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Molecular mass distribution of water-soluble crystallins from the human foetal lens during development

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ABSTRACT

The water-soluble crystallins of twenty human foetal lenses with gestational ages of 112–231 days were analysed by size-exclusion chromatography. The crystallin distribution showed similar patterns for all foetal lenses, but clear changes in the proportions of different crystallins were evident. The distribution showed that the water-soluble part of all the lenses already contained high-molecular-mass material. Also β-crystallins of high molecular mass (β_H), formed by post-translational changes, were detected in all stages. During gestation, the percentage of high-molecular-mass crystallins and of α-crystallins of low molecular mass (α_L) decreased significantly. The total β-crystallins (β_T) and the total γ-crystallins (γ_T) increased significantly. The β_H-crystallins (β_{1+2}) decreased, whereas the β_L-crystallins (β_3) increased significantly. The low M_r crystallins were resolved into three peaks, designated β_s-, γ_H- and γ_L-crystallins. They increased significantly during development. These significant increases of the low M_r crystallins took place exclusively in the developing lens. The rate of protein synthesis of the low M_r crystallins was 23% of the total water-soluble crystallin synthesis rate.

INTRODUCTION

Since the foetal lens is a developing tissue, those crystallins that are newly synthesized primary gene products, such as β_s- and γ-crystallins, should in-

[a]Deceased

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crease in concentration. However, Thomson and Augusteyn [1] found that the γ-crystallins decreased during development, and Thomson et al. [2] reported the same statement. Since these results conflicted with ours [3–6], we analysed twenty foetal crystallin samples by size-exclusion chromatography (SEC) and calculated the percentage distribution, the absolute amounts in milligrams and the rate of synthesis in the foetal lens.

EXPERIMENTAL

Samples

Normal lenses were taken from the eyes of medically aborted foetuses and obtained within 4 h of death. Foetal age was determined to within one week from body measurements by a pathologist, according to Smith [7]. The origins of 29 lenses are specified in Table I. The lens wet weights (LWW) were determined, and single lenses including the lens capsule were homogenized in a microglass Potter homogenizer in 1 ml of distilled water. Homogenates were centrifuged at 11 630 *g* in a Heraeus Christ Biofuge B for 5 min at 4°C. The

TABLE I

ORIGIN OF HUMAN FOETAL LENS MATERIAL

Case No.	Diagnosis	Foetal age (days) and number of eyes[a]	Total number of lenses	Total number of samples
1	Amnion infection syndrome, no malformations	112 (2), 127 (1), 147 (1), 154 (1), 158 (1), 161 (2), 179 (1), 181 (2), 196 (2), 197 (2)	15	10
2	Hypoxia, without pathological changes	126 (1), 182 (2)	3	2
3	Early abortion, with maternal cervix insufficiency	136 (2)	2	1
4	Foeto-foetal transfusion syndrome[b]	178 (2)	2	1
5	Infans mortus, normal development of the foetus	231 (1)	1	1
6	Urethra stenosis	168 (1)	1	1
7	Spina bifida and hydrocephalus	133 (1), 140 (1)	2	2
8	Potter's syndrome[c]	210 (2)	2	1
9	Trisomia 18[d]	120 (1)	1	1

[a]The number of eyes is given in parenthesis.
[b]Donor twin had smaller size in growth and other features as a result of an arteriovenous shunt in the monochorionic placenta.
[c]Primary defect was development of oligohydramnios with bilateral renal agenesis
[d]Extra set of genes, owing to chromosomal maldistribution.

sediments were washed twice in 1 ml of distilled water to remove the water-soluble (WS) crystallins. The sediments and supernatants were lyophilized until 48-h constant weight (mg) was obtained.

Chromatography

SEC was carried out to determine the size distribution of the WS crystallins. A 100-μl volume at a concentration of 1 mg/ml of the WS crystallins was in-

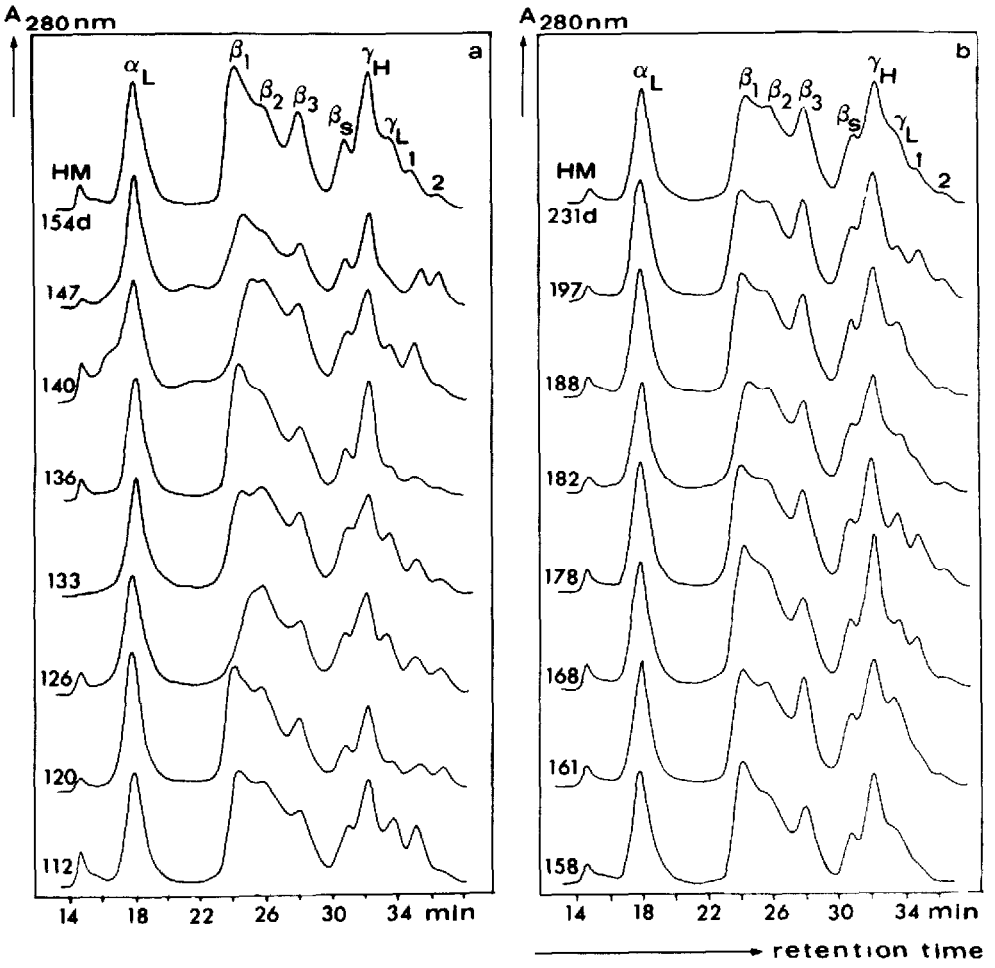

Fig. 1. Size-exclusion chromatography of the WS crystallins of the developing human foetal lens (112–231 days of gestation). Samples: 100 μl of a 0.1% solution. Peaks: HM = high-M_r α-crystallin, $\alpha_L = \alpha$-crystallin of lower M_r; $\beta_1 + \beta_2 = \beta$-crystallins of high M_r; $\beta_3 = \beta$-crystallin of low M_r; β_s = "β-slow"-crystallin; $\gamma_H + \gamma_L = \gamma_T$-crystallins; 1 = a γ-crystallin of lower M_r; 2 = nucleotides. The absorbance was read at 280 nm.

TABLE II

APPARENT MOLECULAR MASSES AND RETENTION TIMES OF HUMAN FOETAL LENS CRYSTALLINS FROM 112 TO 231 DAYS OF GESTATION

The M_r values of the crystallins were determined by calibration of the columns with a standard calibration mixture containing eight proteins. The regression line obtained from the log M_r values (x-axis) and the t_R values (y-axis) of these eight proteins gave $r^c = -0.997$ with $S_{x \cdot y} = \pm 0.054$ (log M_r) and $S_{y \cdot x} = \pm 0.481$ (t_R). S.D.$_1$ is calculated from the $S_{x \cdot y}$ and $S_{y \cdot x}$ of the regression line; S.D.$_2$ is the standard deviation of the t_R. Peak 2 exhibits an $A_{260\,nm} > A_{280\,nm}$ and is designated as containing nucleotides only. BSA = bovine serum albumin. The retention times of the crystallins show a mean standard deviation of 0.15 (from 0.05 to 0.23), with $n = 20$ for each t_R.

Crystallin	M_r	S.D.$_1$	t_R (min)	S.D.$_2$	Standard calibration mixture	M_r	t_R (min)
HM-Crystallin[a]	> 4000 000	> 400 000	14.7	0.1	Thyroglobulin	670 000	18.0
α_L-Crystallin	685 000	170 000	17.9	0.1	Ferritin	450 000	19.9
β_1-Crystallin	133 000	33 000	24.3	0.2	Aldolase	158 000	22.9
β_2-Crystallin	93 000	23 000	25.7	0.1	Di-BSA	136 000	24.3
β_3-Crystallin	52 000	13 000	27.9	0.1	BSA	68 000	26.9
β_s-Crystallin	25 000	6000	30.8	0.1	Ovalbumin	45 000	28.1
γ_H-Crystallin	18 000	4500	32.1	0.1	Chymotrypsinogen	25 000	31.5
γ_L-Crystallin	12 100	3000	33.6	0.2	Cytochrome C	12 500	33.2
Peak 1[a]	8500	2000	35.0	0.3			
Peak 2[a] (nucleotides)	–[b]	–[b]	37.7	0.1			

[a]Component outside the calibration range.
[b]Does not apply.

jected into linked Bio-Sil TSK 400 and 250 columns (30 cm × 0.75 cm I.D. each; Bio-Rad). The elution buffer (pH 6.9) was composed of 0.1 M Na_2SO_4, 0.02 M sodium phosphate (0.012 M $Na_2HPO_4 \cdot 2H_2O$ and 0.008 M $NaH_2PO_4 \cdot 1H_2O$) and 0.02% sodium azide. Elution was performed at a constant flow-rate of 0.8 ml/min using a Bio-Rad Model 401 high-performance liquid chromatographic (HPLC) system. The eluted proteins were detected at 280 nm with a Model 1305 UV monitor (Bio-Rad) and gave the pattern shown in Fig. 1. Quantitative determinations were carried out by peak-area integration by dropping a perpendicular line from the minimum between the peaks.

The molecular masses of the crystallins separated were determined by means of high and low M_r standard gel permeation calibration kits from Boehringer and Pharmacia (Table II). The percentage distribution as calculated was corrected by taking into account the specific absorbances ($A_{280\,nm}^{1\%,1\,cm}$) of the single foetal crystallins, according to Thomson and Augusteyn [1] for high-molecular mass (HM) (10.2), α (8.1), β (23.2) and γ (23.8) and according to Van Dam [8] and Bours [9] for β_s (8.5). The LWW, the WS and water-insoluble crystallin proportions and the crystallin percentages were plotted versus age (Fig. 2). The absolute amounts were calculated from the WS crystallins and the crystallin percentages (Fig. 3). The rates of synthesis of the single crys-

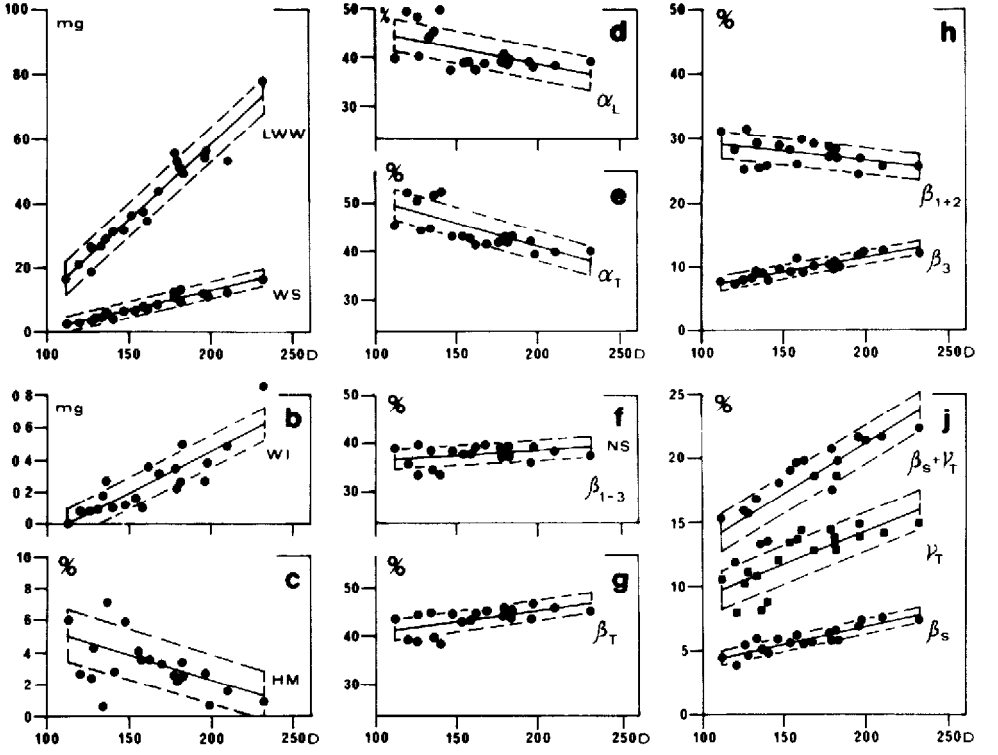

Fig. 2. Proportions of (a) the lens wet weight (LWW) and the WS crystallins, (b) the WI crystallins, and (c–j) the HM-, α_L- α_T-, β_{1-3}, β_T-, β_{1+2}-, β_3-, β_s-, γ_T- and $(\beta_s+\gamma_T)$-crystallins. (α_T=HM+α_L, $\beta_T=\beta_1+\beta_2+\beta_3+\beta_s$). All increases and decreases are significant ($n=20$, NF=18) except that for β_{1-3}. All other crystallins, e.g. HM-, α_L-, α_T-, β_{1+2}-, β_3-, β_s-, β_T-, γ_T- and $\beta_s+\gamma_T$, show t-values > 2.67 with $2p < 1.57\%$.

tallins were calculated from the amounts of crystallins synthesized during the foetal period observed [112–231 days = 119 days (Table III)]. The S.D. of the regression lines, as the evaluation of y from given values of x (Figs. 2 and 3, Table II) and of x from given values of y (Table II) (the residual standard deviations $S_{y\cdot x}$ and $S_{x\cdot y}$) were calculated according to Draper and Smith [10]. The significance of the slope of a regression line is dependent on the correlation coefficient and the number of degrees of freedom of the system (NF = $n-2=18$). In order to estimate whether an increase may be significant or not, the t-test for correlation coefficients was applied according to Draper and Smith [10].

RESULTS

The LWW depicted in Fig. 2a increased from 17.3 mg at 112 days to 73.1 mg at 231 days of gestation ($r^c = +0.961$, the residual standard deviation

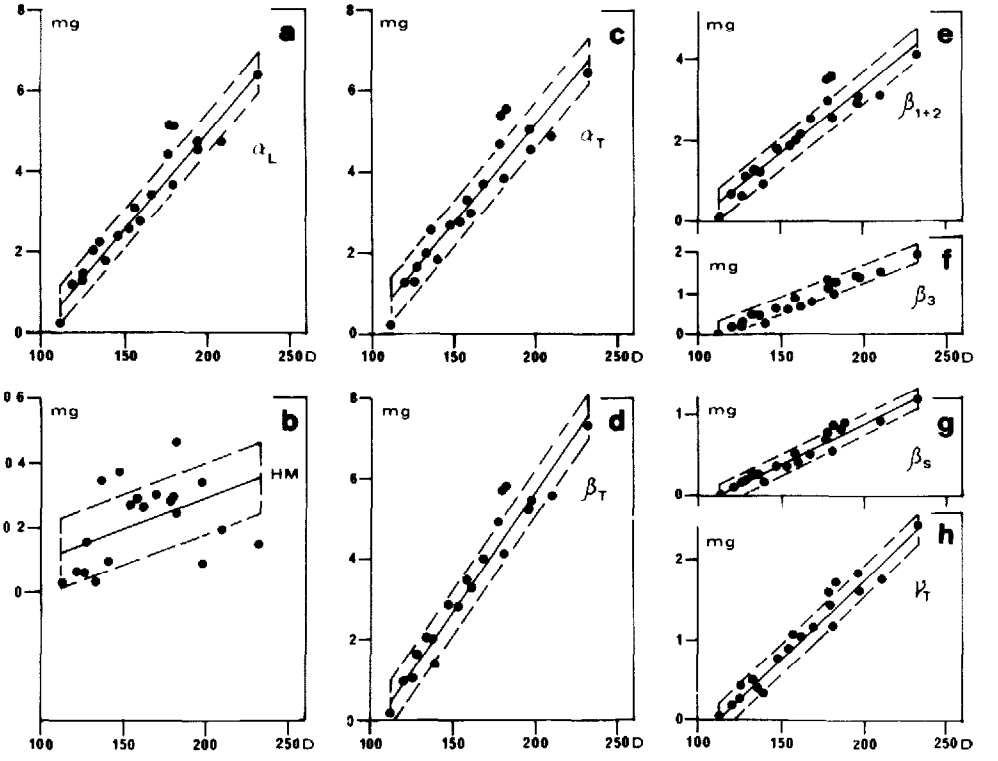

Fig. 3. Proportions of the α_L-, HM-, α_T-, β_T-, β_{1+2}-, β_3-, β_s- and γ_T-crystallins, expressed as mg of crystallin per lens. HM shows an insignificant increase. All other crystallins show significant increases ($n=20$, NF$=18$, $t>11$, with $2p<5\cdot10^{-8}$).

$S_{y\cdot x}=\pm4.5$ mg). The amounts of WS crystallins are also shown in Fig. 2a, and increased from 1.68 to 16.52 mg ($r^c=+0.962$, $S_{y\cdot x}=\pm1.17$ mg). The WI crystallins increased from 0.05 to 0.62 mg ($r^c=+0.852$, $S_{y\cdot x}=\pm0.11$ mg) (Fig. 2b). All these data concern the normal development of the foetal lens.

SEC of the WS crystallins on a series of two columns gave an elution pattern with ten partially resolved peaks (Fig. 1). The foetal crystallins were separated into HM-, α-, β_1-, β_2-, β_3-, β_s- and γ-crystallins. HM-crystallin eluted in the void volume and had a molecular mass greater than $4\cdot10^6$ daltons [11, 12]. It was already detectable in the 112-day foetal lens (Fig. 1). The next crystallin, eluting at 17.9 min, was α-crystallin: no difference in its size was observed as the lens developed.

All fractions eluted from 24.3 to 27.9 min were β-crystallins. The β_1- and β_2-crystallins were β-crystallins of higher M_r and were evaluated as one peak (Fig. 1). The β_3-crystallin at 27.9 min was a β-crystallin of low M_r.

The next group of four peaks belonged to the crystallins of low M_r. The first

TABLE III

RATES OF SYNTHESIS OF HUMAN FOETAL LENS CRYSTALLINS

Rate of synthesis for single crystallins taken from the total WS crystallin synthesis rate is expressed in percent. The data in the first column were taken from the regression lines in Fig. 3. $\beta_T-\beta_s=\beta_1+\beta_2+\beta_3=\beta_H+\beta_L$

Crystallin	Original weight		Rate of protein synthesis and formation		Proportion of total synthesis	
	mg/119 days	$S_{y\cdot x}$	μg/month	S.D.	%	S.D.
HM	0.155	0.117	40	30	1.01	0.76
α_L	5.759	0.495	1472	127	37.53	3 23
α_T	5 914	0.568	1512	145	38.54	3 70
β_{1+2} (β_H)	3.903	0.387	997	99	25.44	2.52
β_3 (β_L)	1.975	0.115	505	30	12.87	0.75
β_s	1.177	0.076	301	19	7.67	0.49
β_T	7 056	0.535	1803	137	45.98	3 48
γ_T	2 374	0.151	607	39	15.47	0 98
$\beta_s+\gamma_T$	3.551	0.213	908	55	23.14	1.39
$\beta_T-\beta_s$	5.878	0.474	1502	121	38.31	3.09
WS	15 344	1.169	3922	299	100 00	1 95

was the β_s-crystallin at 30.8 min. Two discrete γ-crystallin peaks, the γ_H- and γ_L-crystallins, had retention times of 32.1 and 33.6 min, respectively. The next peak, labelled peak 1, may also be a γ-crystallin of lower M_r, whereas the last peak (peak 2) contains nucleotides ($A_{260\,nm}>A_{280\,nm}$) [13].

The retention times of these crystallins and those of a standard calibration mixture were determined and compared in Table II. The apparent M_r values of the crystallins were calculated from a regression line obtained by the use of calibration proteins and their retention times (Table II). Quantitative estimations of the single crystallins were obtained by peak-area integration. The regression lines obtained from the values for the single crystallins were calculated and are shown in Fig. 2c–j. The crystallins of higher M_r, i.e. HM-, α_L- and $\alpha_T=(HM+\alpha_L)$-crystallins and the β-crystallins of higher M_r (β_{1+2}), showed a significant decrease during foetal development (Fig. 2c, d, e and h), whereas the crystallins of lower M_r, the β_3-, β_s- and γ-crystallins, showed a significant increase (Fig. 2h and j). The β_T-crystallins also increased significantly (Fig. 2g). Only the β_{1-3}-crystallins ($\beta_T-\beta_s$) did not increase significantly (Fig. 2f).

In Fig. 3 the crystallin amounts are expressed as mg per lens. There were significant increases for all components, except for HM-crystallin, which showed an insignificant increase (Fig. 3a–h).

The rates of synthesis of the various crystallins are given in Table III, expressed as μg per month and as percentages. The sum of β_H and β_L-crystallins showed the highest rate of synthesis, followed by that of α_L-crystallins. The

rate of synthesis of γ_T-crystallins was twice that of β_s-crystallin. The latter rate may be qualified as high, because it was produced by a crystallin composed of one or two components [14], whereas that of γ_T is produced by a crystallin composed of at least six or eight different components [3].

DISCUSSION

Lens crystallins are a heterogeneous mixture of a large number of native proteins. The molecular masses of the native forms have previously been determined by low-pressure liquid chromatography [13]. When detergents are added, the crystallins break down to subunits of ca. 20 000 daltons [15]. Frequently sodium docecylsulphate (SDS) electrophoresis has been used to analyse crystallins, but since this creates only subunits from these proteins, the results from SDS-electrophoresis cannot be compared with those from our system (Table II). The molecular masses of crystallins from human, rabbit, bovine, rat and chicken lens have been determined on TSK columns and were independently correlated by low-angle laser light scattering [16]. The latter technique shows the advantage of direct molecular mass determination and the ability to determine the proportions of the crystallins directly on a weight basis by measuring the areas under the elution curve obtained with a differential refractive index indicator. We have compared our present M_r values with those obtained by Bindels et al. [16].

The analysis of lens crystallins by SEC often involves errors that are inherent to the method, because the elution volume of the solute is influenced by secondary phenomena other than size exclusion. This is expressed by the comparatively high standard deviations (S.D.) of the M_r values listed in Table II. The quantitation of the crystallins was performed by peak-area measurements. It is clear from the chromatograms of Fig. 1 that most of the peaks are quite poorly resolved and overlap considerably. This is caused by the complexity of the foetal crystallins and of lens crystallins generally under native conditions. Though the drawing of perpendicular lines may introduce errors, because the peak-area measurements are sensitive to flow-rate changes, we could reproducibly measure the peak areas and the retention times of the various crystallins. The flow-rate was maintained within very narrow limits, and the S.D.'s of the retention times of each crystallin were therefore also low, from 0.05 to 0.23 or 0.50% $\pm$ 0.22% of the t_R (Table II).

The elution patterns of the foetal lenses obtained in Fig. 1 were compared with those reported in the literature [1,2,13,17–23]. Foetal lenses contain crystallins that are recently synthesized and should therefore not be markedly affected by aggregation processes. There is one exception, i.e. HM-crystallin. This crystallin is post-synthetically assembled from foetal α-crystallin and is composed of the same subunits that are found in foetal α-crystallin [1]. Foetal HM-crystallin also contains minute amounts of γ-crystallins [3] and probably

also β-crystallins. HM-crystallin eluting in the void volume (Fig. 1) was also demonstrated in the 240-day foetal lens [21] and in the 2-month-old postnatal human lens [20]. Thomson and Augusteyn [1] demonstrated HM-crystallin in 98, 112, 126 and 280-day foetal lenses, but Ringens et al. [18] could detect it only in older lenses. The HM-crystallins show a rate of protein synthesis of 1–2% (Table III). Though this rate was calculated from a significant decrease (Fig. 2c) [3], it is low, but detectable (Table III). This is in contrast to the observations of Thomson and Augusteyn [1], who found an increase of the proportion of HM-crystallin during development. The α-crystallin of M_r 685 000 daltons (Table II) has a relatively low M_r due to its young age. Ringens et al. [18] also found that the foetal α-crystallin had a lower M_r than adult α-crystallin, which was confirmed by Bessems et al. [22] and by Bindels et al. [16]. The α_L-crystallins show a significant relative decrease during development (Fig. 2d) [3], which was also found by Ringens et al. [18] and Thomson and Augusteyn [1]. The rate of synthesis is high (Fig. 3, Table III), and this shows that foetal α-crystallins are continuously synthesized.

The α_L- and β_{1-3} ($\beta_H+\beta_L$)-crystallins show grossly similar proportions of ca. 35–40% during development. It is interesting to note that the synthesis of α_L and of ($\beta_H+\beta_L$)-crystallins are the same at 38% (Table III). Thomson and Augusteyn [1] also found similar proportions of α-crystallins and of what they call the "intermediate M_r crystallins".

We have separated the low M_r crystallins into β_s-, γ_H- and γ_L-crystallins, as did Siezen et al. [20] and Thomson et al. [2]. Our results agree with those of these authors [20]. The proportional rate of synthesis of γ_T-crystallins is 15.5% (Table III). The proportional rate of synthesis of the low-M_r crystallins ($\beta_s+\gamma_T$) is 23%. This contradicts the results obtained by Thomson and Augusteyn [1] and Thomson et al. [2], who stated that during development the synthesis of γ-crystallins decreases progressively. Our results indicate a significant increase of the γ-crystallin concentration (Figs. 2j and 3h and Table III) [3, 4], and hence a high synthesis rate (Table III). During prenatal development the γ-crystallins are still synthesized. This synthesis ceases at about the fifth or sixth year of life, after which the absolute amounts of γ-crystallins decrease [24]. The absolute amounts of crystallins were all observed to increase significantly during development (Fig. 3a–h). Although there are clear differences in the rates of synthesis (Table III), all crystallins are continuously synthesized in the lens during foetal life.

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