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AMINOACYL-TRNA SYNTHETASE ACTIVITY AND PROTEIN
LEAKAGE IN NORMAL AND CATARACTOUS RAT LENSES.

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AMINOACYL-TRNA SYNTHETASE ACTIVITY AND PROTEIN
LEAKAGE IN NORMAL AND CATARACTOUS RAT LENSES

by

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TABLE OF CONTENTS

	page
List of Tables	1-2
List of Illustrations	3-4
Summary	5-7
Introduction	8-17
Materials and Methods	18-25
Results	26-38
Tables and Illustrations	39-90
Discussion	91-99
Bibliography	100-109
Autobiographical Statement	110-111

TABLES

	page
Table A. Incorporation of ^{14}C -Leucine into peripheral zones sRNA with pH 5-enzyme of different origins.	10
Table 1. The percent activity of the methionyl-tRNA synthetase following storage at -20°C and 4°C .	44
Table 2. The distribution of the methionyl-tRNA synthetase in rat lenses.	49
Table 3. Average lens methionyl-tRNA synthetase activity with protein synthetic inhibitors.	50-51
Table 4. Lens methionyl-tRNA synthetase activity in normal, glucose-fed, and galactose-fed rats.	55
Table 5. Lens methionyl-tRNA synthetase activity for animals placed on galactose diets which were discontinued after 3, 8 or 17 days.	58-60
Table 6. Lens methionyl-tRNA synthetase activity in rats fed a 35% xylose diet and controls.	61
Table 7. Lens weights from rats on various diets.	62
Table 8. Average rat weight in grams.	63
Table 9. Food consumption in grams per rat.	64
Table 10. Thymidine C^{14} incorporation into lens DNA after 3-5 days of galactose or xylose feeding.	65-66
Table 11. The effect of inhibitors on thymidine C^{14} uptake into rat lens.	70
Table 12. The lens methionyl-tRNA synthetase activity in 120 gm. galactose-fed rats, and in normal rats.	71

TABLES (Cont.)

	page
Table 13. Se^{75} -selenomethionine-tRNA binding in the presence of unlabelled amino acids.	72-75
Table 14. Methionyl-tRNA synthetase activity in galactose, mixed control and galactose, and control lens supernatants.	76
Table 15. Methionyl-tRNA synthetase activity in lenses of galactose-fed animals and galactose-fed with glucose supplement.	77
Table 16. H^3 -leucyl-tRNA synthetase activity.	80
Table 17. <u>In vitro</u> lens protein leakage from control and galactose-fed rats.	83
Table 18. <u>In vitro</u> leakage of methionyl-tRNA synthetase from normal and galactose (8 days) lenses.	84
Table 19. Leakage of radioactive protein from normal and galactose (11-12 days) lenses.	85
Table 20. In vitro lens protein leakage (control and galactose-fed rats)	86
Table 21. Digestion of collagen gel by enzymes.	88
Table 22. I^{125} lens protein bound to serum gamma globulin in starch gel electrophoresis.	89-90

ILLUSTRATIONS

	page
Fig. A. The activation of an amino acid and its attachment to sRNA.	9
Fig. B. Stages of cataract development.	12
Fig. 1. Effect of incubation time on lens methionyl-tRNA synthetase activity.	39
Fig. 2. The effect of Mg on lens methionyl-tRNA synthetase activity.	40
Fig. 3. Effect of ATP on lens methionyl-tRNA synthetase activity.	41
Fig. 4. Effect of temperature on lens methionyl-tRNA synthetase activity.	42
Fig. 5. The effect of pH on lens methionyl-tRNA synthetase activity.	43
Fig. 6. The effect of tRNA on methionyl-tRNA synthetase activity.	45
Fig. 7. The effect of lens homogenate concentrations on Se^{75} selenomethionine-tRNA binding.	46
Fig. 8. Se^{75} selenomethionine-tRNA binding with unlabeled DL-methionine and DL-selenomethionine.	47
Fig. 9. The apparent K_m value for DL-selenomethionine.	48
Fig.10. Lens methionyl-tRNA synthetase activity in rats ranging from 22-453 days of age.	52
Fig.11. Methionyl-tRNA synthetase activity in rat lens with varied dietary conditions: glucose, galactose and 3 groups returned to purina diet after 3, 8 and 17 days of galactose feeding.	53-54

ILLUSTRATIONS (Cont.)

page

- Fig. 12. Methionyl-tRNA synthetase activity per mg. of lens protein with varied dietary conditions: control, glucose, galactose, and 4 groups returned to purina diet after 3, 8 and 17 days of galactose feeding. 56-57
- Fig. 13. Effect of incubation time on the uptake of C^{14} -thymidine into lens. 67
- Fig. 14. Effect of temperature on the uptake of C^{14} -thymidine into lens. 68
- Fig. 15. Effect of unlabelled thymidine on C^{14} -thymidine uptake into lens. 69
- Fig. 16. The effect of lens homogenate concentrations on H^3 -leucyl-tRNA binding. 78
- Fig. 17. Lens methionyl-and leucyl-tRNA synthetases in normal and galactose-fed rats. 79
- Fig. 18. In vitro leakage of proteins from normal and galactose-fed rats. 81
- Fig. 19. In vitro protein leakage from lenses of young (34 days) and old (10 mo.) rats. 82
- Fig. 20. Disc electrophoretic patterns of in vitro protein leakage from lenses of normal and galactose-fed (13 days) rats. 87

As detected by Se^{75} -selenomethionine binding, rat lens methionyl-tRNA synthetase has been characterized and quantitated. The enzyme activity is relatively constant over a pH range of 6.8-8.6, and optimal binding occurs at 37°C after a 40 min incubation.

Lenses from galactose-fed animals show a significant increase in methionyl- and leucyl-tRNA synthetase activity at 3 days. The methionyl-tRNA synthetase activity is maximum (140% of control) at 3 days while the leucyl-tRNA synthetase reaches a maximum (195% of control) at approximately 8 days. These differences suggest that the enzymes have a cellular regulatory role. The increase in enzyme activity during the development of galactose-induced cataracts appears not to be dependent on the concurrent epithelial cell proliferation since older animals fed galactose show increased DNA synthesis, but no enzyme rise.

With continued galactose feeding, synthetase activity rapidly declines so that at 24 days it is 20-30 percent of the controls. Animals fed galactose for 3 days show normal lens methionyl-tRNA synthetase activity after having been returned to a Purina diet for 2, 12 or 21 days. When galactose is discontinued after 8 days, enzyme activity 7 and 16 days later is less than the control value, but higher than that of the galactose-fed animals. No significant

change in methionyl-tRNA synthetase activity is noted in lenses from animals fed xylose for 31 days.

The loss of synthetase activity in cataractous lenses may in part be due to leakage of lens proteins. Normal rat lenses incubated in vitro show small protein leakage (49 μ g/ 3 hrs) which is probably related to the manipulation of removing the lens and placing it in the incubation medium. Lenses from galactose-fed rats also show similar small leakage until about 8 days. With the appearance of nuclear cataracts leakage increases to approximately (268 μ g/ 3 hrs) and remains at this high level even when lenses from rats fed galactose for 17 or 51 days are tested.

Disc electrophoretic studies indicate that the leaking proteins from normal and galactose-induced cataractous lenses are those proteins which are found in highest concentration in the lens.

Methionyl-tRNA synthetase activity can be detected in the effluent medium from both control and galactose induced cataractous lenses. Significant radioactive protein can be found in the medium when galactose lenses are incubated in Se⁷⁵-selenomethionine. The addition of puromycin inhibits lens protein synthesis and prevents the accumulation of radioactive protein in the medium.

In vivo leakage of lens protein might lead to the formation of autologous antibodies. However, no circulating

lens antibody titres could be detected in the serum of cataractous rats by gel diffusion or by radioimmunoassay.

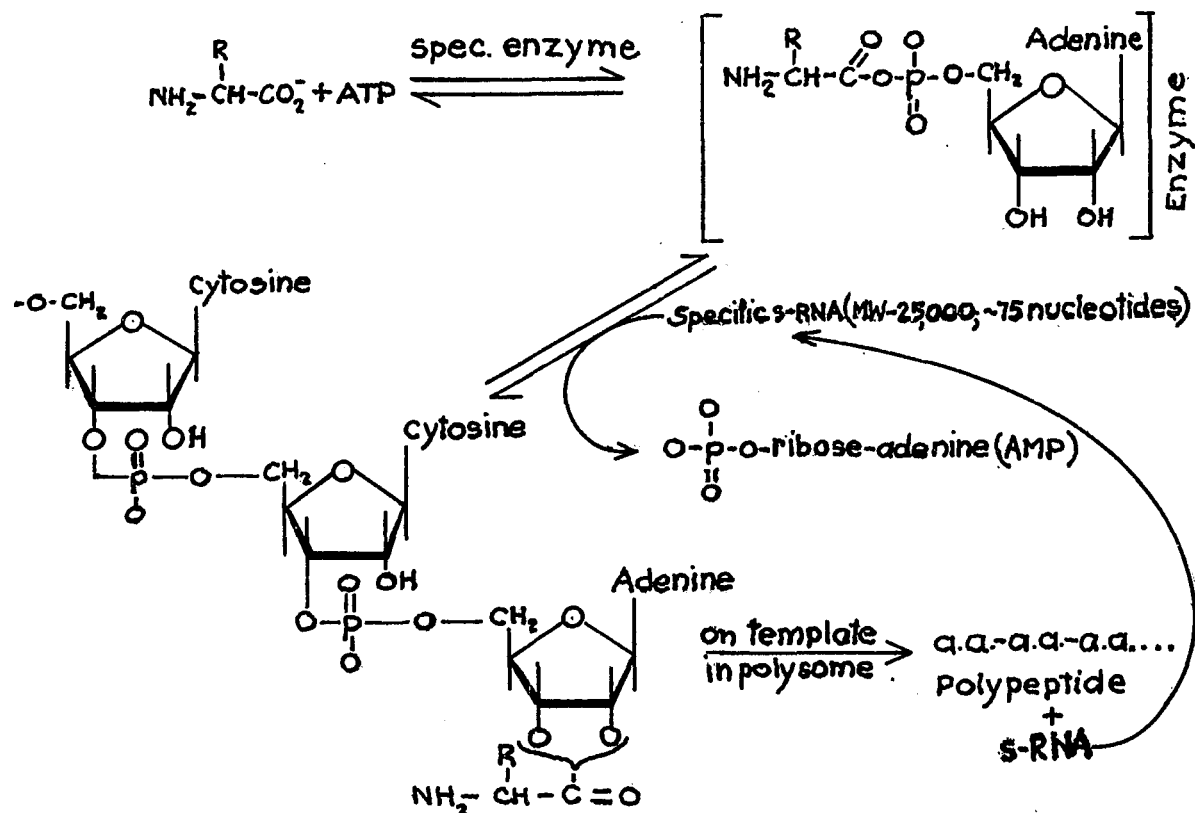
In these investigations methionyl-tRNA synthetase activity was evaluated in the normal, aging and cataractous rat lens by employing an analog of methionine, Se^{75} -selenomethionine, and by modifying existing synthetase assays. An interesting aspect of this work was that an increase in methionyl-tRNA synthetase activity appeared during the early stages of galactose-induced cataractogenesis. The leucyl-tRNA synthetase increase occurred at a slightly later time and was greater. These increases may reflect a cellular regulatory role of these enzymes. Thymidine- C^{14} incorporation studies were carried out in order to compare enzyme and mitotic activities. The decrease in aminoacyl and RNA synthetase activity found in the latter stages of cataractogenesis led to studies of protein leakage. Protein, including enzyme protein, leaked from galactose-induced cataractous lenses during in vitro incubation. Protein leakage may be one of the major dysfunctions of cataractous lenses in situ, or it may be that these lenses are easily damaged during removal.

Aminoacyl synthetase:

The role of aminoacyl synthetases in the biosynthesis of proteins has been well documented (Berg, 1961). These enzymes participate in a 2 step reaction. In the first step, the carboxyl group of the amino acid is activated (acyl group). The reaction is sometimes studied by trapping the

activated amino acids by nucleophilic reagents such as hydroxylamine. This step is reversible and the enzyme may be detected by the incorporation of pyrophosphate- P^{32} into ATP. In the second step the aminoacyl-tRNA complex is formed. The enzyme may now be studied by the radioactivity of the labelled amino acid-tRNA complex (TCA-precipitable). This technique was employed to determine methionyl-tRNA synthetase activity in these investigations.

Fig. A: The activation of an amino acid and its attachment to sRNA.



Reproduced from Ingram, V.M. The biosynthesis of macromolecules. W.A. Benjamin, Inc. N.Y., 1966, p. 106.

Although the methionyl-tRNA synthetase has been characterized in both bacterial systems (Bergman, Berg and Dieckmann, 1961) and mammalian systems (Hoagland, Keller and Zamecnik, 1956) only one paper has been published on aminoacyl-tRNA synthetases in the lens. Virmaux and Mandel (1964) studied the distribution of leucyl-, glycyl- and valyl-tRNA synthetase derived from calf lens epithelium, cortex and nucleus. Enzyme activity in the pH 5 precipitated fraction was measured by the binding of C^{14} -labelled amino acids with tRNA extracted from lens. Enzyme activity was detected in the cortex and epithelium, but not in the nucleus.

Table A.

Incorporation of ^{14}C -Leucine into Peripheric Zones sRNA
with pH 5-Enzyme of Different Origins.

pH 5 fraction derived from	Epithelium+ cortex	Cortex	Nucleus
nmole ^{14}C leu incorporated per mg peripheric s RNA	0.835	0.750	0

Reproduced from Virmaux and Mandel (1964)
Nature 20:297

Because of the convenience of detecting gamma radiation and availability of Se⁷⁵-selenomethionine in high specific activity (364 mc/mM), this acid analogue was used to detect synthetase activity in rat lenses. Other investigators have determined that selenomethionine and methionine are approximate metabolic equivalents. Selenomethionine has been shown to replace methionine in the protein of bacterial systems. (Tuve and Williams, 1961; and Cohen and Cowie, 1957) and in mammalian tissues (Hansson and Blau, 1963; Oldendorf and Kitano, 1963; and Penner, 1966). This analogue has been incorporated into active β -galactosidase (Cowie and Cohen, 1957).

Since Painter (1947) and Klostermann and Painter (1947) first prepared Se⁷⁵-selenomethionine it has also been widely used for medical diagnosis particularly for scanning of the pancreas (Blau and Manske, 1961) and the parathyroid glands (Digiulio and Beierwaltes, 1964) as well as for thyroid uptake (Potchen, Awwas, Edelstein and Dealy, 1966) and intestinal transport studies (Spencer and Blau, 1962).

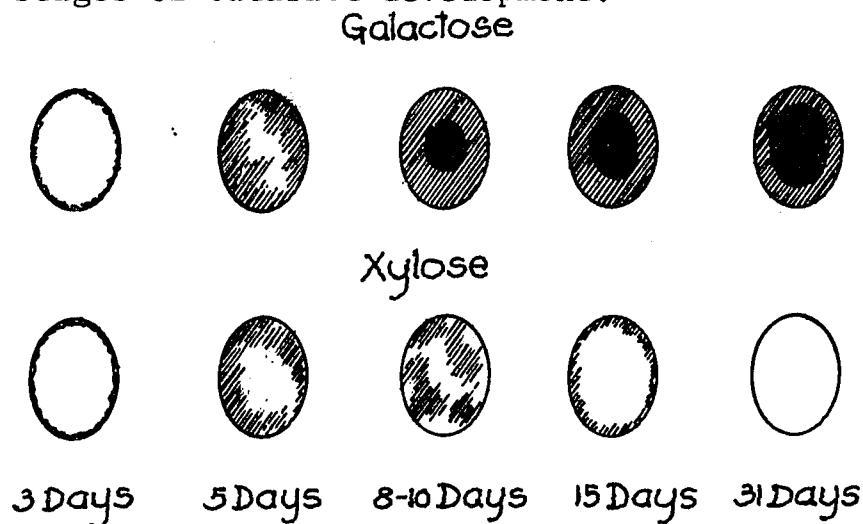
Cataractogenesis:

In human beings, cataracts occur in the disease states, galactosemia and diabetes; in aging; in dietary deficiencies of amino acids and vitamins; by exposure to ionizing radiation; or may be hereditary in origin. Other factors known to induce cataracts are chemical substances: sugars (galactose and xylose),

alloxan, dinitrophenol, mimosine, iodoacetate, mycleran, naphthalene and physical factors: low temperatures, and micro-waves.

Sugar cataracts are easily inducible in laboratory rats. When young animals are placed on diets high in galactose (50%) or xylose (35%) cataractous areas appear after 3 days as narrow opaque rings in the equatorial region of the lens. After 5 days of xylose diet the cataractous area enlarges, but is still confined to the area of the equator, whereas, after 5 days of galactose diet, the opaque area has progressed over most of the lens surface. At 8 days, the surface of the xylose-induced cataractous lens appears to be totally opaque with patchy areas of unequal density. At this time the galactose lens is more uniformly opaque and in some lenses nuclear cataracts can be observed. Once nuclear cataracts are present, the general appearance of the lens shows little change. After 15 days of xylose diet the opaque region has receded to the lens equator and by 31 days the lenses are again grossly transparent.

Fig. B. Stages of cataract development.



Thus, the xylose cataracts are neither progressive nor permanent and few metabolic alterations (a temporary decline in ATP levels and inhibition of hexosemonophosphate shunt) have been found during their course (van Heyningen, 1959; van Heyningen, 1963; Lerman and Heggeness, 1961 and Sigman, Burton, Fontaine and Lerman, 1963).

Gross biochemical alterations are known to occur in the course of galactose-induced cataractogenesis. With the accumulation of metabolites of galactose in the lens, changes occur in the amounts of water, protein and glutathione as well as in amino acid transport, energy metabolism (hexosemonophosphate shunt activity, and ATP concentrations) and in many enzymes of carbohydrate metabolism (Korc, 1961; Lerman, 1960a; Sippel, 1966a; Sippel, 1966b; Sippel, 1967; and Reddy, 1965). As the lens opacity develops, in vivo protein synthesis (Lerman, Devi and Hawes, 1961) and soluble protein content (Dische, Zelmenis and Youlus, 1957; and Lerman, 1962) are lowered.

An increase in the rate of DNA synthesis is also known to occur early in galactose-induced cataractogenesis. Von Sallmann (1957) evaluated the lenticular mitotic activity in young rats fed galactose for a period of 10 days. After the third day of diet, the rate of mitotic increase is at a maximum. Hanna and O'Brien (1960) studied lens proliferation with tritiated thymidine and autoradiography, and found maximum DNA synthesis

after 3-6 days of galactose feeding in 6 month old rats.

Recent studies by Grimes and von Sallmann (1968) using flat mount preparations, indicated increase lens mitotic activity peaking after 4 days of galactose diet while no increase was found in xylose cataracts. It has also been found that RNase activity which is scarce in the normal rat lens appears to significantly increase with visible opacification in sugar cataracts. (Zigman et al, 1963).

Lens proteins:

Lens proteins have been designated according to their solubilities as alpha, beta and gamma crystalline (Mörner, 1894). Purification of each fraction was obtained by combining zinc glycinate and isoelectric precipitation, and fractionation with Sephadex 75 (Spector, 1964). The alpha groups appear to be high molecular weight aggregates (1×10^6) (Bjork, 1963; Bjork, 1964a; Spector, 1965; Francois, Rabney and Wieme, 1955; Resnik, 1957; and Palmer and Papaconstantinou, 1969) with a sulfhydryl content significantly lower than the beta and gamma fractions (Kinoshita and Merola, 1958; and Firfarova and Levdikova, 1962). Beta crystalline represent the largest group, give a number of distinct peaks by sedimentation velocity and have a molecular weight of $5 \times 10^4 - 1 \times 10^5$ (Bjork, 1964b and Spector, 1964). The gamma faction shows homogeneity by ultracentrifugation with molecular weight of approximately 2×10^4 (Bjork, 1960; Crekhovich, Firfarova and Shpikiter,

There have been few studies to determine qualitative changes in lens proteins during the development of galactose-induced cataracts: Holt and Kinoshita, (1968) report changes in electrophoretic mobility of two beta zones after 1 day of galactose feeding, and the appearance of a new beta zone in nuclear cataractous lenses. Many investigators have shown quantitative decreases in gamma and beta regions, while the alpha zone became more diffuse in nuclear opacities with the appearance of a fast alpha fraction (Dische et al, 1957; Wittgenstein and Rowe, 1965; and Holt and Kinoshita, 1968).

Although some investigators have suggested that protein leakage might be involved in the loss of proteins in cataractous lenses, no experimental data have been reported. Recently, Charlton and van Heyningen (1968) showed that normal calf lenses incubated several days in media with altered tonicity (e.g., distilled water) leaked proteins which were predominately of low molecular weight. Since examination of these lenses showed capsules to be intact, the authors assumed that the proteins escaped through the capsule.

Aging:

The production of sugar cataracts is directly related to age: as the animal matures longer periods of time are necessary to induce galactose and diabetic cataracts, while xylose cataracts can only be produced in young animals. Energy

metabolism in the lens, as measured by the hexosemonophosphate shunt and the Embden-Meyherhof pathway, shows a progressive decrease with age (Pitel and Lerman, 1962; Lerman, Donk and Pitel, 1962; Nordmann and Mandel, 1959; and Lerman, 1961).

Protein synthesis as measured by the incorporation of labelled amino acids into protein (Lessinger and Mandel, 1960), and the gamma crystallin protein fraction (Genis-Galves, Maisel and Castro, 1968; and Devi, Friel and Lerman, 1961) decline with aging. The appearance of a new subunit of crystallin occurs with lens maturation and arises from chemical conversion of a pre-existing subunit (Palmer and Papaconstantinou, 1969). As the lens matures the synthesis of soluble lens protein decreases, but the formation of insoluble protein continues at a relatively steady rate. Concurrent with changes in relative protein concentrations is a gradual loss in lens water, and an increase in nuclear fiber volume (Lerman and Zigman, 1965).

Nucleic acid metabolism also shows alterations in the lens with aging. As the lens matures, there is a significant fall in ribosomal RNA and a progressive increase in albuminoid RNA (extracted with insoluble protein). The amount of lens tRNA remains relatively constant with age. (Pitel and Lerman, 1962; Dische et al, 1961; Dische et al, 1956; and Lerman, Devi and Banerjee, 1962). However the level of tRNA

declines when senile cataracts are present (Meraini, Diotti and Santori, 1964). There is also a progressive decrease with age in the turnover rates of $^{32}\text{PO}_4$ and ^{14}C -labelled purines in lens RNA (Zigman et al, 1963). The amounts of lenticular DNA is very low due to the relatively small number of nucleated cells, and remains fairly constant during most of the life span of the lens. In the embryonic lens the entire anterior surface epithelial cells undergo DNA synthesis. After about 8 weeks of age the cell population remains relatively constant (von Sallmann and Grimes, 1966) and the cells undergoing DNA synthesis become confined to the germinative zone, a narrow region anterior to the equator (Hanna, 1965).

Collagenase:

Leakage of lens proteins could be facilitated if the capsule was disrupted. Gross and Lapiere (1962) pioneered in the development of a quantitative assay for collagenolytic activity in tissue fragments cultivated on fibrous collagen gels. The enzyme activity was measured by the increasing area of lysis around the explant, and by the hydroxyproline content of degraded collagen. Since these basic studies, collagenase has been detected in epithelial tissue in wounds (Grillo and Gross, 1967), skin diseases (Eisen, 1967) and alkali treated corneas (Brown, Weller and Wassermann, 1969).

Materials and Methods

Surgical removal of the lens:

Sprague-Dawley rats were sacrificed by decapitation and the eyes enucleated. Lenses were exposed immediately by cutting through the optic disc and then laterally. Tension was then applied to the sclera above and below the place of incision. The lens was removed with a loop and placed into test tubes containing the incubation medium.

Aminoacyl synthetase assay:

Lenses were homogenized in 0.5-1.0 cc 0.05 M Tris buffer, pH 7.8, containing 0.01 M $MgCl_2$ and 0.005 M dithiothreitol (DTT). The final homogenate (12.5 mg lens in 80 μ l) was centrifuged at 30,000 x g in a Beckman Model L-2 ultracentrifuge for 45 min. The supernatant was dialyzed against 0.02 M Tris buffer, pH 7.8, containing 0.005 M reduced glutathione for 4 hr. The above procedures were carried out at 0°C. Lenticular synthetase activity in normal and experimental animals was determined immediately following dialysis.

The reaction mixture, pH 7.6, usually contained 80 μ l of lens dialysate; 26 μ M Tris-Cl; 3.73 μ M $MgCl_2$; 1.32 μ M ATP.K₂; 1.33 μ M DTT; 1.5 mg E Coli, strain B, tRNA; 0.32 m μ M Se⁷⁵-selenomethionine or (0.066 m μ M) H³ leucine and water to make a final volume of 200 μ l. Control tubes from which lens

homogenate, ATP, or tRNA was omitted, ribonuclease was added, or that was heated to 90°C also were run. Following incubation for 10 min. at 37°C the reaction was terminated by the addition of cold 5% trichloroacetic acid (TCA). The mixture was then filtered through a millipore disk, 0.45 μ , washed with cold 5% TCA and Se⁷⁵-selenomethionine-tRNA was counted in a Model RCG3A, well counter (Weller and Green, 1969).

Tritiated leucine-tRNA was precipitated on glass filters, washed with 5% TCA, followed by methyl alcohol, dried and counted in 0.5 ml of Bray's solution in a Mark I, Nuclear Chicago liquid scintillation well counter.

Each assay (done in duplicate) contained 4-6 lenses and groups containing 12-24 lenses were averaged for each experimental point.

Diets:

Four-week-old female rats weighing approximately 40 gm were placed on "galactose" diets (50% powdered Purina Laboratory Chow by weight mixed with 50% D-galactose), "glucose" diets (50% Purina and 50% -glucose), and control (Purina). A series of studies in which rats were placed on 50% galactose diet for 3, 8 or 17 days and then refed Purina Chow also were run. In another series of experiments a similar group of rats was placed on a "xylose" diet (50% Purina and 50% -xylose), and (65% Purina and 35% -xylose), and control (Purina).

Protein analysis:

Protein determinations were carried out by the Lowry, Rosebrough, Farr and Randall (1951) procedure. The final readings were made at 760 m μ on a Beckman DU-2 Spectrophotometer.

Thymidine uptake studies

Lenses were incubated with Krebs-Ringer Phosphate (KRPO₄) (Umbreit, Burris and Stauffer, 1957) containing 3.3 μ c (4.07 μ g) thymidine carrier solution. They were then weighed, homogenized in distilled water, and 1 cc aliquots were placed in planchets and dried. Samples were counted in a Nuclear-Chicago Model 181B. The correction factor for self absorbance of lens tissue was determined by dilution curves and the appropriate value applied to each sample.

In vitro Protein leakage studies:

Lenses (19-28 were averaged for each experimental point) were placed in separate vials containing 1.5 ml of KRPO₄ with 100 mg percent glucose. At the end of 1 hour periods they were transferred to other vials with an ophthalmological loop. In other experiments the transfer of lenses from vial to vial was facilitated by the use of small nicron wire loops with a paraffin cup to hold the lens. A 1 ml sample of the effluent from each time period, along with an aliquot of lens homogenate, was evaluated for protein content by the procedure of Lowry et al (1951). It is assumed that the effluxed material is indeed

protein since dialysis of the effluent caused no less in the Lowry reaction.

In order to study the efflux of newly formed protein, galactose and control lenses were each preincubated in KRPO_4 containing 250,000 c/m of Se^{75} -selenomethionine. These lenses were washed and then transferred every 5 min to new non-radioactive medium. After 12 transfers, all the media for each lens were pooled, precipitated with equal amounts of 10% TCA, filtered through Millipore disk (0.45μ), washed exhaustively with cold distilled water and counted in a well type scintillation counter. In order to test for non-specific binding of the isotope, control tubes, to which Se^{75} -selenomethionine and lens homogenate were added such that the concentrations were equivalent to that found in the pooled media, were also put through millipore filters after the addition of TCA. The controls had an activity less than half of that of the pooled media.

To determine if methionyl-tRNA synthetase activity could be detected in the effluent protein, galactose and control lenses were placed in individual tubes containing 1 ml of KRPO_4 . After incubation for 1 hr, the media were pooled, lyophilized, redissolved in 0.5 cc of 0.05 M Tris-HCL buffer, pH 7.8 containing 0.01 M MgCl_2 and 0.005 M DTT, and dialyzed against 0.02 M Tris-HCL buffer, pH 7.8 containing 0.005 M reduced glutathione for 4 hrs. Methionyl-tRNA synthetase

activity was evaluated as listed in Materials and Methods; aminoacyl synthetase assay.

Disc Electrophoresis:

Effluent lens protein, lens homogenate protein, serum and aqueous and vitreous protein from control and galactose-fed rats were studied by disc electrophoresis. The medium consisted of two separately polymerized layers of polyacrylamide gel. The stacking gel, pH 8.9, contained 2.5% acrylamide, 0.625% N,N'-methylene-bisacrylamide, 0.032M H_3PO_4 , 0.06 M Tris and 0.0005% riboflavin; and the separating gel, pH 9.5, contained 7.5% acrylamide, 0.2% N,N'-methylene-bisacrylamide, 0.06N HCL, 0.38M Tris, 0.07% $(\text{NH}_4)_2\text{S}_2\text{O}_8$, 0.058% tetramethylethylenediamine and 0.004% $\text{K}_3\text{Fe}(\text{CN})_6$. The Tris-glycine buffer, pH 8.4, contained bromphenol blue as a tracking dye. Immediately prior to electrophoresis 200 μl samples (10-600 μg of protein) containing sucrose were layered on the stacking gel. The electrophoresis was run at 5 milliamps per tube for 40 min at 4°C. The gels were fixed in 10% TCA overnight. Coomassie Brilliant Blue stain was then applied for 45 min and destaining was performed in 12.5% TCA (Ornstein and Davis, 1960; Davis 1964 and Ornstein, 1964).

In vivo leakage studies:

To induce antibody titres in non-cataractous rats and rabbits, 0.5 ml of complete Freund's adjuvant mixture containing 0.5 mg of rat lens homogenate protein were made into the front foot pads. Booster injections (0.5 mg protein in 0.5 ml distilled water) administered subcutaneously and blood samples withdrawn by heart puncture between 4 and 8 weeks following the initial immunization. All serums were stored at -20°C . The presence of antibodies to lens protein was determined by the gel diffusion method of Ouchterlony, 1953. Antiseras were diluted with normal serum and reacted against 0.5 mg of lens protein for 1 week at 4°C .

Lens protein was centrifuged at $12,000 \times g$ for 10 min, labelled with I^{125} by the Chloramine T technique (Greenwood, Hunter and Glover, 1963), see (Berson and Yalow, 1966), normal rat serum was added to make a 1% solution, and dialyzed for 4 hrs. against distilled water. Twenty-five μl of I^{125} lens protein, containing 8×10^3 c/m/ 25 protein, was incubated with 25 μl of normal or experimental serum and 50 μl of 0.1 M phosphate buffer, pH 7.4 for 48 hrs at 4°C . The incubation mixtures 50 μl were applied to a 1% starch gel in 0.025M boric acid NaOH buffer, pH 8.9, with a 0.3 M boric acid NaOH buffer, pH 8.2. Verticle electrophoresis was run at 200 volts; 19 milliamps, for 16 hrs at 4°C . The gels were fixed in 10% TCA and stained with

Coomassie Brilliant Blue, following which autoradiographic studies were made.

Preparation of the collagen gel:

Collagen was obtained from the research laboratory of the Japanese Leather Co. through the courtesy of Dr. A.L. Rubin of the Rogosin Laboratory at Cornell University Medical School. The collagen was diluted to a concentration of 0.25% with 0.05% acetic acid, and dialyzed against Ringer's solution for 36 hrs at 4°C. The pH was adjusted to 7.4 with 0.1 N sodium hydroxide. Rigid opalescent gels were formed at 37°C from 4 cc of the collagen solution in sterile lucite tissue culture plates of 35 mm diameters. After 3 hours, Mammalian Tyrodes solution containing amino acids, streptomycin was poured on to the surface of the gel and immediately drained (Brown, Weller and Wassermann, 1969).

Collagenase assay:

Collagenolytic activity was determined by modifying the methods of Gross and Lapiere (1962). The peeled lens capsule and epithelium were immersed momentarily in a Mammalian Tyrodes solution containing streptomycin and amino acids and placed in a sterile tissue culture plate on the surface of a rigid, opalescent collagen gel (Grillo and Gross, 1967). The tissue culture plate was then placed in a 90% oxygen and 10% carbon dioxide moist atmosphere, and incubated at 37°C for 5 days. The plates were observed daily for signs of lysis which,

when present, was recorded as the diameter in millimeters of the lysed area. Trypsin, pepsin, hyaluronidase, chymopapain were incubated at 37°C on gels for 4 days as controls. In these studies the supernatant of the centrifuged collagen gels (15,000 x g for 1½ hrs. at room temperature) which presumably contained the collagen fragments was analyzed for hydroxyproline by the colorimetric method of Neuman and Logan (1950).

Rat lens methionyl-tRNA synthetase was measured by determining the radioactivity in the TCA-precipitable fraction. The radioactivity in this fraction increased with time until 40 min and then decreased slightly (Fig. 1). This decrease probably reflects deacylation since ribonuclease is very low in the normal rat lens (Zigman et al, 1963). The incubation time of all subsequent experiments was fixed at 10 min.

The optimum concentration for Mg and for ATP was determined to be 3.7 μ moles and 1.3 μ moles, respectively, in 200 microliters of reaction mixture (Fig. 2 and 3).

Temperature studies showed an increase in activity from 0-37°C, followed by a sharp decrease at still higher temperatures (Fig. 4).

Although there was little change in enzyme activity from pH 6.8-8.6, optimal complex formation appears between pH 7.8 and 8.0 (Fig. 5). A similar broad pH optimum (6.0-8.8) was found for E. coli. methionyl-tRNA synthetase (Bergman et al, 1961).

The dialyzed lens homogenate showed significant loss in synthetase activity after storage at 4°C or -20°C (Table I). At 4°C the inactivation was approximately 30% higher than that at -20°C. Because of the reduction with storage, all subsequent assays were carried out immediately after dialysis.

The concentration of tRNA was varied in an assay system containing 12.5 mg of lens homogenate in 200 μ l. Synthetase activity increased with increasing addition of tRNA up to approximately 1.0 mg and then leveled off (Fig. 6).

With these optimal conditions (listed under Materials and Methods) lens enzyme activity varies reasonably linearly with increasing concentration of lens homogenate (Fig. 7).

The presence of unlabelled selenomethionine or methionine (1.0 μ mole) significantly decreased the formation of Se^{75} -selenomethionine-tRNA (Fig. 8). Lens synthetase showed a slight preference for methionine since DL-methionine was a better inhibitor than DL-selenomethionine. Mudd and Cantoni, 1957, reported that selenomethionine was bound slightly more efficiently than methionine in the presence of a yeast methionyl synthetase. Synthetase activity was not affected by the addition of DL-phenylalanine, L-lysine or L-glutamic acid (5.0 m moles to the 200 μ l assay mixture).

The apparent K_m for DL-selenomethionine and -methionine was $1.7 \times 10^{-5}\text{M}$ (Fig. 9). The apparent K_m value for tyrosyl-tRNA synthetase purified from hog pancreas was $6.0 \times 10^{-5}\text{M}$ (Schweet and Allen 1958), and the Michaelis constant for purified seryl-tRNA synthetase isolated from rat liver was $5 \times 10^{-4}\text{M}$ for C^{14} serine (Rouge, 1960).

The distribution of the methionyl-tRNA synthetase was studied in rats 83 days of age (Table 2). The peeled

epithelium and capsule contained 37% of the activity, whereas, 63% was found in the remaining lens-cortex and nucleus. This distribution is similar to that reported for the leucyl-tRNA synthetase in calf lens. (Virmaux and Mandel, 1964). When expressed per milligram of protein, the activity in the epithelium and capsule is 37 times that found in the cortex.

The stability of the synthetase was investigated by incubating lenses in KRPO_4 containing glucose and inhibitors for varying time periods (Table 3). Synthetase activity was then determined as listed in Materials and Methods. Inhibition of lens protein synthesis was evaluated by incubating some lenses for an additional 40 min in Se^{75} -selenomethionine, and measuring incorporation into protein (TCA-precipitable fraction). In Study A. following a 3 hr incubation the puromycin treated lenses showed a slight decrease in synthetase activity (85% of control), and Actinomycin D-mannitol and mannitol were 93% and 94% of control. Puromycin, but not Actinomycin D, significantly inhibited Se^{75} -selenomethionine incorporation into TCA-precipitable lens protein (36% of control). In Study B. again a slight decrease in enzyme activity (85% of control) was noted in lenses incubated for 5 hr with puromycin. Rats were injected every 2 hr for a total of $10\frac{1}{2}$ hr with cyclohexamide in Study C. The lenses were then removed from the animals following decapitation and incubat-

ed with cyclohexamide for an additional 4 hr: synthetase activity averaged close to 80% of the control values. Cyclohexamide decreased Se^{75} -selenomethionine incorporation into protein to less than 10% of the control. In Study D. lenses were maintained for 24 hr in TR199 (Hanks base medium) with and without cyclohexamide. No change in activity was found in the treated lenses.

Although in some of these inhibitor studies there may be a slight (not significant) decrease in total lens synthetase activity, the half life of the enzyme is probably at least 24 hrs. The occurrence of stable RNA templates in lenses has been demonstrated by Scott and Bell (1965) Stewart and Papaconstantinou (1967a), Stewart and Papaconstantinou (1967b) and Reeder and Bell (1967).

Figure 10 shows synthetase activity in lenses of rats whose ages ranged from 22 days (17.7 mg per lens) to 453 days (63.0 mg per lens). Enzyme activity per whole lens increases slightly between 22 and 123 days of age and then remains constant. The activity expressed per milligram of protein decreases until 123 days and then appears constant.

Figure 11 compares the synthetase activity per lens in groups of rats given various diets: control, glucose, galactose, and 3 groups returned to the control diet after 3, 8 and 17 days of galactose diet, (the data are tabulated in Table 4). The values for the group given only the

control diet are graphed as the 100% reference standard. The lenses from animals fed glucose showed no significant variation from lenses of control animals. Lenses from galactose-fed rats showed a significant increase in enzymatic activity at days 3 and 4, which returned to the control value at 8 days, then fell in an almost linear fashion until 17 days and remained depressed thereafter. When galactose was discontinued after 3 days, the enzyme activity appeared normal after 2, 12 and 21 days of control diet. When galactose was discontinued after 8 days, the enzyme activity after the next 7 and 16 days of control diet was significantly different from the values of both the control and the galactose-fed animals. Resumption of a normal diet after 17 days of galactose feeding had no noticeable effect on synthetase activity 1 week later. But after two additional weeks (day 44) activity increased slightly.

Lens synthetase activity expressed per milligram of protein is plotted in figure 12 and tabulated in Table 4, and is essentially the same as that expressed per whole per whole lens, except that a statistically significant difference between the control and galactose-fed rats occurs only at day 24.

When animals were fed the normal diet after 3 or 8 days of galactose diet, enzyme activity per milligram of protein

was at the control level. When galactose was discontinued after 17 days, no apparent enzyme recovery was noted 1 week later (Table 5).

The methionyl-tRNA synthetase activity in (35%) xylose-fed rats (Table 6) showed no significant variation from the control value between 1 and 31 days. Animals maintained on a 50% xylose diet showed no change in synthetase activity between 1 and 8 days.

Table 7 represents the average lens weights of the experimental animals. The inhibition of fluid in lenses from rats on a galactose diet has been well documented (Kinoshita, Merola, Dikmak, 1962; and Patterson and Bunting, 1966). The lenses of rats returned to a normal diet after 3, 8 or 17 days show lens weight similar to controls by days 5, 15 and 24 respectively; and at day 44 are less than both the galactose and the control values in all experiments. Patterson and Bunting, (1966) reported fluid inhibition in xylose cataracts, however, in these investigations, changes in weight were seldom found in lenses from rats fed a diet containing either 35% or 50% xylose. Similar results to ours were obtained by van Heyningen (1963).

Table 8 contains the average weights of rats maintained under different dietary conditions. Although the growth rate of animals fed 50% galactose is somewhat less than the control rate, it is close to that of animals maintained on

50% glucose diet. Animals maintained on 50% xylose diet were not continued beyond 8 days since the mortality rate was approximately 50% and some cannibalism was noted. Rats maintained on 35% xylose diet had severe diarrhea for the first week, but appeared to rapidly gain weight when the diarrhea subsided. The average food consumption for rats maintained on various diets; galactose, xylose, glucose and control is given in Table 9.

Thymidine- C^{14} incorporation into DNA was studied in normal, xylose and galactose lenses to evaluate the mitotic activity of these lenses. Experimental lenses could accumulate different amounts of free thymidine- $2C^{14}$ which would consequently affect the radioactivity in the DNA. Therefore, the total uptake of the radioactive nucleoside was determined in all cases in order to evaluate possible differences in transport. Preliminary uptake experiments (effects of time, temperature, carrier concentration and inhibitors) were carried out in normal rat lenses.

There was no incorporation of label into DNA (TCA-precipitable fraction) in lenses evaluated after incubation at $37^{\circ}C$ for 30 minutes. Table 10 shows that after $2\frac{1}{2}$ hrs C^{14} -thymidine incorporation into DNA was normal in lenses from xylose-fed (3-5 days) animals whereas, it was elevated significantly in animals fed galactose for

3-5 days.

Thymidine-2- C^{14} is taken up almost linearly for the first 120 minutes in normal lenses at which time it levels off (Fig. 13). The uptake of C^{14} -thymidine increases with increasing temperature (Fig. 14). At 60°C the lenses appeared cloudy and became totally opaque at higher temperatures. Increasing carrier concentrations to 1.2 μ moles in 0.5 ml. were added to the dose. The effect of unlabelled thymidine on the uptake of C^{14} -thymidine was studied (Fig. 15). There is some leveling of the curve indicating that some saturation might be present. Inhibition studies were carried out with N-ethyl maleimide p-chloromercuribenzoate, iodoacetate, dinitrophenol, mercuric chloride, guanosine and cytidine. The results, in Table 11, indicate that only p-chloromercuribenzoate, and mercuric chloride significantly inhibited uptake.

Table 12 shows that the lens methionyl-tRNA synthetase activity in 120 gm rats fed galactose in contrast to 40 gm rats (Fig. 11). The data indicate that no early rise in enzyme activity (3-5 days on diet) occurs. However, significant increases in incorporation of thymidine C^{14} into DNA were noted. By the 24th day of diet the synthetase activity is 44% of the control value.

It is possible that the enzyme might be altered in its functioning and result in "mischarging". In order to test

this theory, competition experiments were performed in which unlabelled amino acids similar in structure and codon assignments were added to the assay mixture.

Alanine sometimes decreased Se^{75} -selenomethionine binding in lenses of young rats fed galactose for 3 days, but not in controls. No decrease due to the addition of alanine was seen in lenses of normal 547 day old rats (Table 13). The effects of added methionine or selenomethionine decreased similarly the Se^{75} -selenomethionine binding in control and cataractous lenses.

The decline in enzyme activity appeared not to be due to the presence of an inhibitor since mixing the supernatants of galactose and normal lenses gave the expected intermediate values. (Table 14).

Methionyl-tRNA synthetase activity was studied in galactose-fed rats in which the diet was supplemented with glucose. No change in the early pattern of enzyme activity was found. Food consumption was slightly decreased in animals whose diets were supplemented with glucose, but blood galactose levels were the same in both groups (Table 15).

In order to determine if these results were related to only one synthetase, H^3 -leucine binding to tRNA was studied. Lens leucyl-tRNA synthetase activity varies linearly with increasing concentrations of lens homogenate until 4 mg is reached (Fig. 16). Subsequent experiments were carried out

with 2 mg of homogenate.

The leucyl-tRNA synthetase was evaluated in animals fed galactose for 1, 3, 8, 10, 17 and 24 days (Fig. 17). Enzyme activity which was significantly higher at 3 days (129% of control) at 8 days (195% of control), and at 10 days (183% of control) returned to the control level at 17 days and declined to 32% of the control value at 24 days. The c/m/lens and c/m/mg protein are given in Table 16.

The decrease in lens content of aminoacyl-tRNA synthetases as well as other soluble protein might be due to leakage. The leakage of protein from control lenses is small and seems to be diminishing with time (Fig. 18). This efflux pattern is unchanged in lenses from animals fed galactose for 1, 3 or 5 days. The accumulated efflux for 3 hr was 53 μ g. Lenses from animals fed galactose for 8 days showed an increase in efflux: the accumulated efflux from these galactose lenses after 3 hr was 123 μ g. Maximum efflux (268 μ g.) occurs with the appearance of nuclear cataracts and further feeding of galactose (17 or 51 days) does not change the total leakage.

Fig. 19 indicates small differences in in vitro protein leakage between young (20 mg) and old (61 mg) lenses.

The protein efflux per hour from lenses of control and galactose-fed rats (days 1, 3, 5) approximates 0.2% of the total lens protein. The percentage efflux per hour

increases to 0.6% at day 8 and is between $1\frac{1}{2}$ to 2% in nuclear cataractous lenses (Table 17).

Incubation solutions containing effluxed protein were pooled, lyophilized, redissolved in a small volume, and assayed for methionyl-tRNA synthetase activity. The effluent protein from lenses of galactose-fed rats (8 days) and control lenses contained detectable enzyme activity (Table 18).

Incubation solutions from lenses preincubated in Se^{75} -selenomethionine were pooled, protein precipitated with TCA, put through millipore filter and counted. The galactose lenses leaked radioactive protein (Table 19).

Table 20 indicates increased protein efflux from lenses of a group of 60-70 gm rats after 17 days of galactose feeding. No nuclear cataracts were present. When lenses were subject to increased stress (rolling with ophthalmological loop prior to placement in vials) the controls showed a slight increase ($8\mu\text{g}$) for the 1st hr and shortly thereafter returned to "normal" in vitro leakage whereas, the galactose lenses leaked over $100\mu\text{g}$ more for the 1st hr and showed little decline (Table 20).

Disc electrophoretic patterns of lens homogenate protein, and lens effluent protein from control and galactose fed animals are given in Fig 20. Proteins from control lens homogenate formed 13 bands in polyacrylamide

gels. Lens homogenate proteins from animals fed galactose for 5, 7 and 13 days show qualitatively identical patterns. Aqueous and vitreous patterns appear like diluted serum.

Effluent protein from control lenses form 4 bands while galactose effluent proteins form 7 bands after 8 to 13 days of diet. The increased number of bands in the cataractous lens effluent compared to control was related to the increased concentration in this fraction. Actually both effluent patterns (control and cataractous) appeared to be as diluted samples of lens homogenates.

Peeled lens epithelium and capsule from nuclear cataractous lenses contained small, but detectable amounts of collagenase as determined by a 9-11 mm diameter lysis of collagen gels (Table 21). No collagenase activity was demonstratable in control lenses.

If in vivo leakage occurred, autologous lens antibodies might be produced in galactose-fed rats. No antibodies could be detected in serum from rats which had mature cataracts for 4 mos or 1 year. On the other hand sera from rats and rabbits which were previously immunized with rat lens proteins, showed strong precipitin bands after incubation against lens protein on agar diffusion plates and/or slides.

Since booster injections enhanced titres of immunized animals, the possible titres in cataractous rats might be increased by measurable levels by injecting lens homogenates.

Sera removed 7 and 10 days later showed no detectable antibody titres. Appropriate controls were simultaneously run.

Radioimmunoassay experiments were undertaken in order to try to develop a more sensitive technique for detecting lens antibody titres. In this system lens antibodies were allowed to bind to labelled lens proteins and detected by alterations in the chromatographic or electrophoretic properties of the labelled protein-antibody complex. Attempts to label lens proteins proved satisfactory, but the prepared I^{125} -lens protein was unstable and formed microprecipitates. When 1% rat serum was added immediately to the I^{125} labelled lens, the labelled protein was reasonably stable. Starch gel electrophoresis gave satisfactory separation of free and bound antigens. Autoradiographic studies of the starch slab showed 4 major radioactive areas indicating that 4 protein (Or groups of proteins) were labelled. The presence of rabbit or rat antisera significantly increased the radioactivity which remained at the origin. (Table 22A). This radioimmunoassay provided a two-fold increase in sensitivity for antibody detection over the gel diffusion technique, but again no antibody titres could be detected in serum from cataractous rats. Cataractous rats which were "boostered" with lens homogenate showed no significant increase in radioactivity found at the origin (Table 22B).

Fig. 1 Effect of incubation time on lens methionyl-tRNA synthetase activity. Rat weights averaged 140 gms.

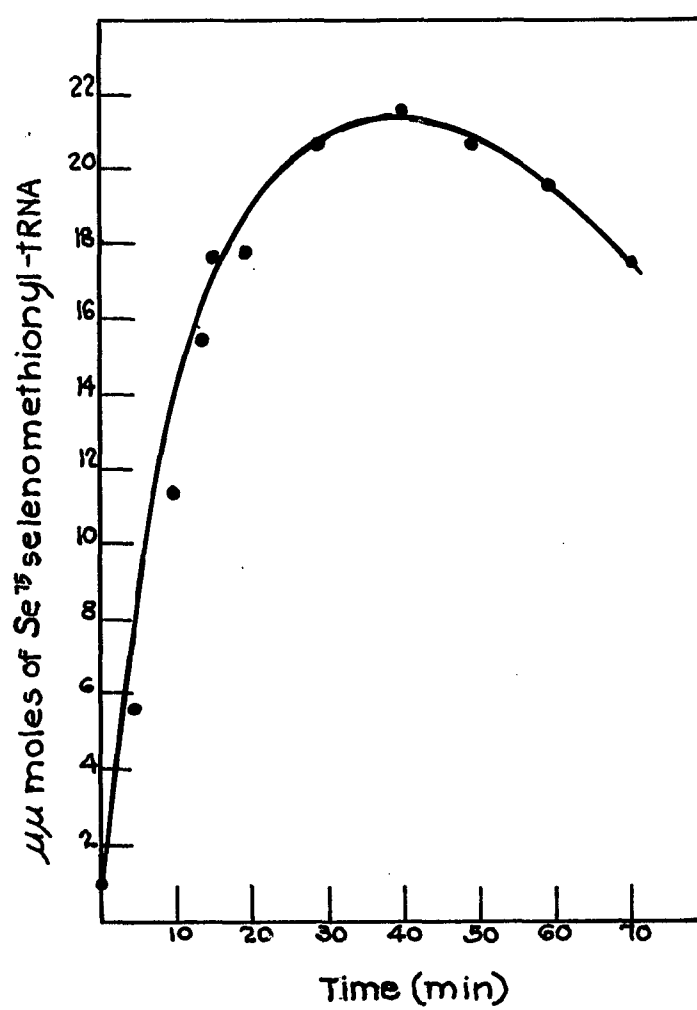


Fig. 2. The effect of Mg on lens methionyl-tRNA synthetase activity. Rat weights averaged 90 gms.

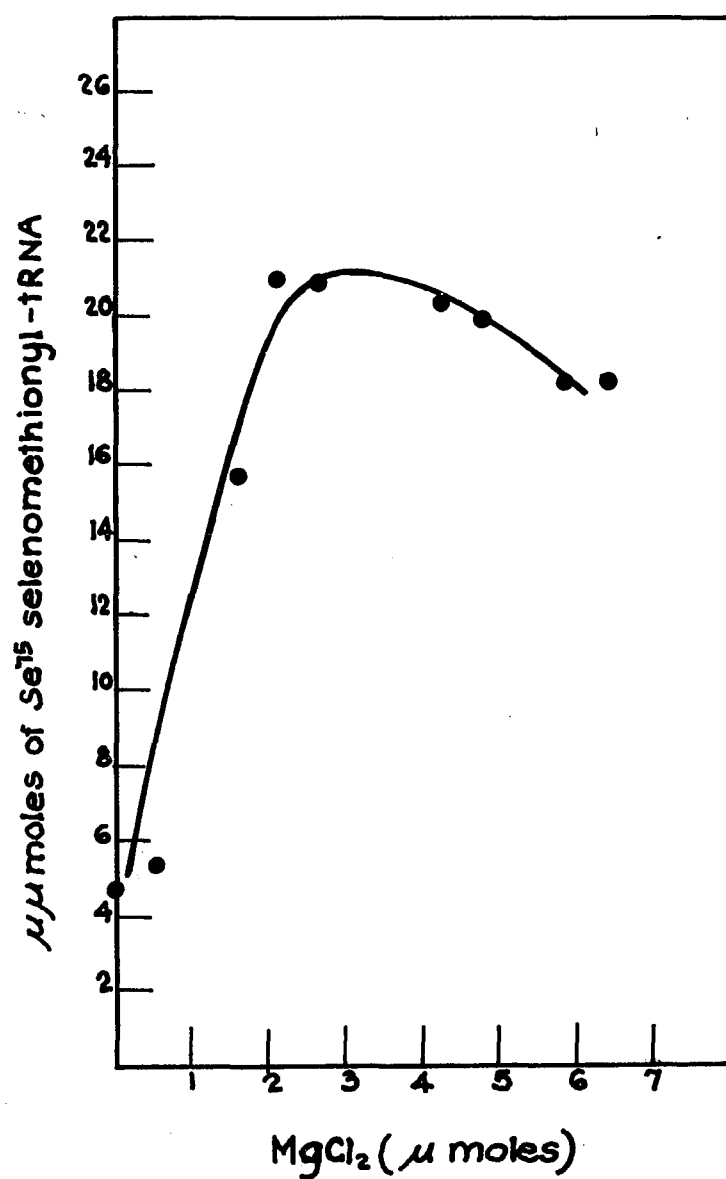


Fig. 3. Effect of ATP on lens methionyl-tRNA synthetase activity. Rat weights averaged 200 gms.

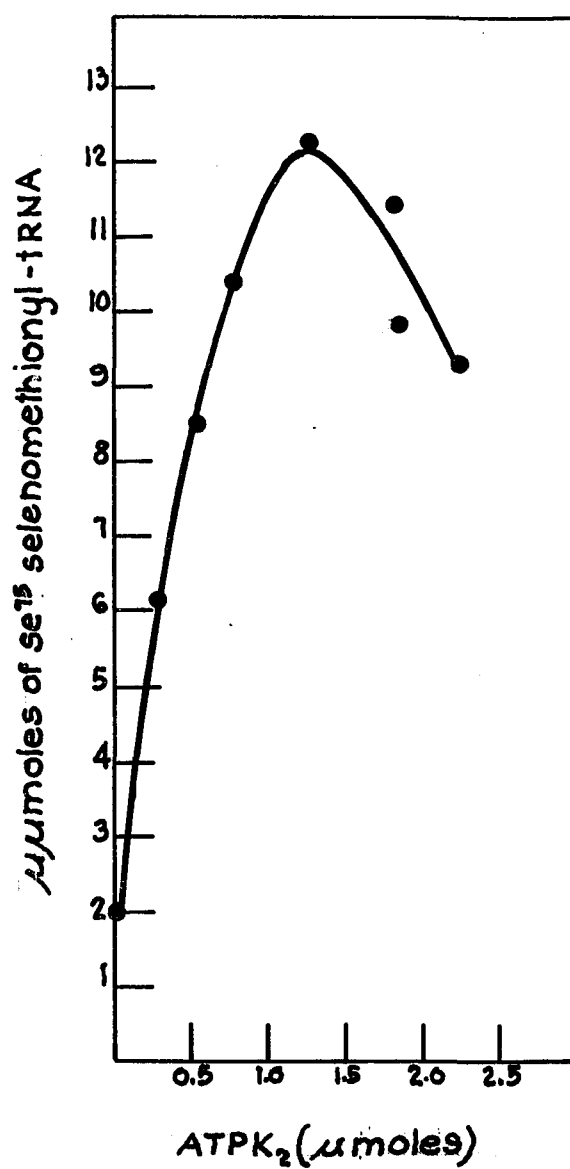


Fig. 4. Effect of temperature on lens methionyl-tRNA synthetase activity. Rat weights averaged 80 gms.

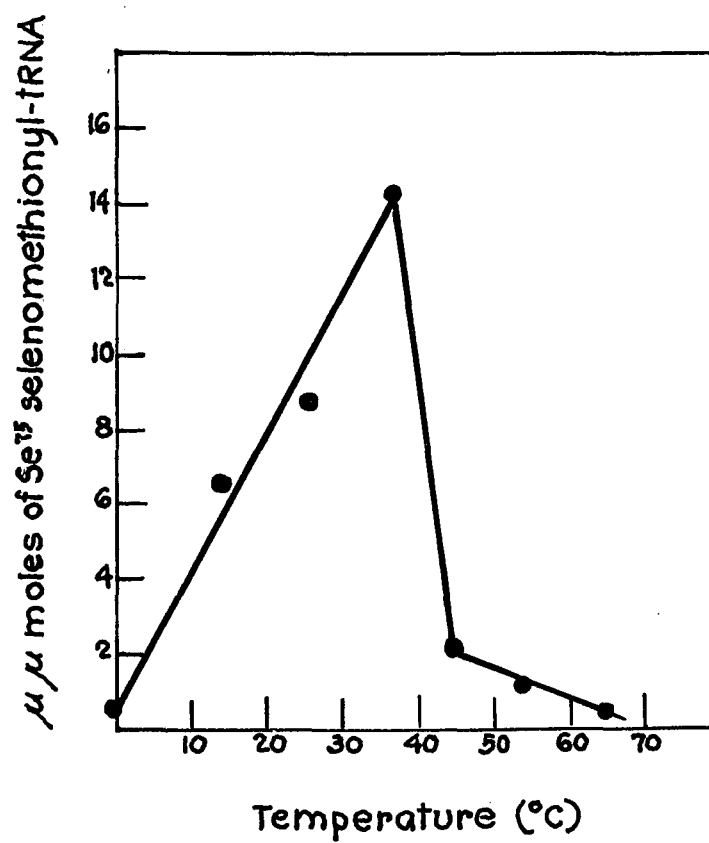


Fig. 5. The effect of pH on lens methionyl-tRNA synthetase activity. Rat weights averaged 110 gms.

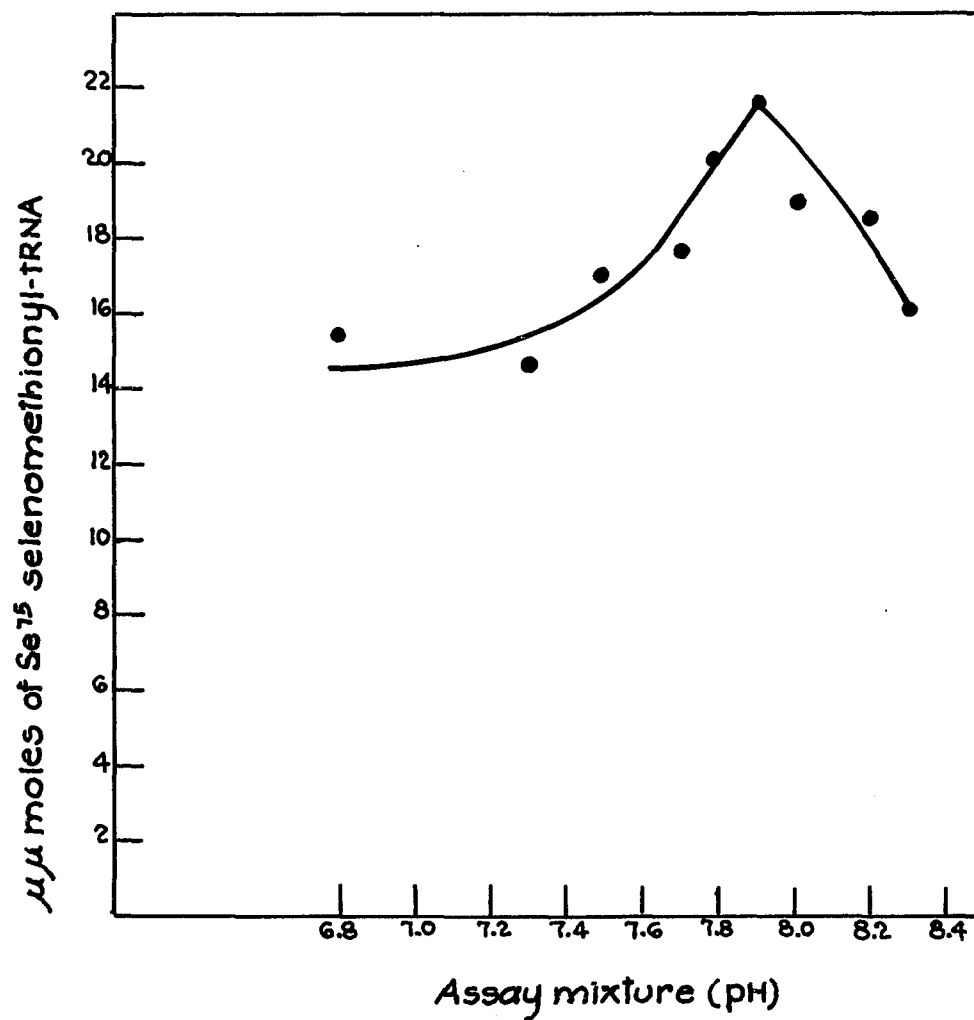


Table I. The percent activity of the methionyl-tRNA synthetase following storage at -20°C and 4°C .

<u>Days</u>	<u>Percent activity remaining at -20°C</u>	<u>Percent activity remaining at 4°C</u>
1	91.0	--
3	80.3	50.6
7	39.5	--
84	24.7	--

Fig. 6. The effect of tRNA on methionyl-tRNA synthetase activity.
Rat weights averaged 40 gms.

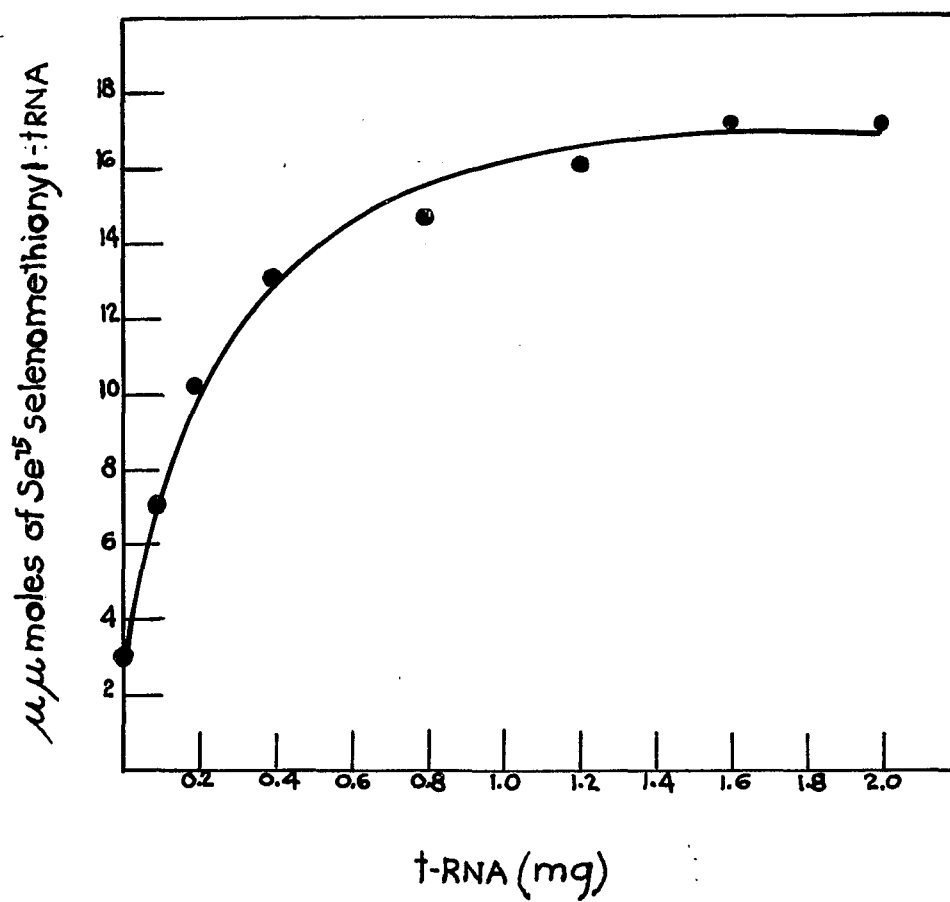


Fig. 7. The effect of lens homogenate concentrations on Se^{75} -selenomethionine-tRNA binding.
Rat weights averaged 40 gms.

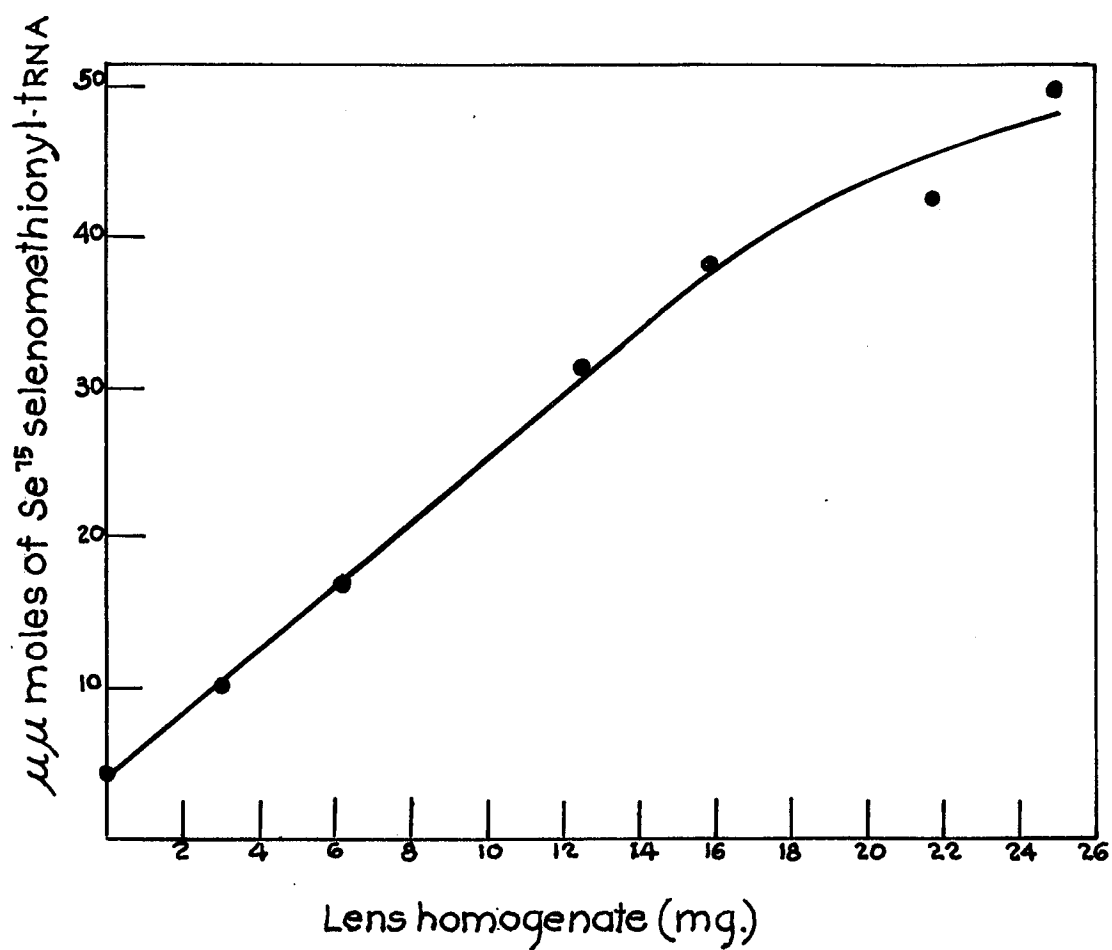


Fig. 8. Se^{75} -selenomethionyl-tRNA binding with unlabelled DL-methionine and DL-selenomethionine. Rat weights averaged 150 gms.

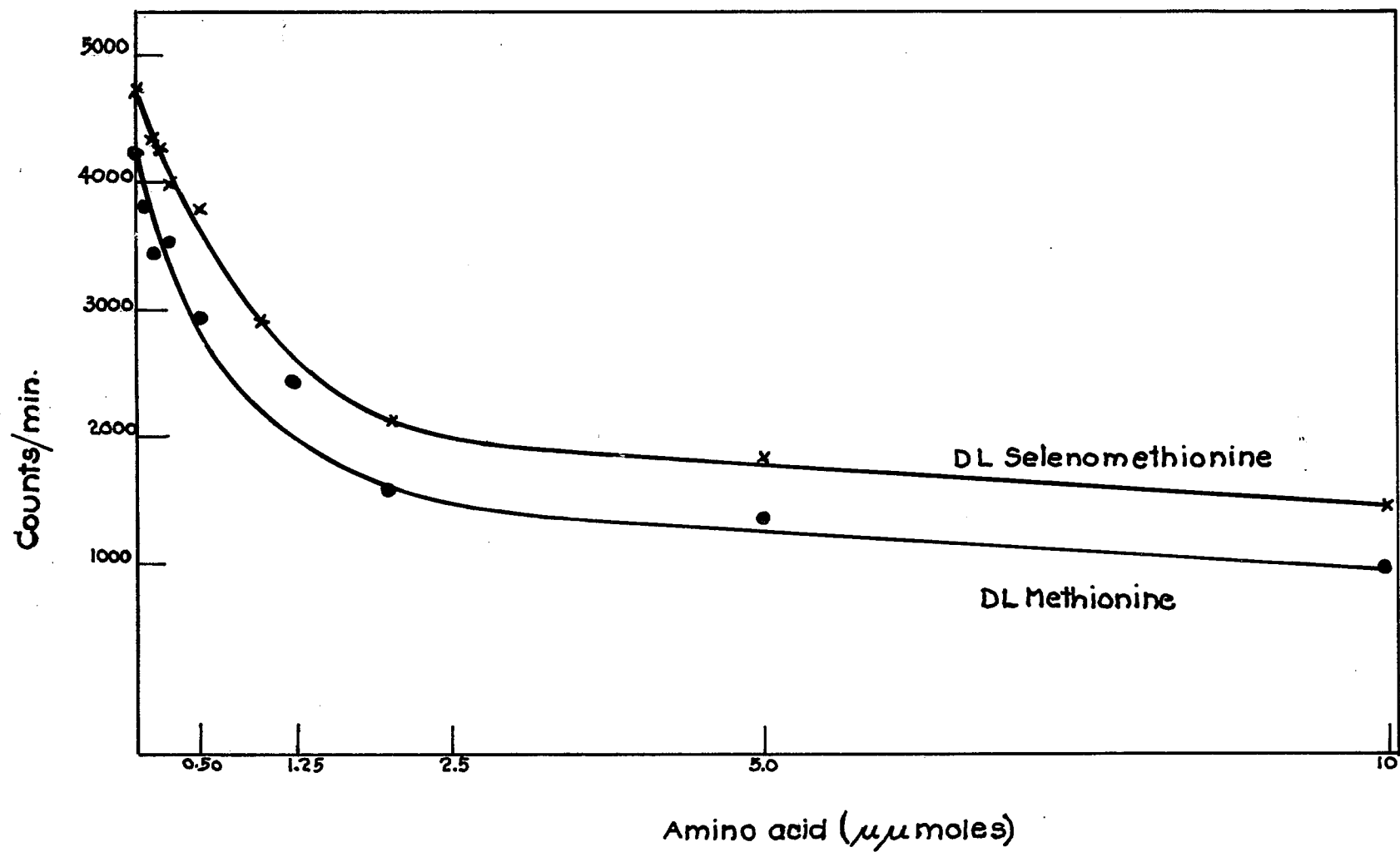


Fig. 9. The apparent K_m value for DL selenomethionine was 1.7×10^{-5} M

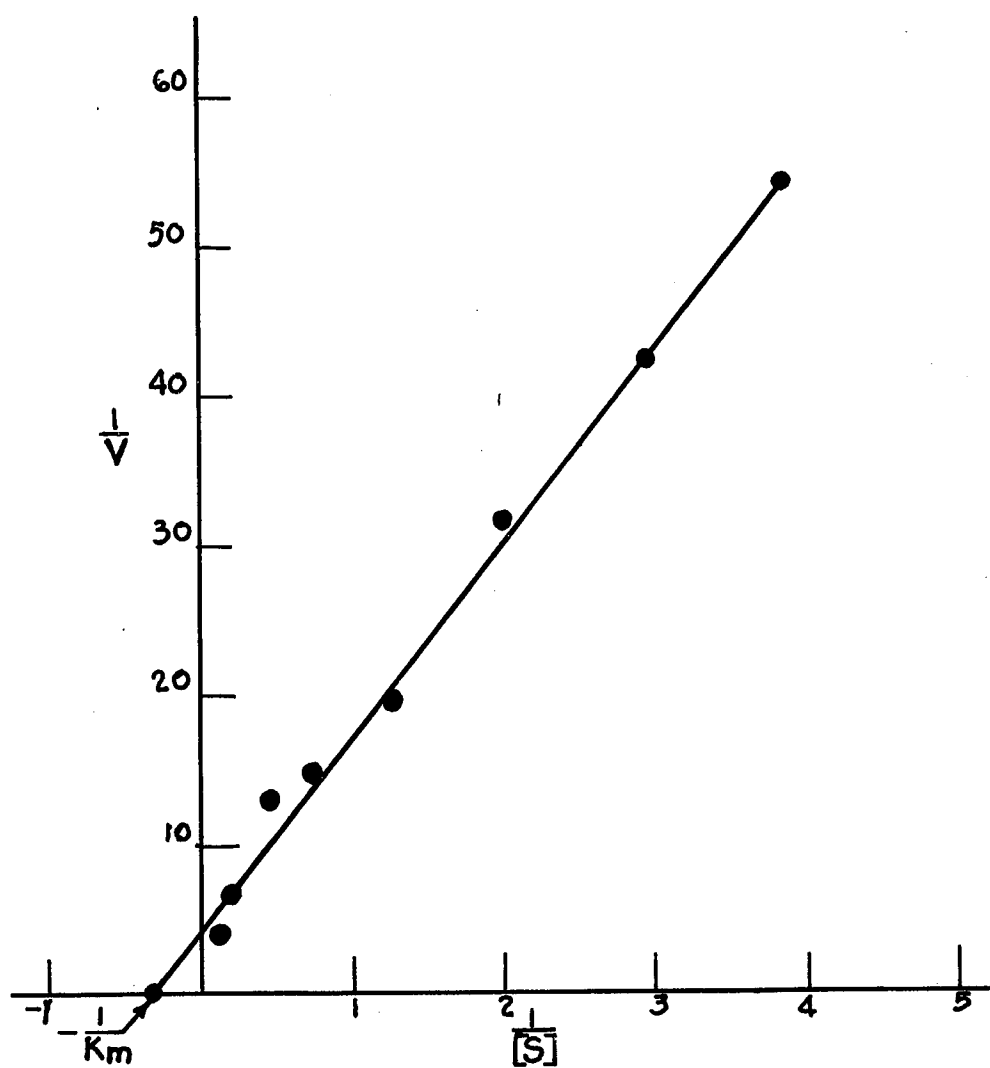


Table 2. The distribution of the methionyl-tRNA synthetase in rat lenses.

	c/m	Percent	c/m/mg. protein
A. Epithelium and capsule	9800	63	25720
B. Cortex and nucleus	5787	37	681
C. A plus B	15587	100	--
D. Total lens	15690		1300

(Rat weights: 230-250 gm.)

Table 3. Average lens methionyl-tRNA synthetase activity with inhibitors of protein synthesis.

A. Lenses incubated with inhibitors in KRPO_4 -glucose solution.

Inhibitors	Synthetase activity c/m/lens 3 hr. incubation	Percent of control	Protein incorporation (TCA precip.) 40 min. incubation	Percent of control	Uptake c/m/lens
1.08 mg. Puromycin	12613	85	340	36	20222
0.2 mg. Actinomycin D in manitol 20 mg.	13815	93	855	85	19431
20 mg. Manitol-control	13871	94	846	89	17455
Control	14918	100	943	100	19931

(Rat weights: 140-150 gm.)

B. Lenses incubated for 5 hours with puromycin in KRPO_4 -glucose solution.

Inhibitors	Synthetase activity c/m/lens	Percent of control
9.4 mg. Puromycin	15895	84
Control	18879	100

(Rat weights: 210-230 gm.)

Table 3. Average lens methionyl-tRNA synthetase activity with protein synthetic inhibitors.

(Cont.)

C. Animals injected with 0.1-0.17 mg. of cyclohexamide every 2 hrs. (total $10\frac{1}{2}$ hrs.) then lenses removed and incubated for 4 hrs. in KRPO_4 -glucose solution containing 0.2 mg. cyclohexamide.

Inhibitor	Synthetase activity c/m/lens	Percent of control
Cyclohexamide	17681	87
Control	20196	100

(Rat weights: 92-100 gms.)

D. Incubation of lenses in TR199 for 24 hrs. with cyclohexamide.

Inhibitor	Synthetase activity c/m/lens	Percent of control	Protein incorporation (TCA precip.) c/m/lens	Percent of control	Se^{75} seleno. Uptake c/m/lens
0.34 mg. cyclohexamide	18701	109	89	94	28459
Control	17133	100	945	100	27357

(Rat weights: 200-230 gms.)

Fig. 10. Lens methionyl-tRNA synthetase activity in rats ranging from 22-453 days of age.

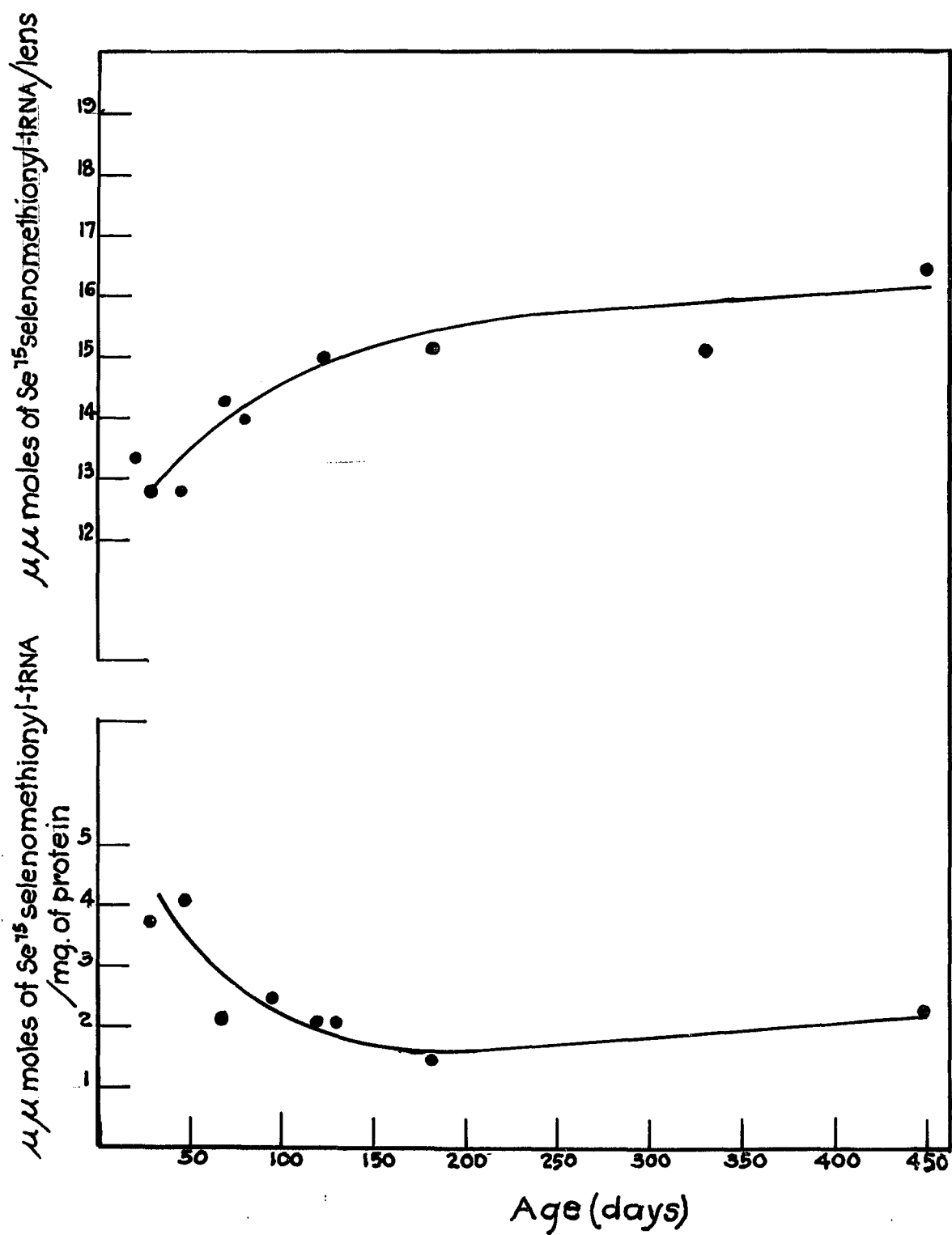


Fig. 11. Methionyl-tRNA synthetase activity in rat lens with varied dietary conditions: control, glucose, galactose and 3 groups returned to purina diet after 3, 8 and 17 days of galactose feeding. 53

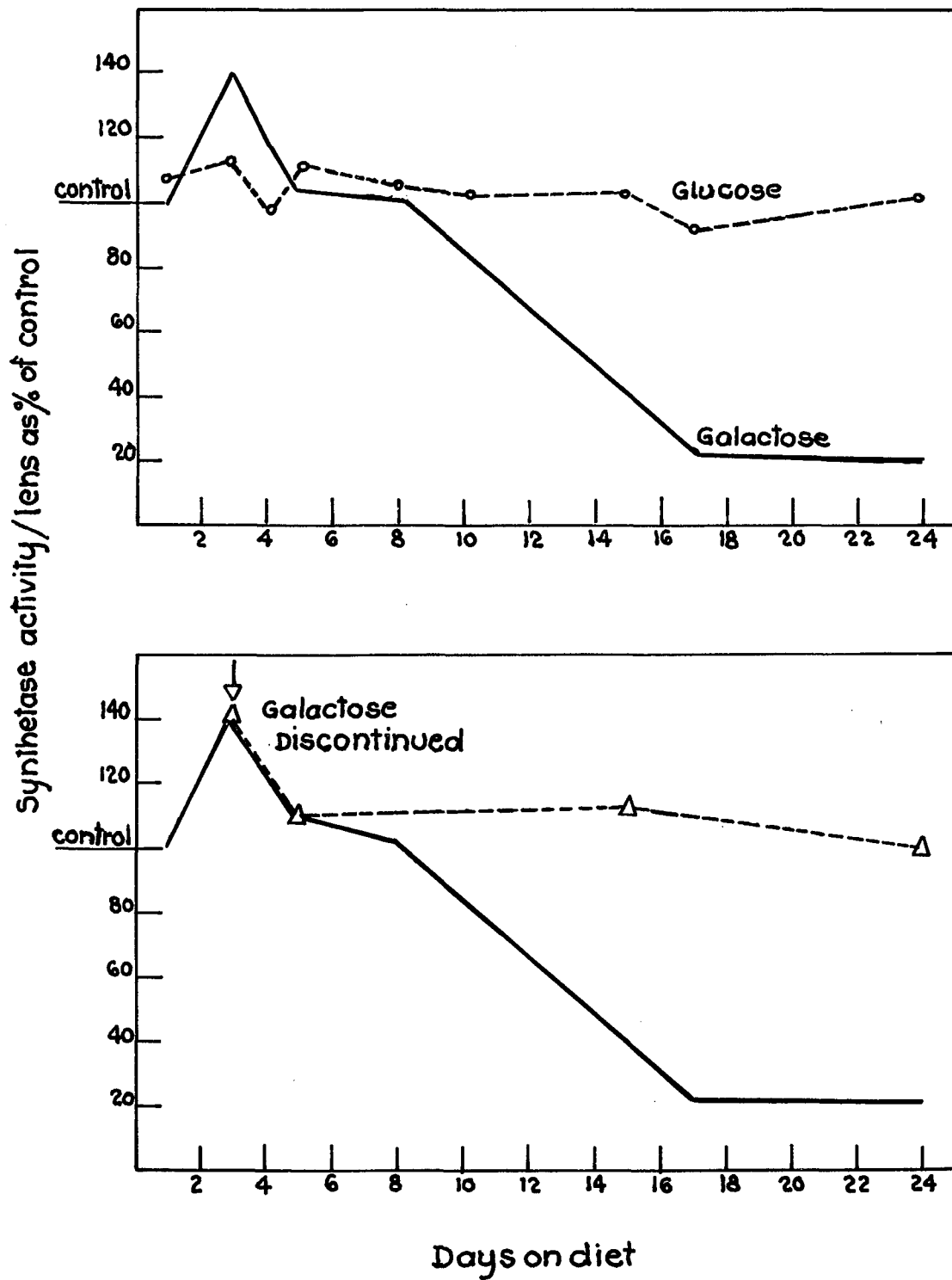


Fig. 11. (Cont.)

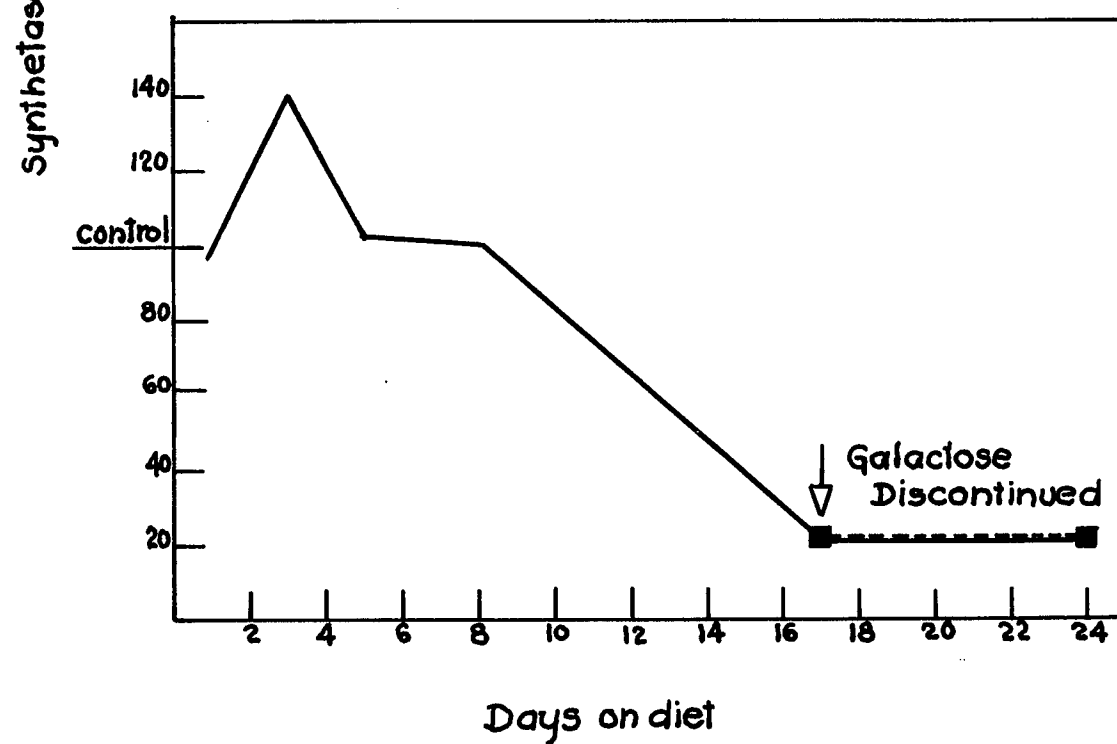
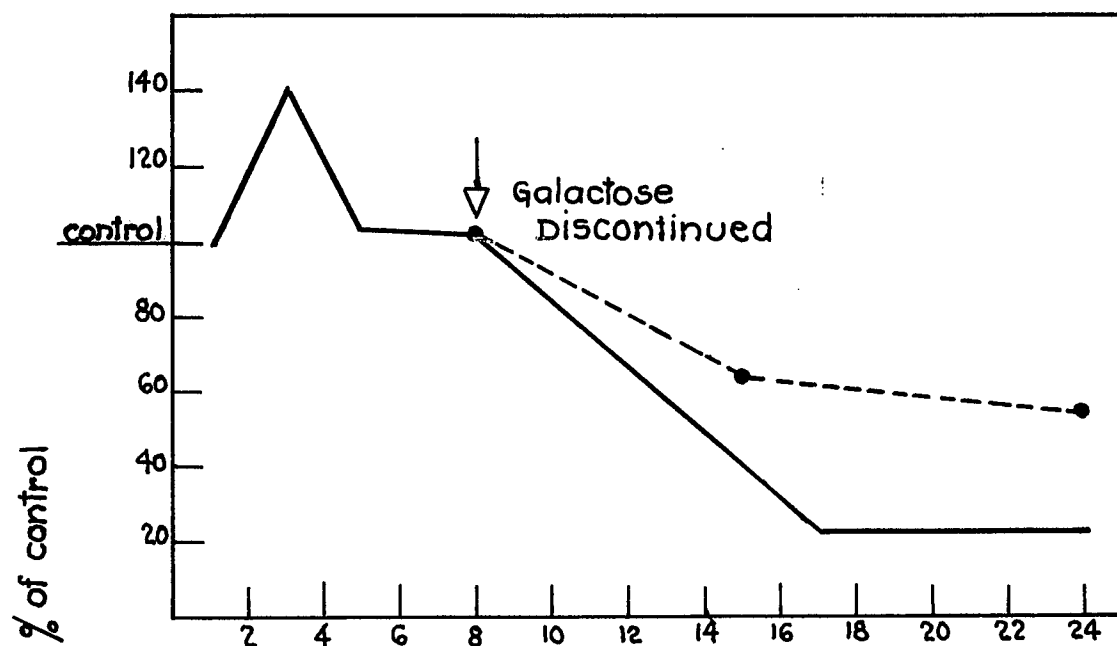


Table 4. Lens methionyl-tRNA synthetase activity in normal, glucose-fed and galactose-fed rats.

Days on Diet	Control			Galactose-fed			Glucose-fed		
	c/m lens	c/m/mg protein	No. of lenses	c/m lens	c/m/mg protein	No. of lenses	c/m lens	c/m/mg protein	No. of lenses
1	11486	2789	12	11296	2893	12	12496	2758	18
3	10717	2892	30	14967	3467	34	--	--	--
	11224	--	--	--	--	--	12835	3122	24
4	11241	2839	11	13384	3276	12	10866	2822	12
5	10317	2222	26	11767	2350	25	11423	2217	18
8	15582	2519	12	12791	2853	12	12736	2431	12
10	12469	2009	18	11118	2032	14	12629	2213	18
15	10064	1644	11	4466	1488	12	--	--	--
	12138	--	--	--	--	--	12404	1854	14
17	11055	1703	8	2326	856	8	10159	1832	8
24	11075	1623	12	1775	642	12	11114	1546	12
44	13935	3415	8	4684	2840	8	--	--	--

Statistical analysis between control and galactose-fed rats: A-c/m/lens;
B-c/m/mg of protein

A. Days on Diet	p Value
1	N.S.
3	p < .01
4	.05 < p < .02
5-15	N.S.
17-24	P < .001

B. Days on Diet	p Value
1-17	N.S.
24	.02 < p < .01

Fig. 12. Methionyl-tRNA synthetase activity per mg. of lens 56 protein with varied dietary conditions: control, glucose, galactose, and 3 groups returned to purina diet after 3, 8, and 17 days of galactose feeding.

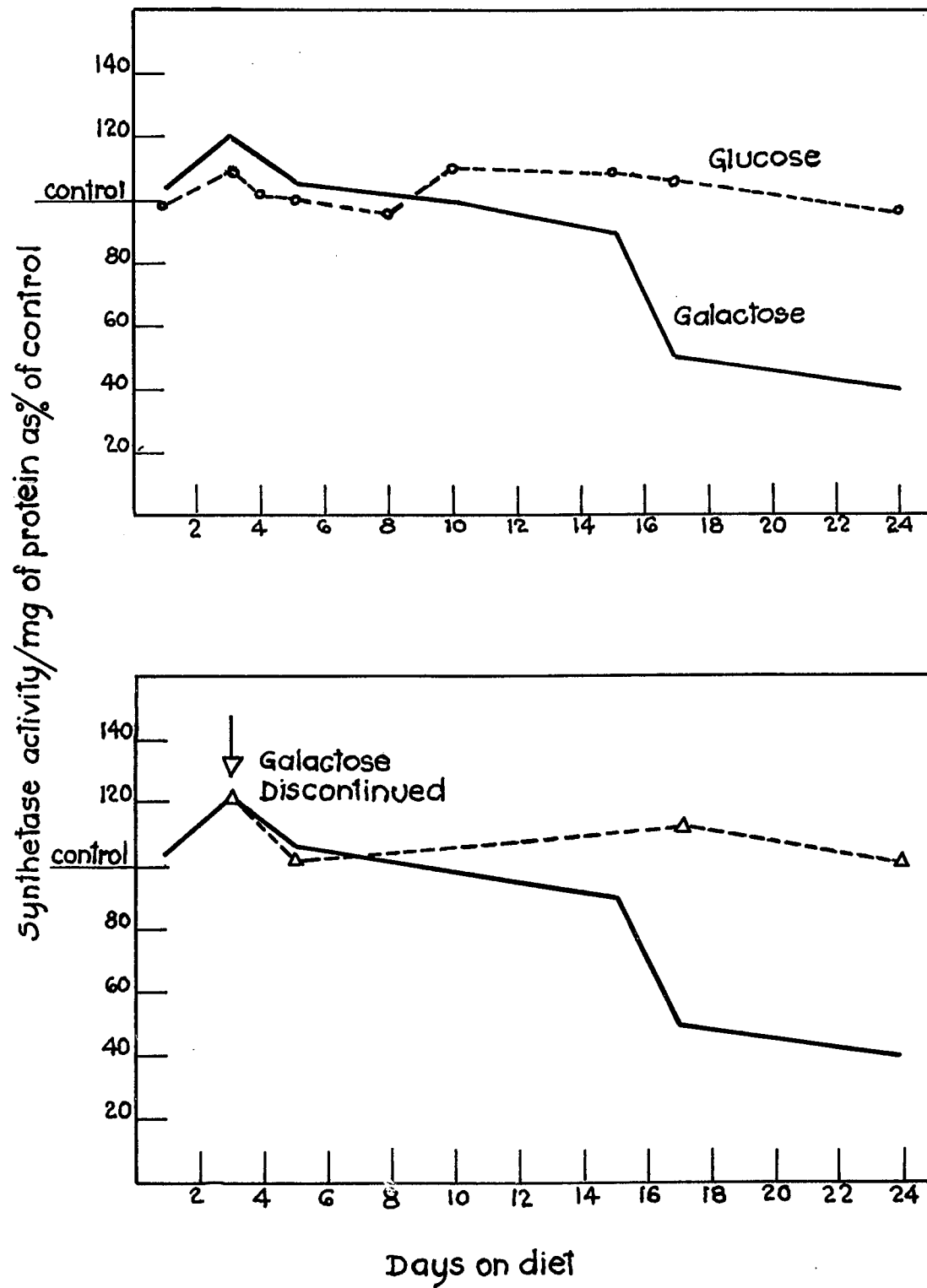


Fig. 12. (Cont.)

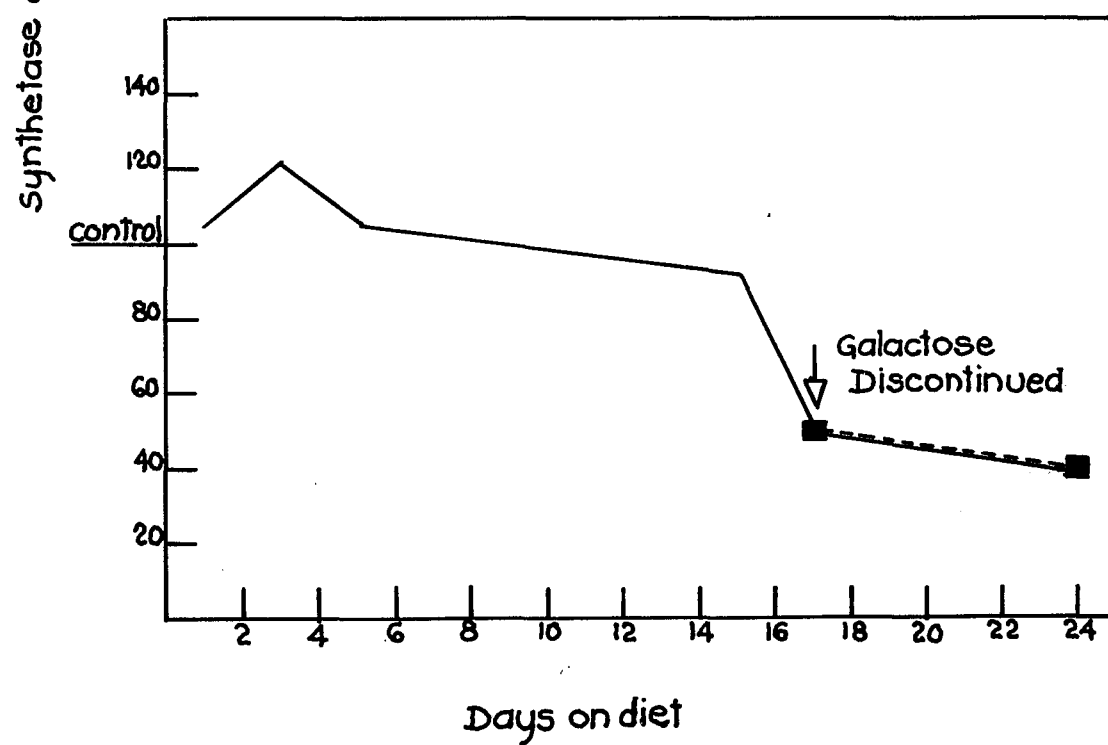
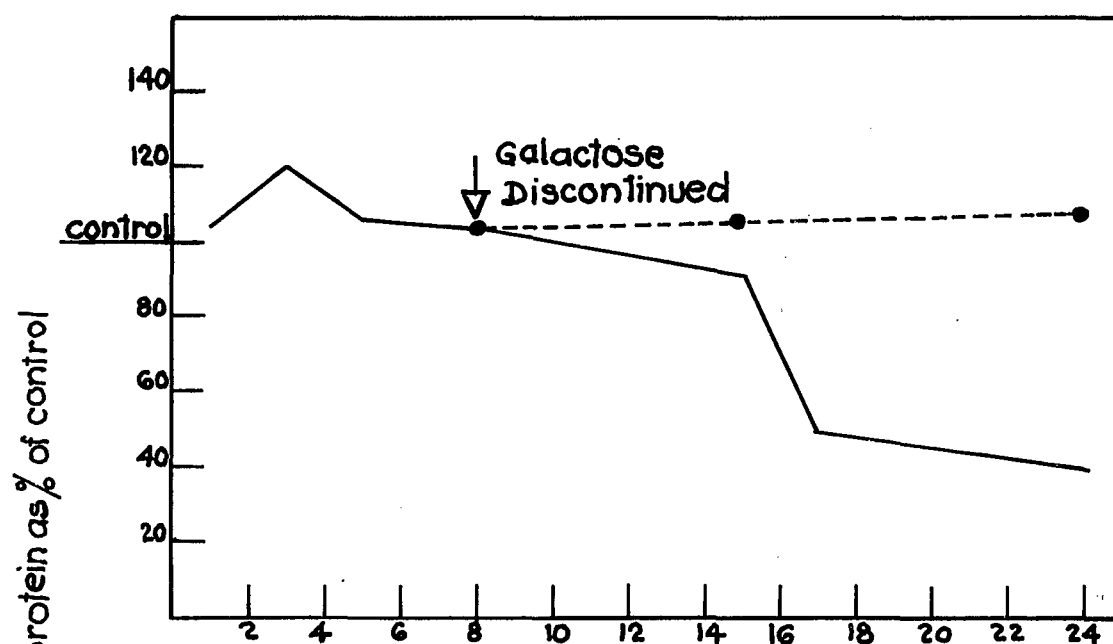


Table 5. Lens methionyl-tRNA synthetase activity for animals placed on galactose diets which were discontinued after 3, 8, or 17 days.

GALACTOSE DISCONTINUED After 3 days				CONTROL			GALACTOSE		
Time of assay (days)	c/m lens	c/m/mg lens protein	No. of lenses	c/m lens	c/m/mg lens protein	No. of lenses	c/m lens	c/m/mg lens protein	No. of lenses
5	10561	3196	18	9929	3128	16			
15	13330	3831	18	12137	3429	20	3653	--	8
24	11750	2468	8	11778	2611	6	3024	1126	8

GALACTOSE DISCONTINUED After 8 Days				CONTROL			GALACTOSE		
Time of assay (days)	c/m lens	c/m/mg lens protein	No. of lenses	c/m lens	c/m/mg lens protein	No. of lenses	c/m lens	c/m/mg lens protein	No. of lenses
15	7649	3329	20	12544	3149	20	4091	2457	12
24	6769	3024	24	12697	2824	22	3286	1383	12
44	11868	3202	6	13935	3415	8	4684	2843	8

Table 5. Lens methionyl-tRNA synthetase activity for animals placed on galactose (cont.) diets which were discontinued after 3, 8, or 17 days.

GALACTOSE DISCONTINUED After 17 Days				CONTROL			GALACTOSE		
Time of assay (days)	c/m lens	c/m/mg lens protein	No. of lenses	c/m lens	c/m/mg lens protein	No. of lenses	c/m lens	c/m/mg lens protein	No. of lenses
24	2693	990	11	11056	2468	8	2110	979	10
44	7788	2903	6	13935	3415	8	4684	2843	8

Statistical analysis of data for the lens methionyl-tRNA synthetase comparing galactose-fed, control and animals fed galactose for 3, 8, or 17 days, for A. c/m/lens

A. Galactose discontinued at day	Assayed at Day	P value	Groups Evaluated
3	15	P < .001	Gal. Disc. vs. Gal.
3	24	P < .01	Gal. Disc. vs. Gal.
8	15	.01 > P > .001	Gal. Disc. vs. Gal.
8	15	.01 > P > .001	Gal. Disc. vs. Gal.
8	24	.01 > P > .001	Gal. Disc. vs. Gal.
17	24	P < .001	Ca. Disc. vs. Contra.

Table 5. Statistical analysis of data of lens methionyl-tRNA synthetase comparing galactose-fed, controls and (cont.) animals fed galactose for 3, 8, or 17 days, B. c/m/mg of protein.

B. Galactose discontinued at day	Assayed at day	P value	Groups Evaluated
3	24	.01 > P > .001	Gal. Disc. vs. Gal.
8	24	.01 > P > .001	Gal. Disc. vs. Gal.

Table 6. Lens methionyl-tRNA synthetase activity in rats fed a 35% xylose diet and controls.

Days on Diet	Xylose Average c/m/lens	Control Average c/m/lens	Xylose Average c/m/mg. prot.	Control Average c/m/mg. prot.	Xylose Number of Lenses	Xylose Number of Lenses
1	12349	12346	--	--	8	8
3	13065	12637	3707	3887	35	32
5	15072	14639	4255	4438	20	20
8	15628	15690	4209	3900	20	20
10	17048	17005	4506	3966	8	8
15	14830	12858	3661	3130	20	18
24	15275	13275	3282	2907	20	16
31	14163	12876	2577	2489	18	12

Table 7. Lens weights from rats on various diets.

DAYS ON DIET	CONTROL		GLUCOSE 50%		GALACTOSE 50%		GALACTOSE 50% DISC. 3 DA.		GALACTOSE 50% DISC. 8 DA.		GALACTOSE 50% DISC. 17 DA.		XYLOSE 50%		XYLOSE 50%	
	wt.	no.	wt.	no.	wt.	no.	wt.	no.	wt.	no.	wt.	no.	wt.	no.	wt.	no.
	mg.		mg.		mg.		mg.		mg.		mg.		mg.		mg.	
1	21.4	42	21.7	30	21.9	24	-	-	-	-	-	-	21.2	18	-	-
3	21.4	42	21.5	18	26.2	18	-	-	-	-	-	-	22.2	12	20.8	12
5	21.9	42	22.5	18	29.0	34	-	-	-	-	-	-	23.4	8	21.0	12
8	23.9	40	24.1	24	31.9	12	-	-	-	-	-	-	26.7	4	23.5	12
10	25.0	18	24.6	18	33.3	14	-	-	-	-	-	-	-	-	-	-
15	26.4	64	26.2	13	33.6	30	26.2	20	-	-	-	-	-	-	25.7	12
17	28.1	8	26.1	10	32.4	8	-	-	-	-	-	-	-	-	-	-
24	29.5	60	29.7	12	31.7	38	28.6	8	29.7	4	31.2	11	-	-	28.4	12
31	33.5	4	-	-	-	-	-	-	-	-	-	-	-	-	29.4	10
44	35.6	8	-	-	30.3	8	-	-	25.1	6	26.6	6	-	-	-	-
51	47.4	6	-	-	34.2	6	-	-	-	-	-	-	-	-	-	-

Table 8. Average rat weight in grams.

Days on diet	Control		Glucose (50%)		Galactose (50%)		Control		Xylose (35%)	
	Weight in gms.	No. of rats	Weight in gms.	No. of rats	Weight in gms.	No. of rats	Weight in gms.	No. of rats	Weight in gms.	No. of rats
1	59	57	56	59	51	70	56	--	51	--
2	--	--	--	--	--	--	60	91	50	107
3	67	47	58	48	53	47	63	77	49	94
4	61	11	51	38	57	47	67	67	51	85
5	77	44	65	--	62	29	69	63	49	81
8	94	29	76	28	67	28	82	52	57	61
10	109	23	86	23	72	22	94	47	62	49
12	---	--	--	--	--	--	104	44	68	48
15	133	8	--	--	90	7	106	38	83	42
17	140	10	106	11	88	11	133	32	84	36
19	---	--	---	--	--	--	136	28	84	33
23	---	--	---	--	--	--	157	31	95	33
24	167	8	131	8	121	8	---	--	--	--
25	---	-	---	-	---	-	163	20	107	17
31	---	-	---	-	---	-	181	--	132	--

Table 9. Food consumption in grams per rat.

Days	Control	Glucose	Galactose	Xylose
1	7.7	8.7	7.3	7.0
2	7.6	6.6	9.1	--
3	8.2	7.9	--	5.1
4	--	8.4	9.4	--
5	8.2	9.7	10.1	--
6	--	--	--	6.8
10	--	--	--	9.7
11	12.3	--	17.2	9.7
12	--	--	--	--
16	--	--	--	12.4
17	--	--	--	12.4
19	18.0	--	21.8	--
20	--	--	--	13.5

Table 10. Thymidine C¹⁴ incorporation into lens DNA after 3-5 days of galactose or xylose feeding. Incubation time was 2½ hrs.

Experiments	Average c/m/lens DNA	Average uptake c/m/lens	No. of lenses	Average c/m/lens DNA	Average uptake c/m/lens	No. of lenses
I	290	29705	6	128	22571	6
II	351	25744	6	212	24182	6
III	116	--	8	65	--	

Average c/m/lens DNA: Gal. vs. Contr.

Exp. I P < .001

Exp. II .001 < P < .01

Exp. III .001 < P < .01

Table 10. Thymidine C¹⁴ incorporation into lens DNA after 3-5 days of
(cont.) galactose or xylose feeding. Incubation time was 2½ hrs.

Experiments	Xylose			Control		
	Average c/m/lens	Average uptake/lens	No. of lenses	Average c/m/lens	Average uptake/lens	No. of lenses
I	213	21656	6	173	20760	6
II	55	--	8	65	--	8

Average c/m/lens DNA: Xylose vs. Contr.

Exp. I P=N.S.

Exp. II P-N.S.

Fig. 13. Effect of incubation time on the uptake of C^{14} -thymidine into lens. Rat weights averaged 85 gms.

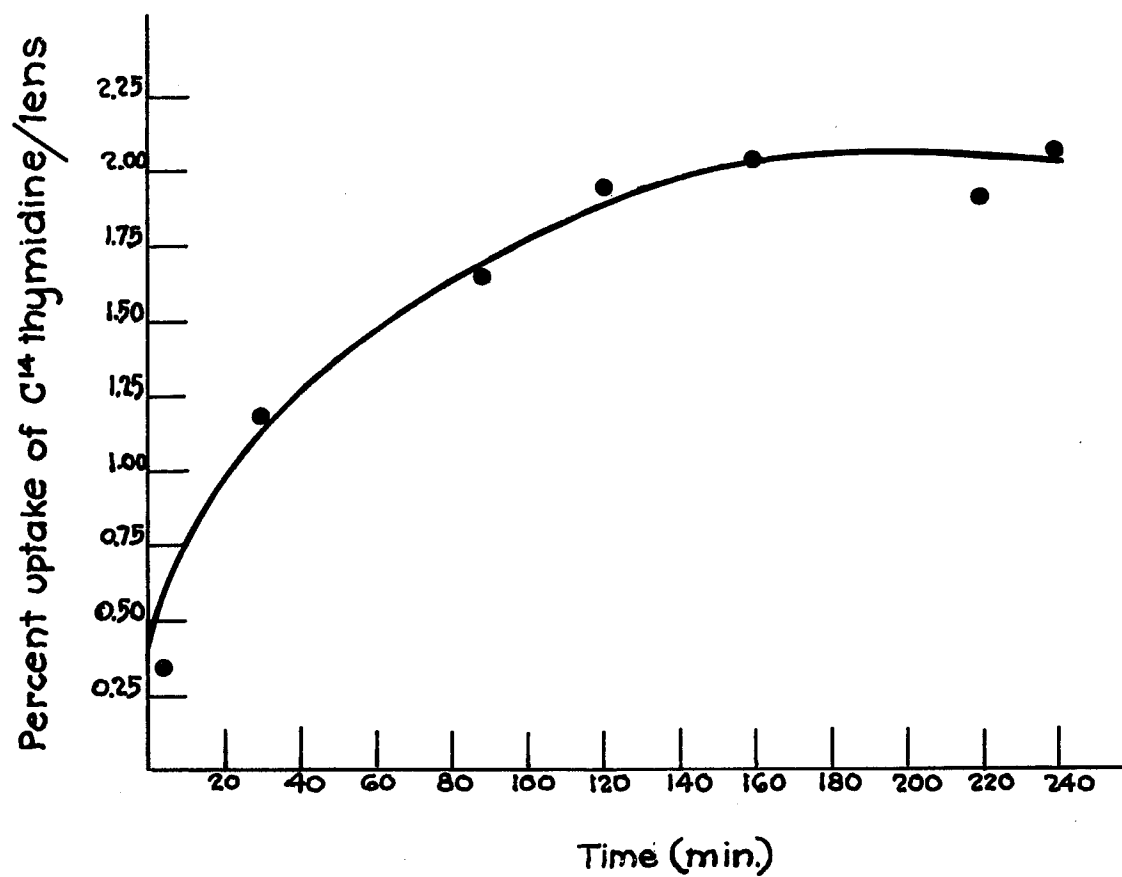


Fig. 14. Effect of temperature on the uptake of C^{14} thymidine into lens. Rat weights averaged 275 gms.

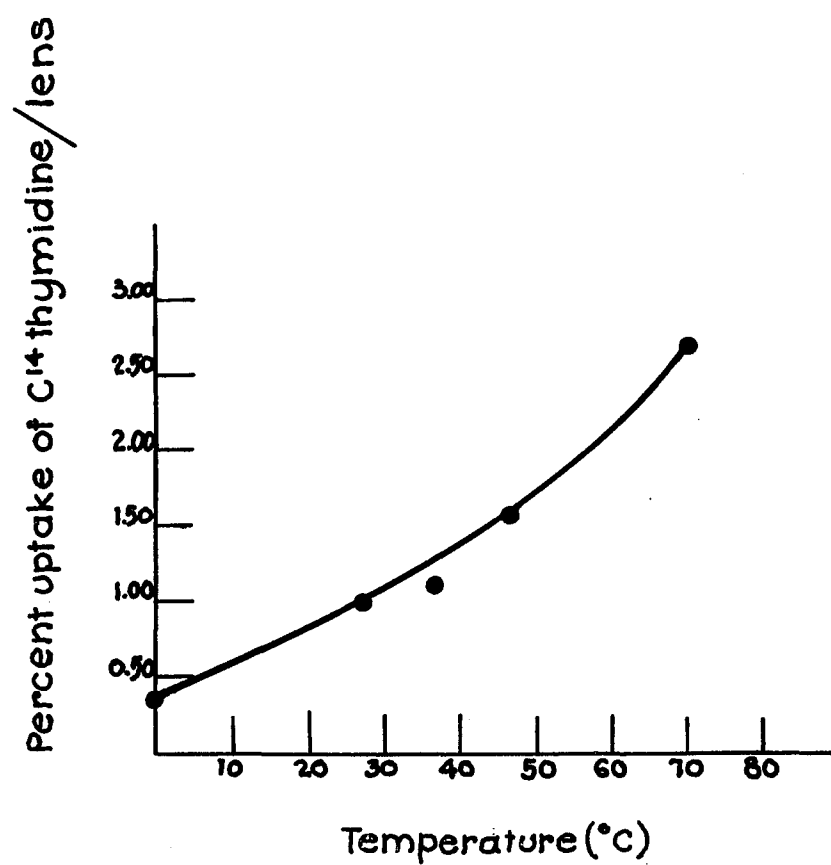


Fig. 15. Effect of unlabelled thymidine on C^{14} -thymidine uptake into lens.

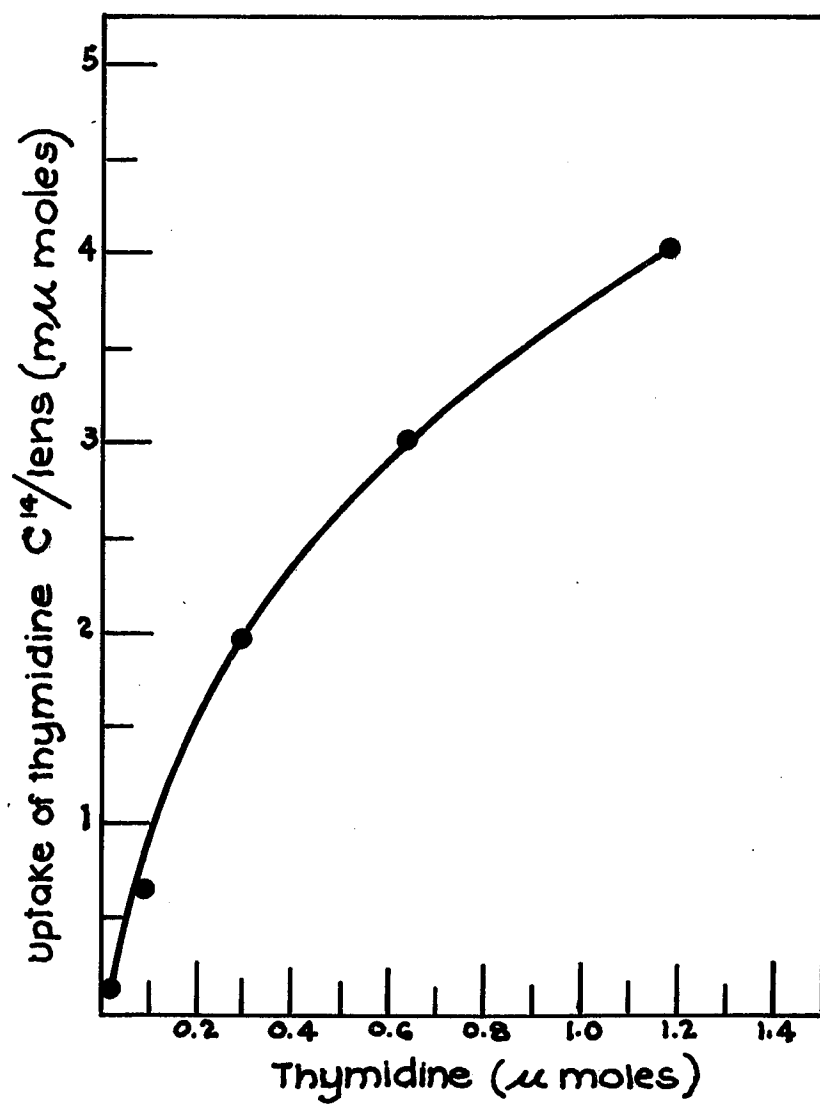


Table 11. The effect of inhibitors on thymidine-C¹⁴ uptake into rat lens.

Inhibitor	Thymidine-C ¹⁴ (0.5 ml. KRPO ₄)	Inhibitor concentration	Percent uptake
Control	4.07 μ g	--	100
Iodoacetate	4.07 μ g	10 ⁻³ M	107
Dinitrophenol	4.07 μ g	10 ⁻³ M	90
p-Chloromercuri- benzoate	4.07 μ g	10 ⁻³ M	55
Mercuric chloride	4.07 μ g	10 ⁻³ M	50
N-ethyl maleimide	4.07 μ g	10 ⁻³ M	88
Guanosine	4.07 μ g	3 x 10 ⁻⁴ M	91
Cytidine	4.07 μ g	3 x 10 ⁻⁴ M	89

Table 12. The lens methionyl-tRNA synthetase activity in 120 gm. galactose-fed rats, and in normal rats.

Day	Average c/m lens	Average c/m/mg. protein	No. of lenses	Percent of control	Average c/m lens	Average c/m/mg. protein	No. of lenses	Percent of control
3	8104	--	6	93	8680	--	6	100
5	13006	2753	6	103	12608	2631	6	100

P-N.S. for average c/m/lens: Gal. vs. Contr.

Table 12a. Thymidine C¹⁴ incorporation into lens DNA after 3-5 days of galactose feeding in 120 gm. rats.

Average c/m/lens DNA	Average uptake/ lens	No. of lenses	Average c/m/lens DNA	Average uptake/ lens	No. of lenses
271.2	33589	12	103	30603	12

P < .001 for average c/m/lens DNA: Gal. vs. Contr.

Table 13. Se^{75} selenomethionine-tRNA binding in the presence of unlabelled amino acids.

Study 1

Amino Acid		Galactose (3 days)		Control	
		c/m/lens	Percent of control	c/m/lens	Percent of control
None	--	20088	123	16493	100
L cysteine	5 μ moles	20547	125	16659	100
DL selenomethionine	5 μ moles	10852	65*	10979	66*
DL alanine	5 μ moles	16600	100*	16390	98
L isoleucine	5 μ moles	19819	120	14971	90
DL phenylalanine	5 μ moles	20040	123	16856	102
DL methionine	5 μ moles	7776	47*	7055	40*

Study 2.

Amino Acid		Galactose (3 days)		Control	
		c/m/lens	Percent of control	c/m/lens	Percent of control
None	--	16717	145	11510	100
L alanine	0.1 μ moles	16182	140	11582	100

Table 13. Se^{75} selenomethionine-tRNA binding in the presence
(cont.) of unlabelled amino acids.

Study 3.

Amino Acid		Galactose (3 days)		Control		1½ year old rats	
		c/m/lens	Percent of control	c/m/lens	Percent of control	c/m/lens	Percent of control
None	--	20306	133	15247	100	19861	100
L alanine	5 μ moles	17708	116*	14640	96	20511	102
L methionine	5 μ moles	6801	44*	5244	35*	8698	45*
--							
None	--	--	--			19133	100
L alanine	50 μ moles	--	--			19330	101

Study 4.

Amino Acid		Galactose (3 days)		Control	
		c/m/lens	Percent of control	c/m/lens	Percent of control
none	--	23817	143	16598	100
L alanine	5 μ moles	23508	141	16490	99
L alanine	500 μ moles	25215	151	16484	99

Table 13. Se⁷⁵ selenomethionine-tRNA binding in the presence
(cont.) of unlabelled amino acids.

Study 5.

Amino Acid		Galactose (3 days)		Control	
		c/m/lens	Percent of control	c/m/lens	Percent of control
None	--	22420	137	16358	100
L alanine	0.1 μ moles	17565	107*	17178	105

Study 6.

Amino Acid		Galactose (3 days)		Control	
		c/m/lens	Percent of control	c/m/lens	Percent of control
None	--	17485	154	11212	100
DL alanine	500 μ moles	17106	152	12830	113
L alanine	500 μ moles	18106	160	--	--
L alanine	500 μ moles	16849	150	--	--
L methionine	500 μ moles	3032	26*	2055	18*

Table 13. Se^{75} selenomethionine-tRNA binding in the presence
(cont.) of unlabelled amino acids.

Study 7.

Amino Acid		Galactose (3 days)	Control	Percent of	
		c/m/lens	Percent of	c/m/lens	control
			galactose		
None	--	17500	100	12591	100
DL selenomethionine	5 μ moles	8825	51*	6737	52*

* indicates significant competitive binding.

Table 14. Methionyl-tRNA synthetase activity in galactose, mixed control and galactose, and control lens supernatants.

Study 1.

<u>Lens supernatants</u>	<u>c/m</u>
Control	6831
Galactose (15 days)	4165
Control + Galactose	
Expected value	5498
Experimental value	6109

Study 2.

<u>Lens supernatants</u>	<u>c/m</u>
Control	6842
Galactose (24 days)	2014
Control + Galactose	
Expected value	4423
Experimental value	4666

Table 15. A. Methionyl-tRNA synthetase activity in lenses of galactose-fed animals and galactose-fed with glucose supplement.

Day	Galactose c/m lens	Percent of control	Galactose plus glucose c/m lens	Percent of control	Control c/m lens	Percent of control
3	14972	130	14497	130	11577	100

B. Glucose consumed per rat.

Day	Grams	Rat (No.)
0-1	3.48	7
1-2	3.40	7
2-3	4.60	7

Fig. 16. The effect of lens homogenate concentrations on H^3 leucyl-tRNA binding. Average rat weights were 70 gms.

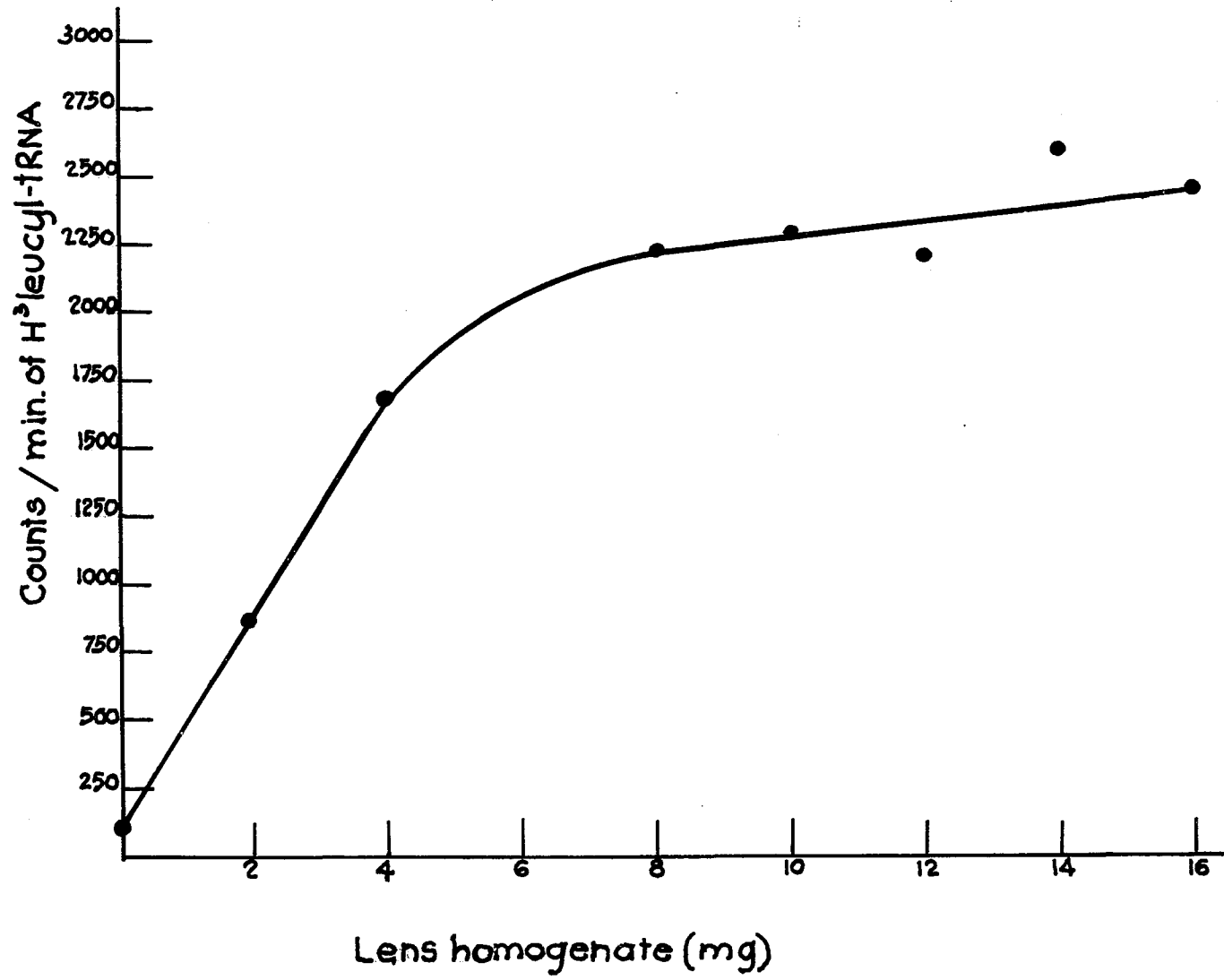


Fig. 17. Lens methionyl- and leucyl-tRNA synthetases in normal and galactose-fed rats.

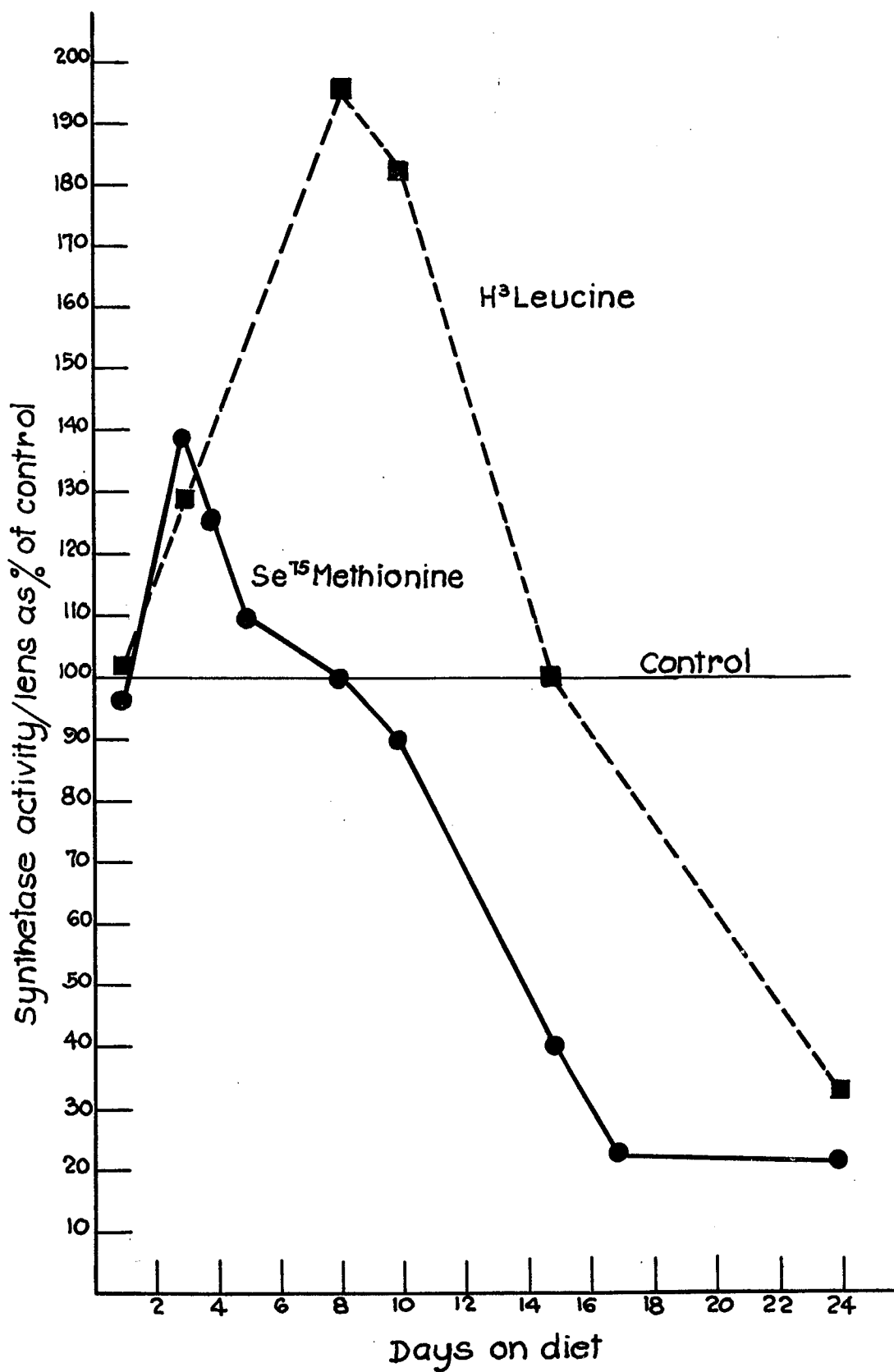


Table 16. H³-leucyl-tRNA synthetase activity.

Days on diet	Galactose			Control		
	c/m/lens	c/m/mg protein	No. of lenses	c/m/lens	c/m/mg protein	No. of lenses
1	747	181	12	721	175	12
3	1111	252	64	856	231	61
8	1025	227	23	524	148	15
10	919	181	12	502	80	8
17	721	--	8	720	--	8
24	306	16	5	964	192	5

Statistical analysis of data for the lens leucyl-tRNA synthetase between control and galactose-fed rats.

<u>Days on diet</u>	<u>P value</u>
1	N.S.
3	P < .01
8	.05 > P > .02
10	N.S.
17	N.S.
24	P=.05

Fig. 18. In vitro leakage of proteins from normal and galactose-fed rats.

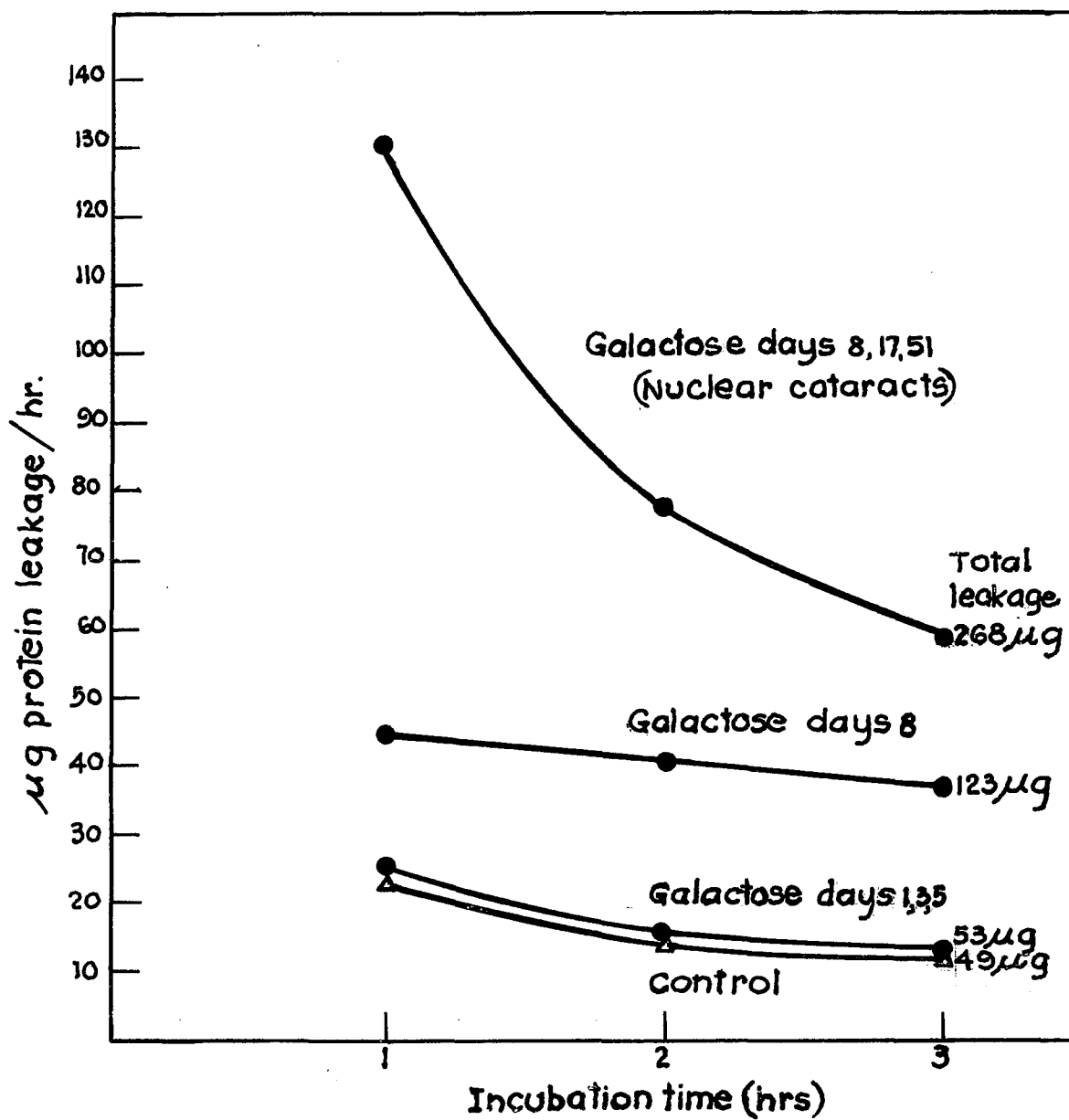


Fig. 19. In vitro protein leakage from lenses of young (34 day) and old (10 mo.) rats.

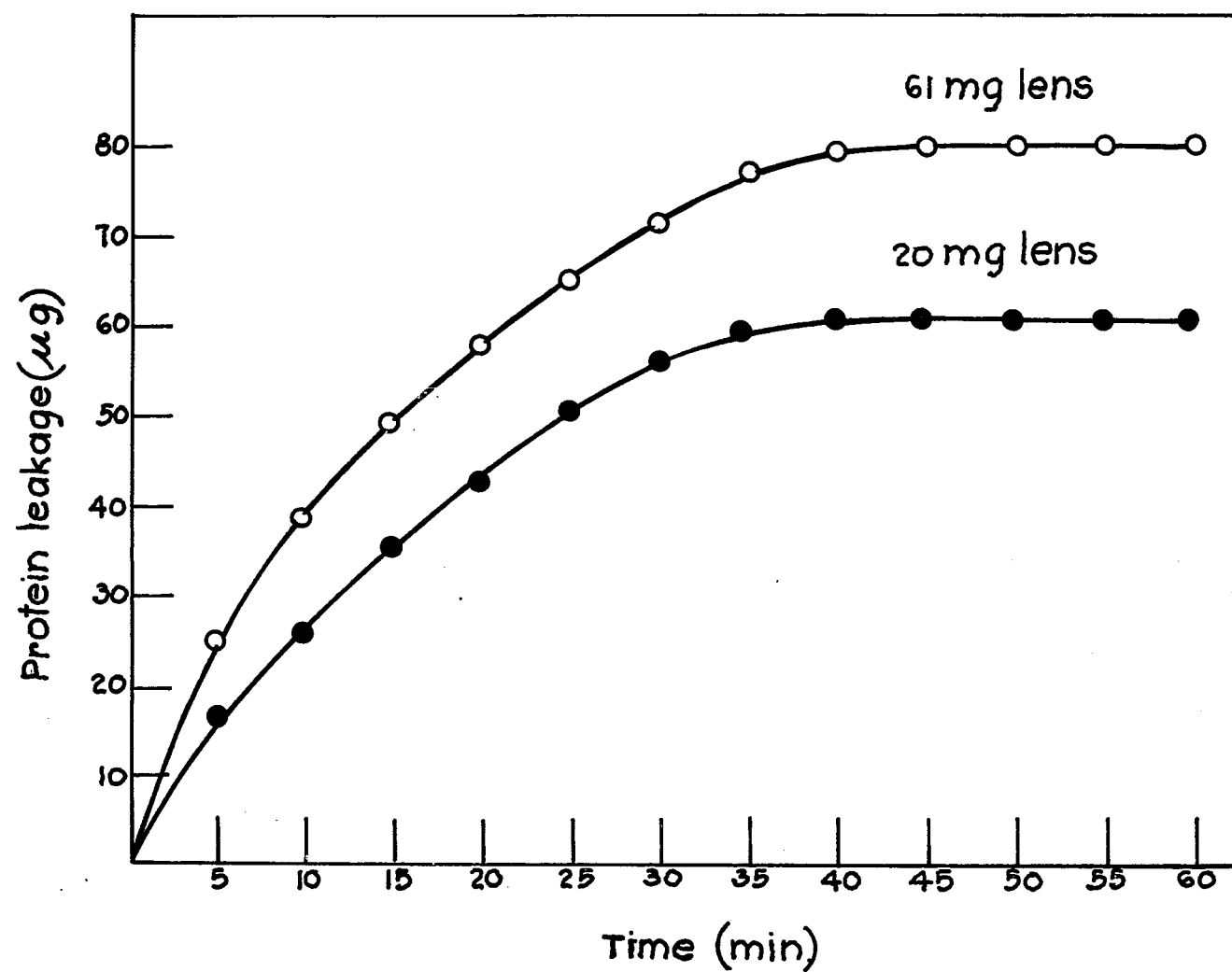


Table 17. In vitro lens protein leakage (control and galactose-fed rats).

Galactose (days on diet)	Hr. (1) (μ g) effluxed	Percent of lens protein	Hr. (2) (μ g) effluxed	Percent of lens protein	Hr. (3) (μ g) effluxed	Percent of lens protein
1, 3, 5	25	0.30	16	0.19	12	0.18
8, 9	45	0.55	41	0.69	37	0.71
<u>17, 51</u>	<u>131</u>	<u>2.01</u>	<u>78</u>	<u>1.51</u>	<u>59</u>	<u>1.28</u>
Controls	23	0.27	14	0.17	12	0.15

Table 18. In vitro leakage of methionyl-tRNA synthetase from normal and galactose (8 day) lenses.

	No. lenses pooled	Effluent protein/lens (μ g)	Effluent synthetase activity/lens (c/m)	Percent of lens synthetase activity
Galactose	29	40	372	1.6
Control	38	23	136	0.6

Table 19. Leakage of radioactive protein from normal and galactose (11-12 days) lenses.

	<u>Se⁷⁵-selenomethionine</u> Average uptake/lens	<u>Effluxed protein</u> in TCA precipitate
*Control	25473	353
*Galactose	13092	791
*Galactose-incubated with puromycin (10^{-2} M)	13951	316
Millipore filter absorption of Se ⁷⁵ -selenomethionine	-	350
Non-specific protein binding to Se ⁷⁵ -selenomethionine	-	390

*Lenses were incubated with 25×10^4 c/m Se⁷⁵selenomethionine for 1 hr.

Table 19. Leakage of radioactive protein from normal and galactose (11-12 days) lenses.

	<u>Se⁷⁵-selenomethionine</u> <u>Average uptake/lens</u>	<u>Effluxed protein in</u> <u>TCA-precipitate</u>
*Control	25473	353
*Galactose	13092	791
*Galactose-incubated with puromycin (10^{-2} M)	13951	316
Millipore filter absorption of Se ⁷⁵ -selenomethionine	--	350
Non-specific protein binding to Se ⁷⁵ -selenomethionine	--	390

*Lenses were incubated with 25×10^4 c/m Se⁷⁵ selenomethionine for 1 hr.

Table 20. In vitro lens protein leakage (control and galactose-fed rats).

Experimental group		Hour (1) efflux (μ g)	Hour (2) efflux (μ g)	Hour (3) efflux (μ g)
Control		25	13	12
Galactose		46	46	46
Control	(lenses were rolled prior to incubation in vials.)	33	16	13
Galactose		148	138	120

Fig. 20. Disc electrophoretic patterns of in vitro protein leakage from lenses of normal and galactose-fed (13 day) rats.

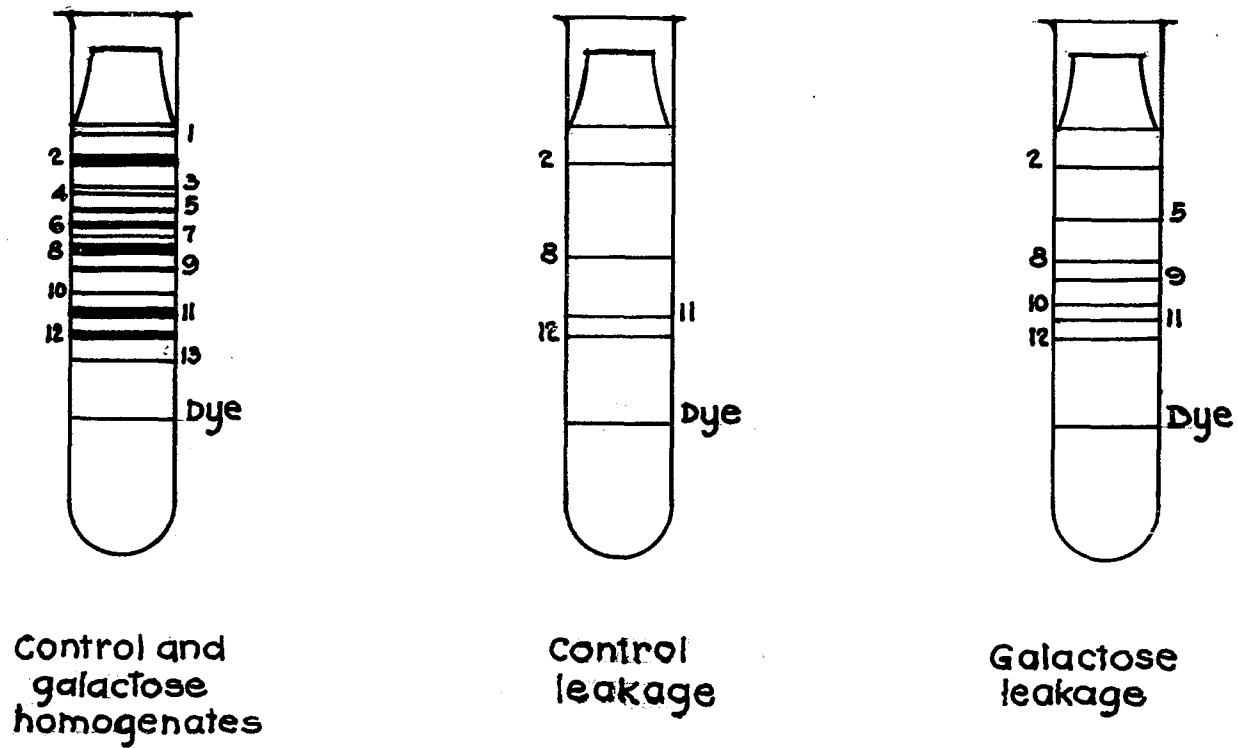


Table 21. Digestion of collagen gel by enzymes.

Control	Lysis in mm.	supernatant Hydroxyproline (μ g)
*Trypsin 50 μ g	None	2.5
*Chymopapain 50 μ g	None	0.05
*Hyaluronidase 50 μ g	None	0
*Pepsin 50 μ g	None	0.08
Control lens	None	0
Nuclear cataractous lens	9-11	2.5

Incubation 4 days at 37°C

*Data from Brown, Weller, and Wassermann (1969).
Arch. Ophth. 81:370.

Table 22. I^{125} lens protein bound to serum gamma globulin in starch gel electrophoresis.

<u>A Experimental animal</u>	<u>Dilution of antisera</u>	<u>c/m/ at origin</u>
Control rabbit	0	2664
Immunized rabbit	0	6025
Immunized rabbit	1:5	5137 *
Immunized rabbit	1:10	3723
Immunized rabbit	1:20	2709
Control rat	0	2531
Immunized rat	0	4988
Immunized rat	1:2	3976 *
Immunized rat	1:5	3229
Immunized rat	1:10	2664

* Indicates sensitivity obtained by Ouchterlony gel diffusion.

Table 22. I^{125} lens protein bound to serum gamma globulin
(Cont.) in starch gel electrophoresis.

<u>B Experimental animal</u>	<u>c/m at origin</u>
control rat - 7 days after lens injection	2407
Control rat - 10 days after lens injection	2907
Galactose rat - 4 mos. * 7 days after lens injection	3303
Galactose rat - 4 mos. * 10 days after lens injection	2330
Galactose rat - 1 yr. ** 10 days after lens injection	3041

* Cataractous rats were fed galactose for 5 weeks
and then returned to a normal diet for 10 weeks.

** Cataractous rats were fed galactose for 5 weeks
and then returned to a normal diet for 47 weeks.

The results presented in this study measure aminoacyl-tRNA synthetase activity since the binding of Se^{75} -selenomethionine to tRNA is almost completely inhibited when homogenate, ATP or tRNA is omitted, RNase is added, or the final mixture is heated at 90°C for 10 min. with 5% TCA. Furthermore, both selenomethionine and methionine inhibit the formation of Se^{75} -selenomethionine-tRNA. Modification of existing conditions for evaluation of the methionyl-tRNA synthetase in the normal, aging and pathological rat lens.

The age studies indicate that the total lens enzyme activity increases slightly between 22 and 123 days, and then appears to plateau throughout 453 days. The epithelial area (von Sallmann and Grimes, 1966) and the soluble protein content (Dische, Borenfreund and Zelmenis, 1956) show similar changes with age. Although the lens grows to three times its size during this time period, only a small increase in enzyme activity occurs.

Enzymatic activity decreases when expressed per milligram of protein indicating that the concurrent increase of other protein is more rapid than that of the synthetase.

There is a significant rise in lens enzyme activity in the early stages of cataractogenesis induced by galactose feeding in young rats. This increase could result from the

lens epithelial proliferation which was first reported by von Sallmann, 1957.

In the studies reported here, rats fed galactose for 3-5 days showed increased lens incorporation of C^{14} -thymidine into DNA (TCA-precipitable fraction that is hydrolyzed with deoxyribonuclease). Methionyl-tRNA synthetase activity and thymidine- C^{14} incorporation studies were also performed on paired lenses from rats (120 gm). No galactose-induced increase in enzyme activity was noted although there was almost a three-fold increase of thymidine incorporation into DNA. It is therefore unlikely that the enzyme rise in lenses from young rats is directly related to cell proliferation.

It was not the purpose of this study to delineate the mechanism of thymidine penetration into the lens. However, certain conclusions can be drawn from these preliminary studies. The uptake of the nucleoside is similar in normal and in galactose lenses. Thymidine uptake is not an active process since the percent uptake approximates the distribution expected in the cell water. Although the temperature studies do not seem to indicate the presence of a heat sensitive carrier, a facilitative transport component might be present since two inhibitors (p-chloromercuribenzoate, and mercuric chloride) are effective, and there is indication that uptake is saturable. Other nucleosides do not significantly affect thymidine uptake.

Since the aminoacyl-tRNA synthetase is intimately associated with protein synthesis and protein alterations occur during cataract formation, it is possible that the elevated enzyme level is associated with the synthesis of new or abnormal protein(s).

It is also possible that the enzyme might be altered in its functioning, thus resulting in "mischarging". In order to test this interesting theory and the specificity of the system, competition experiments were performed using amino acids similar to methionine in structure or codon assignments. It was found that, in lens homogenates from animals fed galactose for 3 days, unlabelled alanine (chromatographically pure) competed with Se^{75} -selenomethionine (in some experiments). The added alanine lowered the binding of Se^{75} -selenomethionine to the value found with control lenses. However, the data repeated only intermittently and could not be construed as reproducible.

The early enzyme rise may be related to a cellular regulatory role of the synthetases. The regulation of RNA synthesis coupled to protein synthesis by synthetases (or indirectly by charged tRNA's) has been postulated by many workers (Ceccarini, Maggio and Barbata, 1967; Eidlic and Neidhardt, 1965a; Eidlic and Neidhardt, 1965b; Morris and DeMoss, 1965; and Williams and Freundlich, 1969). This hypothesis may be supported by the fact that the time

sequence of the galactose-induced rise in methionyl- and leucyl-tRNA synthetase activity differs. The leucyl-tRNA synthetase shows a significant increase (129% of control) after 3 days of galactose ingestion which is similar to the methionyl-tRNA synthetase peak of 140% (of control). The methionyl-tRNA synthetase then rapidly declines, but the leucyl-tRNA synthetase continues to rise to 195% of the control value at 8 days.

When galactose is discontinued after 3 days, synthetase activity (per total lens as well as per milligram of protein) approximates that of the control 2, 12, and 21 days later, suggesting that permanent alterations have not yet occurred. The marked decline in enzymatic activity found with continual galactose feeding may be partially prevented by resumption of a normal diet after 8 days.

The lenses of young animals maintained on galactose for 17 days had nuclear cataracts and returning them to a normal diet for 1 week had no noticeable effect on either lens clarity or enzymatic activity. It was observed, however, that after an additional 3 weeks (day 44) enzymatic activity was close to 50% of the control and the clarity of the lens surface was slightly improved. It appears, therefore, that even in lenses with mature cataracts, some recovery in enzyme activity occurs.

Generally in galactose-induced cataractogenesis, the

methionyl-tRNA synthetase activity is related to lens clarity. Enzyme activity was never observed to return to a normal level in a lens with a nuclear cataract. In early nuclear cataracts, the loss of enzyme activity seemed to be directly related to the degree of opacification.

Although, after 8-10 days of xylose-feeding, there is a significant opacity of the lens (which is again transparent at approximately 3 weeks) there is no noticeable change in methionyl-tRNA synthetase activity.

In young rats when nuclear opacities first appear (usually after 8 days of galactose feeding) the total lens leucyl- or methionyl-tRNA synthetase activity falls rapidly to 20% to 30% of the control value. This decrease in activity may be due to enzyme degradation, decreased enzyme synthesis, the presence of inhibitor(s) and/or protein leakage. The presence of inhibitor(s) appears unlikely since mixing the supernatants of galactose lenses and young lenses gave the expected intermediate value. Under the conditions used in these experiments, lens protein leakage of cataractous lenses has been found in vitro but the significance of this leakage in vivo has not been determined.

A small amount of protein leakage is found when lenses of control and galactose-fed rats (3 week old) are incubated in vitro. After 8 days of galactose feeding there is a significant increase in the lens protein efflux. At this

time, the more apparent the nuclear cataract the greater the leakage. At days 17 and 51, when all lenses have nuclear cataracts, leakage is at a maximum.

When slightly older rats were studied, nuclear cataracts did not develop by 17 days, and the leakage approximated that found in other pre-nuclear cataracts. Protein efflux, therefore, appears to be related to the stage of cataractogenesis. It is possible that the loss of synthetase (as well as other proteins) in cataractous lenses could be due to leakage.

Methionyl-tRNA synthetase activity could be detected in the effluent media of lenses incubated in vitro. Lenses from animals fed galactose for 8 days showed a three-fold increase in enzyme leakage. Furthermore, there is evidence that newly synthesized (Se^{75} -selenomethionine-labelled) protein leaks out of the cataractous lens. This effect is not due to non-specific binding of Se^{75} -selenomethionine since no labelled protein leakage is noted if puromycin is added.

Disc electrophoretic patterns of the in vitro effluent proteins from lenses of normal and galactose-fed rats looked like the patterns obtained from diluted lens homogenates. The effluent proteins from both the control and the galactose lenses appear to be the proteins found in highest concentration in the lens. The leakage from the control lens contain-

ed four reproducible bands which were identical with the most dense homogenate bands. The leakage from the galactose lens (8-13 days) contained seven reproducible bands. This effluent showed more bands simply because there was more protein. The effluent proteins occurred within all three regions (α , β and δ) of total lens homogenate protein.

Leakage from the control lens is small; the total lens protein efflux seldom exceeds 80 μ g., with more of the protein leaking in the first incubation vial than in succeeding vials. This would suggest that leakage of protein from controls is from a limited pool, which is released following small changes to the lenticular cell membranes, either due to surgical manipulation or holes induced by the tearing of zonules during the removal of the lens.

The leakage from cataractous lenses is from a considerably larger protein pool, increases with increasing incubation time, and is enhanced when the lens is disturbed, i.e., when transferred from vial to vial. In these studies Krebs Ringer Buffer and Mammalian Tyrodes solution were used as incubation media. It is possible that an incubation medium could be found which would reduce control leakage; however, the difference observed under the conditions of these experiments indicate that the cataractous lens responds very differently than the normal lens.

There are apparent differences in the response to

trauma between control and galactose-induced cataractous lenses. Rolling of lenses on a moist filter paper prior to placement in vials had only a slight effect on the control lenses while the galactose lenses showed approximately a three-fold increase in efflux.

It was also postulated that any damage to the lens capsule, e.g., by collagenase, might facilitate the escape of lens proteins. Although electron micrographs (Kuwabara, Kinoshita, and Cogan, (1969) indicate that the lens capsule remains intact for 14 days of galactose feeding, it has an uneven density. Small quantities of collagenase were detected in the peeled epithelium of nuclear cataractous lenses, but not in normal lenses. It is possible then that the leakage found in mature cataracts is not only related to cell membrane rupture extruding pools of liquefied protein (Kuwabara, et al, 1969), but that the capsule is being broken down by collagenase.

If cataractous lenses leaked proteins in vivo, antibody titres might be induced. However, a radioimmunochemical assay with a sensitivity approximately two times that of the Ouchterlony gel diffusion method, showed no autologous lens antibodies present in the sera of galactose-induced cataractous rats. If lens antibody titres were present they might be enhanced by injections of lens proteins. However, even 10 days after the injec-

tions of lens homogenate (booster?) to cataractous rats, there was no detectable antibody titre.

Protein leakage from cataractous lenses occurs in vitro probably as a response to trauma. Protein leakage in vivo has not yet been demonstrated. It may be that cataractous lenses lose a significant amount of their soluble protein by this process.

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