur effect has been substantiated in the mammalian retina, but there is much controversy concerning its existence in the retina of poikilotherms. Craig and Beecher (1943) subjected the retinas of mongrel white rats to varying oxygen tensions. In a bicarbonate medium, when the oxygen tension was lowered from 95 % to 5 % there was no significant change in the rate of glucose oxidation by the retinal TCA cycle, but glycolysis

was increased to nearly the anaerobic level, suggesting a Pasteur effect. They concluded that the rate of glycolysis is controlled by oxygen tension rather than by the rate of glucose oxidation via the TCA cycle. Using the Barker and Summerson method for lactic acid determination, de Vincentiis (1951) looked at the effect of high oxygen tension on glycolysis in the fish retina *in vitro*. Two varieties of marine fish were used (*Schylliorhinus* and *Scorpaena scrofa*) and it was found that less lactic acid was produced under aerobic conditions in the retinas of these fish than under anaerobic conditions. This result led de Vincentiis to conclude that oxygen inhibits glycolysis in the fish retina, thereby confirming the existence of the Pasteur effect.

Crabtree Effect

The inhibition of the TCA cycle by glucose is referred to as the Crabtree effect. The only non-neoplastic tissues for which the Crabtree effect has been shown are the retina and leukocytes (Cohen and Noell, 1959). Wu and Racker (1957) proposed a possible mechanism for the action of the Crabtree effect. These workers determined the intracellular concentrations of adenine nucleotides, hexose phosphates and inorganic P_i in ascites tumor cells. The addition of glucose to washed cells resulted in a marked fall in intracellular P_i and ADP. Cohen (1957) postulated that the drop in

inorganic P_i and ADP observed in the Crabtree effect was caused by a stimulation of glycolysis.

A reconstructed system consisting of the glycolytic enzymes and actively respiring liver mitochondria was studied by Gatt, Krimsky and Racker (1956). The addition of glucose to this system resulted in a pronounced inhibition of mitochondrial respiration. These findings suggested that the limiting amounts of ADP are shared by the intra- and extramitochondrial systems, and shuttle back and forth between them. An active glycolytic system may deprive mitochondrial respiration of essential ADP.

The Crabtree effect is absent in the adult rabbit's retina despite the high mitochondrial respiration (Cohen, 1957). Furthermore, it was observed that during postnatal development of the retina, prior to the formation of the visual cells, glucose inhibited mitochondrial respiration as much as 40 %. The data indicated that the adult retina contains a highly active component of mitochondrial respiration which does not share its phosphoralative coefactors with a system of glycolysis of high capacity.

MATERIALS AND METHODS

Experimental Rationale

The experiments were designed to study the carbohydrate metabolism of the teleost retina under different conditions. The contribution of glycolysis to retinal metabolism was determined by measuring lactic acid production. Total glucose utilization was measured to quantitate the contribution of the TCA cycle to retinal metabolism with the assumption that 1 mole of glucose produces 2 moles of lactic acid in the glycolytic scheme. The total amount of glucose utilized subtracted from the amount that could be attributed to glycolysis would give a quantitative estimate of TCA activity. In all determinations a sample containing media without tissue was employed to determine the initial level of glucose and lactic acid in the culture media. The values obtained from the media blank were compared with values obtained from the media in which the tissues were incubated to determine the amount of glucose utilized and lactic acid produced.

Glycolysis was measured at varying time periods under both anaerobic and aerobic conditions. The Pasteur effect was studied by subjecting the retinas to aerobic conditions and measuring the amount of glucose utilized and lactic acid produced and then comparing these results with

glucose utilization and lactic acid production of retinas subjected to anaerobic conditions. The Crabtree effect, inhibition of the TCA cycle by glucose, was studied by measuring glucose utilization and lactic acid production by the retinas during incubation in media of different glucose concentrations. The contribution of lactic acid as a substrate for retinal metabolism was studied. This determination was made by measuring the disappearance of an exogenous source of lactic acid from the culture media in the presence of iodoacetate which blocks the utilization of both exogenous and endogenous glucose.

Experimental Animals

Rainbow trout (Salmo gairdneri) used in this study were obtained from the Michigan Department of Natural Resources at Grayling, Michigan. The 2-2½ year old trout selected were between 9 and 11 inches in length and weighed from 380-480 grams. The fish were kept in fiberglass lined plywood tanks in a constant temperature room at 13 ± 1 C. Dechlorinated tap water constantly flowed into the tanks and was aerated with compressed air filtered through activated charcoal. The photo-period consisted of 15 hours of light and 9 hours of darkness.

Preparation of Tissues

The trout were killed by cervical dislocation and selected tissues removed. The eye was extracted from its orbit by severing the ocular muscles and optic nerve with scissors. The eye was then immediately immersed in a petri dish filled with sterile Ringer solution (Appendix 1). While holding the eye with rat tooth forceps, a small incision was made into the periphery of the cornea with the blade of an iris scissors, the incision being continued along the circumference of the cornea. After removing the cornea, the lens was lifted out of the aqueous humor with small curved forceps. Next, the optic nerve was firmly grasped with iris forceps while a small circular incision was made into the posterior surface of the sclera to one side of the optic nerve with iris scissors. Eye dressing forceps were carefully inserted into the incision and the retina and choroid were worked free from the sclera by gentle scraping.

In some instances cardiac and kidney tissue were removed from the trout. To remove the heart an incision was made from the base of the operculum along the mid-ventral line. After the aorta and vena cavae were cut, the heart was lifted from the pericardial cavity by means of forceps and placed in sterile Ringer solution. Using iris scissors, four thin slices, less than 1 mm in thickness, were cut from the posterior surface of the ventricle. Slicing of the large

tissue mass was necessary to insure the adequate diffusion of the nutrients. The dorsally located opisthonephros kidney was found by continuing the incision posteriorally to the cloaca. Scissors were used to cut through the swim bladder and a piece of the middle portion of the kidney, approximately 10 mm in length and 5 mm in width, was removed and placed in sterile Ringer solution. The loose arrangement of the connective tissue elements and the general flatness of the tissue made further sectioning unnecessary. All surgical instruments which came in contact with the tissues studied were sterilized under 15 psi at 121 C. for 15 minutes in a No. 777 Speed Clave (Wilmot Castle Co., Rochester, N.Y.).

After removal from the Ringer solution the tissues were blotted dry on sterile No. 1 Whatman filter paper. During the blotting process as much of the vitreous body was removed from the retinas as was possible without damaging the retinal cells. All tissues were then weighed on a Roller-Smith (0-500 mg) precision balance (Roller-Smith Co., Newark, N.J.) to the nearest 0.1 mg, and placed in 0.5 ml of the appropriate media. The retinal weights ranged between 290 and 450 mg with never more than 50 mg variation between retinas from the individual fish. The cardiac tissue slices weighed between 70 and 100 mg while kidney tissue weighed between 90 and 140 mg. The media was contained in a sterilized center well (Fig. 2) constructed of Pyrex glass and

FIGURE 2.--Tissue incubation apparatus.

1. Graduated milk dilution bottle (flask) (165 ml).
2. Center well for tissue and media (2.0 ml).
3. Black rubber stopper (No. 2).
4. Syringe needle (18 guage, 2.5" long).
5. 3-way plastic stopcock.

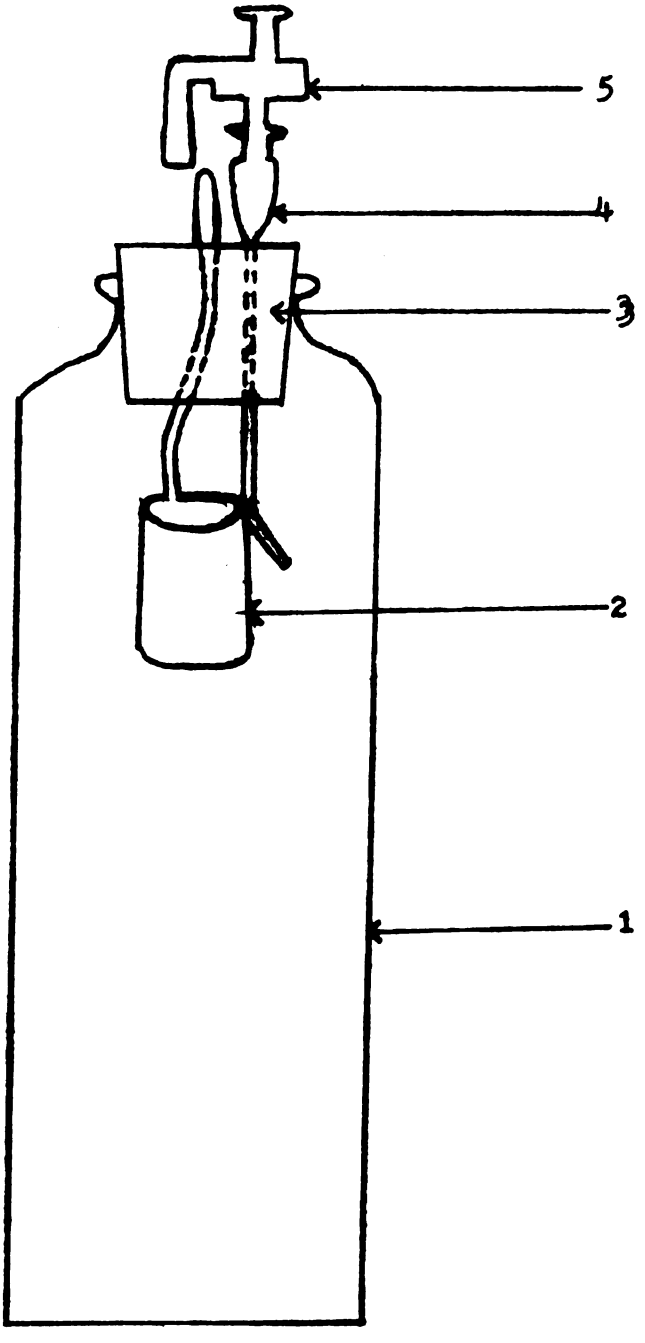

FIGURE 2

having a volume of approximately 2.0 ml. The center well was attached to a No. 2 black rubber stopper which was fitted into a 165 ml capacity graduated milk dilution bottle (No. 1370, Corning Glassware Co., Corning, N.Y.). An 18 guage needle fitted with a 3 way plastic stopcock (No. k-75 Pharmaseal Laboratories, Glendale, California) was permanently inserted into the stopper (Fig. 2). The needle provided the means for gas to enter the incubation flask. The flasks were secured in a wire test tube basket.

Gassing Procedure

Gas mixtures were made by means of a water displacement spirometer having a capacity of 1.9 liters (Fig. 3). The spirometer was attached to a mercury manometer which allowed an accurate determination of the pressure within the incubation flasks. The three-way plastic stopcock was utilized to attach the incubation flasks to a multi-outlet exhaust manifold. The entrance of gas from the spirometer into the partially evacuated incubation flasks was controlled by means of a three-way glass stopcock (Fig. 3). The vacuum was created in the flasks by means of a Cenco-Magavac Vacuum Pump (Central Scientific Co., Boston-Chicago-Toronto). Approximately 95 % of the air was evacuated from the incubation flasks before gas was readmitted. After the flasks were evacuated and the vacuum stabilized at approximately 720 mm

FIGURE 3.--Gassing apparatus.

1. Gas inlet.
2. 3-way glass stopcock.
3. Water displacement spirometer (1.9 liters).
4. Pressure bottle.
5. Pressure bulb.
6. Cenco-Magavac vacuum pump.
7. Acidified water
8. Multi-outlet exhaust manifold.
9. Mercury vacuum gauge.
10. Gas outlet.

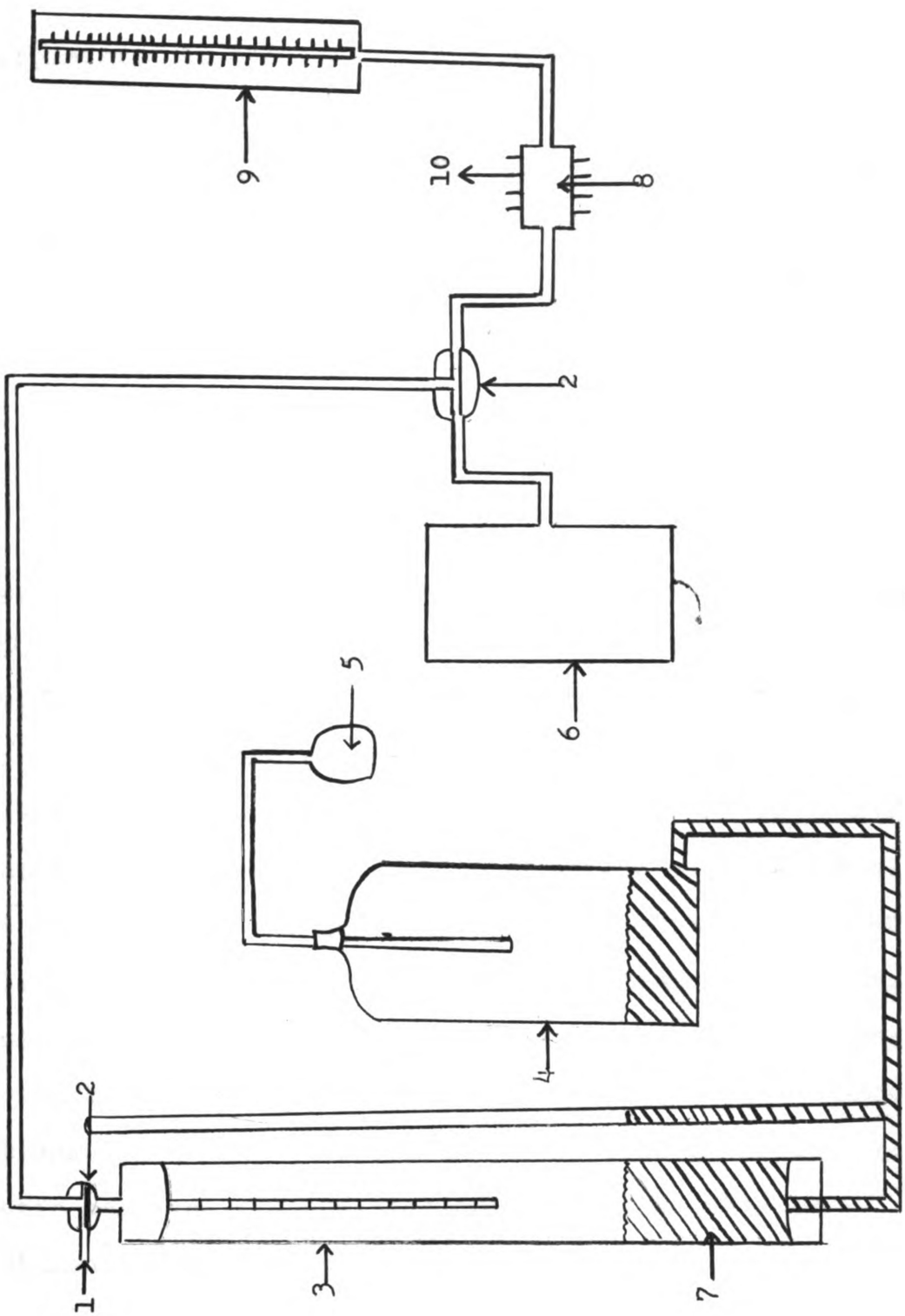

FIGURE 3

Hg below atmospheric pressure, gas was admitted from the spirometer. As the gas mixture entered the incubation flasks the vacuum was reduced to zero so atmospheric pressure existed in the flasks. The gassing procedure as outlined above was repeated three times. When the pressure in the bottles reached atmospheric pressure the plastic stopcocks were closed and the tissues were ready for incubation.

Analysis of the Media

The determination of glucose was by the Glucostat enzymatic micro-method (Worthington Biological Corp.,Freehold, N.J.). During each glucose assay, a glucose blank containing 0 mg% glucose and three standards consisting of 16.6, 25.0 and 33.3 mg% glucose were used for comparison with the unknowns. The color development was carried out at 37 C for exactly 10 minutes. The method of Barker and Summerson(1941) was employed for the determination of lactic acid. A 40 mg % lactic acid standard solution (Sigma Chemical Co., St. Louis, Missouri) was utilized to make up three standards having concentrations of 3.33, 6.66 and 13.33 mg% lactate. A blank containing no lactic acid was also prepared. The lactic acid and glucose standard and blank tubes were combined to facilitate the assays.

Incubation Procedure

Immediately after the samples were gassed they were incubated for variable time periods. All incubations were carried out in a constant temperature room at 13 C. Shaking was accomplished by placing the test tube basket on a sliding platform attached to a steel rod which in turn was attached to a 1/18 H.P. electric speed reducer motor (Bodine Electric Co., Chicago, Ill.). The speed at which the platform was driven was controlled by a Powerstat (Superior Electric Co., Bristol, Conn.) attached to the motor. The speed was adjusted to provide maximum agitation (0.7 cm stroke length at 1.5 cps) of the media without splashing it from the center well. At the completion of the incubation period, 0.3 ml samples of the media were removed from each center well by means of disposable prothrombin pipettes (Scientific Products, Evanston, Ill.) and placed in 15 ml Pyrex centrifuge tubes. The samples were then treated with 2 ml of $ZnSO_4$ and 2 ml of $Ba(OH)_2$ to precipitate any protein present. After the addition of 3.8 ml of distilled H_2O, a 26 fold dilution was produced, bringing the unknowns and standards on to the linear portion of the standard curve. The resulting pH was 7.0.

Procedure for Examining the Effect of Varying Gas Concentrations

Both retinas were removed from the trout and weighed. Each retina was separately placed in 0.5 ml of Modified Medium 199 (Grand Island Biological Co., Grand Island, N.Y.). Modified Medium 199 consists of an Earles base which contains 1.25 gm/l $NaHCO_3$, 0.5 gm/l glucose and no phenol red. The center wells were then tightly stoppered into the milk dilution bottles. Next, the samples were gassed with a gas mixture having a composition of 95 % N_2-5 % CO_2 or 95 % O_2-5 % CO_2. The volume of gas used during gassing was approximately 1355 ml. In the first set of experiments an incubation time of ½ hour was used. After this, 1 hour and finally 3 hour incubation periods were tested.

Samples of cardiac and kidney tissue were weighed and placed in 0.5 ml of modified Medium 199 in separate center wells. Cardiac and kidney tissue were gassed under the same conditions as the retinas. The tissues were then incubated for 1 hour.

Procedure for Studying the Effect of Sonification

The two retinas were removed from the trout, blotted dry and weighed. One intact retina was transferred to a center well containing 0.5 ml of a Phosphate Buffered Saline

(PBS) (Grand Island Biological Co., Grand Island, N.Y.), solution with a 100 mg% glucose concentration. The second retina was placed in a thick-walled Pyrex test tube containing 1.0 ml of PBS with a 100 mg% concentration of glucose. This retina was then homogenized by means of a No. W185C Sonifier Cell Disrupter (Heat Systems Co., Melville, L.I., N.Y.). During the sonification process the test tube was cooled by immersion in ice water. After cellular disruption was completed, 0.5 ml of the solution was transferred to a center well. The center wells were placed in milk dilution bottles and gassed with a mixture of 95 % N_2-5 % CO_2. After gassing, the samples were incubated for a period of 5 hours.

Procedure for Examining the Effect of Various Glucose Concentrations

In this experiment the retinas were incubated in a modified Mammalian Krebs Saline Medium (Appendix 1). The medium was made up to have an osmolarity of 289 mOsm/kg. Enough $NaHCO_3$ was added to the medium to give a pH of 7.6 after the solution was saturated with 99.5 % O_2-0.5 % CO_2 at 13 C. The medium was then divided into five 100 ml portions. To each portion, except the first, varying quantities of anhydrous glucose were added, resulting in five concentrations: 0, 50, 100, 150, and 200 mg% glucose.

The retinas were weighed and placed in 0.5 ml of media of known glucose concentration. After gassing the tissues with 99.5 % O_2-0.5 % CO_2, they were incubated for a period of 1 hour.

Procedure to Study the Effect of Iodoacetate

A solution of 0.5 ml of modified Mammalian Krebs Saline Medium containing 10 mg % lactic acid and 0.001 moles of iodoacetic acid sodium salt (Matheson Co., Cincinnati, Ohio) was placed in three separate center wells. The amount of iodoacetate employed in this experiment ranged from 0.001 to 0.01 moles. The retinas were removed, wet weight determined, and placed in 100 ml of Ringer solution with 10 mg % lactic acid and 0.001 moles of iodoacetate. After a preincubation period of one-half hour the retinas were removed from the Ringer solution and placed in two of the center wells. The samples were then exposed to a 99.5 % O_2-0.5 % CO_2 gas mixture. After a one hour incubation, 0.3 ml of the media was removed from each flask and analyzed for glucose and lactic acid.

Statistical Analysis

Glucose utilization and lactic acid production of the tissues studied were expressed in μg/hr or μg/hr/gm.

During the weighing procedure it was found that not all of the vitreous body could be removed from the retina without destroying the integrity of the cells. Thus, for a more complete interpretation of the data, µg/hr as well as µg/hr/gm wet weights were determined. Statistical analysis to determine the significant difference between the means was carried out by employing the student "t" test. Values considered significant in this study have a calculated α value of 0.05 or less. All regression lines were plotted by the method of least squares.

RESULTS

The project of elucidating the metabolism of the retina was carried out by measuring the glucose utilization and lactic acid production of this tissue under differing experimental conditions. The measurements of glucose utilization gave a quantitative estimate of carbohydrate metabolism. The measurement of lactic acid production determined the degree to which the EMP pathway contributed to metabolism.

Effect of Varying O_2 Concentrations on Retinal Metabolism

One-Half Hour Incubations

In the first series of experiments, utilizing an incubation period of ½ hour, retinas from the trout were exposed to a gaseous environment of 95 % N_2-5 % CO_2 or 95 % O_2-5 % CO_2. There was no apparent change in the physical appearance of the retinas after incubation, but in some cases the modified Medium 199 was grayish in color after ½ hour, probably due to a disruption of the pigmented epithelium of the retina. Under anaerobic conditions (95 % N_2-5 % CO_2) glucose utilization was higher and there was a greater production of lactic acid than under aerobic conditions (95 % O_2-5 % CO_2). The results of the anaerobic

and aerobic experiments were statistically different at the $p < 0.001$ (Table 1).

One Hour Incubations

Utilizing a 1 hour incubation period, there was more glucose utilized and more lactic acid produced under both anaerobic and aerobic conditions than when an incubation period of ½ hour was used. The results also indicate that when glucose utilization and lactic acid production are expressed in µg/hr/gm there is less glucose utilization and lactic acid production under incubation periods of 1 hour than under identical treatments of ½ hour, indicating a slowing of the rate of glycolysis as the incubation time increases (Table 2).

Using a 1 hour incubation time there was more glucose utilized and more lactic acid produced under anaerobic than under aerobic conditions. The results of glucose utilization under anaerobic and aerobic conditions were statistically different at the $p \ll 0.001$ level. Lactic acid production showed a significant difference of $p < 0.001$ between anaerobic and aerobic levels.

Three Hour Incubations

Retinal metabolism under different gaseous environments was tested utilizing an incubation period of 3 hours.

TABLE 1.--Glucose utilization and lactic acid production by retinal tissue utilizing modified Medium 199 for ½ hour at 13C

Treatment	N	Gas Concentration	Glucose utilized (µg/hr)	Glucose utilized (µg/hr/gm)	Lactate produced (µg/hr)	Lactate produced (µg/hr/gm)
Anaerobic	10	95% N_2 - 5% CO_2	386.8 ± 14.1*	1314.7 ± 68.1*	427.6 ± 20.8*	1443.7 ± 59.5*
Aerobic	10	95% O_2 - 5% CO_2	242.6 ± 26.0	744.3 ± 58.9	291.6 ± 19.8	917.7 ± 83.7

N (number of observations)

Mean ± S.E.

* Significant difference between anaerobic and aerobic treatments at $p < 0.001$

TABLE 2.--Glucose utilization and lactic acid production by retinal tissue utilizing modified Medium 199 for 1 hour at 13C

Treatment	N	Gas Concentration	Glucose utilized (µg/hr)	Glucose utilized (µg/hr/gm)	Lactate produced (µg/hr)	Lactate produced (µg/hr/gm)
Anaerobic	6	95% N_2 - 5% CO_2	224.2 ± 7.9*	708.8 ± 39.3*	271.6 ± 11.6*	857.1 ± 45.8*
Aerobic	6	95% O_2 - 5% CO_2	143.9 ± 16.6	433.5 ± 55.5	204.9 ± 7.2	609.6 ± 11.7

N (number of observations)

Mean ± S.E.

* Significant difference between anaerobic and aerobic treatments at $p < 0.01$

The samples of media which contained the retinas were always turbid after 3 hour incubations, but the retinas remained intact based on gross observations. It was found that there was an increase in total µg of glucose utilized and total µg of lactic acid produced over the 1 hour incubation experiments. On a µg/hr/gm basis, however, there was less glucose utilized and lactic acid produced under the 3 hour incubation time than under the 1 hour incubation time (Table 3). This is another indication that the glycolysis of the retina is retarded during longer incubation periods. There was no significant difference in glucose utilization and lactic acid production between anaerbobic and aerobic conditions when a 3 hour incubation period was employed.

The results for glucose utilization as well as lactic acid production were found to be significantly different at each incubation period used under both aerobic and anaerobic conditions. Regression lines showing glucose utilization and lactic acid production under aerobic and anaerobic conditions over the three different incubation periods are shown in Appendix 2.

Effect of Aerobic and Anaerobic Treatment on Cardiac and Kidney Tissue Metabolism

Cardiac and kidney tissue were exposed to aerobic and anaerobic conditions and subjected to 1 hour incubations

TABLE 3.--Glucose utilization and lactic acid production by retinal tissue utilizing modified Medium 199 for 3 hours at 13C

Treatment	N	Gas Concentration	Glucose utilized (µg/hr)	Glucose utilized (µg/hr/gm)	Lactate produced (µg/hr)	Lactate produced (µg/hr/gm)
Anaerobic	10	95% N_2 - 5% CO_2	80.6 ± 4.6*	294.8 ± 58.3*	94.5 ± 5.1*	330.9 ± 50.9*
Aerobic	10	95% O_2 - 5% CO_2	83.7 ± 4.5	245.4 ± 28.3	109.8 ± 3.6	309.7 ± 16.8

N (number of observations)

Mean ± S.E.

*No significant difference between anaerobic and aerobic treatments at $p < 0.05$

in modified Medium 199. The reason for experimenting with cardiac and kidney tissue was to check on the accuracy of the glucose and lactic acid assays, furthermore it was hoped that the metabolism of these tissues could be quantitatively compared with the metabolism of retinal tissue. It is well known that the heart cannot survive without oxygen for more than a few minutes. When the cardiac tissue was exposed to anaerobic conditions the glucose utilization fell to zero and lactic acid production was much lower than under aerobic conditions (Table 4).

Table 4. --Metabolism of cardiac tissue under anaerobic and aerobic conditions in modified Medium 199 for 1 hour at 13 C.

Treatment	N	Gas Concentration	Glucose utilized (µg/hr/gm)	Lactate produced (µg/hr/gm)
Anaerobic	2	95% N_2 - 5% CO_2	0.00*	588.4 ± 69.9*
Aerobic	2	95% O_2 - 5% CO_2	438.5 ± 33.9	1423.6 ± 35.6

N (number of observations)

Mean ± S.E.

* Significant difference between anaerobic and aerobic at $p < 0.01$

The fact that lactic acid is produced in greater quantities anaerobically and aerobically than can be accounted for by

the utilization of glucose from the media leads to the speculation that there is some endogenous substrate in cardiac tissue which is oxidized in addition to exogenous glucose. The results in the case of kidney tissue (Table 5) indicate that for a period of 1 hour the kidney can utilize glucose and produce lactic acid under both aerobic and anaerobic conditions. There is less accumulation of lactic acid under aerobic conditions showing that under these conditions glucose may be routed through the TCA cycle.

Table 5.--Metabolism of kidney tissue under anaerobic and aerobic conditions in modified Medium 199 for 1 hour at 13 C.

Treatment	N	Gas Concentration	Glucose utilized (µg/hr/gm)	Lactate produced (µg/hr/gm)
Anaerobic	2	95% N_2 - 5% CO_2	464.4 ± 111.1	553.2 ± 74.5*
Aerobic	2	95% O_2 - 5% CO_2	440.1 ± 37.4	308.1 ±207.1

N (number of observations)

Mean ± S.E.

* No significant difference between anaerobic and aerobic at $p < 0.05$

Effect of Sonification on Retinal Metabolism

In this experiment a paired experimental design was used. One of the center wells contained an intact

retina in 0.5 ml of Phosphate Buffered Saline (PBS) which contained 100 mg% glucose, the other contained 0.5 ml of a retinal homogenate, which was made by sonifying a whole retina in 1 ml of media. The dilution in the second flask contained an equivalent of ½ the retinal tissue of the flask with the intact retina. After gassing both incubation flasks with a 95% N_2 - 5% CO_2 gas mixture and incubating them for 5 hours, glucose and lactic acid were quantitatively determined (Table 6). The results show that cellular disruption caused by sonification almost completely inhibits glucose utilization and lactic acid production. The intact retina utilized glucose and produced lactic acid with at least a 20X greater magnitude than did the retinal homogenates.

The Effect of Varying Glucose Concentrations on Retinal Metabolism

To test the effect of glucose concentration on retinal metabolism, retinas were subjected to media having different glucose concentrations. The media used was a modified Mammalian Krebs Saline Medium with a bicarbonate buffer to which had been added glucose ranging in concentration from 0 mg% to 200 mg%. The two incubation flasks containing the retinas and one incubation flask containing media alone were then gassed with

TABLE 6.--Effect of sonification on anaerobic retinal metabolism in PBS with 100 mg% glucose for 5 hours at 13C

Treatment	N	Gas Concentration	Glucose utilized (µg/hr)	Glucose utilized (µg/hr/gm)	Lactate produced (µg/hr)	Lactate produced (µg/hr/gm)
Sonified**	2	95% N_2-5% CO_2	8.80 ± 6.20*	109.6 ± 78.6*	0.8 ± 0.6*	10.8 ± 7.20*
Whole	2	95% N_2-5% CO_2	66.5 ± 3.9	863.4 ± 65.2	40.3 ± 0.8	521.8 ± 2.9

N (number of observations)

Mean ± S.E.

* Significant difference between sonified and whole at $p < 0.001$

** All sonified values have been doubled to correct for dilution

99.5% O_2 - 0.5% CO_2. After an incubation period of 1 hour the media was assayed for glucose and lactic acid. At 0 mg% glucose concentration in the media there was no glucose utilization, however, a mean value of 126.2 µg/hr/gm of lactic acid was found in the media for the 6 retinas observed. At each successively higher level of glucose in the media there was a higher glucose utilization and higher lactic acid production (Fig. 4 and 5). When the data was analyzed in terms of a glucose ratio it was found that the ratio reached it's highest value at 100 mg% glucose concentration in the media, indicating the highest TCA cycle activity (Table 7). After the 100 mg% concentration the glucose/lactate ratio began to fall progressively as higher glucose concentrations were used in the media (Fig. 6 and 7).

Table 8 gives a summary of the glucose utilized and lactate produced in µM/hr/gm under the varying experimental conditions. Also incorporated into Table 8 are the percentages of glucose utilized and lactate produced by the EMP pathway and the TCA cycle, assuming these two are the only pathways utilized in glucose degradation.

Effect of Iodoacetate on Retinal Metabolism

The results indicate that there was an accumulation of lactic acid over the 25.3 ± 0.57 mg% initially

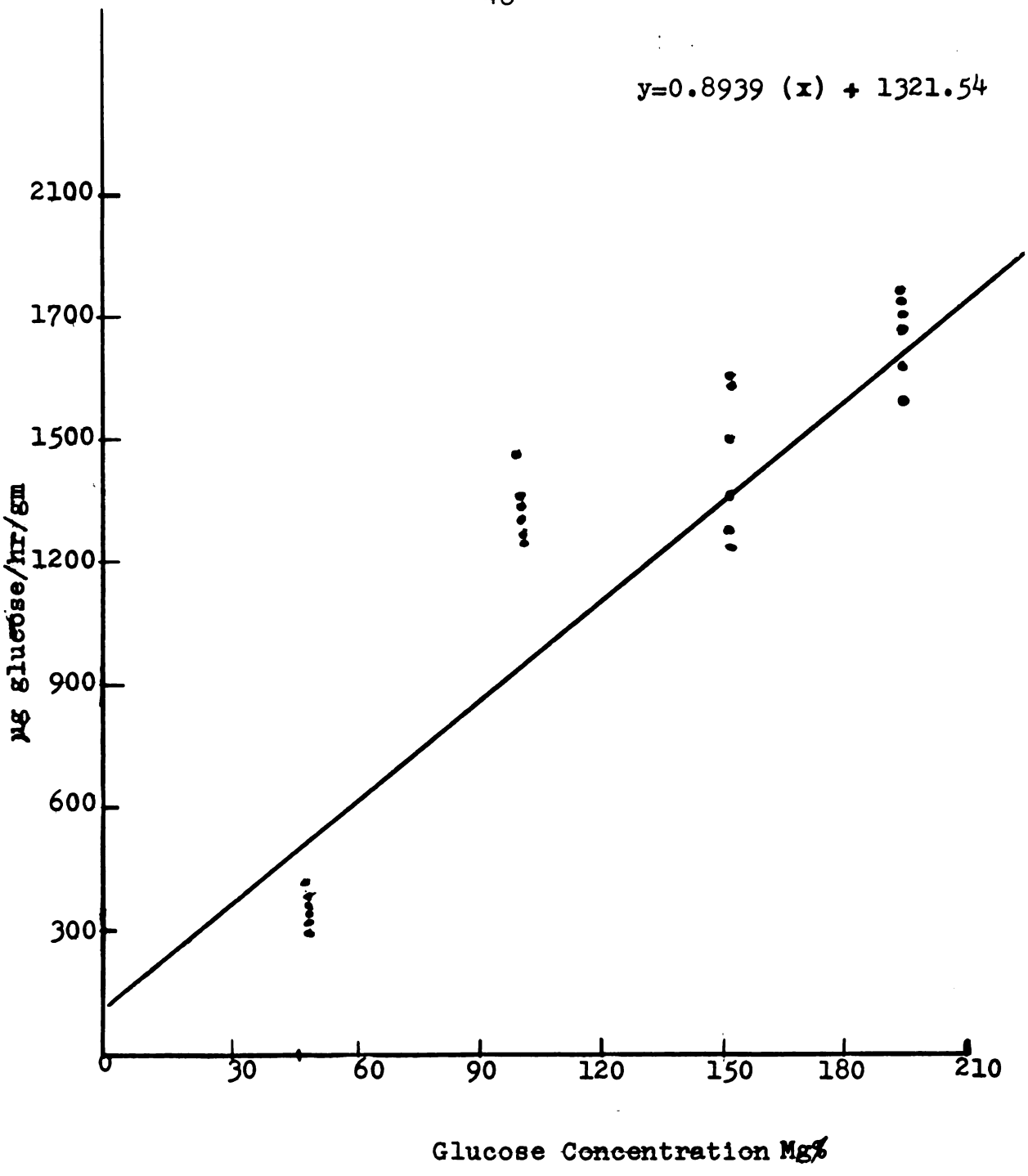

FIGURE 4.-- Regression line showing glucose utilization in a modified Mammalian Krebs Saline Medium ranging in concentration from 50-200 mg% glucose for 1 hour at 13C.

y=0.5383 (x) + 224.783

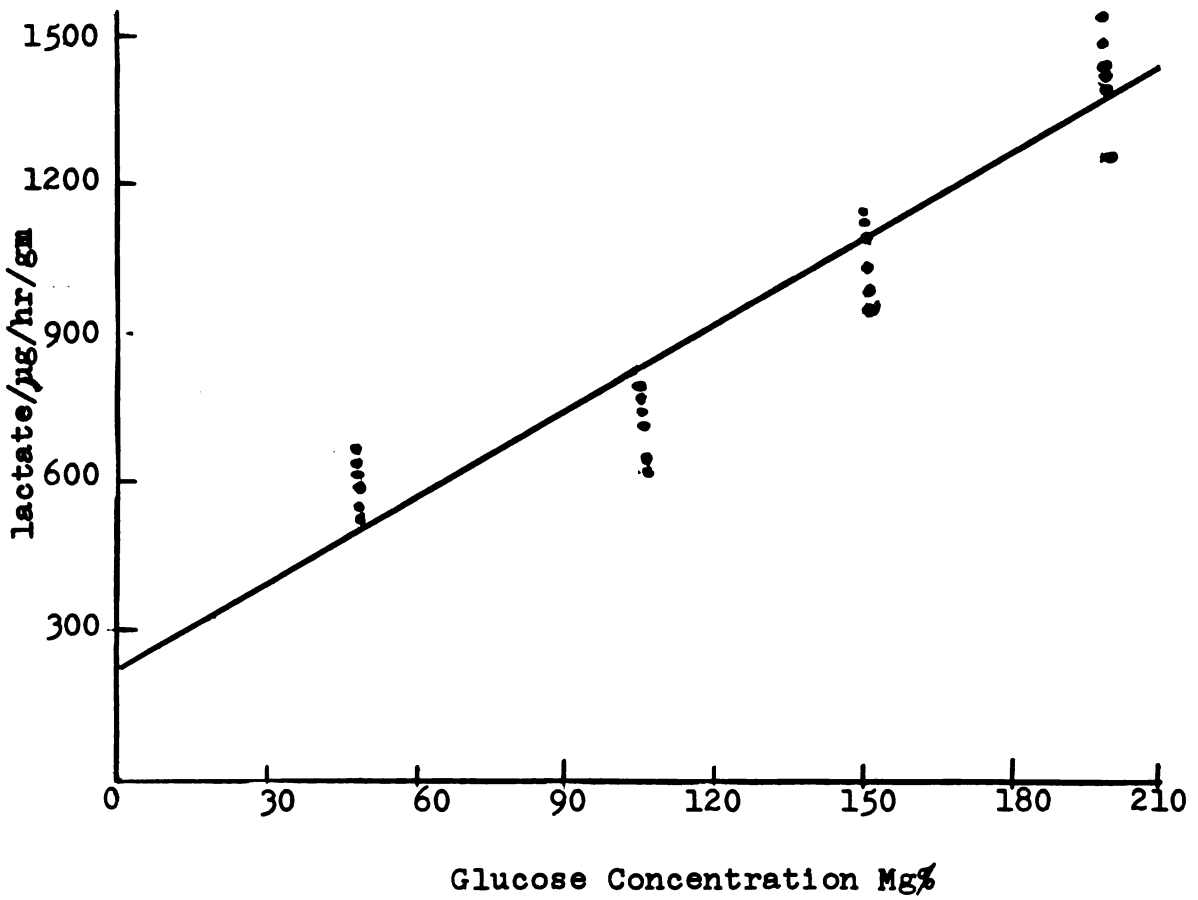

FIGURE 5.--Regression line showing lactic acid production in a modified Mammalian Krebs Saline Medium ranging in concentration from 50-200 mg% glucose for 1 hour at 13C.

TABLE 7.--Effect of varying glucose concentration on retinal metabolism in modified Mammalian Krebs Saline Medium with bicarbonate buffer for 1 hour at 13C

	50mg% Glucose	100mg% Glucose	150mg% Glucose	200mg% Glucose
Glucose utilized µg/hr/gm	340.6 ± 13.1	1395.9 ± 36.7	1448.4 ± 56.6	1813.0 ± 28.9
Lactate produced µg/hr/gm	560.0 ± 09.0	678.6 ± 10.6	1002.7 ± 27.1	1349.1 ± 32.3
$\frac{\text{Glucose}}{\text{Lactate}}$ µg/hr/gm	0.60 ± 0.02	2.05 ± 0.04	1.47 ± 0.03	1.31 ± 0.02

Mean ± S.E. (three observations)

Differences between adjacent values are significant ($p < 0.05$) except values for glucose utilized between 100mg% and 150mg%

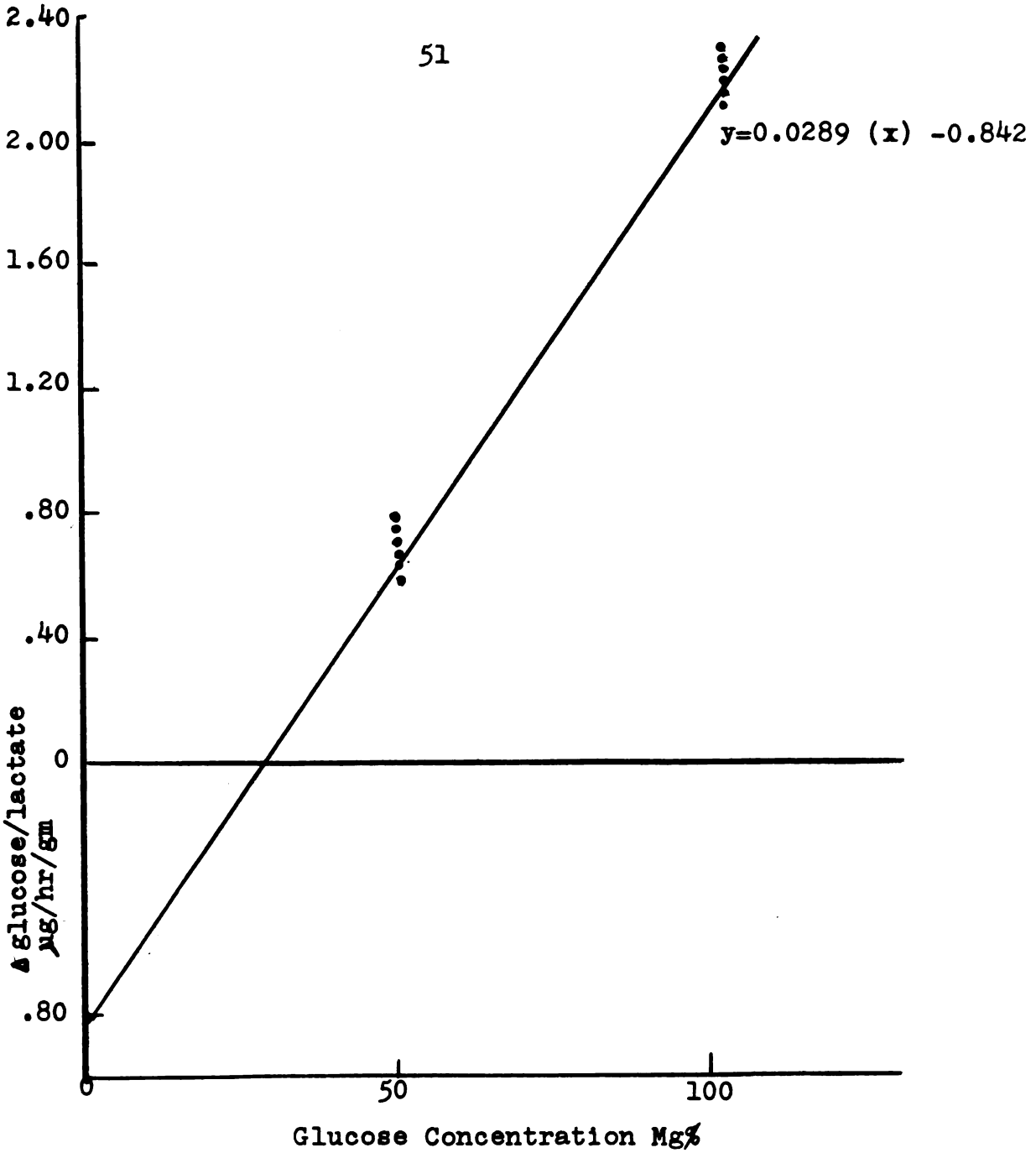

FIGURE 6.--Regression line showing glucose/lactate in a modified Mammalian Krebs Saline Medium ranging in concentration from 50-100 mg% glucose for 1 hour at 13C.

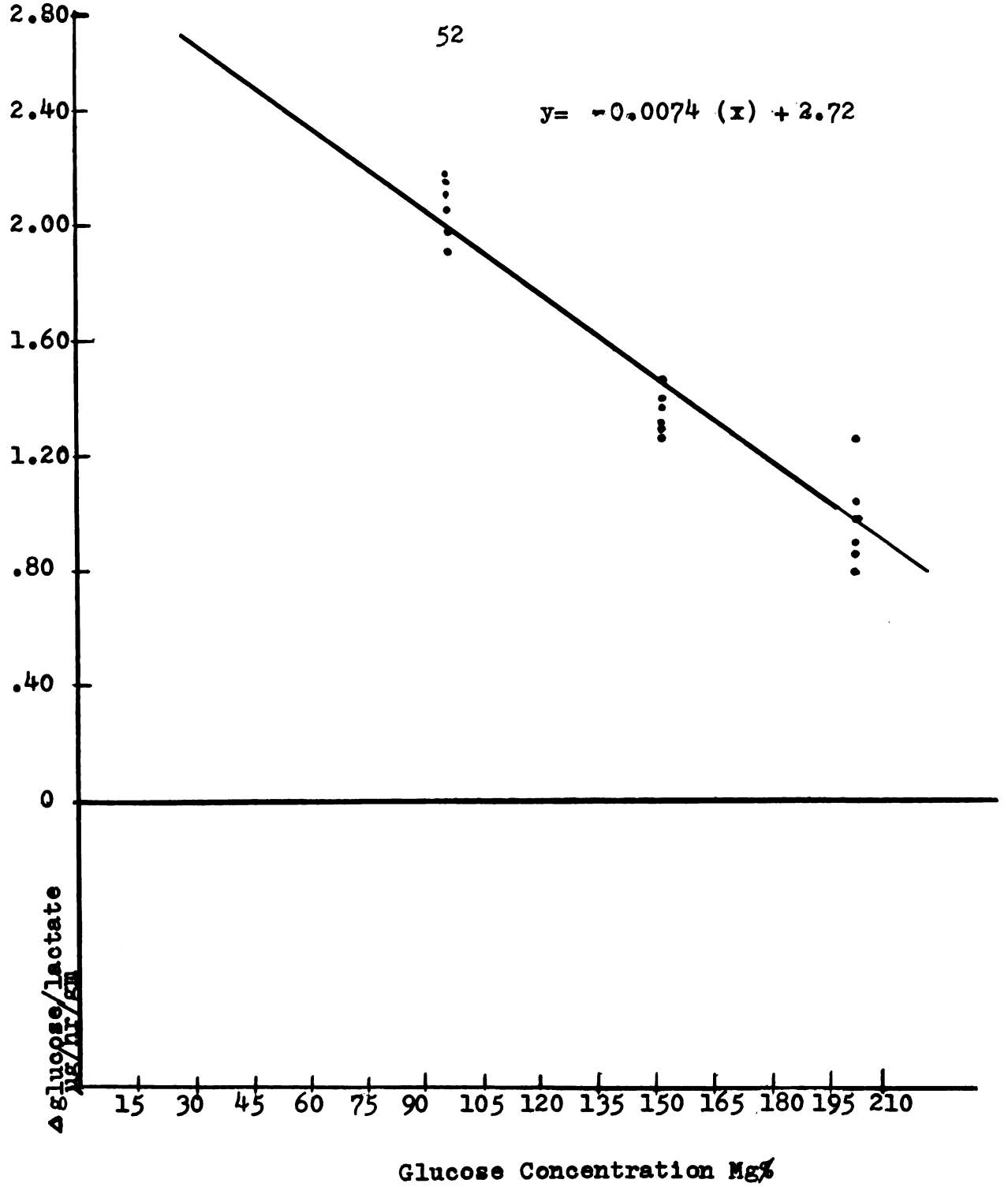

FIGURE 7.--Regression line showing glucose/lactate ratios in a modified Mammalian Krebs Saline Medium ranging in concentration from 100-200 mg% glucose for 1 hour at 13C.

TABLE 8.--Summary of the effects of aerobic and anaerobic conditions and differing glucose concentrations on retinal metabolism at 13C

Media	Time of Incubation and Treatment	Glucose utilized µM/hr/gm	Lactate produced µM/hr/gm	% EMP Pathway Activity	% TCA Cycle Activity
Modified Medium 199	Anaerobic ½ hour	7.30 ±0.37	16.04 ±0.66	109	0
Modified Medium 199	Aerobic ½ hour	4.13 ±0.32	10.19 ±0.93	123	0
Modified Medium 199	Anaerobic 1 hour	3.93 ±0.21	9.52 ±0.50	120	0
Modified Medium 199	Aerobic 1 hour	2.40 ±0.30	6.77 ±0.13	140	0
Modified Medium 199	Anaerobic 3 hours	1.63 ±0.32	3.67 ±0.56	112	0
Modified Medium 199	Aerobic 3 hours	1.36 ±0.15	3.44 ±0.18	126	0
Modified Mammalian Krebs Saline Medium	Aerobic 50mg% Glucose 1 hour	1.89 ±0.07	6.22 ±0.10	164	0
Modified Mammalian Krebs Saline Medium	Aerobic 100mg% Glucose 1 hour	7.55 ±0.20	7.54 ±0.11	49.9	50.1
Modified Mammalian Krebs Saline Medium	Aerobic 150mg% Glucose 1 hour	8.04 ±0.31	11.14 ±0.30	69.2	30.8
Modified Mammalian Krebs Saline Medium	Aerobic 200mg% Glucose 1 hour	10.07 ±0.16	14.99 ±0.35	74.4	25.6

present in the iodoacetate experiments. The accumulation of lactate was very high when 0.001M iodoacetate was utilized (Table 9). As the concentration of iodoacetate was raised to 0.002M the accumulation of lactate in the media was lowered considerably. In the experiments in which there was no glucose, lactic acid or iodoacetate, there was also an accumulation of lactic acid. The pH of the media containing lactate was 7.1 whereas the pH of the media void of lactate was found to be 7.6. During the experiment, with iodoacetate in excess of 0.002M concentrations, the average initial media concentration of 25.3 ± 0.57 (6) mg% showed a significant increase to 28.4 ± 0.48 (8) mg% lactate.

TABLE 9.--Changes in lactic acid concentration in modified Mammalian Krebs Saline Medium fortified with lactic acid in the presence of iodoacetate at 13C for 1 hour

Iodoacetate concentration	N	Initial lactate concentration of media in mg%	Lactate production (µg/hr/gm)
0.000M	6	0	126.26 + 15.63
0.001M	4	25.3 + 0.57	251.75 + 22.8
0.002M	4	25.3 + 0.57	37.17 + 8.08
0.01M	4	25.3 + 0.57	33.17 + 8.2

N (number of observations)

Mean ± S.E.

DISCUSSION

Aerobic glycolysis has been definitely established in the teleost retina as measured in terms of lactic acid production under *in vitro* conditions at a P_{O_2} of 700 mm Hg. It is probable that aerobic glycolysis also occurs *in vivo* at a P_{O_2} in excess of 500 mm Hg, which normally occurs at the teleost retina. The results of the *in vitro* modified Medium 199 experiment show that there is significantly less glucose utilization (41.5%) and lactic acid production (33%) by the teleost retina under aerobic conditions than under anaerobic conditions for periods of 1 hour or less (Table 8). This observation points to the fact that oxygen has an inhibitory effect on retinal metabolism, specifically on glycolysis. It has long been known that oxygen has an inhibitory effect on the metabolism of the mammalian retina. Craig and Beecher (1943) subjected the retinas of white rats to varying oxygen tensions, and found that when the oxygen tension was lowered from 95% to 5% glycolysis was increased to nearly the anaerobic level. The inhibition of retinal metabolism by oxygen is attributed to the phenomenon known as the Pasteur effect.

The Pasteur effect was found to be operational during incubation periods of 1/2 hour and 1 hour. During 3 hour incubation periods there was no significant

inhibition of retinal glycolysis exerted by oxygen. The retarded glycolysis under 1/2 hour and 1 hour incubations can be attributed to a depletion of cytoplasmic inorganic orthophosphate (Pi) and ADP. The increased rate of glucose oxidation by the mitochondrial TCA cycle under aerobic conditions is responsible for depleting the cytoplasm of Pi and ADP.

De Vincentiis (1951) measuring lactic acid production found that glycolysis was inhibited approximately 14% by 100% oxygen in Scorpaena scrofa, a marine teleost. In his experiments the retinas were incubated in vitro but no information is given as to the nature of the incubation medium or the length of the incubation. These results indicate that a Pasteur effect is operative in the retina of the marine teleost.

There are several factors which may explain the decrease in glucose utilization and lactic acid production expressed in µg/hr/gm by the retina as the incubation period is increased. One factor is the decrease in glucose availability with longer incubation periods. The glycolytic rate, and glucose utilization, by the retina may fall as the glucose is depleted from the media. Of the total exogenous glucose amounting to 305 µg the retina consumed 242 µg (79.0%) 224 µg (70.3%) and 193 µg (60.2%) under anaerobic conditions utilizing 3, 1 and 1/2 hour

incubation periods respectively. When glucose was varied from 200 to 50 mg% there was a concurrent decrease in glucose utilization as evidenced in Table 7. Another factor which may slow down glycolysis is the buildup of acid metabolites in the media under longer incubation periods. However, after 3 hour incubations the pH of the modified Medium 199 was 7.0 which is still conducive to the viability of the retinal cells. A third explanation is the possible distruction of cellular integrity. On gross examination, however, the retina appeared to be intact after a 3 hour incubation. Furthermore, modified Medium 199, has been proven to be capable of sustaining teleost ocular tissue for periods in excess of 2 days based on Q_{O_2} measurements.

The EMP pathway was found to be the predominant route for glucose catabolism under both aerobic and anaerobic conditions in modified Medium 199 containing 50 mg% glucose. In Table 8 it is noted that lactate is produced in amounts in excess of that which can be accounted for on the basis of glucose utilization. A plausible explanation for this observation is the presence of endogenous lactate and glycogen. Morath and Hoffert (personal communication) have recently demonstrated with histochemical techniques substantial deposits of highly mobile glycogen in the teleost retina. In addition it is expected

that the retina and attached remnants of the vitreous body contain some endogenous glucose. The total complement of endogenous glucose when added to the incubation media would cause an underestimation of the glucose utilization when the computations were based on the original glucose concentration of the media (610 µg). In addition to underestimating glucose consumption it is possible that lactic acid was overestimated because of the possibility of a small amount of lactate in the retina and attached vitreous. Both of these errors would be additive in overestimating the contribution of the EMP pathway to glucose catabolism. With extended incubation periods the endogenous component will have less influence on the interpretation of the relative rates of the various metabolic pathways. It is known that when 2 moles of lactic acid are produced from 1 mole of glucose that the maximum theoretical activity of the EMP pathway is 100%. It may be possible that the percent of glucose metabolized by the EMP pathway is lower than 100%.

The high EMP pathway percentages in Table 8 are in contrast to the study of Hoffert and Fromm (1970) who found that when the retinas of rainbow and lake trout were incubated in modified Medium 199 with 50 mg% glucose approximately 37% of the glucose was combusted by the TCA cycle and 3.9% of the glucose was metabolized by the HMP

shunt. The apparent conflict in the preferred metabolic pathways in the two aerobic studies may be explained by differences in the experimental procedures and animals. First, Hoffert and Fromm used much smaller trout (27-40g) than were used in this experiment (380-480g). It has been repeatedly shown in the literature that the preferred pathways of carbohydrate metabolism by the retina will vary with age (Cohen and Noell, 1960). The age difference between the two groups of fish is estimated to be 2 1/2 years, with many of the larger trout used in the present study having already reached sexual maturity. Secondly, 1 ml of modified Medium 199 was employed by Hoffert and Fromm with fish having smaller retinas, whereas in this experiment only 1/2 ml of modified Medium 199 was used with much larger retinas. Hoffert and Fromm found significan TCA cycle activity with 1 ml of media whereas in the present work no TCA cycle activity was observed with 1/2 ml of media. Finally, Hoffert and Fromm utilized an incubation period of 24 hours whereas in the present experiment the longest incubation time employed was 3 hours. Perhaps when longer incubation periods are used the glucose is utilized to below some critical level which may be needed for glycolysis and therefore the remaining glucose is oxidized to provide maximum amounts of energy.

Theoretically one would anticipate some TCA cycle activity under aerobic conditions based upon the abundance

of literature on the Q_{O_2} measurements of the vertebrate retina, and Hoffert and Fromm's measurements of glucose to lactate ratios, and C_1/C_6 CO_2 production from labled glucose in the teleost retina. The data as presented for the aerobic measurements in Table 8 for TCA activity is probably an underestimation due to an overestimation of EMP pathway activity for the reasons previously outlined.

The data indicated in Tables 7 and 8 and Figure 7 shows that a Crabtree effect is operative in the teleost retina. When a media consisting of a 150 mg% glucose concentration was used the Crabtree effect became apparent as evidenced by a significant fall in the oxidation of glucose by the TCA cycle. These results seem to confirm the hypothesis set forth by Gatt et al. (1956) for the mechanism of the Crabtree effect. These workers speculated that the limited amounts of ADP are shared by the cytoplasm and the mitochondria, and shuttle back and forth between them. An active glycolytic scheme in the cytoplasm may deprive the mitochondrial TCA cycle of essential ADP. When a 200 mg% glucose concentration was used in the media there was a further reduction in glucose utilization by the TCA cycle, indicating again the operation of the Crabtree effect.

It should be emphasized that there was an increase in lactic acid production with each higher level of glucose

used in the incubation media. This indicates that the glycolytic scheme is operational at the highest glucose concentration utilized. The stimulation of glycolysis would correspond with a drop in intracellular ADP. An ADP decrease in the mitochondria would in turn have an inhibitory effect on the TCA cycle and would explain the Crabtree effect.

As the glucose concentration of the media is increased there is a concurrent increase in TCA cycle activity up to a maximum level at 100 mg% glucose. At a 50 mg% concentration of glucose in the incubation media approximately 1 molecule of glucose is utilized for every 2 molecules of lactic acid produced. This indicates that under these particular conditions glycolysis is operating almost exclusively as a mechanism for the combustion of glucose. When a 100 mg% glucose concentration was incorporated into the media there was a drastic change in the pattern of glucose oxidation. For each glucose molecule metabolized by the glycolytic scheme approximately 4 molecules of glucose are oxidized by the TCA cycle. At a 100 mg% concentration of glucose in the media it appears that the glycolytic machinery in the cytoplasm, although not operating at full capacity, cannot handle the great excess of glucose and therefore a much greater portion is oxidized by the mitochondrial TCA cycle.

The physiological significance of the Crabtree effect is dubious. Normally over the physiological levels of blood glucose one would not encounter a Crabtree effect. It is therefore questionable if the Crabtree effect would ever be observed *in vivo*. It is known that the retina has a fairly constant metabolic rate. Changes in blood glucose would be buffered by the endogenous glycogen present; consequently, the retinal cells can be assured of a constant glucose concentration in their immediate environment, irrespective of blood glucose changes. The Crabtree effect has previously been limited in occurrence to mammalian retinal tissue and leucocytes *in vitro*, but has now been substantiated in the teleost retina.

It was observed that a slightly greater amount of glucose was utilized by retinas incubated in a modified Medium 199 (2.40 μM/hr/gm) than in those incubated in a modified Mammalian Krebs Saline Medium (1.89 μM/hr/gm) for periods of one hour under aerobic conditions (Table 8). Both media utilized a $NaHCO_3$ buffer and contained 50 mg% glucose. After exposing the modified Medium 199 to a gas having a concentration of 95% O_2-5% CO_2 at 13C it was found that the pH equaled 7.0, whereas the pH of the modified Mammalian Krebs Saline Medium was equal to 7.6 after an exposure to 99.5% O_2-0.5% CO_2 with the addition of $NaHCO_3$ at 13C. The 7.6 pH of the modified Mammalian

Krebs Saline Medium approximates the pH of the blood of the rainbow trout, and therefore would seem to be the more physiologically suitable to incubate the retinas in. There have been no values reported for the pH in the posterior chamber of the eye in the vicinity of the retina, however, one might expect the pH to be lower than that of the blood due to the accumulations of lactic acid as a product of anaerobic glycolysis. The slightly higher glucose utilization in the modified Medium 199 may be due then to it's having a physiologically similar pH to that encountered by the retina in vivo.

P_{CO_2} levels of the incubation medium have been shown to have an effect on tissue respiration. Normally aquatic animals have very low arterial P_{CO_2} levels approaching 0.5 vol.%. The reason for this is the high solubility of P_{CO_2} in H_2O. Therefore it was assumed that a P_{CO_2} level of 0.5 vol.% would most closely approximate in vivo conditions. In further studies it would be recommended to adjust the media to a pH of 7.0 in the presence of 0.5 vol.% CO_2 (13C) through the addition of appropriate organic buffers. Avoidance of phosphate buffers would be advantageous because of their known inhibition of retinal metabolism.

The results of the iodoacetate experiments indicate, but do not prove conclusively, that lactic acid is

not utilized as a substrate for metabolism by the teleost retina. At the 0.001 M concentration of iodoacetate employed in the first experiments the high accumulation of lactic acid is due to glycolysis. The 0.001 M iodoacetate is not concentrated enough to stop the utilization of exogenous substrates by glycolysis resulting in the accumulation of lactic acid. When the concentration of iodoacetate was raised to 0.002 M a significant fall in lactic acid accumulation was observed. The fall in lactic acid accumulation shows that the metabolic inhibitor is effective at the 0.002 M concentration. Iodoacetate is known to inhibit glyceraldehyde-P-dehydrogenase resulting in a blockage of the glycolytic scheme.

There are several explanations available for the accumulation of lactic acid in the above experiments. First, there is the possibility of a concentration of endogenous lactic acid in excess of that present in the preincubation media. Secondly, the inhibition of glyceraldehyde-P-dehydrogenase may not be complete. Thirdly, significant pools of intermediate metabolites may exist below glyceraldehyde-P-dehydrogenase which could contribute to the lactic acid production if the necessary coenzymes were present. Finally, the previously mentioned pools may be enriched by intermediates from the HMP shunt which may contribute to the additional lactic acid

accumulation (Figure 1). The summation of all the above sources of lactic acid in the presence of iodoacetate, in amounts in excess of 0.002 M, represents a very minor accumulation of lactic acid. In the presence of a small amount of lactic acid production even moderate amounts of lactic acid utilization would be detected. Under the experimental conditions one would expect maximal lactic acid utilization if the retina is capable of such. It is concluded that the teleost retina is not capable of utilizing lactic acid for the production of significant amounts of biological energy. Futterman and Kinoshita (1959) found contrary evidence suggesting that the mammalian retina can utilize lactic acid as a substrate.

The addition of lactic acid to the modified Mammalian Krebs Saline Medium resulted in a drop of the pH from 7.6 to 7.1. Concurrently the rate of lactic acid production (Table 9) increased. Evidence indicates the pH in the environment of the retina may be substantially lower than that of the plasma (pH 7.6). By lowering the pH through the addition of lactic acid an environment more closely simulating the <u>in vivo</u> state was created, thereby explaining the increased rate of glycolysis.

It was observed by Kornblueth et al. (1953) that glucose utilization of the mammalian retina is dependent on the integrity of the cells. In Kornblueth's study

lactic acid production was not determined. When the retinas of the rainbow trout were sonified, resulting in the rupture of cellular membranes, the glucose utilized over a five hour period was only about 1/9 that of an intact retina (Table 6). Furthermore it was observed that the lactate production of a sonified retina is essentially zero. In some of the experiments retinas were torn into two or three pieces and it was found that the metabolism did not vary from that of intact retinas. These results lead to the conclusion that not only glucose utilization but also lactate production and therefore glycolysis is dependent on cellular integrity. Sonification apparently disrupts glucose catabolism at some intermediate step prior to the formation of lactate, with no mitochondrial activity expected under anaerobic conditions in this experiment.

It has been substantiated that aerobic glycolysis occurs in the teleost retina _in vitro_. Due to the high P_{O_2} generated in the chorid region of the eye it seems likely that aerobic glycolysis also occurs _in vivo_. The importance of aerobic glycolysis to the retina lies in the fact that lactic acid is produced as the predominant end product. It has been proven _in vitro_ that lactic acid is not utilizable as a substrate for retinal metabolism, therefore the lactic acid produced from glycolysis would accumulate in the vicinity of the retina. The presence of this

acid metabolite would lower the pH of the blood in the rete mirabile which in turn would cause a shift of the oxygen dissociation curve to the left resulting in a release of oxygen from hemoglobin. These facts explain the high P_{O_2} encountered in the teleost retina. The high P_{O_2} is essential to provide a diffusion gradient so that the innermost region of the retina has an adequate supply of oxygen. The high P_{O_2} is also important because it provides the needed oxygen for TCA cycle activity which occurs in the retina *in vivo*. A disadvantage of aerobic glycolysis to the retina is that it provides very little usable energy as compared to the great amount produced by TCA cycle activity.

It has been shown that a high P_{O_2} inhibits glycolysis (Pasteur effect) in the teleost retina. The inhibition of glycolysis means less lactic acid formation and accumulation. With less lactic acid present and a resultant higher pH the oxygen dissociation curve will not shift to the left and the hemoglobin of the blood perfusing the retina will retain its oxygen. The retention of oxygen by hemoglobin in turn would mean less oxygen would be released from the blood into the posterior of the eye, resulting in a lower P_{O_2}. Glycolysis would be stimulated by the lower P_{O_2} and lactic acid would therefore accumulate at a faster rate. The extra lactic acid would act on

hemoglobin causing the release of oxygen and a higher P_{O_2} in the eye. The high P_{O_2} generated would cause the Pasteur effect to become operational and the cycle would repeat itself. Thus, it appears that the Pasteur effect is a necessary component of a negative feedback control loop which controls the oxygen tensions around the retina and the rate of glycolysis taking place in the retina.

SUMMARY AND CONCLUSIONS

1. Contrary to current views aerobic glycolysis, as shown by the production of lactic acid, was found to occur in the teleost retina.
2. An inhibition of glycolysis, as measured by glucose utilization and lactic acid production, by oxygen (Pasteur effect) was observed in the teleost retina.
3. There was more glucose utilized (41.5%) and lactic acid produced (33%) under anaerobic conditions than under aerobic conditions.
4. The TCA cycle of the teleost retina was inhibited by levels of glucose in excess of 150 mg% in the incubation media. This so-called Crabtree effect appears to be of little physiological significance.
5. Integrity of the retinal cells is a necessary prerequisite for glycolysis.
6. The teleost retina is not capable of utilizing lactic acid in the presence of iodoacetate for the production of significant amounts of biological energy.
7. The effects of aerobic glycolysis and the inability of the retina to utilize lactic acid were discussed in reference to the oxygen dissociation curve.

8. A negative feedback control loop was postulated to control the P_{O_2} setpoint in the teleost eye. It was further postulated that the Pasteur effect plays an important role as a component in the negative feedback control loop.

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APPENDICES

APPENDIX 1

Incubation Solutions

Composition of Sterile Ringers Solution*

NaCl	7.37 gm/liter
KCl	0.31 gm/liter
$CaCl_2$	0.10 gm/liter
$MgSO_4$	0.14 gm/liter
KH_2PO_4	0.46 gm/liter
$NaHPO_4$	2.02 gm/liter
Osmolarity	298 mOsm/kg

*(Ringer's Injection U.S.P. # 4134 Abbott Laboratories, North Chicago, Ill.)

Composition of Phosphate Buffered Saline (PBS)**

NaCl	8.00 gm/liter
KCl	0.20 gm/liter
Na_2HPO_4 (Anhydrous)	1.15 gm/liter
$KH_2PO_4H_2O$	0.20 gm/liter
$CaCl_2$	0.10 gm/liter
$MgCl_2.6H_2O$	0.19 gm/liter
Glucose	1.0 gm/liter

**(Grand Island Biological Co. Grand Island, N. Y.)

Composition of Modified Medium 199*

Earles Base 199	Standard Formula
$NaHCO_3$	1.25 gm/liter
Glucose	0.5 gm/liter
Phenol Red	not present
Oxygen	95 vol.%
Carbon Dioxide	5 vol.%
pH	7.0
Osmolarity	289 mOsm/kg

*(Grand Island Biological Co., Grand Island, N.Y.)

Composition of Modified Mammalian Krebs Saline Medium

NaCl	6.9 gm/liter
KCl	0.345 gm/liter
$CaCl_2$	0.282 gm/liter
$NaHCO_3$	2.94 gm/liter
$MgSO_4 \cdot 7H_2O$	0.294 gm/liter
KH_2PO_4	0.162 gm/liter
Oxygen	99.5 vol.%
Carbon Dioxide	0.5 vol.%
pH	7.60
Osmolarity	289.25 mOsm/kg

APPENDIX 2
Regression Analyses

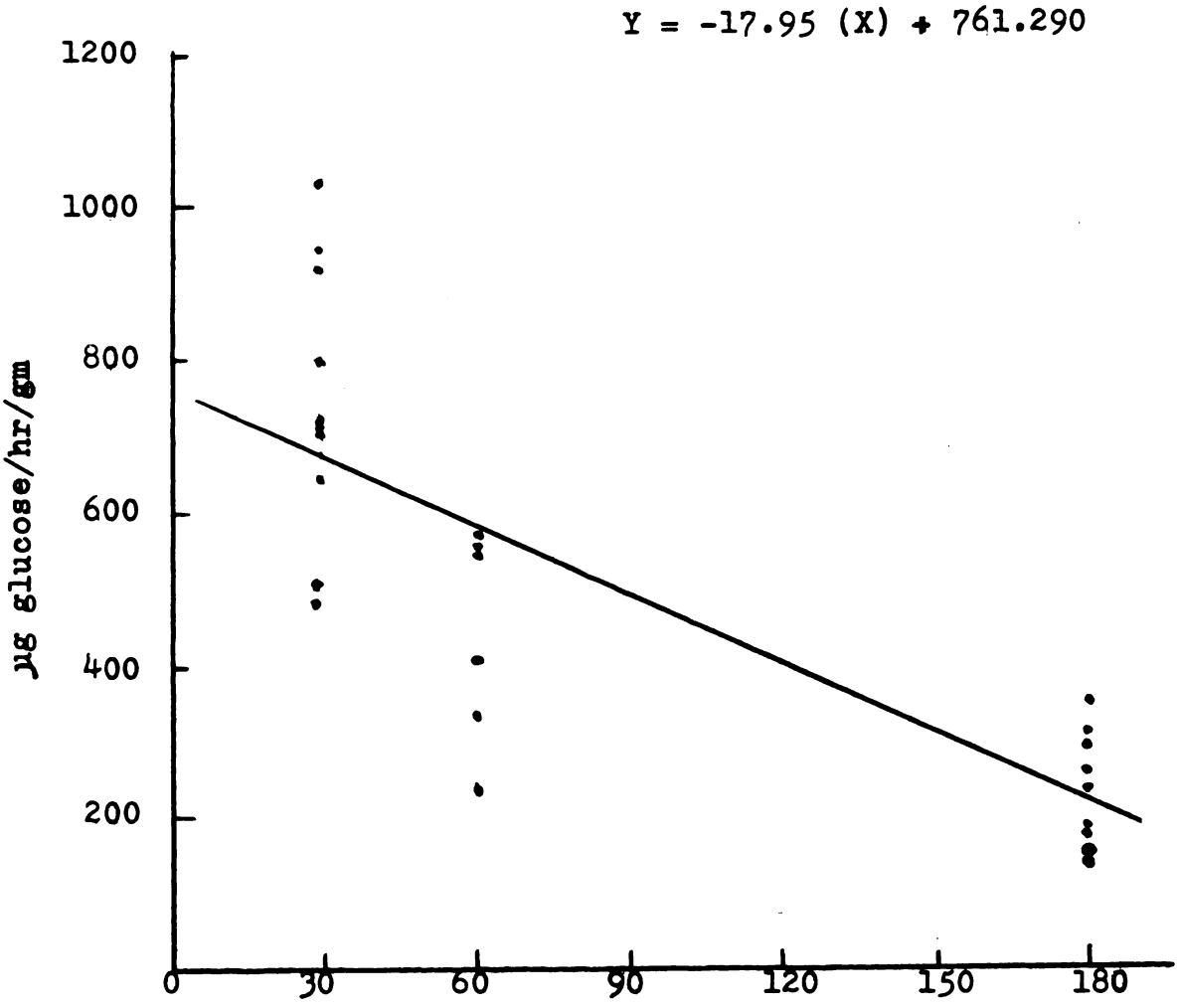

FIGURE 8.--Aerobic glucose utilization (95% O_2 - 5% CO_2) by retinal tissue at 13°C.

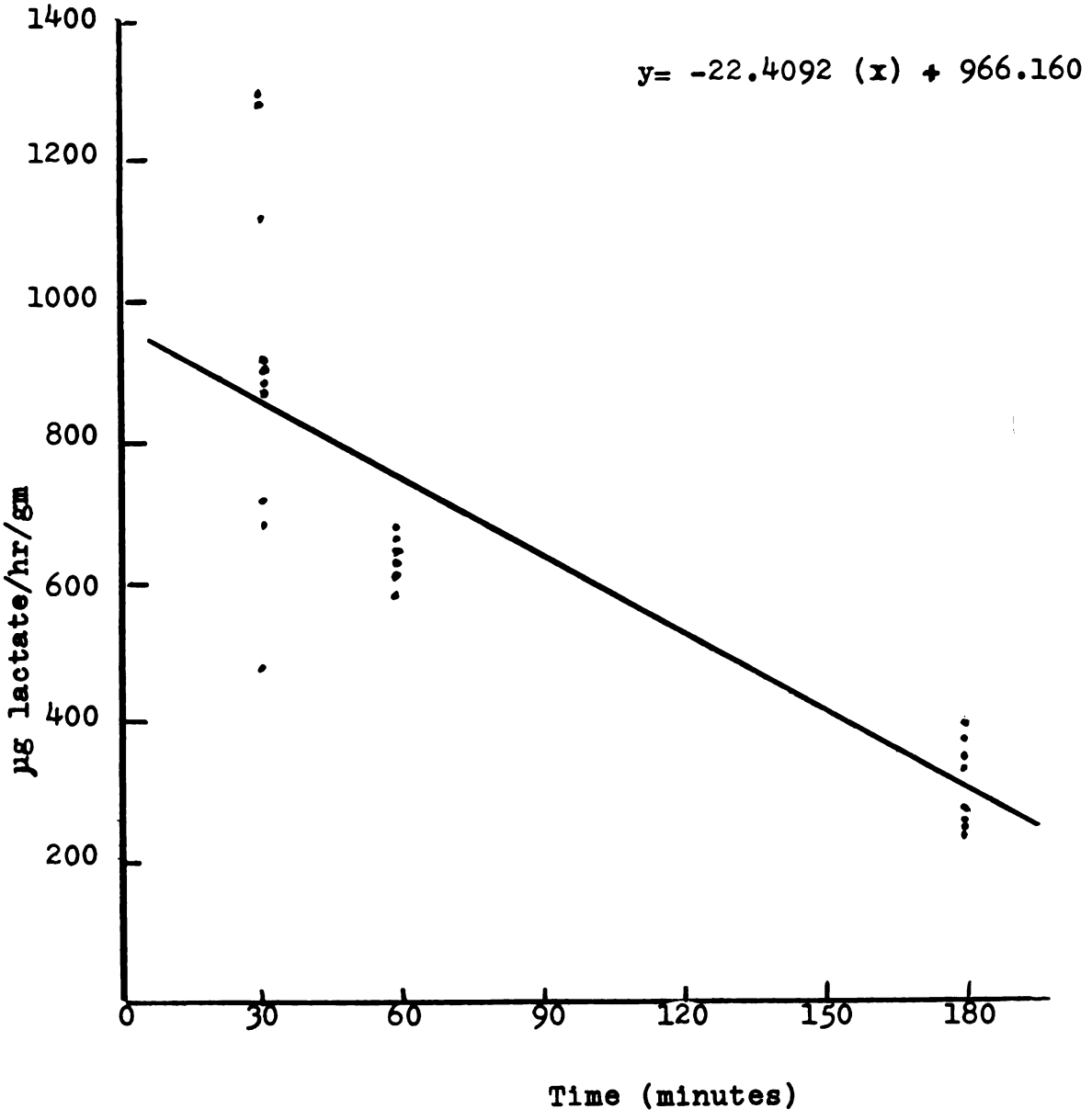

FIGURE 9.--Aerobic lactate production (95% O_2-5% CO_2) by retinal tissue at 13C.

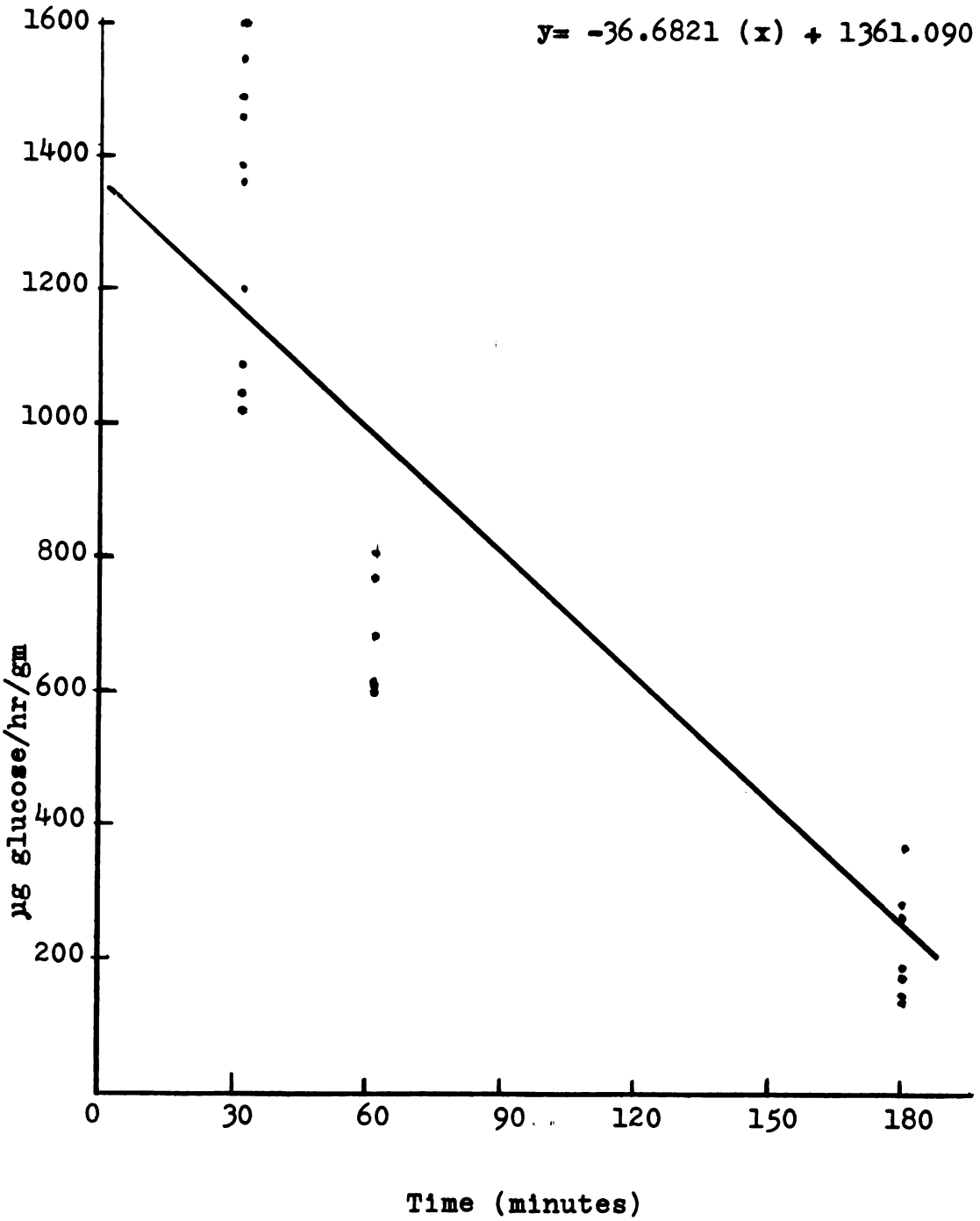

FIGURE 10.--Anaerobic glucose utilization (95% N_2-5% CO_2) by retinal tissue at 13C.

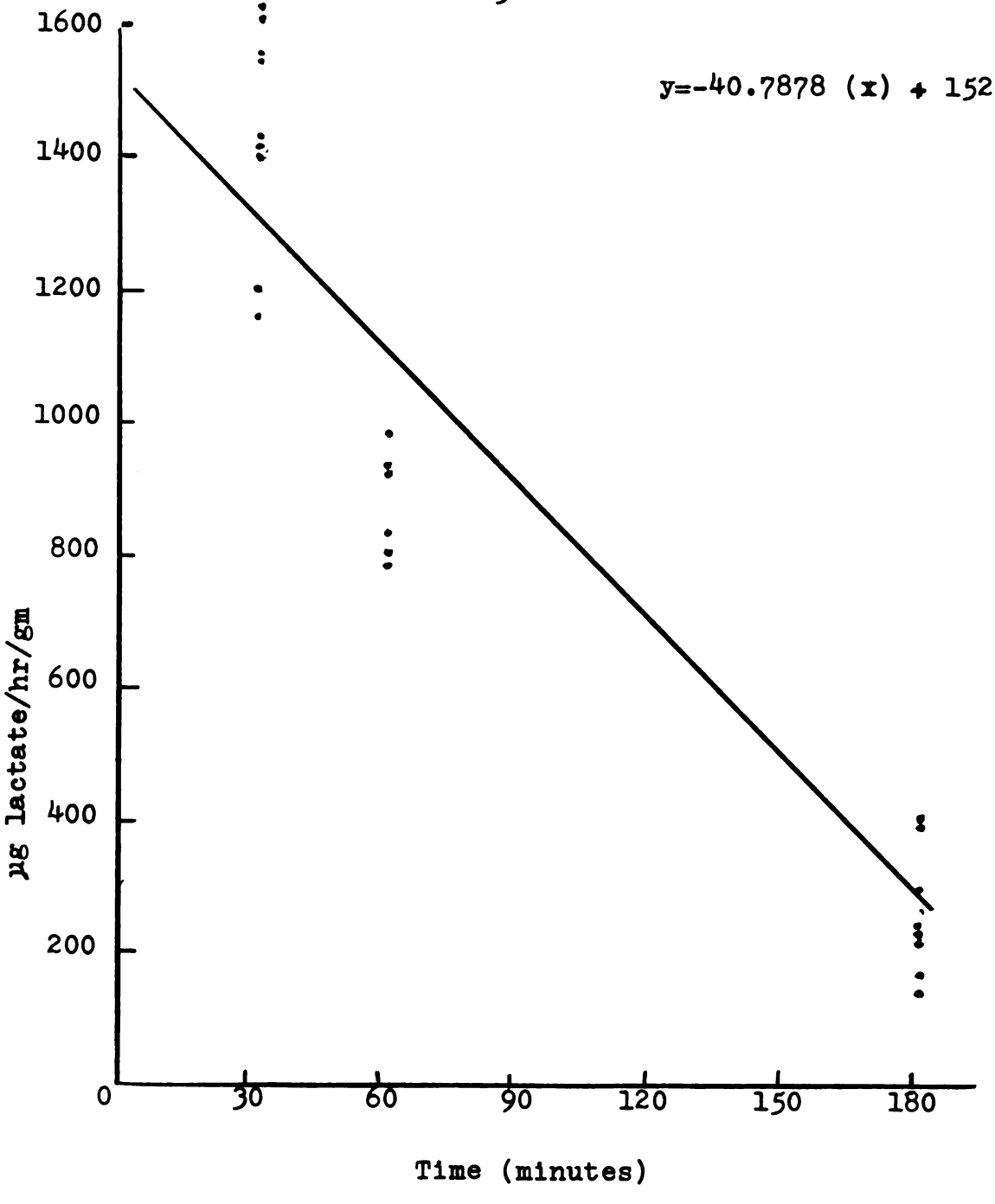

FIGURE 11.--Anaerobic lactate production by retinal tissue at 13C.