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LYSINE TRANSPORT AND PROTEIN INCORPORATION BY THE LENS

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SUMMARY

The rates and interactions between systems for transport, efflux and incorporation into protein of L-lysine were determined in rabbit crystalline lens. L-Lysine was selected as a model amino acid for such studies, as it was not metabolized by the lens. The transport of L-lysine into lens *in vitro* occurred by saturable and "non-saturable" components. Kinetic constants for the saturable fraction were $K_m = 0.7$ mmole/l and $V_{max} = 0.110$ μmole/lens per h. The renewal rate for lens L-lysine was found to be 6 h. Only a small fraction (2.6%) of the total lysine in the lens was incorporated into lens protein at the physiologic levels of L-lysine in the aqueous humor (0.55 mmole/l). L-Lysine incorporation into protein was blocked by puromycin without any effect on the rate of L-lysine accumulated by the lens. A decrease in the rate of L-lysine transported and accumulated by lens resulted in a corresponding decrease in the levels incorporated into lens protein.

INTRODUCTION

Most natural amino acids are actively transported into the lens by specific carrier mediated mechanisms[1,2]. Lens amino acids are either accumulated, metabolized, incorporated into lens protein or efflux from the tissue[3,4] (Fig. 1). The studies on amino acid transport by the lens have centered mainly on α-aminoisobutyric acid, that is not metabolized, nor incorporated into protein. The more complex situations arising from transport of all other amino acids incorporated into protein require investigation. Questions have been raised as to whether transport of amino acids into lens takes place independently from protein synthesis, and whether the levels of an amino acid inside the lens and the rates of transport affect rates of incorporation into lens proteins. This is most significant in the cataracts produced by galactose, xylose or diabetes where there is a marked reduction in the levels of free amino acids[5–7] in the tissue and in the rate of amino acids incorporated into lens protein[8]. An experimental approach to the above mentioned problems is provided by the studies on L-lysine reported here. L-Lysine, a basic amino acid, was considered a model for such studies as it was not metabolized by the lens. To block protein synthesis and L-lysine incorporation into protein puromycin was added to the media. To affect the levels of

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lysine accumulated by lens, the media concentrations were varied or the transport "pump" partially inhibited by ouabain or sodium deprivation. The results were interpreted (a) in terms of Michaelis–Menten kinetics[9], and (b) in terms of transport constants of AKEDO AND CHRISTENSEN[10] for "non-saturable" migration of amino acids[11].

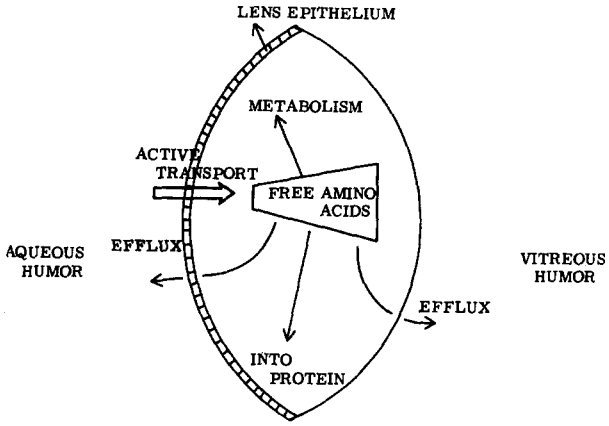

Fig. 1. Schematic diagram of turnover of free amino acids in lens.

MATERIAL AND METHODS

Lenses and incubation media

Rabbit lenses were obtained after enucleation and opening of the eyes through the posterior pole from animals killed by air embolism. The rabbits weighted 2–2.5 kg and their lenses 300–350 mg. Immediately after removal, the lenses were carefully transferred to Kjeldahl round-bottom flasks containing 10 ml of Tyrode's solution and L-[^{14}C]lysine in concentrations of 17 μmoles/l. The Tyrode's solution contained (in mmoles/l) NaCl, 137; KCl, 2.68; $CaCl_2 \cdot 2H_2O$, 2.04; $MgCl_2 \cdot 6H_2O$, 0.49; NaH_2PO_4, 0.416; $NaHCO_3$, 11.9; and D-glucose, 5.50; and was gassed before incubation with O_2–CO_2 (95:5, v/v). Two control flasks without tissue were simultaneously incubated. The lenses were incubated at 30° in a shaking water bath for periods of 2,4 and 21 h. Na^+-free media were prepared substituting by Tris–HCl as previously described[2]. L-[^{14}C]lysine (specific activity 7.5 mC/mmole) was obtained from New England Nuclear, Boston, Mass. Puromycin dihydrochloride and ouabain were obtained from Nutritional Biochemicals Corp., Cleveland, Ohio.

L-[^{14}C]*Lysine in lens water and lens protein*

At the end of the incubation periods the lenses were weighed and transferred to conical glass homogenizers containing 2 ml of 15% trichloroacetic acid. Lens homogenates were centrifuged at 2500 rev./min and 20 μl of supernatants plated for determination of ^{14}C radioactivity. 20-μl samples of initial and final media were similarly plated in planchettes and counted in a Nuclear Chicago gas flow counter. After radioactivity background subtractions, free L-[^{14}C]lysine in the lens was expressed in counts/min per ml of lens water with an average of 66.6% lens water

content. Results of L-[^{14}C]lysine experiments were calculated in lens/media distribution ratios:

$$\frac{L}{M} = \frac{\text{counts/min per ml of lens water}}{\text{counts/min per ml media}}$$

Samples of supernatant and media were plated on silica-gel thin-layer chromatography plates with the corresponding L-lysine standards, and run in a mixture of *n*-butanol–acetic acid–water (80:20:20, by vol.)[12]. 100% of the ^{14}C radioactivity was recovered from the L-lysine spot. Protein radioactivity was determined in precipitates washed in succession with 5 ml of each of the following: (1) 5% trichloroacetic acid; (2) 5% trichloroacetic acid heated for 15 min at 97°; (3) 95% ethanol; (4) 95% ethanol–ether–chloroform (2:2:1, by vol.) and (5) ether. The protein precipitates were dried under a heat lamp, ground in ether and plated on filter paper. 10–20 mg of protein aliquots and an ether–hexane (2:3, v/v) mixture produced an even layering of protein. The samples were counted on a Nuclear Chicago gas flow counter. After self-absorption determination, the results were calculated in:

$$\text{Counts/min per mg} = \frac{\text{counts/min} - \text{background}}{\text{mg of protein} \cdot \text{self-absorption}}$$

Kinetic constants for transport

A_1 or total L-lysine accumulate in lens (μmoles/ml lens water per h) = L/M distribution ratio × lens water (in ml) × L-lysine in media (A_m) (μmoles/ml). For determination of K_D (diffusion constant) and Y (the saturable component) the formulation of AKEDO AND CHRISTENSEN[10] were used. The dissociation constant for accumulation (K_m) and the maximum accumulation rates for L-lysine in conditions of saturation (v_{max}) were found from the values of Y in a double reciprocal plot (Lineweaver–Burk plot).

The total entry rate for L-lysine (in μmoles/ml per h) was found as:

$$\text{Total entry rate} = (Y + K_D \cdot A_m)\, t$$

and the rate of L-lysine efflux from the lens (in μmoles/ml per h) as:

$$\text{Efflux from lens} = \text{total entry rate} - A_1$$

Kinetic constants for incorporation into protein

The rate of L-lysine incorporation into protein was calculated from Michaelis–Menten kinetics[9]. The application of such treatment to calculate kinetic constants for amino acids incorporated into proteins appeared justified in as much as it represents an enzymatic process involving substrate–enzyme relationships dependent on the concentration of substrate (in this case, L-lysine). From the accumulation experiments the various concentrations of free L-lysine in the lens (A_1) were obtained, and the total in lens protein estimated as:

$$\underset{(\mu\text{moles/lens per h})}{\text{L-Lysine in lens protein}} = \frac{\text{counts/min per g lens protein} \cdot \text{g lens protein}}{\text{counts/min per ml lens water} \cdot \text{s.g. lens protein}} \cdot A_1$$

In a double reciprocal plot (Lineweaver–Burk plot)[13], the corresponding values for the K_m (dissociation constant for L-lysine–protein complex) and the v_{max} (maxi-

mum rate of L-lysine incorporation into protein in conditions of saturation) were found. Lens proteins specific gravity (s.g.) values of 1.40 were used in the calculations[14].

RESULTS

L-[^{14}C]*Lysine: Transport into lens and incorporation into lens protein*

A time curve of L-[^{14}C]lysine accumulated by rabbit lens and incorporated into lens protein is shown in Fig. 2. L-[^{14}C]Lysine was accumulated by the lens and reached an average lens/media ratio of 8.2 (S.E. $\pm$ 0.21) after 21 h of incubation.

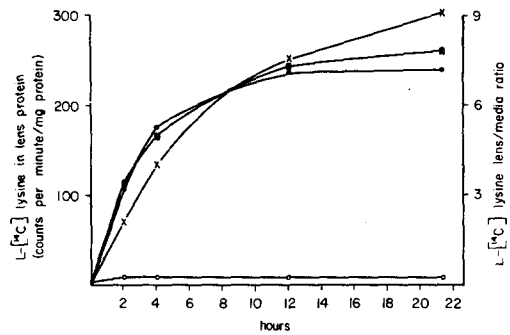

Fig. 2. Time curve of L-[^{14}C]lysine lens/media accumulation ratios (⊗), the L-[^{14}C]lysine incorporation into lens protein (×) at various timed intervals, and the effects of puromycin–HCl (0.9 mmole/l) on the L-[^{14}C]lysine lens/media accumulation ratios (●) and the L-[^{14}C]lysine incorporation into lens protein (○). Each dot corresponds to average of 4 lenses incubated at 37° as described in MATERIALS AND METHODS.

The progressive increases in the L-[^{14}C]lysine lens/media ratio were simultaneous with the incorporation of L-[^{14}C]lysine into lens protein, evidence that the transported amino acid was rapidly incorporated into protein. Furthermore, curves for L-[^{14}C]-lysine accumulated by lens and incorporated into protein paralleled each other, as the higher levels of L-[^{14}C]lysine into the lens resulted in increased incorporations into protein. The lens represents a closed system where: L-[^{14}C]lysine in 0 time incubation media — L-[^{14}C]lysine in final incubation media = L-[^{14}C]lysine free in lens + L-[^{14}C]-lysine in lens protein + L-[^{14}C]lysine metabolized. In a typical balance sheet, shown in Table I, it is evident lysine was not metabolized by the lens in incubations up to

TABLE I

DISTRIBUTION OF L-[^{14}C]LYSINE IN LENS AND MEDIA IN A TYPICAL EXPERIMENT

	Total counts/min in each compartment	
	4 h	*21 h*
Initial media	350 000	350 000
Final media	−301 500	−269 000
Total in the lens	48 500	81 000
Free in lens	40 500	64 000
In lens protein	8 000	17 000

21 h. Radioactive counting of fractions obtained by silicagel thin-layer chromatography of the incubation media and of free lens amino acids in lens after trichloroacetic acid removal with trioctylamine[2] failed to reveal any L-lysine metabolites. Similar results were obtained by W. BARBER who incubated rabbit lenses for 6 h and searched for L-lysine metabolites by ion-exchange column chromatography (personal communication).

Effect of puromycin

Puromycin inhibited L-[^{14}C]lysine incorporation into lens protein without affecting the rate of transport (Figs. 2 and 3). After 4 and 21 h of incubation L-[^{14}C]-lysine in protein was 144 $\pm$ 5 counts/min per mg protein and 307 $\pm$ 8 counts/min per mg protein, respectively. In Tyrode's media with 0.91 mmoles/l of puromycin the corresponding values for 4 and 21 h of incubation with [^{14}C]lysine were 4.2 $\pm$ 0.3 counts/min per mg protein and 3.7 $\pm$ 0.2 counts/min per mg protein, respectively. Low concentrations of puromycin (50 μmoles/l) were required for half inhibitions, and 97 % inhibition of L-[^{14}C]lysine incorporation into lens protein occurred with levels of 0.9 mmoles/l (Fig. 3). Conversely, the lens/media ratio of L-[^{14}C]lysine were not affected by puromycin (Figs. 2 and 3) and L-[^{14}C]lysine lens/media ratios of 5.15–5.57 were found after 4 h incubations with and without puromycin.

Kinetic constants for L-lysine transport into lens and incorporation into protein

In Fig. 4 the rates of L-lysine accumulation by rabbit lens and the corresponding values found for the saturable (Y) and "non-saturable" components at various L-lysine concentrations in the media, are shown. Found values for $K_D = 0.10 \cdot \text{lens}^{-1} \cdot \text{h}^{-1}$. The Lineweaver–Burk plot in the insert of Fig. 3 shows a $K_m = 0.7$ mmole/l and $v_{max} = 0.11$ μmole/lens per h for L-lysine accumulation into lens.

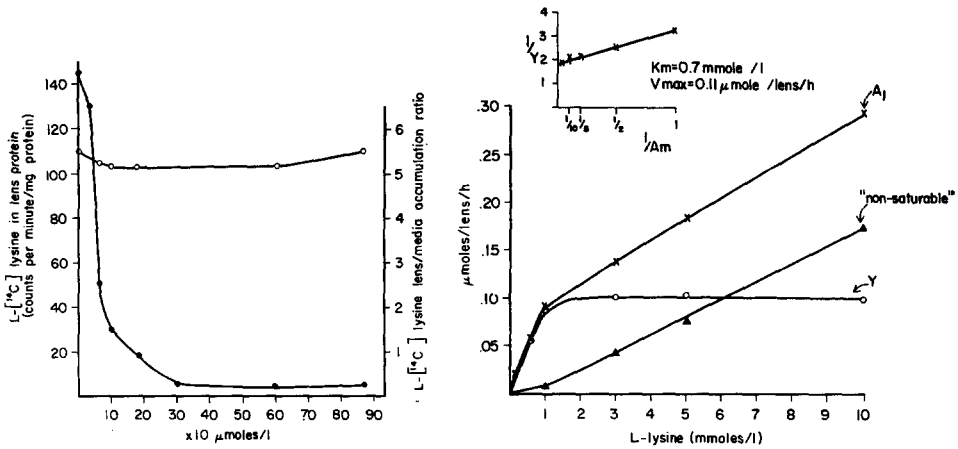

Fig. 3. Effect of puromycin hydrochloride (abscissa) on the L-[^{14}C]lysine lens/media accumulation ratios (○) and L-[^{14}C]lysine in lens protein (●). Each dot corresponds to 4 lenses incubated at 37° for 4 h.

Fig. 4. Rates of L-lysine accumulation by rabbit lens (A_1) and contributions by saturable (Y) and "non-saturable" components, with various L-lysine concentrations in the media. Each dot corresponds to average of 6 lenses incubated at 37° as described in MATERIALS AND METHODS for 4 h.

TABLE II

L-LYSINE RATES OF ENTRANCE AND EFFLUX FROM RABBIT LENSES

Average of 6 lenses in each group, incubated at 37° for 4 h.

A_m (μmoles/ml media)	A_l (μmoles/ml per h)	Y (μmoles/ml per h) From Eqn. 3	Y (μmoles/ml per h) From $Y^* = \frac{v_{max} \cdot A_m}{K_m + A_m}$	Total entry rate (μmoles/ml per h)	Efflux from lens (μmoles/ml per h)
0.5	0.24	0.24	0.23	0.29	0.04
1.0	0.40	0.35	0.32	0.45	0.05
5.0	0.83	0.45	0.48	0.95	0.11
10.0	1.22	0.44	0.51	1.44	0.24

* Refs. 9 and 10.

The experimental values of A_l, Y, total entry rate, and efflux are presented in Table II. Y, the saturable component, was independently calculated according to the Akedo–Christensen equations and from $v_{max} \cdot A_m/(K_m + A_m)$, both values being in close agreement. The efflux figures in Table II indicate at 0.5 μmole/ml concentration in the media, (levels of lysine in posterior aqueous humor = 0.42 mmole/l) 16% of the total accumulated by the lens effluxed per h.

In Fig. 5 the incorporation of L-lysine by lens protein at various L-lysine levels in the lens are shown. A K_m = 0.6 mmole/l and v_{max} = 3.5 nmoles/lens per h were calculated for the L-lysine protein incorporation system into lens.

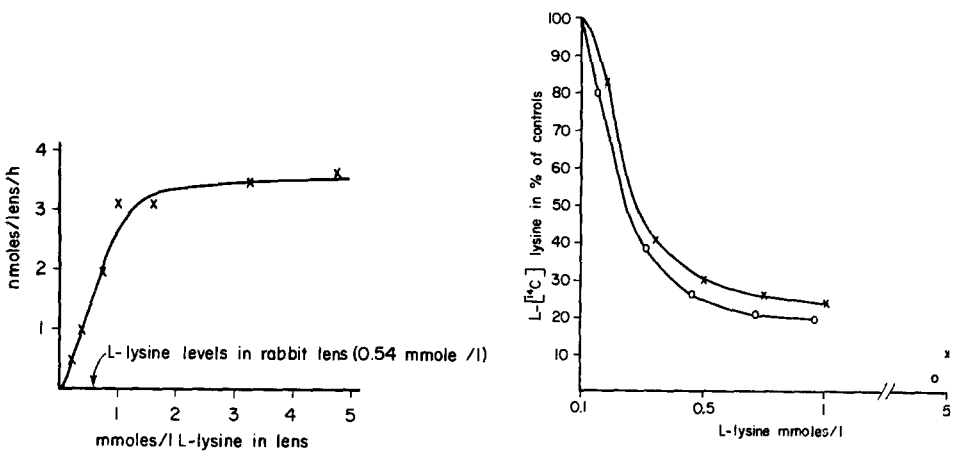

Fig. 5. Rates of L-lysine incorporation into lens protein (ordinate) at various lysine levels in the lens (abscissa). The lysine levels in the lens represented by the experimental values A_l, in the ordinate of Fig. 4. Each dot corresponds to the average of 6 lenses incubated at 37° for 4 h as described in MATERIALS AND METHODS.

Fig. 6. Effect of various concentrations of L-lysine in the media (abscissa) on the transport of L-[^{14}C]lysine (○) into lens and incorporation of L-[^{14}C]lysine into lens protein (×). The concentrations of L-lysine in the lenses were found experimentally (Table II). Each dot corresponds to average of 6 lenses incubated at 37° as described in MATERIALS AND METHODS for 4 h.

Effect of various levels of L-*lysine, Na^+ deprivation or ouabain in the media*

The effects of variations in the L-lysine accumulated by lens on the L-lysine incorporated by protein are reflected in the following results: (a) increased levels of L-lysine in the media resulted in decreased L-[^{14}C]lysine accumulated by the lens, and decreased incorporation into lens protein (Fig. 6); (b) low Na^+ or ouabain in the media inhibited L-[^{14}C]lysine accumulation rates by 22 and 15.5 %, respectively, and the L-[^{14}C]lysine in lens protein by 10 and 11 %, respectively (Table III). Although L-lysine is not very sensitive to Na^+ deprivation effects, the minimal changes in levels of L-lysine transported and accumulated by lens were reflected in the rates incorporated by protein.

TABLE III

EFFECT OF Na^+ REPLACEMENT OR OUABAIN ON THE ACCUMULATION OF L-[^{14}C]LYSINE BY THE RABBIT LENS AND INCORPORATION INTO LENS PROTEIN

Mean ± S.E. of 3 rabbit lenses in each group incubated at 37° for 4 h. *P*, probability; Student's *t* test.

	Accumulated		*Protein incorporated*	
	L/M ratio	*% of control*	*Counts/min per g protein*	*% of control*
Regular Tyrodes	4.87 ± 0.19		125 000 ± 2.800	
Na^+-free (Tris) Tyrodes	3.88 ± 0.15	78 ($P<0.05$)	112 000 ± 2.100	90 ($P<0.05$)
Regular Tyrodes	4.92 ± 0.24		114 000 ± 1.400	
Tyrodes + 0.1 mmole/l ouabain	4.16 ± 0.11	84.5 ($P<0.05$)	100 000 ± 1.200	89 ($P<0.05$)

DISCUSSION

Puromycin, which inhibits protein synthesis by blocking amino acid incorporation into proteins at the tRNA level, has been used in clarifying biochemical mechanisms of hormones and drugs[15]. In addition, puromycin separates biochemical functions related to or dependent on amino acid incorporation into protein by HeLa cells, bacteria, viruses or plants[15]. In transport studies WOOL *et al.*[16] used puromycin to detect the effects of insulin on the accumulation of [^{14}C]amino acids by rat diaphragm and heart muscle[17]. In the present studies, the complete inhibition of L-lysine incorporation into lens protein by puromycin, did not affect the rate of L-lysine transport and accumulation by the tissue. Thus, it is apparent that transport and accumulation of L-lysine by the lens are totally independent of the rate of L-lysine incorporation into protein. Similar conclusions were reached by investigators working on transport of amino acids by rat diaphragm[16] and L-lysine by reticulocytes[21]. However, in embryonic chick bone, puromycin inhibited amino acid transfer to protein and at a later time (2 h) amino acid accumulation by the tissue[18]. The concentrations of puromycin required to abolish incorporation of L-lysine into rabbit lens protein ($1 \cdot 10^{-5}$–$1 \cdot 10^{-3}$ mole/l) were in agreement with values reported for inhibitions of [^{14}C]histidine[19] or [^{14}C]valine[20] incorporation into calf lens protein.

Transport against the concentration gradient and lens/media ratios of 5–6 are reached in 4 h when L-[^{14}C]lysine is present in trace amounts (0.017 μmole/ml) in the media. In the rabbit, the concentration of lysine in lens water is 1.6 that of aqueous humor[1]. At 0.5 mmole/l in the media, the concentration corresponding to that of aqueous humor lysine[1], the A_l/A_m ratio is 1.7 and closely approximates the lysine distribution ratio *in vivo*.

16 and 21 % of the total L-[^{14}C]lysine in the lens was incorporated into protein after 4 and 21 h of incubation. In the 21-h incubations and due to the continuous protein synthesis process, L-[^{14}C]lysine in protein became a larger fraction of the total radioactivity in the lens. Minimal physiological significance can be attached to those distributions as the trace L-lysine levels in incubation media were 1/2000 of the lysine in aqueous humor. The capabilities of the systems for accumulation and incorporation into protein are reflected more precisely in the kinetic constants. The v_{max} for L-lysine transport = 110 nmoles/lens per h and that for protein incorporation = 3.5 nmoles/lens per h. The calculated velocity for transport at the physiologic concentrations of L-lysine in aqueous humor (0.42 μmole/ml)[1] is 0.050 μmole/lens per h while the velocity for protein incorporation at the levels of lens L-lysine (0.55 μmole/ml)[1] is 0.0013 μmole/lens per h. Extrapolating to the lens *in situ*, only 2.6 % of the amino acid L-lysine transported by the lens is incorporated into protein. In the rabbit, the concentrations of L-lysine in the posterior chamber and lens are below the K_m's for transport (0.7 mmole/l) and incorporation into lens protein (0.6 mmole/l). Thus, the K_m's favor both the active transport and incorporation into protein processes. The turnover rate for L-lysine in the lens derived from the efflux rates = 0.16· lens^{-1}·h^{-1} at L-lysine levels in the media of 0.5 μmole/l. Assuming all L-lysine in the lens available for exchange, the renewal rate of L-lysine in the lens would be 6 h.

The calculations of Y, the saturable component, are essential for the determinations of K_m and v_{max} of L-lysine transport. The separation of the "non-saturable" component from the total accumulated by the lens is required in treating such data. "Non-saturable" migration has been discussed in detail by CHRISTENSEN AND LIANG[11]. "Non-saturable" transport was found among a variety of amino acids and tissues such as α-aminoisobutyric acid in rat diaphragm[10]; alanine, taurine and betaine in Ehrlich ascites cells[11], α-aminoisobutyric acid in choroid plexus[22] and lysine in human kidney[23].

The concentration of L-lysine in the lens depends on the extracellular levels of the amino acid and duly regulates the rate of the amino acid entrance into protein. In experiments with low Na^+ or ouabain additions to the media a small fraction, 10–15 % of L-lysine accumulation was found to be dependent on Na^+ or the (Na^+–K^+)-ATPase. However, even such small decreases in the rates of accumulation were reflected in equal decreases of L-[^{14}C]lysine in protein. Thus, the decreased incorporation of amino acids into proteins and protein synthesis in animals with galactose, xylose or diabetic cataracts[5–8] is most likely secondary to the failure in retention of free amino acids by the tissue. The stimulation of liver protein synthesis by elevated intracellular levels of amino acids[24], may occur in lens and large amino acid doses may have possible corrective measures in maintaining protein synthesis by the lens in abnormal situations.

It must be understood that the treatment of the entire system for amino acid incorporation into protein as a single entity or unit is a simplification. The data on L-lysine incorporation into proteins is an approximation as best as can be calculated.

The application of other methods or selective inhibition of L-lysine incorporation into lens may be required to further clarify such complex systems.

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