Studies on the crystalline lens

XII. Turnover of glycine and glutamic acid in glutathione and ophthalmic acid in the rabbit

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The rates of incorporation of ^{14}C-labeled glycine and glutamic acid into glutathione and ophthalmic acid have been determined in cultured rabbit lenses. The average turnover rate of glycine and glutamic acid in glutathione, and of glutamic acid in ophthalmic acid was found to be 1.8 per cent per hour throughout the 24 hour period of culture; the incorporation of glycine in ophthalmic acid also occurred at this rate for the first 9 hours but appeared to decrease thereafter. While the possibility of exchange reactions cannot be ruled out, the similarity of the rates of turnover of both amino acids in each peptide strongly suggest that glutathione and ophthalmic acid are continually being broken down and resynthesized in the lens. The rate of formation of glutathione and ophthalmic acid, assuming incorporation of amino acids represents synthesis, is 21.6 and 0.52 μmoles per 100 Gm. lens per hour, respectively.

Turnover rate of glycine in glutathione in the ocular lens was first studied by Kinsey and Merriam[1] using ^{14}C-labeled glycine. They found half of the amino acid in the tripeptide to be replaced every 29 hours. In the absence of information concerning the turnover rate of the two other constituent amino acids they could not decide whether turnover of glycine in glutathione involves a total breakdown and resynthesis of the latter from its constituent amino acids or an exchange of glycine molecules for those pre-existing in the peptide.

Ophthalmic acid, first observed in bovine lenses,[2] is a structural analogue of glutathione differing only in that the former contains α-amino n-butyric acid while the latter contains cysteine. The synthesis of both peptides appears to involve at least one enzyme in common.[3]

The present investigation is concerned with the determination of the relative rates of turnover of glycine and glutamic acid, in both glutathione and ophthalmic acid, by methods which take into account the rates of entry of the amino acids into the lens. Measurements of the turnover rates of two constituent amino acids are thought to provide better evidence on which to decide whether the glutathione and oph-

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thalmic acid are continually being synthesized.

Methods

All experiments were performed on albino rabbits weighing between 1.8 and 2.3 kilograms. Lenses were cultured for various times by the method of Merriam and Kinsey[4] in 5 ml. of synthetic medium (KEI-4)*[5] containing 0.7 to 0.9 μc of the labeled amino acid per milliliter. Labeled amino acids of high specific activity were used to minimize changes in the concentration of total amino acid in the medium. Sterile techniques were employed when lenses were cultured for more than 5 hours.

Separation of amino acids and peptides and determination of their radioactivity. An automatic amino acid analyzer was used for the separation and quantitative estimation of free amino acids, glutathione, and ophthalmic acid as described elsewhere.[6, 7]

*The concentration of glycine and glutamic acid in the medium was 0.09 and 0.29 mM., respectively. The normally present concentrations of the two free amino acids and the tripeptides in the lens (glycine, 1.79 mM.; glutamic acid, 5.83 mM.; glutathione, 18.4 mM.; ophthalmic acid, .48 mM.) remained approximately constant over the 24 hour culture period.

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The amino acid analyzer was used in conjunction with a continuous Tri-Carb liquid flow detector for measurement of radioactivity. The effluent from the chromatographic column was passed through the detector before reaching the reaction coil of the analyzer. Two spectrometers in the detection unit permit accurate measurement of radioactivity of substances having both high and low activity in the same aliquot since it is possible to use the two scales simultaneously.

Purification of ^{14}C-labeled amino acids. Uniformly labeled glycine and *l*-glutamic acid were obtained from commercial sources.† Both amino acids were of high specific activity (64 mc. to 125 mc. per mmole). The purity of the samples was 99.5 per cent according to the suppliers who based their figures on analyses made with paper chromatography. The possibility that the impurities could give rise to serious errors if any of them was eluted with the tripeptide prompted us to reanalyze the radioactive compounds using column chromatography. The resulting analyses showed that at least one impurity from each amino acid was eluted with ophthalmic acid. The chromatogram (Fig. 1) illustrates how an impurity from the glycine sample, which corresponds in position with ophthalmic acid could introduce a serious error in any estimation of radioactivity present in the peptide. It shows too that ^{14}C-

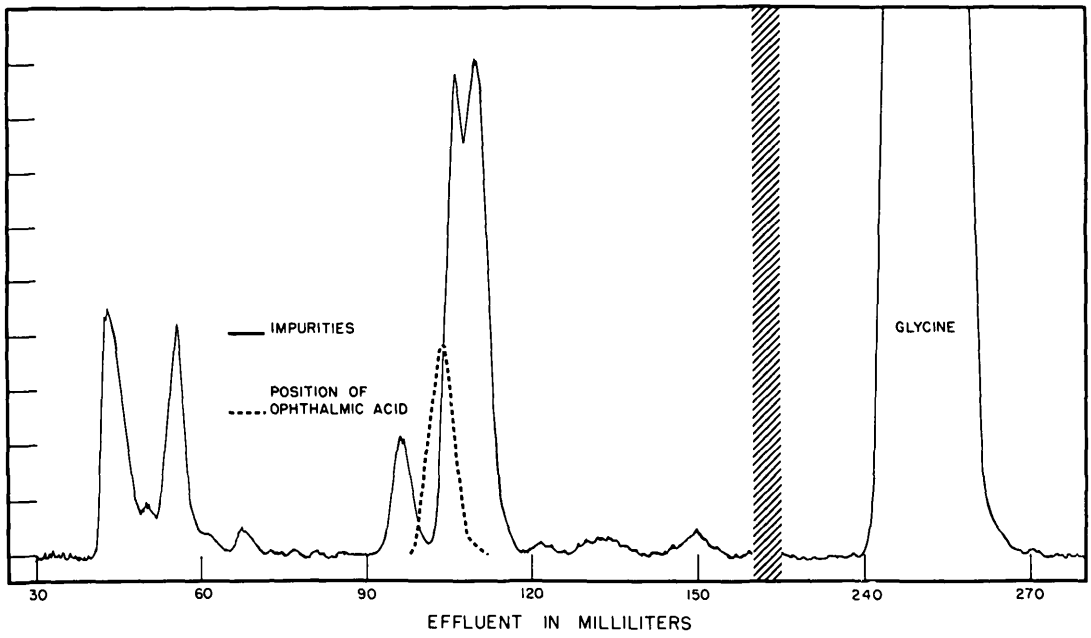

Fig. 1. A radiochromatogram of commercially obtained ^{14}C-labeled glycine showing the correspondence of the location of an impurity with ophthalmic acid. The amino acid was chromatographed on an automatic amino acid analyzer and the effluent fluid monitored continuously with a liquid flow detector for radioactivity.

glycine had 9 peaks other than glycine, accounting for 2.5 per cent of total activity. There were 6 impurities present in the sample of *l*-glutamic acid which amounted to 1.7 per cent of the activity.

The amino acids were repurified in approximately 1 mc. quantities, using a 150 cm. by 3 cm. "preparative" column attached to the automatic amino acid analyzer. Effluent fluid was collected in small fractions and checked for radioactivity by plating small aliquots on copper planchets and measuring the activity with a flow gas–end window type detector. Fractions containing the major portion of radioactivity were combined (200 to 250 ml.).

The following procedure was employed to remove the sodium citrate from the fluid which was used to elute the amino acids: The pH was adjusted to approximately 2.5 with concentrated HCl and the solution was allowed to flow through a column of Dowex 50 (H^+) (100 cm. by 1 cm.) under a pressure of 20 to 30 psi; the resin was 4 per cent cross-linked and had a mesh size of 200 to 400. The column was then washed with distilled water until the pH of the effluent became 4.0. The amino acid was eluted with 1.0N NH_4OH; after approximately 300 ml. of NH_4OH solution was passed through the column, the major portion of the amino acid appeared and was collected in the next 20 to 40 ml. of effluent. Ammonia was removed from the solution by first applying gentle suction and then passing a fine stream of bubbles of nitrogen through it. The volume was adjusted to approximately 50 ml. and the solution was stored at -20° C. Under these conditions no impurities appeared in quantities sufficient to interfere with the determinations made as long as a year after repurification.

Preparation of samples for chromatography. Lenses were cultured separately, and three or more were combined to provide sufficient material to analyze in duplicate the amino acids and peptides by methods outlined previously.[7] The media before and after use for culturing lenses were chromatographed after adjusting the pH to approximately 2.2 with sodium acetate buffer (0.2N, pH 2.2).

Further proof of the presence of ophthalmic acid in rabbit lenses. Because of conflicting reports as to whether ophthalmic acid is present in rabbit lenses[3, 7] this problem was re-investigated. Lenses were cultured in media containing ^{14}C-labeled glycine or glutamic acid, and extracts chromatographed. A peak showing radioactivity was found in the same position on the chromatograms as that obtained when a purified sample of ophthalmic acid was chromatographed. This observation alone, however, does not necessarily indicate the radioactive peak represents ophthalmic acid since traces of other tripeptides containing both glycine and glutamic acid might also occupy the same position. There is evidence for the existence of such compounds, at least in bovine lenses, and Calam[8] has suggested recently the possibility that a derivative of glutathione, S-(α-β-dicarboxyethyl)-glutathione may also exist in bovine lens. To provide further evidence that the peak ascribed to ophthalmic acid does in fact correspond to this compound, an additional experiment was performed.

Lenses were cultured with trace quantities of purified *dl*-α-amino n-butyric acid 3-^{14}C. This amino acid is also a component of ophthalmic acid but is not present in any other peptide thought to occur in the lens. Lens extracts were chromatographed and a single peak of radioactivity was observed in the same position as that shown earlier to correspond to ophthalmic acid, thus further strengthening the argument that this tripeptide is present in rabbit lenses.

Results

The average rate of accumulation of ^{14}C-labeled glycine and glutamic acid in the lens and the extent of the incorporation of the amino acids into glutathione

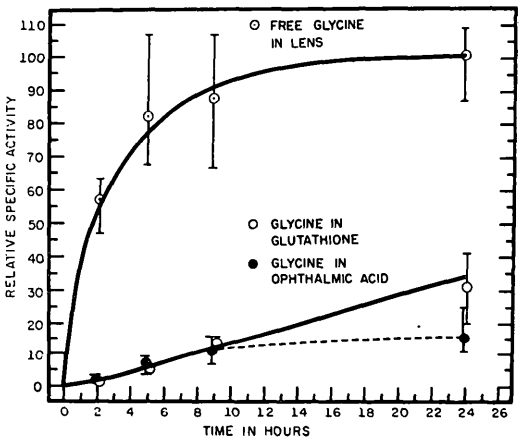

Fig. 2. Relative specific activity of free glycine and of glycine incorporated into glutathione and ophthalmic acid of lenses cultured for various periods. The points are average values weighted for the number of lenses; the vertical bars indicate range. The line representing the specific activity of the free amino acid is drawn as best visual fit, that for the specific activity of the amino acid in the peptides (solid line) is calculated with an analogue computer, where the turnover coefficient *k*, has a value of .018 $hr.^{-1}$ The broken line indicates an apparent decrease in the rate of incorporation of glycine in ophthalmic acid after 9 hours (see text).

Table I. Specific activity of free glycine, relative to that at 24 hours, and of glycine incorporated in the peptides of the lens cultured for various periods

Culture time (hours)	*No. of experiments*	*Glutathione (%)*	*Ophthalmic acid (%)*	*Free glycine (%)*	*Av. lens weight (mg.)*
2	4	1.9*	2.9*	57*	330* (18)
5	4	5.4	7.6	82	312 (15)
9	5	13.9	11.1	87	282 (15)
24	5	30.6	15.7	100	305 (17)

Number of lenses in parentheses.

The data are normalized for the same initial specific activity of glycine in the culture medium.

*Averages, weighted for number of lenses cultured.

Table II. Specific activity of free glutamic acid, relative to that at 24 hours, and of glutamic acid incorporated in the peptides of the lens cultured for various periods

Culture time (hours)	*No. of experiments*	*Glutathione (%)*	*Ophthalmic acid (%)*	*Free glutamic acid (%)*	*Av. lens weight (mg.)*
5	3	1.1*	3.8*	21*	326* (11)
9	2	3.5	3.8	26	307 (6)
15	1	8.5	7.1	111	— (3)
24	3	14.5	12.0	100	328 (10)

Number of lenses in parentheses.

The data are normalized for the same initial specific activity of glutamic acid in the culture medium.

*Averages, weighted for number of lenses cultured.

and ophthalmic acid in rabbit lenses cultured for various periods are shown in Tables I and II. The same data including the range of the values are shown plotted as a function of time in Figs. 2 and 3. The radioactivity of the free glycine and glutamic acid and those present in glutathione and ophthalmic acid have been expressed as a percentage of the average specific activity attained by the free amino acid in the lens at the end of 24 hours of culture.*

The rate of change of specific activity (Sp. Ac.) of the amino acids in the peptides was calculated with an analogue computer as a function of the difference in the specific activity of the free amino acid in the lens and that in the peptide:

$$\frac{d\ \text{Sp. Ac.}_{\text{peptide a.a.}}}{dt} = k\ (\text{Sp. Ac.}_{\text{free a.a.}} - \text{Sp. Ac.}_{\text{peptide a.a.}})$$

where k is the turnover rate of the amino acid in the peptide, and has the dimension of reciprocal time ($hr.^{-1}$); it represents the fraction of the total peptide present which is formed per unit time. The basic assumption in the equation is that the amino acids and peptides are uniformly distributed within the lens.

The lines in Figs. 1 and 2 for the specific activity of the free amino acids in the lens are drawn as best visual fits to the data. The curve for the relative specific activity of amino acids in glutathione and ophthalmic acid is calculated by the computer for a value of k equal to 0.018 $hr.^{-1}$ The data are in reasonable agreement with the calculated line except for the specific activity of glycine in ophthalmic acid at 24 hours. Thus about 2 per cent of glycine and glutamic acid present in glutathione and 2 per cent of glutamic acid present in ophthalmic acid is replaced each hour. The incorporation of glycine in ophthalmic acid for the first 9 hours also occurs at this rate

*The absolute specific activity of free glycine (760 c.p.m. per microgram) in the lens at the end of 24 hours of culture was found to be more than 4 times that of glutamic acid (175 c.p.m. per microgram), indicating that glycine accumulates in the lens much more rapidly than glutamic acid.

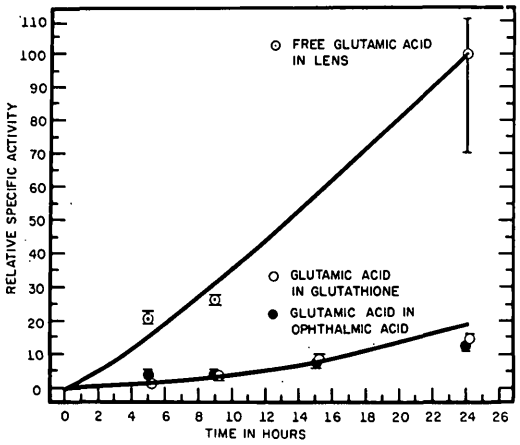

Fig. 3. Relative specific activity of free glutamic acid and of glutamic acid incorporated into glutathione and ophthalmic acid of lenses cultured for various periods. The points are average values weighted for the number of lenses; the vertical bars indicate range. The line for the free amino acid is drawn as best visual fit and that for the specific activity of the amino acid in the peptides is calculated with an analogue computer where the turnover coefficient k, has a value of .018 hr.$^{-1}$.

but appears to decrease thereafter as indicated by the broken line.

Puromycin, an antibiotic known to inhibit peptide bond formation in proteins,[9] when present in the culture media had no effect on the incorporation of glycine in glutathione or ophthalmic acid or on the steady state concentrations of these peptides.

Discussion

The rate of incorporation of glycine and glutamic acid in glutathione was found to be approximately 1.8 per cent per hour. This value is slightly less than that reported by Kinsey and Merriam who found the rate to be 2.4 per cent per hour.[1] While the difference in the rates is not great, the method of calculating turnover rate employed by Kinsey and Merriam involved the rate of approach to isotopic equilibrium of the whole system (lens plus culture medium), and leads to lower values than that used in the present study. Because of this it was suspected that the difference in results might in fact be greater than the values indicated unless other differences in experimental techniques had a compensating effect. Accordingly, a series of experiments, employing identical techniques both for the calculations and isolation of glutathione was performed. When the peptide was isolated, as was done in the previous study, as a cadmium salt, the turnover rate of glycine appeared to increase with the period of culture due to the progressive formation of radioactive material which was precipitated by cadmium and mistaken for glutathione (Table III). This procedure of isolation thus leads to an overestimate of the rate of turnover of glycine, depending on the period of culture employed, which is in part offset by the method employed in calculating turnover rate.

Table III. Incorporation of ^{14}C-labeled glycine into glutathione isolated by chromatography or by cadmium precipitation. Five lenses were cultured in Krebs-Henseleit medium[1] for each time period shown

Culture time (hours)	*% replacement of inert glycine in GSH*	
	Chromatography	*Cadmium precipitation*
2	1.7	1.8
2	1.7	1.7
12	8.4	9.9
24	23.4	31.0
24	24.7	31.8

The biosynthesis of glutathione and ophthalmic acid has been elucidated largely through the work of Snoke and co-workers[10, 11] and Cliffe and Waley[3] on tissue extracts and is thought to occur in the following manner:

A. Glutamic acid + Cysteine → γ-Glutamylcysteine,
γ-Glutamylcysteine + Glycine → γ-Glutamylcysteinylglycine (GSH)

B. Glutamic acid + α-amino n-butyric acid → γ-Glutamyl α-amino n-butyric acid,
γ-Glutamyl α-amino n-butyric acid + Glycine → γ-Glutamyl α-amino n-butyrylglycine (ophthalmic acid)

The incorporation of radioactive amino acids observed in the present study could have occurred through the reactions outlined above or through exchange of labeled for nonlabeled molecules involving no new peptide formation. The average turnover rates for both glycine and glutamic acid in glutathione were the same, suggesting that the amino acid turnover is a measure of direct synthesis rather than exchange, since the latter would be expected to lead to diverse rates of incorporation because of differences in molecular weights and other chemical properties of the amino acids. However, to the extent that glycine and glutamic acid are not similarly distributed, the similarity in their turnover rates would be only coincidental.

The rates of incorporation of glycine and glutamic acid in ophthalmic acid are equal for the first 9 hours although the rate of incorporation of glycine appeared to decrease thereafter (Fig. 2). The explanation for the apparent change in rate, which greatly exceeds the experimental variation of the data is not clear. Disproportionate quantities of peptides and differences in specific activity of labeled amino acids in various parts of the lens throughout the experimental period could account for the observed variation in the rate of incorporation of glycine.

To test whether the apparent decreased rate of incorporation of glycine into ophthalmic acid might be due to differences in the availability of the labeled amino acids or peptides in the lens, the distribution of these substances was determined in various parts of the lens following culture for 24 hours (Fig. 4). The lenses were separated into peripheral portion and central core with a cork borer (8 mm. diameter), and the central core was further divided into an anterior, posterior, and central portion. The respective samples from 6 lenses were pooled and analyzed in the usual manner. The width of the blocks (Fig. 4) is proportional to the weight of the part of the lens analyzed. It is apparent that neither the distribution

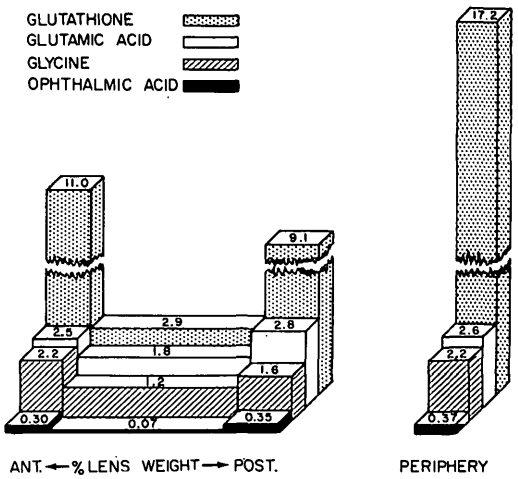

Fig. 4. Concentration (in micromoles per gram lens) of free amino acids, glutathione and ophthalmic acid in different parts of the lens. The width of each block is proportional to the weight of the portion of the lens analyzed.

of the amino acids nor the peptides is uniform throughout the lens. The concentration of all of the compounds studied was shown to be considerably higher in the cortical regions than in the nucleus, an observation also made with respect to glutathione by other investigators.[12, 13] However, the ^{14}C-labeled compounds were also similarly distributed by the end of 8 hours of culture so that the specific activity of the free amino acids was approximately uniform throughout the lens. Thus it seems unlikely that any difference in rate of incorporation could be accounted for on the basis of unequal concentrations of either the labeled amino acids or peptide in the lens.

It is possible that the reduced rate of incorporation of glycine in ophthalmic acid is due in fact to a reduction in the rate of synthesis of the peptide after the first 9 hours. However, the steady state concentration of the total peptide present in the lens was not significantly lower than normal after 24 hours of culture. Moreover, the rate of incorporation of labeled glutamic acid into this peptide did not vary throughout this period.

The absolute rate of formation of glutathione and ophthalmic acid may be calcu-

lated from their steady state concentrations and the turnover rate of the constituent amino acids, assuming that incorporation of amino acids represents synthesis of peptides. Steady state concentration of glutathione and ophthalmic acid in the present experiments was found to be 1,200 ± 250 and 29 ± 5.5 μmoles per 100 Gm. lens, respectively. From these values and the turnover coefficient, k = .018 hr.$^{-1}$ the rate of formation (and breakdown) of the respective peptides, glutathione and ophthalmic acid, is found to be 21.6 and 0.52 μmoles per 100 Gm. lens per hour.

A possible explanation for the large difference in the steady state concentration of the two peptides may be that the γ-glutamyl lactamase present in the lens decreases the net rate of formation of ophthalmic acid by degrading the dipeptide γ-glutamyl-α-amino n-butyric acid.[14]

Finally, the failure of puromycin to affect the incorporation of glycine into glutathione and ophthalmic acid, despite its known capacity to enter the lens,[15] suggests that ribosomal ribonucleic acid is not involved in the synthesis of these tripeptides as it is in proteins. Similar conclusions were reached by Lane and Lipmann[16] from experiments with extracts of pigeon liver and bovine lenses, who showed that ribonuclease had no effect on enzymatic synthesis of glutathione and ophthalmic acid.

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