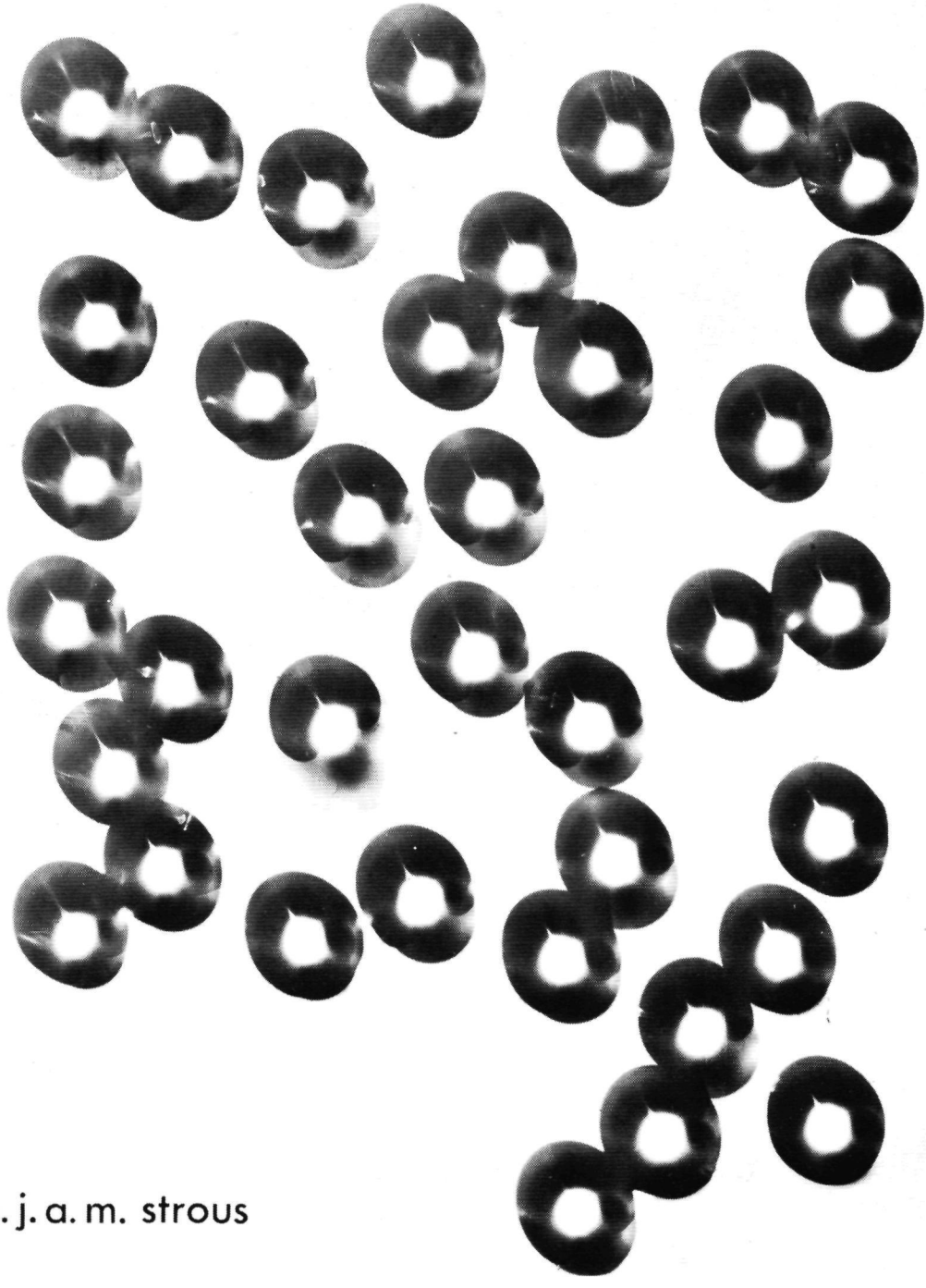
biosynthesis of α-crystallin polypeptides initiation and N-terminal acetylation



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BIOSYNTHESIS OF α-CRYSTALLIN POLYPEPTIDES

INITIATION AND N-TERMINAL ACETYLATION

PROMOTOR:

PROF.DR. H. BLOEMENDAL

BIOSYNTHESIS OF α-CRYSTALLIN POLYPEPTIDES

INITIATION AND N-TERMINAL ACETYLATION

PROEFSCHRIFT

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Omslag : Fotogram van kalfsogen
Ontwerp: Th. Gunsing

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Aan mijn Ouders
Aan Magriet, Marc en Jonas

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CONTENTS

ABBREVIATIONS

ATA	aurintricarboxylic acid
ATP	adenosine-5'-triphosphate
BD-cellulose	benzoylated DEAE-cellulose
DEAE-cellulose	diethylaminoethyl-cellulose
DME	1,2-dimethoxyethane
DNA	deoxyribonucleic acid
DNAase	deoxyribonuclease
DOC	sodium deoxycholate
E.coli	*Escherichia coli*
EDTA	ethylenediamine tetraacetate
EMC	encephalomyocarditis
GTP	guanosine-5'-triphosphate
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
Poly U	polyuridylic acid
RNA	ribonucleic acid
mRNA	messenger RNA
tRNA	transfer RNA
RNAase	ribonuclease
SDS	sodium dodecyl sulphate
TCA	trichloroacetic acid
TEMED	N,N,N,N'tetramethylenediamine
TMV	tobacco mosaic virus
Tris	tris(hydroxymethyl)aminomethane
TYMV	turnip yellow mosaic virus

CHAPTER 1

PROTEIN SYNTHESIS IN EUKARYOTES

Since the literature on protein biosynthesis is so extensive, it would be beyound the scope of this thesis to give a survey of the whole area. Therefore I have confined myself to a short introduction hoping that this will give sufficient background information needed for a better understanding of the experiments described For more detailed reviews on protein biosynthesis I refer e.g. to Lucas-Lenard and Lipmann [95] or Lengyel and Soll [87].

1.1. STRUCTURE AND COMPLEXION OF RIBOSOMES

Since it became clear that protein biosynthesis takes place *in vivo* as well as *in vitro* on ribonucleoprotein particles, termed "ribosomes" [166], these structures have been subjected to intense chemical and physical analysis [112] Ribosomes of prokaryotes can easily be distinguished from those of higher organisms on the base of their sedimentation coefficients, namely 70S and 80S respectively for prokaryotic and cytoplasmic ribosomes of eukaryotes. Ribosomes which are found in chloroplasts and mitochondria resemble bacterial ones with respect to their S-value as well as in their response to certain antibiotics.

Eukaryotic ribosomes are composed of RNA and protein (mass ratio 40 60) and contain 80-90% of the total cellular RNA. The stability of mammalian ribosomes is strongly dependent upon the concentration of cations, especially Mg^{2+}. A magnesium ion concentration of less than 10^{-3} M causes dissociation of the eukaryotic ribosomes into 60S and 40S subunits The RNA components of the ribosomal subunits have been isolated and partly characterized the 40S particle contains an RNA chain of 18S and has a molecular weight of $6x10^5$ daltons, whereas in the 60S subunit two RNA species are present a 28S and a 5S RNA component with molecular weights of $1.5x10^6$ and $4x10^4$

daltons respectively. Presumably also a 7S RNA component is present in eukaryotic ribosomes [7].
The composition of ribosomal protein (total amount 2.0-2.2x10^6 daltons per 80S particle) is rather complex. It has been shown that the 80S ribosomal protein consists of 80-100 components of differing primary structure [49].
Living cells contain ribosomes in structures with different complexities dependent on the activity of the protein synthesizing machinery. In the cytoplasm of fast dividing cells most ribosomes are present in polysomal clusters, whereas a small and rather constant quantity of the ribosomes is found as monosomes or is dissociated into free 40S and 60S particles. It is known that the free subunits are in equilibrium with aggregated subparticles present in polysomes [56, 59]. However, in cells with a low metabolism the amount of free 80S ribosomes is very high as compared with the amount of polysomes. At the moment this phenomenon as well as many other questions concerning the ribosomal cycle are still unsettled.

1.2. MECHANISM OF PROTEIN SYNTHESIS

Proteins consist of one or more polypeptide chains each of which is a polymer of L-amino acids. RNA molecules designated as messenger RNA are the intermediate carriers of information from DNA to protein. In the process of protein biosynthesis the amino acids are activated by specific aminoacyl-tRNA synthetases and attached to specific transfer RNA molecules. The energy for this process is supplied by the hydrolysis of ATP. The aminoacyl-tRNA is bound to the ribosome by base pairing between a specific triplet of nucleotide bases in the aminoacyl-tRNA (anticodon) and a complementary triplet on the messenger RNA (codon). As far as it is known the ribosome per se does not contribute to the specificity of protein synthesis. However, ribosomes might be a target for translational control mechanism [46, 84, 167].

1.2.1. **peptide chain initiation**

Initiation occurs near the 5′ end of mRNA. During translation the ribosomes travel toward the 3′ end at the same time synthesizing a polypeptide from the amino to the carboxyl terminus.

In the process of peptide chain initiation a trigger mechanism seems to be operative. Whereas chain elongation is a repeating process of amino acid addition, probably with a constant velocity, peptide chain initiation is dependent on a delicate mechanism in which initiation factors, a specific tRNA, the ribosomal subunits and an mRNA molecule are involved. At this level regulation of protein biosynthesis has been demonstrated [38, 57, 76, 84, 129, 167].

Peptide chain initiation appears to be essentially the same in prokaryotes as in eukaryotes. As far as it is known all cells contain two species of tRNA, specific for methionine. One of these Met-tRNAs can be formylated by *E.coli* transformylase. This species, designated as $tRNA^{fMet}$, is the initiator tRNA. The other species ($tRNA^{Met}$) is used to insert the methionine residue into internal positions of the peptide chain; this Met-tRNA cannot be formylated enzymatically. In bacteria formylation of the Met-$tRNA^{fMet}$ seems to be an absolute requirement for correct peptide initiation. Eukaryotic cells do not contain cytoplasmic transformylase. However, chain initiation in eukaryotes can function with formylated as well as unformylated Met-$tRNA^{fMet}$. The unique role of $tRNA^{fMet}$ as initiator has not yet been related to the known nucleotide sequences of the molecule [33, 145, 146].

In the binding of the initiator tRNA to the 40S ribosomal subunit several protein factors are involved (initiation factors IF_1, IF_2 and IF_3 in *E.coli*; $IF\text{-}E_1$, $IF\text{-}E_2$ and $IF\text{-}E_3$ in eukaryotes *). The exact order in which the initiation complex is formed in eukaryotes is still unclear. Investigations with purified initiation factors, isolated from reticulocytes [34, 35, 121, 141, 142, 143, 144] or brineshrimp eggs [104, 172] suggest that the first step in initiation is the binding of mRNA to the 40S subunit; this requires protein factor $IF\text{-}E_3$. Met-$tRNA^{fMet}$ joins this complex, requiring factors $IF\text{-}E_1$ and $IF\text{-}E_2$, and GTP, whereafter the 60S subunit is bound. The energy is supplied by the hydrolysis of the GTP. This hydrolysis occurs during or after the binding of the 60S particle. However, more recent observations suggest that the first step of initiation is the formation of a Met-$tRNA^{fMet}$-40S complex [38, 41, 89, 138]; this mRNA independent binding implies a mechanism of polypeptide

*Meanwhile 5-6 protein factors, active in eukaryotic peptide chain initiation have been purified [3].

chain initiation fundamentally different from the mechanism found in bacterial systems.

The codon AUG is the initiation signal (or part of it) in the mRNA and is recognized by the anticodon of $tRNA^{fMet}$. The binding site of Met-$tRNA^{fMet}$ on the ribosome is the peptidyl or P-site. This is confirmed by experiments with puromycin, an analogue of the aminoacyl-adenosine terminus of aminoacyl-tRNA [88]. Only an aminoacyl- or peptidyl-tRNA, bound to the P-site, can react with puromycin. Puromycin can be linked by a peptide bond to the carboxyl end of the methionine residue attached to the initiator tRNA. In contrast Met-$tRNA^{Met}$, inserted in the acceptor or A-site, does not react with puromycin.

Attempts to find a modified initiator tRNA in eukaryotes, analogous to the formylated Met-$tRNA^{fMet}$ in bacterial systems have been unsuccessful [71].

1.2.2. **peptide chain elongation**

Once the 80S complex composed of the 40S subparticle, mRNA, Met-$tRNA^{fMet}$ and the 60S subunit has formed, the machinery is primed for the growth of the polypeptide by a one-by-one addition of amino acid residues. During the growth of a polypeptide chain on a ribosome, the tRNA carrying that peptide, may be located on one of the two binding sites on (or in) the ribosome. The process of peptide chain elongation is GTP dependent and involves three different steps.

1. The formation of a peptide bond between the amino group of the amino acid residue linked to the tRNA on the A-site and the carboxyl end of the peptide linked to the tRNA on the P-site of the ribosome. This reaction, which does not require energy or the assistance of any supernatant factor, is catalyzed by the enzyme peptidyl transferase, which is an integral part of the large ribosomal subunit.
2. The translocation reaction during which the elongated peptide (linked to tRNA) is shifted back from the A-site to the P-site. The deacylated tRNA present on the P-site is removed and the ribosome moves towards the 3′ end of the messenger by one codon shift. This step is dependent on hydrolysis of GTP and catalyzed by transferase II (T_2), an enzyme associated with the 60S subunit.

3. The aminoacyl-tRNA binding reaction which requires the complex transferase I (T_1) (separatable into three components with similar biological activities). The enzymic binding of aminoacyl-tRNA is also dependent on GTP hydrolysis

1.2.3. peptide chain termination

After completion of the polypeptide chain a signal on the mRNA causes the release of the newly synthesized polypeptide and the dissociation of the mRNA-ribosome complex. Protein factors catalyzing the release of completed polypeptides from ribosome-bound peptidyl-tRNA in response to the terminating codons UAG, UAA and UGA have been discovered in mammalian cells. In bacterial systems three of such factors termed R_1, R_2 and S are found They may function in association with GTP. So far no fundamental differences in termination mechanism between prokaryotic and eukaryotic systems have been discovered. A complex of termination factors, isolated from mammalian cells, combine all of the three functions of the factors found in bacteria, moreover the involvement of peptidyl transferase in peptide release is strongly suggested This is shown by experiments with antibiotics which inhibit peptidyl transferase activity as well as peptide release [140].
After polypeptide chain termination the individual components (mRNA, deacylated tRNA, and the ribosomal subparticles) become dissociated and can be re-utilized in the process of protein synthesis.

1.3. POSTTRANSLATIONAL MODIFICATIONS

Protein synthesis is not exclusively restricted to the translation of mRNA into a polypeptide chain. Sometimes a kind of finishing touch is needed in order to enable the protein to function properly There are many kinds of modifications with particular specificities Examples of posttranslational modifications are

- cleavage of precursor polypeptide chains into the native proteins, e.g. EMC viral proteins [25, 147], the light chain of mouse myeloma [96, 159], insulin [151], trypsin [37], collagen [20]

- addition of various groups, e.g. lipids [132], carbohydrates [169] or amino acids [78]
- modification of individual amino acid residues, e.g. by N-α-acetylation [22, 23, 90, 150], N-ε-acetylation [26, 90, 94], hydroxylation [163] or phosphorylation [74, 94].

In most cases not only the mechanism of the modification is unclear but also the significance of modification is poorly understood.

There is one posttranslational or rather postinitiational modification which is inherent in the initiation process: The removal of the N-terminal methionine residue from the growing peptide chain. The methionine residue, donated by the initiator tRNA, is usually removed during chain elongation by a specific ribosome-bound aminopeptidase. In eukaryotes only a few proteins are known which carry an N-terminal methionine residue e.g. haemoglobin β-chains from sheep [21], lens α-crystallin [66] or skeletal muscle phosphorylase [77].

In this thesis especially the initiation process *in vitro* of eye lens α-crystallin is described. One may ask why we have chosen such an unusual system to examine the process of initiation whereas the same process has already been investigated in several other systems like rabbit reticulocytes [12, 29, 34, 35, 36, 53, 70, 73, 171], ascites cells [147], Hela cells [30], wheat germ embryos [86, 99, 162] and *Artemia salina* [104, 172].

There are two main reasons:

Firstly, this study may contribute to our knowledge concerning the universality of the initiation mechanism. So far the process of protein initiation appears to have a universal character; in prokaryotes as well as in eukaryotic systems the initiation of protein synthesis takes place according to an essentially similar mechanism with almost identical requirements.

Secondly, the adult eye lens synthesizes mainly α-crystallin, a well-characterized protein [13]. This protein has interesting features for the study of the initiation mechanism because it is one of the few proteins with a methionine residue in N-terminal position. Moreover this methionine is N-acetylated.

In this thesis several stages in the synthesis *in vitro* of α-crystallin are described [154, 156, 157]. The results confirm the universal character of the initiation mechanism.

CHAPTER 2

ISOLATION AND CHARACTERIZATION OF LENS METHIONYL-tRNA SPECIES

2 1 INTRODUCTION

Initiation of protein synthesis in a prokaryotic system involves N-formyl-methionyl-tRNA^{fMet} [31, 98] In eukaryotic systems the presence of at least two Met-tRNA species has been demonstrated [27, 71, 80, 85, 86, 124, 148, 160] One of the Met-tRNA species from eukaryotes can be recognized by both the homologous methionyl-tRNA synthetase and the synthetase of *E coli* [53, 86, 124, 148, 160] This tRNA, isolated from yeast or mammalian sources, can also be formylated by methionyl-tRNA transformylase present in bacteria [27, 124, 148, 160] With the exception of yeast tRNA^{Met} no other tRNA^{Met} species can be aminoacylated with the *E.coli* synthetase tRNA^{fMet} has been demonstrated to respond to the initiator codons AUG or GUG located near the 5′ end of synthetic polynucleotides [24, 124] and of globin mRNA [34, 35] This is true for formylated as well as unformylated initiator tRNA, either homologous [71] or heterologous (yeast [70] or liver [12]), whereas for the initiation in prokaryotes a formylated methionyl-tRNA is a prerequisite [48] On the other hand tRNA^{Met} has been shown to donate methionine only into internal positions of growing peptides in response to the AUG triplet [53, 148]. These results as well as studies on the enzymic binding of both tRNA species to ribosomes [80, 106, 138] lead to the concept that only tRNA^{fMet} can function as chain initiator in eukaryotic cells whereas tRNA^{Met} is utilized in the elongation of peptide chains

However, the mechanism for preferential recognition of the different codon positions in eukaryotic protein synthesis is unclear Recently evidence has been provided that specific recognition of the Met-tRNA^{fMet} by a ribosome-bound protein factor leads to complex formation in the presence of GTP [138] This ternary complex can react with the 40S ribosomal subunit and mRNA, joining of the 60S subparticle completes the initiation stage This ribosome bound factor does not recognize Met-tRNA^{Met} [80, 106, 162] The

reverse holds for transfer factor T_1, operative in chain elongation, its affinity for Met-tRNA^{Met} is much higher than for Met-tRNA^{fMet} [53, 80, 106, 162] Apparently this model is not absolutely valid in cell-free systems From current studies it becomes clear that under certain conditions Met-tRNA^{fMet} is frequently used for chain elongation [29, 42, 43, 44, 113, 155] (see also section 4.6.).

In this thesis an account will be given of a number of stages in the biosynthesis of α-crystallin In order to study the processes of chain initiation, chain elongation and N-terminal acetylation, it will be necessary to follow the fate of the methionine residues donated to the polypeptide by the different Met-tRNA species This requires the separation of the two tRNA species

In this chapter the isolation and separation of tRNAs specific for methionine from eye lens will be discussed Evidence is provided that the eye lens contains Met-tRNA molecules with similar properties as Met-tRNA preparations from other mammalian cells

2 2 METHODS

2.2 1. **preparation of calf lens tRNA**

Calf eyes were obtained from the slaughterhouse, cooled on ice and transported to the laboratory All further handling was performed at 0-4°C After isolation of the lenses, the capsules containing the epithelial cells and the outer cortices were collected Homogenization of the combined material was carried out with the aid of a teflon homogenizer (2000 rev /min, clearence ± 500μ) in a medium containing 0 005 M magnesium acetate, 0.025 M KCl, 0.05 M Tris-Cl, pH 7 4 and 0 35 M sucrose The homogenate was filtered through 4 layers of cheese cloth and after addition of DOC to a final concentration of 0 5% the filtrate was layered onto a discontinuous sucrose gradient according to the procedure of Bloemendal et al [16] and centrifuged at 78,000 g for 16 hours at 2°C in a rotor 30 of a Spinco preparative ultracentrifuge After centrifugation the supernatant was shaken with one volume of water-saturated phenol in the presence of 1% SDS for 30 min at room temperature The phenol layer was separated from the water layer by centrifugation in a GSA rotor of a Sorvall RC2-B preparative centrifuge To the

water layer again one volume of 80% phenol was added and the extraction was repeated. Then the aqueous layers were combined and the RNA was precipitated with 2.5 volumes of ethanol and 0.1 volume of 2 M potassium acetate, pH 5.0 for 16 hours at -20°C. The precipitate was collected by centrifugation at 4500 g for 20 min in a Martin Christ junior centrifuge and dissolved in a medium containing 0.1 M Tris-Cl, pH 7 4 and 0 15 M NaCl. The solution was stirred with a slurry of DEAE Sephadex A-50 equilibrated in the same medium. The slurry was poured in a column and the column was washed with the starting buffer until no material absorbing at 260 nm could be eluted. Then tRNA was eluted with a buffer containing 0.1 M Tris-Cl, pH 7.4 and 1 M NaCl, precipitated with ethanol (-20°C) and kept overnight at -20°C. Following this procedure about 3 mg of crude tRNA per 1000 eyes was obtained. Transfer RNA concentrations were measured at 260 nm assuming that 1 mg tRNA corresponds with 25 O.D units.

2.2.2. benzoylated DEAE-cellulose chromatography of crude lens tRNA [55]

Crude tRNA (12-16 mg) dissolved in 2 ml of water was applied to a 2.5x25 cm column of BD-cellulose at a flow rate of 15 ml/h. The column was pre-equilibrated with a buffer containing 0.01 M magnesium acetate, 0.005 M 2-mercaptoethanol and 0 3 M NaCl and was operated at 2°C. The tRNAs were eluted with a linear gradient of 300 ml of 0.3 M and 0.7 M NaCl in the presence of 0 01 M magnesium acetate and 0.005 M 2-mercaptoethanol. The effluent was monitored at 260 nm and collected in 2.5 ml fractions Each fraction was essayed for methionine acceptor activity under the following conditions 0.05 M Tris-Cl, pH 7.4, 0.05 M KCl, 0.01 M $MgCl_2$, 0 01 M ATP, 0.005 M-2-mercaptoethanol, 10^{-5} M L-(^{14}C)methionine (spec. act 52 mCi/mmol), 0.1 ml of each column fraction and 0.05 ml of an enzyme fraction (described below) containing aminoacyl-tRNA synthetases from rat liver, in a final volume of 0 25 ml. Then 3 ml of cold 5% TCA was added, the precipitate was collected on glass fiber filters (Whatman GF/c) and washed with 5% TCA. The filters were dried and the amount of radioactivity was determined in a Packard liquid scintillation counter using a toluene based scintillator. The counting efficiency was 75%. Fractions of tRNA capable to accept me-

thionine were pooled and the tRNA was precipitated with ethanol and stored at -20°C.

2.2.3. **aminoacyl-tRNA synthetases from rat liver**

Livers from 2-3 month-old Wistar albino rats were used to prepare the aminoacyl-tRNA synthetases In order to avoid contamination of the preparations by liver glycogen, the animals were starved for 24 hours before sacrificing. The animals were killed by decapitation, the livers were quickly excised and immersed in two volumes of an ice-cold medium containing 0.05 M Tris-Cl, pH 7.4, 0.025 M KCl, 0.005 M magnesium acetate and 0.005 M 2-mercaptoethanol (medium A) Then the livers were minced with scissors and homogenized using a teflon homogenizer. Cell debris, cell organelles and ribosomes were sedimented at 2°C in a Spinco Ti-50 rotor at 220,000 g for 3 hours To the upper two third of the supernatant 2 M KCl was added to a final concentration of 0.25 M and the solution was passed through a 1.5x20 cm column of DEAE Sephadex A-50, equilibrated with buffer A containing 0.25 M KCl The red colored fractions were collected, dialyzed twice against two liters of medium A, frozen in small portions and stored at -70°C

2 2.4. **aminoacyl-tRNA synthetases from** *E.coli*

E.coli B cells (± 20 g) were ground at 2°C in a mortar with twice their weight of alumina (Alcoa A-305). The disrupted cells were extracted with one volume of medium A, the suspension was incubated with DNAase (0.2 μg/ml, RNAase-free) for 5 min at 37°C and the alumina and cell debris were removed by centrifugation in a Sorvall RC-2B centrifuge at 15,000 rpm for 15 min at 2°C. The supernatant was centrifuged in a Spinco preparative centrifuge at 220,000 g for three hours. Aminoacyl-tRNA synthetases were prepared from this supernatant as described for the aminoacyl-tRNA synthetases from rat liver. This enzyme preparation also contained the transformylase activity

2.2.5. acylation of tRNA fractions with (^{35}S)methionine

The tRNA, fractionated on the BD-cellulose column was charged with (^{35}S)methionine (spec. act. 20-30 Ci/mmol). Incubation was performed in a total volume of 0.5 ml containing 1-2 mg tRNA, 0.01 M ATP, 0.05 M Tris-Cl, pH 7.4, 0.05 M KCl, 0.01 M magnesium acetate, 0.05 mCi (^{35}S)methionine and 0.1 ml of crude aminoacyl-tRNA synthetases either from rat liver or from *E.coli*. After incubation 0.1 volume of 2 M potassium acetate, pH 5.0 was added. The reaction mixture was extracted with water-saturated phenol and after separation of the two layers the phenol-layer was extracted with one volume of 0.1 M potassium acetate, pH 5.0. The aqueous layers were combined and 2.5 volumes of 96% ethanol containing 5% 2 M potassium acetate, pH 5.0 were added. After 24 hours at -20°C, the precipitate was collected by centrifugation and washed three times with cold ethanol. The ethanol was removed by flushing with nitrogen, the pellet was dissolved in 0.3 ml of 0.005 M potassium acetate, pH 5.0 and tRNA was stored at -20°C. Under these conditions the tRNA contained 5000-30,000 cpm/μg.
In cases that formylation of the (^{35}S)Met-tRNA was required the incubation was performed as described above with an *E.coli* synthetases preparation except that 2×10^{-4} M leucovorin was added as a formyl donor. To determine the percentage of formylation 1 μl of N-formyl-(^{35}S)Met-tRNA was treated with 0.1 M KOH for 15 min at 37°C and then analyzed by descending paper chromatography on Whatman nr 1 in n-butanol-water-acetic acid (90:25:10, by volume). One centimeter strips were cut out from the chromatogram and counted in a liquid scintillation counter. Unlabeled methionine and formylmethionine were co-chromatographed and stained with platinic iodide [45].

2.2.6. T_1 RNAase digestion

Digestion of tRNA charged with (^{35}S)methionine was carried out in a total volume of 5 μl containing 0.1 M EDTA, 0.01 M potassium acetate, pH 5.2 and 5 μg T_1 RNAase. The solution was incubated in a sealed capillary tube at 37°C for 20 min. The incubation mixture was immediately spotted on Whatman 3 MM paper and electrophoresis was performed at pH 3.8 for 3 hours at 35 V/cm in a Savant electrophoresis tank cooled with Varsol. The electro-

pherogram was dried and autoradiographed using a Kodak X-ray film (type RHP Royal X-omat) for 24 hours. Paper strips containing too little radioactivity to be autoradiographed were cut into 1 cm pieces and counted in a liquid scintillation counter using a toluene based scintillator.

2.2.7. binding of Met-tRNA species to ribosomes in the presence of ApUpG

The essays were carried out following the procedure of Nirenberg and Leder [111]. The binding reaction mixtures contained in a total volume of 0.04 ml 1.0 A_{260} units of washed *E.coli* ribosomes, 0.05 A_{260} units of ApUpG, 0.1 M Tris-Cl, pH 7.5, 0.05 M KCl, 0.005 M 2-mercaptoethanol and 0.5 pmol of either (^{35}S)Met-tRNA$_I$, or formyl-(^{35}S)Met-tRNA$_I$, or (^{35}S)Met-tRNA$_{II}$, or (^{35}S)Met-tRNA$_{III}$ (1 pmol = 40,000 cpm). The components were allowed to react at 37°C for 8 min at the Mg^{2+} concentration indicated. After incubation the mixtures were diluted with 3 ml of ice-cold incubation medium (having the same ion concentrations as the reaction mixtures) and filtered through a nitrocellulose membrane. The filters were washed 3 times with 3 ml of buffer, dried and the radioactivity was determined in a liquid scintillation counter.

2.3. SEPARATION OF LENS tRNAs CODING FOR METHIONINE

Chromatography of tRNA isolated from bovine eye lens on benzoylated DEAE-cellulose results in a clear separation of three fractions with methionine acceptor activity, until further notice designated as tRNA$_I$, tRNA$_{II}$ and tRNA$_{III}$ (fig. 1).

The three species are detected when the column fractions are assayed for methionine acceptor activity with crude liver aminoacyl-tRNA synthetases. However, only one fraction (tRNA$_I$) can also be acylated with methionyl-tRNA synthetase isolated from *E.coli*. The three fractions (1-2 mg each) are pooled separately and precipitated with ethanol. Transfer RNA$_I$ is esterified with (^{35}S)methionine using *E.coli* aminoacyl-tRNA synthetases, tRNA fraction II and III are charged in the presence of rat liver synthetases. The (^{35}S)Met-tRNAs are deproteinized and recovered. The radioactivity of Met-

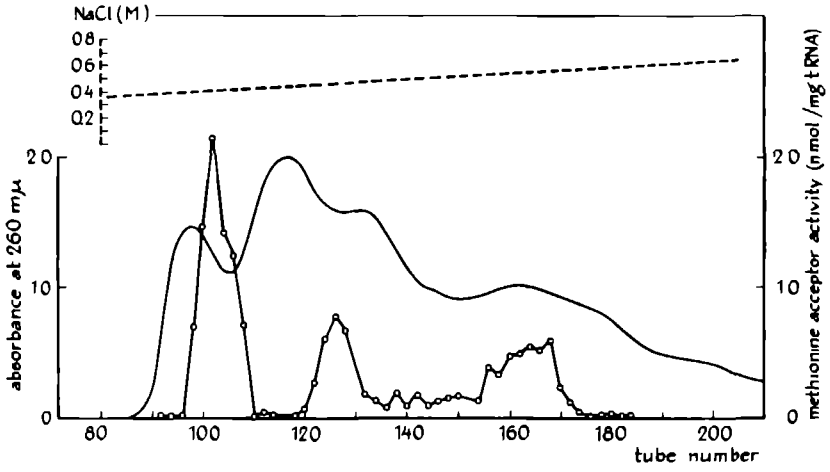

Fig.1. Chromatography of crude eye lens tRNA on BD-cellulose. The column was loaded with 15 mg of tRNA and was developed at 2°C as described in section 2.2.2. ——— absorbance at 260 nm, o——o methionine acceptor activity as assayed with crude rat liver aminoacyl-tRNA synthetases. Fractions 95-110 correspond to $tRNA_I$, fractions 120-135 to $tRNA_{II}$ and fractions 145-170 to $tRNA_{III}$.

$tRNA_I$ amounts 800,000 cpm per A_{260} unit and of Met-$tRNA_{II}$ and Met-$tRNA_{III}$ 200,000 cpm per A_{260} unit.

In order to further identify the tRNA species 1 μl samples of the different (^{35}S)Met-tRNAs are subjected to digestion with T_1 RNAase and the products are analyzed by paper electrophoresis.

Fig. 2 shows the distribution of radioactivity of the (^{35}S)methionyl-oligonucleotides after electrophoresis. The analysis of the (^{35}S)Met-oligonucleotide derived from Met-$tRNA_I$ is shown in C; lanes D and E represent the patterns of the T_1 RNAase digests of (^{35}S)Met-$tRNA_{II}$ and (^{35}S)Met-$tRNA_{III}$. It can be seen that the (^{35}S)Met-$tRNA_I$ preparation is not contaminated with (^{35}S)Met-$tRNA_{II}$ or (^{35}S)Met-$tRNA_{III}$. This confirms the finding of others that *E.coli* Met-tRNA synthetases are capable to esterify only one of the mammalian cytoplasmic $tRNA^{Met}$ species [53, 124, 160]. If the $tRNA_I$ fraction is methionylated with rat liver synthetases, analysis of the T_1 RNAase products reveals contamination with minor amounts of Met-$tRNA_{II}$ and/or Met-$tRNA_{III}$ (not shown).

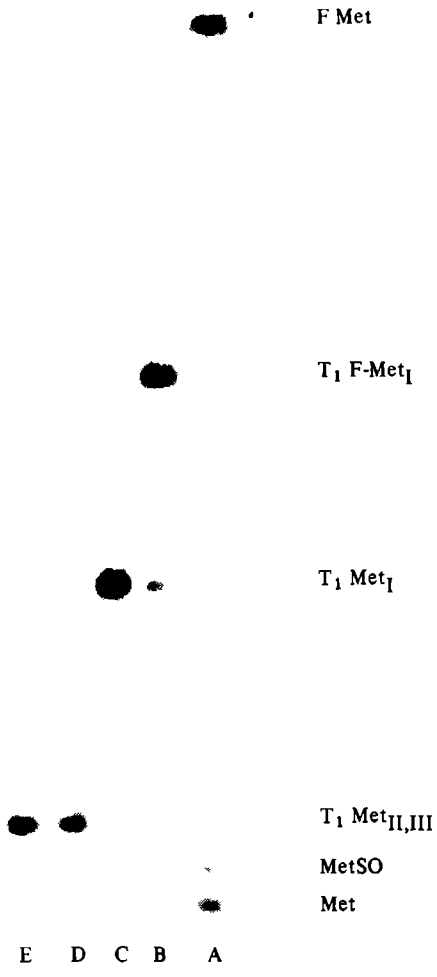

Fig 2. Electrophoresis of (^{35}S)methionine labeled T_1 RNAase digests The (^{35}S)methionyl-oligonucleotide fragments derived from different lens tRNA species were separated by high voltage paper electrophoresis at pH 3 8 as described in section 2 2 6. Oxidized methionine is designated as MetSO, f-MetSO is not shown. The zones marking the methionyl-oligonucleotides, derived from the different (^{35}S)Met-tRNAs are indicated in the picture Autoradiography was performed for 24 hours

Further it is clear that at the 3′ end the $tRNA_{II}$ and $tRNA_{III}$ have a number of nucleotide sequences in common. Moreover it is apparent that these two tRNA species contain only a very small amount of the Met-$tRNA_I$ fraction.
Upon aminoacylation of the Met-$tRNA_I$ species in the presence of the formyl donor leucovorin using crude *E.coli* aminoacyl-tRNA synthetases the Met-$tRNA_I$ can be formylated completely (the *E.coli* synthetase preparation contains the enzyme Met-tRNA-transformylase). Fig. 2B shows the analysis of the T_1 RNAase digest of the formylated (^{35}S)Met-$tRNA_I$; this preparation apparently contains still some non-formylated Met-$tRNA_I$. In fig. 2A the radioactivity pattern of the formyl-(^{35}S)Met-$tRNA_I$ species after alkaline hydrolysis of the formyl-methionine from the tRNA is shown. The radioactivity co-migrates with chemically synthesized F-Met. It has been impossible to formylate Met-$tRNA_{II}$ as well as Met-$tRNA_{III}$ in the presence of *E.coli* transformylase.

2.4. CHARACTERIZATION OF LENS METHIONYL-tRNA SPECIES

Studies with cell-free systems derived from *E.coli* [54, 131], wheat germ [162] and L-cells [88] have shown that the initiator (Met-$tRNA^{fMet}$) binds better to ribosomes at low Mg^{2+} concentrations than the non-initiating Met-$tRNA^{Met}$. In table I the effect of different magnesium ion concentrations on the binding of (^{35}S)Met-tRNA species from lens to *E.coli* ribosomes in the presence of the triplet nucleotide ApUpG is depicted.

Mg^{2+} conc (mM)	Met $tRNA_I$ (cpm)		F Met $tRNA_I$ (cpm)		Met $tRNA_{II}$ (cpm)		Met $tRNA_{III}$ (cpm)	
	+AUG	−AUG	+AUG	AUG	+AUG	−AUG	+AUG	AUG
5	35	50	305	290	45	60	130	160
8	365	40	630	300	55	55	210	240
12	2100	45	2230	490	200	65	340	260
15	3315	215	4630	910	680	440	630	310
20	4010	685	4965	1600	1465	570	1200	365

Table I Binding of eye lens methionyl tRNA species to ribosomes in the presence of ApUpG

It is obvious that Met-tRNA$_I$ and formyl-Met-tRNA$_I$ do bind much more efficiently than Met-tRNA$_{II}$ and Met-tRNA$_{III}$. The difference is especially significant at the lower magnesium ion concentrations. It appears further that the ability to bind Met-tRNA$_I$ is not affected by formylation of the NH_2 group of methionine.

The magnesium ion concentration dependence of the acylation reaction for the three tRNA fractions is shown in fig. 3.

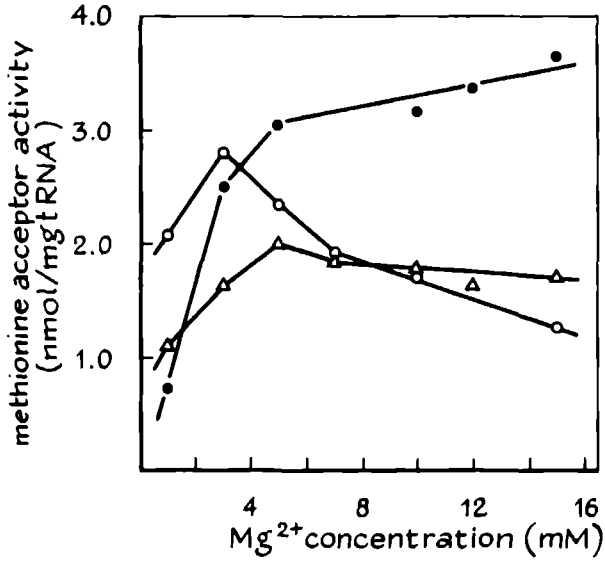

Fig.3. Effect of Mg^{2+} concentration on the acylation reaction. The incubations contained in a total volume of 0.15 ml 0.004 M ATP, 0.04 M Tris-Cl, pH 7.4, 0.05 M KCl, 1.3×10^{-5} M (^{14}C)methionine, 50-150 μg crude aminoacyl-tRNA synthetases from rat liver or *E.coli* and 10 μg eye lens tRNA. The incubations were performed at 37°C for 15 min. o—o—o methionine acceptor activity of tRNA$_I$ essayed with rat liver synthetase; •—•—• methionine acceptor activity of tRNA$_I$ essayed with *E.coli* synthetase and △—△—△ methionine acceptor activity of tRNA$_{II}$ and tRNA$_{III}$ essayed with liver synthetase.

It can be seen that the aminoacylation of tRNA$_I$ using aminoacyl-tRNA synthetases from different sources occurs to nearly the same extent, but the

optimum is found at different Mg^{2+} concentrations. Using rat liver synthetases the characteristics of methionylation of $tRNA_I$ on the one hand and $tRNA_{II}$ and $tRNA_{III}$ on the other hand are also different; aminoacylation of $tRNA_I$ shows a discrete optimal magnesium ion concentration (3 mM), while the Mg^{2+} concentration for the acceptor activity of $tRNA_{II}$ and $tRNA_{III}$ does not seem to be critical at these concentrations.

2.5. DISCUSSION

The present results demonstrate that three chromatographically distinct methionyl-tRNAs can be found in the bovine eye lens, Met-$tRNA_I$, Met-$tRNA_{II}$ and Met-$tRNA_{III}$, the latter two having very similar biological properties. Met-$tRNA_I$ behaves in a different way and resembles initiator tRNA from other mammalian tissues.

In view of the similarities between the tRNA species with methionine acceptor activity isolated from lens and other mammalian systems, it is justified to designate the different lens tRNA species in accordance with the general accepted nomenclature, viz. $tRNA_I$ as $tRNA^{fMet}$, $tRNA_{II}$ as $tRNA_1^{Met}$ and $tRNA_{III}$ as $tRNA_2^{Met}$ [72].

The definite proof for the specificities of the different Met-tRNA species will be given in the chapters 3 and 4 of this thesis.

In experiments on the initiation of protein synthesis in eukaryotic systems formylated (^{35}S)Met-$tRNA^{fMet}$ has often been used to label the N-termini of newly initiated proteins [70, 107, 113, 147]. Hence it is worthwhile to point out that, although Met-$tRNA^{fMet}$ does not occur in a formylated form in the cytoplasm of eukaryotes, formyl-Met-$tRNA^{fMet}$ can function as initiator in cell-free systems derived from higher organisms [71]. Labeling of proteins using F-(^{35}S)Met-$tRNA^{fMet}$ has the advantage that the N-terminal methionine cannot be removed from the polypeptide by the methionine splitting aminopeptidase, because the enzyme deformylase –naturally occurring in prokaryotes– is not present in the cytoplasm of eukaryotes [70].

The method described in this chapter for the isolation and separation of the different methionyl-tRNAs can easily by applied for the preparation of similar tRNA species from other tissues.

CHAPTER 3

CHARACTERIZATION OF A CELL-FREE SYSTEM DERIVED FROM EYE LENS CELLS

3.1. INTRODUCTION

The elucidation of biochemical reaction mechanisms is greatly favored by the use of cell-free systems. In contrast to organ culture or intact cells, in cell-free systems the number of variable parameters can be reduced and some of them can be changed. A fairly crude system is sometimes satisfactory for the verification of a particular hypothesis.

In this chapter the preparation of a crude cell-free system derived from calf lens tissue is described. The requirements for this system are:

1. The cell-free system should be capable to initiate the synthesis of new polypeptides.
2. The protein synthesizing machinery should keep functioning for a certain period of time as it did before cell rupture. This means that the same proteins should be synthesized in the same relative amounts.

The first requirement is very important as we want to investigate the initiation and elongation of α-crystallin, a major eye lens protein which has been well characterized [13]. Experiments to be described in this chapter will elucidate whether or not these two requirements have been met.

3.2. THE CHEMISTRY OF EYE LENS PROTEINS

For the identification of polypeptide chains synthesized *in vitro* it is necessary to know a number of characteristics of the native proteins. It has been shown in calf lenses that both epithelial and cortex fiber cells are highly specialized in synthesizing crystallins, especially α-crystallin.

We shall restrict ourselves chiefly to the description of this protein. α-Crystallin is a protein (average molecular weight 800,000 daltons) which is composed of aggregated polypeptide chains [15]. Four different polypeptides occur:

two acidic (αA_1 and αA_2) and two basic (αB_1 and αB_2) chains [133, 136 165]. The amounts in which they are present in the aggregates decrease in the order αA_2, αB_2, αA_1 and αB_1 [15]. The αA_1 chain is almost identical to αA_2 as has been shown by fingerprinting techniques [116]. It has been postulated that the αA_2 polypeptide is a precursor of the αA_1 chain [14].

The molecular weights of αA_1 and αA_2 are approximately 19,000, while the molecular weight of αB_1 and αB_2 is estimated at 22,000 by SDS gel electrophoresis and several other techniques [4].

The α-crystallin polypeptides can be characterized on the base of charge by electrophoresis on urea gels [134, 136]. Electrophoresis on acidic urea gels resolves the polypeptides of α-crystallin into two groups of polypeptides, αA and αB, whereas electrophoresis on basic urea gels resolves all four polypeptides. In fig. 4 the electrophoretic behavior of the α-crystallin polypeptides under different circumstances is given schematically.

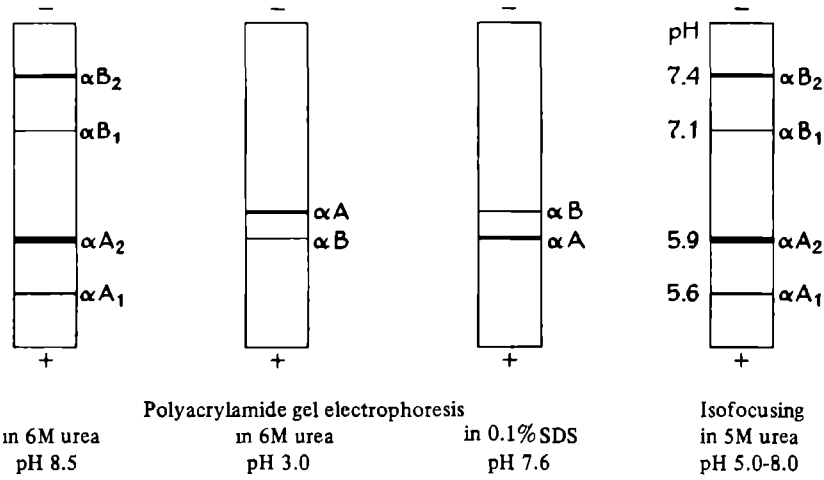

Fig.4. Electrophoretic behavior of α-crystallin polypeptides.

Recently the complete amino acid sequence of the αA_2 polypeptide chain has been elucidated [116]. Most interesting for our study is the N-terminal sequence, which is identical for the four polypeptide chains, namely N-acetyl-Met-Asp-Ile-Ala [67] Specific cleavage of the N-terminal peptide of α-crystallin polypeptides can be obtained after proteolytic digestion with different enzymes (table II).

ENZYME	AMINO ACID SEQUENCE	REFERENCES
pronase	acetyl-Met-Asp	[66]
subtilisin	acetyl-Met-Asp-Ile-Ala	[67]
thermolysin	acetyl-Met-Asp	[32]
trypsin (only for α A)	acetyl-Met-Asp-Ile-Ala-Ile-Gln--His-Pro-Trp-Phe-Lys	[32]

Table II

In the αA_2 chains two methionine residues are present. After aminoethylation of the cysteine residue present in the αA_2 chains, trypsin releases two methionine-containing peptides which can be identified by paper chromatography [116].

The other water-soluble structural lens proteins (β- and γ- crystallins) have not been so well characterized The molecular weights of their polypeptide chains are in the region of 25,000. The β-crystallins are most probably N-acetylated too [105], while the γ-crystallins have a free glycine residue in N-terminal position [19]. Native β-crystallin can be found in aggregates up to 200,000 daltons (β-high) and 80,000 daltons (β-low).

3.3.1. the cell-free system

Fresh calf eyes (40-60 per preparation) were obtained from the city slaughterhouse and transported on ice to the laboratory within 30 min. The epithelial part and outer cortices were collected immediately at 0°C. One volume of cold deionized water was added and homogenization was carried out with a tight fitting teflon homogenizer. The homogenate was centrifuged in a Sorvall RC-2B preparative centrifuge at 15,000 g for 10 min at 2°C. The supernatant (12-15 ml) was carefully collected, frozen in liquid nitrogen in 0.3 ml aliquots and stored at -70°C. Because of the very high protein concentration of the lysate and the presence of slow sedimenting fiber like structures contaminated with iris-derived material, it was difficult to obtain a clear supernatant. Sometimes Nonidet P-40 was added before homogenization in order to dissolve membrane structures. This resulted in a cleaner preparation without affecting the biological activity.

3.3.2. conditions for *in vitro* incubation

Synthesis of lens protein *in vitro* was performed in incubation mixtures containing 1 mM ATP, 0.5 mM GTP, 5 mM 2-mercaptoethanol, 0.01 M creatine phosphate, 50 μg/ml creatine-phosphokinase, 0.01 M Tris-Cl, pH 7.4, 0.050 M KCl and 3 mM magnesium acetate. These concentrations are not corrected for ions and other components already present in the lysate. Depending on the type of experiment either 50 μCi of (^{35}S)methionine plus the 19 remaining unlabeled amino acids, 1.5×10^6 counts/min of one of the (^{35}S)Met-tRNA fractions or 10 μCi of a ^{14}C amino acid mixture supplemented with the 6 missing amino acids (Asp, Cys, Glu, His, Met and Trp at 20 μM each) was added. One ml of incubation mixture contained 0.6 ml lens lysate.
Incubations were performed at 30°C. For measurement of the insoluble radioactivity routinely 0.01 ml samples were taken at zero time and after incubation, diluted with 0.5 ml of 0.1 M KOH and incubated for 15 min at 37°C. Trichloroacetic acid (5%) was added, the precipitate was collected on glass fiber filters and washed with 5% TCA. Then the filters were dried and the

radioactivity was counted in a liquid scintillation counter using a toluene based scintillator. If the reaction mixture was used for further identification e.g. by column chromatography or proteolytic digestion, 0.1 volume of 0.3 M EDTA and pancreatic RNAase (final concentration 0.1 mg/ml) were added and the incubation was continued for 15 min.

3.3.3. conditions for *in vivo* incubation

Lenses were carefully removed from fresh calf eyes without injuring the collagenous capsule, and rapidly immersed in cold Hanks' salt solution. Incubation was performed at 37°C in a label-medium, which contained 1 volume of TC-199, 2 volumes of Hanks' salt solution, 10% serum and a mixture of ^{14}C amino acids (final concentration 1 μCi/ml) supplemented with the 6 missing amino acids (Asp, Cys, Glu, His, Met, Trp).
After 4 hours of incubation the lenses were washed with cold Hanks medium, the capsules, to which the epithelial cells tightly adhere, were removed and the outer cortices were collected and homogenized; the insoluble material was removed by centrifugation. The protein solution was dialyzed and lyphilized.

3.3.4. fractionation of the eye lens proteins

The water-soluble structural eye lens proteins can be separated into four fractions namely α-, β-high, β-low and γ-crystallins by gel filtration on Sephadex G-200 [135]. The protein (± 150 mg) dissolved in 2 ml of a medium containing 0.1 M Tris-Cl, pH 7.8, 1 M NaCl and 10^{-4} M EDTA was applied on the column (120x2 cm), equilibrated with the same buffer, and eluted at a flow rate of 10 ml/h. The protein content was determined at 280 nm. Fractions of 4 ml were collected; for determination of the radioactivity 0.1 ml fractions were taken and 0.2 mg bovin serum albumin was added as carrier. The protein was precipitated with 5% TCA, collected on glass fiber filters and the radioactivity was determined with a liquid scintillation counter. The protein fractions were pooled, dialyzed against 2 liters of aqueous 0.01 M 2-mercaptoethanol for 48 hours and lyophilized.

3.3.5. N-terminal peptide analysis

For the determination of the N-terminus of α-crystallin the following proteolytic enzymes have been used: pronase, thermolysin, subtilisin and in the case of pure αA_2 trypsin (see table II, p. 24). For a final identification of the N-terminal di- and tetrapeptide digestion with carboxypeptidase A was performed. Both the dipeptide and the tetrapeptide yielded Ac-Met. The analysis of Ac-Met, the di- and tetrapeptide were routinely performed by high voltage paper electrophoresis at pH 6.5. For this purpose the digests were dissolved in 20-50 μl of distilled water and applied on Whatman 3 MM paper. Electrophoresis was carried out in a water-cooled Savant electrophoresis apparatus at 50 V/cm in acetic acid-pyridine-water (6:200:794, by volume), pH 6.5 for 2 hours. After electrophoresis the paper was dried, cut into 1 cm strips and the radioactivity was counted in a liquid scintillation counter using a toluene based scintillator. Reference peptides were stained for methionine using platinic iodide [45].

- Pronase digestion [66].

The protein was suspended in 1 ml of 0.1 M NH_4HCO_3, pH 8.0 and 0.002 M $CaCl_2$ containing 0.2 mg of pronase. After digestion for 6-24 hours at 37°C the pH was adjusted to 4-5 with acetic acid and the solution was either lyophilized or treated with Dowex-50 (H^+) in order to remove unblocked amino acids and peptides. For this treatment one volume of a Dowex-50 suspension was added to the digest and mixed thoroughly; the Dowex-50 resin was removed by centrifugation and washed with one volume of water. The water layers were combined and lyophylized.

- Subtilisin digestion [67].

Subtilisin digestion was carried out at 37°C in 0.1 M ammonium bicarbonate buffer, pH 8.0 for 6 hours at an enzyme concentration of 0.1 mg/ml. As reference peptide the N-terminal tetrapeptide prepared from native α-crystallin was used.

- Carboxypeptidase A digestion [67].

Digestion with carboxypeptidase A was performed on the N-terminal di- and tetrapeptides of α-crystallin, isolated from the paper electropherograms. One milligram of diisopropyl fluorophosphate-treated carboxypeptidase A was added to 1 ml of 2% NH_4HCO_3 containing the peptides. Digestion was carried out at pH 8.0 and 37°C. The reaction was stopped after 24 hours by lowering the pH to 3 using acetic acid.

3 3 6 polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis was performed on alkaline gels at pH 8 5 in 8 0x0 5 cm cylindrical glass tubes at 2 5 mA/gel for 4 hours. The gels contained 10% acrylamide, 0 2% N,N' methylene-bisacrylamide, 0 006% TEMED, 0 004% potassium ferricyanide, 0 07% ammonium persulphate, 6 M urea, 0 025 M Tris and 0 2 M glycine The electrode buffer contained 0 025 M Tris, 0 2 M glycine, 6 M urea and 1% 2-mercaptoethanol Pre-electrophoresis was carried out at 2 5 mA/gel for one hour Per gel 0 05-0 2 mg protein, dissolved in 0 03 ml electrode buffer containing 10% sucrose, was applied After electrophoresis the gels were stained with Amido Black for 20 min. The Amido Black solution was made up of 2 5 g of Amido Black, 250 ml of methanol, 35 ml of acetic acid and 215 ml of water The gels were destained electrophoretically in 2% acetic acid

In order to determine the radioactivity the gels were sliced longitudinally, dried down under vacuo on filter paper and autoradiographed with Kodak X ray film, type RHP-Royal X-omat

If a quantitative determination of the radioactivity in the different polypeptides was required, the gels were cut into 1 mm slices, which were solubilized with 0 1 ml of Protosol at 60°C for 16 hours After addition of a toluene based scintillator they were counted in a liquid scintillation counter Before slicing the gels were monitored at 540 nm min in a Gilford spectrophotometer, adapted with a gel scanner

3.4 EFFECT OF IONIC CONDITIONS ON THE INCORPORATION OF (^{35}S)METHIONINE INTO PROTEIN USING DIFFERENT tRNA SPECIES

Studies in mammalian cell-free systems from e.g ascites cells [101, 102] or rabbit reticulocytes [36, 141] indicate that the ionic conditions needed for reliable cell-free protein synthesis are rather stringent, especially the potassium and magnesium ion concentrations are important

In the experiments described here different (^{35}S)Met-tRNA species are used In order to interprete the data it is necessary to anticipate some results, which will be discussed in one of the next chapters At the ionic concentrations

routinely used (85 mM KCl and 5 mM Mg^{2+}) $tRNA^{fMet}$ functions specifically as protein chain initiator whereas $tRNA^{Met}$ donates its methionine exclusively to internal positions of the peptide chain

As Met-$tRNA_1^{Met}$ and Met-$tRNA_2^{Met}$ show identical labeling characteristics when used in the cell-free system, in most cases only one of the two species has been tested

The effect of the magnesium ion concentration on the incorporation into lens proteins of methionine donated by Met-$tRNA^{fMet}$ as well as by Met-$tRNA^{Met}$ is shown in fig 5. With regard to the experimental part it is worth-

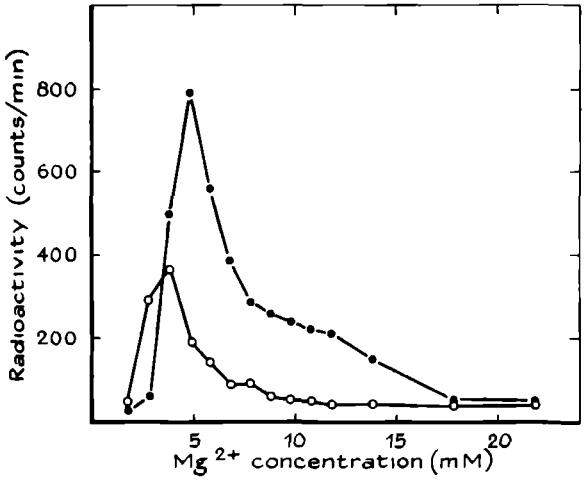

Fig 5 Effect of magnesium ion concentration on the incorporation of methionine into lens protein using different (^{35}S)Met-tRNA species. Incubations were performed in a total volume of 0 05 ml Conditions were as described in section 3 3 2 After incubation 0.02 ml samples were withdrawn, treated with 0 5 ml of 0 1 M NaOH, the protein was precipitated with TCA, washed and collected on glass fiber filters The Mg^{2+} concentrations mentioned in the figure is composed of the contribution of Mg^{2+} ions present in the lysate plus the amount added •——• Incorporation using (^{35}S)met-$tRNA^{fMet}$, 2×10^6 cpm/ml, reaction time 20 min o——o Incorporation using (^{35}S)Met $tRNA^{Met}$, 10^6 cpm/ml, reaction time 30 sec

while to draw the attention to the following points The time needed to synthesize completed α-crystallin chains is about 4 minutes This conclusion can be drawn from the observation that after 4 min of incubation at 30°C N-terminally labeled α-crystallin polypeptides are present in the 220,000 g supernatant As this finding is consistent with the results of others concerning the translation rate in cell-free systems as well as in intact cells [36, 141], we may assume that the translation rate for lens proteins is of the same order of magnitude, at least during the first four minutes If we want to take the incorporation rate of methionine donated by Met-tRNA^{Met} as a measure for chain elongation, we have to determine the elongation rate in a period of time when it is independent of the initiation rate This is the case during a reaction time which is short in relation to the time needed for the synthesis of a whole polypeptide chain Therefore we have measured the incorporation of (^{35}S)methionine using (^{35}S)Met-tRNA^{Met} for an incubation time of 30 sec It appears from fig 5 that the optimal Mg^{2+} concentration for initiation is 5 mM while the optimal concentration for elongation is slightly lower The curve for methionine incorporation using (^{35}S)Met tRNA^{fMet} shows a plateau between 8 and 12 mM Mg^{2+}, while the incorporation via (^{35}S)Met-tRNA^{Met} decreases slowly and more regularly at higher magnesium ion concentrations The irregularity in the curve of Met tRNA^{fMet} will be further discussed in chapter 4 If the incorporation of (^{35}S)methionine, using Met tRNA^{Met}, is determined as a function of the magnesium ion concentration for a longer incubation time ($\geqslant$ 3 min) the incorporation becomes dependent on chain initiation as the optimal Mg^{2+} concentration shifts to the optimum of initiation namely 5 mM (not shown)

Fig 6 exhibits the effect of potassium ion concentration at 5 mM Mg^{2+} on cell free protein synthesis The optimal incorporation using (^{35}S)Met-tRNA^{fMet} is at about 0 060 M while optimal incorporation via (^{35}S)Met-tRNA^{Met} occurs at about 0 085 M KCl Incorporation via the initiator tRNA is more critical i e the curve shows a sharp optimum and incorporation decreases abruptly at higher KCl concentration, the presence of 150 mM KCl abolishes initiation entirely The effect of the K^+ concentration on chain elongation is not so dramatic, at 200 mM KCl the incorporation is still 30% In fact the free Mg^{2+} concentration is indirectly related to the KCl concentration [97] It has been shown that there is a weak association of Cl^- with Mg^{2+}, the stability constant for the complex $MgCl^+$ being about 3 5 M^{-1} [58] From

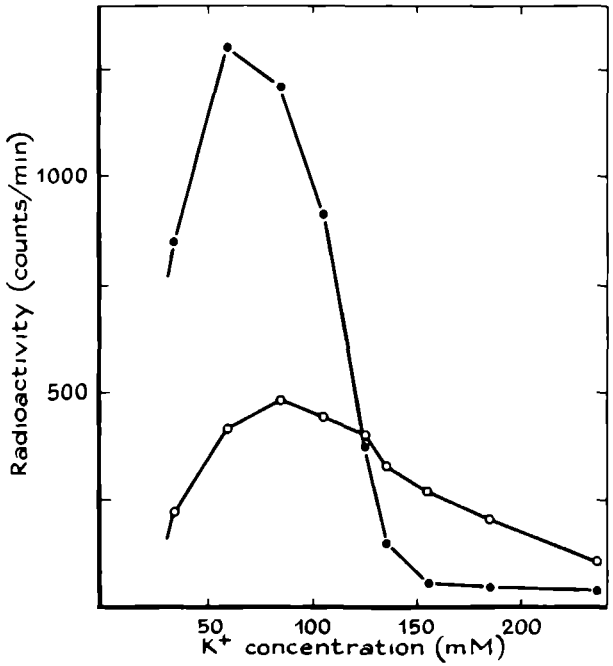

Fig 6 Effect of potassium ion concentration on the incorporation of methionine into lens protein using different (^{35}S)Met-tRNA species. Incubations were performed in a total volume of 0 05 ml Conditions were as described in section 3 3 2 After incubation 0 02 ml samples were withdrawn, treated with 0 5 ml of 0 1 M NaOH, the protein was precipitated, washed with TCA and collected on glass fiber filters The Mg^{2+} concentration was 5 mM The K^+ concentrations mentioned in the figure were composed of the contribution of K^+ present in the lysate plus the amount added •——• Incorporation using (^{35}S)Met-tRNA^{fMet}, 2x10^6 cpm/ml, reaction time 20 min o——o Incorporation using (^{35}S)Met-tRNA^{Met}, 10^6 cpm/ml, reaction time 30 sec

stability constants of complexes as Mg-creatine phosphate, Mg-ATP, Mg-GTP and $MgCl^+$ one can calculate that in a medium containing 5 mM Mg^{2+}, 1 mM ATP, 0.5 mM GTP, 10 mM creatine phosphate and 0.1 M KCl the concentration of free magnesium ions would be about 2 mM. Therefore we have to note that the reported optimal Mg^{2+} concentrations have only empirical meaning as long as the free Mg^{2+} concentration in the cell-free system has not been estimated.

We have also made an attempt to determine the influence of the pH on the incorporation of methione into protein It appears that, using a Tris-Cl buffer system at a concentration of 0 05 M, no sharp optimum can be detected between pH 7 and 8, both with Met-tRNA^{fMet} and Met-tRNA^{Met} It is quite remarkable that at this Tris concentration, which is five times higher than routinely used the incorporation via Met-tRNA^{fMet} as well as via Met-tRNA^{Met} drops to a level of one third If a Tris concentration of 0 1 M is used, the incorporation via tRNA^{fMet} (chain initiation) is completely abolished, while chain elongation is reduced to 20%

3 5 PROTEIN SYNTHESIS IN THE CELL-FREE AND IN INTACT LENSES

In order to establish whether or not the lens lysate is still capable to synthesize eye lens proteins as it does before cell rupture, the cell-free system (1 ml) is incubated for 60 min as described in section 3 3 2 using a mixture of ^{14}C-labeled amino acids The reaction mixture is passed through a column of Sephadex G 200 This results in a clear separation of the four classes of water soluble eye lens proteins, namely α , β high, β-low and γ-crystallins (fig 7A) From the distribution of radioactivity it is clear that α , β low and γ crystal lins have been synthesized in relative equal amounts However, β-high synthesis is exceptionally low as compared to the other crystallins The same conclusion can be drawn, if the analysis is performed with protein labeled in organ culture (fig 7B)

In table III the specific activities of the protein fractions are summerized

Further analysis of the newly synthesized proteins, separated on the Sephadex G-200 column is carried out with the aid of analytical polyacrylamide gels containing 6 M urea at pH 8 5 (fig 8) As the specific activity of the polypeptide chains is rather low, it is impossible to decide whether all polypeptides made both *in vitro* and *in vivo* have been synthesized in comparable amounts However, from the distribution of radioactivity among the main components of the α , β-low and γ-crystallin fractions we may conclude that these polypeptides are indeed synthesized to the same extent *in vivo* as well as *in vitro*

Because of the occurrence of an equal amount of methionine residues (2) in the four α crystallin polypeptides, α-crystallin labeled with (^{35}S)methionine

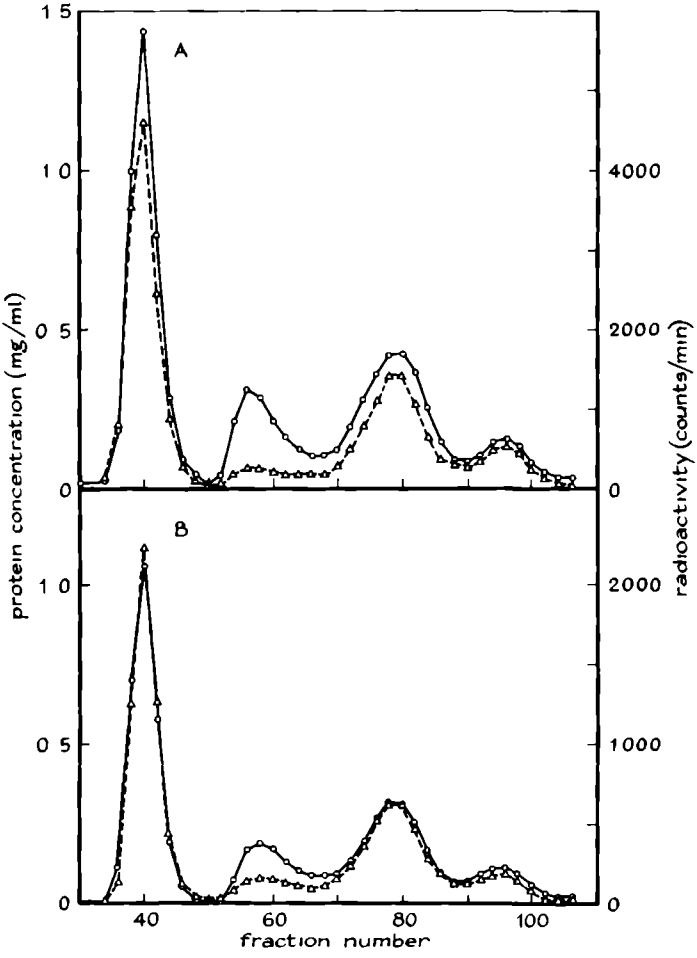

Fig 7 Gel filtration on Sephadex G-200 of lens proteins labeled with a ^{14}C-amino acid mixture The protein concentration was calculated from the extinction at 280 nm by assuming $E_{280}^{1\%} = 8\ 2$ for α-crystallin and 22 for the other crystallin fractions [79] o—o Protein concentration (mg/ml) △– △ Radioactivity (cpm/ml) A elution profile of lens proteins labeled in the cell-free extract, B elution profile of lens proteins labeled in intact lenses

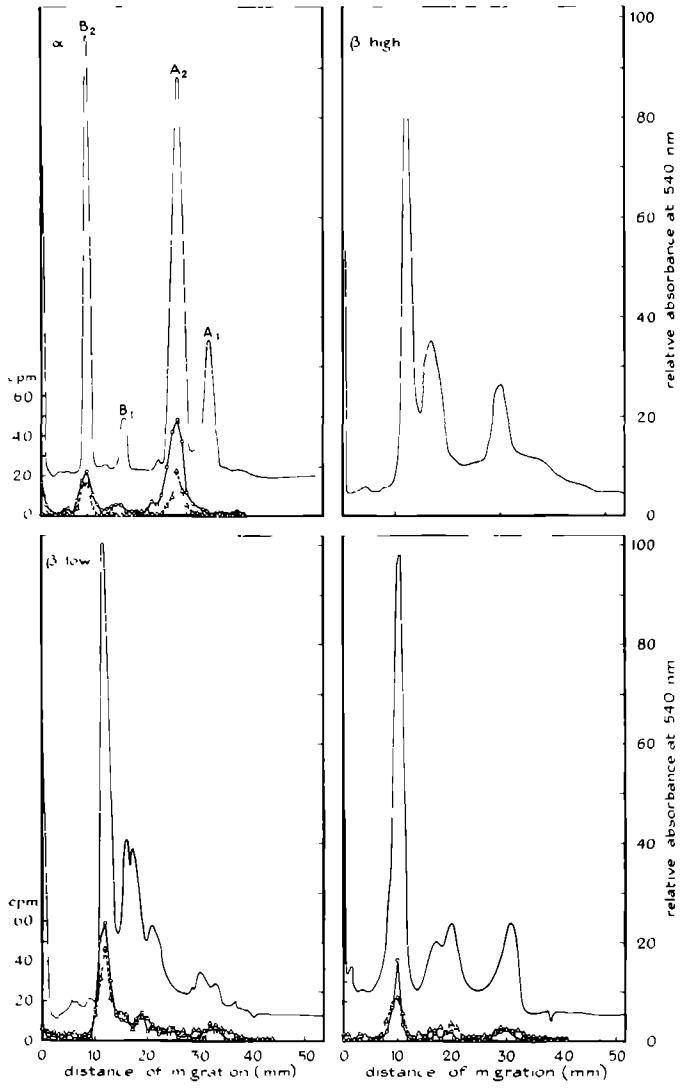

Fig.8. Electrophoretic analysis on basic urea gels of eye lens polypeptides. Gel electrophoresis was carried out as described in section 3.3.6. Per gel 0.15 mg of protein, isolated as described in fig. 7 was applied. ——— Optical density profile scanned at 540 nm; o—o—o distribution of radioactivity after labeling with ^{14}C-amino acids in the cell-free system; △--△--△ distribution of radioactivity after labeling with ^{14}C-amino acids in lens culture. Radioactivity in the β-high fraction was under detection level.

in lens culture has been used to compare the synthesis of these polypeptides mutually Fig 9 shows that the B_2 and A_2 chains have almost the same specific activity. Synthesis of A_1 cannot be detected whereas only a low amount of radioactivity in the position of B_1 is observed Similar results have been obtained with α-crystallin labeled in the cell-free system

From all these labeling experiments it seems to be justified to conclude that the protein synthesizing machinery of the intact eye lens functions identically in the cell free system.

protein fraction	Relative amount of protein present in the lysate	^{14}C-amino acids (cpm/mg)	
		in vitro	*in vivo*
α	40	3200	2100
β-high	23	800	800
β low	30	3100	2000
γ	7	3200	1700

Table III

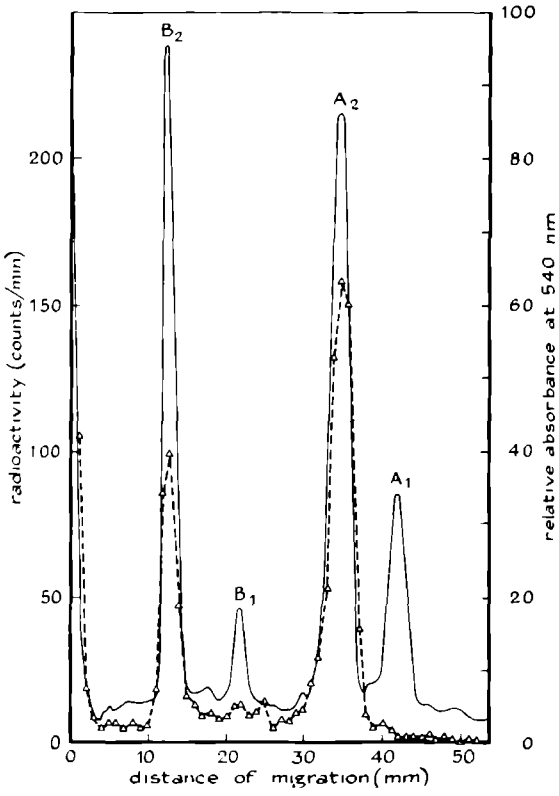

Fig.9. Polyacrylamide gel electrophoresis of α-crystallin labeled with (^{35}S)methionine. Gel electrophoresis was performed as described in section 3.3.6. α-crystallin labeled in lens culture and isolated by Sephadex G-200 gel filtration was separated in four polypeptides. ——— Optical density scanned at 540 nm. △- -△- -△ Distribution of radioactivity.

3.6. RATE OF METHIONINE INCORPORATION FROM DIFFERENT MET-tRNA SPECIES INTO PROTEIN

The three Met-tRNA species have been tested for their capacity as methionine donors in lens protein synthesis. A more detailed account of Met-tRNA specificity will be given in chapter 4.

The incubation conditions are different from the conditions routinely used in this study for *in vitro* protein synthesis. In order to prevent incorporation of (^{35}S)methionine via deacylation and recharging, the pH of the incubation mixture is kept at 7.0 using a HEPES buffer system. The incorporation of methionine into TCA precipitable material as function of time is demonstrated in fig. 10.

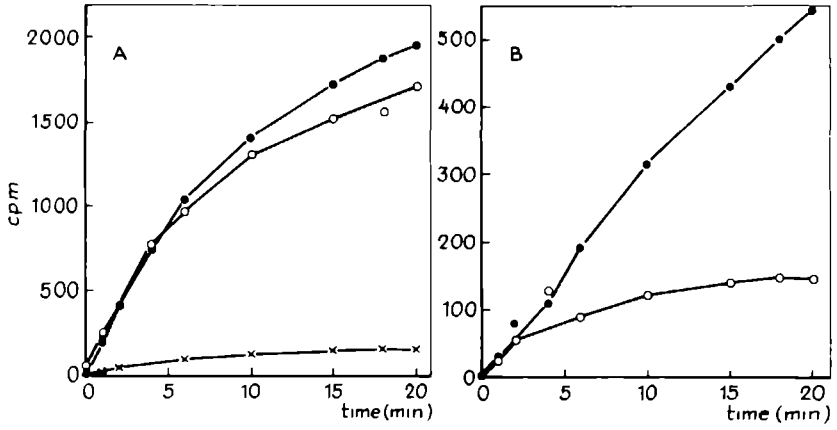

Fig.10. Transfer of (^{35}S)methionine from (^{35}S)Met-tRNA species into protein. 1.4x10^5 counts/min of (^{35}S)Met-tRNAs derived from eye lens tissue were incubated at 37°C in a final volume of 0.22 ml containing 0.12 ml of eye lens extract. All reactions contained 1 mM ATP, 0.5 mM GTP, 5 mM 2-mercaptoethanol, 10 mM creatine phosphate, 1 mg/ml creatine phosphokinase, 25 mM HEPES (pH 7.0), 80 mM KCl, 5 mM magnesium acetate and 0.1 mM of all twenty amino acids except methionine. 0.02 ml samples were withdrawn at the indicated intervals and mixed with 0.5 ml of cold water. KOH was added to 0.1 M, after incubation at 37°C for 10 min, one volume of 10%TCA was added. The precipitate was collected on glass fiber filters, washed with 5%TCA, whereafter the filters were dried and counted. A. x—x (^{35}S)Met-tRNA^{fMet}, o—o (^{35}S)Met-tRNA$_1^{Met}$, •—• (^{35}S)Met-tRNA$_2^{Met}$. B. o—o (^{35}S)Met-tRNA^{fMet}, •—• formyl-(^{35}S)Met-tRNA^{fMet}.

Although in each reaction mixture the same amount of radioactivity is present, the incorporation by (^{35}S)Met-tRNA^{fMet} is low as compared to the incorporation by (^{35}S)Met-tRNA^{Met}.

Enzymic formylation of (^{35}S)Met-tRNA^{fMet} results in an enhancement of incorporation into protein Formyl-Met-tRNA^{fMet} can donate its formyl-methionine only to the N-terminal position of a newly initiated polypeptide chain If the incorporation via Met-tRNA^{fMet} is transient, then the N-terminal methionine residue donated by tRNA^{fMet} has to be removed from the polypeptide chain during peptide growth. So it is quite comprehensible that formylation of (^{35}S)Met-tRNA^{fMet} enhances the incorporation of radio-activity as the cytoplasm of mammalian cells is not capable to deformylate the peptides, newly initiated by F-(^{35}S)Met-tRNA^{fMet}

In order to verify the reliability of the incorporation experiments the effect of a number of antibiotics on the incorporation of methionine by the differ-ent tRNA species have been studied The results are summerized in table IV

Additions	(^{35}S) methionine donor Met-tRNA^{fMet}	Met-tRNA$_1^{Met}$	Met-tRNA$_2^{Met}$
	(318 cpm)	(2145 cpm)	(1515 cpm)
None	100%	100%	100%
ATA (2×10^{-4}M)	15%	60%	61%
Cycloheximide (0.2 mg/ml)	2%	2%	3%
Chloramphenicol (0.1 mg/ml)	105%	109%	95%

Table IV Effect of antibiotics on protein synthesis Reactions as described in the legend to fig. 10 Incubations were performed at 37°C for 10 min in a total volume of 0 03 ml

It is obvious that chloramphenicol, an antibiotic which inhibits protein biosynthesis on 70S ribosomes [47, 127], does not inhibit the incorporation of methionine via either of the tRNA species. Therefore the conclusion can be drawn that no mitochondrial peptide formation takes place and that bacterial infection may be excluded. Aurintricarboxylic acid at a concentration of 2×10^{-4} M inhibits initiation preferentially [153]. It appears from table IV that addition of this compound to the incubation mixture mainly interferes with incorporation by Met-tRNA^{fMet}. On the other hand, addition of cycloheximide results in an almost complete inhibition of incorporation independently of the type of Met-tRNA used. As the later antibiotic, at the concentration used, inhibits initiation as well as translation on 80S ribosomes [114] the effect observed can easily be understood.
The results presented in this section suggest that Met-tRNA^{fMet} functions as initiator of protein synthesis in the eye lens system.

3.7 FORMYL-METHIONYL-tRNA^{fMet} AS INITIATOR OF α-CRYSTALLIN

When formylated (^{35}S)Met-tRNA^{fMet} is incubated with the crude lens cell-free system as described in the legend of fig. 10, the sequence formyl-Met-Asp is found in the NH_2-terminus of the newly formed polypeptides (fig. 11).
In order to ascertain the identity the material co-migrating with F-Met-Asp is eluted from the paper, treated with HCl and the resulting peptides are analyzed (fig. 11B). Hydrolysis with 0.5 M HCl at 90°C removes only the formyl group, whereas an acetyl group cannot be split off [70]. Removal of the formyl group results in the appearence of methionyl-aspartic acid. The radioactivity in the position of F-Met on the electropherogram is due to formyl-methionine that has not reacted. This material is not completely removed after incubation. The radioactivity migrating slower than formyl-Met in fig. 11A is on the one hand due to the tetrapeptide F-Met-Asp-Ile-Ala, which can also be split off from the α-crystallin polypeptides, on the other hand to formyl-peptides derived from other polypeptides initiated in this system.
Our finding that upon incubation of the lens lysate with formyl-(^{35}S)Met-tRNA^{fMet} a radioactive dipeptide analogous to the native one can be identified allows the conclusion that the lens cell-free system is capable to initiate new α-crystallin polypeptide chains.

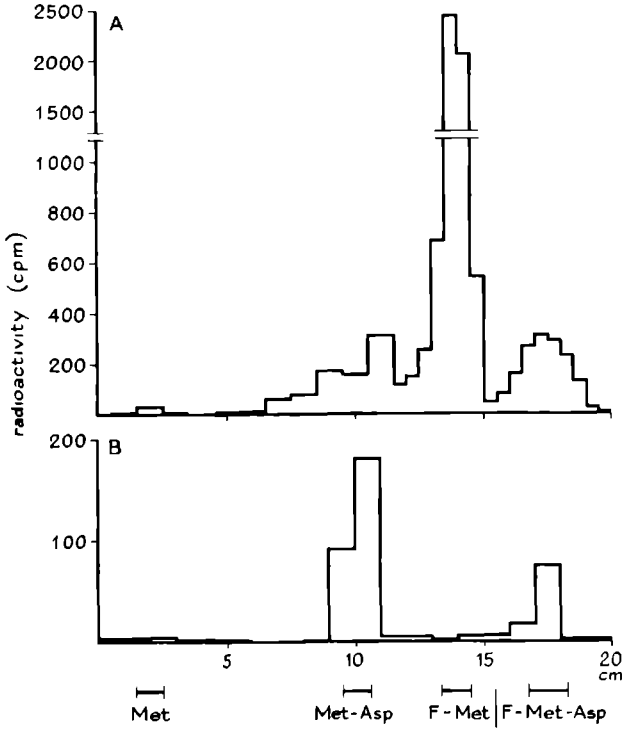

Fig 11 Analysis of blocked peptides derived from lens protein, labeled with formyl-(^{35}S)Met-tRNA^{fMet} After 20 min of incubation at 37°C as described in the legend to fig 10 pancreatic RNAase and EDTA were added and the incubation was continued for 10 min The protein was precipitated with 5% TCA centrifuged and washed with 5% TCA and ethanol The precipitate was suspended in 1 ml of 0 1 M NH_4HCO_3 and digested with pronase The blocked peptides and amino acids were isolated by Dowex 50 treatment and analyzed by high voltage paper electrophoresis at pH 6 5 (A) The radioactive material migrating together with unlabeled Γ-Met-Asp was cut out from the paper and treated with 0 5 M HCl for 20 min at 90°C to split off the formyl residues After lyophilization the deformylated product was again analyzed by electrophoresis at pH 6 5 (B)

3 8 DISCUSSION

Optimal ionic conditions as found for the eye lens cell-free system correspond to those described for other mammalian systems like ascites tumor [101] and reticulocytes [137].

The data described in section 3.4. show that both the magnesium and potassium ion concentrations are critical for chain initiation It is noteworthy that the optimal K^+ and Mg^{2+} concentrations are different for chain initiation and elongation Chain initiation seems to require a lower potassium and a higher magnesium ion concentration than chain elongation

In section 3.5. it is demonstrated that under optimal ionic conditions the cell-free system is capable to synthesize the same polypeptides as intact lenses Analysis of the newly synthesized lens proteins reveals that the synthesis of polypeptides of the protein aggregate, designated as β-high crystallin, occurs in small amounts as compared with β-low, although both proteins have some polypeptide chains in common [62] Assuming that crystallin synthesis in lens culture is an accurate picture of the *in vivo* process, the β-high formation seems to take place at a much lower rate It cannot be excluded that the β-high aggregate arises from β-low and that β-high crystallin formation possible reflects an aspect of lens aging.

Another aspect of the biosynthesis of crystallins needs discussion

Are the two minor polypeptides of α-crystallin (A_1 and B_1) products of direct genetic expression? It is generally accepted now that A_1 is generated from A_2 by a posttranslational modification [14, 39] The experiments described in this chapter (see fig 9) sustain this conclusion since no radioactivity co-migrates with the A_1 polypeptide on polyacrylamide gel However, the synthesis of B_1 is still questionable In fig 9 a small amount of radioactivity is co-migrating with B_1 It remains to be seen whether the B_1 polypeptide is separable from this radioactivity As structural studies on B_2 and B_1 revealed a large similarity in amino acid and peptide composition [75] and so far no messenger RNA coding for αB_1 could be detected [9], it is most likely that also αB_1 arises from αB_2 as a result of a slow posttranslational conversion.

Formylation of Met-tRNA^{fMet} results in an enhancement of incorporation of radioactivity into the protein by a factor 5 (section 3 6) In a cell-free system derived from rabbit reticulocytes, synthesizing mainly globin, incorporation of F-Met derived from formyl-Met-tRNA^{fMet} is high as compared with labeling via the unformylated Met-tRNA^{fMet} species [70] This is comprehensible as the N-terminal methionine is only transiently incorporated into N-terminal positions of the globin polypeptides However, in the lens system about half of the methionine residues donated by Met-tRNA^{fMet} are permanently

bound (it will be shown in chapter 5 that the N-terminal methionine residue of α-crystallin is not removed at any stage in the process of protein synthesis). On the basis of the foregoing arguments the enhancement of incorporation by formylation of Met-tRNA^{fMet} (as compared with the unformylated species) cannot be explained. The explanation should be search in the experimental conditions. The experiments as described in the sections 3.6. and 3.7. are carried out at pH 7.0 in the presence of a HEPES buffer system. When (^{35}S)Met-tRNA^{fMet} as well as formyl-(^{35}S)Met-tRNA^{fMet} are present in equal amounts in the reaction mixture (as described in section 3.3.2.), mainly (^{35}S)Met-tRNA^{fMet} is used for α-crystallin chain initiation. This leads us to the suggestion that conditions which are optimal for incorporation of methionine via Met-tRNA^{fMet} are far from being optimal for the utilization of formyl-Met-tRNA^{fMet} if the HEPES buffer system is used.

The fact that upon incubation using formyl-(^{35}S)Met-tRNA^{fMet} the dipeptide F-Met-Asp can be identified demonstrates clearly that the cell-free system is capable to initiate new α-crystallin polypeptides. It also gives some information about the initiation of the polypeptide chains of α-crystallin. We *can now partly discriminate between the following possibilities:*

1. Initiation starts with a special acetyl-Met-tRNA.
2. Initiation is achieved by the initiator Met-tRNA^{fMet}; the methionine residue is not removed from the nascent chain and at a certain moment the N-terminus is acetylated.
3. Initiation occurs via Met-tRNA^{fMet}; after initiation another methionine residue donated by Met-tRNA^{Met} elongates the chain. After the insertion of 20-30 amino acids the N-terminal methionine as observed with globin, is removed by a specific aminopeptidase. Thereafter the penultimate methionine is acetylated.

The third possibility can be excluded by the finding that the use of F-Met-tRNA^{fMet} results in the synthesis of α-crystallin polypeptides with an N-terminal sequence formyl-Met-Asp; if the initiation would occur according to the mechanism described in 3. one should have found the dipeptide F-Met-Met as the N-terminal dipeptide of α-crystallin polypeptides.

Between hypothesis 1. and 2. we cannot discriminate yet. Despite of intensive search acetylated Met-tRNA could never be detected in lens extracts. Moreover chemically acetylated Met-tRNA^{fMet} failed to initiate new α-crystallin chains. This renders the first possibility rather unlikely. An experimental

approach to the determination of the moment of acetylation will be given in chapter 5.

CHAPTER 4

ROLE OF METHIONYL-tRNA IN THE SYNTHESIS OF THE αA_2 CRYSTALLIN CHAIN

4.1. INTRODUCTION

As earlier mentioned (compare chapter 3) it has been claimed that different blocked amino acids play a role in the initiation of eukaryotic protein biosynthesis: N-acetyl-serine in histon and ovalbumine initiation [91, 109]. Further blocked cysteine and pyrrolidone carboxylic acid have also been suggested in this context [5, 118, 130]. In all these cases an initiation mechanism differing from the general accepted one would be required. This ambiguity in eukaryotic protein initiation requires further intensive study of each of these rather specialized systems.

The major part of the structural eye lens proteins (crystallins) is N-terminally blocked. α-Crystallin, which makes up approximately 40% of the soluble lens protein, carries an N-terminal methionine residue. Examination of the initiation mechanism of α-crystallin will show whether its initiation follows the general mechanism with Met-tRNA^{fMet} as initiator or that it has to be classified among the (doubtful) exceptions of the general rule.

In this chapter we describe the role of the different lens Met-tRNA species in the synthesis of the A_2 polypeptide chain of α-crystallin.

4.2. METHODS

4.2.1. isolation and aminoethylation of the αA_2 polypeptide chain

The separation of the αA_2 chain from the other polypeptides in the lens system or from globin in the reticulocyte system was carried out on preparative polyacrylamide gels. Gel electrophoresis was performed in 1.2x9 cm cylindrical glass tubes at 5 mA/gel for 16 hours. The gels were prepared as described in section 3.3.6. Per gel 4-5 mg of lyophilized protein dissolved in

0.2 ml of electrode buffer was applied. After electrophoresis a thin longitudinal slice was stained with Amido Black and destained electrophoretically. The zone containing the αA_2 polypeptide was cut out from the unstained part of the gel, put into a dialysis tube with 10 volumes of distilled water, minced and dialyzed against water containing 0.01 M 2-mercaptoethanol. The gel fragments were removed by filtration and the protein was lyophilized. The purified αA_2 polypeptide chain was aminoethylated [123] in order to get identifiable methionine containing peptides after digestion with trypsin. For this purpose the polypeptides were dissolved in 2 ml of a solution containing 7 M deionized urea, 1.3% 2-mercaptoethanol, 0.5 M Tris-Cl, pH 8.6 and 4 mg EDTA, which was flushed with argon for 5 min. The solution was left to stand for 2 hours at 20°C and aminoethylation was started by addition of 0.05 ml of ethylenimine. After two hours at room temperature the aminoethylated protein was purified by gel filtration on a Sephadex G-25 column in 0.5% formic acid and lyophilized.

4.2.2. enzymic digestion

Digestion of the αA_2 polypeptide isolated by preparative gel electrophoresis was performed with trypsin. The aminoethylated chains were digested (1 mg trypsin per 50 mg protein) at 37°C for 5 hours in 0.1 M ammoniumbicarbonate, pH 8.9. The resulting peptides were lyophilized, dissolved in water and subjected to descending paper chromatography on Whatman 3 MM paper, eluted with a mixture of n-butanol-acetic acid-pyridine-water (60:12:48:40, by volume) for 16 hours. After drying the paper was cut into one cm strips, which were counted in a liquid scintillation counter using a toluene based scintillator.

4.2.3. conditions for heterologous *in vitro* synthesis of the αA_2 chain

In order to synthesize the A_2 chain in a heterologous system 14S lens messenger RNA, coding for αA_2 isolated from eye lens polysomes was translated in a cell-free system derived from rabbit reticulocytes [8, 9, 10]. The lysate was prepared according to Lockard and Lingrel [92]. The reaction mixtures (1

ml) contained 0 4 ml of the reticulocyte lysate, 0 01 M Tris-Cl, pH 7.4, 0.002 M $Mg(OAc)_2$, 0 1 M ammonium acetate, 1 mM ATP, 0 2 mM GTP, 0.015 M creatine phosphate, 0.06 mg of creatine phosphokinase, 0.1 mM of all amino acids except methionine, 0.05 mCi (^{35}S)methionine (spec. act. 20 Ci/mmol) and 0 02 mg 14S mRNA. Incubation was performed at 30°C. After incubation 0 1 ml of 0.25 M EDTA, 0 1 mg pancreatic RNAase and 0 3 mg α-crystallin was added and the mixture was kept at 30°C for 15 min. Globin was prepared by adding the incubation mixture dropwise to 15 volumes of 2 5% oxalic acid in acetone under vigorous mixing. The precipitated protein was washed three times with cold acetone and once with di-ethylether. The precipitate was dried at room temperature

4.3. TRANSFER OF METHIONINE FROM DIFFERENT MET-tRNA SPECIES INTO LENS PROTEINS

As described in the previous chapter after labeling the eye lens cell-free system with formyl-(^{35}S)Met-tRNA^{fMet} it is possible to isolate a radioactive dipeptide identical to the N-terminal dipeptide of α-crystallin, except that the blocking group is a formyl instead of an acetyl group. So far we have concluded that our system is capable of initiating new polypeptide chains and that the methionine residue present in N-terminal position in native α-crystallin is donated by the initiator tRNA. Now we want to examine the capabilities of the different Met-tRNA species in labeling the polypeptide chains made in the eye lens cell-free system. For that reason polypeptides labeled *in vitro* via one of the (^{35}S)Met-tRNA species are analyzed by polyacrylamide gel electrophoresis at pH 8.5 in the presence of 6 M urea Fig. 12 shows the electrophoretic patterns of the stained gels as well as their autoradiographs. Labeling of the polypeptides is performed with (^{35}S)methionine and with the different (^{35}S)Met-tRNA species. In all these cases the stained protein patterns are identical, however, the autoradiographs reveal that (^{35}S)Met-tRNA^{fMet} donates its label almost completely into the αA_2 and αB_2 chains (fig 12B) The radioactivity migrating faster than A_2 is presumably due to carbamylation of A_2, the band just above the A_2 is probably caused by dimerization of A_2.

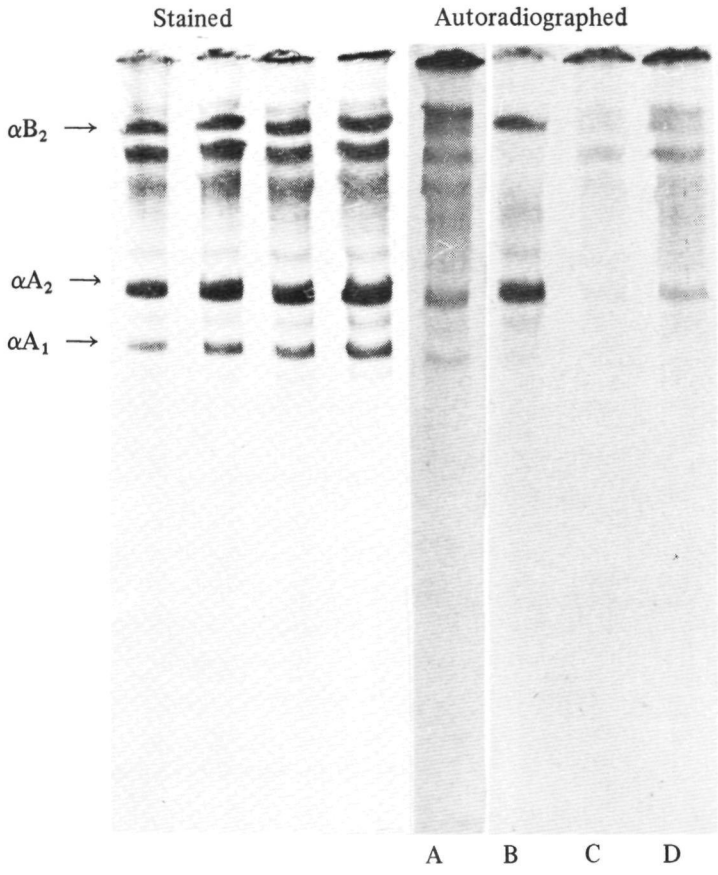

Fig.12. Electrophoretic analysis and autoradiography on basic polyacrylamide gels of the polypeptides synthesized in the eye lens cell-free system. Polypeptides were labeled with (^{35}S)methionine (gel A), with (^{35}S)Met-tRNA^{fMet} (gel B), with (^{35}S)Met-tRNA$_1^{Met}$ (gel C) and with (^{35}S)Met-tRNA$_2^{Met}$ (gel D). Polyacrylamide gel electrophoresis was performed as described in section 3.3.6. Per gel 0.15 mg of protein was applied. The dried gels were autoradiographed for 14 days.

The autoradiographs of the gels A, C and D represent radioactivity patterns of incubations labeled with (^{35}S)methionine, (^{35}S)Met-tRNA$_1^{Met}$ and

(^{35}S)Met-tRNA$_2^{Met}$ respectively. It can be seen that nearly all bands coincide with radioactivity.

In order to investigate whether or not the label donated by tRNA^{fMet} is indeed incorporated in the N-terminal position of the αA_2 and αB_2 chains the lens cell-free system is incubated with (^{35}S)Met-tRNA^{fMet} under conditions of protein synthesis. The sequence Ac-(^{35}S)Met-Asp, which is known to be the N-terminal sequence of native α-crystallin chains can be identified after digesting the labeled polypeptides with pronase (fig. 13A). This is achieved by

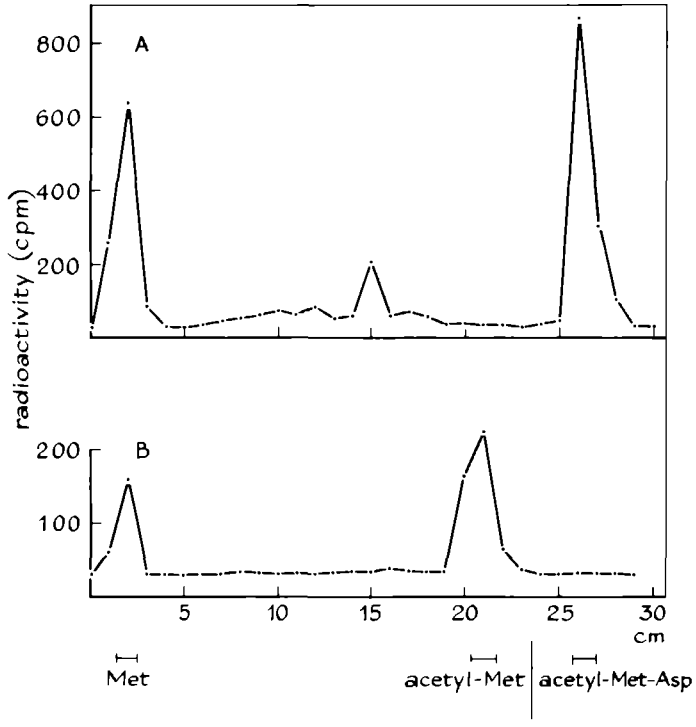

Fig.13. Identification of peptides after pronase digestion. (^{35}S)Methionine-labeled protein from a lens lysate was digested with pronase as described in section 3.3.5. at 37°C for 6 hours The peptides were lyophilized and analyzed by paper electrophoresis at pH 6.5 (A). The radioactivity migrating together with unlabeled acetyl-methionyl-aspartic acid was eluted from the paper and treated with carboxypeptidase A (see section 3.3 5.). The resulting material was analyzed by the same technique (B).

elution from the paper of the material at the position of Ac-Met-Asp and electrophoresis after digestion with carboxypeptidase A This treatment causes a shift of the radioactivity to the position of acetyl-methionine (fig 13B) The radioactivity in the methionine region is probably due to deacetylation of Ac-Met

Label in the Ac-Met-Asp region is only observed after incubation with (^{35}S)Met-tRNA^{fMet}, no radioactivity can be detected in this peptide when the incubation is carried out with one of the (^{35}S)Met-tRNA^{Met} species The radioactivity near the starting zone in fig 13A has probably to be attributed to free methionine, still present in the digest despite of extensive washing with TCA

From these results and the previous finding that formyl (^{35}S)Met-tRNA^{fMet} gives rise to the sequence F-(^{35}S)Met-Asp in α-crystallin we conclude that the N-terminal methionine of α-crystallin is donated exclusively by tRNA^{fMet}

4 4 TRANSFER OF METHIONINE FROM DIFFERENT MET tRNA SPECIES INTO THE αA_2 POLYPEPTIDE

Newly synthesized αA_2 chains are useful for the study of the specificity of each of the three Met-tRNAs. These chains contain two methionine residues, one N-terminally, the other one in position 138 After aminoethylation of the A_2 chain tryptic digestion yields two methionine-containing peptides which can easily be characterized [116] During handling the methionine residue in the tryptic peptides have been sulfoxidized, partly in the internal and completely in the N-terminal peptides As sulfoxidation brings about a lowering of the R_f values of the peptides in the eluting system used, two spots for the internal and one for the N-terminal peptides are found

Fig 14 shows the distribution of label upon chromatography of the tryptic peptides obtained after incubation with (^{35}S)methionine (A), (^{35}S)Met-tRNA^{fMet} (B), (^{35}S)Met-tRNA$_1^{Met}$ (C) and (^{35}S)Met-tRNA$_2^{Met}$ (D) The radioactive material in fig 14A has the same chromatographic behavior as the native methionine-containing tryptic peptides of the αA_2 chains [116]

The results depicted in fig 14B prove once more that the methionine residue present in N-terminal position is donated exclusively by Met-tRNA^{fMet}

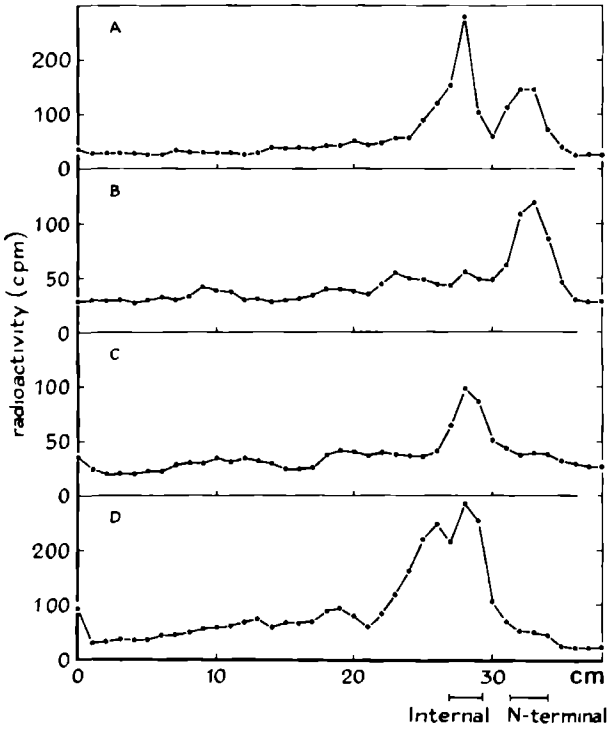

Fig 14. Paper chromatography of the methionine-containing tryptic peptides of A_2 chains A peptides obtained after (^{35}S)methionine labeling, B peptides obtained after labeling with (^{35}S)Met-tRNA^{fMet}, C peptides obtained after labeling with (^{35}S)Met-tRNA$_1^{Met}$ and D peptides obtained after labeling with (^{35}S)Met-tRNA$_2^{Met}$.

Moreover it is demonstrated here that tRNA^{Met} supplies the methionine only to the internal peptide (fig. 14C and D).

4.5. INITIATION OF αA_2 BIOSYNTHESIS IN A RABBIT RETICULOCYTE CELL-FREE SYSTEM

One of the systems most intensively studied in regard to the initiation of mammalian protein biosynthesis is the rabbit reticulocyte system [12, 29, 35,

59, 70, 71, 73, 171]. As far as the role of the different Met-tRNA species is concerned it has clearly been shown that in the synthesis of globin, Met-$tRNA^{fMet}$ is the initiator and that methionine is removed from the nascent chain leaving valine as N-terminal amino acid

Since messenger RNA coding for the αA_2 chain is at our disposal we are able to compare the initiation mechanism of α-crystallin in the homologous (lens) system with the mechanism in the heterologous rabbit reticulocyte system [8, 10]. The translation of 14S lens mRNA in the reticulocyte cell-free lysate results in a newly synthesized peptide chain with the same properties as native A_2 chains [9] When formyl-(^{35}S)Met-$tRNA^{fMet}$ is used as a radioactive precursor, following pronase digestion the N-terminal dipeptide F-Met-Asp can be demonstrated [10]. Lens F-Met-$tRNA^{fMet}$ can also serve as initiator of globin synthesis. In the latter case after pronase digestion, F-Met-Val is identified [10]

As has been shown previously in the lens system Met-$tRNA^{fMet}$ initiates the αA_2 chain. To answer the question what happens with the N-terminal methionine of the A_2 chain when the chain is synthesized in a reticulocyte system, we have incubated 14S lens messenger coding for αA_2 in the reticulocyte lysate using (^{35}S)methionine as a radioactive label After incubation at 30°C for 60 min the αA_2 chains are isolated by preparative gel electrophoresis in urea and digested with subtilisin. Fig. 15 shows that the presence of 14S lens mRNA in the incubation results in the formation of a polypeptide from which subtilisin releases a compound with an electrophoretic behavior similar to the peptide Ac-Met-Asp-Ile-Ala which is identical to the N-terminal tetrapeptide of α-crystallin (fig 15B) Radioactivity is also found in the position of methionine which is most probably due to digestion of the internal methionine peptide of αA_2. The radioactive material in the region between the tetrapeptide and methionine has not further been characterized. Upon incubation without 14S messenger RNA no radioactivity is found in the region of the tetrapeptide (fig. 15A)

A comparable result can be obtained after digestion with trypsin. Fig. 16 shows that the methionine-containing tryptic peptides, isolated from a reticulocyte lysate incubated with (^{35}S)methionine in the presence of 14S lens mRNA, have the same chromatographic behavior as the reference peptides derived from calf lens αA_2. A control, incubated without 14S mRNA does not show identical peptides. The inequality in amount of radioactivity present

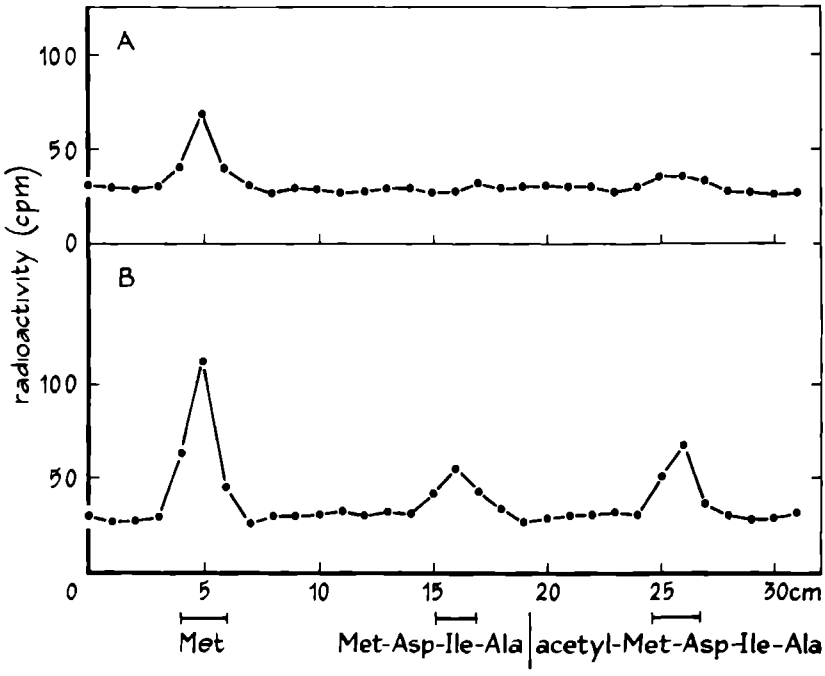

Fig 15 Identification of the N-terminal tetrapeptide of the αA_2 chain by paper electrophoresis The incubation was carried out in a reticulocyte lysate at 30°C for 60 min as described in section 4 2 3 Thereafter carrier α-crystallin was added to the mixture, the αA_2 chain was separated by polyacrylamide gel electrophoresis, isolated and subjected to digestion with subtilisin The analysis was performed by high voltage electrophoresis at pH 6 5 A Incubation minus 14S lens mRNA B incubation in the presence of 14S mRNA

in the internal and the N-terminal peptide may be due to uncomplete digestion or to the presence of contaminating chymotryptic activity

The acetylated state of the N-terminal tryptic peptide is further confirmed by digesting this peptide with pronase This yields material co-migrating with Ac-Met-Asp

From these data the conclusion is justified that the initiation of the biosynthesis of the αA_2 polypeptide follows the same mechanism in the lens system as in the reticulocyte system The N-terminal methionine is not removed, moreover the reticulocyte lysate is able to acetylate the α-crystallin poly-

peptides, although the acetylating mechanism is not operative in globin synthesis.

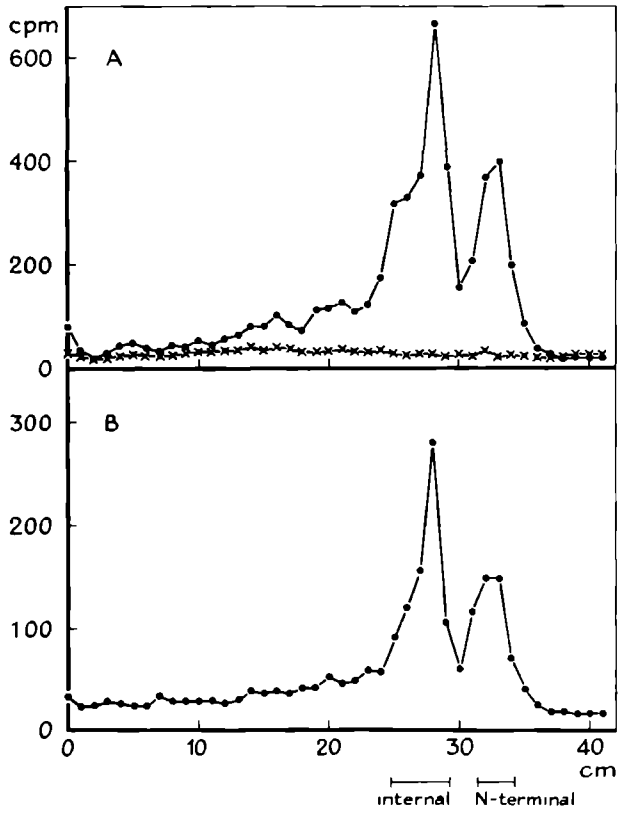

Fig.16 Paper chromatographic analysis of the methionine containing tryptic peptides of polypeptide chains synthesized in a reticulocyte lysate under the direction of 14S lens messenger RNA The peptides were chromatographed as described in 4 2 2 A •–•–• Distribution of radioactivity after incubation with 14S lens mRNA x–x–x Distribution of radioactivity after incubation without 14S mRNA B Distribution of radioactivity obtained with methionine labeled tryptic peptides derived from native αA_2 chains

4.6. SPECIFICITY OF MET-tRNA^{fMet}

In the preceding sections we have shown that the tRNA species are rather specific. However, reports have been published from which it appears that under certain conditions the use of Met-tRNA^{fMet} gives rise to a diminished specificity in chain initiation [29, 42, 43, 44, 113].

The degree to which the initiator tRNA can serve as a donor of internal methionine moieties is strongly influenced by the availibility of Met-tRNA^{Met} in the reaction mixture [29] and by the ionic conditions of the medium [43]. The utilization of Met-tRNA^{fMet} in chain elongation seems to be dependent upon the affinity of Met-tRNA^{fMet} to the elongation factor T_1 which binds the aminoacyl-tRNA to the A-site of the ribosome It has been shown that the magnesium ion concentration strongly affects this affinity.

As already mentioned in section 3 4 in the lens system the optimal Mg^{2+} concentration for chain initiation is 5 mM. The curve in fig 5 exhibits a plateau between 7 and 12 mM Mg^{2+} In the experiments described here an attempt has been made to elucidate this phenomenon

From previous experiments it can be concluded that, if (^{35}S)Met-tRNA^{fMet} is used in the lens system the radioactivity is almost completely transferred to the N terminus of α-crystallin polypeptides To see whether the magnesium ion concentration influences the specificity of the initiator tRNA lens lysate is incubated using (^{35}S)Met-tRNA^{fMet} as a radioactive label at two different Mg^{2+} concentrations (5 and 11 mM). In both cases α-crystallin is isolated by gel filtration on Sephadex G-200 and the purified α-crystallin is subjected to digestion with pronase The paper electrophoresis of the resulting peptides is shown in fig 17

After incubation at 5 mM Mg^{2+} the label is exclusively incorporated in the N-terminal dipeptide Ac-Met-Asp (fig 17A), whereas after incubation at 11 mM magnesium the radioactivity is recovered exclusively in the position of methionine, probably resulting from the internal methionine peptide of α-crystallin (fig 17B). In fig 17C the distribution of radioactivity is depicted after pronase digestion of α-crystallin labeled with (^{35}S)methionine In this case both the N-terminal peptide and the peptide containing the internal methionine residue are equally labeled

A second approach has been made to investigate the specificity of Met tRNA^{fMet} in dependence on the Mg^{2+} concentration Incubations are per-

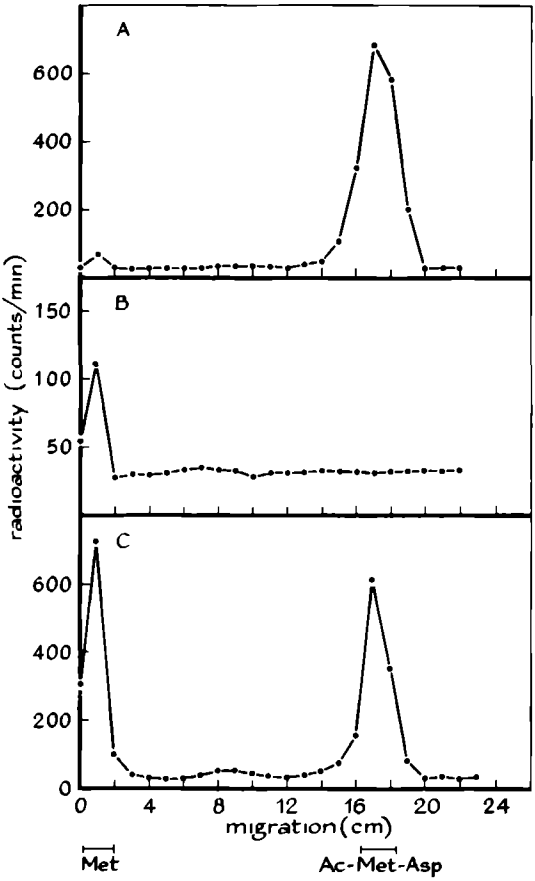

Fig 17 Identification of α-crystallin peptides after digestion with pronase Incubations (0 5 ml) were carried out as described in section 3.3 2 with exception of the Mg^{2+} concentration, which was varied After incubation at 30°C for 30 min the reaction mixtures were passed through a Sephadex G-200 column (as described in section 3 3 4) The peak containing α-crystallin was pooled and the protein was digested with pronase I lectrophoresis was performed at pH 6 5 Staining with platinic iodide revealed a sulfur containing spot in the digest of α-crystallin with the same electrophoretic behavior as synthetic Ac-Met-Asp A Incubation carried out at 5 mM Mg^{2+}, B incubation carried out at 11 mM, C incubation carried out at 5 mM Mg^{2+} using (^{35}S)methionine In cases A and B (^{35}S)Met-tRNA^{fMet} was used as radioactive label

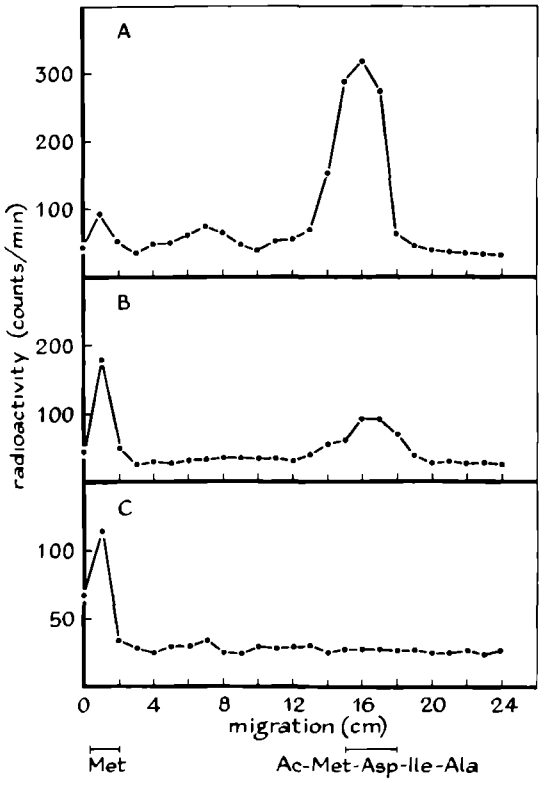

Fig.18. Specificity of initiator tRNA in the synthesis of eye lens proteins. Incubations (0.2 ml) were performed as described in section 3.3.2. at three Mg^{2+} concentrations. (^{35}S)Met-tRNA^{fMet} was used as radioactive precursor. After incubation at 30°C for 30 min 0.02 ml of 1 M KOH was added and the incubation was continued for 15 min. Two ml of 5% TCA were added; the precipitate was spun down, dissolved in 2 ml of 0.1 M KOH and precipitated again with TCA. This treatment was repeated twice. Then the precipitate was washed with ethanol and dried. Digestion with subtilisin was performed as described in section 3.3.5. and the resulting peptides were analyzed by paper electrophoresis at pH 6.5. Chemically synthesized Ac-Met-Asp-Ile-Ala was used as a reference. The magnesium ion concentrations used were in A 5 mM, in B 7 mM and in C: 12 mM.

formed at 5, 7 and 12 mM Mg^{2+}. After incubation the reaction mixtures are treated with 0 1 M KOH, the protein is precipitated with 5% TCA and extensively washed with TCA and ethanol. Then the protein is subjected to digestion with subtilisin, and the resulting peptides are analyzed by paper electrophoresis at pH 6.5 (fig. 18) It can be seen that by raising the Mg^{2+} concentration the amounts of radioactivity in the tetrapeptide region decrease in favor of the radioactivity at the starting zone.

In order to rule out the possibility that at higher Mg^{2+} concentrations the acetylating mechanism is inhibited leaving a free methionine at the N-terminus which may be split off by pronase as well as by subtilisin, we have also analyzed thermolytic peptides In the next chapter it will be demonstrated that thermolysin cleaves Ac-Met-Asp and Met-Asp from acetylated and unacetylated α-crystallin chains respectively. Analysis of the thermolytic peptides does not reveal any Met-Asp when the incubation is performed at 12 mM, only radioactivity with the same electrophoretic behavior as the internal peptide can be detected

These data support the hypothesis that at magnesium ion concentrations higher than 7 mM a loss of specificity of the initiator tRNA occurs, while the capacity of the protein synthesizing machinery to initiate new polypeptide chains is abolished.

4.7 DISCUSSION

From the experiments described in this chapter one may conclude that

1. The Met-tRNA species derived from lens tissue behave in a similar was as reported for Met-tRNAs from other mammalian systems Gel electrophoresis of the newly synthesized α-crystallin polypeptides shows that only the A_2 and B_2 chains are labeled when (^{35}S)Met-tRNA^{fMet} is used as a precursor. Examination of the tryptic peptides of the αA_2 chain reveals that the label is present exclusively in N-terminal position Furthermore it proves that (under the conditions routinely used) the three Met-tRNA species have specific functions

   However, Met-tRNA^{fMet} may lose its specificity as chain initiator. The reported utilization of Met-tRNA^{fMet} for chain elongation in cell-free systems may be explained in three ways

a) Ionic conditions influence the complex constant for the formation of a complex between the transfer factor T_1 and initiator tRNA.

b) The $tRNA^{fMet}$ molecules from different eukaryotes or even from different organs of the same species are different in their structures which causes a variation in the affinity for the T factor.

c) The T_1 elongation factors from different species exhibit different affinity for Met-$tRNA^{fMet}$.

With the exception of the initiator tRNA from yeast, which seems to differ in structural respect from mammalian $tRNA^{fMet}$ species [125, 128], there are no data available supporting the idea that structural differences between initiator tRNAs from different eukaryotic sources exist.

More is known about the role of elongation factor T_1. Several workers have studied the complex formation between initiator tRNA, GTP and the transfer factor, binding to the A-site of mammalian ribosomes in the presence of poly-AUG or endogenous messenger RNA [42, 43, 128]. In fact the crucial point seems to be the degree of recognition of Met-$tRNA^{fMet}$ by the polymerizing enzymes. This affinity for Met-$tRNA^{fMet}$ is influenced by the availability of Met-$tRNA^{Met}$ and by ionic conditions.

In section 4.6. it has been demonstrated that the specificity of the initiator tRNA is strongly influenced by the magnesium ion concentration. In fig. 5 the plateau in the curve for methionine incorporation via Met-$tRNA^{fMet}$ (between 8 and 12 mM Mg^{2+}) is a range of Mg^{2+} concentrations where Met-$tRNA^{fMet}$ provides methionine for both initiation and elongation.

The donation of methionine via Met-$tRNA^{fMet}$ to internal positions in the polypeptide chain should probably be considered to be non-physiological and a consequence of the complex formation between transfer factors and initiator tRNA.

2. Both the lens lysate and the rabbit reticulocyte cell-free extract possess the capacity to acetylate newly formed α-crystallin chains. Other studies in our laboratory revealed that translation of lens messenger RNA in frog oocytes also results in N-terminally acetylated αA_2 chains [11]. However, the stage at which acetylation occurs is still uncertain. The experiments described in this chapter do not definitely exclude the

possibility that the acetyl-methionine is provided by Ac-Met-tRNA^{fMet}, although in spite of an intensive search for this species in the lens as well as in the reticulocyte system no blocked Met-tRNA could be found.
However, since the initiation process in eukaryotic systems appears to be quite similar for different proteins including lens crystallins, the available data suggest that N-terminal acetylation is not an integral part of the initiation mechanism.
The universality of the acetylating mechanism is strongly suggested by the finding that α-crystallin chains have been acetylated in totally different systems like calf lens lysate, rabbit reticulocyte lysate and frog oocytes.

CHAPTER 5

N-TERMINAL ACETYLATION OF αCRYSTALLIN

5.1. INTRODUCTION

Concomitantly with the elucidation of the primary structure of proteins the list of proteins carrying an N-acetylated N-terminal amino acid is growing (see table VI in section 5 6) It appears that bacterial as well as eukaryotic cells are capable of acetylating a number of their proteins Therefore N-terminal acetylation of proteins seems to be a general phenomenon in living cells. However, studies on acetylation in relation to other events in protein biosynthesis hitherto failed to elucidate the exact role of acetylation or the stage at which it occurs

In earlier studies on the initiation of protein biosynthesis in eukaryotes it was suggested that the synthesis of some proteins might start with an acetylated amino acid, like N-acetyl-valine (rabbit globin [52]), N-acetyl-glycine (ovalbumin [110]) and N-acetyl-serine (histone [91]). However, since it is generally accepted now that the initiation of protein biosynthesis in prokaryotes as well as in eukaryotes takes place according to an universal mechanism in which N-acetyl-amino acids are not involved, we have to interprete the N-terminal acetylation as a postinitiational modification of polypeptide chains.

The transition of the *E.coli* ribosomal protein L_{12} to L_7 by acetylation of the N-terminal serine is a recent example of this process [22, 23].

In this chapter it will be demonstrated that acetylation of α-crystallin is no part of the initiation mechanism.

5.2 METHODS

5.2 1 **preparation and fractionation of ribosome-bound peptides**

Incubation of the lens lysate supplemented with lens polysomes and

(^{35}S)Met-tRNA^{fMet} was carried out under conditions for amino acid incorporation as described in section 3.3.2 The total volume was 5 ml After incubation at 30°C for 8 min, cycloheximide was added to a final concentration of 0 2 mg/ml The incubation mixture was chilled in ice and layered over 2 ml of 1 M sucrose in 0.1 M Tris-Cl, pH 7.4, 0.15 M KCl and 0.005 M magnesium acetate. Centrifugation was performed at 200,000 g for 2 hours in a Ti-50 rotor of a Spinco preparative ultracentrifuge at 2°C The ribosomal pellet was suspended in 1 ml of water, adjusted to pH 10-11 with NaOH and incubated at 30°C for three hours This treatment caused cleavage of amino acids and nascent peptides from tRNA in the ribosomal complex The suspension was adjusted to pH 3 with formic acid and the insoluble material was removed by centrifugation About 80% of the peptides and amino acids was found in the supernatant. The solution containing the amino acids and nascent peptides was applied on a Sephadex G-25 "superfine" column (70x1.2 cm), equilibrated with 0.5% formic acid The G-25 column was previously calibrated with α-crystallin, glucagon and valine One ml fractions were collected. Aliquots of 0.1 ml were taken from the even fractions for measurement of radioactivity. The fractions were pooled as indicated in fig. 23, lyophilized and subjected to digestion with thermolysin

5.2.2. **chemical preparation of the tetrapeptide Met-Asp-Ile-Ala**

For the determination of the N-terminus of peptidyl chains, bound to the ribosomes via tRNA, it was necessary to know the specificity of several proteolytic enzymes towards the acetylated as well as the unacetylated substrate Since it was impossible to obtain the unblocked α-crystallin polypeptide chains the unacetylated N-terminal tetrapeptide was synthesized chemically. Benzyloxycarbonyl-L-aspartic acid-β-t-butyl ester, the dipeptide L-isoleucyl-L-alanine and N-t-butyloxycarbonyl-L-methionine-N-hydroxysuccinimide ester were used for the synthesis of this peptide Thin layer chromatography was performed on 5x20 cm silica gel plates GF_{254}. The following solvent systems were used n-butanol-acetic acid-water, 10 1 3 (system A) and chloroform-methanol-17% ammonia, 5 5 1 (system B)

Benzyloxycarbonyl-L-aspartic acid-β-t-butyl-α-N-hydroxysuccinimide ester (A) [68, 170]. N,N-dicyclohexylcarbodiimide (1.27 g, 6.16 mmol) was added to an ice-cold solution of N-hydroxysuccinimide (0.684 g, 5.95 mmol) and β-t-butyl-benzyloxycarbonyl aspartate (2 g, 5.86 mmol) in dry dioxane (10 ml). The mixture was stirred at 0°C for 4 hours and kept in a refrigerator overnight. The urea was removed by filtration and washed with dioxane; the combined filtrate and washings were evaporated *in vacuo*. The residue was recrystallized twice from isopropylalcohol and dried over calcium chloride *in vacuo*, yielding 1.65 g or 3.94 mmol (67%); mp 152°C.

Benzyloxycarbonyl-L-aspartyl-(β-t-butyl ester)-L-isoleucyl-L-alanine (B) [2]. A solution of the N-hydroxysuccinimide ester A (1.65 g, 3.94 mmol) in 35 ml of DME was added under mixing to a solution of L-isoleucyl-L-alanine (0.88 g, 4.35 mmol) and potassium bicarbonate (0.87 g, 8.7 mmol) in 15 ml of water. After stirring overnight the mixture was poured into 150 ml of ice-2N sulfuric acid. The resulting gum was dissolved in 60 ml of ethyl-acetate, washed with water (2x40 ml) and with 20 ml of brine; the ethyl-acetate layer was dried over natrium sulfate for 60 min, filtered through a glass filter and concentrated to 10 ml by evaporation. Dilution with 50 ml of ether and addition of hexane at 0°C resulted in a crystalline precipitate, 1.68 g or 3.2 mmol (84%), mp 94-96°C, migrating as one component in system A.

L-aspartic-(β-t-butyl ester)-L-isoleucyl-L-alanine (C) [6]. A solution of the tri-peptide B (1.53 g, 3.9 mmol) in about 200 ml of an ethanol-water mixture (in a ratio that the solution was just saturated) was treated with 300 mg of Pd/C as catalyst and hydrogen was bubbled through this suspension for two hours. The catalyst was filtered on a Celite bed and the filtrate was concentrated and dried. This yielded 1.08 g or 2.9 mmol of a white powder, homogeneous, when chromatographed in system B.

N-t-butyloxycarbonyl-L-methionyl-L-aspartic-(β-t-butyl ester)-L-isoleucyl-L-alanine (D) [2]. To a solution of C (1.08 g, 2.9 mmol) and potassium bi-carbonate (0.58 g, 5.8 mmol) in 40 ml of water a solution of 0.65 g (2.6 mmol) N-t-butyloxycarbonyl-L-methionine-N-hydroxysuccinimide ester in 70 ml of DME was added while stirring. After stirring overnight the DME was evaporated and the water layer was acidified with citric acid. The resulting

precipitate was washed with water and dried to yield 1.45 g (2.4 mmol, 92%) of a white powder, migrating as one component in system A[α]$_D^{23}$ = -35 5 (c = 1, methanol).

L-methionyl-L-aspartyl-L-isoleucyl-L-alanine (E). In order to remove the protecting groups 1.35 g of D (2.25 mmol) was treated with 90% trifluoroacetic acid at 22°C for 30 min. The solution was poured in ether, the resulting precipitate was isolated by centrifugation, dissolved in a small volume of 1 M acetic and passed through a column (15x2 cm) of Dowex-1 (CH_3COO^-) equilibrated in 1 M acetic acid. The eluent was concentrated and lyophilized, yielding 0.96 g or 2.15 mmol (96%) of a white powder. Analysis by thin layer chromatography in system A and by paper electrophoresis at pH 6 5 revealed that the material was almost homogeneous; a minor ninhydrin positive component (less than 3%) was present which migrated slower than the tetrapeptide. No attempt was made to remove this contamination.

Acetyl-L-methionyl-L-aspartyl-L-isoleucyl-L-alanine [2]. Part of the tetrapeptide E was acetylated with the aid of acetyl-N-hydroxysuccinimide ester A solution of 23.4 g (0.20 mmol) acetyl-N-hydroxysuccinimide ester in 5 ml DME was added with stirring to a solution of 100 mg of E (0 22 mmol) and potassium bicarbonate (44.6 mg, 0.446 mmol) in 3 ml of water. Stirring was continued overnight, DME was evaporated and the water layer was lyophilized.
The synthetic product appeared to have the same chromatographic and electrophoretic properties as the tetrapeptide of native α-crystallin isolated after digestion of the protein with subtilisin and Dowex-50 treatment.

5.2.3. specificity of proteolytic enzymes

With the two tetrapeptides chemically synthesized as described in section 5.2.2. the specificity of five proteolytic enzymes was tested pronase, subtilisin, thermolysin, chymotrypsin and pepsin Digestion with pronase and subtilisin was carried out as described in section 3.3.5. Thermolytic digestion was performed as described in 5.2 4. For digestion with chymotrypsin the procedure of Schoenmakers et al. [134] was followed and digestion with pepsin was according to the procedure of Tang [161].

In table V the results of these assays are summerized.
It can be concluded that only thermolysin exhibited the specificity required for identification of the acetylated as well as the unacetylated N-terminal dipeptide of α-crystallin.

Enzyme	Substrate	Product(s)
Pronase	Met-Asp-Ile-Ala	Met, Asp, Ile and Ala
Subtilisin	,,	Met, Met-Asp, Met Asp-Ile, Met-Asp-Ile-Ala
Thermolysin	,,	Met-Asp
Pepsin	,,	none
Chymotrypsin	,,	none
Pronase	Ac-Met-Asp-Ile-Ala	Ac-Met-Asp
Subtilisin	,,	none
Thermolysin	,,	Ac-Met-Asp
Pepsin	,,	none
Chymotrypsin	,,	none

Table V Specificity of proteolytic enzymes

5 2 4 **thermolysin digestion and product analysis**

Thermolysin crystals were suspended in 0 2 M ammonium acetate buffer pH 8 5 and 0 005 M $CaCl_2$ at a protein concentration of about 2 mg/ml, 0.1 M KOH was added until the enzyme was dissolved completely, the pH was adjusted to pH 8.5 with 0 1 M acetic acid The enzyme solution was stored at -20°C in small batches To protein samples, dissolved in the same buffer (0.5-1 0 ml), thermolysin was added to a final concentration of 20-50 μg/ml and digestion was performed at 37°C for 4-6 hours. After digestion the peptides were lyophilized, dissolved in distilled water and subjected to paper electrophoresis at pH 6 5 (see section 3.3 5) or at pH 4.5 in acetic acid-pyridine-water (50 50 900, by volume) When unblocked peptides had to be removed the digested material was treated with Dowex-50(H^+). Radioactivity on the electropherograms was quantitated by cutting the paper into one cm

strips which were counted in a liquid scintillation counter Reference peptides were stained for methionine using platinic iodide [45]

5 3 INITIATION OF PROTEIN BIOSYNTHESIS INHIBITED BY SPARSOMYCIN

The presence of an acetyl group on the N-terminal methionine donated by Met-tRNA^{fMet} would be detected by examination of the products formed, when sparsomycin inhibits the growth of newly initiated chains [164] For this purpose a crude cell-free system supplemented with additional lens polysomes is incubated under conditions for amino acid incorporation as described in section 3 3 2 Sparsomycin is added at a concentration of 0.14 mM in order to inhibit peptide formation (^{35}S)Met tRNA^{fMet} is used as radio active label After incubation at 30°C for 10 min the ribosomes are isolated by centrifugation through a 1 M sucrose layer, the RNA is extracted from the pellet with phenol at pH 5 0, precipitated with ethanol and digested with T_1 RNAase The products are subjected to paper electrophoresis at pH 3 8 (fig 19)

The total radioactivity has the same mobility as the oligonucleotide product of the T_1 RNAase digestion of (^{35}S)Met tRNA^{fMet} No radioactivity can be detected at the position of the T_1 digestion product of acetyl-Met tRNA^{fMet} In another series of experiments the nascent chains labeled by transfer of methionine from (^{35}S)Met-tRNA^{fMet} are treated with trimethylamine and also subjected to paper electrophoresis at pH 6 5 Apparently almost all ^{35}S label is present as methionine Hence the conclusion is justified that dipeptide formation is inhibited almost completely at the sparsomycin concentration used When (^{35}S)Met tRNA^{Met} is used under identical conditions as described for (^{35}S)Met-tRNA^{fMet} the amount of radioactivity attached to the ribosomes is negligible indicating that the process of elongation is blocked

These results demonstrate that the N-terminal acetylation of α-crystallin is not at the stage of initiation

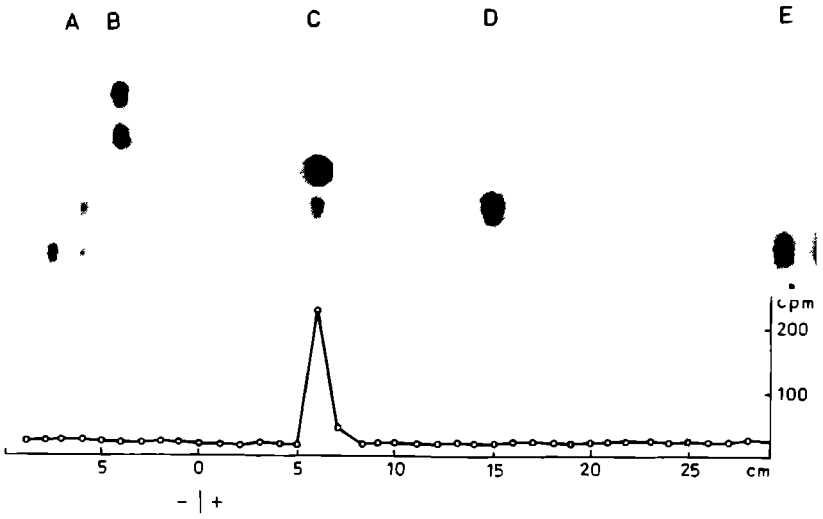

Fig 19 Electrophoresis of (^{35}S)methionine labeled T_1 RNAase digests The following samples were subjected to high voltage paper electrophoresis at pH 3 8 for 3 hours at 40 volts/cm The lower part of the figure represents the RNAase T_1 digest of (^{35}S)Met $tRNA^{fMet}$ isolated from ribosomes after incubation in a crude lens cell free system The lysate was supplemented with lens polysomes in the presence of 0 14 mM sparsomycin The upper part of the figure is an autoradiogram of the following T_1 RNAase digests (from top to bottom) (^{35}S)Met-$tRNA_2^{Met}$ (^{35}S)Met-$tRNA_1^{Met}$, (^{35}S)Met $tRNA^{fMet}$ and formyl-(^{35}S)Met-$tRNA^{fMet}$, the lowest lane represents the radioactivity distribution after electrophoresis of the alkaline treated F (^{35}S)Met $tRNA^{fMet}$ Zone A represents free methionine and methionine sulfoxide, zone B is the (^{35}S)methionyl-oligonucleotide derived from (^{35}S)Met-$tRNA^{Met}$, zone C is the (^{35}S)Met-oligonucleotide derived from (^{35}S)Met-$tRNA^{fMet}$, zone D is the F-(^{35}S)Met-oligonucleotide derived from F-(^{35}S)Met-$tRNA^{fMet}$ respectively acetyl-(^{35}S)Met-$tRNA^{fMet}$ (chemically acetylated) and zone E is the position of formyl methionine (formyl methionine sulfoxide is not visible)

5 4 STATE OF NEWLY FORMED α-CRYSTALLIN POLYPEPTIDES

Once it is concluded that acetylation is not an integral part of polypeptide chain initiation, one should find an appropriate substrate in order to investi gate the very moment of acetylation Our previous studies revealed that Met-$tRNA^{fMet}$ starts the synthesis of α-crystallin and that the N-terminal methio-

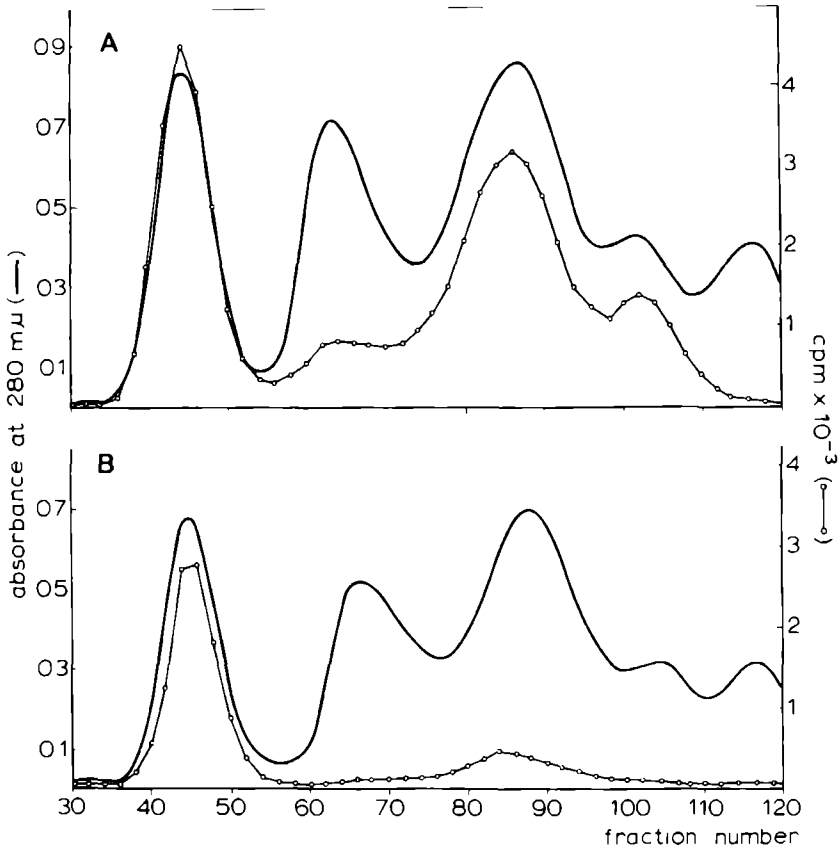

Fig 20 Gel filtration of a lens lysate on Sephadex G 200 The incubation mixture (final volume 2 ml) was passed through a Sephadex G-200 column as described in section 3 3 4 To determine the radioactivity aliquots of 0 1 ml were precipitated with 5% trichloroacetic acid, filtered on glass fiber filters and the radioactivity was counted in a liquid scintillation counter The A_{280} elution profile reflects the separation of 4 classes of crystallins according to molecular weight α-, β-high, β-low and γ-crystallin A Incubation mixture labeled with (^{35}S)methionine, B incubation mixture labeled with (^{35}S)Met-tRNA^{fMet}

nine residue, carrying an acetyl group in the native protein, is not removed during chain growth. However, the question remains whether or not α-crystallin is the only water soluble lens protein which can be labeled with (^{35}S)Met-tRNA^{fMet}.

We have incubated the cell-free system with (^{35}S)Met-tRNA^{fMet} at 30°C for 1 hour. After alkaline treatment and dialysis to remove the free (^{35}S)methionine, the incubation mixture is subjected to gel filtration on Sephadex G-200. It appears that the methionine donated by the initiator tRNA is incorporated almost exclusively into α-crystallin (fig. 20B). A very small amount of radioactivity can be detected in the β-low crystallin region, indicating that still another polypeptide in the eye lens system can be labeled with (^{35}S)Met-tRNA^{fMet}. No radioactivity can be found in the fractions of the G-200 column which should contain proteins with a molecular weight of 20,000 (the molecular weight of the individual polypeptide chains of α-crystallin [18]). This is in agreement with association experiments which show that the polypeptide chains of α-crystallin reaggregate fast and quantitatively into large complexes of about 8×10^5 daltons [17].

Fig. 20A shows the radioactivity pattern if (^{35}S)methionine is used. It can be seen that in the latter case each of the four classes of crystallins is synthesized, albeit in varying amounts.

In order to identify the radioactivity present in the α and β-low region after labeling with (^{35}S)Met-tRNA^{fMet}, the corresponding column fractions are pooled, dialyzed against water, lyophilized and subjected to polyacrylamide -6 M urea gel electrophoresis at pH 8.5. Fig. 21 shows the stained gels as well as their autoradiographs. It can be seen that both αA_2 and αB_2 are labeled, whereas no radioactivity is present in the A_1 and B_1 region of the gel. The radioactivity coinciding with the β-low crystallin fractions on the G-200 column appears to be present as a minor component amongst several other β-crystallin polypeptide chains.

These results are in accordance with data obtained from labeling experiments described in section 4.3.

Thermolysin is a useful tool for determination of the N-terminus of α-crystallin as it cleaves the N-terminal dipeptide Met-Asp in acetylated as well as in unacetylated state. Analysis of the thermolytic digestion products of α-crystallin which has been labeled with (^{35}S)Met-tRNA^{fMet} and purified on Sephadex G-200 reveals that all N-terminal methionine residues are ace-

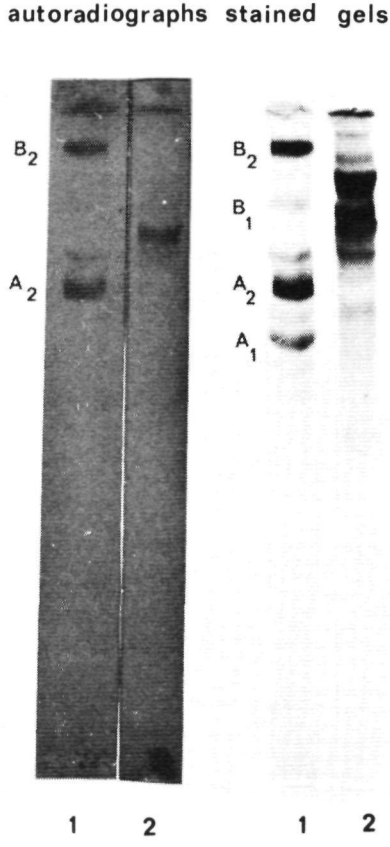

Fig.21. Electrophoretic analysis on basic polyacrylamide gels of polypeptides labeled with (^{35}S)Met-tRNA^{fMet} in the cell-free system. The lens proteins were separated on a Sephadex G-200 column (compare fig. 20); fractions of the two regions containing radioactivity (α and β-low) were pooled, dialyzed, lyophilized and electrophoresed. The dried slices of the gels were autoradiographed for three weeks. Gel 1: α-crystallin; gel 2: β-low crystallin.

tylated. No radioactivity can be detected in the Met-Asp region of the electropherogram (fig. 22A).

Therefore, since all polypeptide chains composing the α-crystallin aggregate

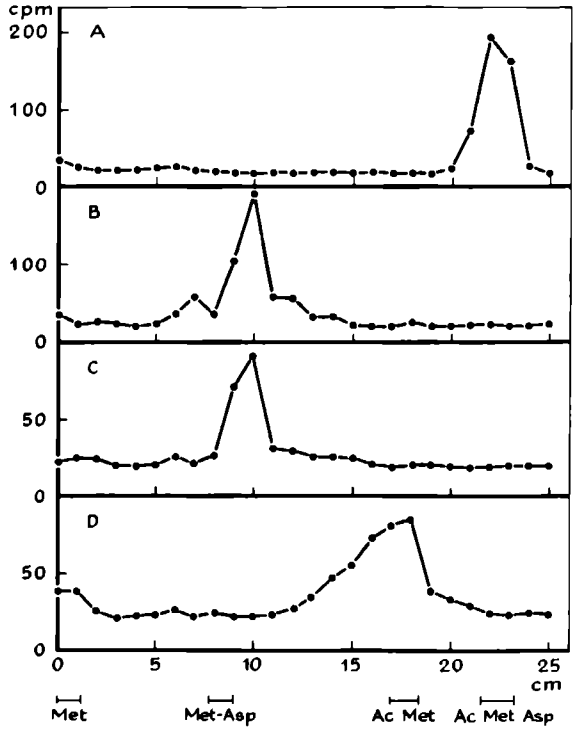

Fig 22 Paper electrophoresis of (^{35}S)Met tRNA^{fMet} labeled peptides at pH 6 5, derived from eye lens proteins The proteins were isolated by chromatography on Sephadex G 200 (see fig 20B)

A Thermolytic digestion of α-crystallin

B Thermolytic digestion of β-low crystallin

C Identical to B after digestion the peptide solution was treated with a Dowex-50 (H^+) suspension, the water layer containing NH_2-blocked peptides and amino acids was lyophilized and electrophoresed

D Identical to B, but after thermolytic digestion the peptides were subjected to digestion with pronase for 24 hours and electrophoresed

are acetylated in N-terminal position, the conclusion seems to be justified that the acetylation must have occurred before incorporation of the newly formed chains into the α-crystallin aggregate

An attempt has also been made to analyze the radioactivity present in one of the polypeptides of β-low crystallin (figs 22B, C, D) Electrophoresis at pH 6 5 reveals that after thermolytic digestion the radioactive label migrates slightly faster than the dipeptide Met-Asp (fig. 22B), the radioactivity cannot be bound to Dowex-50 (H^+) resin indicating that this peptide does not possess a free amino group (fig 22C). Exhaustive digestion with pronase, followed by paper electrophoresis results in a shift to the acetyl-methionine position (fig. 22D). This indicates that the polypeptide in question is probably a β-crystallin chain with an acetylated methionine residue in N-terminal position

5 5. N-TERMINAL ACETYLATION, A RIBOSOMAL EVENT

In order to study the possibility of acetylation during chain elongation, ribosome-bound peptides are examined for the presence of an N-terminal acetyl group After 8 min of incubation the ribosomes are isolated by centrifugation through a layer of sucrose The ribosome-bound peptides are dissociated from the ribosomes as described in section 5 2 1. The nascent chains are fractionated according to their length on a Sephadex G-25 "superfine" column The elution profile of the peptides is shown in fig 23 Fraction I consists of completed or nearly completed chains, fraction II contains peptides larger than glucagon (about 4000 M W) and fraction III consists of peptides with a molecular weight of about 3000 Radioactivity eluted behind fraction III is mainly due to free methionine and some other unidentified components The three fractions are lyophilized and the material is digested with thermolysin. To the supernatant, containing the released polypeptides, RNAase is added to destroy the remaining (^{35}S)Met-tRNA^{fMet} and the solution is dialyzed against water and also treated with thermolysin

Results of the analyses are shown in fig 24 In fig 24A the distribution of radioactivity after digestion of the completed chains is depicted. It appears that all polypeptides are acetylated at that stage Figs 24B, C, D represent the radioactivity patterns of fraction I, II and III, respectively It can be seen that going from larger to smaller peptides the amount of radioactivity in the acetyl-methionyl-aspartic acid region decreases as compared to the amount of radioactivity in the region of methionyl-aspartic acid of the electropherogram

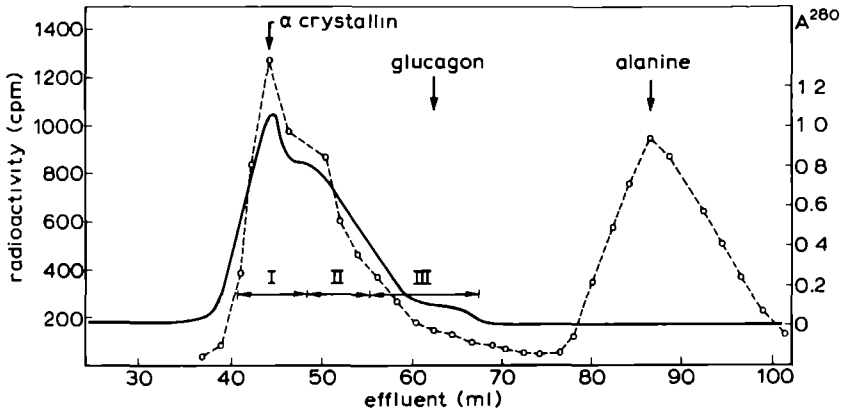

Fig.23. Elution profile of ribosome-bound peptides from a Sephadex G-25 column. Peptides and amino acids isolated from ribosomes were solubilized by alkaline treatment, applied on the column and eluted with 0.5% formic acid; 0.1 ml fractions were used for measurement of radioactivity. ——— A_{280nm}; o–o–o radioactivity. Radioactivity was derived from (^{35}S)Met-tRNA^{fMet}.

In fig. 24B the ratio Ac-Met-Asp to Met-Asp is about 3:1; in fig. 24D this ratio is about 1:1. The radioactivity migrating between Ac-Met-Asp and Met-Asp is due to degradation of acetyl-Met-Asp to acetyl-Met and the radioactivity in the position of methionine in figs. 24B, C, D is probably a result of degradation of Met-Asp. The radioactivity present in the starting zone (fig. 24A) is due to free methionine which has not been removed during dialysis. A relatively low amount of radioactivity due to N-terminal methionine is also present in the β-low crystallin region (compare fig. 20B). The enzymic studies, described in section 5.4., reveal that this radioactivity does not contribute to the radioactivity found in the Met-Asp and Ac-Met-Asp region which is exclusively derived from α-crystallin chains.

The results described justify the conclusion that N-terminal acetylation occurs during chain elongation.

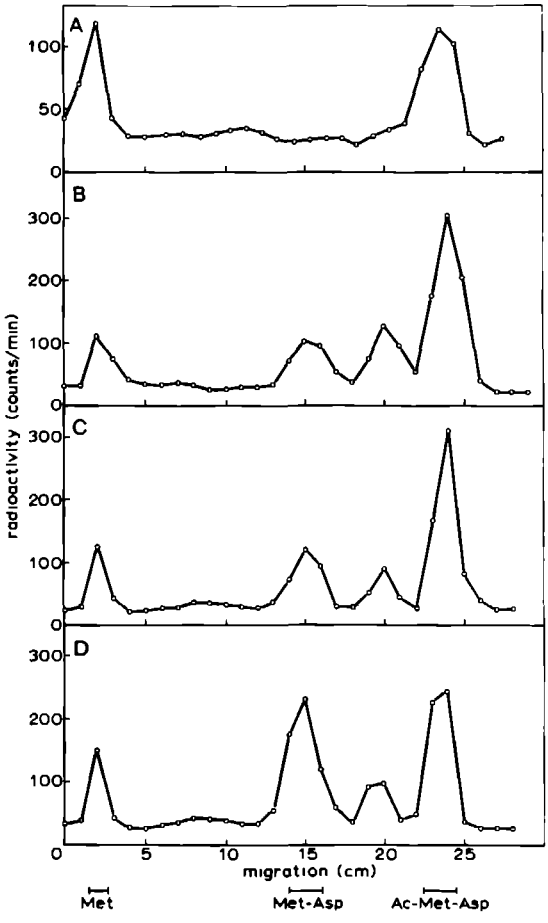

Fig.24. Electrophoretic analysis of the N-terminal dipeptides of α-crystallin after digestion with thermolysin. Five ml of a reaction mixture containing (^{35}S)Met-tRNA^{fMet} was incubated at 30°C for 8 min as described in section 3.3.2. Uncompleted chains were fractionated according to their molecular weight (compare fig. 23). Each fraction was digested with thermolysin and analyzed by high voltage paper electrophoresis at pH 6.5. A: distribution of radioactivity after digestion of completed polypeptides; B, C and D: distribution of radioactivity after digestion of the fractions I, II and III from the Sephadex G-25 column (compare fig. 23).

The results show that acetylation of the N-terminus of α-crystallin takes place after initiation and before completion of the chains In our experiments advantage is taken from the fact that despite of the use of a crude cell-free system (S-15), it is possible to label the N-terminal amino acid of α-crystallin specifically.

From our data one may also conclude that besides α-crystallin only one other component, synthesized in the cell-free system, possesses a methionine residue in N-terminal position It is known from studies on the structure of bovine eye lens crystallins that three of the four classes are blocked –presumably acetylated– N-terminally namely α ,β-high and β-low whilst γ-crystallin has a free amino acid. Taking these facts into consideration one may argue that N-terminal acetylation is not dependent on a single (N-terminal) amino acid (i c methionine). Apparently a more complicated signal e.g. a certain amino acid sequence or a secondary structure is required

Acetylation of α-crystallin which occurs during chain elongation suggests that this process is ribosome-bound This does not necessarily exclude the occurrence of a soluble enzyme catalyzing acetylation whether or not in the presence of ribosomes In this connection it has to be mentioned that we have not succeeded to enzymatically acetylate neither the dipeptide Met-Asp nor the chemically synthesized tetrapeptide Met-Asp-Ile-Ala. For this purpose the crude lens lysate has been used as enzyme-source and (^{14}C)acetyl-CoA as a radioactive precursor It is also impossible to acetylate in this manner the CNBr fragment representing the N-terminal polypeptide of α-crystallin (12,000 M.W.) with aspartic acid as N-terminal amino acid.

Since the N-terminal methionine residue, which becomes acetylated during peptide growth, is not removed from the chains of α-crystallin this protein has to be considered as a rather unique one At the moment at least four different amino acids other than methionine are known which may occur N-terminally acetylated (table VI). Assuming that the same initiation mechanism with Met-tRNA^{fMet} is operative in the synthesis of all proteins on 80S ribosomes, it would seem that removal of the N-terminal methionine residue by a special ribosome-bound amino-peptidase generally occurs before the growing peptide chain becomes accessible to acetylation However, cleavage of the methionine residue may be prevented by the nature of the adjacent amino acid residue(s)

1.	Hemoglobin fetal F_1 (human)	Ac-Gly-His-Phe	[139]
2.	Ovalbumine	Ac-Gly-Ser-Gly	[108]
3.	Cytochrome c (chicken)	Ac-Gly-Asp-Ile	[28]
4.	Cytochrome c (human)	Ac-Gly-Asp-Val	[103]
5.	Hemoglobin (carp)	Ac-Ser-Leu-Ser	[64]
6.	Histone F2a2 (calf thymus)	Ac-Ser-Gly-Arg	[120]
7.	Myosin (rabbit muscle)	Ac-Ser-Ser-Asp	[115]
8.	Apoferritin (horse spleen)	Ac-Ser-Ser-Gln	[158]
9.	Melanocyte-stimulating hormone (pig)	Ac-Ser-Tyr-Ser	[60]
10.	TMV coat protein	Ac-Ser-Tyr-Ser	[51]
11.	Cytochrome c (wheat)	Ac-Ala-Ser-Phe	[152]
12.	Enolase (rabbit muscle)	Ac-Ala-Gly-Lys	[168]
13.	Keratin (sheep)	Ac-Ala-Cys-Cys	[61]
14.	Fibrinopeptide (bovine)	Ac-Thr-Glu-Phe	[50]
15.	Lactate dehydrogenase (dogfish)	Ac-Thr-Ala-Leu	[1]
16.	α Crystallin (bovine)	Ac-Met-Asp-Ile	[65]
17.	TYMV coat protein	Ac-Met-Glu-Ile	[126]
18.	Tropomyosin (rabbit muscle)	Ac-Met-Asp-Ile	[149]

Table VI. N-terminal sequence of acetylated proteins

which may determine the specificity of the splitting enzyme. In this connection it has to be mentioned that in *E.coli* a ribosome-bound aminopeptidase cleaves all methionine dipeptides tested with exception of Met-Asp [100]. The only other hitherto known N-terminal acetylated methionine residues occur in the coat protein of TYMV particles [126] and in tropomyosin from rabbit muscle [149]. It is remarkable that the corresponding N-terminal tripeptides are quite similar to N-acetyl-Met-Asp-Ile derived from α-crystallin.

Table VI shows that of the twenty amino acids only five occur in the N-acetylated state At this moment it is impossible to find a link between N-terminal acetylation and the function of the proteins listed One explanation for the fact that α- and presumably β-crystallins are acetylated could be that N-terminal acetylation warrants protection against enzymic degradation. In general a plausible explanation for N-terminal acetylation may be derived from the theory of molecular evolution On this basis it is reasonable to

assume that neutralization of the terminal NH_3^+-group results in an advantagous conformation in contrast with the unblocked state.

Acrylamide	Union Chimique Belge
Amido Black	E. Merck AG
labeled amino acids	The Radiochemical Centre, Amersham
unlabeled amino acids	Sigma
ATA	E. Merck AG
ATP (dipotassium salt)	C.F. Boehringer & Soehne
ApUpG	Miles Laboratories Inc.
N-t-Boc-L-Met-N-hydroxy-succinimide ester	Bachem (Switzerland)
Carboxypeptidase A	Sigma
Chymotrypsin	BDH Chemicals Ltd
Creatine phosphate	C.F. Boehringer & Soehne
Creatine phosphokinase	C.F. Boehringer & Soehne
Cycloheximide	Koch-Light Laboratories Ltd.
Dowex-50	Fluka AG
Ethylenimine	Fluka AG
GTP (dipotassium salt)	C.F Boehringer & Soehne
HEPES	BDH Chemicals Ltd
L-Ile-L-Ala	Bachem (Switzerland)
Leucovorin	Lederle Laboratories Div.
N,N'Methylene-bisacrylamide	Schuchardt, Munchen
Nonidet P-40	Boom NV, Meppel, Netherlands
Pepsin	Sigma
Phenol	E. Merck AG
Pronase P	Serva
SDS	Sigma
Sephadex	Pharmacia
Subtilisin	Nutritional Biochemical Corp.
Thermolysin	Calbiochem
T_1 RNAase	C.F. Boehringer & Soehne
Trypsin	Worthington Biochemical Corp.
Z-L-aspartic acid-β-butyl ester	Bachem (Switzerland)

SUMMARY

An attempt has been made to verify whether or not the biosynthesis of the lens protein α-crystallin follows the pathways established for other mammalian proteins α-Crystallin has been chosen for a number of reasons

a) This protein is built up by four related polypeptide chains each of which containing two methionine residues One methionine is located in the chain, the other one in N-terminal position.

b) The N-terminal methionine is acetylated

The study of initiation and N-terminal acetylation are the main topics of this thesis.

The experimental approach was as follows A cell-free extract has been prepared from those parts of the calf eye lens which have biosynthetic activity. This preparation contains all factors required for protein biosynthesis. In fact only cell debris and nuclei have been removed.

The study of initiation demands a specific tRNA species which can be loaded with methionine. This tRNA has also been isolated from eye lenses. In addition two other methionyl-tRNAs have been found which promote the incorporation of methionine into polypeptide chains The three methionyl-tRNAs can be separated on special columns The properties of the three tRNAs are similar to methionyl-tRNAs from other eukaryotic sources. After labeling with (^{35}S)methionine of high specific radioactivity the three tRNAs have been used to transfer the label to α-crystallin chains

The initiator tRNA (Met-tRNA^{fMet}) can be formylated with the aid of an enzyme isolated from *E.coli*. Using the formyl-(^{35}S)Met-tRNA^{fMet} as radioactive precursor in the lens lysate it has been possible to show that the N-terminus of α-crystallin becomes labeled and contains the N-terminal dipeptide formyl-Met-Asp At low magnesium ion concentration (5 mM) the initiator tRNA acts exclusively as methionine donor for N-terminal positions

The two other methionyl-tRNAs insert the amino acid only internally This has been demonstrated by analysis of newly synthesized α-crystallin polypeptides. The conclusion has been drawn that the biosynthesis of α-crystallin occurs in analogy with other eukaryotic proteins However, the N-terminal methionine is not split off after termination and release of the polypeptides It has further been observed that the N-terminal acetylation of α-crystallin takes place while the peptide chains are still on the ribosomes. No obvious

relation exists between acetylation and the process of peptide chain initiation. Additional results of the present studies are:

- A cell-free extract from rabbit reticulocytes programmed with lens messenger RNA is capable of acetylating N-terminally newly synthesized α-crystallin polypeptides. This suggests that in general eukaryotic systems may acetylate proteins provided that these proteins have certain (hitherto unknown) characteristics.
- In the eye lens a β-crystallin polypeptide is synthesized which also carries an N-terminally acetylated methionine.
- The αA_1 and αB_1 chains are practically unlabeled even in cases when very highly radioactive precursors have been used in the incubation mixtures. Since for both kinds of chains no messengers have been detected and the amino acid content and sequence of B_2 and B_1, and A_2 and A_1 respectively are very similar it has been suggested that B_1 arises from B_2 and A_1 from A_2 after synthesis.
- The specificity of the initiator tRNA is lost at magnesium ion concentrations higher than 5 mM.
- The synthesis of the β-high crystallin fraction takes place to a much lesser extend than the synthesis of the other water-soluble lens proteins. The latter protein has some features in common with β-low crystallin.

In het onderzoek beschreven in dit proefschrift is getracht de biosynthese van het structurele ooglenseiwit α-crystalline te plaatsen in het algemene kader van biosynthetisch onderzoek, verricht aan andere dierlijke eiwitten. De keuze is gevallen op α-crystalline omdat, behalve het feit dat dit eiwit goed gekarakteriseerd is, het vanuit biosynthetisch oogpunt enkele aspecten bezit die bijzonder interessant zijn:

a) α-Crystalline is opgebouwd uit 4 verwante polypeptideketens die elk twee methionine residuën bevatten, één N-terminaal en het andere in de polypeptideketen.
b) Het N-terminale methionine is geacetyleerd.

Bestudering van de initiatie van de biosynthese en de acetylering vormen de belangrijkste onderwerpen uit dit proefschrift.

De experimentele benadering is als volgt: Uit die gedeelten van de kalfsooglens, die biosynthetisch actief zijn, is een celvrij extract bereid. Dit lysaat bevat alle componenten die nodig zijn voor eiwit synthese; slechts de celwanden en eventuele celkernen zijn verwijderd.

Voor het bestuderen van de initiatie is een initiator tRNA specifiek voor methionine nodig. Dit tRNA is eveneens geïsoleerd uit runderooglenzen. Deze blijken nog twee andere tRNAs te bevatten die de inbouw van methionine in de peptideketen bewerkstelligen. Met behulp van kolomchromatografie kunnen de verschillende tRNAs gescheiden worden. Aan de hand van gegevens bekend over methionyl-tRNAs geïsoleerd uit andere dierlijke systemen kan worden geconcludeerd dat de Met-tRNAs verkregen uit lenscellen wat hun biologische activiteit betreft, gelijk zijn aan die uit andere weefsels. De tRNAs worden met radioactief (^{35}S)methionine van hoge specifieke activiteit beladen. Met behulp van deze radioactieve Met-tRNAs kunnen α-crystallineketens worden gemerkt.

Het blijkt mogelijk het initiator tRNA (Met-tRNA^{fMet}) enzymatisch te formyleren met behulp van transformylase geïsoleerd uit *E.coli* bacteriën. Na "labeling" van het lenslysaat met dit formyl-(^{35}S)Met-tRNA^{fMet} kan worden aangetoond dat α-crystalline N-terminaal gemerkt is en het dipeptide formyl-(^{35}S)Met-Asp bevat. Verder onderzoek brengt aan het licht dat bij lage Mg-ionenconcentratie (5 mM) methionine afkomstig van initiator tRNA uitsluitend als NH_2-terminaal aminozuur in α-crystalline aanwezig is. De twee ande-

re Met-tRNAs plaatsen hun methionine alleen intern in de peptideketens Dit kan worden aangetoond na analyse van de tryptische peptiden.
De conclusie is dan ook dat de biosynthese van α-crystalline precies verloopt volgens het stramien bekend uit de gegevens van onderzoek aan de synthese van andere eukaryotische eiwitten, het N-terminale methionine residue, afkomstig van Met-tRNA^{fMet}, wordt echter in het geval van α-crystalline niet verwijderd
Verder kan worden vastgesteld dat N-terminale acetylering van α-crystalline plaats vindt terwijl de groeiende polypeptideketens zich nog op het ribosoom bevinden De N-terminale acetylering staat echter los van de initiatie
Bovendien levert het onderzoek de volgende resultaten op
- Een celvrij extract van konijnen-reticulocyten, voorzien van lensboodschapper RNA, is in staat nieuw gevormde α-crystalline polypeptiden N-terminaal te acetyleren. Dit leidt tot de suggestie dat elk eukaryotisch systeem eiwitten kan acetyleren, mits deze eiwitten, vooralsnog onbekende, intrinsieke kenmerken bezitten
- In de ooglens wordt een β-crystalline polypeptideketen gesynthetiseerd die N-terminaal een geacetyleerd methionine residue draagt
- Zelfs bij zeer hoge "labeling" van de lenseiwitten, zowel in het celvrije extract als in intacte cellen, worden de α-crystalline polypeptiden A_1 en B_1 weinig of niet gemerkt Aangezien voor deze polypeptiden geen messenger RNAs zijn gevonden en de aminozuursamenstelling en -sequentie van respectievelijk B_2 en B_1, en A_2 en A_1 grote overeenkomsten vertonen, lijkt het aannemelijk dat B_1 en A_1 ontstaan uit B_2 en A_2 als gevolg van veranderingen die optreden na de synthese
- De specificiteit van het initiator tRNA gaat verloren in de celvrije incubatie bij magnesium-ionenconcentraties boven 5 mM.
- De synthese van de polypeptideketens voorkomend in β-high crystalline vindt in geringe mate plaats terwijl dit eiwit wat peptidesamenstelling betreft enkele overeenkomsten vertoont met β-low crystalline, dat wel normaal gesynthetiseerd wordt

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STELLINGEN

I

Bij het onderzoek naar de vorming van een initiatiecomplex met eukaryotische ribosomale subeenheden is ten aanzien van het in fase brengen van het mRNA op het ribosoom ten onrechte analogie verondersteld met het mechanisme in prokaryoten.

D.P. Weeks, D.P.S. Verma, S.N. Seal, and A. Marcus, Nature, **236**, 167-168 (1972)

II

Het fragmentatiepatroon van bacteriofaag fd-RF DNA, verkregen na digestie met het endonuclease uit *H.Influenzae* (H-I), zoals beschreven door Takanami en Kojo, sluit de aanwezigheid van twee verschillende restrictie-enzymen niet uit.

M. Takanami, and H. Kojo, FEBS Lett., **29**, 267-270 (1973)

III

De zuiverheid van trypsine na chromatografie over ovomucoid-Sepharose is door Feinstein onvoldoende aangetoond.

Gad Feinstein, FEBS Lett., 7, 352-355 (1970)

IV

De 'rapid essay' van Smith en Wigle om de initiatie van de eiwitsynthese te meten kan in het algemeen niet als snel worden gekwalificeerd; bovendien is de methode slechts in een enkel zeer specifiek geval bruikbaar.

A.E. Smith, and D.T. Wigle, Eur.J.Biochem., **35**, 566-573 (1973)

V

De experimentele gegevens op grond waarvan Fager en medewerkers beweren dat retinaldehyde in rhodopsine is gebonden aan lysine zijn onvoldoende, omdat slechts 20% van de chromophoor kan worden geïdentificeerd als N-retinyllysine; bovendien kan onder hun experimentele omstandigheden het gebruik van natriumcyanoboorhydride als reductiemiddel aanleiding geven tot denaturatie van rhodopsine.

R.S. Fager, P. Sejnowski, and E.W. Abrahamson, Biochem.Biophys.Res.Commun., 47 1244-1247 (1972)

VI

Het is onwaarschijnlijk dat de reproductie van RNA-tumorvirussen in het mitochondrion plaats vindt.

J. Kára, M. Dvorák, and H. Cerná, FEBS Lett., **25**, 33-37 (1972)
N.J. Richert, and J.D. Hare, Biochem.Biophys.Res.Commun., **46**, 5-10 (1972)

VII

De bewering van Tyuma en medewerkers dat uit hun analyse van het oxygenatie-evenwicht van hemoglobine onder invloed van diphosphoglyceraat volgt, dat het derde en vierde zuurstofmolecuul simultaan worden gebonden, moet gezien hun eerdere resultaten als 'wishful thinking' worden beschouwd.

I. Tyuma, K. Imai, and K. Shimizu, Biochem., **12**, 1491-1498 (1973)

VIII

Het is door Williamson en medewerkers onvoldoende bewezen dat hoog moleculair RNA van muizen-erythroblasten transleerbare messengergedeelten bevat of vormt.

R. Williamson, C. Drewienkiewicz, and J. Paul, Nature New Biol., **241**, 66-68 (1973)

IX

Zolang deskundigheid niet voor iedereen in voldoende mate beschikbaar is en de (op zich zelf juiste) bezwaren tegen beleidsvoornemens worden afgedaan met een vraag naar een alternatief, zal de democratisering niet van de grond komen.

X

Het succes van Wiegels campagne ten aanzien van de lastenverzwaring voor de middengroepen berust op het feit dat het grootste deel van de Nederlandse bevolking zich in enigerlei opzicht met Wiegels middengroep kan identificeren.

Nijmegen, 25 oktober 1973 G.J.A.M. Strous