THE PHOTODYNAMIC ACTION OF METHYLENE BLUE ON THE CATION BALANCE OF THE ISOLATED LENS

by

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INTRODUCTION

The lens of the eye is a multicellular structure but in many respects it behaves as a single cell with the capusle assuming the role of cell membrane. For example, like most cells, the lens normally maintains a high concentration of potassium and a low concentration of sodium although bathed in fluids of high sodium and low potassium content. Until fairly recently, the consensus has been that the cellular barriers of the lens were impermeable to the positive ions. Studies with radioisotopes, however, have demonstrated that the cations of the lens readily exchange with those of the fluid environment.[1,2] It follows then, that each ion must be continuously secreted against a concentration gradient, and such a transport system has been identified. Furthermore, it seems likely that maintenance of normal hydration of the lens requires an active sodium pump.[3]

A knowledge of the mechanisms by which the active transport of cations across the lens surface is mediated is of more than academic interest. Failure of cation control leads to increased lenticular hydration and the development of an opacity or cataract. Thus cataractous lenses have lost potassium and gained sodium and water.[4] Therefore, exploration of the factors controlling lenticular cation balance should be of value in helping to elucidate both the pathogenesis of cataract and the mechanisms governing cation transport in other tissues. With respect to the latter consideration, the lens offers several technical advantages over other tissues for cation transport studies. One, it is easy to achieve artificial conditions simulating the in vivo situation because the lens is not dependent on a circulating blood supply for its nutrition. Two, the difficulties encountered in tissues where correction must

be made for extracellular fluid are obviated. Three, quantitative analyses can be made on a single lens and this is analogous to analyzing a single cell.

When one examines the anatomical relationships of the lens, it does not seem surprising that it acts as a single cell. The lens is bathed by the aqueous humor from which it derives its nutrients and into which it excretes its waste products. In this respect, it resembles a cell which is nourished from the extracellular fluid. The lens capsule which is rich in mucopolysaccharide acts as a membrane encasing the entire structure. On the anterior surface directly under the capsule is found the lens epithelium, considered to be metabolically the most active portion of the lens.[5,6]. Lens fibers with different metabolic activity make up the balance of the lens substance.* Consequently within the lens as in individual cells there is compartmentalization of metabolic pathways, each part, however, being essential for the maintenance of the whole.

Active transport of cation requires an expenditure of energy which is supplied by the metabolism of the lens. This can be shown in a variety of ways but is best demonstrated by the reversible, cold-induced cation shift.[3,8,9] When the lens is immersed in a balanced salt solution approximating the composition of aqueous humor and the metabolic rate is reduced by chilling, potassium leaves the lens and sodium and water enter.

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*The lens is unique in that it does not desquamate old tissue[6] as new cells form. Instead, the cells lengthen and are pushed backward and inward to form lens fibers. As a result, the central portion of the lens (the nucleus) is composed of densely packed old fibers of low metatolic activity. Fibers located on the periphery (the lens cortex) are younger and possess greater metabolic activity.[7]

If, then, the metabolic activity of the lens is restored by raising the temperature to 37°C. migration of potassium into and extrusion of sodium and water from the lens occurs. These temperature reversible studies have proved to be a convenient and sensitive tool for observing various influences which effect the cation transport of the lens and to a certain extent the viability of the lens itself.

The energy used for active transport is probably derived from the metabolism of carbohydrates. Enzymes or intermediates of the Embden-Meyerhof glycolytic cycle,[10-13] the tricarboxylic acid cycle,[14,15] and the hexose monophosphate shunt[16,17] have been demonstrated. Kinoshita has shown that in the lens, the major portion of the glucose is metabolized only as far as lactic acid via the pathway of anaerobic glycolysis and that the hexose monophosphate shunt is the preferred aerobic pathway.[17] The absolute glucose requirement of the lens is low and dependent to a considerable extent on the age of the lens. Young lenses are able to maintain normal cation balance in a medium which is low in or depleted of glucose considerably better than older lenses.[18] If conditions are made even more unphysiologic by excluding oxygen, even the young lens loses its ability to maintain the normally observed cation relationship with its environment.

That the active transport of sodium from and potassium into the lens occurs across a barrier at or near the lens surface has been inferred from the following in vitro observations. When the lens capsule is altered by physical means, such as trauma or actual dehiscence, the cation content approaches equilibrium with the extralenticular fluid and hydration occurs.[7,8] Moreover, depletion of calcium from the medium permits equilibration with the bathing fluid.[7,9] In general it is well known that

animal membranes demonstrate greater permeability to solutes when calcium is omitted from the surrounding medium.

The investigation described here is concerned with the effect of methylene blue on cation transport of the lens. Our interest in the problem was aroused by recent reports of the deleterious action of methylene blue on ocular tissues. Constant found that lenses maintained in vitro in a medium containing methylene blue developed vacuoles and displayed altered mitotic activity in the epithelium.[19] Since these same changes are observed in cataract formation, methylene blue can be considered to induce cataracts in vitro. In addition, Philpot has reported that many dyes,[20] including methylene blue, caused hydration of the cornea when injected intraocularly and this observation has been confirmed in our laboratory. Inasmuch as many conditions causing hydration of the cornea also cause hydration of the lens, it seemed reasonable to investigate the effects of methylene blue on hydration and cation transport in the lens in the hope of gaining further insight into the mechanisms governing these processes.

EXPERIMENTAL

Tissue

Paired rabbit lenses were used throughout. Unless otherwise indicated, they were obtained from a local slaughter house. The rabbits were decapitated and the eyes removed immediately. In most cases, they were immersed in physiological saline in tubes packed in ice for transport to the laboratory. Occasionally, when lenses were to be incubated without prior refrigeration, the eyes were brought to the laboratory at ambient temperature.

Lenses were removed from the eye as quickly as possible with aseptic precautions being observed. The eyes were opened posteriorly by dividing the sclera into quarters, and these sections of sclera were then turned back exposing the vitreous body and posterior surface of the lens. The vitreous was pulled away gently with a lens loop, and the zonules, which hold the lens in place, carefully cut with a pair of fine scissors. Extreme care was taken to avoid touching the lens since even slight trauma impairs the ability of the lens to maintain its normal cation balance.

Cation Studies

For the most part, the reversible cold-induced cation shift was used. For these studies, the lenses were placed on their posterior surfaces in tubes containing 3 ml. of a balanced salt solution (Appendix I) approximating the ionic composition of aqueous humor. Unless otherwise stated, the tubes were flushed with a gas mixture composed of 95%O_2-5%CO_2, tightly stoppered, and placed in an ice chest at 0°C. for 40 to 44 hours. Following refrigeration, one lens was removed for analysis and the other lens was incubated at 37°C. for 6 hours* to determine recovery from the

*Previous studies have indicated that a new steady-state has been reached at the end of six hours and that a more prolonged period of observation is not necessary.[9]

cold-induced cation shift. (The lenses were in darkness during the period of refrigeration. During the incubation at 37°C. the lenses were exposed to ambient light unless otherwise noted.)

Occasionally lenses were incubated at 37°C. for 24 hours without previous refrigeration. In general, this type of study is not as sensitive an indicator of the factors determing cation balance as the temperature reversible studies, but it does provide a more direct experimental procedure.

For analysis, the lenses were blotted to remove excess moisture, rapidly weighed in tared silica crucibles on a Mettler microbalance, and dried at 105°C. for 40 hours.* The water content was calculated from the difference between the wet and dry weights. The lenses were then charred under infra-red lights with the aid of a drop of concentrated sulphuric acid, and ashed in a muffle furnace at 475°C. for 18 to 22 hours or until no charred ash was visible.

Lens ash was taken up in hot distilled water and transferred quantitatively to a 25 ml. volumetric flask. Lithium sulfate was added to provide a final concentration of 150 ppm of lithium. The sodium and potassium analyses were carried out on a Baird Associates flame photometer with an internal standard. To insure accuracy, known sodium and potassium solutions were analyzed each time. Results were calculated as mEq/1000 gm. of lens water.

Results of the cold-induced cation shift experiments are expressed as per cent recovery and indicate the ability of the lens to return toward normal by excreting sodium and concentrating potassium following a period of equilibration in the cold. It is calculated from the formula $\frac{Cr - Ci}{Cr - Cn} \times 100$

*Previous studies had indicated that constant weight is attained in 40 hours of drying at 105 C.[8]

in which Cr indicates the cation concentration of the refrigerated lenses, Ci the cation concentration in lenses refrigerated at 0°C for 40-44 hours followed by incubation at 37°C. for 6 hours, and Cn the cation concentration in fresh lenses which have been neither refrigerated nor incubated.

<u>Gas Consumption Studies</u>

Eyes were obtained from 3 to 4 kilogram rabbits killed in the laboratory by air embolism. Lenses were removed in the manner previously described, weighed and placed in the flasks. The construction of the flasks made it impossible to place the lens on its posterior surface so a random distribution of positioning must be assumed.

An Aminco Warburg apparatus was used for gas consumption studies. The flasks were of approximately 17 ml. capacity with a center well and a side arm. Four ml. of medium* were placed in the main compartment and 0.6 ml. of a Pardee's solution[21] in the center well. (Appendix II)** Fluted filter paper strips were inserted in the center well to increase the absorbing surface.

Two water thermobarometers and two blanks containing medium and Pardee's solution were run with each experimental situation. In experiments where light was to be excluded the flasks were wrapped in aluminum foil. Flasks which were to receive lenses were packed in ice until they were attached to the manometers and placed on the machine. After the

*Appendix I.

**A phosphate buffer is used for oxygen consumption studies whenever possible. However, control studies had shown that phosphate buffers did not support cation transport as well as our usual medium which has a bicarbonate concentration approximating that found in the aqueous humor, (30 mEq./l) Pardee's solution is a reversible CO_2 buffer system designed to maintain a constant atmospheric CO_2 tension.[22,23] This it does with reasonable efficiency and is valid within limits when the oxygen consumption is high. Actually the oxygen consumption of the lens is low[6] and the results obtained cannot be interpreted as absolute values of oxygen consumption.

flask and manometer assemblies had been attached to the instrument for a few minutes, they were removed and the ground glass joints were reseated to assure a gas-tight connection. Flasks were then gassed with $95\%O_2$-$5\%CO_2$ for a 15 minute equilibration period, the height of the fluid in the manometer was adjusted and zero readings were taken at $37^{o}C$. Gaseous exchange was measured over a 2 hour period. At the end of this time the lenses were quickly removed for phosphate determinations. The usual corrections for changes due to temperature, pressure and medium were made and the results are expressed as micro liters of gas exchanged per lens per 2 hours.

Phosphate Determinations

In vascular tissues specimens must be frozen almost instantaneously to preserve creatine phosphate. Therefore, several techniques were explored to determine the best procedure for handling the lenses to avoid loss of the labile components of the phosphate fraction. In one method the intact eye was placed in acetone-dry ice and frozen immediately. The lens was removed from the eye while still frozen and weighed in a tared tube containing frozen trichloracetic acid. The lens was triturated with a glass rod and as the lens-acid mixture thawed it was refrozen before trituration was continued. In a second method, the lens was removed as for cation studies, weighed rapidly and placed in a tube containing frozen trichloracetic acid, and immediately frozen by placing the tube in dry ice. The lens was then triturated as above. Last, the lens was removed as for cation studies, weighed, and quickly homogenized in ice cold trichloracetic acid in a Potter-Elvjehm homogenizer. Subsequent handling was the same in all procedures.

No significant difference was observed in the inorganic phosphate, creatine phosphate, or ATP-ADP fractions of lenses initially handled in

the ways described above. For this reason the third technique was routinely employed. Apparently speed in handling is not so important with the lens as with other tissues, probably because the lens is an avascular structure having a low metabolic rate. (Table I)

The lens-acid mixture was then thawed and frozen three times during a ten minute extraction period, centrifuged for one minute, and filtered into tubes packed in dry ice. To determine the different phosphate fractions the filtrate was treated in the method described in Unbreit's Manometric Techniques.[21]

The filtrate was thawed and an aliquot was immediately pipetted into a calibrated centrifuge tube and quickly neutralized with 20% NaOH using phenolphthalein as an indicator. The true inorganic phosphate was precipitated with calcium, leaving creatine phosphate in solution, and the phosphate determination made on the precipitate. Since creatine phosphate is unstable in acid solution, phosphate determination of the crude filtrate gives an apparent inorganic phosphate value which actually contains the true inorganic phosphate plus the creatine phosphate. The difference between the apparent inorganic phosphate and the true inorganic phosphate is considered to be creatine phosphate. Another aliquot was hydrolyzed in 1 N hydrochloric acid for seven minutes at 100°C.; the difference between this phosphorus value and the apparent inorganic phosphorus value is considered to be the ATP-ADP fraction. Total phosphate was obtained by digestion of the filtrate with concentrated hydrochloric acid and oxidation with 30% hydrogen peroxide at 150°C.

The method of Gomori[24] was used for determining the amount of phosphorus in the various fractions. Color was developed by molybdate sulphuric acid reagent and Elon reducing reagent. After a 60 minute color

developing period the determinations were read in a Coleman Jr. spectrophotometer at 690 millimicrons. Results were expressed as mg. per 100 gm. of wet lens.

RESULTS

Cation Content of Normal Lenses

In order to obtain a yardstick for measuring deviations from the normal, a series of lenses were analyzed for sodium, potassium and water immediately upon removal from the eye. These figures for fresh lenses represent the concentrations normally found in lens tissue. Another series of lenses, designated as control lenses, were subjected to the reversible, cold-induced cation shift procedure described previously. The data are tabulated in Table II. Note that the lens lost approximately 35 mEq. of potassium and gained an equivalent amount of sodium during refrigeration. During the subsequent incubation at 37°C., this sodium was excreted and the potassium concentration returned toward normal, attaining approximately 100% recovery.

Effect of Methylene Blue

Lenses were bathed in the usual balanced salt solution containing varying concentrations of methylene blue and subjected to the reversible cation shift technique. Dye concentrations of 5×10^{-5} M and above abolished cation recovery and caused hydration of the lenses.* A methylene blue concentration of 1×10^{-5} M inhibited cation recovery only slightly and lower concentrations had no effect. (Fig. 1)

Effect of Light

Methylene blue has been shown to exhibit a photodynamic action on many biological systems. For instance, upon exposure to methylene blue

*Dye content of the commercial reagent was not taken into account in calculating the amount to use. Since the reagent used assayed 89% methylene blue, the actual molar concentration of the dye is somewhat less than indicated.

in the light, some bacteria[25] are killed, some bacteriaphages[26] and viruses[27] are inactivated, whole blood loses its ability to clot[28] and erythrocytes[29] are hemolyzed. These actions of methylene blue are not manifest in the dark.

The primary event in photodynamic action is the absorption of a quantum of light by the sensitizing material,[31] in this case methylene blue, to form an active dye molecule. Not all activated molecules will react with a substrate but no molecule can react without absorption of light. The action spectrum of methylene blue is in the visible range and corresponds to its absorption spectrum.

To determine whether the deleterious effects of methylene blue on the lens were photodynamic in nature, lenses bathed in 5×10^{-5} M methylene blue were subjected to the cold-induced cation shift in the absence of light. (Fig. 2) These lenses recovered significantly from the stress placed upon them by chilling in contrast to the previous experiments carried out in ambient light, in which cation recovery was abolished by the same concentration of methylene blue.

Further study of the light sensitivity was accomplished by incubating lenses without prior refrigeration at 37°C. for 24 hours. Ambient light was permitted during the day, and at night light was supplied by a 40 watt bulb approximately 14 inches from the lenses. The ambient light varied by as much as 100% but roughly it corresponded to 4.0×10^2 ergs/cm²/sec. Normal lenses maintain their cation content during a 24 hour incubation period at 37°C. Indeed, the potassium concentration tends to increase during this period, with a resulting increase in total base. Lenses exposed to methylene blue in the dark showed the same general behavior. (Fig.3) However, lenses exposed to methylene blue in the light for 24 hours at

$37^{\circ}C$ gained sodium and lost potassium, the total base, however, increasing.

To determine if this was an induction period in the light followed by further change in the dark, lenses were incubated at $37^{\circ}C$. as above. Both lenses of a pair were exposed to light for 6 hours. At that time, one lens was removed for analysis and the other lens was incubated for an additional 18 hours in the dark by wrapping the tubes with black electrician's tape. The lenses analyzed at the end of 6 hours had also concentrated potassium and little further change occured in the subsequent 18 hours. Therefore, whatever the nature of the photodynamic action, it seems to require an induction period before the results are detectable in the cation shift. A similar induction period is seen when erythrocytes are treated with enzyme poisons such as fluoride.[31]. It is also evidenced when erythrocytes are exposed to rose bengal in the light.[32]

Further investigation of the light requirement was carried out by reducing methylene blue to its colorless state with sodium hydrosulfite. Since photodynamic action can occur only when light is absorbed by the sensitizing molecule, if methylene blue were reduced to its colorless form, considerably less light would be absorbed and hence photodynamic activity should be reduced. To achieve a satisfactory experimental situation, certain alterations in our usual procedure were required. If the reduced solution was flushed with the usual $95\% O_2$-$5\% CO_2$ mixture, reduced methylene blue was promptly oxidized. However, when atmospheric air was employed this oxidation was not evident for several hours.

Two control situations were therefore necessary. One served as a control for the altered gas phase; the other served as a control for the sodium hydrosulfite, receiving as much hydrosulfite as was added to the methylene blue medium. Reducing the oxygen tension from that of

95% oxygen to atmospheric air had no appreciable effect on cation recovery. The hydrosulfite itself was toxic to the lens system, but nevertheless the lenses recovered appreciably in the presence of reduced methylene blue. (Fig. 4)

Effect of Oxygen

Results obtained thus far indicate that the major action of methylene blue is photodynamic in nature, i.e., light is essential for the reaction. One of the outstanding characteristics of photodynamic activity as observed in other systems, is its enhancement by molecular oxygen. This was investigated with the lens.* Methylene blue medium was equilibrated with 95% N_2-5% CO_2, the tubes were overlaid with the same gas mixture, and studies of the temperature reversible shift carried out in ambient light as previously described. These lenses recovered noticeably from the cold-induced cation shift. (Fig. 5) Certainly, the presence of oxygen enhances the photodynamic action of methylene blue on cation transport in the lens.

Effect of Catalase

No one has yet been able to define the exact role played by oxygen in this complex photodynamic reaction. Predominant thinking at this time favors the concept that an essential material, i.e. protein, complexes with the activated dye or that protein and dye form a complex which is activated and the material is then oxidized in the presence of

*Actually the requirement for oxygen by the lens has not been definitely established. The relative importance of the Embden-Meyerhof cycle would suggest that the requirement should be minimal. Conflicting results have been obtained in our laboratory on the oxygen requirement for cation transport in the lens. Early data indicated a definite requirement.[3] However, other workers in the same laboratory have found cation transport to be little altered under anaerobic conditions. The reason for the discrepancy has not been resolved. The data given here are my own.

molecular oxygen.[30]. However, in the absence of readily oxidizable material, and in some cases in its presence, it has been shown that hydrogen peroxide is formed.[33,34] Since the lens protein is contained within the capsule and is not readily available for complexing with the extralencicular dye it seemed that toxic concentrations of hydrogen peroxide might possibly be formed in the bathing medium. If this were the case, addition of catalase should block the toxic effect of methylene blue by destroying the hydrogen peroxide. To test this, 10 units of catalase were added to the methylene blue medium in which the lens was immersed and the temperature reversible cation shift measured in the usual manner under ambient light. The catalase appeared to improve cation recovery to a slight extent. (Fig. 6) However, in dilute solutions catalase is quite unstable and readily denatured, making it seem likely that addition of the catalase just prior to the 6 hour incubation period at 37°C instead of before the 40 hour refrigeration might provide a more favorable experimental situation. Presumably hydrogen peroxide would be formed exclusively during the incubation at 37°C because it is at this time that the lenses are exposed to light. Significant cation recovery occured in this experiment. (Fig. 7) Increased recovery was also obtained when boiled catalase was added although it was not so great. (Fig. 8) Probably the protective effect of catalase is not due solely to enzyme activity or to added protein but to a combined effect of both.

On the surface it appears that some hydrogen peroxide is formed in the incubating solution and is toxic to the lens. However, this has not been substantiated by situations in which hydrogen peroxide was incorporated into the medium. Following refrigeration at 0°C and prior

to incubation at 37°C. 0.1 ml. of hydrogen peroxide of such strength as calculated to give a 1×10^{-6} M or a 1×10^{-5} M* solution was added. No inhibition of cation recovery was observed. (Fig. 9)

There are several possible explanations for this. First, the substance is unstable and the actual concentration might be lower than the calculated value. Second, a little hydrogen peroxide being produced continuously might prove more harmful to the lens than a great deal added all at once. Third, the site of accumulation or production may be quite critical.** Fourth, hydrogen peroxide may be without effect.

Oxygen Consumption Studies

Methylene blue is know to stimulate oxygen consumption in erythrocytes,[37,38] acites tumor cells[39] and other tissues. It is also a well known uncoupling agent of oxidative phosphorylation. For these reasons studies of the gaseous exchange and phosphate fractions of the lens were made.

As noted earlier, the bicarbonate buffer provides a much more physiological medium for the lens than does a phosphate buffer. Indeed, phosphate buffer appeared to be toxic to cation transport activity. For this reason our usual bicarbonate medium was used. The results obtained with this system cannot be considered as the actual oxygen uptake and are significant only when compared with data obtained under like conditions. When immersed in the bicarbonate solution or in the methylene blue solution

*Higher concentrations were not employed because it was felt that hydrogen peroxide concentration would always be considerably less than that of the dye.

**The lens contains catalase,[35] however an assay of catalase in other ocular structures has shown minimal amounts in aqueous and vitreous,[36] If one assumes that catalase is found where hydrogen peroxide is formed it can be inferred that the exterior of the lens is not normally exposed to hydrogen peroxide.

with light excluded, no consumption of gas by the lens could be measured. Actually they appeared to have given off gas. This can probably be attributed to the production of acid metabolites and consequent release of CO_2 from the medium which did not equilibrate with Pardee's solution immediately. However, there was a satistically significant difference between the results obtained in these two situations and the results obtained when the lenses were exposed to methylene blue in the light. ($p<0.05$) In the latter situation, gas consumption was observed. (Fig.10) These data can be interpreted as indicating a photodynamic stimulation of oxygen uptake by lenses exposed to methylene blue solution and are therefore, comparable to data obtained using other tissue.

Phosphate Determinations

To maintain cation balance, energy must be expended by the lens. The immediate energy would reasonably be obtained from high energy phosphate. In view of the uncoupling action of methylene blue, it seemed important to determine whether there was a decrease in high energy phosphates in the lens. Analysis of the phosphate fractions of the lenses used in the oxygen consumption studies did not show a significant difference in total phosphate, high energy phosphate, or inorganic phosphate. (Table III)

However, changes in phosphate might not become apparent in two hours in a tissue metabolizing as slowly as the lens. Hence, a series of lenses were incubated for 24 hours at 37°C in 5×10^{-5} M methylene blue, one of the pair in the light and one in the dark. Lenses exposed to light had less high energy phosphate and more inorganic phosphate than had the lenses from which light had been excluded. (Table IV)

DISCUSSION

Our results leave little doubt that the deleterious action of methylene blue on the cation transport is for the most part photodynamic in nature. First, methylene blue in a concentration which had previously abolished cation transport in lenses exposed to light, had very little effect on cation recovery when light was excluded. Second, lenses equilibrated for a prolonged period in methylene blue in the dark at 37°C. were able to maintain their normal cation content, while lenses exposed to methylene blue in the light lost potassium and gained sodium. Third, lenses incubated in colorless (reduced) methylene blue showed much better recovery than those incubated in equimolar concentrations of oxidized methylene blue.

(Blum attributes this protective action of a reducing agent to its interference in an oxidizing process;[40] we look upon it as simply another way of showing that light is essential. In any event, the reduced dye is colorless and absorbs little light in the visual spectrum. Therefore the primary step in the photodynamic process, the absorption of light, is absent or at least considerably reduced.)

It is interesting that some substances, by the absorption of light, can cause photochemical reactions which lead to cataract formation. This may be significant since some theories of cataract formation suggest that light itself is a deleterious agent. It also suggests that some of the observed reactions of dyes on biological material are really photodynamic in nature. Certainly the vacuoles found by Constant[19] can be interpreted as such.

A requirement for oxygen for the photodynamic effect of methylene blue on cation transport has also been demonstrated. The harmful action of methylene blue is not demonstrable in the absence of

oxygen. This result was not unexpected because most photodynamic systems either require oxygen for the reaction or the reaction is enhanced by the presence of oxygen.[30]

One can envisage the normal cation balance as resulting from active transport against a concentration gradient and passive leakage along a concentration gradient. Therefore, any change in cation concentration reflects alteration of one or both regulatory mechanisms. It is possible that methylene blue exerts its effect on both processes, if indeed, they can be separated. For the purpose of exposition, we will say that methylene blue acts in one of two fashions. Either it alters active transport by actually reducing the amount of energy produced or by interfering with utilization of the energy available, or it alters the characteristics of the barrier so that leakage increases.

In any event, the photodynamic effect on cation balance seems to be a surface phenomenon. During incubation in methylene blue solution, the blue dye concentrates at the lens surface and little is found in the lens nucleus. (This cannot be taken as positive evidence that no methylene blue has reached the nucleus because the possibility of reduction of the dye to leucomethylene blue must be considered.[41]) Other workers have shown that the dye is adherent to cell membranes. Ferlin and Hedrick,[42] working with yeast cells, were able to elute the dye from the surface of the cells by heavy metals and acid solutions. Freeman and Geise,[43] and Davson and Ponder[32] also support the theory of surface action in agents which exert a photodynamic effect.

Davson found that the permeability of the red cell to potassium was increased by exposure to rose bengal in the light. He theorized that this occurred because the membrane was oxidized by the activated

dye molecule. This dye molecule could oxidize several molecules of substrate simply by absorbing another quantam of light after losing the previous one in an oxidation process. Following this line of reasoning, he explained his observation of an initial slow leakage of potassium from the red cell followed by a period of more rapid loss, ultimately leading to the breakdown of the cell. This could also explain our finding a negligible change in cation concentration in lenses exposed to the light for 6 hours followed by 18 hours in the dark at 37°C. as opposed to the accumulation of sodium and reduction of potssium found in lenses exposed for a full 24 hours to methylene blue in the light.

By analogy then, one must credit the possibility that the photodynamic effect of methylene blue in the lens is due to an oxidation of a barrier which restricts the passive movement of cation along a concentration gradient. This barrier could be a protein, and proteins are known to be photodynamically oxidized by methylene blue. Weil[44-47] and co-workers have shown the most readily oxidized amino acids to be the cyclic histidine, tryptophan, and tyrosine, and the sulfur containing cysteine, and methionine. Oxidation of these amino acids in essential proteins and enzymes could alter the characteristics of the barrier.

The active transport mechanism is dependent upon energy and this is intimately tied up with phosphorus metabolism,[48,49] particularly as it relates to high energy phosphates, the immediately available source of energy of the lens. Considering glucose as a measure of energy input it seems likely that the energy input is not reduced when the lens is subjected to methylene blue, either in the dark or light. Harris, et al[50] have shown that there is increased glucose uptake in

both cases. Methylene blue also stimulates the uptake of glucose in the erythrocyte.[37,38,51] *

While the energy input does not seem to be reduced it is conceivable that the available energy required for cation transport might be reduced. Strong supportive evidence is given by the fact that there is considerably less high energy phosphate available to lenses which have been exposed to methylene blue in the light than to those exposed to methylene blue in the dark. To our knowledge this is the first demonstration that the uncoupling activity of methylene blue requires light. It is tempting to say that active cation transport is reduced in these lenses because of a decrease in the amount of available energy. However, in a system as complex as this, conclusions must be guarded.

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*It has been shown that the increased glucose consumption proceeds via the hexose monophosphate shunt.[51,52] The energy derived from this path is probably from the oxidation of TPNH. In most tissues, the electrons pass through the cytochrome system but it has been suggested that glutathione[53,54] may be a part of the electron transport system in the lens.

SUMMARY

1. Methylene blue exerts a deleterious photodynamic action on the cation recovery of rabbit lenses following a cold-induced cation shift. This is characterized by being light and oxygen dependent.

2. Catalase appeared to protect against this photodynamic action. However, no deleterious effect of hydrogen peroxide in the concentrations employed was noted.

3. Comparative studies were made of gas consumption of lenses bathed in bicarbonate buffer and in methylene blue medium in the presence and absence of light. The results can be interpreted as indicating a photodynamic stimulation of oxygen consumption.

4. Phosphate determinations were made in lenses bathed in methylene blue in the presence and absence of light. Less high energy phosphates were found in lenses exposed to the light.

5. It is concluded that the photodynamic action of methylene blue on the cation transport of the lens is exerted in one of two fashions. Either the characteristics of the barrier are altered so that passive leakage of cation increases, or active transport is altered because of a decrease in immediately available energy.

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APPENDIX I

COMPOSITION OF BASIC MEDIUM

Constituient	Mg/100 cc
Glucose	200.0
NaCl	689.8
KCl	38.0
$MgCl_2 \cdot 6\ H_2O$	10.0
$CaCl_2 \cdot 2\ H_2O$	26.5
$NaH_2PO_4 \cdot HOH$	2.5
$NaHCO_3$	250.0

This medium contains Na^+ 148 mEq/1, K^+ 5 mEq/1, HCO_3^- 30 mEq/1.

Unless otherwise stated medium was equilibrated and pH adjusted to 7.25-7.50 with $95\%O_2$-$5\%CO_2$.

Methylene blue medium was made by dissolving the dye in the basic medium. Unless otherwise noted the dye concentration was 5×10^{-5} M.

APPENDIX II

PARDEE'S SOLUTION

Constituient	Amount
Diethanolamine	6 ml.
Thiourea	15 mg.
$KHCO_3$	3 gm.
6 N HCl	4.8 ml.
Distilled Water	5.2 ml.

TABLE I

Effect of Various Techniques on the Phosphate Fractions of Lenses Prepared Initially in the Manner Described

Procedure	Phosphorus - mg/100 gm. Wet Weight					mg./lens
	Inorganic	Creatine Phosphate	ATP-ADP	High Energy	Total Acid Soluble	High Energy Phosphate
Lens frozen during entire procedure	7.8 ± 0.5	1.1 ± 0.3	15.4 ± 2.8	16.5	31.6 ± 2.1	.0488
Lens weighed, then frozen during remainder of procedure	7.4 ± 2.0	1.4 ± 0.9	15.0 ± 2.2	16.4	38.4 ± 4.8	.0518
Lens not frozen. Homogenized in cold TCA*	8.1 ± 0.7	1.7 ± 0.8	19.0 ± 2.1	20.7	39.4 ± 1.6	.0520

*These lenses were smaller than lenses in the other two groups so it was felt that a truer picture of high energy phosphate would be given if figured per lens. This is found in the last column.

TABLE II

Concentration of Sodium and Potassium in Fresh Lenses, Control Lenses Bathed in Balanced Salt Solution for 40-44 hours at 0°C. and Control Lenses Bathed in Balanced Salt Solution for 40-44 hours at 0°C. followed by 6 hours at 37°C.

Procedure	Potassium mEq/1000 Gm. Water	Per cent Recovery	Sodium mEq/1000 Gm. Water	Per cent Recovery	Water
Fresh lenses	123.4 ± 3.7		22.3 ± 4.9		66.5 ± 1.3
Control lenses 0°C. for 40 hr.	88.8 ± 15.4		59.2 ± 15.4		66.8 ± 1.0
0°C. for 40 hr. plus 37°C. for 6 hr.	125.1 ± 7.7	105	24.3 ± 5.2	95	66.0 ± 1.0

TABLE III

Phosphate Fractions of Rabbit Lenses Determined After Two Hours Incubation at 37°C. in Indicated Medium

Procedure	Phosphorus - mg/100 gm. Wet Weight					
	Inorganic	Creatine Phosphate	ATP-ADP	High Energy	Organic Acid Soluble	Total Acid Soluble
Balanced Salt Solution	5.0 ± 1.0	1.2 ± 1.3	18.0 ± 2.4	19.2 ± 2.2	32.1	37.1 ± 5.0
Methylene Blue 5×10^{-5}M Light excluded	5.8 ± 1.4	2.9 ± 1.7	18.0 ± 3.8	20.9 ± 4.0	36.1	41.9 ± 3.7
Ambient light	6.8 ± 2.0	2.1 ± 2.0	18.2 ± 2.1	20.3 ± 2.6	34.5	41.3 ± 2.9

TABLE IV

Effect of Light on Phosphate Fractions of Rabbit Lenses Bathed in a Medium Containing 5×10^{-5} M Methylene Blue for 24 hours at 37°C.

Procedure	Phosphorus - mg/100 gm. Wet Weight					
	Inorganic	Creatine Phosphate	ATP-ADP	High Energy	Organic Acid Soluble	Total Acid Soluble
Ambient Light	12.4 ± 4.4	1.2 ± 1.1	15.5 ± 3.5	16.7 ± 3.8	32.6	45.0 ± 7.6
Light Excluded	9.9 ± 1.7	1.1 ± 1.0	19.2 ± 3.3	20.3 ± 3.4	33.9	43.8 ± 7.8
	$p < 0.01$			$p < 0.01$		

Figure 1. Effect of varying concentrations of methylene blue on cation recovery of rabbit lenses during incubation at 37°C. following a cold induced cation shift. (Each bar represents the average figure of at least 9 pair of lenses.)

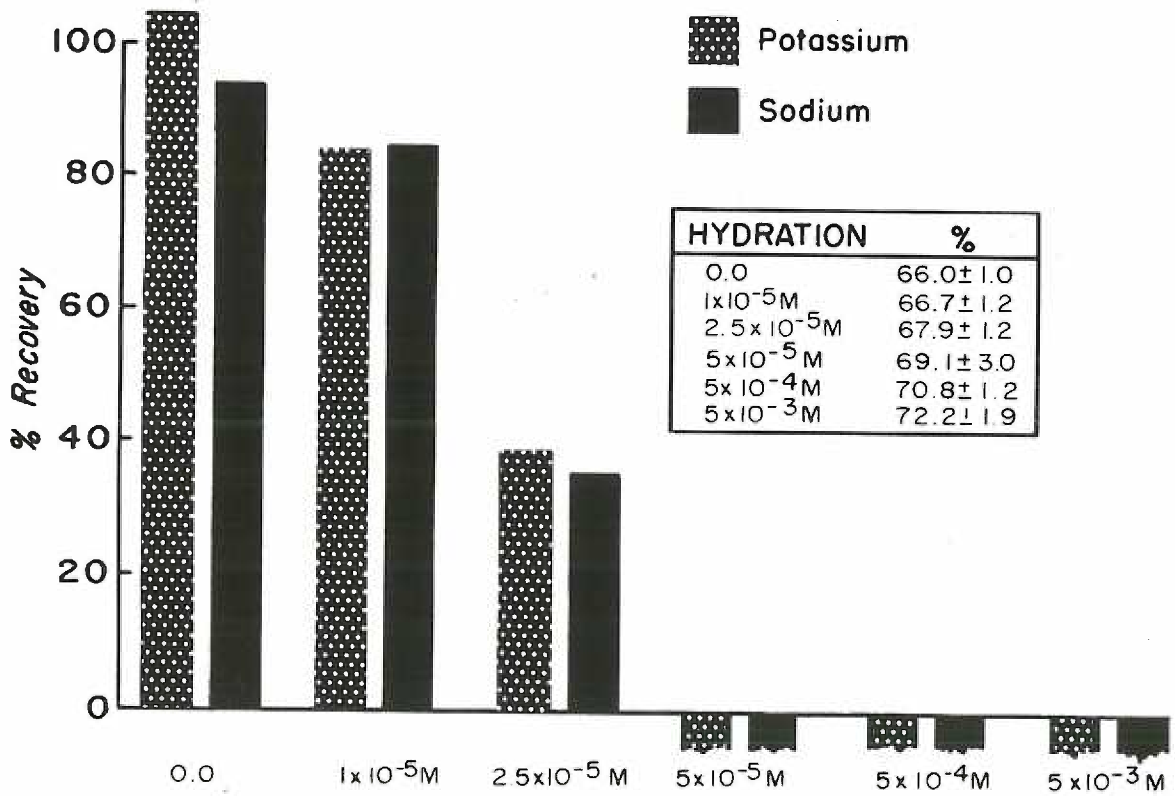
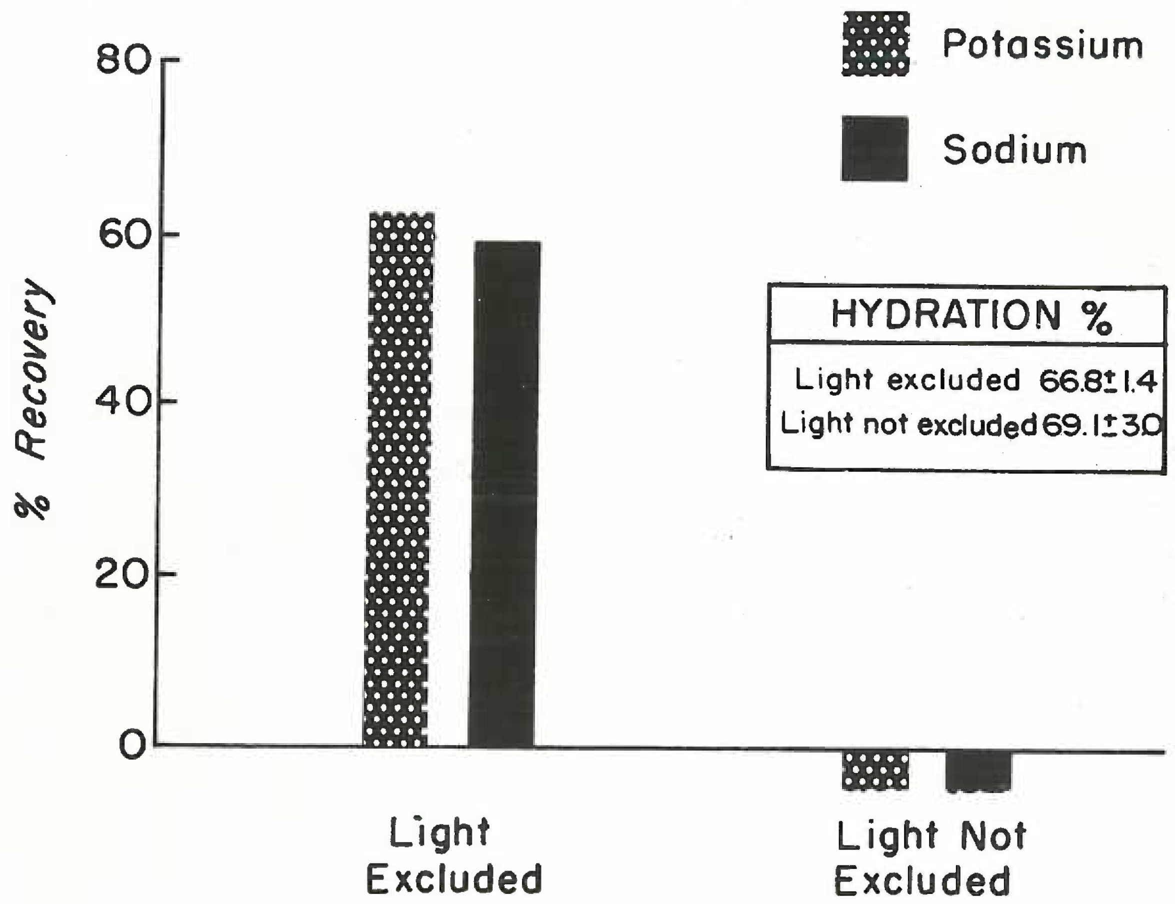
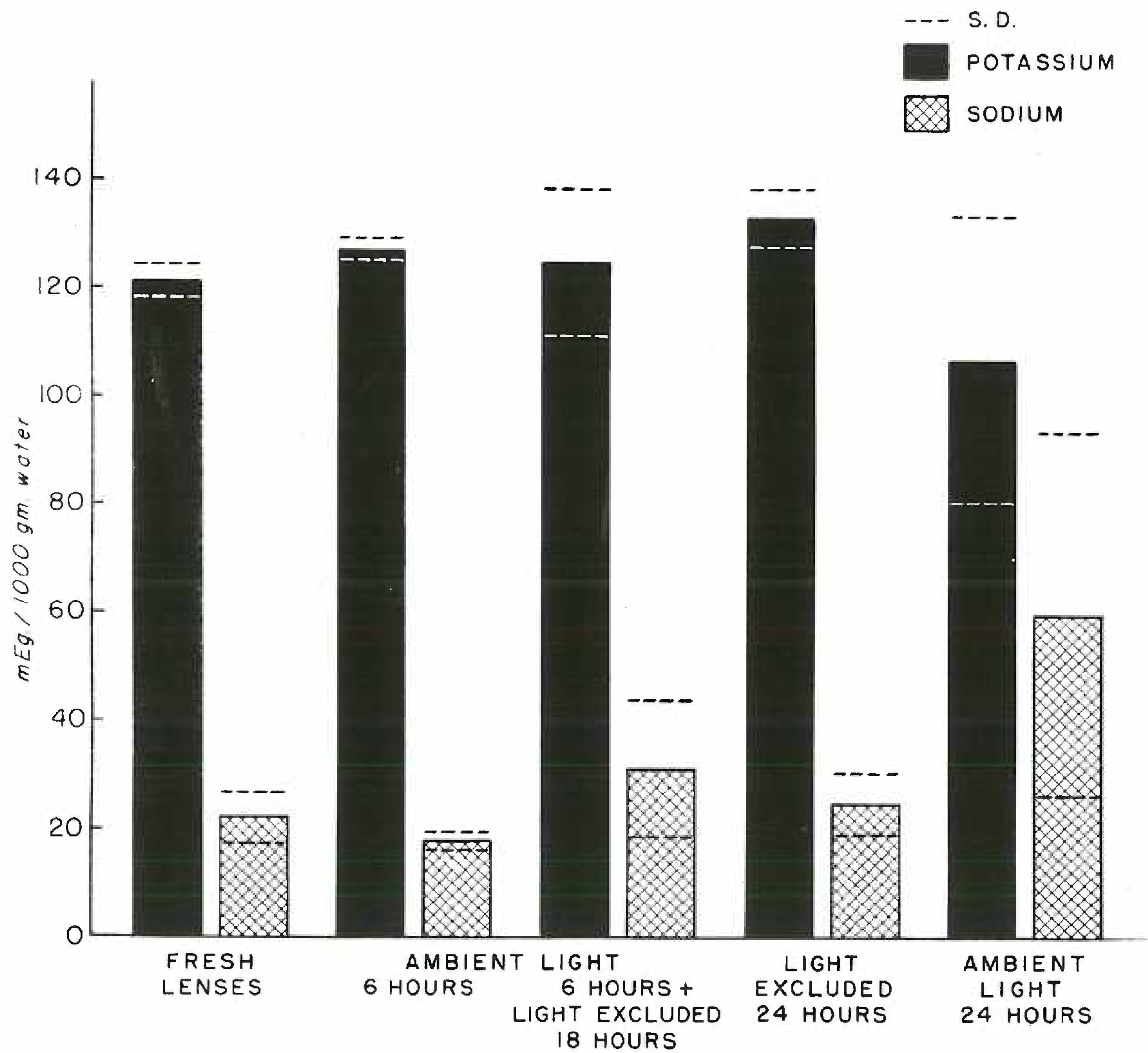

100
80
60
40
20
0
% Recovery
Potassium
Sodium
HYDRATION %
0.0 66.0± 1.0
1x10-5M 66.7± 1.2
2.5x10-5M 67.9± 1.2
5x10-5M 69.1±3.0
5x10-4M 70.8± 1.2
5x10-3M 72.2± 1.9
0.0
1x10-5M
2.5x10-5M
5x10-5M
5x10-4M
5x10-3M

Figure 2. Effect of ambient light on cation recovery of rabbit lenses during incubation at 37°C. following cold-induced cation shift. Bathing medium contained 5×10^{-5} M methylene blue. (Each bar represents the average of at least 9 pair of lenses)



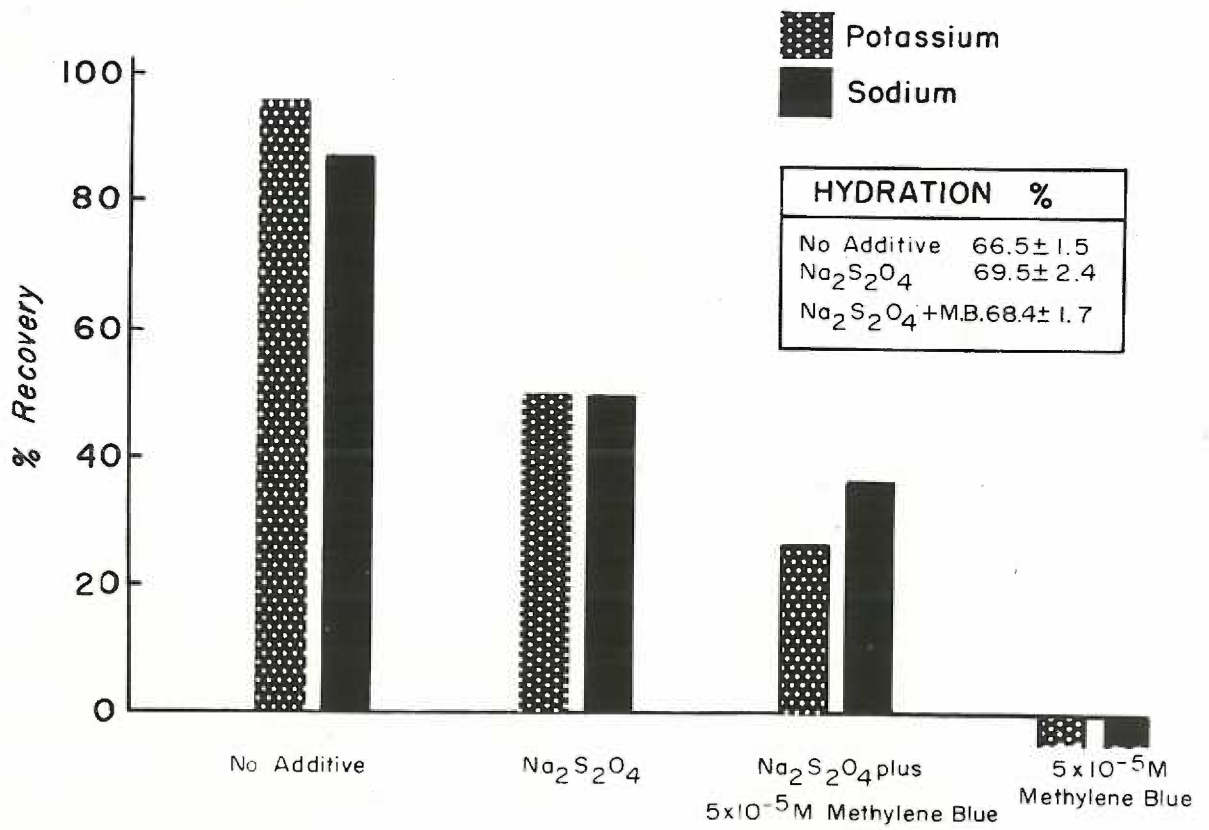
Potassium
Sodium
HYDRATION %
Light excluded 66.8±1.4
Light not excluded 69.1±3.0
80
60
40
20
0
% Recovery
Light Excluded
Light Not Excluded

Figure 3. Effect of ambient light on cation concentration of rabbit lenses bathed in medium containing methylene blue at 37°C. for the specified time under the imposed conditions. (Each bar represents an average of at least 9 lenses.)



--- S. D.
POTASSIUM
SODIUM
mEg / 1000 gm water
140
120
100
80
60
40
20
0
FRESH LENSES
AMBIENT LIGHT 6 HOURS
6 HOURS + LIGHT EXCLUDED 18 HOURS
LIGHT EXCLUDED 24 HOURS
AMBIENT LIGHT 24 HOURS

Figure 4. Effect of ambient light on cation recovery of rabbit lenses during incubation at 37°C. following cold induced cation shift. Bathing medium contained 5×10^{-5} M methylene blue reduced with sodium hydrosulfite. (Each bar represents the average of at least 9 pair of lenses.)



Potassium
Sodium
HYDRATION %
No Additive 66.5± 1.5
$Na_2S_2O_4$ 69.5± 2.4
$Na_2S_2O_4$+M.B. 68.4± 1.7
100
80
60
40
20
0
% Recovery
No Additive
$Na_2S_2O_4$
$Na_2S_2O_4$ plus 5×10^{-5}M Methylene Blue
5×10^{-5}M Methylene Blue

Figure 5. Effect of oxygen on cation recovery of rabbit lenses during incubation at 37°C. following cold-induced cation shift. (Each bar represents an average of at least 9 pair of lenses.)

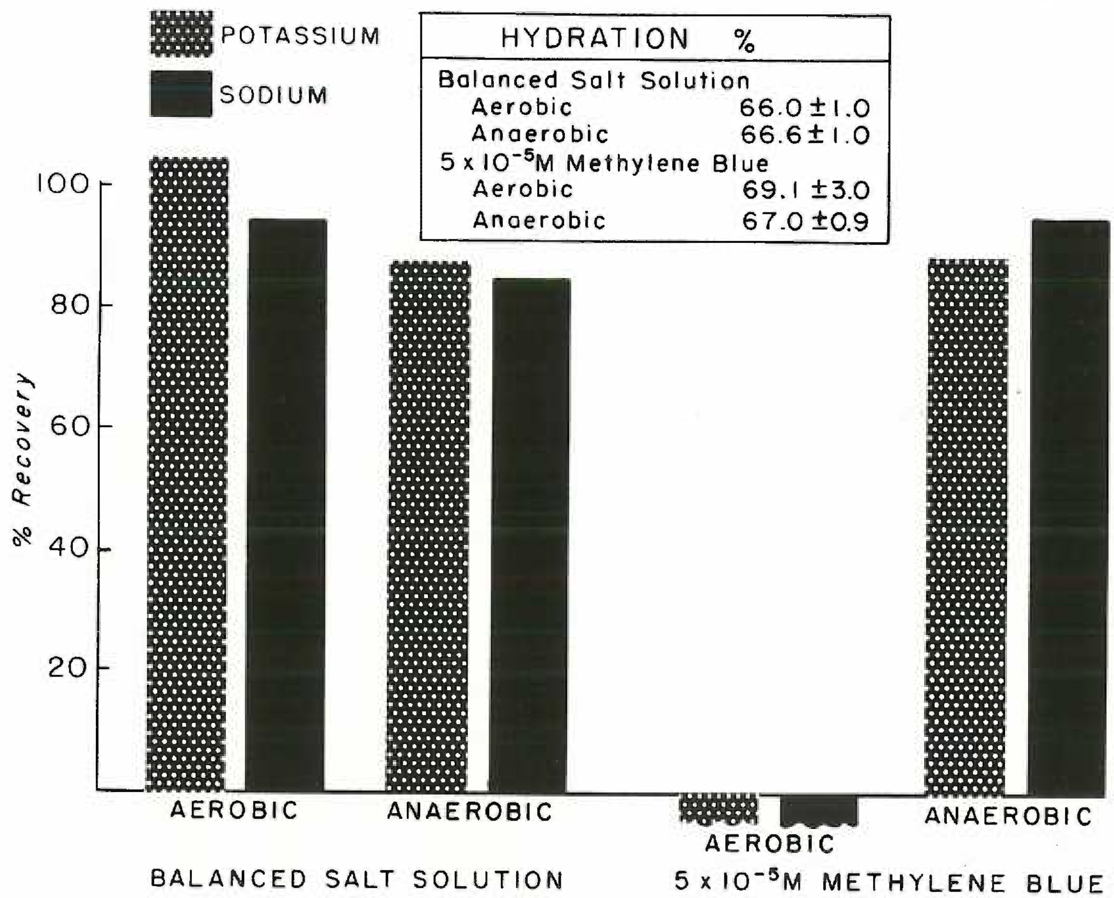
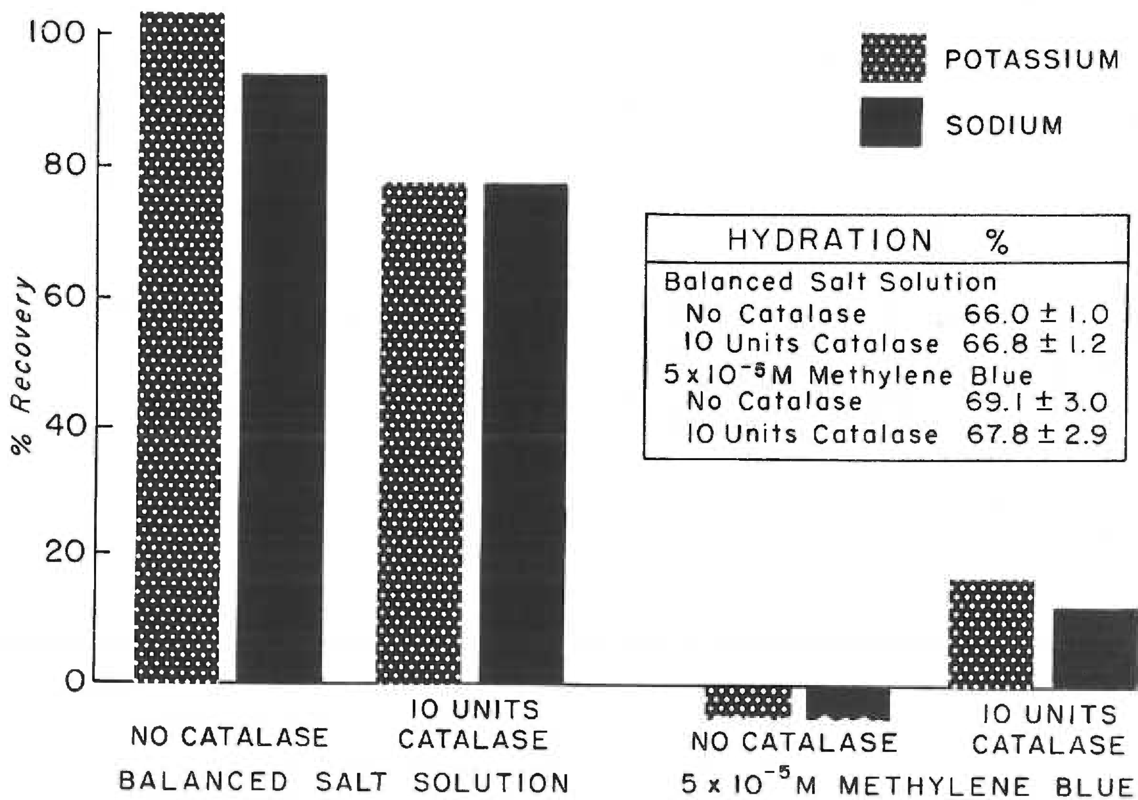
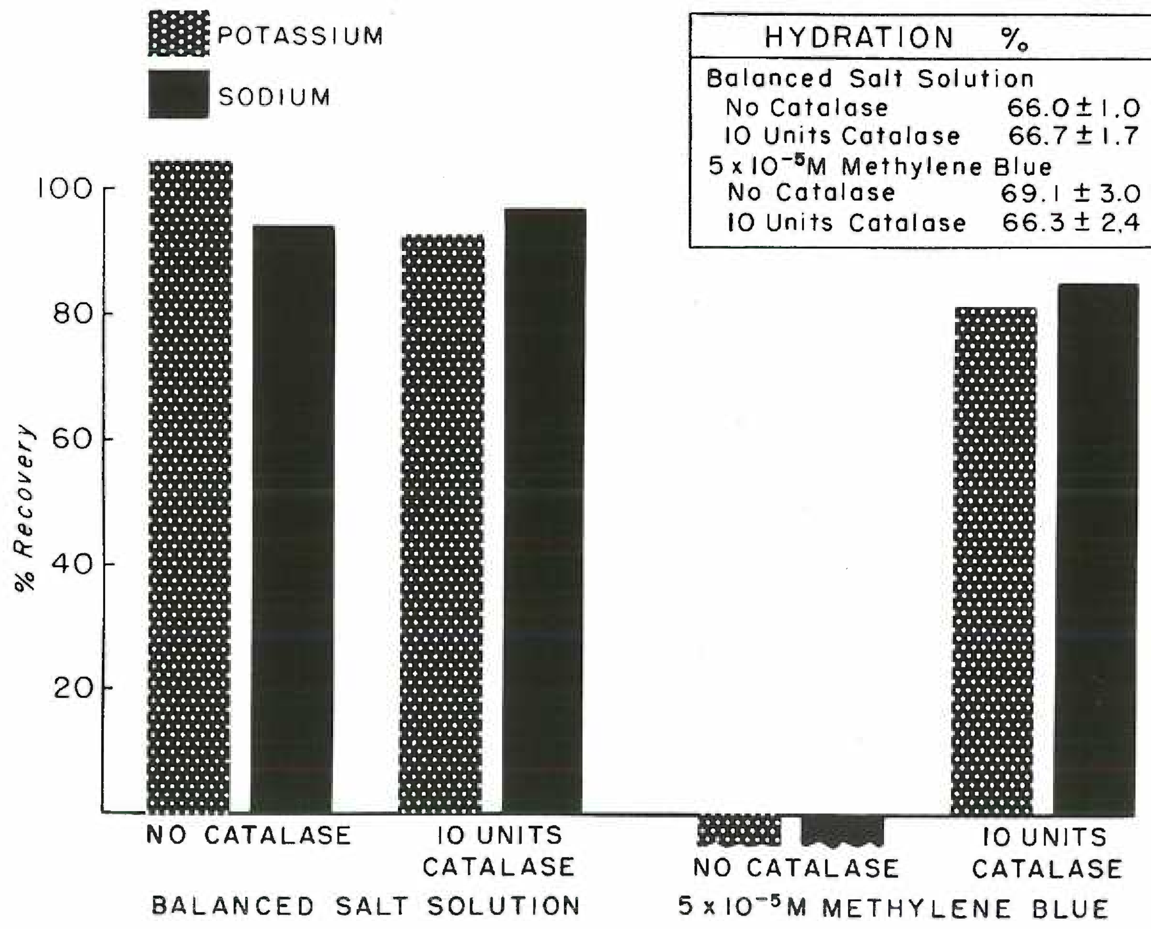

POTASSIUM
SODIUM
HYDRATION %
Balanced Salt Solution
Aerobic 66.0 ± 1.0
Anaerobic 66.6 ± 1.0
5 x 10^{-5}M Methylene Blue
Aerobic 69.1 ± 3.0
Anaerobic 67.0 ± 0.9
100
80
60
40
20
% Recovery
AEROBIC
ANAEROBIC
AEROBIC
ANAEROBIC
BALANCED SALT SOLUTION
5 x 10^{-5}M METHYLENE BLUE

Figure 6. Effect of catalase on cation recovery of rabbit lenses during incubation at 37°C. following cold-induced cation shift. Ten units of catalase was added to the medium prior to 40 hour refrigeration period. (Each bar represents the average figure of at least 9 pair of lenses.)



100
80
60
40
20
0
% Recovery
POTASSIUM
SODIUM
HYDRATION %
Balanced Salt Solution
No Catalase 66.0 ± 1.0
10 Units Catalase 66.8 ± 1.2
5 x 10⁻⁵ M Methylene Blue
No Catalase 69.1 ± 3.0
10 Units Catalase 67.8 ± 2.9
NO CATALASE
10 UNITS CATALASE
BALANCED SALT SOLUTION
NO CATALASE
10 UNITS CATALASE
5 x 10⁻⁵ M METHYLENE BLUE

Figure 7. Effect of catalase on cation recovery of rabbit lenses during incubation at 37°C. following cold induced cation shift. Ten units of catalase was added to the medium just prior to the 6 hour incubation period at 37°C. (Each bar represents the average figure of at least 9 pair of lenses.)



POTASSIUM
SODIUM
HYDRATION %
Balanced Salt Solution
No Catalase 66.0 ± 1.0
10 Units Catalase 66.7 ± 1.7
5 x 10⁻⁵M Methylene Blue
No Catalase 69.1 ± 3.0
10 Units Catalase 66.3 ± 2.4
% Recovery
100
80
60
40
20
NO CATALASE
10 UNITS CATALASE
BALANCED SALT SOLUTION
NO CATALASE
10 UNITS CATALASE
5 x 10⁻⁵M METHYLENE BLUE

Figure 8. Effect of boiled catalase on cation recovery of rabbit lenses during incubation at 37°C. following cold induced cation shift. Ten units of boiled catalase was added to the medium just prior to the 6 hour incubation period at 37°C. (Each bar represents the average figure of at least 9 pair of lenses.)

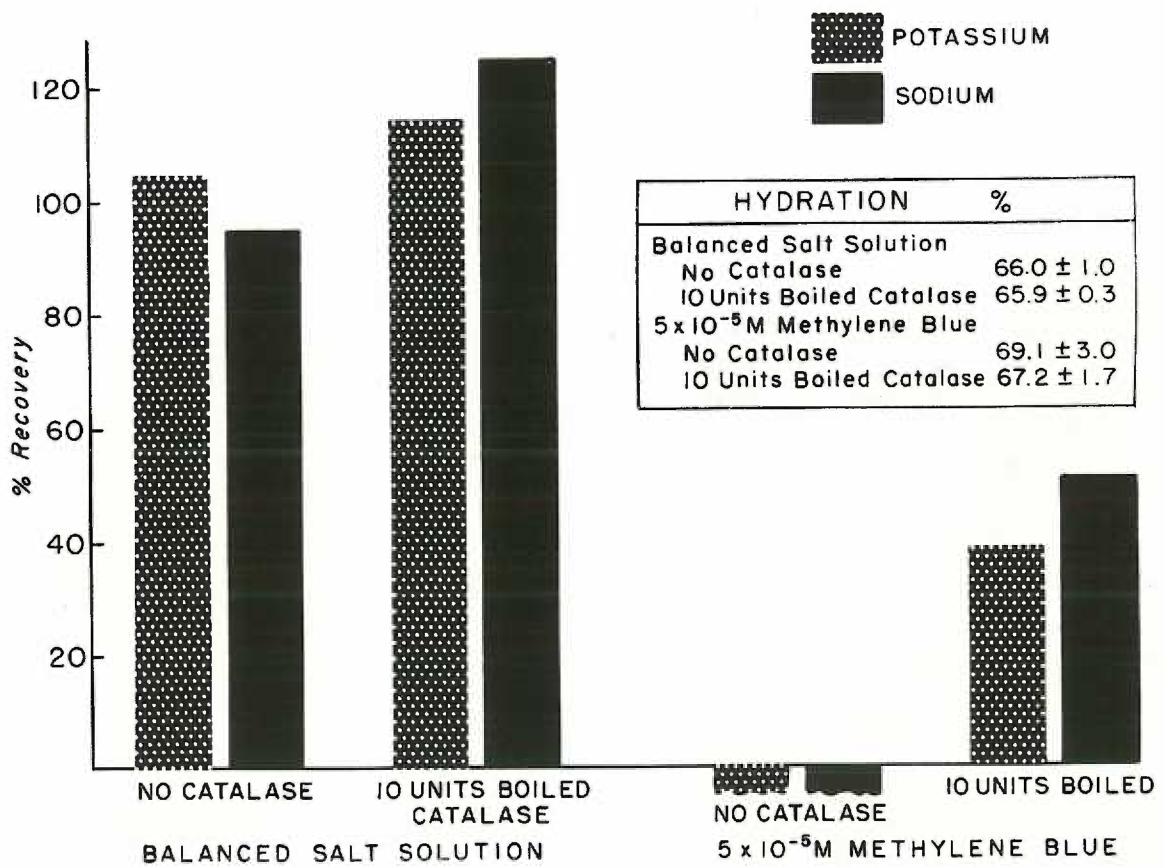
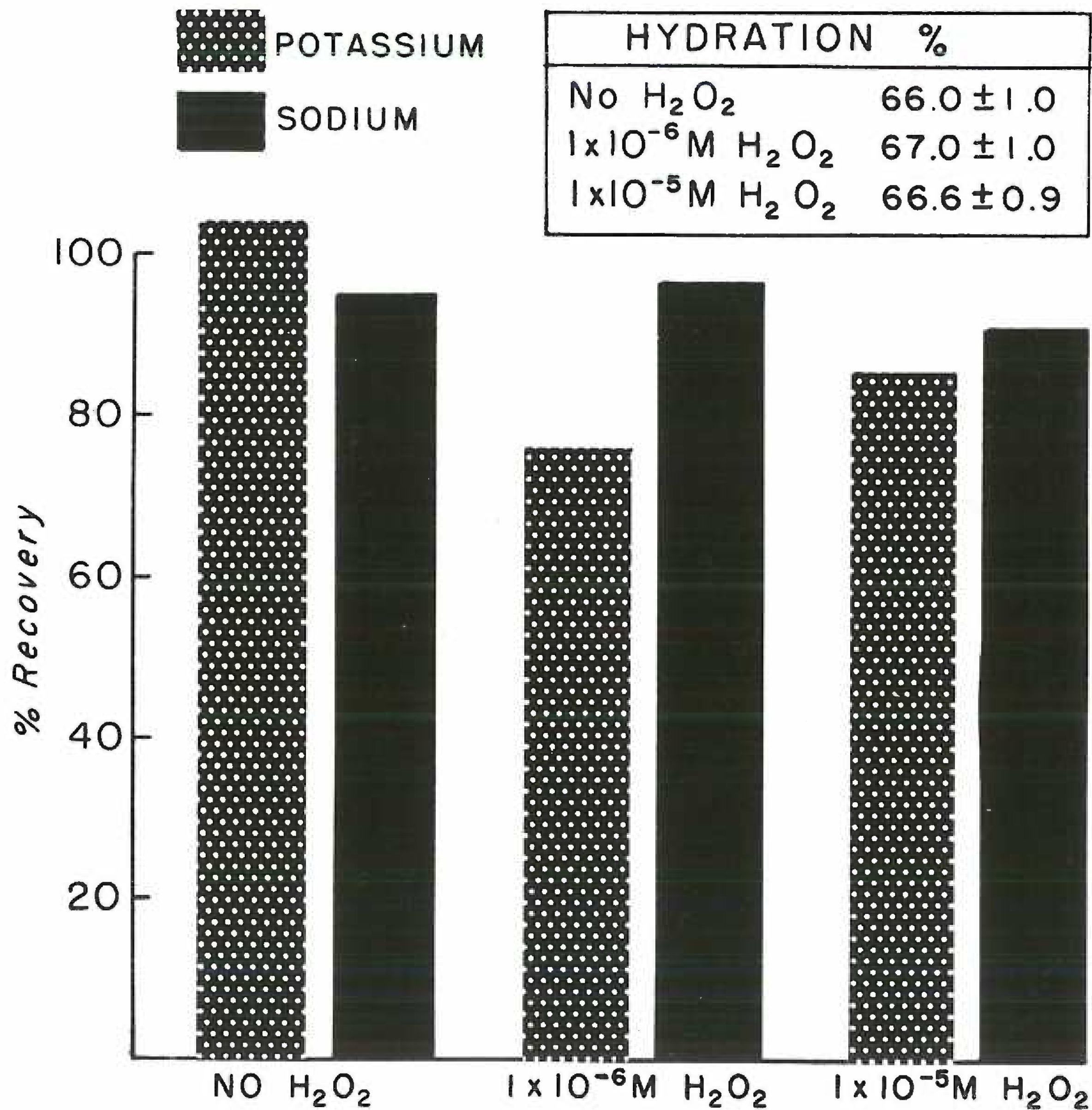
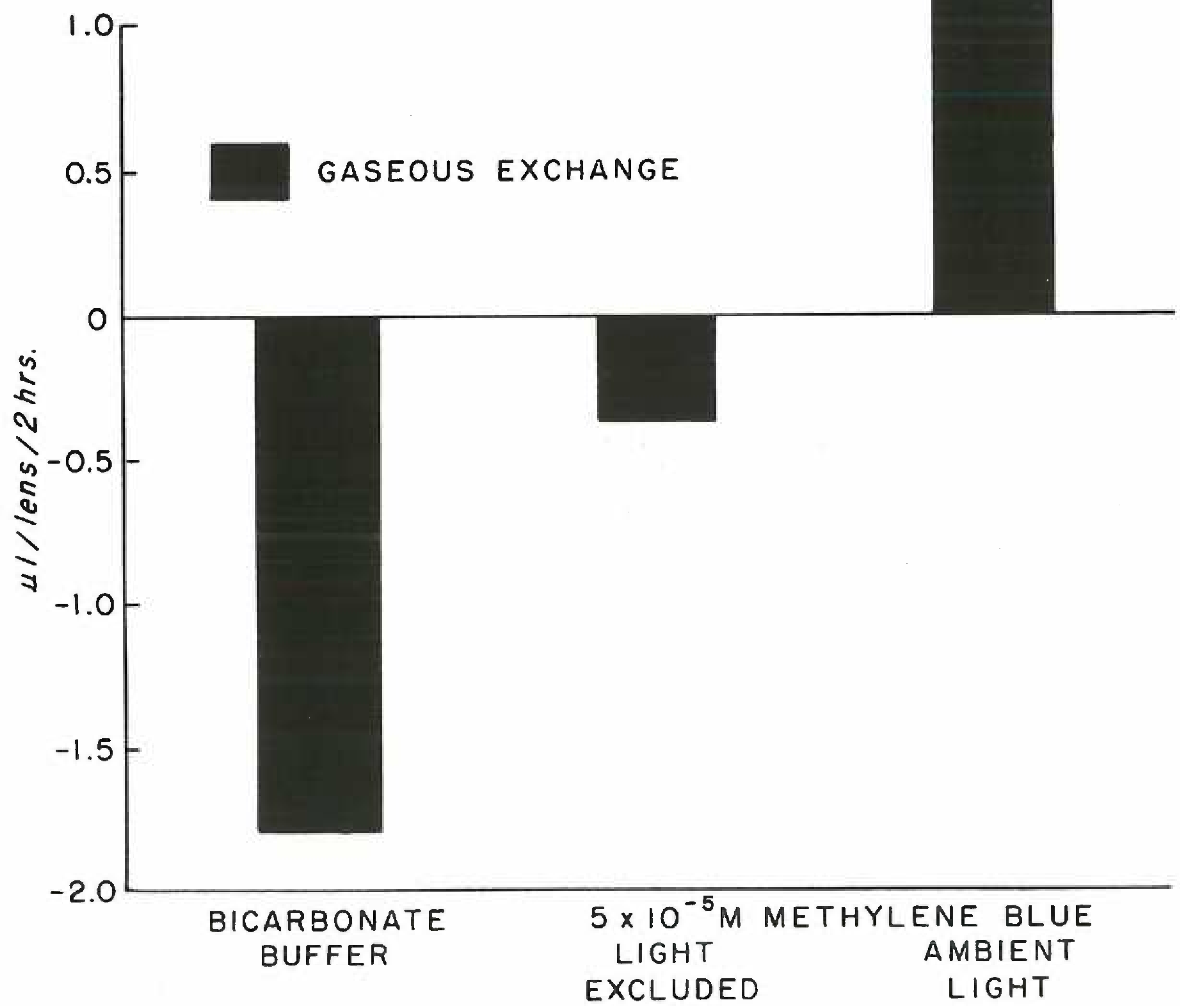

POTASSIUM
SODIUM
120
100
80
60
40
20
% Recovery
HYDRATION %
Balanced Salt Solution
No Catalase 66.0 ± 1.0
10 Units Boiled Catalase 65.9 ± 0.3
5 x 10⁻⁵M Methylene Blue
No Catalase 69.1 ± 3.0
10 Units Boiled Catalase 67.2 ± 1.7
NO CATALASE
10 UNITS BOILED CATALASE
BALANCED SALT SOLUTION
NO CATALASE
10 UNITS BOILED
5 x 10⁻⁵M METHYLENE BLUE

Figure 9. Effect of hydrogen peroxide on cation recovery of rabbit lenses during incubation at 37°C. following cold-induced cation shift. Hydrogen peroxide was added just prior to the 6 hour incubation period at 37°C. (Each bar represents the average figure of at least 8 pair of lenses.)



POTASSIUM
SODIUM
HYDRATION %
No H_2O_2 66.0 ± 1.0
1×10^{-6} M H_2O_2 67.0 ± 1.0
1×10^{-5} M H_2O_2 66.6 ± 0.9
100
80
60
40
20
% Recovery
NO H_2O_2
1×10^{-6} M H_2O_2
1×10^{-5} M H_2O_2

Figure 10. Gaseous exchange of lenses in designated bicarbonate containing medium with Pardee's solution in center well. Gas phase 95%O_2-5%CO_2. Temperature 37°C. Time 2 hours. (Each bar represents the average of ten lenses.)


1.0
0.5
0
-0.5
-1.0
-1.5
-2.0
µl/lens/2 hrs.
GASEOUS EXCHANGE
BICARBONATE
BUFFER
5 x 10^{-5}M METHYLENE BLUE
LIGHT
EXCLUDED
AMBIENT
LIGHT