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BIOCHEMICAL STUDIES ON THE OCULAR LENS IN RELATION TO CATARACTOGENESIS

PROGRESS REPORT # 8

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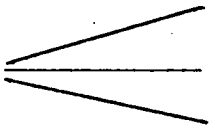
DISCLAIMER

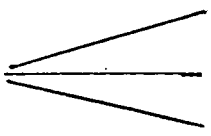
TRANSPORT SYSTEMS IN THE LENS AND CILIARY PROCESSES

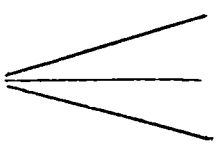
Transport mechanisms across the blood aqueous barrier and into the lens have been extensively studied in this laboratory. One of the major objectives of the program supported by the AEC is investigation of the biochemical and transport mechanisms in the lens. Insofar as the metabolic needs and the elimination of metabolites of this avascular organ depend on the composition of the intraocular fluids, knowledge of the mechanisms of transport across blood aqueous barriers is likewise of importance to our understanding of lens physiology. Accordingly, some of the studies during the current year deal with parallel investigations in the lens and ciliary body.

Cations in the Lens

Measurement of accumulation of radioactive isotopes in cultured lenses and in isotope-free media was obtained on over a 1000 lenses under the following conditions;

^{42}K 
- Nonlabeled K Conc. 0, 1, 5, 10 mM
- Nonlabeled Rb Conc. 0, 1, 5, 10 mM
- Nonlabeled Cs Conc. 0, 1, 5, 10 mM

^{86}Rb 
- Nonlabeled K Conc. 0, 1, 5, 10 mM
- Nonlabeled Rb Conc. 0, 1, 5, 10 mM
- Nonlabeled Cs Conc. 0, 1, 5, 10 mM

^{137}Cs 
- Nonlabeled K Conc. 0, 1, 5, 10 mM
- Nonlabeled Rb Conc. 0, 1, 5, 10 mM
- Nonlabeled Cs Cons. 0, 1, 5, 10 mM

The experiments provided the data on cross saturation needed to evaluate the Michaelis-Menten constants for these cations and the Vmax of the carrier responsible for active transport into the lens. They also made it possible to determine the relative permeability coefficients for the cations and their turnover rate in the lens. These evaluations were made on the basis of a mathematical model of the pump-leak hypothesis of transport described in the accompanying reprint entitled "Kinetics of the pump-leak system of transport in the ocular lens, derived from classic enzyme kinetics and diffusion theory." It is assumed that a carrier interacts with substrate or inhibitor ions by Michaelis-Menten kinetics both of which compete for the active site of a carrier; and the passive movement of each ion takes place by diffusion across both a uniform electric as well as a chemical gradient.

Values, calculated on the basis of the model for parameters governing the pump, support the conclusion that these cations are actively transported into the lens by a carrier-mediated system which has a single kind of active site and which preferentially transports potassium and rubidium with respect to cesium. The electric forces have a significant effect on transport of these ions, increasing influx by about 15 per cent and decreasing efflux by over 60 per cent. The computed values of the permeability coefficient for the leak are consistent with the hypothesis that permeability of the lens for the three ions is dependent on absorptive rather than frictional forces, i.e., on the anionic field strength of the membrane that limits diffusion. The values are incompatible with any model based on purely structural considerations of the lens membrane in which transport is assumed to occur by sieving through hydrated pores.

Details of this investigation and the conclusions are provided in the enclosed preprint of a paper published in Investigative Ophthalmology (9:769-784 1970, reprints are not yet available). Preprint removed

Taurine in the Lens

Studies on intraocular transport of taurine in both the ciliary body and in the lens have been discussed in last year's AEC report. The details of this investigation are given in the attached reprint which was published during the present grant period. The transport system for taurine has been characterized and its relation to membrane ATPase established. The transport system appears to be highly specific for β-amino acids and differs in many respects from the transport system for neutral amino acids in general, and more specifically from that of α-AIB.

Myoinositol in Lens and Ciliary Body

Myoinositol is an important component of biological membranes and is present in unusually high concentrations in most ocular tissues; its concentration decreases in the lenses of galactose-fed animals. Inositol has been implicated in the transport of certain amino acids across membranes and phosphatidyl inositol is thought to play a role in membrane permability although the mechanism is unknown. In view of the apparent importance of myoinositol in transport across membranes, we have undertaken extensive investigations concerning the mechanisms which may be responsible for maintaining high concentration of this substance in the lens and intraocular fluids.

These investigations dealt with the accumulation of labeled inositol in the ciliary body and in the lens in vitro as well as transport of this substance across the blood aqueous barrier in vivo. The transport system for myoinositol in both the ciliary body and in the lens has been characterized. While the transport system for myoinositol in both tissues is similar, there are certain important differences. For example, the myoinositol transport system is affected by a number of neutral amino acids in the lens while it is not in the ciliary body.

Studies also dealt with the role of sodium ion in the transport of myoinositol in the lens and ciliary body. When the sodium ion is replaced by equivalent amounts of various other cations or by sucrose, the transport process was affected differently, suggesting that in addition to the possible effect of the lack of sodium ion on the transport mechanism, the substituent ions may affect the transport system. These investigations raise important questions with regard to the role of sodium ions in the transport of amino acids and carbohydrates in several other tissues where a deficiency of sodium ions was shown to affect transport of these substances. Since the details of these investigations are recorded in the accompanying reprints, only brief summaries of the findings are given here.

[2-^{3}H] Myoinositol accumulates in rabbit ciliary body-iris preparations in vitro against a concentration gradient. The transport system is energy and temperature dependent (Q_{10} = 2.3) and demonstrates saturation kinetics (K_m = 0.9 mM.). It is inhibited by ouabain, iodoacetate, phlorhizin, and shows requirements for Na^{+}, K^{+}, and Ca^{++}. The transport system appears highly specific; various amino acids, ascorbic acid,

glucoascorbic acid, and sugar alcohols were without effect on the accumulation of inositol. The stereoisomer scylloinositol was found to inhibit the transport of myoinositol. D- and L-chiroinositols had no effect. The phosphate esters of inositol competitively inhibited transport of myoinositol. The steady state concentrations of inositol in the lens, ciliary body-iris, aqueous humors, and plasma were determined. The concentration in posterior aqueous was higher than in the anterior aqueous and still higher than in plasma. Evidence is presented to show that the transport of inositol across the blood-aqueous barrier in vivo is a carrier-mediated process and it is suggested that the high concentration of inositol in the aqueous humors is the result of active transport from plasma into posterior aqueous.

The characteristics of myoinositol transport in the lens were studied by culturing them in a medium containing the tritium-labeled compound. The accumulation in the lens takes place against a concentration gradient and is inhibited by ouabain, iodoacetate, phlorhizin, and at reduced temperature (Q_{10} = 3). The energy for transport is derived from glycolysis. The transport system follows Michaelis-Menten kinetics (V_{max} = 0.02 µmoles per lens per hour, K_m = 0.4 mM.) and requires the presence of Na^+ and K^+. Although a relationship between Na^+ concentration and inositol transport was observed, the lenses in Na^+ deficient media could not be maintained in a physiological state so that the decreased transport cannot be ascribed solely to the effect of this cation. Similar to the observations made in the ciliary body, the transport system in the lens is inhibited by inositol mono- and hexaphosphates and shows the same

specificity for the stereoisomers of inositol. Unlike the findings in the case of ciliary body, inositol accumulation in lens is inhibited by several amino acids. The reciprocal inhibition of α-aminoisobutyric acid transport by inositol, however, could not be demonstrated. See reprints for details.

BIOELECTRIC POTENTIAL IN THE LENS

We have now excellent evidence that the lens acts as a pump-leak system involving active transport of ions in and out of this organ balanced by passive diffusion across chemical gradients. Associated with these gradients is a bioelectric potential of some 40 millivolts (inside negative) and a translens potential, under short circuit conditions, of about 25 millivolts (anterior side positive).

The bioelectric potential is important in the present studies because a knowledge of its magnitude is required to characterize the carrier systems responsible for active transport and to evaluate the permeability coefficients for various ions in the lens. To make these evaluations, for the 1A cations K, Rb, and Cs, as described in a preceding section of this report, the bioelectric potential of lenses has been determined following culture in both a medium resembling aqueous humor, and in one in which these ions were substituted for Na in varying concentrations. In addition, the translenticular potential has been measured on intact and decapsulated lenses under varying circumstances with the object of clarifying the source of the potentials existing in the lens.

Potentials were measured by inserting glass microelectrodes (1-2 meg ohms) filled with 3 M CKl into normal or decapsulated lenses to a depth of 1 or 2 mm or by applying cotton wick electrodes to the exposed anterior or posterior surface of the lens. Reference electrodes in both instances were placed in media bathing the lens. Appropriate electrical connections were made through calomel cells to an NFl Bioelectric

Instruments high impedance amplifier. The potential differences were read on a microvolt ammeter.

The bioelectric potentials determined with the microelectrodes were measured at 37°C in rabbit lenses bathed in vitreous humor or a complete synthetic medium (KEI-4) and found to be approximately -40 mv. The potential was maintained essentially constant in lenses cultured for 24 hours.

The bioelectric potential of lenses cultured for varying periods in media in which the carrier system for K was partially saturated with 50 mM K, Rb, or Cs, is shown in Fig. 1. The potential is seen to decrease from an average of -42 mv to approximately 28 mv within the first hour of culture in all three media. In the medium containing K, it remains steady for as long as 24 hours whereas in those containing Rb or Cs it declines further, reaching a level of about -17 and -13 mv, respectively, at 24 hours.

The wick, or translens potential, which is -23 mv in freshly removed lenses, also decreases after culture in all of the media, but much more rapidly in the one containing 50 mM K (Fig. 2). Like the bioelectric potential it remains constant up to 24 hours whereas in lenses cultured in the media containing Rb or Cs the translens potential continues to fall.

The bioelectric potential of lenses cultured for 24 hours in media containing K, Rb, or Cs, is shown as a function of concentration of these ions in Fig. 3. The potential decreases linearly with concentration of K to 50 mM, an effect that other experiments show continues up to concentrations prevailing inside fibers, viz. 120 mM, at which point it is -9 mv. The potential falls more rapidly with concentration of Rb

or Cs than K, but only in the range of 0 to 10 mM. With higher concentrations the effect on all 3 ions appears to be the same.

The lens potential of lenses cultured for 24 hours also decreases linearly with increasing concentrations of all three cations (Fig. 4). The effect of each ion appears to be the same within the experimental error.

The fiber potential alone as determined in decapsulated lenses in the presence of 50 mM Rb and Cs is maintained at approximately -19 mv for at least 5 hours a culture (Fig. 5). The potential of decapsulated lenses cultured in medium containing K at the same concentration decreases slightly with time. The transmembrane potential of decapsulated lenses is zero.

A concentration of 10^{-7} M ouabain has no appreciable effect on the bioelectric potential, but at 10^{-5} M ouabain the potential decreases significantly after a delay of several hours (Fig. 6).

While the immediate purpose of this investigation was, as previously stated, to acquire information needed to evaluate the carrier systems responsible for the transport and diffusion of cations in lens, as already described, some conclusions can be drawn on source of the potential. It appears that the bioelectric potential represents the sum of two approximately equal potentials (22 and 19 mv) across the epithelium and fiber membranes, respectively. Thus the removal of the epithelium results in a reduction of the bioelectric potential to about 22 mv, and abolishes the translens potential entirely.

The bioelectric and translens potentials are both reduced by increasing the concentration of the three cations in the media whereas similar changes had no significant effect on the fiber potential. These observations suggest that it is only the potential across the epithelium that is affected by changes in the ionic composition of the media bathing the lens.

The cations apparently do not exert their effect on potential through reduction in the amount of sodium present in the media since both the rate of change of and the magnitude of the potential differs when equal concentrations of K, Rb, and Cs are substituted for sodium. Further work on the significance of potential with regard to the mechanisms involved in the transport of ions is contemplated.

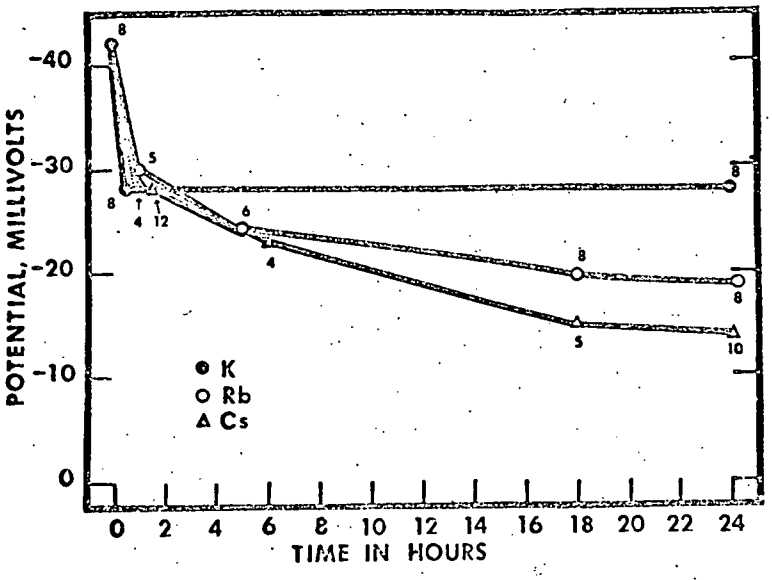


Fig. 1: Bioelectric potential of lenses cultured in KEI-4 medium in which a concentration of 50 mM of potassium, rubidium, or cesium is substituted for an equivalent concentration of sodium.

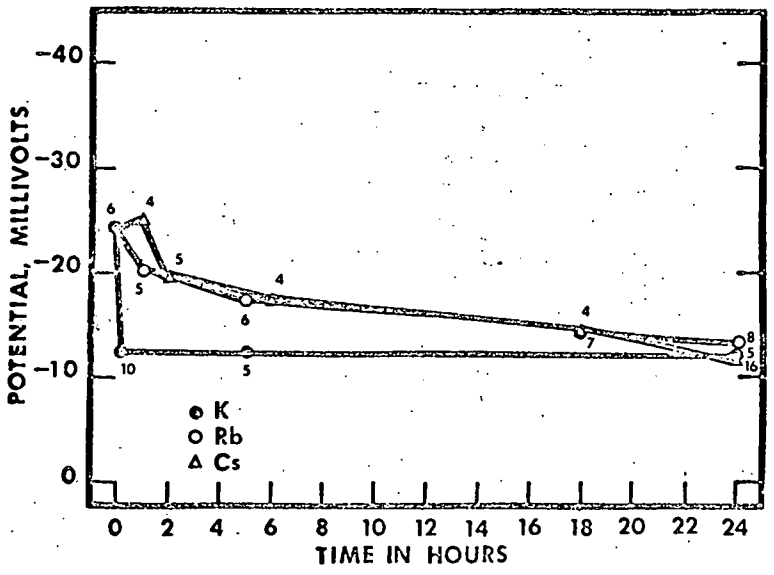


Fig. 2: Translens potential of lenses cultured in KEI-4 medium in which a concentration of 50 mM potassium, rubidium, or cesium is substituted for an equivalent concentration of sodium.

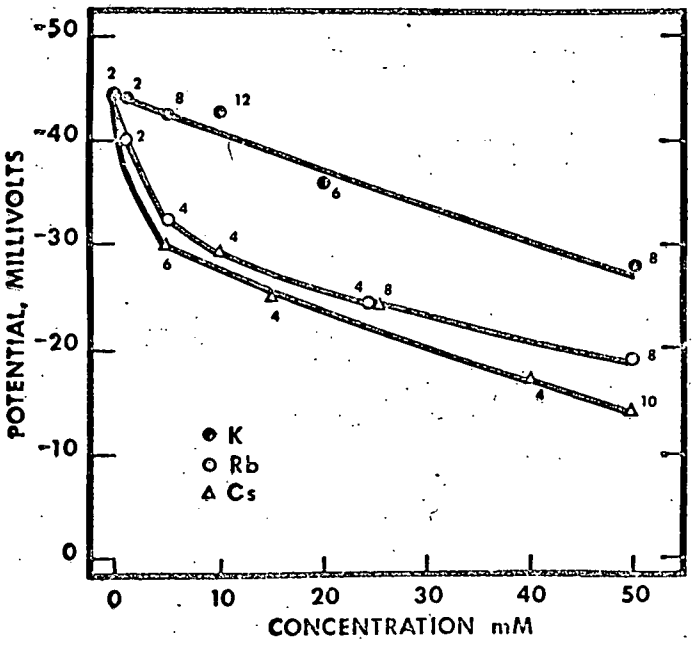


Fig. 3; Bioelectric potential of lenses cultured for 24 hours in KEI-4 medium in which various concentrations of potassium, rubidium, or cesium were substituted for equivalent concentrations of sodium.

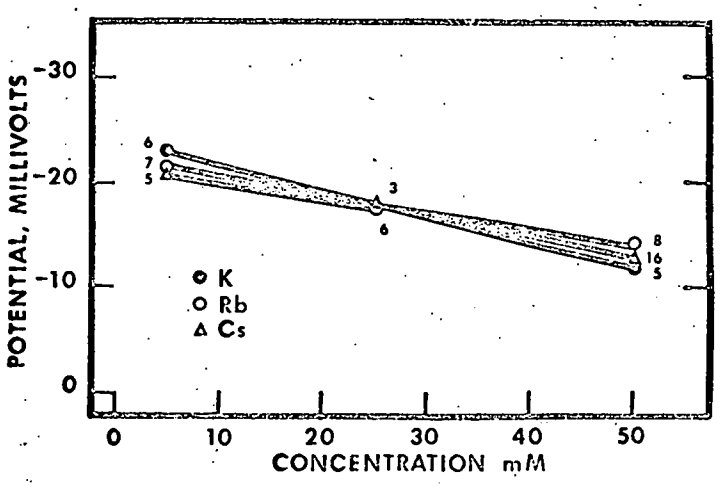


Fig. 4: Translens potential of lenses cultured in KEI-4 medium in which various concentrations of potassium, rubidium, or cesium were substituted for equivalent concentrations of sodium.

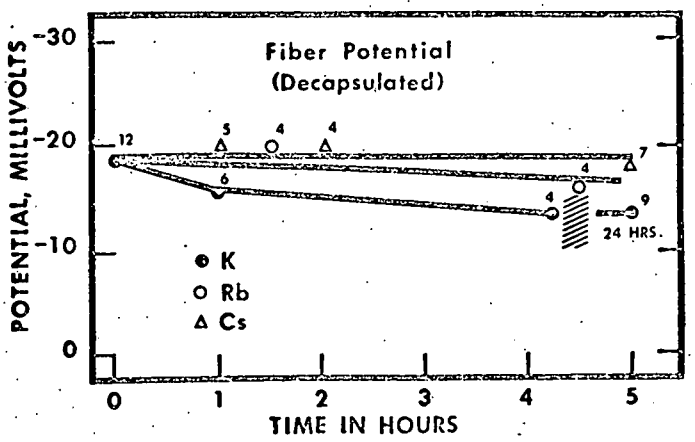


Fig. 5: Lens fiber potential of decapsulated lenses cultured in KEI-4 medium in which a concentration of 50 mM of potassium, rubidium, or cesium is substituted for an equivalent concentration of sodium.

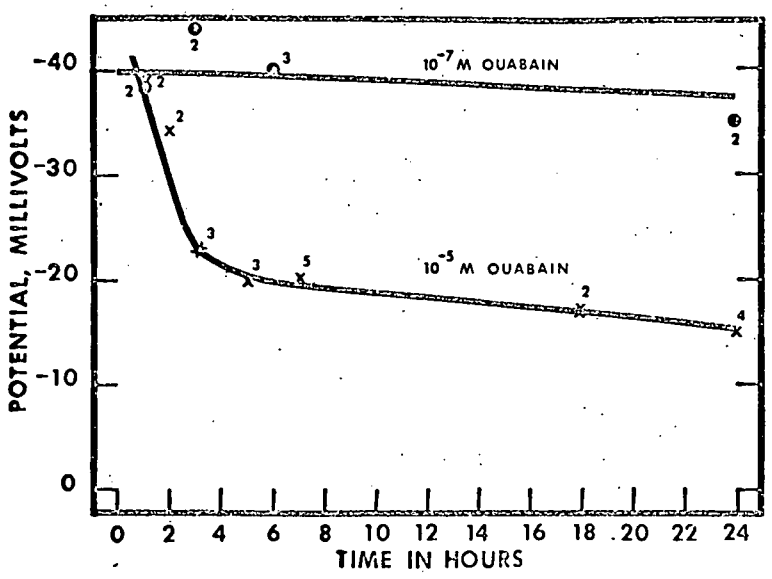


Fig. 6: Lens fiber potential of lenses cultured in KEI-4 medium containing ouabain.

ROLE OF GLUTATHIONE IN CATARACTOGENESIS

It is a common feature of all types of cataracts that the level of glutathione decreases in the lens. It has repeatedly been observed that one of the earliest changes that takes place in the lens of galactose-fed animals is in the level of glutathione which is observed long before the onset of any opacity. The mechanism by which glutathione is lost from the lens has never been adequately explained. Studies were, therefore, undertaken to investigate this problem as stated in the earlier report.

Preliminary studies indicated that when lenses are cultured in an adequate medium, very little glutathione was found to leave the lens, confirming the earlier observations of Kinoshita. It was further observed that following irradiation of the lenses in vitro there was no significant increase in the rate of efflux of glutathione. While the possibility remains that the loss of glutathione from the lens may be affected after a latent period following irradiation, it became apparent that the problem of the loss of glutathione from the lens, not only under conditions leading to cataract formation, but even in the normal lens, needed investigation at a more fundamental level.

In studies supported by AEC in this laboratory, it was demonstrated that approximately 2% per hour of total glutathione in the lens is continuously being synthesized. Since the steady state level of glutathione remains constant through the experimental period, it is presumed that the synthesis is balanced by a similar rate of degradation of the tripeptide. However, to date there is no convincing evidence that lens contains peptidases which are capable of degrading glutathione since gamma glutamyl bond

of glutathione appears to be resistant to the peptidase activity of the lens. The alternative possibility that glutathione may be continuously lost by leakage out of the lens is also not substantiated by the preliminary experiments on the efflux of the tripeptide.

Reports have recently appeared that there is an increased efflux of glutathione from cataractous lenses in the form of oxidized glutathione. On the basis of such studies, it has been postulated that glutathione leaves the lens primarily as GSSG, i.e. the oxidized compound, possibly involving active transport of the oxidized peptide. Experimental evidence is lacking at the present time for such a hypothetical mechanism. Moreover, the possibility that cataractous lenses become more permeable to glutathione molecule cannot be excluded.

In the light of this background, a number of exploratory studies have been undertaken. In order to increase the sensitivity of the method employed for measuring the efflux of glutathione, the tripeptide in the lens was labeled by culturing lenses in the presence of ^{14}C glycine. The efflux of glutathione was measured by following the radioactivity present in glutathione molecule after the GSH and GSSG were separated by ion exchange chromatography.

In nearly all lenses that were incubated either in a physiologic medium, or hypotonic Tyrode's to enhance glutathione efflux, small amounts of oxidized glutathione appeared in the medium. A typical experiment on the efflux of glutathione from intact lenses incubated in hypotonic medium is shown in Figures 7a, 7b,and 7c. There is no evidence for the presence of GSH, but a progressive increase in the amount of GSSG in the incubation medium is observed. These experiments suggest two possibilities. One, that reduced

glutathione may be bound in some form within the lens because of the reactive sulfhydryl groups and that only GSSG may leak out of the lens. The second possibility is that glutathione leaves the lens in the reduced form but gets oxidized within the incubation medium.

In contrast to the extremely slow rate of efflux of glutathione from intact lenses (only 3% of lens glutathione was found in the medium after 24 hour incubation), the loss of glutathione was much more rapid from decapsulated lenses (Fig. 8). Nearly 25% of total glutathione in the lens was recovered in the medium within one hour of culture and almost 40% at the end of four hours. This may mean that either the capsule and epithelium or possibly the lens fiber membranes, if they are damaged in the process of removing the capsule, may serve as a barrier to the diffusion of glutathione from the lens. The studies with decapsulated lenses further showed that the bulk of glutathione found in the medium at the end of the first hour of incubation was present in the reduced form and only 5% of total glutathione was present in the oxidized form. With increasing time of incubation, there was a gradual increase in the recovery of oxidized glutathione and a proportionate decrease or leveling off of GSH. These results suggest that from decapsulated lenses glutathione leaves in the form of reduced compound and the oxidation must take place outside the lens. Secondly, glutathione may not be tightly bound to the proteins.

Question then remains whether from an intact lens, glutathione is lost as a reduced or oxidized compound. This problem may be approached by studying the efflux of glutathione from lenses in which the intracellular (intralenticular) distribution of reduced and oxidized glutathione is altered. While it is easy to alter the relative amounts of reduced and oxi-

dized glutathione in a cell, it is difficult to maintain the cell in a physiologic state since the agents may bring about other changes in addition to the oxidation of glutathione. Recently, Kosower has introduced a compound known as "diamide" which is said to oxidize selectively the sulfhydryl groups of GSH. This compound may serve as a very important tool in studying the role of reduced and oxidized glutathione within the lens. One of the suggested functions of the high level of glutathione in the lens is to maintain the protein sulfhydryl in a reduced state and since there is a reduction in the glutathione levels in situations leading to lens opacities, it has been speculated that reduced level of glutathione may bring about changes in the physical characteristics of the proteins. With the availability of a compound which is known to oxidize sulfhydryl groups of glutathione selectively, a number of worthwhile experiments can be pursued in order to delineate the role of GSH in maintaining lens clarity.

Preliminary experiments indicate that there is a greater efflux of glutathione primarily in the form of GSSG from lenses which have been treated with the specific glutathione oxidant, "diamide". Considerably more information is needed before one can conclude that glutathione is lost from the lens primarily as the oxidized compound. Moreover, it remains to be demonstrated if there exists a mechanism for the transport of GSSG out of the lens, a mechanism that has been suggested for the red blood cell. These studies will be continued during the coming year.

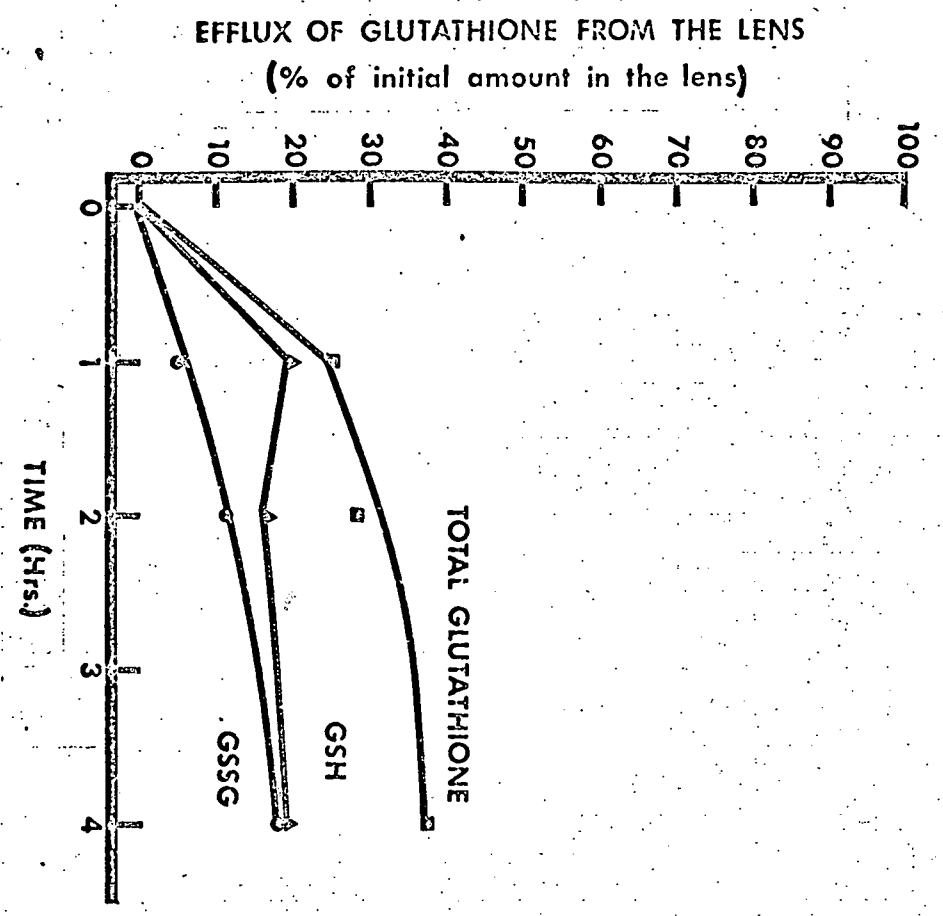
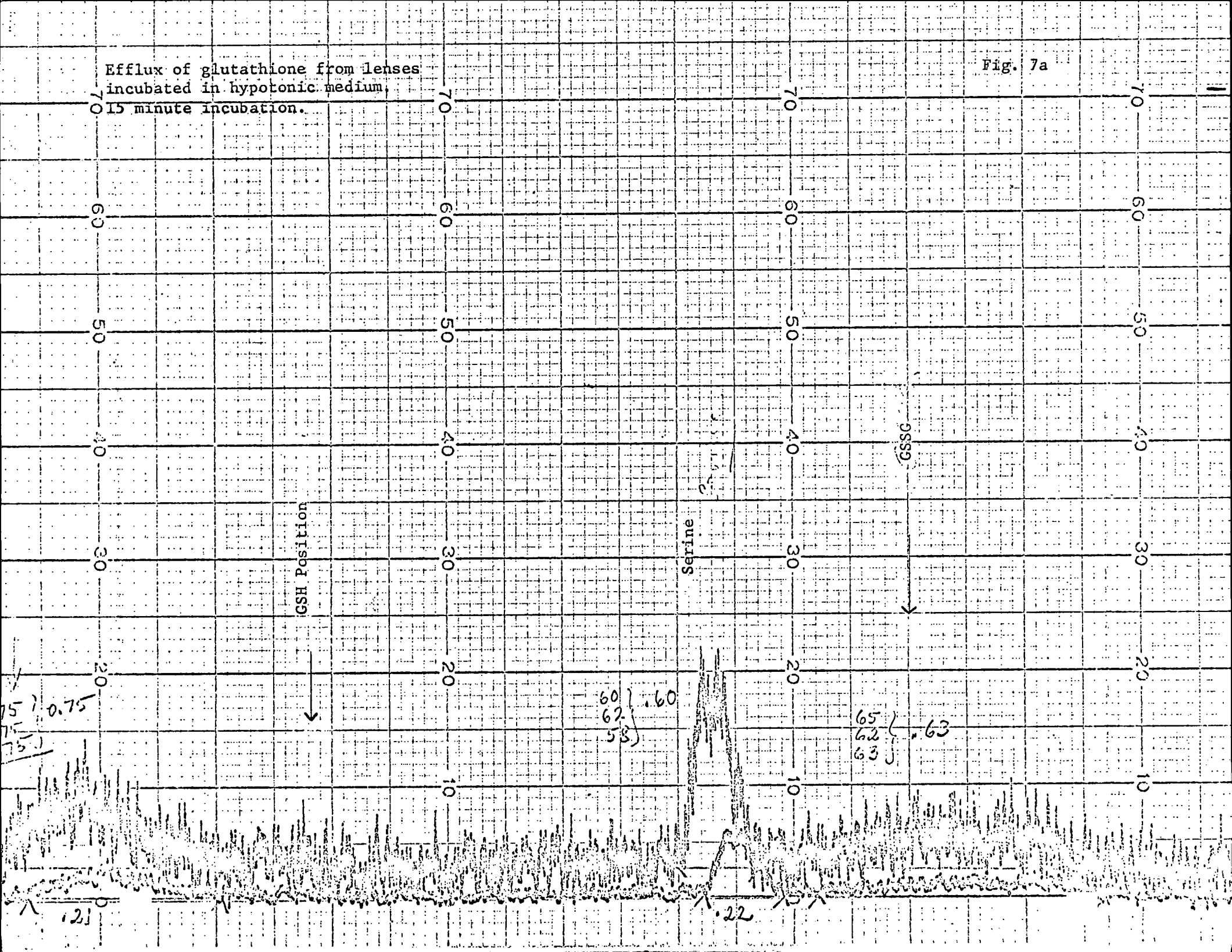


Fig. 8 - Efflux of glutathione from decapsulated lenses. Glutathione was labeled in intact lenses by culturing them in KEI-4 medium in the presence of C^{14} glycine. Following incubation, capsule and epithelium were removed and efflux of glutathione into a normal Tyrode's medium was followed by analyzing the media for reduced and oxidized glutathione. The values for reduced, oxidized and total glutathione are all expressed as the concentration of the reduced compound.

Efflux of glutathione from lenses incubated in hypotonic medium.
15 minute incubation.

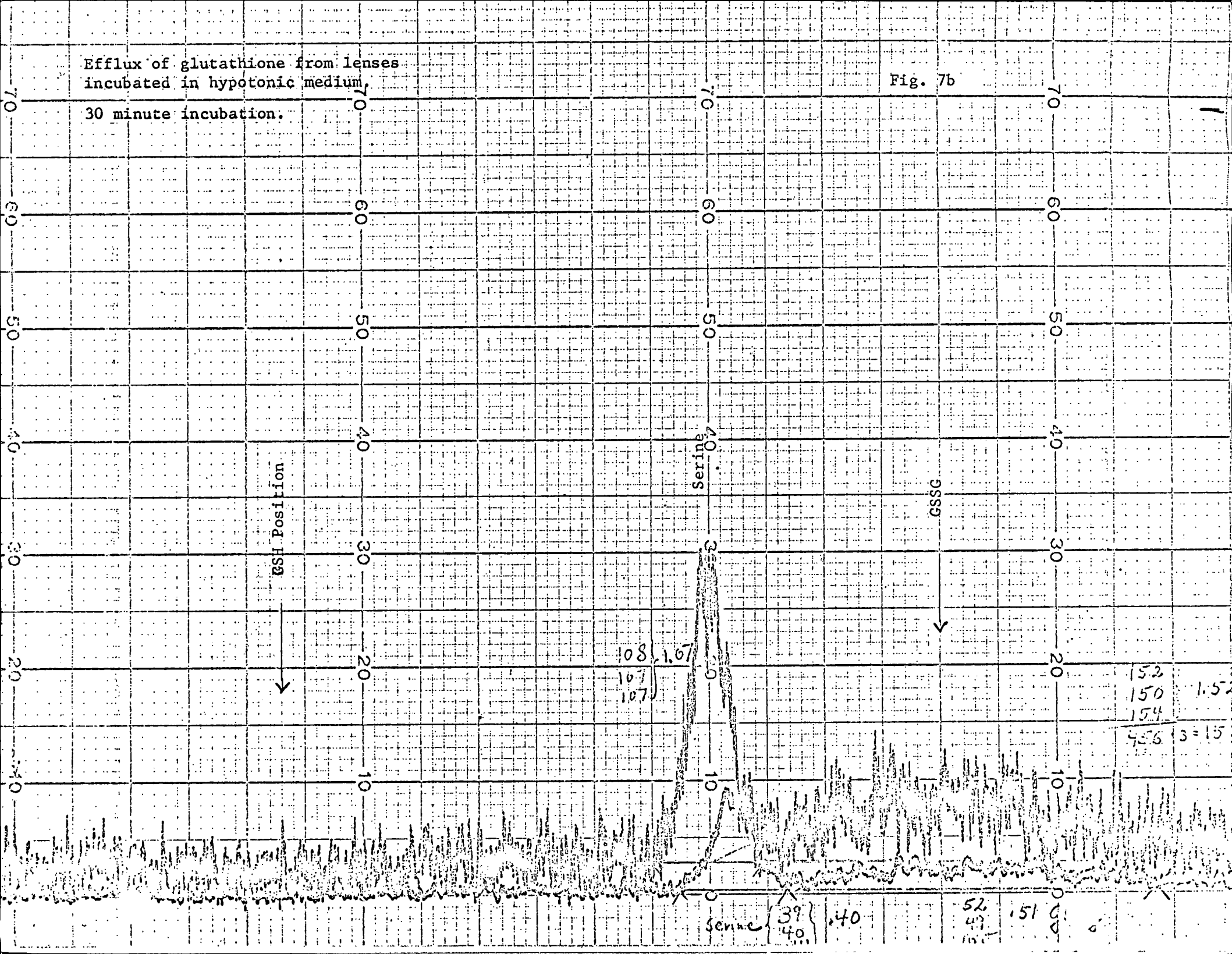
Fig. 7a



Efflux of glutathione from lenses incubated in hypotonic medium.

30 minute incubation.

Fig. 7b



Efflux of glutathione from lenses incubated in hypotonic medium.

1 hour incubation.

Fig. 7c