# GLUCOSE METABOLISM AND MAINTENANCE OF TRANSPARENCY IN OCULAR TISSUE OF RAINBOW TROUT

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This is to certify that the

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Maintenance of Transparency in
Ocular Tissue of Rainbow Trout

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Paul O. Fromm
Major professor

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ABSTRACT

GLUCOSE METABOLISM AND MAINTENANCE OF TRANSPARENCY IN OCULAR TISSUE OF RAINBOW TROUT

By

Lance E. Olson

Glucose metabolism in the rainbow trout lens and cornea was investigated by the amounts of $C^{14}O_2$ produced during incubation of tissues with 2-C-$^{14}$-pyruvate, glucose-1-$C^{14}$ or glucose-6-$C^{14}$ as the labeled precursors. Tissues were incubated in phosphate buffered saline containing a labeled precursor for 4 hours at 13 C, and the $C^{14}O_2$ yield was determined by counting in a liquid scintillation system. Tissue radioactivity was measured after solubilization in Hyamine hydroxide for 18 hr at 60 C.

Glucose appears to enter the trout lens and cornea by passive diffusion which is a different mechanism than is postulated for similar mammalian tissues. Neither lesioning the lens capsule nor use of specific inhibitors of the citric acid cycle (TCA) on the lens or cornea changes the rate of entry into the tissues. The entrance of pyruvate into the tissue can be decreased when the tissues were subjected to NaCN.

Lance E. Olson

In the tissue glucose is oxidized to $CO_2$ by means of glycolysis and the TCA cycle in the lens and cornea but more $CO_2$ is produced in the cornea.

The hexosemonophosphate (HMP) shunt also oxidized glucose to $CO_2$ but accounted for only a fraction of the total $CO_2$ production by the lens or cornea.

Glucose was oxidized primarily through energy yielding reactions of the TCA cycle at the environmental temperature of rainbow trout (13 C). As the temperature rises, the HMP shunt responds in both types of tissues with a greater increase in activity than the TCA cycle. Viability of the tissue was judged by the maintenance of transparency, and this increase in activity of the HMP shunt lasted to 23 C in the cornea but persisted to 33 C in the lens, the respective temperatures where the tissues become opaque.

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By

Lance E. Olson

A THESIS

Submitted to
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Dedicated to my wife, Jan, for her
understanding, tolerance, and sacrifice to
make this project possible.

## ACKNOWLEDGMENTS

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The author is also indebted to the National Institutes of Health for the support of this work through grant PO. 04125 from the National Institute of Neurological Disease and Blindness.

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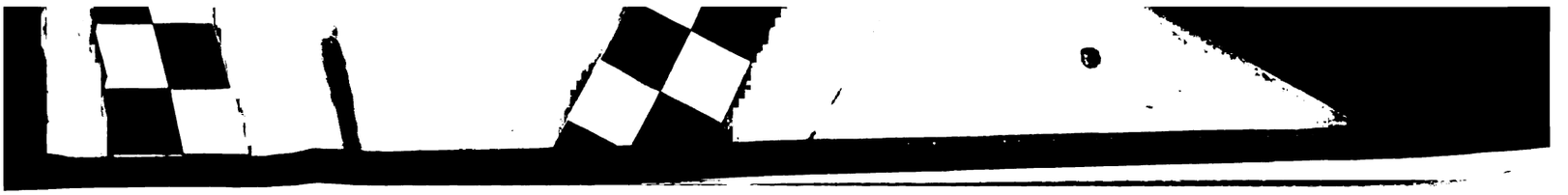

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# INTRODUCTION

Hatchery reared lake trout are known to develop corneal and lenticular lesions during and after the second year of life which makes them useless for stocking lakes (Allison, 1963; Hoffert and Fromm, 1965). A similar condition is known to exist in hatchery reared rainbow trout. These lesions, which usually involve keratoconus, corneal opacity, and cataracts, closely resemble pathological conditions of mammalian species.

The low oxygen demands of poikilothermic ocular tissues make them well suited for in-vitro studies. In addition, the animals are readily available, and offer a large ocular tissue weight to body weight ratio which makes tissues from even small fish acceptable for experimental purposes.

The lens and cornea are avascular, therefore their metabolic needs must be satisfied from the aqueous and vitreous environment. Thus, information derived from experiments using these tissues in-vitro in media similar to their natural environment probably reflects the activity which occurs under in-vivo conditions. Through similar reasoning, one would also expect their metabolic activity to be low.



A comparison of the oxygen consumption and glucose utilization of mammalian ocular tissues supports this conclusion. However, there must be a minimum amount of energy needed to maintain viability. Certainly, the primary function of the crystalline lens and cornea is to retain their transparency. Although we do not have a complete understanding, it appears that the maintenance of lens and cornea transparencies depends on the utilization of biological energy.

The free fatty acid content of the lens is extremely low similar to that of the cornea (Krause, 1935). The aqueous, which serves as a nutrient media for the two ocular tissues, is also low in free fatty acids. Thus, unlike many other tissues, the lens and cornea are unable to derive little, if any, energy from the oxidation of free fatty acids, hence, the importance of glucose as the major substrate for the production of energy in the lens and cornea.

Carbohydrate metabolism in these tissues is restricted by the nature of their environment. The aerobic phase of glucose metabolism would not be expected to play an important role in tissues which are avascular and exist in an environment of low oxygen content. Even if oxygen were readily available, these tissues must remain colorless as well as transparent; therefore, they

cannot contain large amounts of pigmented oxidative enzymes of the cytochrome system or those of the riboflavin containing group.

This study is an endeavor to provide a knowledge of normal metabolic function which is the basis for pathological investigation by answering the following questions:

1. Is transparency a function of metabolic energy?
2. Is the tricarboxylic acid cycle (TCA) functioning in the lens and cornea of rainbow trout?
3. Does the hexosemonophosphate (HMP) shunt operate in the lens and cornea as an alternate pathway of energy production?
4. If present, what are the activities of the respective pathways in the catabolism of glucose for the production of energy?
5. What effect does temperature change have on the activities of the various pathways?
6. What is the mechanism of entry for glucose and pyruvate into the lens and cornea?

# LITERATURE REVIEW

## General Metabolism

Glucose is metabolized anaerobically in the mammalian lens and cornea via two main pathways: anaerobic glycolysis (Embden-Meyerhof pathway) and the hexosemonophosphate shunt (HMP). The Embden-Meyerhof glycolytic pathway is familiar to most people and will not be described in detail. Glucose-6-phosphate (G-6-P) undergoes a series of steps to form pyruvic acid. Pyruvic acid in the presence of an active citric acid cycle (TCA) is completely oxidized to $CO_2$ and water (Figure 1), but where the oxygen tension is low, or an inactive TCA cycle exists, it is reduced almost exclusively to lactic acid by means of an active lactic dehydrogenase and DPNH formed at an earlier stage (Langham, 1954). Fonner, Hoffert and Fromm (1969) have found that ocular tissues of rainbow trout contain an active lactic dehydrogenase.

As the name indicates, anaerobic glycolysis can and does operate in the absence of molecular oxygen. The HMP shunt actually involves a dehydrogenation of G-6-P and oxygen is not directly utilized in the reaction.

Figure 1.--Changes in carbon atom di
during metabolism of glucose.[a]

G6P - Glucose-6-phosphate

Xu5P - Xylulose-5-phosphate

R5P - Ribose-5-phosphate

S7P - Sedoheptulose-7-phosphate

GAP - Glyceraldehyde-3-phosphate

E4P - Erythrose-4-phosphate

H6P - Hexose-6-phosphate

[a]S. Hollman. 1964. Non-glycolytic
metabolism of glucose (Academic Press, New

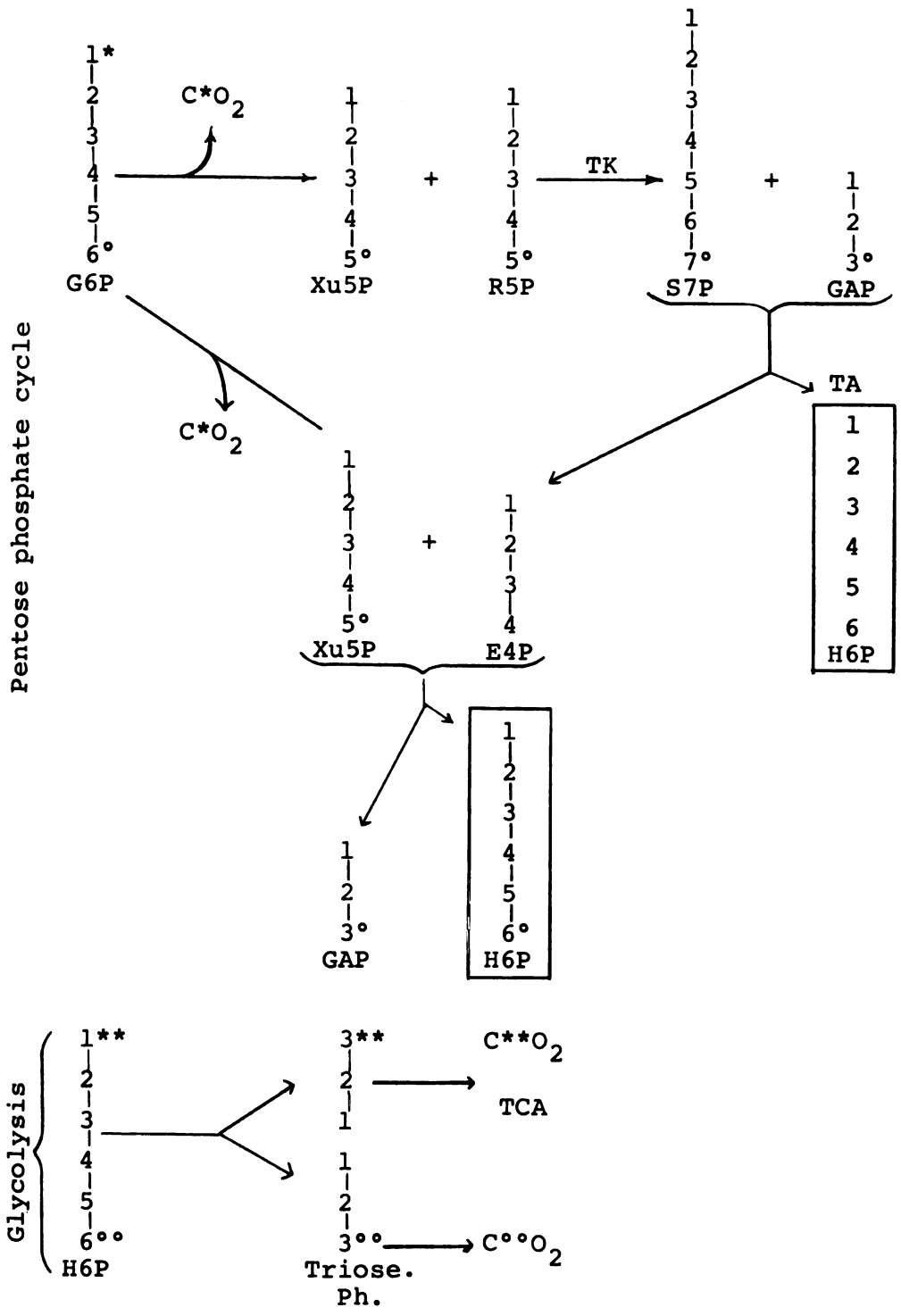
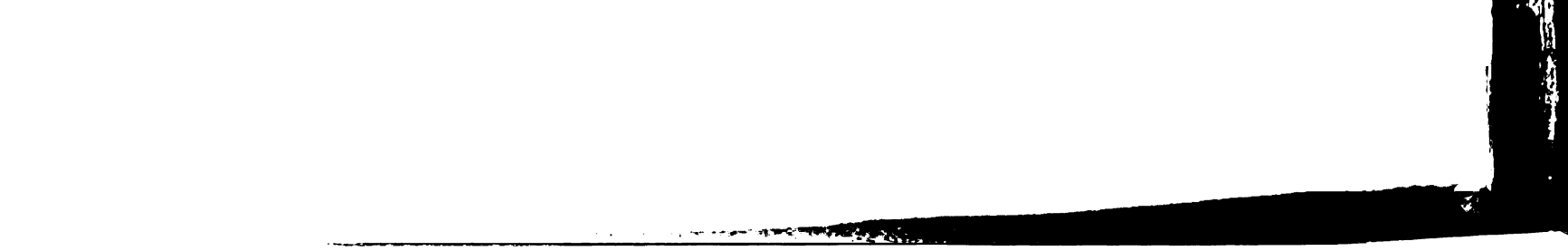

Pentose phosphate cycle
G6P
C*O2
Xu5P
R5P
TK
S7P
GAP
TA
H6P
C*O2
Xu5P
E4P
H6P
GAP
Glycolysis
H6P
C**O2
TCA
C°°O2
Triose.
Ph.

Figure 1.





However, the eventual oxidation of the reduced TPNH (which carries the hydrogen removed from G-6-P) may require oxygen directly or indirectly.

The first step in the HMP shunt, the phosphorylation of glucose to G-6-P, is common also to anaerobic glycolysis. This compound is then oxidized in two stages with the concomitant reduction of the coenzyme triphosphopyridine nucleotide (TPN) occurring during both reactions. The first reaction is a simple oxidation of G-6-P to 6-phosphogluconic acid. Oxidative decarboxylation then takes place at carbon 1 of 6-phosphogluconic acid to yield ribulose-5-phosphate and to liberate $CO_2$ (Figure 1). The ribulose-5-phosphate goes through a series of intermediate steps eventually becoming D-glyceraldehyde-3-phosphate or fructose-6-phosphate. These compounds are an integral part of the Embden-Meyerhof pathway and offer a reentry to it from the HMP shunt. The stoichiometric analysis of the shunt may be illustrated in the following equation:

$$6G\text{-}6\text{-}P + 12TPN \rightarrow 5G\text{-}6\text{-}P + Pi + 6CO_2 + 12TPNH + 12H^+$$

Thus, the shunt yields $CO_2$ and an abundance of TPNH as its primary products.

While the reoxidation of DPNH is directly associated with the generation of ATP by the mitochondria, there is as yet no known mechanism whereby TPNH can be

directly oxidized via a similar cytochrome system with the concurrent production of ATP.

## Lens

Interest of early investigators was centered on the optical properties of the lens and perhaps the first important work on its integrity was that of Wagemann (1891). After ligation of the posterior ciliary arteries he observed permanent histological changes of the lens and concluded that the integrity of the lens depended on a continuous supply of oxygen, of nutrients, or both.

Since the lens is avascular, all its needs must be supplied by the aqueous and the vitreous body. The $P_{O_2}$ of the aqueous in mammalian species is considerably lower than that in the blood (Heald and Langham, 1956). This limits the availability of oxygen to the mammalian lens and other tissues supplied by the aqueous. But, Fairbanks (1969) recently has found that the rainbow trout eye concentrates $O_2$ to a level of 445 mm Hg $P_{O_2}$ measured behind the retina. A gradient was present and showed a $P_{O_2}$ of 103 mm Hg in the vitreous body.

Sippel (1962) confirmed Kleifeld and Hockwin's (1959) statement that oxygen uptake by the lens is directly dependent on $P_{O_2}$ in the media, and extended it to say that lens respiration increases abruptly to plateau at a level of 140-230 mm Hg $P_{O_2}$ in dry gas, then rises

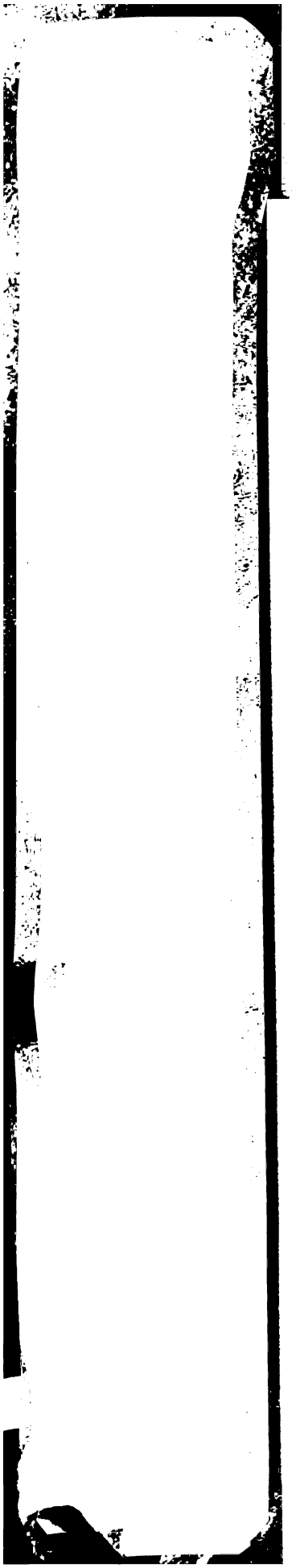


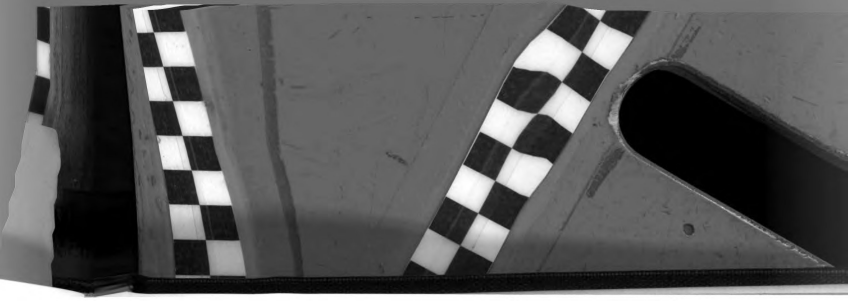
continuously through to 730 mm Hg $P_{O_2}$. He suggests that this biphasic curve may indicate that two oxidation reactions may be involved. Kronfeld and Bothman (1928), Fisher (1931), Michail and Vancea (1932) all observed that anaerobic as well as aerobic glycolysis occurs in the mammalian lens to supply the needed energy.

The use of various known metabolic inhibitors expanded the information on oxygen consumption of the lens.

Data of Field et al (1937) suggest that 2,4,-dinitrophenol may be the etiologic agent in the development of cataracts in a small percentage of cases after its therapeutic administration. Ely and Robbie (1950) exposed the lens to various levels of cyanide in the incubation media. A concentration of $10^{-4}$M HCN inhibited oxygen uptake by 50%, but about 9% of the respiration of the rabbit lens is resistant to a level of $10^{-2}$M HCN. They believe that one mole of cyanide inhibits one mole of the active heavy metal containing enzyme of the crystalline lens.

Attention was shifted by the investigators from the measurements of gross metabolic activity to experiments designed to elucidate and assay intermediary metabolites of different metabolic pathways used to produce energy. Glucose, the major substrate for the







production of energy, must first pass through the lens capsule and then encounter the second biological barrier consisting of the cell membrane. Studies by Harris, Hanschild and Nordquist (1955) on accumulation of glucose within, and utilization of glucose by rabbit lenses treated with various enzyme inhibitors indicate that glucose does not enter the lens solely by simple diffusion but also by some active process. They think that one site (or barrier) at which this process occurs lies at or in close proximity to the lens capsule and there may be more than one barrier. Experimenters have also attempted, without success, to establish the role of insulin in the transport of glucose in the lens. Levari, Wertheimer and Kornbluth (1964) investigated the effects of various concentrations of glucose in the media on metabolism. Incubation of intact rat lenses in increasingly higher concentrations of glucose resulted in a proportional increase in liberation of $C^{14}O_2$. Lactic acid production was stimulated as glucose concentration increased up to 1.0 mg/ml but higher concentrations had little affect.

Once inside the cell, glucose is phosphorylated by the hexokinase reaction which involves the utilization of ATP. The product, G-6-P, can then directly enter the HMP shunt with the resultant removal of the first carbon atom and the formation of a five carbon residue, or it

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can undergo transformation to its isomer, fructose-6-phosphate, and continue in the glycolytic pathway.

In 1955 Green, Bocher and Leopold published the first of several articles describing the fate of glucose during anaerobic metabolism of the crystalline lens. They concluded from their experiments that the necessary intermediates for the formation of lactic acid from glucose were present in the lens, and that one of the rate limiting steps for the pathway was the limited amount of hexokinase enzyme present in the tissue.

Recognizing the fact that the lens has a low oxygen consumption does not allow one to make any a priori assumptions as to the utilization of glucose by the lens. According to Ely (1951), lactic acid, pyruvic acid, and various intermediates of the citric acid cycle were oxidized when used as substrates for bovine lens homogenates. However, the addition of cytochrome C was essential for maximal aerobic oxidation. This is the first evidence pointing to the low activity of the citric acid cycle in the lens. Harris, Hanschild and Nordquist (1954) proved that oxidation is essential for the maintenance of viability of the lens. This information led Kinoshita (1955) to investigate the effectiveness of the citric acid cycle in aerobic metabolism. He speculated that if the function of the TCA cycle is to completely oxidize pyruvate to $CO_2$, an indication of the activity of

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this cycle might be gained by studying how effectively the lens would oxidize 2-$C^{14}$-pyruvate to $C^{14}O_2$. Using 2-$C^{14}$-pyruvate, the radioactive carbon is incorporated in the middle of this three carbon compound and thus the yield of $C^{14}O_2$ is taken as an index of the complete oxidation of the pyruvate. He found that bovine lenses were unable to utilize 2-$C^{14}$-pyruvate to any appreciable extent during four hours of incubation and concluded that the citric acid cycle is relatively inactive in the lens.

In the second part of the experiment Kinoshita applied the fact that the metabolic fate of the carbon-1 and carbon-6 atoms of glucose differs depending on how glucose is metabolized (Figure 1). If glucose were metabolized via the glycolytic and citric acid pathways exclusively, the carbon atoms 1 and 6 of glucose become the methyl carbon of pyruvate and subsequent oxidation by the citric acid cycle would result in C-1 and C-6 of glucose appearing as $CO_2$ simultaneously. If glucose were metabolized via the HMP shunt, the C-1 atom of glucose would appear as $CO_2$ much earlier than C-6, since there is preferential cleavage of the C-1 of glucose. To test this approach, the lens was incubated with either glucose-1-$C^{14}$ (G-1-$C^{14}$) or glucose-6-$C^{14}$ (G-6-$C^{14}$) and the amount of $C^{14}O_2$ produced was compared. Results from three different mammalian species, cat, rabbit, and bovine established that a G-1-$C^{14}$/G-6-$C^{14}$ ratio of $C^{14}O_2$ recovered

was approximated 40:1. Thus, the lens exhibits a preferential oxidation of the C-1 of glucose to $CO_2$, which strongly suggests the participation of the HMP shunt.

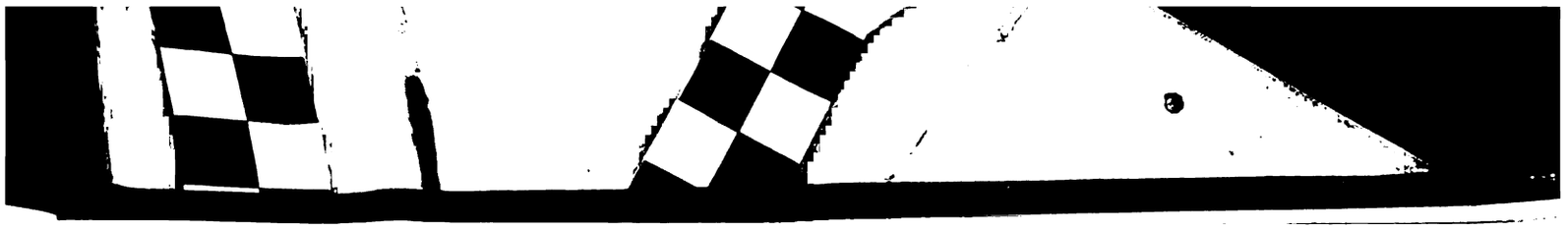
A more quantitative estimate may be made by measuring the radioactivity incorporated in lactic acid when labeled C-1 or C-6 glucose is used as the substrate. By the HMP shunt, the carbon-1 atom of glucose is directly converted to $CO_2$. Therefore, it seems reasonable to assume that the activity recovered in the lactate from G-1-$C^{14}$ is derived solely from glycolysis. Conversely, the radioactivity recovered in lactate from G-6-$C^{14}$ is that contributed from both the direct oxidative and glycolytic pathways. The ratio of $C^{14}$-lactate from G-1-$C^{14}$/G-6-$C^{14}$ is the approximate fraction of glucose metabolized via glycolysis. Kinoshita's (1955) value for the bovine lens was 0.79. This means that for every four molecules of glucose utilized by glycolysis, one molecule of glucose is metabolized via the HMP shunt.

Kinoshita and Wachtl repeated the experiment in 1958. This time one lens per flask was incubated for twenty-four hours. The results were similar. Labeled $CO_2$ was formed at a rate 44 times faster using G-1-$C^{14}$ than when G-6-$C^{14}$ was used. The radioactive lactic acid data demonstrates that 14 percent of the glucose was oxidized by the direct oxidative pathway.



The use of differentially labeled radioactive glucose, as mentioned above, provided a needed tool in the research of carbohydrate metabolism. Lerman's (1961) interest was in the formation of metabolic pathways during development and the relation of the age of the animal to carbohydrate metabolism. The C-1/C-6 ratio of $C^{14}O_2$ from the lens in his experiments showed marked change correlated with age as did the TPNH/TPN ratios. The DPNH/DPN ratio was similar in both young and mature rat lens. The conclusion was that the HMP shunt diminished considerably in activity in the lens as the animal ages. Later, Lerman, Donk and Pitel (1962) established with fetal rat lenses that aerobic glycolysis and HMP shunt of glucose oxidation are both functioning on the 17th day of gestation, but the TCA cycle is not operative until 20-21 days. A steady increase in amount of lactic acid per lens from day 17-21 indicates that glycolysis is functioning.

What happens to the metabolic scheme of carbohydrates in a pathological tissue? Lerman (1959) experimentally induced cataracts by two different methods. He fed rats a high proportion of galactose in the diet, and in 1962 he produced cataracts following an alloxan caused diabetic condition. The radioactive $CO_2$ data indicates that the oxidation of G-6-$C^{14}$ occurs at about normal rate or may increase slightly. Carbon dioxide





from G-1-$C^{14}$ is less than normal after four days of either treatment and remains low.

In 1965 Van Heyningen reported the separation of ten substantially labeled compounds of various metabolic pathways from protein free extracts of incubated lenses. Her technique involved the use of electrophoresis, paper chromatography, and radioautography. In the absence of oxygen the radioactivity was prevalent in lactic acid, alpha-glycerophosphate, and glycerol. Aerobic conditions caused the radioactivity of the lens proteins to be 2-4 times greater than in the absence of oxygen. Finally, the radioactivity of all labeled compounds was predominant in the outer part (cortex).

In the mammalian lens the biological energy necessary for the maintenance of transparency and repair is supplied primarily by the reactions that break down glucose to lactic acid. To enter the lens from the aqueous humor, glucose must first cross a barrier, believed to be located in the area of the capsule or epithelium, at the expense of energy. Once inside the low levels of enzymes associated with the TCA which aerobically oxidizes glucose restricts the lens metabolism mainly to anaerobic glycolysis, the low $P_{O_2}$ in aqueous may also restrict the TCA cycle. The HMP shunt is functional and although it produces most of the $CO_2$ liberated by the mammalian lens, only a minor part of the total glucose

used is metabolized by this pathway. Lactic acid which is produced in the lens freely diffuses into the aqueous where it is present in higher concentrations than the blood.

The trout lens differs from mammalian lenses by existing in an environment with an oxygen tension which is greater than that of the blood instead of low oxygen tension (compared to blood) found in mammalian eyes (Fairbanks, 1968).

## Cornea

### Nutrition

The basic metabolites of the cornea, glucose and oxygen are of great interest. Equally important is the removal of breakdown products of metabolism, particularly carbon dioxide and lactic acid. These substances can move into and out of the cornea across three surfaces: the anterior and posterior surfaces of the cornea, and the corneal scleral junction called the limbus.

How much of the needed materials are supplied by way of the limbus is a moot point. According to the kinetics for diffusion of sodium, at a point 6 mm from the limbus, 99% of that entering the cornea from the blood will be lost to the aqueous humor. The kinetic conditions for the movement of glucose and oxygen are

less favorable than for sodium. These calculations are made without regard to the amount that will be metabolized along the way. It is unlikely that any of the nourishment from the limbal blood vessels ever reaches the center of the cornea.

The rear surface of the mammalian cornea is bathed by the continuously circulating aqueous. The anterior surface is covered by a thin film of tears which separates it from the air. The environment of the anterior surface of the teleost cornea is the water in which it lives. Whereas the mammalian cornea may be supplied to some extent by its tears, the environment is unlikely to contribute much to the metabolic needs of the teleost cornea. In fact, the hypotonic environment of freshwater species places a great osmotic burden on the cornea.

Measurements on the excised rabbit cornea hint that the primary resistance to the diffusion of oxygen is within the stroma (Heald and Langham, 1956). From this it has been calculated that the oxygen diffusing into the cornea from the aqueous humor is inadequate to supply the respiratory requirements of the epithelial layer. This leaves only the anterior supply route to meet the oxygen needs of the epithelium. For mammals, this seems reasonable partly because of the large pressure

gradient of oxygen between the surrounding air and the cornea, and partly because of the ease of diffusion of oxygen through the tear layer.

Noting that water has no glucose, and regarding the limbal supply as insignificant, the sole source of glucose to the teleost cornea is the aqueous humor. The concentration of glucose in the rabbit aqueous is approximately 100 mg% (Giardini and Roberts, 1950). This is not greatly different from that in the tissue fluid of the stroma. Consequently, there must be some mechanism other than simple diffusion across the endothelium to satisfy the cornea. The small amount of glucose contributed by tears is inadequate, so the aqueous appears responsible as the major supply route to the epithelium of the mammalian cornea.

Carbon dioxide and lactic acid are the main metabolic end products in the cornea and both appear to be eliminated by simple passive diffusion. Carbon dioxide, like oxygen, is fat soluble in its unionized form and consequently it penetrates cellular membranes readily. Because most of the carbon dioxide is formed in the epithelium, it is likely that the anterior surface of the cornea would be the primary escape route (Redslob and Trembley, 1933).

A lactic acid concentration gradient (of about 25 mg%) exists favoring the stroma of the cornea over the



aqueous humor. According to Langham (1954), this gradient is sufficiently large to permit the unmetabolized lactic acid to leave the cornea by passive diffusion across the endothelium.

Metabolism

Respiration of the cornea, like that of the lens, has been measured numerous times under various conditions. The results agree well. The oxygen uptake by the cornea does not seem to vary when subjected to different conditions. Langham's (1954) results agreed even when incubation proceeded in the absence of a liquid incubation medium. Figures for the respiratory quotient seem to point toward carbohydrate utilization exclusively, until challenged recently by Haberich and Dennhardt (1965). They reported an R.Q. of 0.8 which would indicate some utilization of protein and possibly some fat as a source of energy.

Langham (1952) denuded the cornea and measured the respiration of its parts. Respiration for the epithelium and perhaps the endothelium is comparable to that of some of the more active tissues of the body. The stroma in the rabbit and in the ox can be considered relatively inert. Pontocaine, chlorbutanol, cyanide, iodoacetate, pentobarbital, atropine, and cocaine in varying degrees of effectiveness all inhibit the oxygen

uptake of the bovine cornea (Herrmann, Moses, Friedenwald, 1942). Kinoshita (1959) later established that pontocaine blocked the citric acid cycle and HMP shunt, but did not affect anaerobic glycolysis.

Two pathways are established for metabolism of glucose in the cornea: anaerobic glycolysis supported to some extent by the TCA cycle, and the HMP shunt.

The aerobic pathway of glucose metabolism in the cornea has been investigated by Kinoshita and Masurat (1959). From the relative rates of oxidation of the three carbon atoms of pyruvic acid, it appears that the TCA cycle is functioning in the cornea. However, its role is limited, evidenced by the large accumulation of lactate in-vivo and by the relatively small amount of pyruvate oxidized by this tissue. Lactic acid that is not oxidized diffuses out of the tissue.

Anaerobic production of lactic acid in rabbit and bovine corneas with a sufficient amount of other necessary nutrients available is about four times greater than the aerobic production (Herrmann and Hickman, 1948c; Langham, 1954). The major portion occurs in the cellular epithelium and the endothelium. The stroma has a low rate of production. The denuded cornea retains about one-third of its ability to oxidize lactate but loses 90% of its capacity to oxidize glucose when incubated with these substrates in radioactive form (Kuhlman and Resnik, 1959).



In 1964 Kinoshita and Masurat produced evidence for the HMP shunt in bovine corneal epithelium. Measurements of the activity of glucose-6-phosphate and 6-phosphogluconate dehydrogenases, the formation of pentose phosphate and carbon dioxide from phosphogluconate, and the heptose formation from pentose phosphate convlusively established the pathway. Kinoshita, Masurat and Helfant (1955) quantitatively estimated the contribution of the HMP shunt to the total oxidation of glucose. They reported that 65% of glucose was metabolized via the conventional glycolytic scheme, and 35% by the shunt. Four years later, Kuhlman and Resnik (1959) indicated that even more, up to 70% of the oxidation of glucose by the cornea proceeds by the HMP shunt. They also added a large amount of unlabeled lactate, anticipating that the specific activity of the intermediates beyond this step would be reduced. The time interval was brief enough to prohibit the system from attaining an equilibrium. The radiochemical yield of $C^{14}O_2$ from G-6-$C^{14}$ decreased 86% and that from G-1-$C^{14}$ only fell 30%. This indicates that the predominate pathway of $CO_2$ production from carbon one of glucose does not pass in equilibrium with the lactate pool.

How are the various pathways related and what is the mechanism of control designating the method of glucose oxidation to be used? The participation of the



direct oxidative pathway in the utilization of pyruvate was demonstrated by studying the effect of it on the anaerobic oxidation of G-1-$C^{14}$ by corneal epithelium (Kinoshita, 1957). Addition of unlabeled pyruvate to the incubation media stimulated the oxidation of G-1-$C^{14}$ to an eight fold increase in the production of $C^{14}O_2$ over that produced from media lacking additional pyruvate. Increase in the 2-$C^{14}$-glucose oxidation to $C^{14}O_2$, but not that of G-6-$C^{14}$ added additional support for the involvement of the HMP shunt in the utilization of pyruvate. There seems to be an interrelation between the conversion of pyruvate to lactate and the TPN-linked dehydrogenases of the direct oxidative pathway seems like the probable explanation for the stimulation of the C-1 oxidation of glucose. Lactic acid dehydrogenase of the corneal epithelium seems capable of functioning to some extent with TPN; however, DPN is the preferred coenzyme (Kinoshita, 1957).

The results indicate that C-1 oxidation of glucose to carbon dioxide under anaerobic conditions in the presence of pyruvate is greater than that under aerobic conditions without added pyruvate. It therefore appears that in the corneal epithelium, the rate of re-oxidation of TPNH is the rate limiting step in the HMP shunt.

Maintenance of constant water content in lens and cornea is an energy consuming process dependent on metabolic activity. Since a shift in water content of the tissue is an important process in corneal pathology, a decrease in metabolic production of energy is indicated (Schwartz, Danes and Leinfelder, 1954). The type of process for maintenance of hydration or the site of action is unclear; however, it may be associated in some way with the maintenance of transparency. Smelser (1961) thinks that control of corneal hydration in fish is different from mammals. The elasmobranch cornea is not hydrophilic and does not swell in any aqueous media. The degree of swelling is greater in fish adapted to the sea and less in carp which live in a hypotonic medium. Since these poikilothermic tissues do not show a temperature reversal effect, perhaps there is no metabolic control. He offers the explanation that the swelling pressure is balanced by a high colloid osmotic pressure consisting largely of mucopolysaccharides.

Antimycin A was introduced by Strong (1958) as a potential systemic antifungal agent. However, when it was found to inhibit cytochrome C of the electron transport chain its systematic use was limited, and it is now marketed as Fintrol (Ayerst, New York) for use by fish conservationists as a fish eradication agent in lakes

before restocking. Rodonski and Wendt (1966) and Foye (1969) noted that one of the effects of the compound on many of the dying fish was the presence of cloudy eyes.

In summary, the cornea also appears to utilize glucose exclusively which enters primarily from the aqueous through the posterior surface of the tissue. Carbon dioxide and lactic acid, both metabolic end products, appear to be eliminated by simple passive diffusion. Once within the cells, glucose is phosphorylated by the hexokinase enzyme then can enter either the glycolytic or HMP shunt pathways, which are both functional in mammalian corneas. The TCA cycle has a limited ability to oxidize the products of glycolysis, lactate and pyruvate, to $CO_2$. The lactate that is not oxidized diffuses out of the tissue. Anaerobic production of lactic acid in mammalian corneas is about four times greater than the aerobic production of lactate.

The HMP shunt is active and may account for up to 70% of the metabolism of glucose by the mammalian cornea. The rate limiting step of the HMP shunt seems to be the rate of reoxidation of TPNH which is a primary product of the pathway. Lactic dehydrogenase of corneal epithelium seems capable of functioning to some extent with TPNH; however, DPNH is the preferred coenzyme.

# MATERIALS AND METHODS

## Experimental Animals

The rainbow trout (Salmo gairdneri) used in these experiments were obtained from the Michigan Department of Natural Resources at Grayling, Michigan. Trout approximately two years old, weighing between 100-250 g, were selected. The fish were transported from the hatchery to the East Lansing campus in a galvanized metal tank lined with non-toxic paint. The tank was housed in a polystyrene-lined plywood box to maintain constant water temperature, and was fitted with an agitator for aeration.

The laboratory holding facilities consisted of fiberglass-lined plywood tanks supplied with a continuous flow of dechlorinated water at one end with an overflow spout at the other end. The photoperiod was 15 hours light and 9 hours darkness each day and the water temperature was 13 C. Constant aeration was provided by activated charcoal-filtered air lines.

## Removal of Tissues

Fish were killed by a sharp blow to the top of the head. With the fish on its side, a small incision

was made in the ventral limbal area of the cornea with one blade of an iris scissors. Rat tooth forceps were used to hold the cornea fixed at the initial incision, while the cornea was cut around the entire periphery at the limbus anterior to the iris. The excised cornea was placed in iced cold blooded Ringers solution (Appendix 1). Small curved forceps were then placed under the lens which was gently lifted out of the eye and also placed in the iced Ringer bath.

The tissues were carefully washed in the iced solution, blotted dry on No. 1 Whatman filter paper, weighed on a Roller-Smith precision balance to the nearest 0.1 mg, and placed in the appropriate media. Suspensory ligaments attached to lenticular tissue were removed with a scalpel during the blotting process. Damage to the lens capsule was infrequent but visibly manifest. If damage occurred, all tissues from that fish were discarded. The entire blotting and weighing procedure deprived the tissues of media for less than one minute. Surgical instruments, syringes, needles, center wells, and media were all sterile.

Weights of the tissues ranged from 80-120 mg for the lenses and 40-60 mg for the corneas, but in any one experiment, the variation in weight between contralateral lenses or between corneas was 2.0 and 4.0 mg respectively.

## Isotope Studies

### Apparatus

Liquid scintillation counting vials of 20 ml capacity were used as the closed incubation system. The apparatus was modified from the design of Hostetler et al (1966) to include a No. 2 black rubber stopper inserted in the neck of the vial to complete the enclosure. The rubber stopper also served as support for the center well. A local glassblower constructed the center well from pyrex glass to have a volume of approximately 2.0 ml. An 18-gauge needle 2 inches long and fitted with a B-D (No. MS09) spring loaded stopcock (Beckton, Dickinson and Co., Rutherford, N.J.) was permanently inserted through the stopper to provide an entrance to the center well for the addition of acid (Figure 2).

The tissue and media were contained within the center well suspended from the stopper. This allowed the $CO_2$ absorber to remain in the bottom of the counting vial after removal of the center well. The possibility of error encountered during transfer of the $CO_2$ absorber in other procedures was eliminated.

### General Procedure

In all experiments chemically defined Phosphate-Buffered-Saline (PBS) obtained from the Grand Island

Figure 2.--Closed incubation system for collection of $C^{14}O_2$.

1. Liquid scintillation vial (20 ml).
2. Glass center well for tissue and media.
3. Black rubber stopper (No. 2).
4. Syringe needle (18 gauge, 2.5" long).
5. B-D spring loaded stopcock (No. MS09).

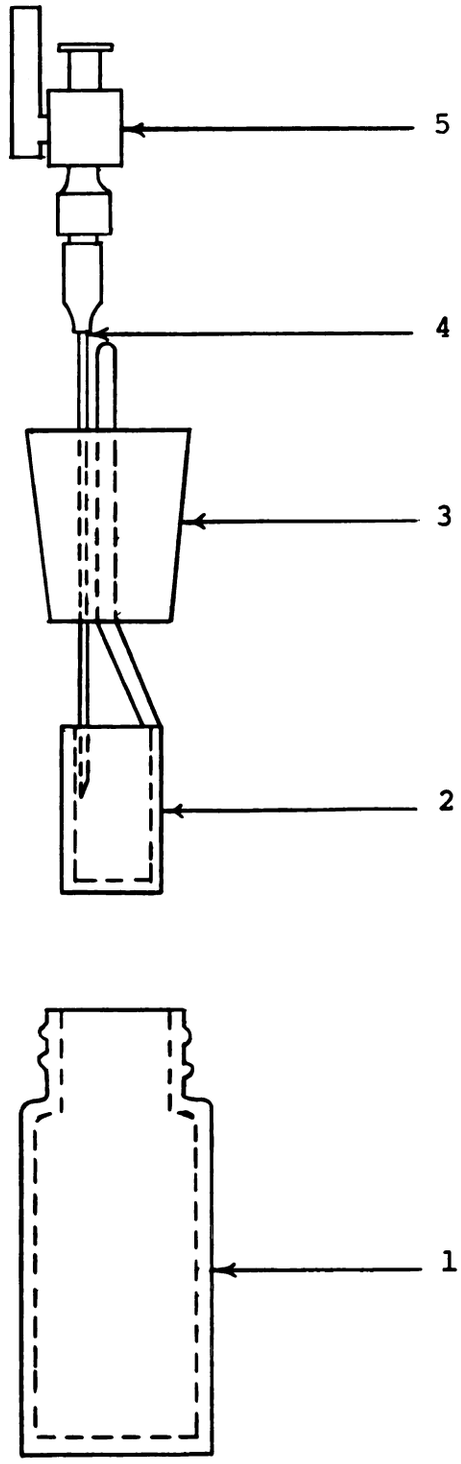



Figure 2

Biological Co., Grand Island, New York, was used as incubation media for the tissues (Appendix 1). A 0.4 ml volume of 5 gm/100 ml dextrose (Abbott Laboratories, Chicago, Ill.) was added to 19.6 ml of PBS resulting in a concentration of 100 mg/100 ml media. The final constituent of the media was 5μc of a $C^{14}$ labeled compound. Each center well contained 0.7 ml of incubation media, and all solutions used were sterile.

After the tissues were added to the center wells, the entire chamber was flushed with 100% oxygen for approximately 10 seconds and then the vessels were sealed and shaken for 4 hours at the desired temperature. Shaking was accomplished by a cam-driven 24 cm diameter circular table which oscillated about its center. The cam was cut with an instantaneous decceleration phase which caused the spring loaded table to rapidly return and strike a rubber stopper, thereby creating agitation of the media. The distance the table was allowed to return was the maximum allowable without splashing media from the center well and was empirically determined to be approximately 1 cm at the periphery of the table. The cam speed caused about 50 impulses per minute, and the table held 12 incubation chambers.

At the termination of the incubation period, 0.2 ml of Hydroxide of Hyamine 10-X (Packard Instrument Co., Downers Grove, Ill.) was injected into the liquid

scintillation vial through the side of the stopper thereby preventing loss of $C^{14}O_2$. Initially, 1.0 ml of Hyamine was used to absorb the $C^{14}O_2$ in the vial, but successively reducing the amounts to 0.2 ml showed that this amount was adequate to absorb all $CO_2$ produced. This provided a saving in materials with much less quenching of the counting solution.

The reactions were terminated at this time by injection of 0.1 ml of 0.67 N HCl through the 18-gauge needle into the center well. To correct for the approximately 0.1 ml dead space in the needle, acid was injected into the needle by a 1.0 ml tuberculin syringe until it was visible at the needle tip. Then an additional measured 0.1 ml was injected from the syringe into the center well. The vessels were shaken for an additional hour to absorb into the Hyamine the $C^{14}O_2$ evolved. The center wells containing the tissue and incubation media were then removed and treated as described below.

Fifteen milliliters of a liquid scintillation counting solution were added to each vial and the $C^{14}$ activity was determined with a Mark I Liquid Scintillation System (Nuclear-Chicago Corporation, Des Plains, Ill.). Originally, counting solution I (Appendix 2) was used, but later the ingredients were decreased to conserve materials. Counting solution II (Appendix 2) was used in the final experimentation and did not alter either

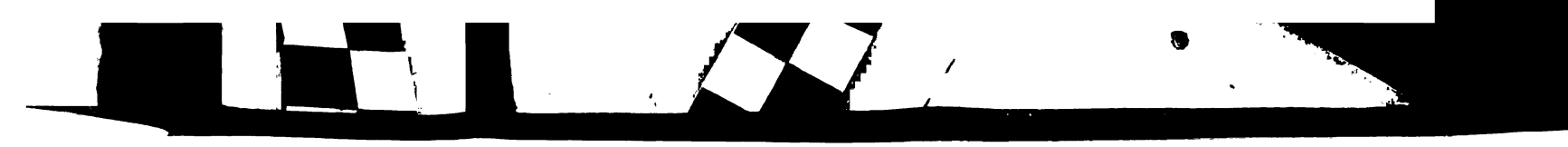


Figure 3.--Shaking apparatus containing incubation vessels.

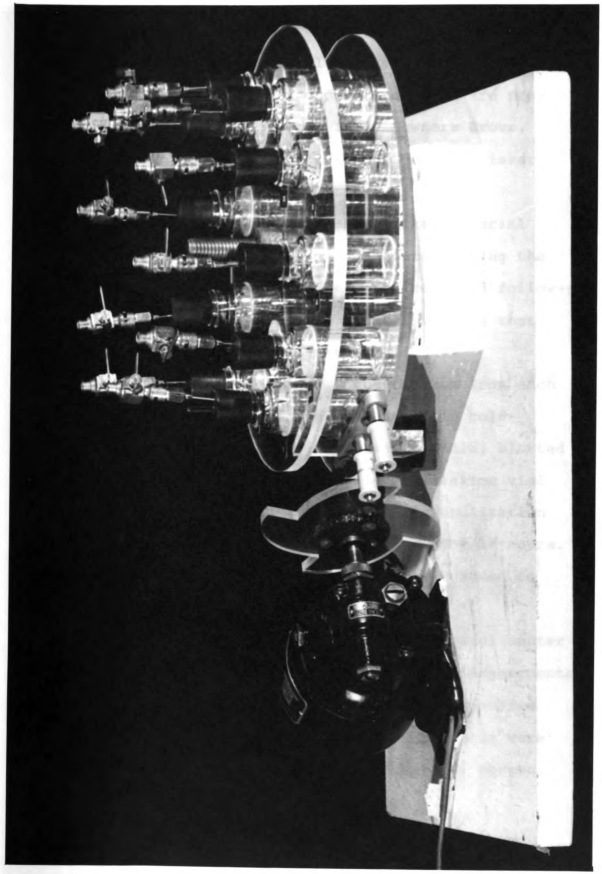


Figure 3.

counting efficiency or water solubility for the procedure. All scintillation chemicals and counting vials are products of Packard Instrument Co., Inc., Downers Grove, Ill. The dioxane used was certified grade from Fisher Scientific Co., Fair Lawn, New Jersey.

Corrections for the "carbonate like" material (Merlevede, Weaver, and Landau, 1963) contaminating the G-1-$C^{14}$ were made by measuring the $C^{14}O_2$ released following acidification of the media in control vessels that had been incubated without tissues.

After collection of $C^{14}O_2$, the tissues from each center well were removed, rinsed thoroughly in cold-blooded-Ringer solution to elminate excess media, blotted dry on filter paper, and placed in a scintillation vial containing 1.0 ml of Hyamine Hydroxide. Solubulization of the tissues was aided by heating to 60 C for 18 hours. Finally, 15 ml of scintillation cocktail were added to each vial and tissue activity was counted.

A 0.1 ml aliquot of media from two control center wells without tissue and two randomly selected experimental vessels was counted for activity to determine specific activity (dpm/mg) of the media. All $C^{14}$ compounds were purchased from New England Nuclear Corporation, Boston, Mass.

Procedure for Studies with Antimycin A

Antimycin A was donated for experimental use by the Veterinary Medical Division of Ayerst Laboratories Incorporated, New York, N.Y. The compound is only slightly soluble in polar solvents; therefore, it was delivered in an acetone solvent. The acetone solvent from the original solution was evaporated from a measured volume and was replaced with 100% ethanol to make a concentration of 2.6 µg/µl ethanol. One ml of the solution was added to 1.6 ml of sterile distilled water making a concentration of 1 µg/µl 62% ethanol. This is the concentration of water at which the inhibitor began to precipitate. Five µl (5 µg) of this solution were injected in-vivo into the anterior chamber of the left eye with a micropipet attached to a microburet.

A control solution of 1.0 ml of 100% ethanol in 1.6 ml of water without inhibitor was made and 5.0 µl were injected into the opposite eye. The eyes were examined periodically for 72 hours after the time of injection.

In-vitro paired experiments consisted of adding Antimycin A to the media for the tissues of one side at a concentration of 2.6 µg/ml. Both 1-$C^{14}$ and 6-$C^{14}$ radioisotopes of glucose were used in-vivo as well as in-vitro.

## Procedure for Temperature Studies

To determine the effect of changing temperature on the tissue production of $C^{14}O_2$ from G-1-$C^{14}$ and G-6-$C^{14}$, 4 hour experiments were conducted at temperatures of 4, 13, 23, 33, and 43 C. The procedure for tissue preparation was the same as described above, and to obtain the desired temperature the shaking apparatus containing the incubation vessels was placed in a Model 82 Fisher Low Temperature Incubator (Instrument Division, Fisher Scientific, Pittsburg, Pa.). The temperature of the chamber was monitored using a YSI (Yellow Springs Instrument Co., Yellow Springs, Ohio) Model 432 Thermistor and Model 43TD Telethermometer. The temperature fluctuated no more than 1 C of the desired temperature.

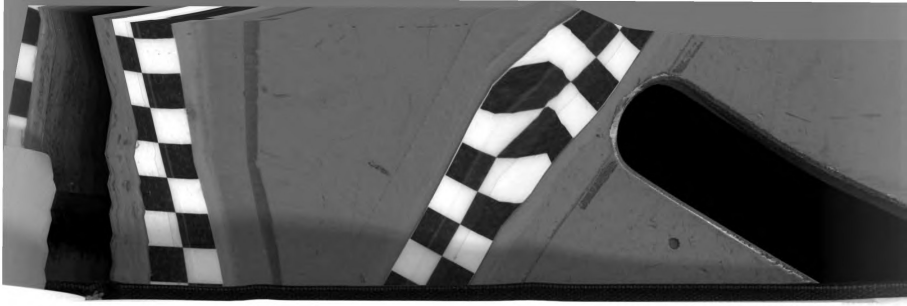
## Procedure for Counting Radioactivity

The Mark I Liquid Scintillation System was used to count all experimental samples for $C^{14}$ radioactivity. The amplifier gain (or attenuation) on channel C was adjusted to provide highest efficiency (counting rate) for a given sample between the selected lower and upper discriminator levels. The least quenched standard of a series of quenched standards (Nuclear-Chicago Corporation) was used for adjustment. The maximum machine efficiency was 91% (see operating manual for Mark I system for details).

Counting efficiencies for all samples ranged between 40-85% with the majority counted at 50-80% level. Efficiency for the tissue activity was at the lower end of the range due to the quenching effect of Hyamine which turned yellow when heated to 60 C for 18 hours. The $C^{14}O_2$ counting efficiency was high because of the small amount of Hyamine used. All samples were counted for 40 minutes or 100,000 counts, whichever occurred first. A statistical error of less than 3% resulted from the counting procedure (operating manual for Mark I System).

To correct for varying amounts of sample quenching, the upper level discriminator on channel B was lowered so channel B monitored the lower 30% of the counting rate of channel C. A channels ratio quench correction standard curve was then plotted using the series of quenched standards. Machine counting efficiency was plotted on the ordinate against channels ratio ($C^{14}$ cpm channel B/$C^{14}$ cpm channel C) which are plotted on the abscissa. With the channels ratio (B/C) printed by the counter, the counting efficiency was obtained from the quench correction standard curve (Appendix 2). All counts were corrected to 100% efficiency (dpm).

$$\text{Total counts (dpm)} = \frac{\text{Machine counts (cpm)}}{\text{\% efficiency}} \times 100$$





Statistical Analysis

Statistical analysis of data from the paired experiments used throughout was calculated using the non-parametric Walsh test for small (N) sample numbers. The term significant when used hereafter indicates a calculated p value of less than 0.05.

## Microrespirometer Studies

### Apparatus

A multiple-unit constant-pressure microrespirometer was constructed for oxygen consumption studies. Winterstein (1912) introduced the constant-pressure principle and Scholander (1941) added modifications to measure changes in gas volume with a micrometer-controlled burette attached to each respirometer unit. Reineke (1961) connected a series of respirometer units into a manifold, permitting measurement of gas volume changes of all units with a single micrometer-controlled burette to further improve the design. Construction of the microrespirometer was patterned after Reineke's design, and included the following alterations:

1. A three-way stopcock was inserted to provide a short circuit across the manometer, thereby eliminating the possibility of ejecting the fluid from the manometer when instantaneously subjected to a large pressure

difference between the manifold and the thermobarometer flask.

2. Nine respirometer units were included, permitting the use of paired experiments with a blank unit remaining.

3. Interchangeable syringe microburets were used to assure maximum sensitivity.

4. Respirometer units, individually constructed and connected to each other by ground glass ball-joints, provided easy removal and exchange if damaged.

Each individual respirometer unit is basically a U-tube manometer with one arm connected to an incubation flask, and the other arm attached to a thermobarometer compensating flask. A piece of glass tubing served as a segment of a common manifold, and a connection was made from this segment to the side of the manometer with the incubation flask. A two-way stopcock was inserted in this connection to separate the unit from the common manifold, creating a closed incubation system. This connection provided a route for addition of gas from the common manifold to the incubation flask, replacing the oxygen consumed by the tissue during incubation. The forementioned three-way stopcock was introduced to provide a direct connection, when turned to the appropriate position, from the thermobarometer to the incubation flask, by-passing the manometer. The unit was made of

Figure 4.--Individual respirometer unit.

1. Manometer.
2. Standard 7/20 joint to connect incubation flask.
3. Standard 14/20 joint to connect compensation flask.
4. Three-way stopcock.
5. Manometer by-pass.
6. Two-way stopcock.
7. Segment of common manifold.
8. Standard 12/2 ball and socket joint.

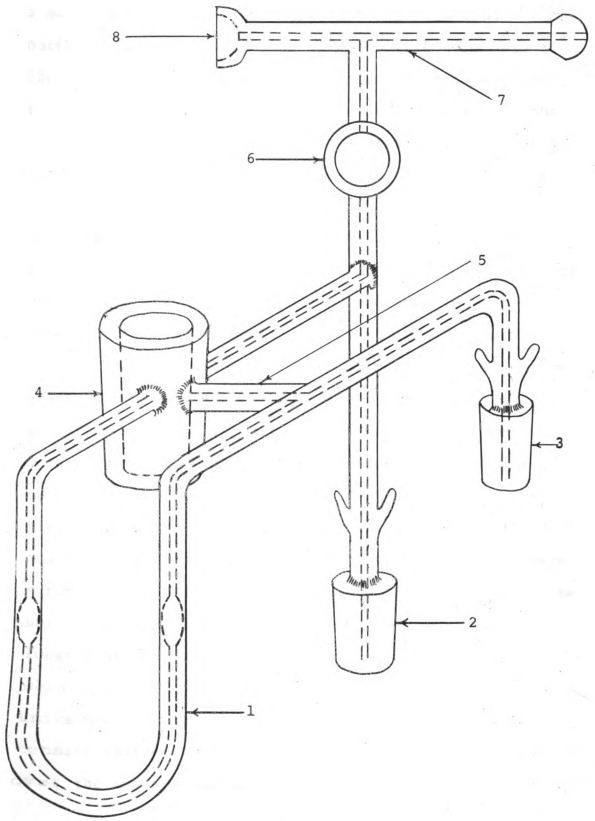



Figure 4.

2 mm (i.d.) capillary tubing and stopcocks having a 2 mm bore. Standard taper joints of size 17/20 were used at the incubation flask connection to accommodate Warburg reaction vessels. A 15 ml Erlenmeyer flask used as the compensating flask was joined to the unit by a 14/20 joint. Manometer fluid (Kerosene, colored with Sudan III) was injected into each manometer with a small-bore polyethylene tube connected to a syringe. Manometers were filled to 1 cm below the expansion bulbs, and zero points were marked with narrow pieces of adherent tape on the left arm of each manometer, level with the meniscus.

A local glassblower geometrically fabricated the glassware to fit like an inverted U over an acrylic plastic bench-type support. The 9 respirometer units were coupled together by 12/2 ball and socket joints on each manifold segment to form a common manifold. The first unit on the left was fitted with a two-way stopcock and the extreme unit on the right was fitted with a male syringe adapter holding a 20-gauge needle. With all nine units assembled in a row on the bench, flasks were located at the rear for accessibility, and manometers were in front to facilitate visualization. Individual units were firmly held to the acrylic plastic bench by tension exerted from two pieces of rubber tubing stretched over the two arms extending from the manometer and fastened

on both sides. The bench was mounted on wheels to allow shaking motion.

During operation, the bench with glassware included, was completely submerged in a constant temperature water bath, which was also constructed of acrylic plastic. This tank rested on a plywood superstructure built with a platform at each end of the tank. One platform rigidly supported an electric motor (Bodine Electric Co., Chicago, Ill.) used for shaking the bench. It was rated a 1/18 hp at 5000 rpm, on line voltage, but a Powerstat (Superior Electric Co., Bristol, Conn.) was inserted in the line to regulate motor speed. The motor was connected to the movable bench through a removable bronze rod, which was fixed to an eccentric bronze ball-joint on the reduction shaft of the motor, and extended to a similar metal joint on a vertical plastic projection rising from the bench. The joints were easily separated for removal of the bench from the water. Mounted on the other platform was a dial-type syringe microburet (model No. SB2, Micro-Metric Instrument Co., Cleveland, Ohio). A microburet syringe, calibrated at 0.500 µl per micrometer division, yielded maximum accuracy, but other syringes were interchangeable, thus increasing versatility of the apparatus. Communication from the microburet syringe to the common manifold was through a small-bore polyethylene (PE100) tube, fitted

tightly over 20-gauge hypodermic needles on both the common manifold and the microburet syringe.

Water bath temperature was maintained by keeping the entire apparatus in a controlled temperature room at 13 0.5 C. Space was provided in the water bath to accommodate a thermostatically controlled heating unit and a motor-driven stirring propeller.

Measurement of Oxygen Consumption

Nine ml of distilled water were placed in each compensating flask. Tissues prepared as described were placed in the Warburg vessels, each of which contained 2.0 ml of Krebs-Ringer-Phosphate incubation media with a concentration of 100 mg/100 ml glucose (Appendix 1). A filter paper wick and 0.2 ml of 10% KOH were added to the center well of the reaction vessel. The side arm stoppers of the Warburg vessels were inserted and turned to allow passage of gas from the vessel. All vessels then were attached to the respirometer. Preparation of the respirometer to receive the vessels consisted of opening the manifold stopcock to atmospheric pressure, positioning the three-way stopcock to short-circuit the manometer, and opening each respirometer unit to the common manifold.

The flasks were then gassed simultaneously with 100% oxygen for approximately three minutes. Oxygen entered through a rubber hose from a tank to the stopcock

end of the common manifold, and gas left via the open side arm of each vessel. After the gassing procedure, the side arm of each reaction vessel was closed and the entire apparatus was placed in the constant temperature bath. The polyethylene tubing was connected to the syringe microburet on one end, and the shaking rod fixed to the bench at the other.

The tissues and media were allowed to equilibrate for 30 minutes while shaking at a frequency of 100-110 cycles per minute and a stroke of 2.8 cm. This time and frequency promoted adequate oxygenation of the media so that oxygen diffusion could be excluded as the limiting factor in any results (Umbreit, Burris and Stouffer, 1964).

To start the incubation following equilibration, stopcocks were first closed to separate the units from the manifold, and then the three-way stopcocks were turned so the manometers were connected to the reaction vessels and compensating flasks. Time, temperature, and barometric pressure were noted at that time. The manifold remained open to atmospheric pressure.

At the end of the 24 hour incubation, the shaker was stopped, the syringe was filled to the capacity of the micrometer, and the manifold was closed from the atmosphere. The initial setting on the micrometer was recorded, then the first unit was opened to the manifold.

The manometer fluid was restored to the zero point by manipulating the micrometer. The new micrometer reading was recorded. A change in gas volume was measured directly by the difference in micrometer readings. The first unit was closed and measurements were made successively on the remaining units as described. The syringe was recharged with oxygen from the manifold and the above cycle repeated as often as necessary.

Complete compensation for pressure changes due to temperature and barometric pressure variations was accomplished by using the first unit as a thermobarometer containing media only. Adjustment of this unit as described above will set the pressure in the manifold equal to the pressure in the compensating flasks; thus, the readings are automatically corrected for the remaining units. The volume of gas consumed by the tissue in each unit was corrected to STP using 13 C and the original barometric pressure as the initial conditions.

Before removal of the apparatus from the water bath, the three-way stopcocks were always turned so the manometers were eliminated from the circuit to prevent expulsion of the manometer fluid.

## Comparison of Results with the Microrespirometer

Oxygen consumption of rat liver slices in the constructed microrespirometer was compared with values

for liver slices measured by Reineke (1961) when he used his apparatus and the Warburg microrespirometer simultaneously.

Rats were killed by a sharp blow to the head. The livers were quickly removed, and rinsed in a chilled Ringer-Phosphate buffer. Liver slices were cut 0.5 mm thick with a Stadie-Riggs hand microtome and replaced in the buffer. Pieces of tissue (80-140 mg) were blotted, rapidly weighed, and placed in reaction vessels containing Ringer-Phosphate buffer with glucose. Triplicate samples from each liver were dried for 12 hours at 100 C to determine per cent dry weight. Eight samples of liver tissue from each of three rats were run, totaling 24 determinations. Measurements were made at 15 minute intervals over a period of 60 minutes. The temperature of the apparatus was controlled at 37 C. The $Q_{O_2}$ values compare well with those obtained by Reineke (1961) (Appendix 3).

Analysis of Media

Duplicate aliquots of Krebs-Ringer-Phosphate buffer were taken for glucose determination prior to incubation. After completion of the incubation period, samples were again taken from each of the vessels to be analyzed for glucose. The Glucostat enzymatic micromethod (Worthington Biochemical Corp., Freehold, N.J.)

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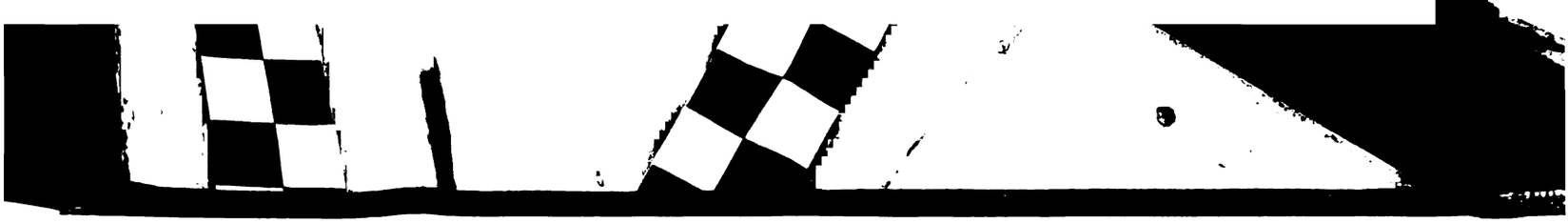



with glucose oxidase was used for all glucose determinations.

Lactic acid was determined at the completion of the experiment by the colorimetric micromethod of Barker and Summerson (1941).

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# RESULTS

## General Aspects of Metabolism of Corneal and Lenticular Tissues

Viability of tissues in-vitro was demonstrated by measuring oxygen consumption, lactic acid production and glucose utilization. The in-vitro oxygen consumption of lenses and corneas of rainbow trout eyes was found to continue at a constant rate over a period of as long as 48 hours (Appendix 3). In studies of $CO_2$ production tissues were removed and placed in separate vials which contained the labeled precursor. Tissues from one eye served as a control for tissues taken from the contralateral eye and no statistical difference in rate of $C^{14}O_2$ production was noted (Appendix 3).

Data presented below are for corneal tissue and lenses only and in all cases paired (right and left eye) samples were run.

## Production of $C^{14}O_2$ from 2-$C^{14}$-pyruvate: Effect of Inhibitors

The inhibitors were added to the media bathing the tissues and the data are presented in Table 1. Sodium cyanide ($10^{-3}$M) caused nearly complete inhibition of

$C^{14}O_2$ production by both lenses and corneas whereas $10^{-3}$M potassium iodoacetate had no effect.

TABLE 1. Effect of inhibitors on production of $C^{14}O_2$ from 2-$C^{14}$-pyruvate by ocular tissues of trout at 13 C

| Tissue | | $C^{14}O_2$ (dpm/100 mg wet tissue) | |
|---|---|---|---|
| | | No inhibitor | Inhibitor |
| Lens | NaCN($10^{-3}$M) | 3989.4±1332.9(5)[a] | 5.7±2.1(5)[b] |
| | KIAA($10^{-3}$M)[c] | 3660.1±996.3(5) | 3927.6±1365.8(5) |
| Cornea | NaCN($10^{-3}$M) | 31,974±14,124(5) | 17.8±12.3(5)[b] |
| | KIAA($10^{-3}$M) | 29,455±11,477(5) | 31,886±5,661(5) |

[a]Mean ± standard error (observations)
[b]Significant at the p = less than 0.05 level
[c]Potassium iodoacetate

## Formation of Labeled $CO_2$ from G-1-$C^{14}$ and G-6-$C^{14}$

### Untreated Tissues

Data presented in Table 2 show that the $CO_2$ production by the cornea is about 20 times greater than that by lenses. Both tissues produced over twice as much labeled $CO_2$ from the G-1-$C^{14}$ as from G-6-$C^{14}$. These differences were statistically different at the p = 0.01 level.

TABLE 2.--Comparison of $C^{14}O_2$ from G-1-$C^{14}$ and G-6-$C^{14}$ by untreated tissues in PBS at 13 C

| | $C^{14}O_2$ (dpm/100 mg wet tissue) | | |
|---|---|---|---|
| Tissue | G-1-$C^{14}$ | G-6-$C^{14}$ | $\frac{\text{G-1-C}^{14}}{\text{G-6-C}^{14}}$ |
| Lens | 154.3±17.9(18)[a] | 66.3±7.4(16) | 2.33 |
| Cornea | 3589.8±77.2(18) | 1295.9±17.1(17) | 2.77 |

[a]Mean ± standard error (observations)

Effect of Cyanide

In this series, experiments were performed using G-1-$C^{14}$ and G-6-$C^{14}$ separately as the original source of $C^{14}O_2$. Sodium cyanide ($10^{-3}$M) caused a complete suppression of $C^{14}O_2$ formation from G-6-$C^{14}$ by both corneas and lenses. In contrast, when G-1-$C^{14}$ served as the substrate, lesser but significant ($p = 0.03$) reductions of 70% and 60% occurred in the lenses and corneas respectively. None of the tissues used exhibited any visible change in physical appearance or loss of transparency during the course (4 hours) of these experiments.

TABLE 3.--Effect of NaCN ($10^{-3}$M) on $C^{14}O_2$ production from G-1-$C^{14}$ and G-6-$C^{14}$ by tissues in PBS at 13 C

| | | $C^{14}O_2$ (dpm/100 mg wet tissue) | | |
|---|---|---|---|---|
| Tissue | Substrate | No inhibitor | inhibitor | $\frac{\text{inh.}}{\text{No inh.}}$X100 |
| Lens | G-1-$C^{14}$ | 94.8±12.6(5)[a] | 28.6±4.5(5) | 30.1 |
| Cornea | G-1-$C^{14}$ | 3250.1±391.7(5) | 1294.1±187.4(5) | 39.8 |
| Lens | G-6-$C^{14}$ | 71.3±8.0(5) | 0.0±0.0(5) | 0.0 |
| Cornea | G-6-$C^{14}$ | 1008.1±99.1(5) | 1.6±0.7(5) | 0.1 |

[a]Mean ± standard error (observations)

Effect of Anoxia

The paired experimental design was again used. One incubation chamber received the tissue and media which had been previously deoxygenated by bubbling $N_2$ through it. The chamber was then gassed with $N_2$. The other chamber containing tissue and oxygenated media was flushed with 100% oxygen prior to being sealed for incubation.

A lack of oxygen significantly ($p = 0.03$) reduced $C^{14}O_2$ production from G-1-$C^{14}$ in the lens and cornea by 75% and 67% respectively. Production of $C^{14}O_2$ from G-6-$C^{14}$ by the lens showed a 90% decrease under anoxic conditions, but formation by the cornea was

reduced only 52% (Table 4). All tissues were transparent at the end of the four hour incubation period.

TABLE 4.--Effect of anoxia on $C^{14}O_2$ production from G-1-$C^{14}$ and G-6-$C^{14}$

| | | $C^{14}O_2$ (dpm/100 mg wet tissue) | | |
|---|---|---|---|---|
| Tissue | Substrate | Aerobic | Anaerobic | $\frac{\text{Anaer.}}{\text{Aer.}}$ X100 |
| Lens | G-1-$C^{14}$ | 238.9±56.9(5)[a] | 57.7±16.9(5) | 24.1 |
| Cornea | G-1-$C^{14}$ | 4979.5±343.1(5) | 2121.1±139.7(5) | 42.6 |
| Lens | G-6-$C^{14}$ | 75.9±21.2(5) | 7.3±4.4(5) | 9.6 |
| Cornea | G-6-$C^{14}$ | 1361.7±189.0(5) | 652.0±120.8(5) | 47.8 |

[a]Mean ± standard error (observations)

Effect of Antimycin A
In-vivo and In-Vitro

In this experiment 5 µg of Antimycin A were injected in-vivo into the anterior chamber of one eye. After an interval, tissues were removed and $C^{14}O_2$ formation from labeled precursors was measured. Antimycin A was added directly to the media for another part of the experiment to compare its in-vitro effect on the $C^{14}O_2$ produced.

Antimycin A, when administered either in-vivo or in-vitro, nearly abolished $C^{14}O_2$ formation from G-6-$C^{14}$ by both lenticular and corneal tissues (Table 5). When G-1-$C^{14}$ was the precursor, each tissue reduced $C^{14}O_2$ production to the same extent, independent of the method of administration of the inhibitor. Lenses subjected to Antimycin A reduced $C^{14}O_2$ production by 19% and corneas by approximately 46%.

TABLE 5.--Effect of Antimycin A in-vivo and in-vitro on $C^{14}O_2$ yield from G-1-$C^{14}$ and G-6-$C^{14}$

| | $C^{14}O_2$ (dpm/100 mg wet tissue) | | |
|---|---|---|---|
| G-1-$C^{14}$ | No inhibitor | Inhibitor | $\frac{\text{inh.}}{\text{No inh.}}$X100 |
| Lens in-vitro[a] | 173.1±33.0(4)[b] | 139.6±23.4(4) | 80.6 |
| Lens in-vivo[c] | 184.9±35.1(4)[b] | 149.2±23.6(4) | 80.6 |
| Cornea in-vitro | 3340.3±475.5(4) | 1841.7±138.8(4) | 55.1 |
| Cornea in-vivo | 3131.0±83.3(4) | 1665.2±181.8(4) | 53.1 |
| G-6-$C^{14}$ | | | |
| Lens in-vitro | 61.3±8.9(4) | 0.7±0.2(4) | 1.1 |
| Lens in-vivo | 54.9±12.9(4) | 1.5±0.3(4) | 2.7 |
| Cornea in-vitro | 1682.3±123.0(4) | 15.0±4.4(4) | 0.9 |
| Cornea in-vivo | 1367.9±302.2(4) | 114.9±29.3(4) | 8.3 |

[a]Mean ± standard error (observations)

[b]Antimycin A concentration of 2.6 µg/ml in media

[c]Antimycin A (5 µg) injected into anterior chamber of the eye

The addition of 5 μg of Antimycin A to the anterior chamber of trout eyes resulted in a change of the physical characteristics which were evident by 48-72 hours after injection. Eyes in treated fish appeared swollen and a slight loss of pigment was evident in the iris and the limbus of the corneas. Lenses were opaque to the extent that they were clearly visible from the exterior and they showed an approximate 20% gain in weight. Corneas appeared cloudy and were more affected at the periphery than in the center. Most unusual was the presence of a gas bubble in the anterior chamber of all the fish injected with inhibitor. Contralateral eyes of the fish which were injected with an equal volume of a solution similar in content to the experimental solution but lacking Antimycin A, showed no visible changes for a period of 72 hours after injection.

Effect of Temperature

Experiments contrasting the $C^{14}O_2$ production from G-1-$C^{14}$ and G-6-$C^{14}$ were performed at temperatures from 4-43 C.

With an increase in temperature, formation of $C^{14}O_2$ from G-6-$C^{14}$ by lenses rose rapidly and reached a maximum at 23 C. Above this temperature there was an equally rapid fall in $C^{14}O_2$ production (Figure 5). With G-1-$C^{14}$, $C^{14}O_2$ production sharply increased between 13 and 23 C, then leveled off to reach a maximum at about

33 C (Figure 5). At this temperature about 17 times more $C^{14}O_2$ was formed from G-1-$C^{14}$ than from G-6-$C^{14}$ metabolism. Lenses retained their transparency after 4 hours at temperatures up to 33 C but clouding occurred in 45 to 60 minutes after the start of incubation at 43 C.

TABLE 6.--Effect of temperature on $C^{14}O_2$ production from G-1-$C^{14}$ and G-6-$C^{14}$ from tissues in PBS at 13 C

| | $C^{14}O_2$ (dpm/100 mg wet tissue) | | | |
|---|---|---|---|---|
| Temp. | Lens | | Cornea | |
| | G-1-$C^{14}$ | G-6-$C^{14}$ | G-1-$C^{14}$ | G-6-$C^{14}$ |
| 4 C | 135.3±31.6[a] | 21.4±7.2 | 1404.5±234.3 | 262.0±59.9 |
| 13 C | 162.4±8.0 | 47.5±1.4 | 3512.8±102.1 | 1278.9±833.4 |
| 23 C | 609.2±38.5 | 96.0±5.2 | 9628.4±1046.6 | 3568.5±503.0 |
| 33 C | 616.8±79.5 | 35.6±8.9 | 4576.2±677.0 | 666.4±126.1 |
| 43 C | 559.1±39.5 | 29.7±10.5 | 1239.4±232.2 | 110.9±13.7 |

[a]Mean of 4 observations ± standard error

The formation of $C^{14}O_2$ from G-1-$C^{14}$ by corneas rose rapidly when temperature was increased and, like lenses, showed a peak production at 23 C with a sharp decline above this temperature. The profile is similar for $C^{14}O_2$ formation from G-6-$C^{14}$ but about one-third the magnitude at 23 C (Figure 5). At temperatures above 23 C,

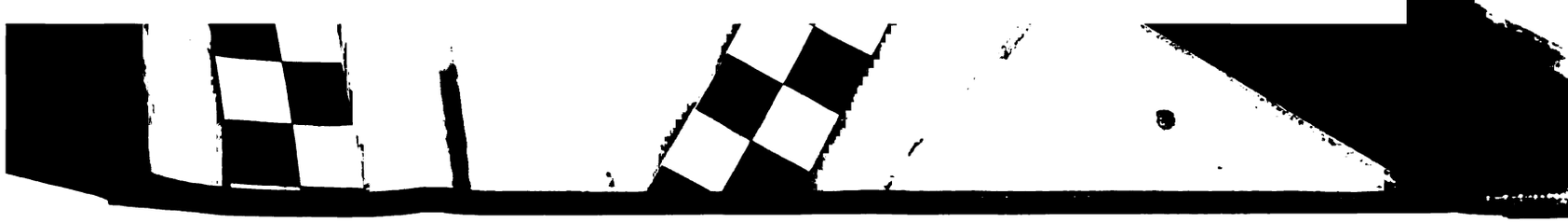


Figure 5.--The effect of changing temperature on the $C^{14}O_2$ formation from G-1-$C^{14}$ and G-6-$C^{14}$ from the lens and corneas incubated in PBS.

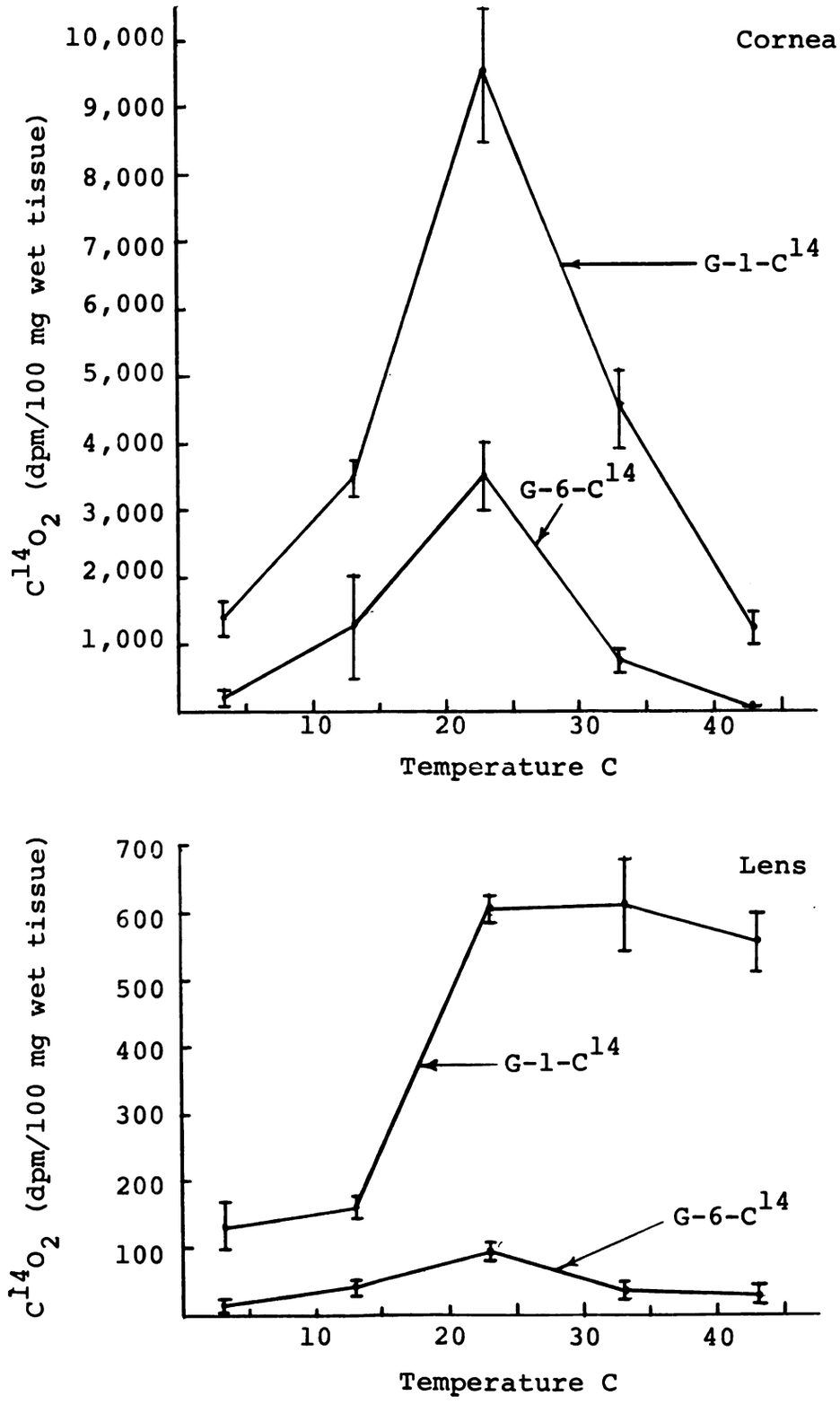

Cornea
10,000
9,000
8,000
7,000
6,000
5,000
4,000
3,000
2,000
1,000
$C^{14}O_2$ (dpm/100 mg wet tissue)
G-1-$C^{14}$
G-6-$C^{14}$
10
20
30
40
Temperature C
Lens
700
600
500
400
300
200
100
$C^{14}O_2$ (dpm/100 mg wet tissue)
G-1-$C^{14}$
G-6-$C^{14}$
10
20
30
40
Temperature C

Figure 5.

corneas became cloudy and their edges assumed a scalloped appearance shortly after the incubation period began.

Effect of Capsule Damage

The lens capsule was cut with a sharp scalpel. As the cut was made, the contents within the lens appeared to extrude out of, and enlarge the opening as if the capsule had been under tension. The $C^{14}O_2$ production from five of these lenses was compared to that from intact lenses. Data in Table 7 show no significant change in $C^{14}O_2$ production after the capsule was damaged.

TABLE 7.--Effect of capsule damage on $C^{14}O_2$ production and $C^{14}$ tissue content of lens incubated in G-1-$C^{14}$

| | Activity (dpm/100 mg wet tissue) | |
|---|---|---|
| Lens | Intact | Damaged |
| $C^{14}O_2$ | 192.3±29.0(5)[a] | 207.2±25.2(5) |
| Tissue $C^{14}$ | 9,843±471(5) | 10,538±527(5) |

[a]Mean ± standard error (observations)

Effect of Inhibitors on Tissue $C^{14}$ Activity After Incubation with G-1-$C^{14}$

In experiments to determine the effects of various inhibitors on the presence of $C^{14}$ activity in the tissues after incubation, the tissues were solubilized

in 1.0 ml of Hyamine. The activity was then counted in a liquid scintillation system.

TABLE 8.--The $C^{14}$ activity in ocular tissue after incubation with G-1-$C^{14}$ and various inhibitors

| | Tissue $C^{14}$ activity (dpm/100 mg wet wt.) | |
|---|---|---|
| Lens | No inhibitor | Inhibitor |
| NaCN ($10^{-3}$M) | 12,108±708(4)[a] | 11,061±664(4) |
| Antimycin A (2.6 µg/ml) | 12,613±510(4) | 9,714±296(4) |
| $N_2$ atmosphere | 13,810±321(4) | 12,255±564(4) |
| Cornea | | |
| NaCN ($10^{-3}$M) | 50,383±1237(4) | 44,708±476(4) |
| Antimycin A (2.6 µg/ml) | 41,086±2260(4) | 42,642±2761(4) |
| $N_2$ atmosphere | 53,738±965(4) | 52,462±1401(4) |

[a]Mean ± standard error (observations)

No significant difference in activity was seen when the radioactivity of the tissue subjected to NaCN ($10^{-3}$M), Antimycin A (2.6 µg/ml), or an $N_2$ environment, was compared to the activity of the untreated controls (Table 8). The activity present in the corneal tissue was approximately four times that in the lens when calculated on a per unit volume basis.

## Effect of Inhibitors on the $C^{14}$ Activity in Tissues After Incubation with 2-$C^{14}$-pyruvate

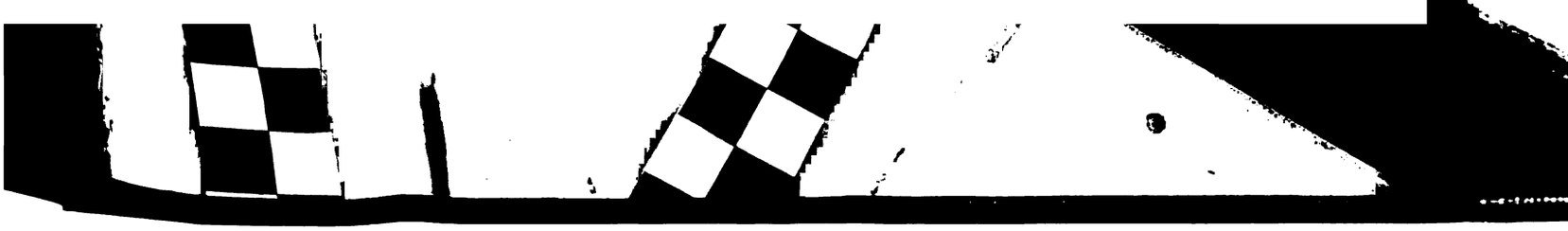
Tissues were incubated with 2-$C^{14}$-pyruvate with and without the presence of inhibitors and the $C^{14}$ activity contained in each tissue at the end of the experiment was determined.

TABLE 9.--Effect of inhibitors on $C^{14}$ activity in ocular tissue after incubation with 2-$C^{14}$-pyruvate

| | Tissue $C^{14}$ activity (dpm/100 mg wet tissue) | | |
|---|---|---|---|
| Lens | No inhibitor | Inhibitor | $\frac{\text{Inh.}}{\text{No inh.}} \times 100$ |
| NaCN ($10^{-3}$M) | 47,363±6197(4)[a] | 21,051±1769(4) | 44.4[b] |
| KIAA ($10^{-3}$M) | 41,995±3575(4) | 38,043±4362(4) | 90.5 |
| Cornea | | | |
| NaCN ($10^{-3}$M) | 121,751±23,559(4) | 57,943±4358(4) | 47.5[b] |
| KIAA ($10^{-3}$M) | 92,746±7061(4) | 77,532±6629(4) | 83.1 |

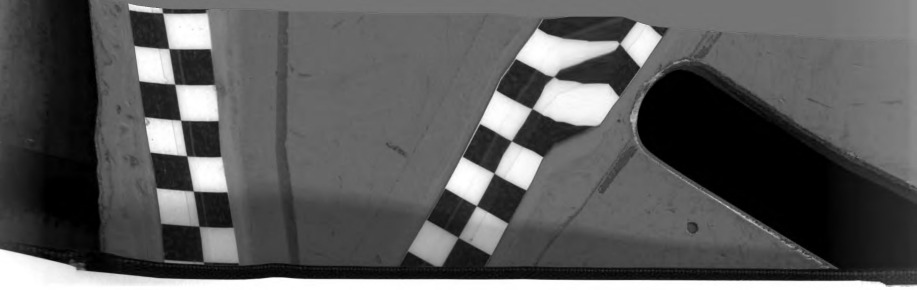
[a]Mean ± standard error (observations)
[b]Significant at the p = 0.03 level

The addition of cyanide to either the lens or cornea caused a significant reduction in tissue radioactivity to approximately one-half of the amount present in the contralateral tissue. No significant difference





was apparent when potassium iodoacetate ($10^{-3}$M) was added to the media. The cornea without inhibitor contained roughly 2.5 times more $C^{14}$ activity than the lens.



## DISCUSSION

Biochemical studies reported here have produced data to show that oxidation of glucose to $CO_2$ by the citric acid cycle and via the hexosemonophosphate (HMP) shunt occurs in lenticular and corneal tissues of rainbow trout. Potassium iodoacetate, an inhibitor of glycolysis, had no effect on $CO_2$ production from pyruvate. Production of $CO_2$ from glucose was blocked by known inhibitors of the electron transport chain (ETC), which is necessary for the operation of the citric acid cycle.

It is established that the citric acid cycle is operational in lenses of mammals. Recently Fonner, Hoffert and Fromm (1969) reported that (based on histochemical data) the presence of the citric acid cycle in ocular tissues of rainbow trout was equivocal. The presence of intermediates of the cycle in a tissue is not unequivocal proof that the cycle is operating in the tissue since many of the enzymes identified and substrates used are involved in other known cellular reactions.

Using labeled glucose (G-6-$C^{14}$) as a substrate, it was found that the formation of labeled $CO_2$ by both lenses and corneas was inhibited when NaCN, Antimycin A

or anoxia were used. Many workers have found that cyanide severely reduces lens respiration (Herrmann and Moses, 1945; Ely and Robbie, 1950; Hockwin, Kleifeld and Arens, 1956), but Kinoshita (1955) reported only 35% inhibition of glucose oxidation.

For illustrative purposes, calculations were made using specific activities. The amount of $C^{14}O_2$ produced from 2-$C^{14}$-pyruvate indicated that about 0.06 µg pyruvate/100 mg wet tissue was oxidized to $CO_2$ by the lens and roughly 0.46 µg/100 mg tissue by corneas. A ratio of the amount of $C^{14}O_2$ produced to the amount of 2-$C^{14}$-pyruvate present in the tissue indicates that the cornea is far more active in the oxidation of pyruvate than is the lens of rainbow trout. Similar results have been reported for mammalian corneas by Kuhlman and Resnik (1959) who found that when excess lactate was added to the incubation media as an unlabeled competitor, the $CO_2$ yield from G-6-$C^{14}$ by mammalian corneas dropped some 86%. From their experiments with labeled lactate they concluded that the efficiency of lactate oxidation was comparable to that for glucose.

When trout corneas were incubated with G-6-$C^{14}$ under anaerobic conditions the production of $C^{14}O_2$ was only reduced to about 48% of that which occurred aerobically. If the ETC were truly ineffective due to oxygen

lack, then an alternate pathway for the oxidation of the 6-$C^{14}$ atom of radio-glucose must exist in the cornea. It is possible that all oxygen was not removed from the media and that the tissue contained some oxygen at the start of the experiment; hence, complete inhibition was not evident under the "anaerobic conditions" as described. Both cyanide and Antimycin A did; however, completely inhibit the ETC and production of $C^{14}O_2$.

The metabolic fate of the carbon-1 and carbon-6 atoms differs depending on how glucose is metabolized. If a tissue metabolized glucose via the glycolysis-citric acid cycle exclusively, carbon atoms 1 and 6 of glucose become the methyl carbon of pyruvate and the complete oxidation of pyruvate would result in these atoms appearing as $CO_2$ in equal amounts. The HMP shunt, on the other hand, preferentially removes carbon 1 of glucose to form $CO_2$.

Based on the above principle, Kinoshita (1955) and Kinoshita and Wachtl (1958) incubated mammalian lenses with G-1-$C^{14}$ and G-6-$C^{14}$ and measured the relative contributions of the two metabolic pathways. They found that the C-1 atom of the glucose molecule was oxidized to $C^{14}O_2$ at 41 times the rate of oxidation of the C-6 atom, and that some 85% of the total $CO_2$ produced is via the HMP shunt. If glucose had been metabolized by the glycolytic-citric acid cycle exclusively, then the ratio

would have been 1 instead of 41. With trout tissues it was found that those incubated with G-1-$C^{14}$ yielded more $C^{14}O_2$ in all cases than those incubated with G-6-$C^{14}$. Ratios of C-1-$CO_2$/C-6-$CO_2$ were 2.33 for the lens and 2.77 for the corneas which, when compared with data from mammals, indicates that the HMP shunt is much less active in fish than in mammalian ocular tissues.

By making some additional calculations using data presented in Table 2 a more quantitative relationship between activities of the HMP shunt and the citric acid cycle in trout lens can be shown. The 66.3 dpm recovered from G-6-$C^{14}$ represents the amount of glucose oxidized to $CO_2$ via the citric acid cycle. Since there are six carbon atoms in glucose, the total $CO_2$ production via the citric acid cycle would be 6 X 66.3 or 397.8 dpm/100 mg wet tissue. The 154.3 dpm listed as coming from G-1-$C^{14}$ includes labeled $CO_2$ produced by both the HMP shunt and the citric acid cycle; hence, the total amount of $CO_2$ produced by the HMP shunt is represented by 154.3 - 66.3 or 88.0 dpm/100 mg wet tissue. Therefore, in the trout lenses the $CO_2$ produced via the HMP shunt represents about 1/5 (or 22%) of the total $CO_2$ produced by this tissue. Similar calculations for trout corneas show the HMP shunt activity accounts for about 1/3 of the total $CO_2$ produced by this tissue. These results differ from data for

comparable mammalian tissues which indicates that the HMP shunt accounts for about six times more $CO_2$ than the citric acid cycle or about 85% of the total produced.

All evaluations of the activity of the two pathways were made without consideration for the effect of recycling of compounds through the HMP shunt with the subsequent rearrangement of isotopic carbon atoms. Katz and Wood (1960) estimated the amount of randomization of $C^{14}$ which will occur in the glucose-6-phosphate at steady state when glucose-$C^{14}$ is metabolized via a complete pentose cycle. They have shown that the extent of randomization of $C^{14}$ is proportional to the fraction of the total metabolism of glucose-$C^{14}$ that proceeds by the pentose cycle. The level of activity of the HMP shunt appears to be low in the lens and cornea of rainbow trout; therefore, recycling of the HMP shunt would be of minor significance.

Data on the effect of temperature indicates that the lens responds to increases in temperature by increasing $CO_2$ production from G-1-$C^{14}$ to a much greater extent than from G-6-$C^{14}$. In other words, the HMP shunt activity shows a greater response to increased temperature than citric acid cycle activity. At the preferred temperature of trout, which is about 13 C, $C^{14}O_2$ production from the HMP shunt is about 3 times greater than citric acid cycle $C^{14}O_2$ production. At 33 C it is 17 times greater. This

figure is somewhat comparable to the ratio of 41 reported above for mammalian corneas at 37 C. Above 33 C the HMP shunt activity in fish lenses declines and at these temperatures they become opaque during incubation. Activity of the HMP shunt appears to be quite sensitive to temperature and as temperature goes up, it may become the prime contributor of the energy required to maintain the normal transparency of the lens. It is possible that above 33 C, the HMP shunt cannot produce enough energy to maintain lens transparency.

The cornea responds to temperature changes somewhat differently than the lens. As previously stated, the citric acid cycle is initially more active in the cornea and it responds to increased temperature by increasing activity up to 23 C. At this temperature the ratio ($C\text{-}1\text{-}CO_2/C\text{-}6\text{-}CO_2$) remains at approximately 3. As the temperature is increased from 23 C to 43 C the $C^{14}O_2$ yield from $G\text{-}1\text{-}C^{14}$ falls rapidly and above 23 C changes in the opacity along with other physical changes occur in the cornea. Perhaps transparency could be maintained at elevated temperatures if the HMP shunt could respond with increased activity above 23 C. One is tempted to speculate that at temperatures normally encountered the trout cornea depends primarily on the citric acid cycle for energy production, and that the added increment of energy necessary to maintain transparency at high

temperatures is dependent upon increased HMP shunt activity. Corneal HMP shunt activity appears more limited in its response to increased temperature than is the HMP shunt of trout lenticular tissue.

The differences noted between trout and mammalian eyes may represent significant adaptations permitting normal function under very different conditions. Trout eyes seem to depend mainly on the activity of the citric acid cycle and very little on the HMP shunt for production of usable energy. They are able to maintain normal transparency in lens and cornea at temperatures approaching 0 C. Clouding of these normally transparent structures at high temperature coincides with decreases in HMP shunt activity. In mammalian ocular tissues HMP shunt activity appears to be the prime provider of usable energy and may represent a metabolic adaptation permitting maintenance of transparency at higher temperatures. Mammalian ocular tissues become opaque at lower temperatures (Harris, Gehrsitz, and Nordquist, 1953), but the way various metabolic reactions are involved is not known.

The constant temperature chamber used for the temperature studies had a limited working space and all preparation of tissues and media had to be carried out at room temperature (23 C). This may have introduced a source of error into the data from experiments run at temperatures different than room temperatures because

there was no equilibration of the incubation vessel to the experimental temperature level before the experimentation period began. The experimental period was started when the tissue was placed in the media at room temperature and the vessels were sealed. The metabolic rate of the tissue during the time of changing temperature would be different from the rate of metabolism at the desired temperature. In the experiment at 43 C, a temperature which is known to denature some protein enzymes, a rate of metabolism existed during the period of temperature change which might not have been present at all if equilibration of the tissue to 43 C had occurred prior to the start of the experimental period.

Based on the production of $C^{14}O_2$ it was found that disruption of the lens capsule did not alter the metabolism of this tissue. The technique used to produce lesions was admittedly crude and the extent of lenticular damage was variable and difficult to ascertain. The fact that no change was observed under these conditions suggests that the capsule of the trout lens is not an impenetrable barrier against the inward diffusion of glucose. Ely (1949) has reported that the isolated capsule and nucleus of bovine lens has a negligible respiration; however, he also found that rupturing of the capsule increased the oxygen uptake 2-4 times. Rae (1968) showed that damage

to the capsule caused a nearly 50% reduction of the potential difference of the lens of rainbow trout. He contends that the capsule is a limiting membrane to ion diffusion.

Glucose that entered the tissue was determined by counting the $C^{14}$ activity of the solubilized tissue after completion of the experimental period. At that time the $C^{14}$ may have been incorporated into metabolic intermediates, but it was assumed that the radiolabeled carbon entered as part of glucose molecules. Calculations made from the specific activity of the media provided information on the amount of glucose that entered the tissue.

The concentration of glucose in the media was 90.8 µg/µl. The glucose in lenses weighing about 100 mg was 21.8 µg but the volume of tissue in which this was contained is subject to discussion. If one assumes that glucose is evenly distributed throughout the lens, then a 100 µl volume (assuming 1 mg = 1 µl) lens would have an internal concentration of 21.8 µg/100 µl. A diffusion gradient would then exist which would favor entry of glucose into the tissue. However, the teleost lens differs from its mammalian counterpart in that it is a sphere, does not change shape for accommodation, and has a greater percentage dry weight. The increased dry weight is manifest in a nucleus which is fibrous, extremely hard, and resistant to digestion by Hyamine at 60 C for 18 hours.

Beads of undissolved lenticular nuclei, differing in size, remained in the scintillation vial after 18 hours digestion but their presence did not affect the counts. This leads one to believe that the beads did not contain radioactive glucose so glucose could not have penetrated the lens to this depth in 5 hours.

If one assumes that glucose entrance into the lens occurs solely by a passive diffusion process, a volume within the lens can be calculated in which 21.8 µg of glucose is distributed to obtain an equilibrium concentration with the media. This volume was found to be 24.0 µl. Further assuming that this volume is evenly distributed over the surface of the lens, then its thickness would be some 230 microns and would represent the depth of penetration of passively diffusing glucose. Rae (1968) showed that the potential difference of the lens which is dependent on the diffusion of ions into the lens, existed to a depth of only about 100 microns from the surface.

Neither lesioning the capsule nor the addition of inhibitors affect the entry of glucose into the tissue. This differs from the results of Harris, Hanchild, and Nordquist (1955) who showed that the accumulation of glucose within the mammalian lens could be altered by various enzyme inhibitors. Perhaps the energy dependent

system for uptake of glucose is an adaptation by the mammalian lens to meet the greater energy demands necessary for maintenance of transparency.

The concentration of glucose in trout corneas was found to be equal to that of the incubation media. The anterior surface of the trout cornea has been shown to be impermeable to ions and very likely to glucose as well (Edelhauser, Hoffert, and Fromm, 1966). The endothelium has been described as incomplete and poorly developed in comparison to mammalian lenses (Hoffert and Fromm, 1965). Therefore, equilibrium concentration of glucose in the cornea could be achieved by entry through both the endothelium and the limbal area where the insicision was made. The use of inhibitors failed to alter the rate of entry into the cornea.

When trout tissues were incubated in a medium which contained pyruvate at a concentration of 0.75 µg/100 ml, lenses had mean concentrations of pyruvate of 0.68 µg/100 mg wet tissue whereas corneas accumulated pyruvate to a concentration of 1.75 µg/100 mg. The fact that after poisoning with $10^{-3}$M NaCN the amount of radioactivity in both lenses and corneas was about half the concentration attained in non-poisoned tissues suggests the presence of an energy dependent mechanism for pyruvate transport in both tissues. Cyanide could alter the amount

of pyruvate entering a tissue by inhibiting a membrane transport system that is directly responsible for transfer of pyruvate or cyanide could cause a buildup of endogenous unlabeled pyruvate thereby reducing a membrane diffusion gradient causing less labeled pyruvate to enter by passive diffusion. The low concentration of pyruvate in the lens may result from pyruvate being confined to a limited lenticular volume as suggested above for glucose. Also, since cyanide blocks the ETC, pyruvate could enter the tissue at a normal rate and then be converted into lactic acid. The trout lens does contain lactic dehydrogenase (LDH) and any lactate formed would be a freely diffusable substance which could leave the lens carrying the $C^{14}$ label with it.

In summary, glucose appears to enter the trout lens and cornea as a result of a different mechanism than is postulated for similar mammalian tissues. Neither lesioning the lens nor use of specific inhibitors for the TCA cycle on the lens or cornea changes the rate of entry, whereas, specific inhibitors used with mammalian tissues show a marked reduction of glucose entry. Inside the tissue, glucose is oxidized primarily through reactions of the TCA cycle at the environmental temperatures of rainbow trout. As the temperature rises, the HMP shunt responds in both types of tissues with a greater increase



in activity than the TCA cycle. This increase in activity lasted to 23 C in the cornea but persisted to 33 C in the lens, the respective temperatures where the tissues became opaque.

## CONCLUSIONS

1. The TCA cycle is present and actively oxidizes pyruvate to $CO_2$ in both the lens and cornea, but the activity of the cycle is greater in the cornea than the lens.
2. The HMP shunt is present, but accounts for only about 1/5 of total $CO_2$ production by the lens, and 1/3 of total $CO_2$ production by the cornea.
3. The activity of the TCA cycle in both the lens and cornea increases with rising temperatures to 33 C, then declines to 43 C; however, the cornea responds with a greater magnitude of increase than does the lens.
4. The HMP shunt is able to increase activity with rising temperatures to 23 C in the cornea before a decrease occurred, while shunt activity increased with temperature to 33 C in the lens.
5. Entrance of glucose into the lens or cornea is not dependent on energy production of the TCA cycle.
6. Pyruvate enters the lens and cornea aided by an active TCA cycle.



7. In-vivo treatment with Antimycin A which causes opacities in both the lens and cornea also results in substantial reduction of energy produced from the series of reactions for the breakdown of glucose.

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# APPENDICES

APPENDIX I

INCUBATION SOLUTIONS

Composition of Phosphate Buffered Saline (PBS)

| | |
|---|---|
| NaCL | 8.00 gm/liter |
| KCL | .20 gm/liter |
| $Na_2HPO_4$ (Anhydrous) | 1.15 gm/liter |
| $KH_2PO_4H_2O$ | .20 gm/liter |
| $CaCL_2$ | .10 gm/liter |
| $MgCL_2 \cdot 6H_2O$ | .19 gm/liter |

Composition of Krebs-Ringer-Phosphate Solution

| Constituent | Concentration (M) | Parts Mixed For Whole Medium (ml) |
|---|---|---|
| NaCL | 0.154 | 232 |
| KCL | 0.154 | 8 |
| $MgSO_4$ | 0.154 | 2 |
| $CaCL_2$ | 0.110 | 6 |

The mixture is adjusted to pH 7.4 by the addition of 0.1 N NaOH. To this is added 12 ml of M/15 phosphate buffer, pH 7.4. (This is prepared by mixing 80.8 ml of M/15 $Na_2HPO_4$ with 19.2 ml of M/15 $KH_2PO_4$.) To avoid precipitation the calcium solution and phosphate buffer were

not brought together in less than final volume. The completed solution however was stable.

Glucose was added to this medium in the amount of 0.4 ml of 5% solution to 19.6 ml of medium. The resulting medium contains in millimoles per liter approximately the following: $Na^{+}$, 140; $K^{+}$, 5; $Ca^{++}$, 2.5; $Mg^{++}$, 1; $Cl^{-}$, 144; P, 3 and $SO_4^{--}$, 1, giving a total ionic strength of 0.158 (umbreit, Burris, and Stouffer, 1964).

## APPENDIX II

### COUNTING RADIOACTIVITY

Liquid scintillation counting solution I

| | |
|---|---|
| Naphthalene | 100 g |
| POP | 10 g |
| POPOP | 250 mg |

Add Dioxane to make 1 liter.

Liquid scintillation counting solution II

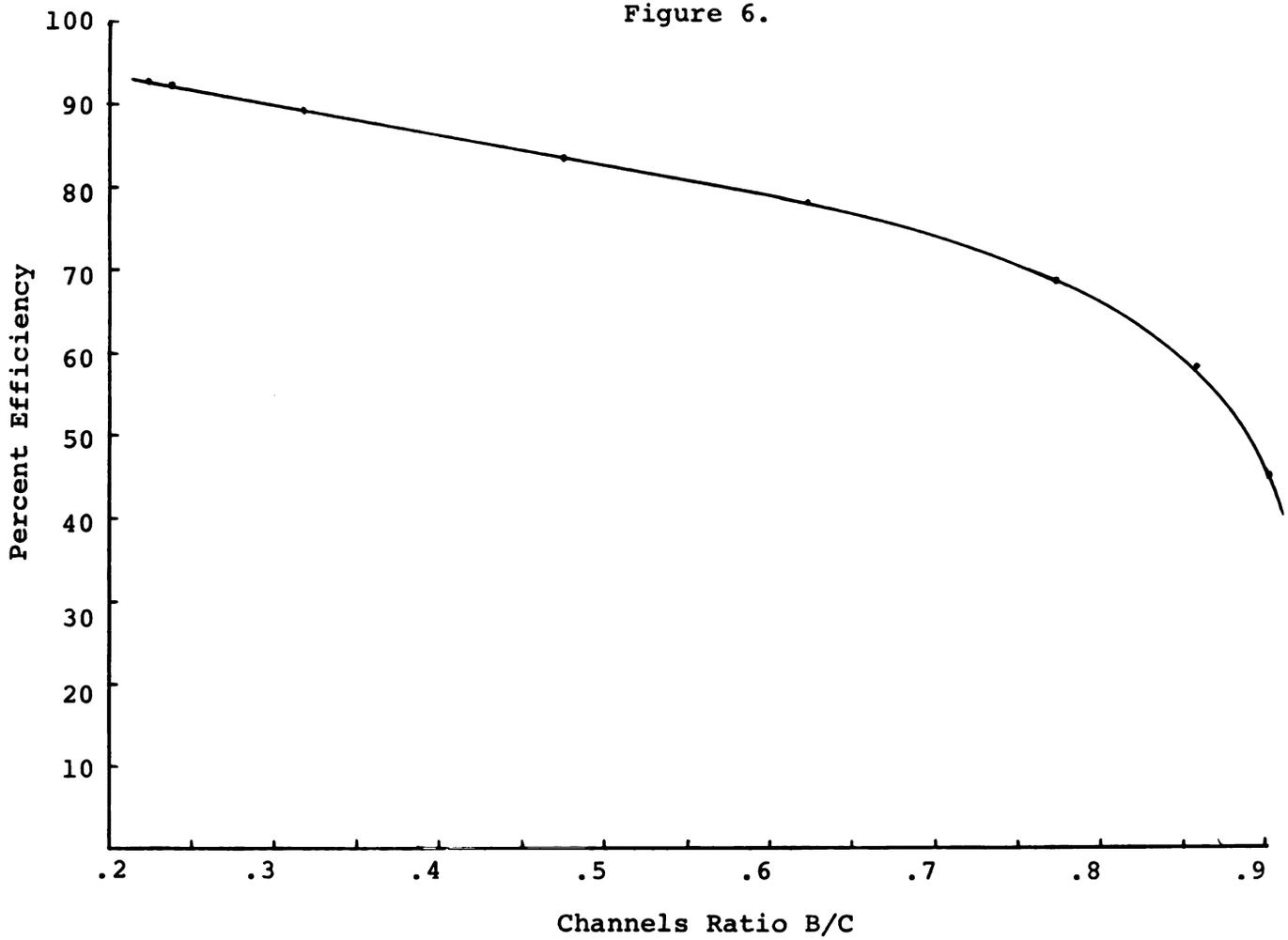
| | |
|---|---|
| Naphthalene | 80 g |
| POP | 5 g |
| POPOP | 100 mg |

Add Dioxane to make 1 liter.



Figure 6.--Sample channels ratio quench correction curve for varying degrees of quenching.

Figure 6.


100
90
80
70
60
50
40
30
20
10
Percent Efficiency
.2
.3
.4
.5
.6
.7
.8
.9
Channels Ratio B/C



## APPENDIX III

### TABLES

TABLE 10.--Comparison of $C^{14}O_2$ production from bilateral ocular tissues using uniformly labeled glucose-$C^{14}$

| | $C^{14}O_2$ (dpm/100 mg wet tissue) | |
|---|---|---|
| | Right tissue | Left tissue |
| Lens | 265.8±53.0(6)[a] | 300.5±41.5(6) |
| Cornea | 2839.3±523.7(6) | 3067.2±516.3(6) |

[a]Mean ± standard error (trials)

TABLE 11.--Comparison of $QO_2$ values obtained from constructed respirometer with literature values for liver slices

| | Mean $QO_2$ S.E. | | | |
|---|---|---|---|---|
| | Rat 1 | Rat 2 | Rat 3 | Mean |
| Constructed apparatus | 4.63±0.26* | 5.71±0.32 | 3.89±0.31 | 4.74±0.37 |
| Reineke's values | | | | |
| Warburg | | | | 4.73±0.20 |
| New design | | | | 4.86±0.19 |

[a]Mean ± standard error of 8 observations

TABLE 12.--Metabolic data from respirometer studies

| | Cornea | Lens |
|---|---|---|
| $QO_2$ @ 13 C. | | |
| (µl/gm wet wt-hr) | 52.67±2.63(35)[a] | 11.82±0.95(36) |
| (µl/gm dry wt-hr) | 315.95±15.35(34) | 26.20±1.81(36) |
| Glucose utilization | | |
| Aerobic (mg/gm-hr) | 0.28±0.02(49) | 0.14±0.01(33) |
| Anaerobic (mg/gm-hr) | 0.37±0.08(13) | 0.18±0.03(15) |
| Lactic acid production | | |
| Aerobic (mg/gm-hr) | 0.21±0.03(50) | 0.30±0.03(51) |
| Anaerobic (mg/gm-hr) | 0.84±0.13(15) | 0.47±0.09(15) |
| Per cent dry weight | 12.73±1.48(50) | 46.98±0.20(51) |

[a]Mean standard error (observations)