THE POLYPEPTIDE CHAIN COMPOSITION OF
β CRYSTALLIN

P. HERBRINK

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INTRODUCTION

The vertebrate eye lens is an avascular tissue surroun-
ded by a capsule.It is composed of an outer,single layer of
epithelial cells on the anterior side of the organ,a zone of
differentiation at the equator containing cells that are de-
veloping into fiber cells and fiber cells in the inner part
of the lens (fig.1.1).The epithelial layer can be divided in-
to two zones:the germinative zone containing cells with a high
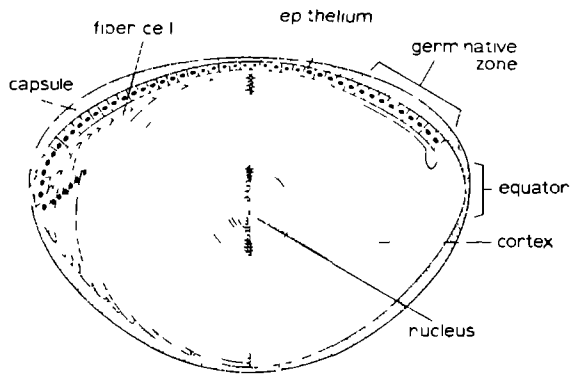
Fig.1.1 Schematic drawing of the vertebrate eye lens.
(picture taken from Van Kleef,1975)

rate of mitosis and the central zone with cells with a low

mitotic rate.At the equator dividing cells are elongating to
fiber cells which are laid down layer upon layer during the
life of the animal.During the process of elongation fiber
cells loose their nuclei.

Since Mörners (1894) classification of the water-soluble
eye lens proteins in α-,β- and γ-crystallin,much work has been
done on the characterization of the crystallins.As very recent-
ly an excellent review on the structural proteins of the mam-
malian lens has been published (Harding and Dilley ,1976),
only a brief survey on α- and γ-crystallin will be given.

α-Crystallin,the most thoroughly studied soluble lens
protein,is a population of aggregates,having mainly a molecu-
lar weight of 8 x 10^5 dalton and $S_{20,w}$ values ranging from
16 to 24 S (Bloemendal et al.,1972a).About 40 % of the wa-
ter-soluble eye lens proteins consists of α-crystallin (Van
Kamp,1973).The iso-electric point of α-crystallin is approxi-
mately 5 (Bloemendal and Ten Cate,1959, Bours,1971, Van Kleef,
1975).It is now well accepted that α-crystallin is built up
by four polypeptide chains,termed αA_1,αA_2,αB_1 and αB_2 (Schoen-
makers et al.,1969,Van Kamp et al.,1971) with a molecular
weight of approximately 20,000 dalton (Spector et al.,1971a,
Bloemendal, 1972b).The primary structure of the αA_2 and αB_2
chain has been elucidated by Van der Ouderaa et al., (1973,1974a).
Both chains have an acetylated N-terminal methionine (Hoenders
and Bloemendal,1967).A homology of 57 % was found between the
αA_2 and αB_2 chain (Van der Ouderaa et al.,1973,1974a).O.D. mea-

surements revealed that there was less than 0.01 % α-helical
structure in both native α-crystallin and its subunits and
that approximately 55-63 % of the total conformation consists
of β-structure (Li and Spector,1974).

The αA_1 chain,which has the same molecular weight and
amino acid composition as the αA_2 chain,is believed to origi-
nate from the αA_2 chain by deamidation (Bloemendal et al,1972c,
Stauffer et al.,1974).There are some indications that the con-
version of αA_2 to αA_1 is a two step deamidation process (Van
Venrooy et al,1974).The same amino acid composition and mole-
cular weight has also been found for the αB_1 and αB_2 chains.
It is,therefore,likely that the αB_1 chain originates from the
αB_2 chain by deamidation.However,until now no definite proof
has been given.

α-Crystallin is subject to several post-synthetic modi-
fications.Low molecular weight α-crystallin is converted to
high molecular weight α-crystallin(Spector et al.,1971b,Van
Kleef,1975).The subunits of α-crystallin are exposed to de-
amidation and specific degradation processes (De Jong et al,
1974,Van Kleef 1975).

γ-Crystallin,the crystallin of lowest molecular weight
(approximately 20,000 dalton) and the highest iso-electric
point (7.1 to 8.1,Bours,1971),comprises about 20 % of the
water-soluble proteins of calf lens (Björk,1961).It is found
in larger amounts in the nucleus than in the cortex.Björk
(1964a) separated calf γ-crystallin into four fractions by

ion-exchange chromatography on sulpho-ethyl-Sephadex.All frac-
tions have a free N-terminal amino acid (glycine), a low
content of hydrophobic residues and a high content of cysteine.
The primary structure of the calf γ -crystallin fraction II
has been elucidated by Croft (1972).

β-Crystallin,the fraction with a molecular weight inter-
mediate between α- and γ-crystallin,has much less thoroughly
been investigated.Initially β-crystallin was believed to be a
single protein (Hesselvik,1939,Viollier et al.,1947),as a re-
sult of studies using free electrophoresis.Electrophoresis on
paper(François et al.1953) showed a separation into two com-
ponents.Ultracentrifugal studies (Björk, 1960) yielded at
least two fractions.Immunoelectrophoretical studies of β-crys-
tallin (Manski,1961) revealed 3 to 5 bands.Spector (1964,
1965) fractionated β-crystallin by chromatography on DEAE-
cellulose,resulting in mainly four fractions.These fractions
had similar amino acid compositions with the exception of ma-
jor differences in methionine.Björk (1964b) reported the frac-
tionation of β-crystallin by gel filtration on Sephadex G-100.
Four fractions were obtained.Each fraction showed,in addition
to some minor peaks,one major peak in the ultracentrifuge.The
sedimentation coëfficients were 13.6 , 9.6 ,4.9 and 4.2 S,res-
pectively.On the basis of immunoelectrophoresis none of the
fractions was homogeneous.

Testa et al.(1965) separated β-crystallin into two
fractions B and C by gel filtration on Biogel P-300.Immuno-

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electrophoresis of β-crystallin yielded at least eight bands.
Fraction B and C were heterogeneous as judged by free electro-
phoresis.Sedimentation analyses of fraction B gave a symmetri-
cal peak.The sedimentation coëfficient decreased when the pH
was lowered from 8.2 to 7.1 and 5.0 ,the values being 8.2 S ,
7.9 S and 4.4 S respectively.After readjusting the pH from 5.0
to 7.1 ,the sedimentation coëfficient remained unaltered.Immu-
noelectrophoresis and immunodiffusion experiments showed the
presence of at least 5 clearly distinguishable precipitation
bands.Fraction B turned out to be oxidation sensitive.Sedimen-
tation analysis of fraction C revealed a single symmetrical
peak with sedimentation coëfficients of 3.4 S at pH 5.0 ,3.4 S
at pH 7.1 and 3.8 S at pH 8.2. Immunoelectrophoresis showed
the presence of at least 3 precipitation bands.By chromatogra-
phy on SE-Sephadex an incomplete separation of fraction B into
six subfractions was obtained.

A further fractionation of fraction B was achieved by
Armand et al.(1970) using an organo mercurial polysacharide
Sephadex column.Three fractions were obtained.The first two
fractions to be eluted,designated bB_1 and bB_2 ,with molecular
weights of 118,000 and 147,000 dalton respectively,appeared
to be homogeneous in immunoelectrophoretical and ultracentri-
fugal studies.Optical rotatory dispersion and infra red stu-
dies indicated the absence of α-helical structure and the pos-
sible presence of some β conformation in both proteins.Harmsen
et al.(1966) observed that the β-crystallins consist of a

mixture of α-helix,random coil and an unknown structure,pro-
bably β-conformation.

β_s-Crystallin,a low molecular weight β-crystallin,was
first isolated by Van Dam (1966).This compound,as well as
another β-crystallin,called β_2-crystallin (Van Dam,1968),
was purified from the γ-crystallin fraction,which was obtai-
ned after chromatography of cortical lens extract on Sephadex
G-75.Both β_s and β_2 were considered to belong to β-crystallin,
since the proteins nad a higher electrophoretic mobility than
the γ-crystallins and a blocked N-terminal amino acid,which
is common to the other β-crystallins (Mok and Waley ,1968).
The molecular weignt as calculated from tne amino acid compo-
sition was found to be 28,000 dalton for both proteins (Van
Dam,1966).Bours (1973) reported an iso-electric point of
7.0 for β_s-crystallin.Tne N-terminal residue of bovine β_s-
crystallin has been claimed to be N-acetyl-tryptophan (Croft,
1973).It has been suggested that β_s-crystallin plays a role
in lens development (Harding and Dilley ,1976).

According to Bours (1971) tne β-crystallins nave iso-
electric points between 5.7 and 7.0.

Upon addition of dissociating reagents like urea,guani-
dine-HCl or sodium dodecyl sulphate,the β-crystallins,like α-
crystallin,drop in molecular weight to an average of 25,000
dalton (Bont et al.,1962 ,Bloemendal et al.,1962).A similar
result is obtained after lowering the pH below 3.

Spector and Katz (1966) obtained six fractions with

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different amino acid composition by chromatography of β-crystallin on DEAE-cellulose in the presence of 6 M urea.However, no evidence was presented that the six fractions contained homogeneous polypeptides.

Zigler and Sidbury (1973) investigated the polypeptide composition of fraction B and C ,isolated by chromatography of lens extract on Sephadex G-200.Fraction C had a molecular weight of approximately 52,000 dalton.Sodium dodecylsulphate gel electrophoresis yielded two bands with molecular weights of approximately 24,000 and 27,000 dalton.After blocking the sulfhydryl groups with N-ethylmaleimide,polyacrylamide gel electrophoresis in the presence of 6 M urea yielded eight distinct polypeptide chains.The molecular weight of fraction B was calculated to be approximately 210,000 dalton.Sodium dodecylsulphate gel electrophoresis of this fraction revealed four bands.More than 80 % of the protein material was of either 24,000 or 27,000 molecular weight.Two lesser components had a molecular weight of approximately 31,000 and 35,000 dalton.After blocking the sulfhydryl groups with N-ethylmaleimide, electrophoresis in the presence of 6 M urea yielded eight bands corresponding in mobility to the bands found for fraction C, plus at least five other bands with a lower mobility.On the basis of these figures Zigler and Sidbury suggest that fraction C exists as a dimer in the native state,whilst fraction B consists of eight polypeptide chains.Further each native molecule of fraction B is thought to contain one polypeptide chain from

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the 31,000 or 35,000 molecular weight species.

Most of the work published on β-crystallin aimed to isolate a homogeneous β-crystallin aggregate,which,however, seems to be a rather difficult problem,since all β-crystallin aggregates are apparently closely related.

Only a few reports have been presented on the polypep-tide composition of β-crystallin (Spector and Katz,1966, Zigler and Sidbury,1973).Knowledge of the polypeptide com-position of α-crystallin (Schoenmakers et al.,1969,Van Kamp et al.,1971) and elucidation of their primary structure (Van der Ouderaa et al.,1973,1974a) has been of great help instudying the biosynthesis of the αA_2 chain.Exact data con-cerning the polypeptide composition of β-crystallin and che-mical characterization of these chains therefore is also a prerequisite for biosynthetical studies on this class of crys-tallins.

The aim of the present investigations was to characte-rize β-crystallin by its polypeptide composition and the elu-cidation of the primary structure of the principal basic β-chain (βB_p).The latter study represents the first endeavour of sequence determination of the β-crystallins.

CHAPTER II

ISOLATION AND PARTIAL CHARACTERIZATION OF β_H AND β_L

Several methods for the isolation of soluble lens pro-
teins have been developed.Originally α-crystallin was prepa-
red by iso-electric precipitation,β-crystallin by salting out
procedures,whilst γ-crystallin comprised the remaining soluble
protein (Woods et al.,1927, Burky et al., 1928a, Burky et al.,
1928b).Björk (1964b) applied gel filtration on Sephadex G-75,
followed by column electrophoresis on cellulose to isolate β-
crystallin.Bloemendal and Ten Cate (1959) used starch-block
electrophoresis for the separation of the soluble lens proteins.
Other techniques described are: chromatography on DEAE-cellulo-
se (Spector, 1960, Papaconstantinou et al.,1962, Björk,1963,
Spector,1965);precipitation in the presence of Zn-ions fol-
lowed by gel filtration on Sephadex G-75 (Spector,1964);con-
tinuous flow electrophoresis (Woods and Burgess,1961, De Groot
et al., 1970) and zonal centrifugation followed by gel filtra-
tion (Bloemendal et al.,1972a).However,the most convenient
way to separate the water-soluble lens proteins is gel filtration
on Sephadex G-200, Biogel P-300 or Biogel A5M (Testa et al.,
1965, François et al., 1965, Van Dam, 1967, Schoenmakers et al.,

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1968,Hoenders and Van Kamp, 1972).These fractionation pro-
cedures yield four rather well-defined fractions,namely α-,
β_H-,β_L- and γ-crystallin.On Biogel A5M an additional peak
(very high molecular weight α-crystallin) is observed (Van
Kamp, 1973, Van Kleef,1975).We prepared β-crystallin by gel
filtration of lens extract on Sephadex G-200 according to
Van Dam (1967) with some modifications.

2.1. METHODS

2.1.1. Preparation of lens extract.

        The eyes of 2-3 month old calves were obtained fresh
from the slaughterhouse and the lenses removed.After removal
of the epithelial cells the lens cortices were dissolved in
distilled water by gentle stirring during one hour.The nuclei
were decanted and the remaining turbid solution was centrifu-
ged at 15,000 x g for 20 minutes.The clarified solution was
dialyzed against distilled water and lyophilized.The dry ma-
terial,referred to as lens extract,was stored at -20° C.

2.1.2.Purification of β-crystallin.

        Purification of β-crystallin was achieved by gel fil-
tration of the lens extract on Sephadex G-200 (Van Dam, 1967).
The swollen material was equilibrated with 0.1 M Tris-HCl,
pH 7.5 ,containing 1 M NaCl and 0.005 M EDTA. Samples contai-
ning 500 mg lyophilized lens extract in a volume of 5 ml were

applied onto the top of the column (100 x 4 cm).The elution
rate was 20 ml per nour.The effluent was monitored at 278 nm
with an LKB Uvicord II.Occasionally the absorbance at 280 nm
was determined in a Beckman DB spectrophotometer.The fractions
were dialyzed against distilled water,lyophilized and stored
at -20° C.

2.1.3.Amino acid analysis.

Amino acid analysis was conducted on a Beckman Multi-
chrom automatic amino acid analyzer using a single column.
Samples of protein (0.2 mg) were hydrolyzed under vacuum
at 110° C with 6 N HCl for 24, 48 and 72 hours.Cysteine was
determined after carboxymethylation with monoiodoacetic acid
(Schoenmakers et al., 1969).Tryptophan was measured accor-
ding to Benzce and Schmid (1957).

2.1.4.Sedimentation analysis.

Sedimentation coëfficients of the two β-crystallin frac-
tions were determined in a Spinco model E analytical ultra-
centrifuge.Ultraviolet adsorption optics at 280 nm in combi-
nation with an automatic scanner were used.The sedimentation
coëfficients of the top fractions of the two β-crystallins
were determined in 0.1 M Tris-HCl, pH 7.5 ,containing 1 M NaCl
and 0.005 M EDTA.

2.1.5. Immunoelectrophoresis.

Immunoelectrophoresis was carried out on 3 % agar gels essentially according to Scheidegger (1955),using Veronal buffer ,pH 8.6 ,$\Gamma/2$= 0.08. The antiserum against cortical lens extract was prepared in rabbits according to Dandrieu (1972). The first day 1 mg of protein,dissolved in 1 ml of 0.9 % NaCl, was mixed with 1 ml of complete Freund's adjuvant and 0.5 ml portions were injected subcutaneously under the shoulder blades and in the flanks.The third day 2 mg of protein,dissolved in 1 ml of 0.9 % NaCl,was mixed with 1 ml of complete Freund's adjuvant,followed by injection of 0.5 ml portions as described for the first day.The fifth day the procedure of the first day was repeated.After 20 days the rabbits were bled and the antiserum was collected after centrifugation.

2.1.6. Polyacrylamide gel electrophoresis.
2.1.6.1. Basic polyacrylamide gel electrophoresis.

Polyacrylamide gels at pH 8.5 containing 6 M urea were prepared according to Bloemendal (1967).The final acrylamide concentration was 7.5 %. 8.6 g urea, 3 ml of N,N,N',N'-tetramethylethylenediamine (9.46 % v/v in 0.0184 M Tris-glycine buffer,pH 8.5), 6 ml acrylamide-bisacrylamide (30-0.8 %) and 3 ml of a 0.03 % solution of potassium ferricyanide were mixed with water to a final volume of 22.1 ml.After deaeration 1.9 ml of a 1 % ammonium persulphate solution was added.The solution was poured into cylindrical tubes (9 x 0.6 cm) and allowed to

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polymerize for 30 minutes.The electrophoresis buffer used was

0.0184 M Tris-glycine,pH 8.5,containing 6 M urea.To the buffer

in the catnodal appartment dithioerythritol was added to a fi-

nal concentration of 0.04 %.After a pre-electrophoretic run of

1 hour,samples,dissolved in electropnoresis buffer containing

dithioerythritol and 10 % sucrose,were layered on top of the

gels.The gels were run for 30 minutes at 100 V,followed by 200

V for four and a half hours.After removal of the gels from the

tubes,they were stained according to the method of Malik and

Berry (1972).

2.1.6.2.Sodium dodecylsulphate gel electrophoresis.

        Sodium dodecylsulphate gel electrophoresis was performed

according to Laemrli (1970).Usually 13 % acrylamide gels were

prepared.However,in a few cases 10 % acrylamide has been used.

Eleven ml of an acrylamide-bisacrylamide solution (30-0.8 %)

(when 10 % gels were prepared 8.3 ml was used),6.25 ml of 1.5 M

Tris-HCl,pH 8.8,0.125 ml 10% N,N,N',N'-tetramethylethylenedi-

amine and 7.3 ml distilled water (10 ml for 10 % gels) were

mixed and deaerated.0.025 ml of a 10 % sodium dodecylsulphate

solution and 0.075 ml 10 % ammonium persulpnate solution were

added and the mixture was poured into siliconated glass tubes

(9 x 0.6 cm).The gels were then layered with a small volume

of a 0.1 % sodium dodecylsulphate solution on top.After poly-

merization 200 μl of a stacking gel solution,containing 1.0

ml acrylamide-bisacrylamide (30-0.8 %),2.5 ml 0.5 M Tris-HCl

pH 6.8 , 0.1 ml 10 % sodium dodecylsulphate, 6.25 ml of distil-
led water, 0.05 ml N,N,N',N'-tetramethylethylenediamine and 0.1
ml 10 % ammonium persulphate,was applied and allowed to poly-
merize.Samples,dissolved in a solution of 2 % sodium dodecyl-
sulphate,10 % sucrose , 0.04 % dithioerythritol and 0.0625 M
Tris-HCl,pH 6.8 containing bromophenol blue,were applied to
the gels within two hours after polymerization of the stacking
gel.Before layering the samples on the gels,they were heated
for two minutes in a boiling water bath.The electrophoresis
buffer used was 0.0184 M Tris-glycine,pH 8.5,containing 0.1
% sodium dodecylsulphate.Electrophoresis was performed at a
constant current of 1 mA per gel during the first hour,fol-
lowed by 3 mA per gel until the bromophenol blue band had rea-
ched the bottom of the gel.After removal of the gels from the
tubes, they were stained during one hour at 37 $^\circ$ C with Coomas-
sie Brilliant Blue R 250 (0.2 % solution in methanol:distil-
led water:acetic acid = 50:50:14).The gels were destained in
a solution of methanol:acetic acid:distilled water = 5:7:88.

For a few special runs all solutions were mixed with urea
to a final concentration of 6 M.

2.1.6.3.Isoelectric focusing.

Isoelectric focusing was performed essentially as des-
cribed by Wrigley (1968).To a mixture of 3 ml 1 % N,N,N',N'-
tetramethylethylenediamine 11 ml acrylamide-bisacrylamide so-
lution (30-0.8 %),1.1 ml carrier ampholine solution (LKB,

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40 %, pI range 5-8) and 16.2 g urea, distilled water was ad-
ded to a final volume of 45 ml.After deaeration the mixture
was cooled and 3 ml of a 1 % ammonium persulphate solution was
added.The solution was poured into glass tubes and allowed to
polymerize.The tubes were put in the electrophoresis appara-
tus and the gels were layered with an ampholyte layer contai-
ning 6 M urea, 5 % sucrose,0.04 % dithioerythritol and 0.25%
ampholine solution (LKB, 40 %, pI range 5-8).The anodic com-
partment (top) contained 0.2 % sulphuric acid,whilst the
cathodal compartment (bottum) was filled witn 0.4 % triethyl-
amine.After a prerun at 360 V during 30 minutes,the protein,
dissolved in a solution of 6 M urea, 10 % sucrose and 0.04 %
dithioerythritol was applied to the gel through the ampholyte
layer.After electrophoresis for four hours at 360 V,the gels
were removed from the tubes and stained according to the method
of Malik and Berry (1972).

2.2.RESULTS

2.2.1 Isolation and partial characterization of β_H and β_L.
        The fractionation of the water-soluble proteins from
calf lens cortex is shown in fig.2.1. α-Crystallin,having the
highest molecular weight,moves ahead of two fractions,desig-
nated as β_H (high molecular weight β-crystallin) and β_L
(low molecular weight β-crystallin).The fraction behind
β_L contains γ-crystallin.The immunoelectrophoretic behavior
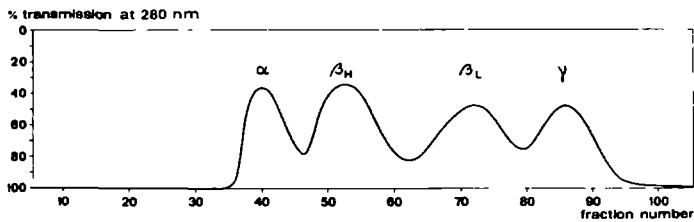
Fig.2.1.Gel filtration of the water-soluble eye lens proteins
on Sephadex G-200.For experimental details,see text.

of the individual fractions is visualized in fig.2.2.Only α-
crystallin appears as a single precipitation arc,while the
other fractions show heterogeneity.In the γ-fraction the fast
moving anodal band (FM-crystallin ,Van de Broek et al.,1973)
is clearly visible.Sedimentation analysis of β_H yielded one
major peak with a small additional peak.The $S_{20,w}$ value of the
major peak was 7.8 S.Sedimentation analysis of β_L yielded one
symmetrical peak with an $S_{20,w}$ value of 3.5 S.

The amino acid composition of β_H and β_L is given in ta-
ble 2.I.The most striking feature is the great similarity be-
tween β_H and β_L,major differences only being found in the va-
lues for cysteine and methionine.For comparison the amino acid

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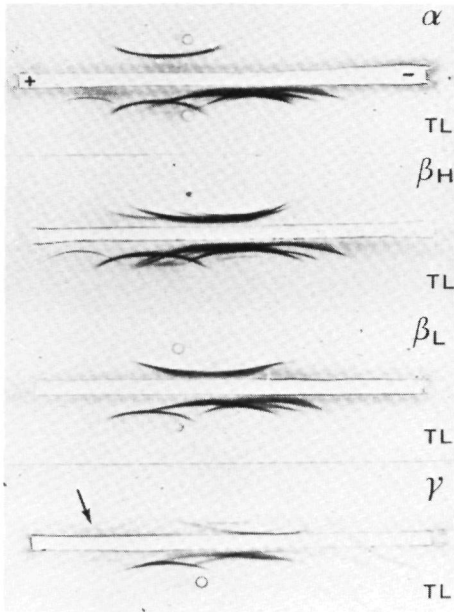
Fig.2.2.Immunoelectro-
phoretic behavior of the
isolated α-, β_H-, β_L- and
γ-crystallin fractions.
The upper hole on each
slide contained the lens
protein fraction as in-
dicated,the lower holes
the total water soluble
lens extract.After elec-
trophoresis the central
wells were filled with
antiserum against total
lens extract and diffu-
sion was allowed for 16
hour.TL stands for total soluble lens protein.

composition of fraction B and C (comparable to β_H and β_L)
as obtained by Zigler and Sidbury (1973) is also shown.

2.2.2.Characterization of β_H and β_L by polyacrylamide gel
electrophoresis under disaggregating conditions.
2.2.2.1. Basic urea gels.

    The considerable difference in polypeptide composition
of the fractions obtained after gel filtration on Sephadex

TABLE 2.I

Amino acid analyses of β_H and β_L

Amino acid	β_H mol %	β_L mol %	β_H' mol %	β_L' mol %
aspartic acid	7.3	8.5	7.4	7.9
threonine	2.9*	3.0*	2.9	2.9
serine	7.9*	8.1*	8.4	9.9
glutamic acid	14.6	14.6	15.5	15.5
proline	6.0	5.9	6.8	6.4
glycine	9.3	9.1	10.4	10.4
alanine	5.4	4.9	5.6	5.3
cysteine	1.7**	0.9**	2.8	2.1
valine	6.1***	6.3***	5.1	5.5
methionine	1.8	1.0	1.7	1.6
isoleucine	3.3***	3.5***	2.7	2.7
leucine	5.3	6.2	5.4	6.0
tyrosine	4.1	4.1	4.4	4.4
phenylalanine	4.8	4.3	4.9	4.4
tryptophan	4.5****	3.8****	2.0	1.3
lysine	4.3	5.4	4.0	4.5
histidine	4.1	4.5	4.0	4.6
arginine	6.7	6.1	6.0	5.7

*   Extrapolated to zero time hydrolysis.

**   Estimated as carboxymethylcysteine.

***   Values for 72 hour hydrolysis.

****   Determined according to Benzce and Schmid (1957).

'   From Zigler and Sidbury (1973).

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G-200 is revealed after electrophoresis on basic polyacryl-
amide gels containing 6 M urea (fig.2.3).Like α-crystallin,
β_H and β_L consist of several polypeptide chains.Unlike the
well-resolved patterns of α- and γ-crystallin,the patterns of
β_H and β_L are rather complex.A number of β bands migrate with
identical mobility in both fractions.β_H and β_L have one pre-
dominant band in common,for which the nomenclature βB_p was
proposed (Herbrink and Bloemendal,1974, Bloemendal and Her-
brink, 1974),where B stands for basic and p for principal.
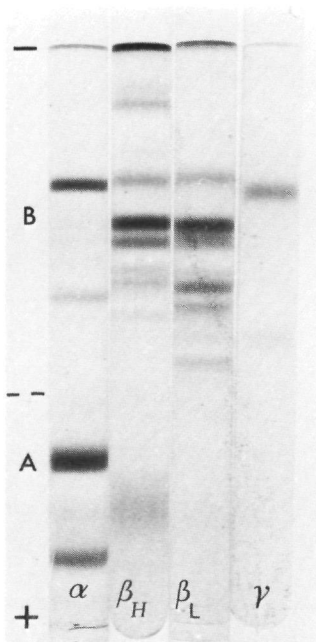
Fig.2.3.Basic polyacryl-
amide gel electrophoresis
in the presence of 6 M
urea of α-,β_H-,β_L- and
γ-crystallin.For experi-
mental details,see text.

The main difference in polypeptide composition between β_H
and β_L is the occurrence of two minor basic and an acidic

chain in β_H and two chains in the neutral region in β_L.

2.2.2.2. Isoelectric focusing.

The polypeptide composition was also studied by poly-acrylamide gel electrophoresis in a pH gradient ranging from pH 5 to 8 in the presence of 6 M urea.The results are shown in fig.2.4.A number of polypeptide chains of β_H and β_L appear
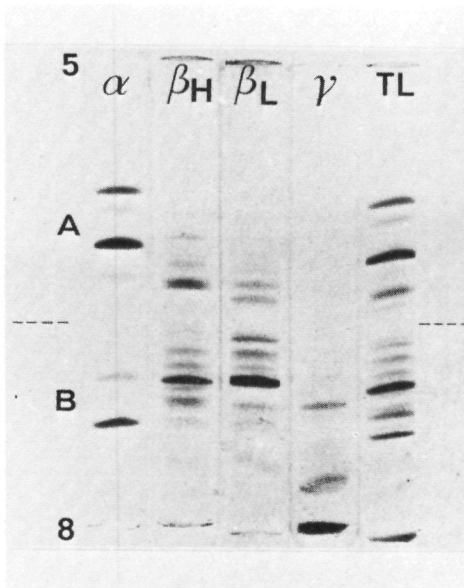
Fig.2.4.Isoelectric focusing of lens proteins.

to have the same isoelectric points.The βB_p chain is clearly visible.The isoelectric focusing method yields a better reso-lution of the individual zones,especially in the acidic region. However,in our hands the number and intensity of the bands

obtained for β_H and β_L are not very reproducable.

2.2.2.3. Sodium dodecylsulphate gel electrophoresis.

Electrophoresis on polyacrylamide gels containing sodium dodecylsulphate reveals the existence of several size classes of β-crystallin polypeptide chains (fig.2.5).The minimum number of polypeptide chains of different size is five for β_H and four for β_L.Molecular weights were determined by running bovine serum albumin,ovalbumin,chymotrypsin and ribonuclease on a separate gel.
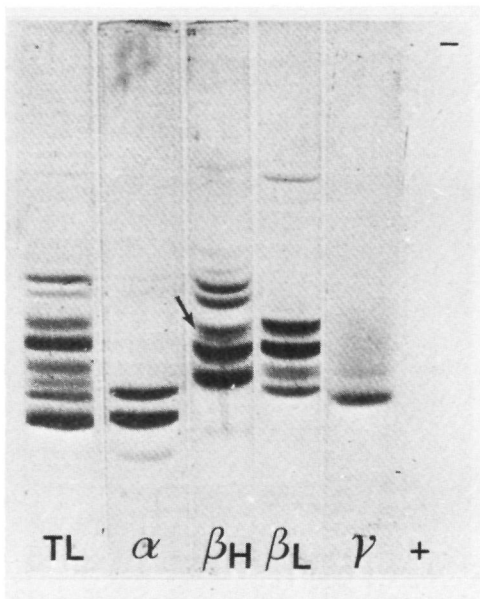
Fig.2.5.Sodium dodecyl-sulphate gel electrophoresis of lens proteins. TL stands for total soluble lens protein.

Molecular weights determined on 10 and 13 % Laemmli gels showed some discrepancy (table 2.II).At any rate from the

TABLE 2.II

Approximate molecular weights of crystallin chains

α-crystallin			β$_H$			β$_L$			γ-crystallin		
13 %	10 %	13 %	13 %	10 %	13 %	13 %	10 %	13 %	13 %	10 %	13 %
gels	gels	gels	gels	gels	gels	gels	gels	gels	gels	gels	gels
		6 M urea			6 M urea			6 M urea			6 M urea
21,500	19,500	19,500	37,500	31,000	31,000	30,500	26,000	24,000	20,500	17,800	19,000
18,500	18,500	18,500	35,500	29,000	30,000	27,500	24,500	22,500			
			30,500	26,000	24,000	25,000	21,000	22,000			
			27,500	24,500	22,500	23,500	19,500	21,000			
			25,000	21,000	21,000						

sodium dodecylsulphate gels one may conclude that β_H and β_L also show heterogeneity on basis of the size of their poly-peptide chains.

The electrophoretical analysis on sodium dodecylsulpha-te gels containing 6 M urea is shown in fig.2.6. As far as α-crystallin is concerned,the result is comparable to that described by Van der Ouderaa et al. (1974b).For β_H at least
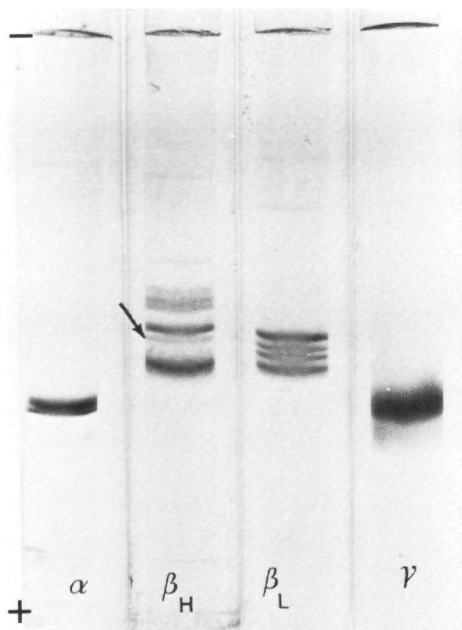
Fig.2.6.Sodium dode-cylsulphate gels con-taining 6 M urea of lens proteins.

five bands are found,whilst β_L yields four bands.However,on basis of the intensity of the bands it seems likely that one of the polypeptide chains (arrow) migrates with a different mobility in the presence of 6 M urea.The approximate molecu-

lar weights estimated in the sodium dodecylsulphate-6 M urea
gels are also shown in table 2.II.

2.3.DISCUSSION

Several authors (Testa et al.,1965, Van Dam and Ten
Cate, 1966, Schoenmakers et al., 1968, Hoenders and Van Kamp,
1972, Zigler and Sidbury, 1973, Herbrink and Bloemendal,1974,
Bloemendal and Herbrink, 1974) have reported the separation
of bovine β-crystallin into two fractions of differing mole-
cular size (see fig.2.1.).Zigler and Sidbury (1973) used
the nomenclature B and C for the two β-crystallin fractions.
However, the designation β_H and β_L seems more rational,since
it gives an indication concerning the molecular size of the
two main β-crystallin classes,isolated by gel filtration.Of
tne two β fractions,isolated from the gel filtration column,
in particular β_H is heterogeneous as can be shown by immuno-
electrophoresis (fig.2.2).This finding is in agreement with
the observations of Testa et al. (1965) and Armand et al.
(1970),who found four distinct bands after immunoelectropho-
resis of fraction B isolated on Biogel P-300.Immunoelectro-
phoresis of β_L yiels mainly one band.However from the tailing
at the end of the precipitation arc some heterogeneity can
be inferred.Testa et al. (1965) found at least three preci-
pitation bands upon immunoelectrophoretic analysis of fraction
C.

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The sedimentation coëfficients found for β_H and β_L are in reasonable agreement with those reported by other investigators (Testa et al.,1965, Armand et al.,1970).Analytical gel filtration of β_{II} and β_L yielded an approximate molecular weight of 210,000 dalton for β_H and 52,000 dalton for β_L (Zigler and Sidbury,1973).

The amino acid composition of β_H and β_L shows a striking similarity between both proteins.Our results are in good agreement with those reported by Zigler and Sidbury (1973, see table 2.I), except for the values of cysteine,methionine and tryptophan.

From the results of polyacrylamide gel electrophoresis it is clear that the polypeptide composition of β_H and β_L is rather complex.On the basis of the basic polyacrylamide gels containing 6 M urea the composing chains of the β-crystallins,as in the case of α-crystallin,can be divided into an A (acidic) and a B (basic) group (fig.2.3).The most striking feature of the gel electrophoresis patterns in the presence of 6 M urea is the occurrence of a common predominant band in both β_H and β_L,called the principal basic β band (βB_p).Moreover,it can be observed that a number of faint bands migrate with identical mobility in both fractions.This finding is in agreement with the work of Zigler and Sidbury (1973),who reported that after blocking the sulfhydryl groups with N-ethylmaleimide eight bands of β_H and β_L have the same electrophoretic mobility on basic polyacrylamide gels contai-

ning 6 M urea.They also reported that β_H contains six other bands.The mean discrepancy between the basic gels of Zigler and Sidbury and the results presented here is the fact that Zigler and Sidbury find all the β_L bands present in the β_H fraction.From fig.2.3 ,however,it is clear that at least two bands in the neutral region of β_L are not present in β_H.

On basis of the alkaline urea gels the minimum number of polypeptide chains seems to be nine for β_H and eight for β_L.

Electrophoresis on polyacrylamide gels containing sodium dodecylsulphate reveals the existence of several size classes of β polypeptide chains (fig.2.5).A number of polypeptide chains of β_H and β_L appear to have identical molecular weights. The same number of polypeptide chains was found on sodium dodecylsulphate gels containing 6 M urea (fig.2.6).However,probably due to a better unfolding of the chain,one of the polypeptide chains migrates with a different mobility (see arrow, fig.2.5 and 2.6).The same phenomenon has also been found for the αB chains (Van der Ouderaa et al.,1974b).Apparent differences in molecular weights,obtained by sodium dodecylsulphate gel electrophoresis,have been reported for the α and β chain of hemoglobin in some species (Weber et al.,1972, Fyhn and Sullivan,1975),several strains of cucumber virus and for tobacco mosaic virus and myoglobin,which have very similar molecular weights (Tung and Knight, 1972),as well as for parvalbumins of several species (Sullivan et al., 1975).These

apparent differences in molecular weight have been ascribed
to irregularity in the binding of sodium dodecylsulphate, due
to the presence of proline or other amino acids containing
bulky side chains (Sullivan et al.,1975).

The sodium dodecylsulphate gel patterns of figs 2.5
and 2.6 differ from those reported by Zigler and Sidbury
(1973,1976).These authors observed four to six bands for
β_H and two to four bands for β_L.A possible explanation for
this discrepancy is the fact that they used sodium dodecyl-
sulphate gels containing phosphate buffer according to Weber
and Osborne (1969).

ISOLATION AND PARTIAL CHARACTERIZATION OF SIX POLYPEPTIDE

CHAINS OF β-CRYSTALLIN

In the previous chapter it was shown that β_H and β_L consist of several polypeptide chains.A number of them migrate with identical mobility when subjected to gel electrophoresis. From this deservation it seems likely that β_H and β_L share a number of polypeptide chains.However,for a definite proof the polypeptide chains have to be purified both from β_H and β_L and then characterized.This chapter deals with the purification and partial characterization of a number of polypeptide chains from β_H and β_L.

3.1.METHODS

3.1.1.Isolation of β_H and β_L.

Isolation of β_H and β_L was performed as described in the previous chapter by gel filtration of cortical lens extract on Sephadex G-200.

3.1.2.Purification of the polypeptide chains.

Routinely 200 mg of salt-free β_H or β_L were dissolved in 5 ml starting buffer.The starting buffer,containing 0.005 M

Tris and 6 M urea (free of isothiocyanate ions) was adjusted to pH 7.7 by addition of 0.1 N HCl,after which dithioerythritol was added to a final concentration of 0.02 %.For the isolation of the principal basic β-chain (βB_p) for sequence studies,in a later stage,about 1 g of β_H or β_L was dissolved in 5-10 ml of starting buffer.The samples were applied to a column (40 x 1.6 cm) of DE-52 (Whatman),thoroughly equilibrated with the same buffer.After washing the column with initial buffer,a linear gradient was applied consisting of 350 ml 0.005 M Tris-HCl,6 M urea,pH 7.7 ,containing 0.02 % dithioerythritol and 350 ml 0.030 M Tris-HCl,6 M urea,pH 7.7 ,containing 0.02 % dithioerythritol. The βB_p chain has also been purified by chromatography of total lens extract (1.5 g) on DE-52 using a linear gradient from 0.005 M Tris-HCl,6 M urea,pH 7.7 ,0.02 % dithioerythritol to 0.020 M Tris-HCl,6 M urea,pH 7.7 ,0.02 % dithioerythritol.Elution was carried out at 4° C at an average flow rate of 20 ml per hour.The effluent was monitored at 280 nm with an LKB Uvicord II.Protein fractions were pooled,desalted on Sephadex G-25 in 0.1 M ammonia and lyophilized.

3.1.3.Polyacrylamide gel electrophoresis.

Alkaline urea polyacrylamide gel electrophoresis and sodium dodecylsulphate gel electrophoresis were performed as described in chapter II,sections 2.6.1 and 2.6.2.

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3.1.4.Amino acid analysis.

Amino acid analyses were performed as described in chapter II,section 2.1.3,except that cysteine was determined as cysteic acid after performic acid oxidation (Hirs,1956).

3.1.5.Tryptic peptide mapping.

Protein (2.5 mg per ml) was dissolved in 1 % NH_4HCO_3 , pH 8.9.Trypsir (0.025 mg) was added and digestion was allo- wed to proceed for 2 hr at 37° C.At the end of this period the digestion mixture was frozen and lyophilized.The peptide mixture was dissolved in 50 µl of electrophoresis buffer,pH 6.5 (pyridin:acetic acid:water=25:1:225,by volume) and sub- jected to high voltage electrophoresis,which was performed on Whatman 3 MM at 47 V/cm and 35 mA for 75 minutes.Thereafter descending chromatography in n-butanol-acetic acid-water-py- ridin (75:15:60:50,by vol.)was carried out for 18 hr.After drying the peptides were detected with a 0.2 % (w/v) ninhy- drin solution in acetone containing 1 % (v/v) pyridine and 1 % (v/v) acetic acid.

3.2.RESULTS

3.2.1.Nomenclature of the β-chains.

In the previous chapter the β-crystallin chains were divided into a basic and an acidic group,indicated by the capitals B and A behind the symbol β (fig.2.3).This is in

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fig.3.1.Chromatography of β_H (a) and β_L (b) on DE-52.

β_H βB_1^* βB_2 βB_p βB_{2-5} $\underline{\beta B_4}$ $\underset{\sim}{\beta B_5}$ βA β_L
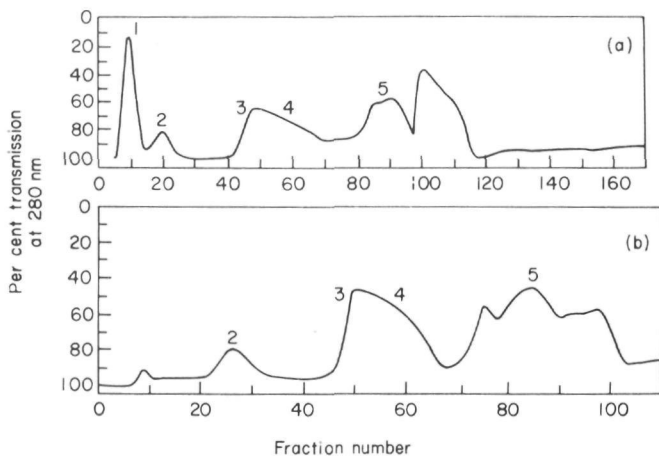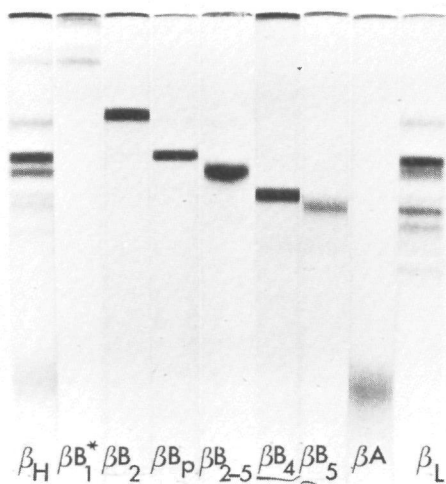
fig.3.2.Alkaline urea polyacrylamide gels of the purified chains of β_H and β_L. * The symbol βB_1 stands for the two highly basic polypeptide chains of β_H.

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accordance with the generally accepted nomenclature of the α-
crystallin chains αA and αB (Bloemendal,1969).A further sub-
division of α-chains was based on their electrophoretic be-
havior in alkaline polyacrylamide-urea gels.At this moment a
similar approach for the β-crystallin chains seems to be less
meaningful since the interrelationship between the individual
chains is not yet fully understood.One should also keep in
mind that a few polypeptide chains occur only in minor quan-
tities which even may vary in different experiments.

For these reasons the β-crystallin polypeptide chains,
with the exception of βA,are designated in correspondence
with their position in the elution profile from the DE-52 co-
lumns (compare figs 3.1.a and 3.1.b).

3.2.2.Purification of the βB_2,βB_3 (= βB_p),βB_4,βB_5,βB_{2-5} and
      βA chains.

Fractionation of these chains has been achieved by chro-
matography of β_H and β_L on DE-52.Typical elution diagrams are
shown in figs 3.1.a and 3.1.b.The numbered fractions were ana-
lyzed on basic urea gels (fig.3.2).The first peak eluted du-
ring chromatography of β_H contains the more basic polypeptides
of β_H.The second peak yielded pure βB_2.The third peak sometimes
consists of pure βB_p chains,but often βB_4 is eluted together
with the βB_p chains.If this is the case βB_4 can be isolated by
rechromatography using a linear gradient ranging from 0.005
to 0.020 M Tris-HCl,pH 7.7 ,containing 6 M urea and 0.02 %

ditnioerythritol.The βB_5 has been obtained pure by rechroma-
tography of the fifth peak.In the case of chromatography of
β_L the βB_{2-5} chain has also been purified from the fifth peak.
However,the isolation of βB_{2-5} and βB_5 is not very reproducible.
The βA chain has been isolated from β_H by applying a second
gradient (0.03 to 0.06 M Tris-HCl,pH 7.7 ,containing 6 M urea
and 0.02 % dithioerythritol) after elution of the more basic
chains from the DE-52 column.The result is shown in fig.3.3.a.
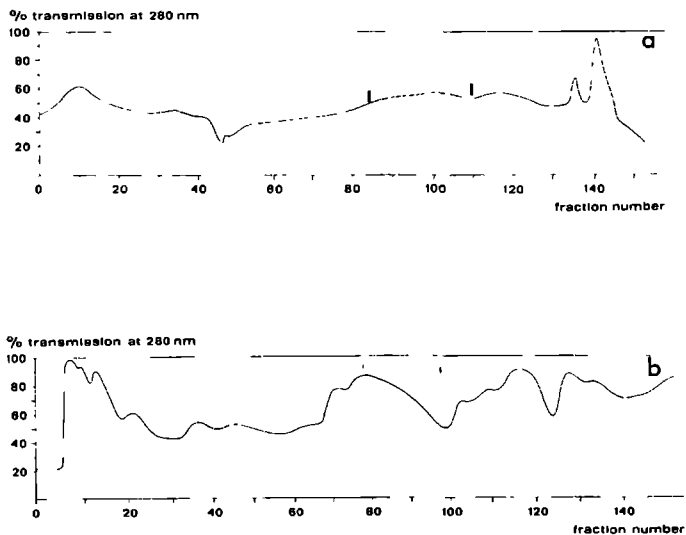
fig.3.3.   a. Purification of the βA chain by applying a se-
cond gradient after elution of the more basic chains of β_H.
              b.Chromatography of total lens extract on DE-52.

The fraction between bars contained pure βA.The elution profile
after chromatography of total lens extract on DE-52 is shown
in fig.3.3.b.The fraction between bars contained either pure

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βB_p chains or βB_p plus βB_4.

3.2.3.Characterization of the chains.

3.2.3.1.Determination of the molecular weights by sodium do-
decylsulphate gel electrophoresis.

All purified chains were subjected to sodium dodecyl-
sulphate gel electrophoresis (fig.3.4).It is clear that the
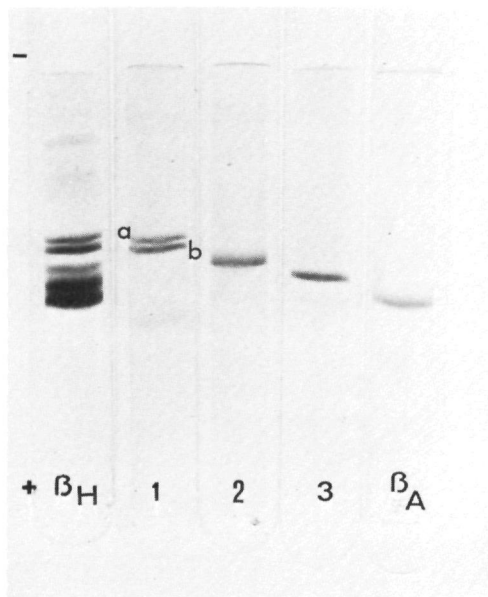
fig.3.4.Sodium dodecyl-
sulphate gel electropho-
resis of the purified
chains of β-crystallin.

1. βB_1 ; 2. βB_2, βB_{2-5}, βB_5 ;

3. βB_p , βB_4 .

first peak eluted during chromatography of β_H on DE-52,consists
of the two "high" molecular weight chains of β_H,designated
βB_{1a} and βB_{1b}.The βB_p-chain ,as well as the βB_4-chain,has a
molecular weight of approximately 24,500 dalton,whereas the
βB_2, βB_{2-5} and βB_5 chain reveal a molecular weight of approxi-
mately 26,000 dalton (see table 2.II,values for 10 % gels).

TABLE 3.I

Amino acid analyses of the β chains

Amino acid	$\beta B_2, \beta B_{2-5}, \beta B_5$ (mol %)	$\beta B_p, \beta B_4$ (mol %)	βA (mol %)
Aspartic acid	7.7	8.3	6.3
Threonine	2.0[*]	3.4[*]	3.7[*]
Serine	7.1[*]	8.4[*]	8.4[*]
Glutamic acid	13.6	15.8	15.4
Proline	5.0	6.8	3.4
Glycine	9.1	9.3	10.1
Alanine	6.0	4.2	5.8
Cysteine	1.0[**]	1.0[**]	1.9[**]
Valine	6.2[***]	6.9[***]	6.8[***]
Methionine	0.9	1.0	1.9
Isoleucine	2.8[***]	3.1[***]	2.3[***]
Leucine	7.3	5.0	6.4
Tyrosine	3.8	4.4	3.6
Phenylalanine	4.2	4.0	6.6
Tryptophan	6.1[****]	2.0[*****]	3.6[****]
Lysine	4.2	6.0	3.2
Histidine	4.6	4.1	5.1
Arginine	8.5	5.0	7.0
% Hydrophobic residues[******]	24.2	18.5	22.5

[*]   Extrapolated to zero time hydrolysis.

[**]   Estimated as cysteic acid.

[***]   Values for 72 hr hydrolysis.

[****]   Determined according to Benzce-Schmid (1957).

[*****] Determined according to Gaitonde-Dovey (1970).

[******] Isoleucine, Leucine, Phenylalanine, Tyrosine, Tryptophan.

The βA-chain has a molecular weight of approximately 21,000 dalton.These molecular weights were obtained irrespectively of whether the chains were derived from either β_H or β_L.

Sodium dodecylsulphate gel electrophoresis in the presence of 6 M urea revealed approximately the same molecular weights for most β-chains,apart from $\beta B_2, \beta B_{2-5}$ and βB_5,for which a lower value was found (22,500 dalton).

3.2.3.2.Amino acid composition of tne β-chains.

Hydrolysis of tne β-chains was carried out for 24, 48 and 72 hours.Tne amino acid composition of the different polypeptide cnains is depicted in table 3.I.No differences were found in the amino acid corposition of the corresponding chains of β_H and β_L.Moreover,the βB_p and βB_4,as well as the $\beta B_2, \beta B_{2-5}$ and βB_5 chain revealed tne same amino acid composition.

3.2.3.3.Tryptic peptide mapping.

After tryptic peptide mapping again no difference was found between tne corresponding chains of β_H and β_L.The tryptic peptide map of βB_p is snown in fig.3.5.βB_4 yields tne same peptide nap.Tre peptide maps of βB_2 and βB_5 (figs 3.6.a and 3.6.b) differ mainly in two spots (dasned arrows).The difference in electrophoretic benavior between these spots might well be due to a difference in two acidic groups.Tne tryptic peptide map of βB_{2-5} (fig.3.7) lacks tnese two spots. However,trere is a spot somewhere in the midale.Tne tryptic
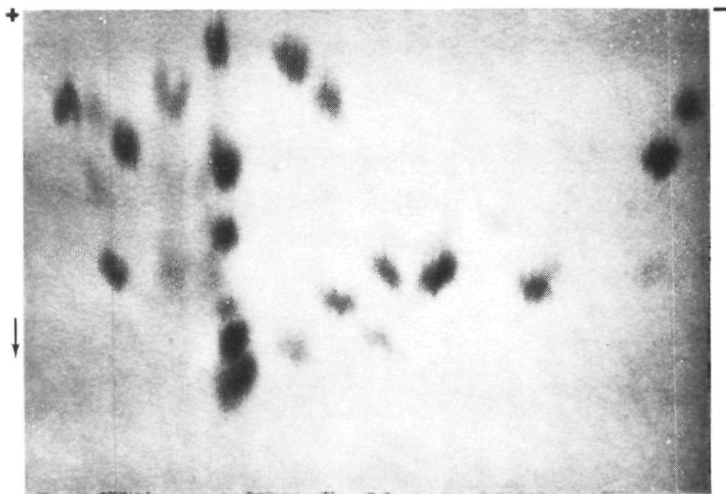
fig.3.5.Map of the tryptic peptides from the βB$_p$ and βB$_4$ chain. Since both finger prints are identical only one map is shown.
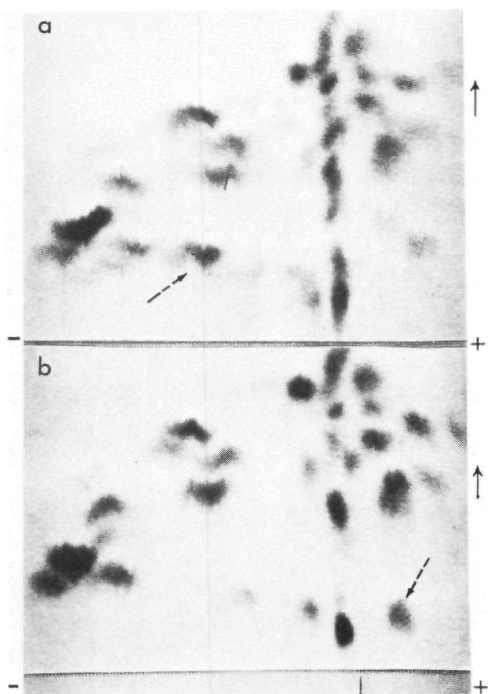
fig.3.6.Map of the tryptic peptides from the βB$_2$ (a) and the βB$_5$ (b) chain.

peptide map of βA is shown in fig.3.8.

fig.3.7.Map of the tryptic peptides from the βB_{2-5} chain.

fig.3.8.Map of the tryptic peptides of the βA chain.

3.3.DISCUSSION.

From the results presented in this chapter it is clear that the polypeptide composition of β-crystallin is less complex than was concluded initially from the results of alkaline gel electrophoresis.It is clear that β_H and β_L share a number of polypeptide chains.Four polypeptide chains with a different electrophoretic mobility,isolated from β_H,appear to have the same molecular weight,amino acid composition and tryptic peptide map as the corresponding chains isolated from β_L.Moreover,a close relationship between a number of chains (βB_p and βB_4; βB_2,βB_{2-5} and βB_5) is suggested by a striking similarity in amino acid composition,molecular weight and tryptic peptide map.It would,therefore,not be surprising if the β-crystallin polypeptides,as in the case of the αA_2 and αB_2 chain,are subjected to deamidation.If this is the case,the βB_4 chain would originate from the βB_p chain by a single deamidation of one asparagine or glutamine.The βB_5 chain would originate from the βB_2 chain by a two-step deamidation with the βB_{2-5} chain as intermediate.

The βA chain might also be subjected to deamidation processes.As shown in fig.3.3.a the second gradient (0.03 to 0.06 M Tris-HCl,6 M urea,pH 7.7 ,containing 0.02 % dithioery-thritol),applied during chromatography of β_H,yields also a number of peaks.Whereas the tryptic peptide maps of some of these fractions were very similar to the map of βA,these peaks

yielded single bands on alkaline urea gels,which revealed a
greater electrophoretic mobility than the βA chain.

It should be noted that the purification of most β-
crystallin chains is not easily and reproducibly achieved.
A possible reason might be the susceptibility of the isolated
β-crystallin chains to rapid deamidation.This is best illus-
trated by the following example:by chromatography of β_H and
β_L on DE-52 βB_p can be purified from the third peak.However,
this peak is often contaminated with βB_4.When rechromatography
using a less steep gradient was performed,two clearly separated
fractions were obtained.The second fraction contained pure βB_4,
whilst the first again contained βB_p plus βB_4.It,therefore,
seems that,at the moment,conditions under which deamidation
occurs,cannot easily be controlled.

For the βB_2,βB_{2-5} and βB_5 chains a different molecular
weight,calculated by sodium dodecylsulphate gel electrophoresis,
was found in the presence of 6 M urea.As discussed in chapter
2.3 these differences might well be ascribed to irregularity
in the binding of sodium dodecylsulphate,due to the presence
of proline or other amino acids containing bulky side chains.
From the amino acid compositions presented in table 3.I,however,
no significant difference in the amount of hydrophobic residues
can be found between the β-crystallin polypeptide chains.

BIOSYNTHESIS OF β-CRYSTALLIN CHAINS IN VITRO

From the results presented in the previous chapter it
is clear that a close relationship exists between a number of
polypeptide chains of β-crystallin.The question arose whether
some of these polypeptide chains result by a deamidation pro-
cess.If this is the case only a limited number of β-chains
would be direct genetic translation products.The same pheno-
menon has been observed for the polypeptide chains of α-crys-
tallin.From the results of biosynthetic studies it has been
concluded that of the two acidic chains only the αA_2 chain
is under direct genetic control (Bloemendal et al,1972c,Strous
1973).There are also indications that the same relation exists
between the two basic chains,αB_1 and αB_2 (Strous,1973).

A first endeavour to a biosynthetic study of β-crystallin
has been presented by Strous (1973).He reported that in the
lens cell-free system or in lens culture in the presence of
[^{35}S]-methionine or a [^{14}C]-amino acid mixture incorporation
in β_L was found whilst incorporation in β_H was very low.It was
suggested that β_H arises from β_L and that β_H formation possibly
reflects an aspect of lens aging.The same assumption was made
by Van Kamp (1973),based on studies on the distribution of

lens crystallins.Going from cortex to nucleus a gradual de-
crease in β_L content with a concomitant increase of β_H was
found.

    In this chapter an attempt is described to verify which
β polypeptide chains are the result of direct genetic trans-
lation.Moreover,by reaggregation studies further evidence will
be presented that β_H shares a number of polypeptide chains
with β_L.

4.1.METHODS

4.1.1.Isolation of calf lens polyribosomes.

    Calf lens polyribosomes were isolated as described by
Berns and Bloemendal (1974).About 5 mg of polyribosomes per
1000 lenses were obtained.Sucrose gradient centrifugation
revealed that the major part of the polyribosome preparation
from cortex cells had an absorbance profile corresponding with
an optimum of eight ribosomes per polyribosome.

4.1.2.Synthesis of lens protein in vitro.

    Rabbit reticulosytes were prepared as described by Evans
and Lingrel (1969) and lysed by addition of water.A 30,000
x g supernatant fraction of these lysed cells was used as cell-
free system and incubations were performed at 30° C for 1 hour.
The reaction mixture contained per ml : 0.5 ml of reticulosyte
cell-free extract,1 µmol ATP,0.2 µmol GTP,5 µmol 2-mercapto-
ethanol,10 µmol creatine phosphate,50 µg creatine phosphokinase,

50 μmol Tris-HCl,pH 7.4 ,100 μmol KCl,3 μmol magnesium acetate and 0.1 μmol of 19 amino acids.40 μCi $[^{35}S]$-methionine was added as only labeled amino acid.Polyribosomes were added in a concentration of 400 μg per ml.

4.1.3.Gel filtration of translation products.

Gel filtration on Sephadex G-200 was performed as described in chapter 2,section 2.1.2.To one part of the incubation mixture 50 mg of cortical lens extract,dissolved in 1 ml elution buffer,was added and the solution was centrifuged for 20 minutes at 15,000 x g.The supernatant was applied to the column (100 x 2.5 cm).The other part of the incubation mixture was first centrifuged at 15,000 x g during 20 minutes.The supernatant was then added to 50 mg of cortical lens extract,dissolved in 1 ml elution buffer and subsequently applied to the column.Fractions of 6 ml were collected.For determination of radioactivity 0.6 ml portions were treated with 0.6 ml 10 % TCA.The precipitated protein was collected on millipore filters and the radioactivity determined in a liquid scintillation counter.

4.1.4.Reaggregation of lens crystallins.

Reaggregation of lens crystallins was performed essentially as described by Bloemendal et al. (1975).The incubation mixture,containing radioactive translation products,was divided into two parts.One part was first centrifuged for 20 minutes at 15,000 x g.The supernatant was then added to 50 mg of cor-

tical lens extract in 25 ml 0.05 M Tris-HCl,pH 7.6,containing

0.0514 M sodium chloride,0.001 M EDTA and 6 M urea.The solution

was allowed to stand for 30 minutes at 4° C and subsequently

dialyzed against several volumes of distilled water.After lyo-

philization the sample was dissolved in elution buffer and

applied to a column (100 x 2.5 cm) of Sephadex G-200.The

other part of the incubation mixture was added to 50 mg of

cortical lens extract in 25 ml of the same buffer as described

above.The same procedure as described above was followed,ex-

cept that the dry material obtained after lyophilization,was

dissolved in 1 ml elution buffer and centrifuged for 20 minutes

at 15,000 x g before application to the Sephadex G-200 column.

Determination of radioactivity was performed as described in

section 4.1.3.

4.1.5.Analysis of translation products.

         The translation products were analyzed by alkaline urea

gel electrophoresis using polyacrylamide gel rods (see chapter

2.1.6.1) and by sodium dodecylsulphate gel electrophoresis

according to Laemmli (1970) with the modification that a slab

gel instead of rods was used.The gel was 12 cm long and contained

13 acrylamide,0.4 % methylene-bisacrylamide and 0.1 % sodium-

dodecylsulphate.In this method a stacking gel was applied.Stai-

ning and destaining were performed as described by Weber and

Osborne (1969).For the detection of the labeled proteins the

procedure of Bonner and Laskey (1974) was used in combination

with the arying procedure described by Berns and Bloemendal
(1974).

4.2.RESULTS

4.2.1.Synthesis of lens proteins in vitro.

In order to establish whether β_H and β_L are synthesized in vitro in the cell-free system,the incubation mixture,after centrifugation,was added to cortical lens extract and passed through a column of Sephadex G-200.The result is shown in fig. 4.1.The same elution and radioactivity profile was obtained
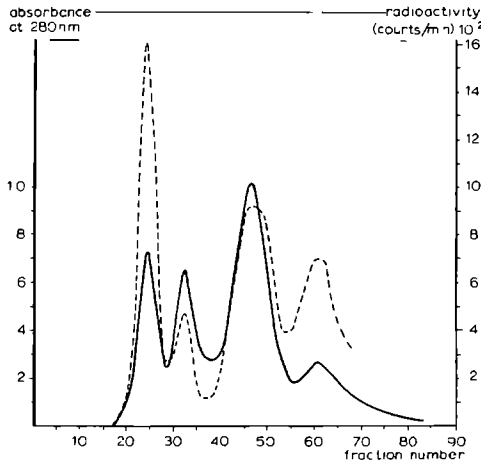
Fig.4.1.Gel filtration on Sephadex G-200 of lens proteins labeled witn [^{35}S]-methionine.The solid line represents the elution profile based on the extinction at 280 nm.The dashed line represents tne radioactive elution profile.

when the incubation mixture was first added to cortical lens extract and tnen centrifuged.It is clear that β_H synthesis is low as compared to other crystallins.Tne radioactivity found under the β_L peak is partly due to newly syntresized hemoglobin.

The fractions,obtained by gel filtration on Sephadex

G-200 were analyzed by sodium dodecyl sulphate gel electro-
phoresis and alkaline urea gel electrophoresis and subsequent-
ly subjected to scintillation autoradiography.From the results
of the sodium dodecylsulphate gel (fig.4.2) it is clear that,
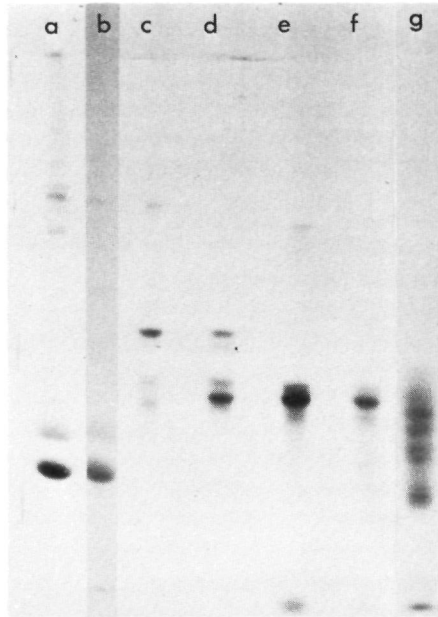
Fig.4.2.Autoradiography of sodium dodecylsulphate gels
containing labeled lens crystallin fractions.a.α-crystallin;
b.α-crystallin after reaggregation;c.β_H ;d.β_H after reaggre-
gation; e. β_L ; f. β_L after reaggregation ; g. γ-crystallin
after reaggregation.

although radioactivity under the β_H peak is very low,there
is some synthesis of β_H chains.The main radioactivity can be
found in one of the "high" molecular weight chains of β_H,de-
signated βB_{1a},whereas some radioactivity is also present in
the 26,000 and 21,000 dalton component.The sodium dodecylsul-

phate gel of β_L shows at least three labeled bands, the βB_p band (24,500) containing most of the radioactivity.

By alkaline urea gel electrophoresis (fig.4.3a) little or no radioactivity could be found in β_H. However, some radioactivity remains on top of the gel, probably due to the "high" molecular weight chain of β_H, which has not penetrated the gel. Moreover, some faint label can be found in the βB_2 and βA chain. Autoradiography of the basic urea gel of the β_L fraction clearly shows incorporation of [^{35}S]-methionine in βB_2, βB_p, βB_{2-5}, βB_4 and a chain in the neutral region.
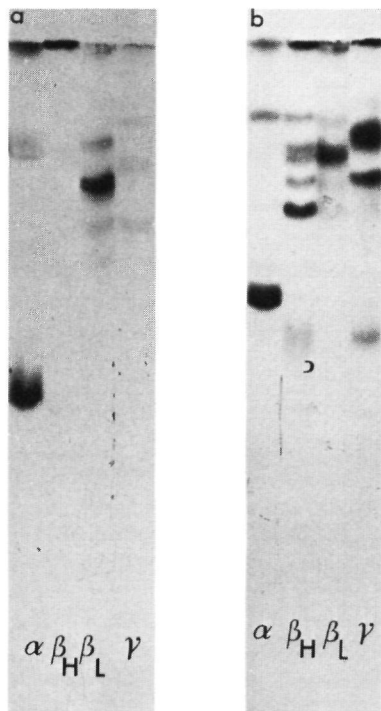
Fig.4.3. Autoradiography of basic urea gels containing labeled lens crystallin fractions. a. Fractions obtained after gel filtration of the incubation mixture on Sephadex G-200.

b. Fractions obtained after gel filtration of the incubation mixture after re-aggregation.

4.2.2.Reaggregation of lens crystallins in the presence of newly synthesized [^{35}S]-methionine containing polypeptide chains.

As shown by Bloemendal et al. (1975) under proper conditions correct reaggregation of lens crystallins after dissociation in 6 M urea can be obtained.Since β_H and β_L share a number of polypeptide chains and de novo synthesis of β_H chains is very low,it seemed interesting to repeat these experiments using the products resulting from the incubation in the cell-free system.

The results of the gel filtration on Sephadex G-200 after reaggregation is shown in fig.4.4.Irrespective of whether
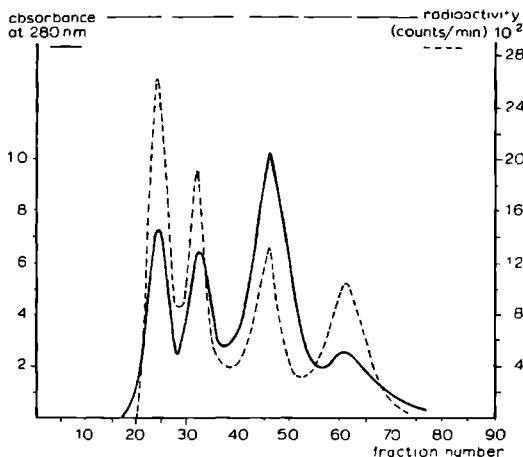
Fig.4.4.Gel filtration of lens proteins containing [^{35}S]-methionine after reaggregation.The solid line represents the absorption at 280 nm.The dashed line represents the radioactivity elution profile.

the incubation mixture was centrifuged before or after reaggregation,the same result was obtained.A significant increase in radioactivity is found under the β_H peak as compared to the results presented in chapter 4.2.1.

The results obtained by sodium dodecylsulphate gel elec-
trophoresis (fig.4.2) show that all the polypeptide chains
of different size of β_H have been incorporated in the reaggre-
gated β_H (compare fig.2.5),the 31,000 dalton (βB_{1a}) and the
24,500 dalton (βB_p plus βB_4) band containing most of the
radioactivity.

Analysis on alkaline urea gels (fig.4.3b) revealed the
same radioactive pattern for β_L as described in section 4.2.1.
For β_H a completely different pattern is obtained.Radioacti-
vity can be found in βB_2,βB_p,βB_{2-5},βA and a chain in the neu-
tral region,whereas the radioactivity on top of the gel proba-
bly represents βB_{1a}.In contrast to the radioactive pattern
obtained for β_L,the most intensive band in the pattern of β_H
is not the βB_p band,but a band in the neutral region.

4.3.DISCUSSION

From the results presented in section 4.2.1 it is clear
that,although β_H synthesis in the cell-free system is low as
compared to other crystallins,de novo synthesized β_H is found.
Autoradiography of the sodium dodecylsulphate gels (fig.4.2)
revealed that the βB_{1a},the 26,000 dalton and the 21,000 dalton
chain are labeled (values for 10 % gels,compare table 2.II).
No radioactivity can be detected in the 24,500 dalton band
(βB_p plus βB_4).The relative proportion of the labeled chains
is not in agreement with the relative proportion found in na-
tive β_H.This might be due to a higner methionine content of

the βB_{1a} chain.Since the latter chain has not yet been purified,
the amino acid composition is still unknown.Hence a definite
statement about the relative proportion of newly synthesized
β_H chains cannot be made.Autoradiography of the alkaline urea
gels of β_H (fig.4.3a) shows little or no radioactivity.A faint
blackening can be found at the position of the βB_2 and βA chain
Some radioactivity remains on top of the gel,probably due to
βB_{1a} which has not entered the gel.The newly synthesized β_H
aggregate might,therefore,be built up by the βB_{1a},βB_2 and βA
chain.

It is clear that of the β crystallins mainly β_L is syn-
thesized in the cell-free system.This finding is in agreement
with the results of Strous (1973),who reported the same
radioactive elution profile after gel filtration on Sephadex
G-200 of crystallins,newly synthesized in the lens cell-free
system.

From the reaggregation experiments the conclusion can be
drawn that a number of chains of β_L are incorporated in β_H
after reaggregation.This is a confirmation of earlier findings
(chapter 3) that β_H and β_L share a number of polypeptide
chains.All β_H polypeptide chains observed on the sodium dodecyl-
sulphate gel pattern are labeled (fig.4.2).The origin of the
βB_{1b} chain remains unclear,since it could not be detected in
newly synthesized β_H.However,there are some indications (Ver-
morken,personal communication) that the βB_{1b} chain originates
from the βB_{1a} chain.Gel autoradiography of β_L reveals radio-

activity in the 26,000,24,500 and 19,500 dalton band.The re-
maining 21,000 dalton band (compare fig.2.5 ,chapter 2)
might be overshadowed by the very intense 24,500 dalton band.
Autoradiography of the alkaline urea gel profile of reaggrega-
ted β_L yields the same picture as obtained for newly synthesi-
zed β_L.In contrast,for β_H a totally different pattern is obser-
ved.Most of the polypeptide chains of β_H after reaggregation
contain radioactivity.Further the most intensive band is not
βB_p but a chain in the neutral region.

From the results presented in this chapter it is difficult
to draw definite conclusions about the biosynthesis of β crys-
tallin chains.The question whether only a limited number of
polypeptide chains of different electrophoretic mobility,found
on basic urea gels,are direct translation products cannot be
answered.Most of these chains show radioactivity,perhaps as
a result of rapid deamidation.The same phenomenon may be res-
ponsible for the occurrence of most of the radioactivity in
a chain in the neutral region instead of in the βB_p chain
after reaggregation of β_H.

CHAPTER   V

SOLID-PHASE EDMAN DEGRADATION.HIGH YIELD ATTACHMENT

OF PROTEIN FRAGMENTS TO AMINATED SUPPORTS

In the solid-phase Edman degradation peptides or pro-
teins,bound to a solid support, can be sequenced automatically
by cleaving amino acids from their N-termini in a stepwise
manner. The Edman degradation can be considered as a repeated
three step process (fig.5.1). In the first step (I)
phenylisothiocyanate is coupled at high pH to the α-amino
group of the N-terminal amino acid,yielding a phenylthiocar-
bamyl (PTC) peptide. In the second step (II), performed
at low pH, a cyclization reaction followed by cleavage occurs,
resulting in a shortened peptide and a thiazolinone,which in
the third step (III) is converted to a phenylthiohydantoin
(PTH).The latter is then identified.

The solid-phase Edman degradation demands a reliable pro-
cedure for the attachment to a solid support of chemically or
enzymatically obtained protein fragments.The reliability of
the attachment method depends on the specificity of the activa-
tion reaction to which the peptide has to be subjected.C-ter-
minal activation is preferred and derivatization of the peptide

$$\text{(phenyl)}-N=C=S \quad + \quad H_2N-\underset{R_1}{CH}-\underset{O}{C}-NH-\underset{R_2}{CH}-\underset{O}{C}\text{---peptide}$$

I | pH 8-9

$$\text{(phenyl)}-NH-\underset{S}{C}-NH-\underset{R_1}{CH}-\underset{O}{C}-NH-\underset{R_2}{CH}-\underset{O}{C}\text{----peptide}$$

II | TFA

$$\text{(phenyl)}-NH-C=N \qquad + \qquad H_2N-\underset{R_2}{CH}-\underset{O}{C}\text{----peptide}$$

with ring: S, CHR₁, C, O

H⁺, H₂O | III

ring structure: N-C=S, C NH, O CHR₁

PTH-amino acid

fig.5.1

$$H_2N-\underset{R_1}{CH}-\underset{O}{C}\text{---------------------------}NH-CH-COOH$$
CH₂
CH₂-OH

TFA

$$H_2N-\underset{R_1}{CH}-\underset{O}{C}\text{---------------------}NH-CH-CH_2$$
C   CH₂
O   O O

H₂N-P

$$H_2N-\underset{R_1}{CH}-\underset{O}{C}\text{---------------------------}NH-CH-C-NH-P$$
CH₂-CH₂-OH

fig. 5.2

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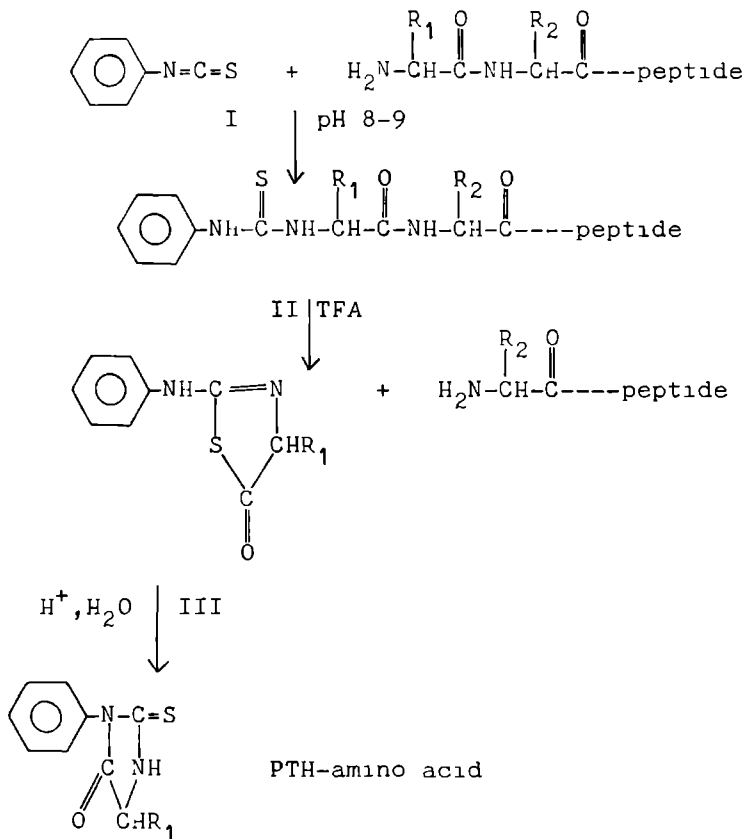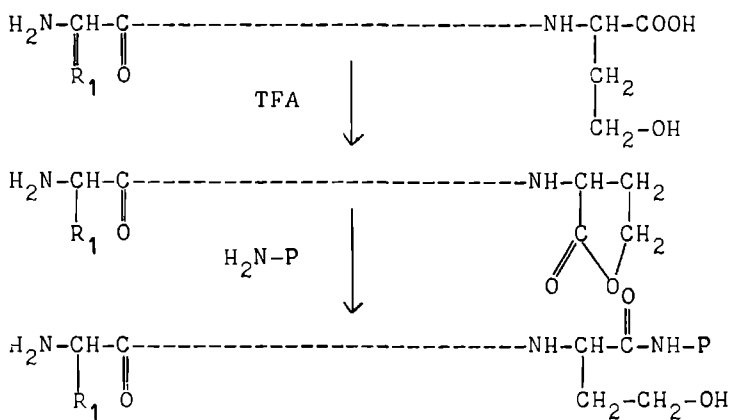
side chains gives rise to complications during the degradation process.

A number of procedures for the coupling of peptides and proteins to a solid support have been described.There are three basic approaches:1.Attachment through C-terminal homoserine lactone;2.Attachment by activation of the C-terminal carboxyl group and 3.Attachment through side-chain amino groups.

1.Attachment through C-terminal homoserine lactone.

The only procedure that warrants a reasonable specific C-terminal activation consists of the generation of homoserine lactone residues by treatment of purified fragments from a cyanogen bromide degradation with trifluoroacetic acid.Tne weak activation as occurring in the lactone ring appears to be sufficient to undergo a rapid aminolysis with a polymer substituted with a primary amine (Horn and Laursen,1973,fig.5.2).The application of this elegant method which results in sufficiently stable peptide-support adducts, is hampered by the relative scarcity of methionyl residues in proteins,wnich moreover should not be clustered.Tne advantages of this method are:no separate end group determination,no gaps in the sequence and high coupling yields.

2.Attachment by activation of the C-terminal carboxyl-group.

A number of methods have been recommended for the attachment of peptides and proteins to aminated supports by

$$X-NH-\underset{\underset{R_1}{|}}{CH}-\underset{\underset{O}{\|}}{C}------------NH-\underset{\underset{R_n}{|}}{CH}-COOH$$

carbonyldiimidazol

$$X-NH-CH-\underset{\underset{R_1\ O}{}}{C}------------NH-CH-\underset{\underset{R_n\ O}{}}{C}-N$$

$$NH_2-P$$

$$X-NH-CH-\underset{\underset{R_1\ O}{}}{C}------------NH-CH-\underset{\underset{R_n\ O}{}}{C}-NH-P$$
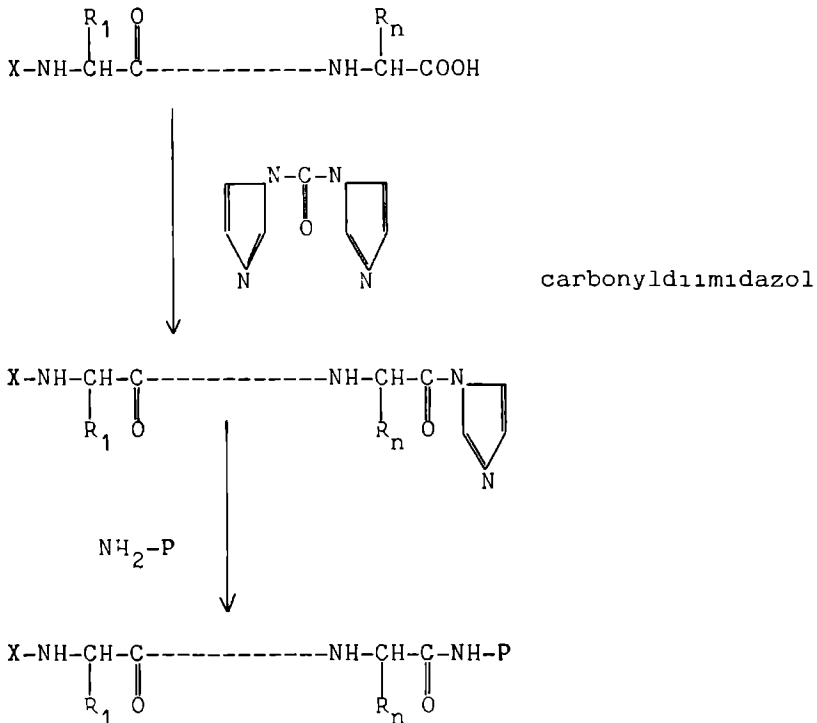
fig.5.3

their C-terminal carboxyl group.

The procedure ,described by Laursen (1971) utilizes
carbonyldiimidazol to activate the C-terminal carboxyl group
(fig.5.3).This method requires protection of the amino groups
and the use of anhydrous solvents,which is a serious drawback
since many peptides are insoluble in these solvents.Another
disadvantage is the activation of side-chain carboxyl groups,
which also become attached to the resin,yielding gaps in the

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sequence.In the case of aspartic acid cyclic imide formation

occurs,which prevents further degradation.Although high cou-

pling yields ($>$ 80 %, Laursen,1971) have been reported,the

method has not widely been practised.

Another procedure (Previero et al.,1973) describes

the activation of the C-terminal carboxyl group with carbo-

diimides (fig.5.4) under conditions which should favor se-

lective blocking of the side chain carboxyl groups.However,

$$H_2N-CH-C-------------------NH-CH-C-NH-CH-COOH$$

with R_1 O under $C=O$ groups R_m R_n

'R-N=C=N-R''   carbodiimide

$$H_2N-CH-C-------------------NH-CH-C \quad C=O$$

R_1 O R_m $NH-CHR_n$ with substituent $O-C \begin{smallmatrix} NR' \\ NHR'' \end{smallmatrix}$

$$H_2N-CH-C----------NH-CH-C \quad C=O \quad + \quad HNR'-C-HNR''$$

R_1 O R_m $N----CHR_n$ O

H_2N- P

$$H_2N-CH-C-------------------NH-CH-C-NH-CH-C-NH- P$$

R_1 O R_m O R_n O

fig. 5.4

fig.5 .5

60
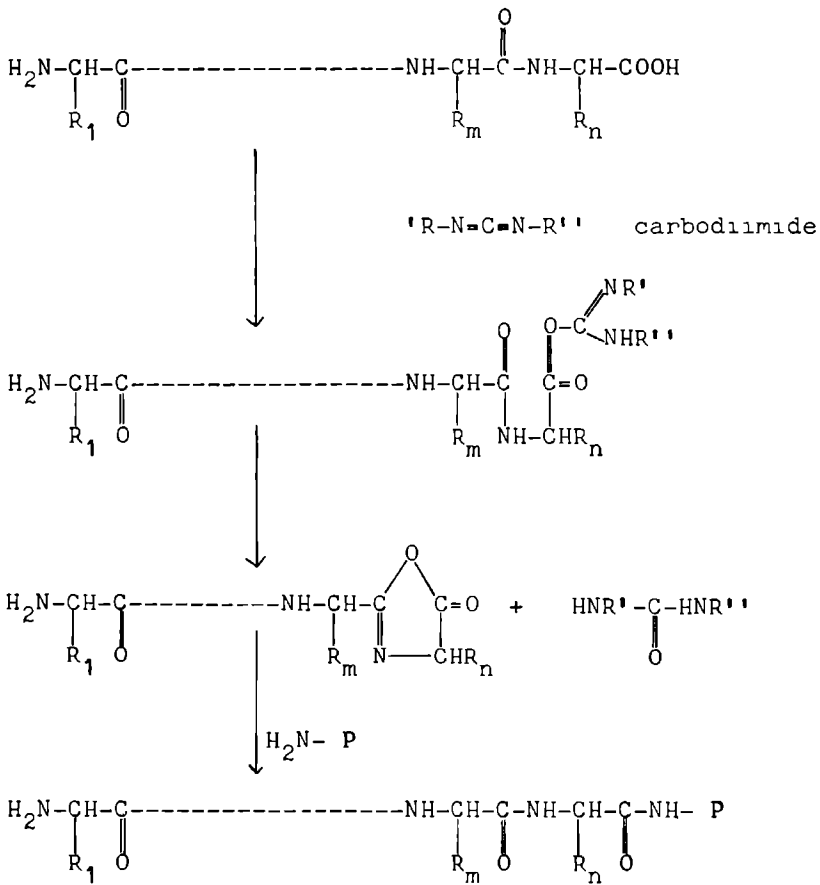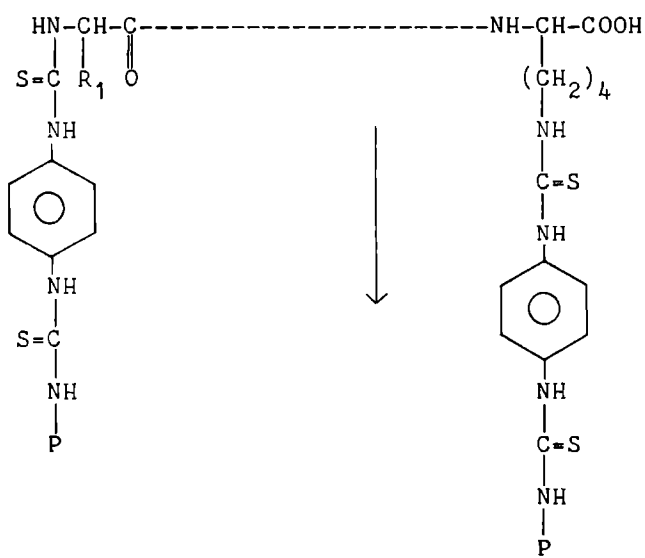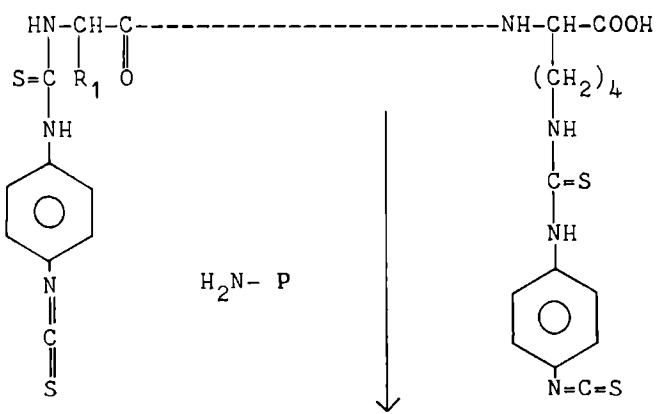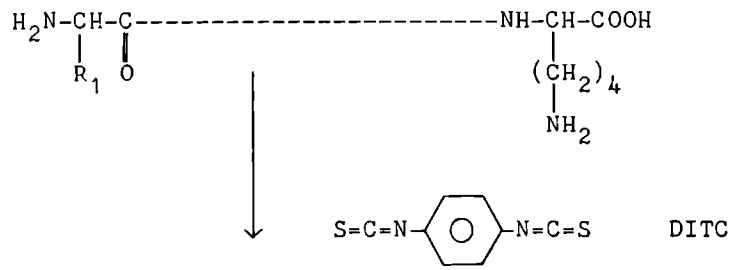
the amino groups have to be protected first.Using other conditions (Schellenberger et al.,1972, Laursen et al.,1975) all the carboxyl groups become attached to the support.In this case blocking of the amino groups is not necessary.An advantage of this method is that coupling can be performed in aqueous solutions.However,attachment yields are quite variable (10-80 %) and unpredictable (Laursen,1975,Wachter et al., 1975),presumably by lack of selectivity.

3.Attachment by side-chain amino groups.

Peptides or proteins bearing side-chain amino groups can be coupled to aminated supports by the use of p-phenyl-di-isothiocyanate (DITC) as a bifunctional reagent (Laursen et al.,1972,fig.5.5).A great excess of the reagent must be used to prevent cross-linking or even polymerization of peptides.The N-terminal amino acid has to be determined separately.The di-isothiocyanate method is restricted to peptides containing lysine,aminoethylcysteine or ornithine (conversion of arginine by hydrazinolysis).

In an attempt to combine the merits of the homoserine lactone method and the di-isothiocyanate procedure,the usefulness of p-isothiocyanato-benzoyl-DL-homoserine lactone as a bifunctional reagent was investigated.

5.1.EXPERIMENTAL

5.1.1. Materials and methods.

For attachment experiments horse heart cytochrome c was

used;for primary modifications and sequence studies melittin
(Serva,Heidelberg) was chosen as the model and the technique
was applied to several tryptic peptides of the βB_p chain of
β-crystallin.One example will be described.Further reagents,
apart from N-methylmorpholine and phenylisothiocyanate,which
were of ' sequenal grade ' (Pierce),were of ' pro-analyse '
quality.The support,controlled pore glass (Corning CPG-10,
200-400 mesh,75 $\overset{o}{A}$ mean pore diameter) was aminated with N-
(2-aminoethyl)-3-aminopropyltrimethoxysilane.The substitu-
tion of the support was carried out according to Wachter et
al.,(1973) with the applications of Bridgen (1975).Control-
led pore glass (5 g) was boiled with 6 N HCl for 15 minutes.
The glass beads were then washed with acetone on a sintered
glass filter and dried over night at -110°C.Acetone (20 ml)
containing 1 ml of N-(2-aminoethyl)-3-aminopropyltrimethoxy-
silane was added and the solution was stirred for 48 hours at
45° C.The yellow product was washed on a sintered glass filter
with acetone and methanol and subsequently dried in a vacuum
desiccator in the presence of P_2O_5.Spectrophotometric deter-
mination of the amount of primary amino groups was carried out
with 2,4,6-trinitrobenzenesulphonate.Per mg 170 neq amino
groups were present.Automatic sequential degradation was per-
formed with the Sequemat 10 K (Sequemat Inc.,Watertown,M.A.)
and the LKB 4020 solid-phase peptide sequencer.Amino acid
analyses were performed on the Beckman Multichrom automatic
amino acid analyzer.

fig. 5.6

63
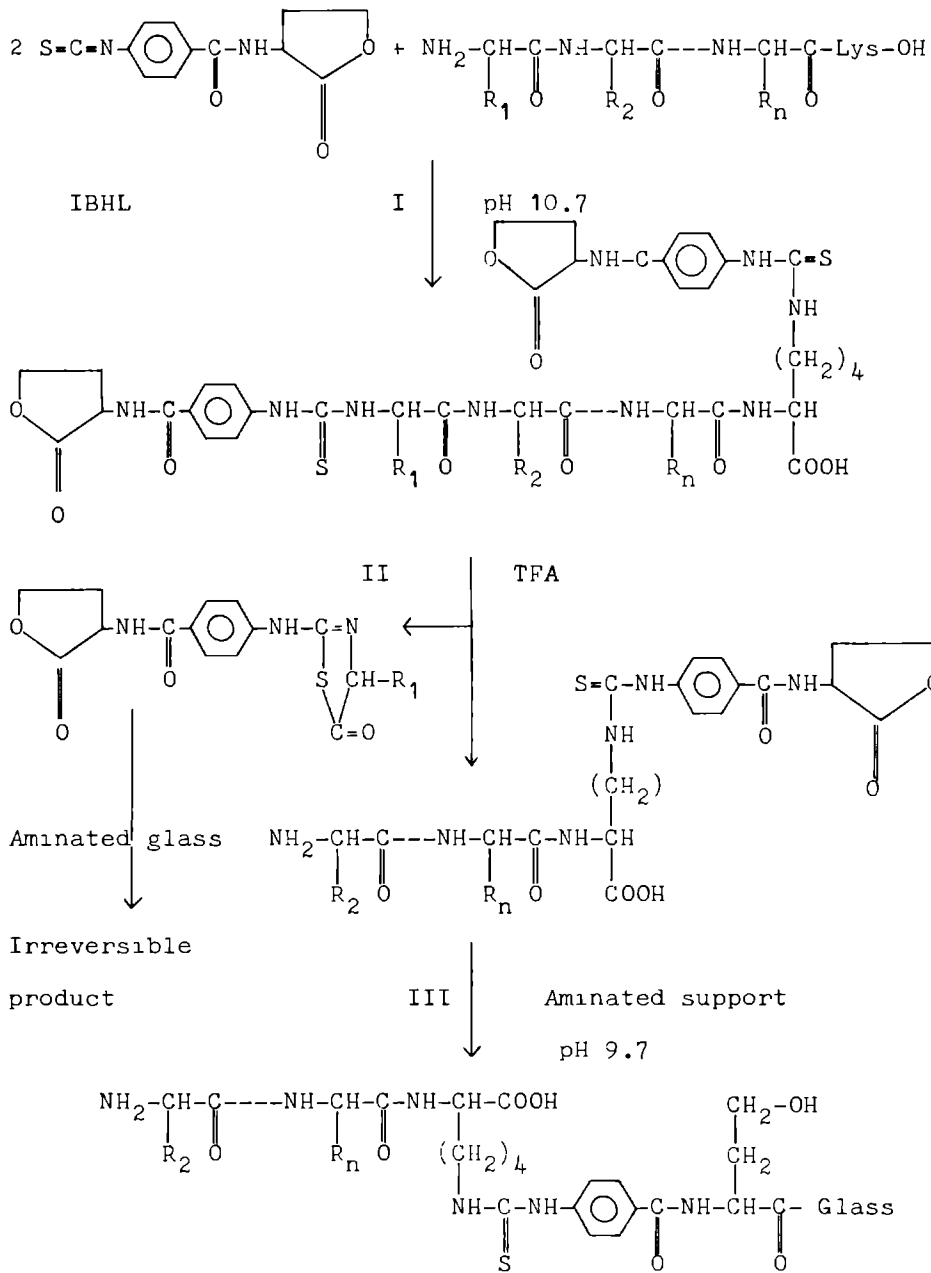
5.1.2.Derivatization and attachment of peptides using N-(p-isothiocyanato-benzoyl)-DL-homoserine lactone (IBHL, Tesser et al.,1976) (see fig.5.6).

Melittin was dissolved in a mixture of N-methylmorpholine and water (1:1 , pH 10.7) and in the same mixture containing sufficient trifluoroacetic acid to give pH 9.5.Into two series of test tubes 100 μl samples of the solution were pipetted,each sample comprising about 100 nmol of peptide. Varying amounts of IBHL dissolved in 100 μl of dimethylformamide were added (molar ratio increasing from 1 to 10) and the tubes flushed with nitrogen.Reaction was allowed to proceed for 1/2 hour at room temperature,followed by another 1/2 hour at 45° C.The mixtures were then evaporated in vacuo and subjected to paper chromatography (Whatmann 3 MM) using n-butanol,pyridine,water,acetic acid (75:60:50:15 by volume) as the solvent system.Spots were detected by U.V. fluorescence and with the ninhydrin and chloridine/tolidine tests.

For attachment of peptides to the aminated support a known tryptic hexapeptide of the βB_p chain of β-crystallin and melittin were treated with IBHL at pH 10.7 as described,but an intermediate concentration of IBHL was chosen,corresponding to two equivalents for each amino group present in the peptide, to ensure full modification.The evaporated peptide derivatives, comprising 100-200 nmol,were dissolved in 1 ml anhydrous trifluoroacetic acid (fig.5.6 ,step II),heated for 30 minutes at 45° C ,and again evaporated in vacuo in the presence of KOH.

The residues were coupled to 100 mg of aminated glass (fig.
5.6 ,step III) using the conditions of Horn and Laursen (1973)
with some modifications.The lactonized peptide-IBHL adduct,
dissolved in 100 µl of dimethylformamide,was added to 100 mg
of aminated glass support,suspended in 200 µl of dimethylfor-
mamide.The tube was then rinsed with another 100 µl of dime-
thylformamide and 50 µl of N-methylmorpholine-water (1:1),
acidified to pH 9.7 with trifluoroacetic acid,was added.When
the peptide-IBHL adduct is poorly soluble in dimethylformamide,
N-methylmorpholine-water (1:1),pH 9.7 ,can be used as solvent.
The mixture was stirred for two hours at 45° C.Thereafter 100
µl of a 50 % (v/v) solution of phenylisothiocyanate in ace-
tonitril was added.The tube was flushed with nitrogen and the
mixture was stirred for another hour at 45° C.The peptide-glass
adduct was then washed with dimethylformamide (two times),
methanol (two times) and ether.The product was dried in a
vacuum desiccator in the presence of P_2O_5.

For experiments with cytochrome c, 0.1 % (v/v) aqueous
triethylamine was used for derivatization of tne amino groups.
Coupling to the glass support was carried out in a similar way
as described for melittin and the hexapeptide of βB_p.The cou-
pling efficiency was determined by amino analysis of hydro-
lysates prepared from the peptide glass adducts containing the
βB_p hexapeptide and melittin.The amount of cytochrome c retai-
ned by the support was determined by spectrophotometric exami-
nation (410 nm) of the supernatant after centrifugation.

5.1.3. Automatic Sequencing.

The dry support was mixed with glass beads and subjected to degradation as described by Laursen (1971).Liberated thiazolinones were converted to phenylthiohydantoins by incubation in 20 % trifluoroacetic acid at 80° C for 10 minutes.The samples were purified by filtration through a column of Dowex-50 (X_2, 200-400 mesh) in methanol or by extraction with ethylacetate. The phenylisothiohydantoins were identified by thin layer chromatography and gas chromatography as described by Van der Ouderaa et al.(1973).

5.2. RESULTS

N-(p-isothiocyanato-benzoyl)-DL-homoserine lactone (IBHL) reacts readily with the amino groups of the three compounds,which were chosen as models.The reaction proceeded rapidly at pH 10.7 and did not leave unaltered starting material when equimolar amounts were reacted for one hour.At pH 9.7 the reaction had a much slower course.Even in the presence of an excess of reagent (2.5 equivalent per amino group) the reaction did not reach completion in that time.The subsequent reaction with 2-aminoethyl-3-aminopropyl glass gave excellent results (100% coupling) with the hexapeptide from βB_p and with cytochrome c (95 %).With melittin the incorporation was lower, but still satisfactory (60 %).The Edman degradation of the adducts of melittin and the hexapeptide of βB_p had the expec-

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ted course.The amino acid sequence of the latter proved to be

Glu-Thr-Gly-Val-Glu-Lys. C-terminal lysine was not eluted from

the column and the N-terminal glutamic acid was established

by the Dansyl-Edman method.The same holds for the lysyl resi-

due in position 7 of melittin,but this point of fixation could

be passed without decrease in yield of the liberated thiazo-

linones,indicating multiple fixation (positions 21 and/or 23)

of the hexacosapeptide amide.From this model 20 residues were

removed stepwise.The average yield per cycle amounted to 90 %.

5.3. DISCUSSION

The here advocated reagent for Solid-phase Edman degra-

dation combines the advantages of the proposed methods of Horn

and Laursen (1973) and Laursen et al.(1972).It offers a so-

lution for the general scarcity of methionine in proteins,which

restricts application of the first technique,and it eliminates

some drawbacks of the second.The reagent is thought for rapid

derivatization of protein fragments terminated by lysine,amino-

ethylcysteine or ornithine or bearing an amino side chain near

to the C-terminus.The risk of cross-linking of peptides by a

bifunctional reagent bearing two groups of equal reactivity

(as in 1,4-di-isothiocyanatobenzene) is greatly diminished.

This makes application of large excess of the bifunctional re-

agent unnecessary.Moreover,in general derivatives of homose-

rine lactone are stable compounds which can be reactivated by

a simple treatment with an anhydrous acid.This allows,if de-sired,an extensive purification prior to sequencing.A drawback of the second method (Laursen et al.,1972) which cannot be eliminated is the inherent blocking of the N-terminus,which remains to be determined in a separate experiment.

THE PARTIAL PRIMARY STRUCTURE OF THE PRINCIPAL BASIC β CHAIN

(βB_p)

In this chapter the elucidation of parts of the amino acid sequence of βB_p is described.Since no differences in amino acid composition,molecular weight and tryptic peptide map could be found between βB_p and βB_4,both the pure βB_p fractions as well as fractions containing βB_p plus βB_4 were used as starting material for sequence studies.

6.1.METHODS

6.1.1.Isolation of βB_p.

βB_p was isolated from β_H,β_L or total lens extract as described in chapter 3,section 3.1.2.

6.1.2.Aminoethylation.

Protein (10 mg/ml) was dissolved in 0.2 M Tris-HCl, pH 8.6 ,containing 6 M urea,0.005 M EDTA and 0.2 % 2-mercaptoethanol.After keeping the solution for one hour at room temperature 0.02 ml ethyleneimine per 10 mg protein was added.After another hour at room temperature excess 2-mercaptoethanol was added.The mixture was desalted over Sephadex

G-25 equilibrated in 0.1 N NH$_3$ and the protein fraction lyophilized.

6.1.3.Cyanogen bromide cleavage.

Cyanogen bromide cleavage (Gross,1967) of aminoethylated βB$_p$ was performed in 70 % formic acid at a protein concentration of 10 mg/ml,using an equal weignt of cyanogen bromide (Eastman).After 24 hours at room temperature in the dark a second amount of cyanogen bromide was added and the mixture was allowed to react for another 24 hours.The solution was diluted ten times with distilled water and evaporated to dryness by rotatory evaporation.

6.1.4.Separation of the cyanogen bromide fragments.

Separation of tne cyanogen bromide fragments was accomplished by gel filtration on a column (94 x 1.3 cm) of Sephadex G-75,equilibrated with 20 % acetic acid at an elution rate of 20 ml/h.Effluents were monitored at 280 nm.

6.1.5.Sodium dodecylsulphate gel electrophoresis.

Scdium dodecylsulphate gel electrophoresis,using 13 % acrylamide gels according to Laemmli (1970),was performed as described in chapter 2.1.6.2.

6.1.6.Enzymic digestions.

Digestion with trypsin (Worthington) was carried out

in 0.1 % ammonium hydrogen carbonate,pH 8.9 at a protein con-
centration of 10 mg/ml.Trypsin was added at an enzyme-substrate
ratio of 1:100.After digestion at 37° C during 2 hours the
mixture was frozen in dry ice-acetone and lyophilized.

Digestion with chymotrypsin (Boehringer) was performed
at 37° C in 0.1 % ammonium hydrogen carbonate,pH 8.9 for one
hour using an enzyme-substrate ratio of 1:100.

For thermolytic digestion protein (10 mg/ml) was dis-
solved in 0.2 M ammonium acetate,pH 8.5 ,containing 5 mM $CaCl_2$.
After addition of thermolysin (1 % w/w,Calbiochem) digestion
was carried out at 37° C for one hour.

Digestion with Staphylococcus aureus protease (Miles)
was performed in 0.5 M NH_4HCO_3,pH 7.8 at room temperature
during 30 hours using an enzyme-substrate ratio of 1:50.

6.1.7.Isolation of peptides.

Analytical peptide maps of enzymic digests were pre-
pared as described in chapter 3,section 3.1.5.Preparative
peptide mapping using 1.0 - 2.0 µmol of protein was performed
under the same conditions as described for the analytical
maps,with the exception that the electrophoresis time was
increased to two hours.Peptides were located by reaction with
fluorescamine (Hoffmann La Roche,0.00125 % solution in ace-
tone containing 0.05 % pyridine).For elution of the peptides
the paper was cut into small pieces and eluted by centrifu-
gation with 10 % acetic acid.

6.1.8.Amino acid analysis.

Amino acid analyses were performed with a Beckman Multichrom amino acid analyzer using a single column and with the Rank Hilger Chromaspek.For determination of homoserine the temperature of the column of the Beckman Multichrom was lowered to 50° C,whilst the pump speed was decreased to 70 ml/h.The tryptophan content of βB_p , βB_p-CB_1 and βB_p-CB_2 was determined by the methods of Bredderman (1974) and of Gaitonde and Dovey (1970).

Hydrolysis of βB_p and its cyanogen bromide fragments was performed for 24, 48 and 72 hours at 110° C in evacuated tubes.Peptides were hydrolyzed in 6 N HCl at 110° C for 22 h.

6.1.9.Sequence Analysis.

Sequence analysis was performed by the dansyl-Edman procedure as described by Gray and Smith (1970) and by solid phase sequencing using the IBHL-method (Herbrink et al.,1975) and the homoserine lactone coupling (Horn and Laursen,1973). Identification of dansyl amino acids was performed by thin-layer chromatography on polyamide sheets (5 x 5 cm) (Woods et al.,1967).Amino acid phenylthiohydantoins were identified by thin layer chromatography and by gas chromatography as described by Van der Ouderaa (1974).

6.1.10.Amide Assignment.

Determination of amide groups was performed by identifi-

72

cation of phenylthiohydantoins of glutamine and asparagine by thin-layer chromatography and from the electrophoretic mobilities of peptides at pH 6.5 (Offord,1966).

6.1.11.Peptide Nomenclature.

Since the complete amino acid sequence of βB_p has not yet been elucidated,the peptide nomenclature remains more or less arbitrarily.Since amino acid analysis of βB_p showed the presence of 12 lysine and 10 arginine residues,the numbering of the tryptic peptides was made backwards from the C-terminal peptide T_{23}.The numbering of the tryptic peptides T_5 to T_{13} is arbitrarily.

Cyanogen bromide fragments are indicated by the pre-scription CB,thermolytic peptides by Th,chymotryptic peptides by C and peptides obtained by digestion with S.aureus protease by SP.

6.2.RESULTS.

6.2.1.Cleavage with cyanogen bromide and purification of cya-nogen bromide fragments.

Reaction of unmodified βB_p with cyanogen bromide resulted in poor cleavage results (approximately 60 %).Much better results were obtained when βB_p was aminoethylated before clea-vage with cyanogen bromide.The cyanogen bromide fragments were fractionated as shown in fig.6.1.Since the βB_p chain contains two methionyl residues three fragments are obtained.The first
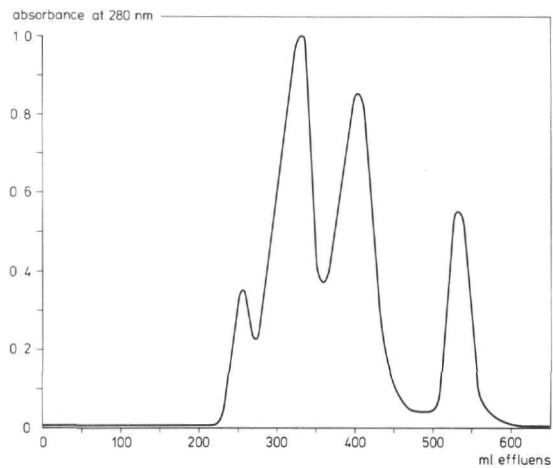
Fig.6.1.Gel filtration of cyanogen bromide fragments of βB_p
on Sephadex G-75 equilibrated with 20 % acetic acid.
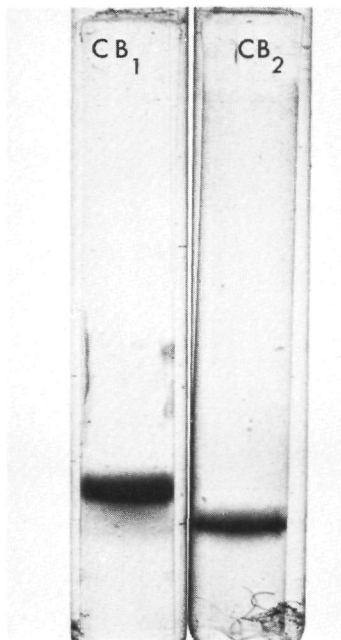
Fig.6.2.Sodium dodecylsul-
phate gel electrophoresis
of βB_p-CB_1 and βB_p-CB_2.

peak contains unreacted βB_p.A similar elution profile (measured at 230 and 280 nm) was found by gel filtration on Sephadex G-75 using 0.1 N NH_3 as the eluent.However,since fractionation using acetic acid as eluent gave a better separation,the latter was preferred.The purity of the cyanogen bromide fragments was checked by sodium dodecylsulphate gel electrophoresis using 13 % polyacrylamide gels according to Laemmli (1970) (fig.6.2).From the results of sodium dodecylsulphate gel electrophoresis a molecular weight of approximately 13,000 dalton was calculated for the largest cyanogen bromide fragment,whilst the fragment eluted in the third peak revealed a molecular weight of approximately 9,000 dalton. This leaves a molecular weight of approximately 2,000 dalton for the smallest cyanogen bromide fragment.

6.2.2.Allignment of cyanogen bromide fragments.

The position of the cyanogen bromide fragments was obtained by end group determination.

Since cleavage with cyanogen bromide converts methionine into homoserine ,the absence of homoserine should identify the C-terminal cyanogen bromide fragment.Amino acid analysis showed that,in contrast to the 13,000 and 9,000 dalton fragments, the smallest fragment lacks homoserine.The smallest fragment, therefore,is the C-terminal fragment and called CB_3.Since no free N-terminal amino acid could be found for the βB_p chain, the position of the two larger fragments could be determined

by the finding of a free N-terminal amino acid.By the use of dansylchloride glutamic acid was found as the N-terminal amino acid of the 9,000 dalton fragment.No free N-terminal amino acid could be detected for the 13,000 fragment and the 2,000 dalton fragment.In the latter case this turned out to be due to the presence of pyrrolidone carboxylic acid.Therefore,the sequence of the three cyanogen bromide fragments should be: 13,000 dalton fragment (CB_1)- 9,000 dalton fragment (CB_2)- 2,000 dalton fragment (CB_3).

The results of amino acid analysis of the three cyanogen bromide fragments are summarized in table 6.I.From the amino acid analysis of cyanogen bromide fragments obtained after cleavage with cyanogen bromide of unmodified βB_p,it turned out that only CB_1 contains cysteine.

6.2.3.Partial amino acid sequence of CB_1.

6.2.3.1.Tryptic peptides.

After digestion with trypsin of aminoethylated CB_1 a number of peptides could be purified by fingerprinting (fig. 6.3).Their amino acid compositions are given in table 6.II. Peptide T_{5a} sometimes can be found in the tryptic peptide map of AE-CB_1.Amino acid sequences of the tryptic peptides of AE-CB_1 are shown in fig.6.4.Peptide T_1 turned out to contain a blocked N-terminus.Since βB_p is believed to be acetylated,T_1 is considered to be the N-terminal tryptic peptide. The C-terminal sequence of this peptide was deduced from

Table 6.I. Amino acid compositions of βB_p and its cyanogen bromide fragments.

Amino acid	βB_p mol/ 22,537 g	nearest inte- gral number	CB$_1$ mol/ 13,022 g	nearest inte- gral number	CB$_2$ mol/ 8,079 g	sequence	CB$_3$ mol/ 1436 g	sequence
Aspartic acid	16.6	17	9.9	10	6.6	7		
Threonine*	6.8	7	5.7	6	1.2	1		
Serine*	16.8	17	9.4	9	6.2	6	1.9	2
Glutamic acid	31.8	32	20.1	20	11.0	10	2.0	2
Proline	13.6	14	9.0	9	4.1	4	1.1	1
Glycine	18.6	19	9.8	10	8.0	8	1.1	1
Alanine	8.4	8	5.0	5	2.2	2	1.0	1
Cysteine**	1.8	2	1.8	2				
Valine***	13.8	14	6.1	6	8.0	8		
Methionine	1.8	2		(1)		1		
Isoleucine***	6.2	6	4.2	4	1.9	2		
Leucine	10.0	10	6.9	7	3.2	3		
Tyrosine	8.8	9	3.0	3	5.8	6		
Phenylalanine	8.0	8	4.8	5	2.2	2	1.0	1
Lysine	12.0	12	9.3	9	3.0	3		
Histidine	8.2	8	3.9	4	2.4	2	2.0	2
Arginine	10.0	10	4.1	4	5.4	5	1.1	1
Tryptophan****	4.0	4	2.3	2	1.0	1	n.d.	1
Total		199		116		71		12

*Extrapolated to zero time hydrolysis. **Determined as cysteic acid. ***Values for 72 h hydrolysis. ****Determined according to Gaitonde-Dovey (1970) and Bredderman (1974).

TABLE 6.II

Amino acid compositions of the tryptic peptides of AE-CB$_1$. Molar ratios are given. No corrections were made for hydrolytic losses.

Peptide	Asp	Thr	Ser	Glu	Pro	Gly	Ala	Val	Ile	Leu	Tyr	Phe	Lys	His	Arg	Trp	number of residues	mobility
T$_1$	1.9	1.1	1.0	2.8	3.3	1.3	1.8			1.0			2.1	0.7			17	0.20
T$_2$				3.4		1.1		1.0				1.6	0.9				8	-0.26
T$_3$				1.1	0.9	1.1						+			0.9		5	0
T$_4$	0.9		2.1	1.0											0.9	+	6	0.26
T$_5$	1.1	1.0	2.9		1.2			0.9	2.0				0.9	1.0			11	0.23
T$_{5a}$	1.0	0.9	2.4		1.0			0.9	2.0				1.1	1.8			12	0.40
T$_6$	1.2		0.9	2.2				1.0					0.8	0.9			7	0
T$_7$	2.6	2.2	0.6	1.4	1.0	1.2			0.9	1.0	1.0	0.9	0.9				13	0
T$_8$		1.0	2.1		1.0			1.0					0.8				6	-0.34
T$_9$	2.7	1.6	4.0	2.8	0.9	1.9	1.4	2.5		1.0					1.2	+	21	0
T$_{10}$	1.0			1.2	3.1	0.7				1.1		1.8					9	0.51
T$_{11}$										1.0		1.0					2	0.60
T$_{12}$															1.0		1	0.93
T$_{13}$													1.0				1	1.0

Fig.6.4

Amino acid sequences of tryptic peptides of AE-CB$_1$.Sequences were determined by the solid-phase method using IBHL (⟶),the dansyl-Edman procedure (⟶) or both (⟶).In a few cases data from thermolytic,chymotryptic and S.aureus protease cleavage of AE-CB$_1$ were used.

$$T_1 \quad CH_3-\overset{\overset{\textstyle O}{\|}}{C}-(Asx,Thr,Ser,Glx,Pro_3,Gly,Ala,Lys\ )-Leu-Glu-Gln-Ala-Asn-His-Lys$$

⟵--------C1-------------
⟶ ⟶ ⟶ ⟶
⟵----------SP1-------

T_2 Gly-Glu-Gln-Phe-Val-Phe-Glu-Lys
⟶ ⟶ ⟶ ⟶ ⟶ ⟶
⟵-------SP2--------⟶
⟶ ⟶ ⟶ ⟶

T_3 Gly-Glu-Tyr-Pro-Arg
⟶ ⟶ ⟶ ⟶ ⟶

T_4 Asn-Ser-Trp-Ser,Gln-Arg
⟶ ⟶

T_5 Thr-Asp-Ser-Leu-Ser-Ser-Leu-Arg-Pro-Ile-Lys
⟶ ⟶ ⟶ ⟶
⟵--Th1----⟶
⟶ ⟶
⟵------C3------
⟶ ⟶ ⟶ ⟶

T_6 Val-Asp-Ser-Gln-Glu-His-Lys
⟶ ⟶ ⟶ ⟶ ⟶ ⟶

T_7 Ile-Thr-Leu-Tyr-Glu-Asn-Pro-Asn-Phe-Thr-Gly-Ser-Lys
⟶ ⟶ ⟶ ⟶ ⟶ ⟶ ⟶ ⟶
⟵------C4--------⟶
⟶ ⟶ ⟶ ⟶

T_8 Glu-Thr-Gly-Val-Glu-Lys
⟶ ⟶ ⟶ ⟶ ⟶ ⟶

T_{10} Gly-Lys-Pro-Gln-Pro-Ile-Asn-Pro-Lys
⟶ ⟶ ⟶ ⟶ ⟶ ⟶ ⟶

T_{11} Leu-Lys
⟶ ⟶
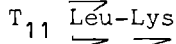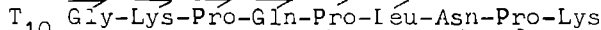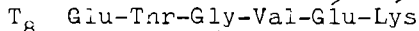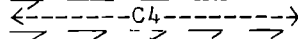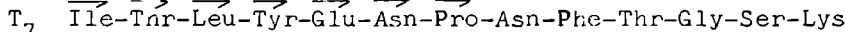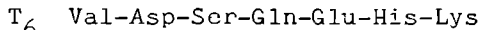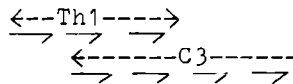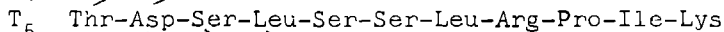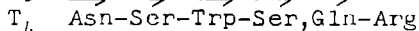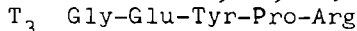
results obtained after digestion of AE-CB$_1$ with chymotrypsin and S.aureus protease.The reason for considering these peptides as overlap peptides between T$_1$ and T$_2$ lies in the presence of histidine in both fragments.CB$_1$ contains four histidine residues,one in T$_1$,one in T$_6$ and two in SP3 (see fig.6.5) Since the sequence of the chymotryptic peptide C1 did not match with the sequence of T$_6$ or SP3,it was concluded to be part of T$_1$.The amino acid sequence of T$_2$,T$_5$,T$_6$,T$_7$,T$_{10}$ and T$_{11}$ has been determined by the dansyl-Edman procedure (⟶),the solid-phase method using IBHL (⟶) or both (⟶).For sequence elucidation of T$_5$ the results obtained after digestion of AE-CB$_1$ with thermolysin and chymotrypsin were also used.The N-terminal part of T$_4$ was sequenced by the dansyl-Edman method.
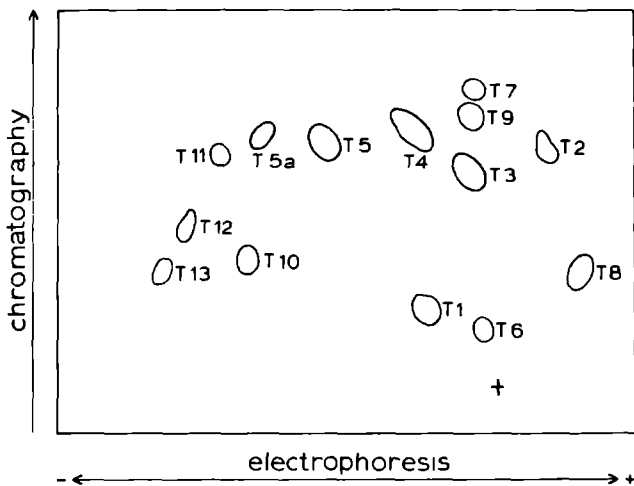
Fig.6.3.Tryptic peptide mapping of AE-CB$_1$.

TABLE 6.III

Amino acid compositions of chymotryptic,thermolytic and Staphylococcal protease peptides of AE-CB$_1$.Molar ratios are given.No corrections were made for hydrolytic losses.

Peptide	Asp	Thr	Ser	Glu	Pro	Gly	Ala	Val	Met	Ile	Leu	Tyr	Phe	Lys	His	Arg	Trp
C$_1$	1.2			4.0		1.1	1.1						0.8	1.1	0.6		
C$_2$	1.0	1.2	2.0	1.0	1.1		0.9						0.9	1.0		0.8	+
C$_3$	1.3	1.2	2.8	1.4	1.0		1.1		0.7					1.8	0.7	1.0	
C$_4$	1.9		1.1	1.3								0.8					
Th$_1$					1.0						1.1					0.9	
Th$_2$		0.9	1.9		1.0								0.9		2.3		
SP$_1$	1.1		1.8		1.0	1.2								0.8	0.7		
SP$_2$			2.0					1.1					1.9				
SP$_3$	0.9	1.2	2.4		1.1								0.8		1.6		

6.2.3.2.Chymotryptic,tnermolytic and S.aureus protease peptides.

A number of peptides were purified by peptide mapping after digestion with chymotrypsin,thermolysin or S.aureus protease.The amino acid compositions are given in table 6.III. Sequential analysis was performea by tne dansyl-Edman procedure.The results are snown in fig.6.5.Amide assignment in C1 was made from tne position on tne peptide map,the sequence of T_2 and the preference of S.aureus protease to cleave at the C-terminal side of glutamic acid.Amide assignment of Th2 and SP3 was also made from the position on tne peptide map and the preference of S.aureus protease for cleavage at the C-terminal side of glutamic acid.Amide assignment of C3 was made from the position on the peptide map.

Fig.6.5 Amino acid sequences of peptides obtained after digestion of AE-CB$_1$ with thermolysin,chymotrypsin or S.aureus protease.Sequences were determined by the dansyl-Edman procedure (⟶).

C1   Glu-Gln-Ala-Asn-His-Lys-Gly Glu-Gln-Phe
       ⟶ ⟶ ⟶ ⟶   ⟶ ⟶

C2   Val-Phe-Glu-Lys-Gly-Glu-Tyr-Pro-Arg-Asn-Ser-Trp
       ⟶ ⟶ ⟶ ⟶ ⟶ ⟶ ⟶

C3   Arg-Pro-Ile-Lys-Val-Asp-Ser-Gln-Glu-His-Lys-Gly,Gln
       ⟶ ⟶ ⟶ ⟶ ⟶ ⟶ ⟶ ⟶

C4   Glu-Asn-Pro-Asn-Phe
       ⟶ ⟶ ⟶ ⟶ ⟶

Th1   Leu-Arg-Pro
       ⟶ ⟶ ⟶

Th2   Pne-His-Gly-His-Ser-Gln-Glu
       ⟶ ⟶ ⟶ ⟶ ⟶

SP2   Gln-Phe-Val-Pne-Glu
       ⟶ ⟶ ⟶ ⟶

SP3   Asn-Phe-His-Gly-His-Ser-Gln-Glu
       ⟶ ⟶ ⟶ ⟶

6.2.3.3.Partial sequence assembly of CB$_1$.

From the results obtained by digestion of AE-CB$_1$ with thermolysin,chymotrypsin and S.aureus protease a number of overlaps between tryptic peptides could be obtained (see fig.6.6).

T$_1$-T$_2$-T$_3$-T$_4$.As discussed above,T$_1$ is considered to be the N-terminal peptide of CB$_1$ since it lacks a free N-termi-nal amino acid.Overlap between T$_1$ and T$_2$ was established by the sequence of C1 and the amino acid composition of SP1.The linkage between T$_2$,T$_3$ and T$_4$ was deduced from the finding of C2.

T$_5$-T$_6$.The junction T$_5$-T$_6$ was established after sequencing C3.

Fig.6.6.Partial sequence assembly of AE-CB$_1$.Overlap sequences were determined by the dansyl-Edman procedure (⟶).

$$CH_3-\overset{\overset{O}{\|}}{C}-(\ Asx,Thr,Ser,Glx,Pro_3,Gly,Ala,Lys\ )-Leu-Glu-Gln-Ala-$$

T$_1$

← -----C1----
⇀ ⇀ ⇀
←--SP1--

T$_2$ T$_3$

Asn-His-Lys-Gly Glu-Gln-Phe-Val-Phe-Glu-Lys--Gly-Glu-Tyr-Pro-

---------------------------→ ←-------------C2--------------
⇀   ⇀ ⇀   ⇀ ⇀ ⇀ ⇀ ⇀ ⇀ ⇀
---------------------→   T$_4$
Arg-Asn-Ser-Trp-Ser, Gln-Arg

-------------→   T$_5$ T$_6$
Thr-Asp-Ser-Leu-Ser-Ser-Leu-Arg-Pro-Ile-Lys-Val-Asp-Ser-Gln-
                    ←-----------------C3--------------
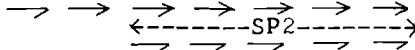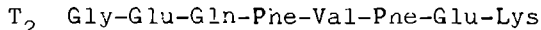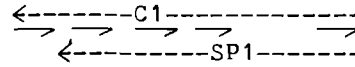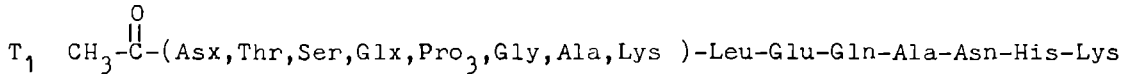   ⇁ ⇁ ⇁ ⇁ ⇁ ⇁ ⇁ ⇁
Glu-His-Lys-Gly,Gln

------------------→
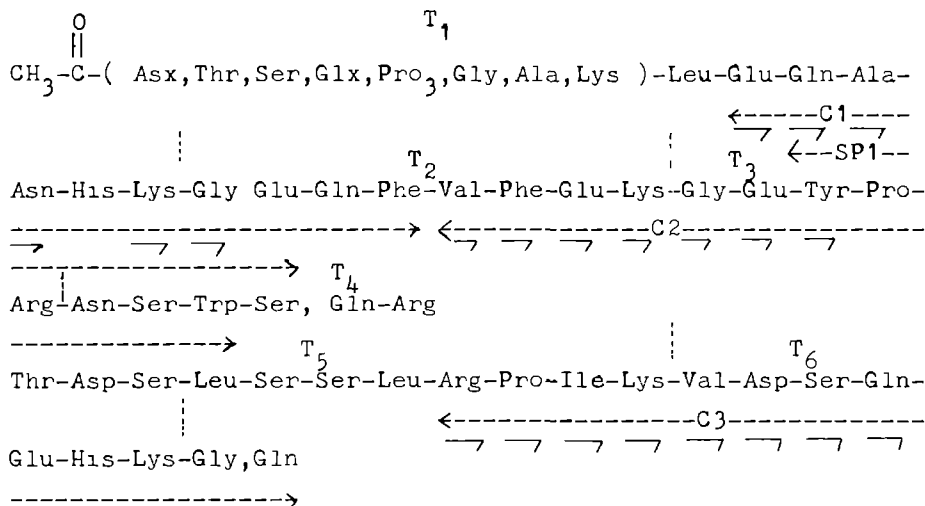
TABLE 6.IV

Amino acid compositions of the tryptic peptides of CB_2.Molar ratios are given.No corrections for hydrolytic losses were made.

Peptide	Asp	Thr	Ser	Glu	Pro	Gly	Ala	Val	Ile	Leu	Tyr	Phe	Lys	His	Arg	Trp	number of residues	mobility
T_{14}	3.1		1.1	3.5	1.2	1.2	1.0	1.8	0.6		0.8	1.0	1.4	2.2			18	−0.44
T_{15}		1.8						2.2							1.0		5	0.51
T_{16}		0.9	1.0	2.3	1.0	2.6		1.8			1.2				1.2	+	15*	0.17
T17			2.0			1.1			2.8	+		1.2					8	0
T_{18}	1.0					1.0					0.9	1.2					4	0
T_{19}	2.1		1.6	3.1	2.0	2.1	1.2	2.0				1.0			1.0		16	−0.22
T_{20}															1.0		1	1.0
T_{21}								1.0							1.0		2	0.65
T_{21a}								0.9							2.1		3	1.0

* Sequence determination of T_{16} yielded 3 Tyrosines.Amino acid analysis yielded a to low value for Tyrosine,due to destruction during hydrolysis often observed for peptides isolated from Whatman 3 MM paper.

6.2.4.The amino acid sequence of CB_2.

6.2.4.1.Tryptic peptides.

    After digestion with trypsin all the tryptic peptides
of CB_2,except the C-terminal peptide,could be isolated by
peptide mapping (fig.6.7).The amino acid composition of the
tryptic peptides of CB_2 is given in table 6.IV.In a few cases
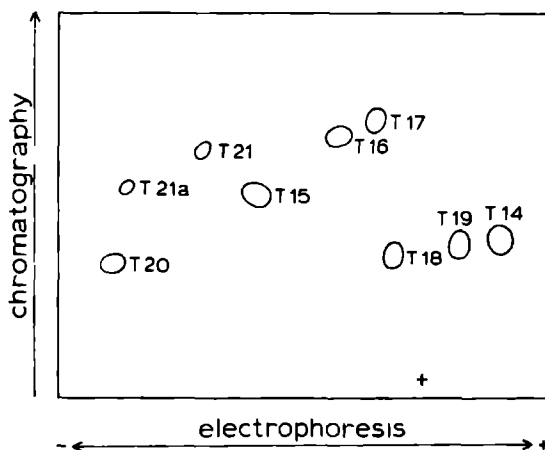
Fig.6.7.Tryptic peptide map of CB_2.

the peptide T_{21a} was also found in the tryptic peptide map
of CB_2.Amino acid sequences were determined by the dansyl-
Edman procedure ($\longrightarrow$),the IBHL-method ($\longrightarrow$) or both pro-
cedures ($\longrightarrow$).Tne results are shown in fig.6.8.For the larger
peptides (T_{14},T_{16} and T_{19}) data from chymotryptic and ther-
molytic digestion of total CB_2 were also used.Before attach-

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Fig.6.8.Amino acid sequences of the tryptic peptides of CB_2.Sequences were determined by
the solid phase method using IBHL ($\longrightarrow$),the dansyl-Edman procedure ($\longrightarrow$) or both ($\longrightarrow$).
In a few cases data from thermolytic and chymotryptic cleavage of total CB_2 are used.

T_{14} Gln-Val-Ile-Asp-Asp-Asp-Val-Pro-Ser-Phe-His-Ala-His-Gly-Tyr-Glu-Gln-Lys
$\to$ $\to$ $\to$ $\to$ $\to$ $\to$ $\to$ $\to$ $\to$ $\to$ $\quad$ $\to$ $\quad$ $\to$ $\to$ $\to$ $\to$
$\qquad\qquad\qquad\qquad\qquad\qquad\qquad$ ←-Tn3-→ ←--------Th4----------------→
$\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad$ ←-----C6------→

T_{15} Val-Ser-Ser-Val-Arg
$\to$ $\to$ $\to$ $\to$

T_{16} Val-Gln-Ser-Gly-Thr-Trp-Val-Gly-Tyr-Gln-Tyr-Pro-Gly-Tyr-Arg
$\to$ $\to$ $\to$ $\to$ $\to$ $\qquad$ $\to$ $\to$ $\to$ $\to$ $\to$
$\qquad\qquad\qquad\qquad\qquad$ ←---C7---→ $\quad$ ←--------C8------→

T_{17} Gly-Leu-Gln-Tyr-Leu-Leu-Glu-Lys
$\to$ $\to$ $\to$ $\to$ $\to$ $\to$ $\to$

T_{18} Gly-Asp-Tyr-Lys
$\to$ $\to$ $\to$ $\to$

T_{19} Asp-Ser-Gly-Asp-Phe-Gly-Ala-Pro-Gln-Pro-Gln-Val-Gln-Ser-Val-Arg
$\to$ $\to$ $\to$ $\to$ $\to$ $\to$ $\to$ $\to$ $\to$ $\to$ $\to$ $\to$
$\qquad\qquad\qquad$ ←-----------C13--------------→ $\quad$ ←--C14-----
$\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad$ $\to$ $\to$ $\to$

T_{20} Arg

T_{21} Ile-Arg
$\to$ $\to$

T_{21a} Arg-Ile-Arg
$\to$ $\to$ $\to$
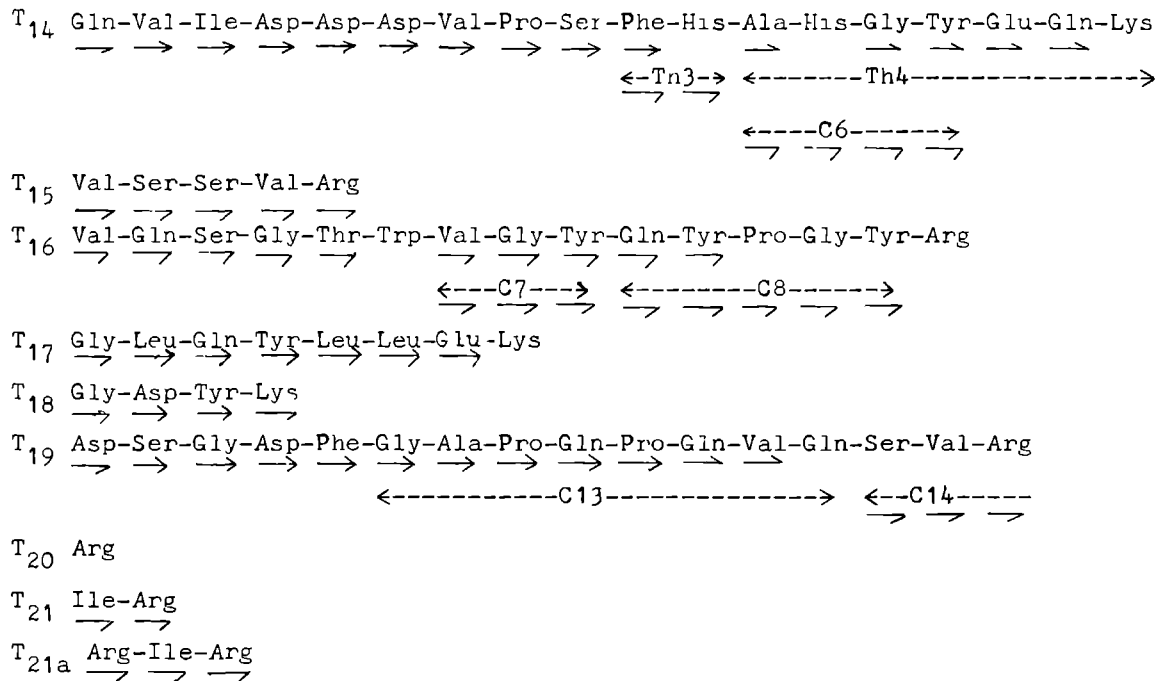
ment to the solid support T_{19} was treated with hydrazine (64%
solution in distilled water, 10 minutes at 80° C) in order
to convert arginine to ornithine. By use of the solid phase
Edman degradation T_{19} could be sequenced up to valine in po-
sition 12. The sequence of T_{19} has been completed by the chymo-
tryptic peptides C13 and C14.

6.2.4.2. Chymotryptic, thermolytic and S. aureus protease pep-
tides of CB_2.

In order to obtain overlap fragments for the allignment
of the tryptic peptides CB_2 was degraded with thermolysin,
chymotrypsin and S. aureus protease. A number of peptides were
purified by preparative peptide mapping. The amino acid compo-
sition of these peptides is given in table 6.V. The amino acid
sequences, as determined by the dansyl-Edman method are given
in fig. 6.9. The sequence of SP7 has been determined by solid
phase Edman degradation after coupling through the C-terminal
homoserine lactone (Horn and Laursen, 1973).

6.2.4.3. Sequence assembly of CB_2.

The complete amino acid sequence of CB_2 is shown in fig.
6.10. The overlap of T_{14}, T_{15} and T_{16} has been established by
solid phase Edman degradation after coupling of total CB_2 to
aminated glass by the IBHL-method. Identification of phenyl-
thiohydantoin amino acids could be performed up to glycine
at position 36. Overlap between T_{16} and T_{17} was determined by

TABLE 6 V

Amino acid compositions of chymotryptic,thermolytic and Staphylococcal protease peptides of CB_2.Molar ratios are given.No corrections for hydrolytic losses were made.

Peptide	Asp	Thr	Ser	Glu	Pro	Gly	Ala	Val	Ile	Leu	Tyr	Phe	Lys	His	Arg	mobility
C_5	3.4		0.9	1.1	1.1		1.5	0.8			1.0		1.0			-0.50
C_6						0.9	0.9				0.6		1.2			
C_7						1.1		0.9								
C_8				1.1	0.9	1.1					1.9					
C_9				1.1		1.1				1.0	0.8			1.0		
C_{10}	1.2			1.4		1.1				1.6	0.5	1.1				-0.26
C_{11}	1.9	0.9				1.2						1.0	1.2			-0.28
C12	3.1		1.0	4.0	2.4	3.0	1.1	1.0		1.8	0.9	1.0	1.8			
C_{13}				3.4	1.7	1.0	1.0	1.0								
C_{14}	0.8	0.8					1.1	0.8						3.4		
Th_3												0.9	1.1			
Th_4			2.2			1.1	1.0				0.4		1.0	1.1		0.41

TABLE 6.V continued

Peptide	Asp	Thr	Ser	Glu	Pro	Gly	Ala	Val	Ile	Leu	Tyr	Phe	Lys	His	Arg	mobility
Th5			0.9	1.2		0.5		2.0							1.0	
Th6			1.3	0.7		1.1					0.9					
Th7						1.0									1.0	
Th8	2.0		0.9	1.3		2.2				1.0	0.8		1.8			−0.50
Th9				2.2	2.1	0.8	1.0					1.0				
Th10										0.9					2.1	
SP4	2.5		0.9	2.4	0.8	1.1	1.2	1.5	0.6		0.5	0.7		1.6		
SP5		1.0	2.6	5.2	1.0	4.5		4.4		2.9	2.6		0.8		1.9	
SP6	3.4		1.8	3.7	1.8	3.0	1.1	1.0			0.7	1.0	1.6			
SP7	1.1		0.3	0.5				0.8	0.8						3.2	

Fig.6.9.Amino acid sequences of peptides obtained by diges-
tion of CB_2 with chymotrypsin,thermolysin and S.aureus pro-
tease.Seqences were determined by the dansyl-Edman procedure
(——→) or by the solid phase method after coupling through
homoserine lactone (——→).

C5   Gln-Val-Ile-Asp-Asp-Asp-Val-Pro-Ser-Phe-His
      →   →   →

C6   Ala-His-Gly-Tyr
      →   →   →   →

C7   Val-Gly-Tyr
      →   →   →

C8   Gln-Tyr-Pro-Gly-Tyr
      →   →   →   →   →

C9   Arg-Gly-Leu-Gln-Tyr
      →   →   →   →   →

C10   Leu-Leu-Glu-Lys-Gly-Asp-Tyr
      —   —   —   —

C14   Ser-Val-Arg-Arg-Ile-Arg-Asn
      →   →   →   →   →   →   →

Th3   Phe-His
      →   →

Th5   Val-Arg-Val-Gln-Ser-Gly
      →   →   →   →   →

Th6   Tyr-Gln-Tyr-Pro-Gly
      →   →   →   →   →

Th7   Tyr-Arg-Gly
      →   →   →

Th8   Leu-Glu-Lys-Gly-Asp-Tyr-Lys-Asp-Ser-Gly-Asp
      →   →   →   →   →

Th9   Pne-Gly-Ala-Pro-Gln-Pro-Gln
      →   →   →   →

Th10 Val-Arg-Arg
      →   →   →

SP7   Ser-Val-Arg-Arg-Ile-Arg-Asn-Homoser.
      ⇢   ⇢   ⇢   ⇢

the finding of the chymotryptic peptide C9 and the thermoly-
tic peptide Th7.The sequence T_{14}-T_{15}-T_{16}-T_{17} is in good agree-
ment with the amino acid composition of SP5.The junction T_{17}-
T_{18} is arrived at by the sequence of C10 and Th8.The order of

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Fig.6.10 Proposed amino acid sequence of CB_2. Overlap sequences were determined by the dansyl-Edman procedure (⟶) or by solid phase Edman degradation (⟶).

```
                                    10      T14                                    |      20    T15
Gln-Val-Ile-Asp-Asp-Asp-Val-Pro-Ser-Phe-His-Ala-His-Gly-Tyr-Glu-Gln-Lys-Val-Ser-Ser-Val-
  →   →   →   ⇢   ⇢   ⇢   →   →       →       →       ⇢   →   ⇢   ⇢   →       →       ⇢   →
  ←-----------------------SP4-------------------------------→  ←----------------------
```

```
    |                          30      T16                              |      40    T17            ⇐--
Arg-Val-Gln-Ser-Gly-Thr-Trp-Val-Gly-Tyr-Gln-Tyr-Pro-Gly-Tyr-Arg-Gly-Leu-Gln-Tyr-Leu-Leu-
  →   →       →       →   →   →       →   →   →       →   →                                    ⇒
------------------------------------SP5----------------------------------------------------
```

```
-------Th5--------→                                    ←---Th7--→                          ←--
  →   →       →   →                                      →   →   →                            ⇐
    |    T18      50 |     T19                            ←-----C9------→  ←-------          ⇒
                                                        60  →   →   →   →   ←--⇒--⇒
Glu-Lys-Gly-Asp-Tyr-Lys-Asp-Ser-Gly-Asp-Phe-Gly-Ala-Pro-Gln-Pro-Gln-Val-Gln-Ser-Val-Arg-
--→ ←----------------------------SP6----------------------------------------------→  ⇐--⇒--
```

```
--------Th8------------------------→                                              ⇐-Th10-
  →   →   →   →                                                                     →   →
------C10---------→ ←-------C11-----------→                                        ←----------
⇒--⇒--⇒--⇒--⇒                                    -----------C12----------------→   →   →   →
```

```
T20  T21      T22a
Arg-Ile-Arg-Asn-Homoser.
```

```
-------SP7------------→
  ⇢       ⇢
--→
```

```
-----C14------→
  →   →   →   →
```
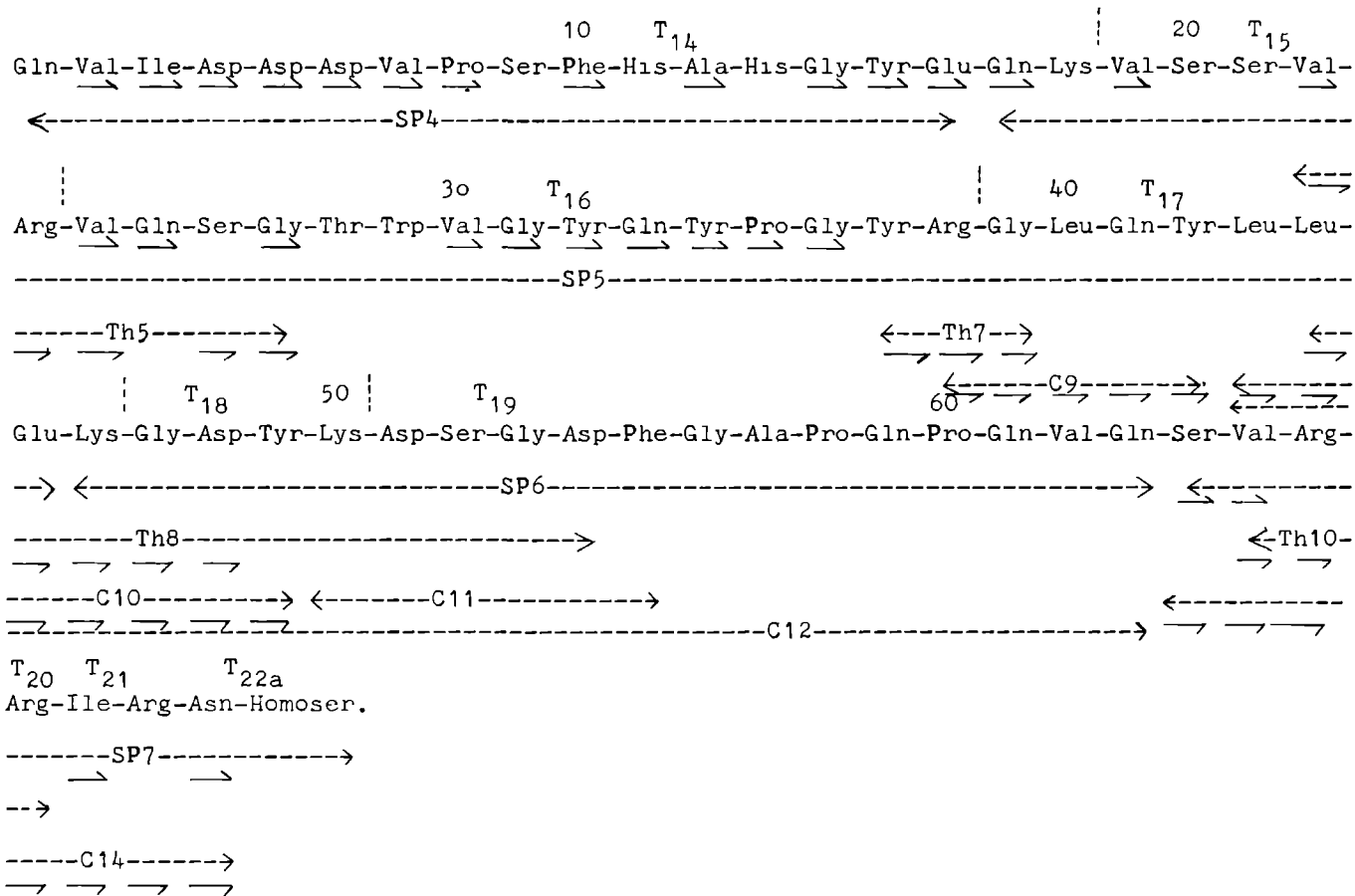
$T_{18}-T_{19}$ has been established by the sequence of C11 and Th8.
The order $T_{17}-T_{18}-T_{19}$ is in agreement with the amino acid
composition of SP6.However,two serines were found instead
of one.The overlap $T_{17}-T_{18}-T_{19}$is confirmed by the amino acid
composition of C12.The overlap $T_{19}-T_{20}-T_{21}-T_{22a}$ has been found
by the sequence of C14,Th10 and SP7.Amide assignment of T14
was determined by the identification of phenylthiohydantoins
using thin-layer chromatography.Thin-layer chromatography of
the phenylthiohydantoin of the residue in position 17 identi-
fied it as glutamic acid.However,from the electrophoretic mo-
bility of Th4 it was concluded that Th4 contains one glutamine
and one glutamic acid.Positioning of glutamic acid has been
performed on basis of the preference of S.aureus protease for
cleavage next to glutamic acid.The N-terminal residue of CB_2
has been found to be glutamine on the basis of the electropho-
retic mobility of C5.Amide assignment of Gln at position 25
and 33 was on basis of the mobility of T_{15}.Glutamine at posi-
tion 41,glutamic acid at position 45 and aspartic acid at po-
sition 48 and 54 were determined by thin-layer chromatography
of their phenylthiohydantoin derivatives.Amide assignment of
aspartic acid at position 51 was made from the electrophore-
tic mobility of C11.Amide assignment of glutamine at position
59,61 and 63 was made on basis of the electrophoretic mobility
of C13.Amide assignment of asparagine at position 70 was made
from the electrophoretic mobility of C14.

6.2.5.Tne amino acid sequence of CB_3.

Since CB_3 contains a blocked N-terminal residue,no di-
rect Edman degradation or dansyl–Edman degradation could be
performed.The sequence of CB_3 was elucidated after aigestion
with chymotrypsin.After high voltage electrophoresis at pH
6.5 three peptides could be isolatea.Two were ninhydrin po-
sitive,the third one was ninhydrin negative but showed fluo-
rescence under U.V. light,indicating the presence of trypto-
phan.Tne amino acid compositions are shown in table 6.VI.Amino
acid sequences of C15 and C16 were determined by the dansyl-
Edman procedure.The results are shown in fig.6.11.C14 was
considered to be the N-terminal fragment of CB_3,since it lacks
a free N-terminal amino acid.C16 was placed at the C-terminal
side of CB_3,since it did not contain a hydropnobic residue.
The sequence of C15-C16 has been confirmed by the isolation of
a tryptic peptide (T_{23}) from the peptide map of βB_p (fig.
6.12).The amino acid composition of T_{23} is shown in table 6.VI.
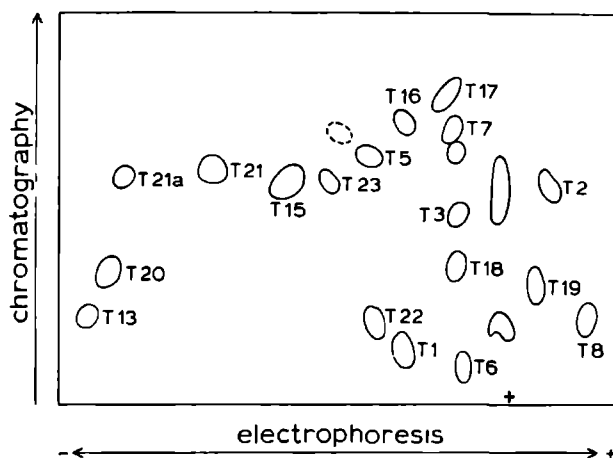
Fig.6.12.
Tryptic peptide
map of βB_p.

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TABLE 6.VI

Amino acid compositions of the tryptic peptides T_{22} and T_{23} and of the chymotryptic peptides of CB_3. Molar ratios are given. No corrections for hydrolytic losses were made.

Peptide	Asp	Thr	Ser	Glu	Pro	Gly	Ala	Val	Met	Ile	Leu	Tyr	Phe	Lys	His	Arg	Trp	number of residues	mobility
T_{22}	0.8			2.0				0.9							0.7	1.3	+	7	0.36
T_{23}		1.9		0.9	1.0	1.0							1.0		1.2			7	0.22
C_{14}			1.0														+		
C_{15}			1.1			1.1	0.8						1.2		0.8	1.2			
C_{16}		1.3			1.0										0.6				

Fig.6.11.Amino acid sequences of T_{22},T_{23} and of chymotryptic peptides of CB_3.Sequence was determined by the dansyl-Edman procedure($\longrightarrow$).

T_{22} Asn-Met-Glu-Trp-His-Gln-Arg
            $\rightarrow$ $\rightarrow$ $\rightarrow$ $\rightarrow$ $\rightarrow$

T_{23} Gly-Ala-Phe-His-Pro-Ser-Ser
            $\rightarrow$ $\rightarrow$ $\rightarrow$ $\rightarrow$ $\rightarrow$ $\rightarrow$ $\rightarrow$

C15   His-Gln-Arg-Gly-Ala-Phe
       $\rightarrow$ $\rightarrow$ $\rightarrow$ $\rightarrow$ $\rightarrow$ $\rightarrow$

C16   His-Pro-Ser-Ser
       $\rightarrow$ $\rightarrow$ $\rightarrow$ $\rightarrow$
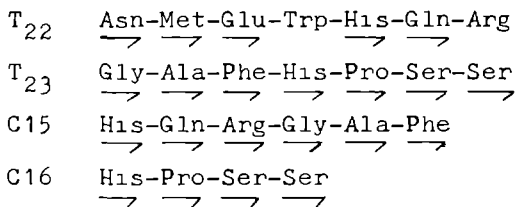
The amino acid sequence,determined by the dansyl-Edman pro-cedure,is given in fig.6.11.The total amino acid sequence of CB_3 is shown in fig.6.13.Amide assignment of C15 was performed on the basis of the electrophoretic mobility.

Fig.6.13.Proposed amino acid sequence of CB_3.

$$T_{23}$$

Pyr-Glu-Trp-His-Gln-Arg-Gly-Ala-Pne-His-Pro-Ser-Ser-OH
                        $\rightarrow$ $\rightarrow$ $\rightarrow$ $\rightarrow$ $\rightarrow$ $\rightarrow$ $\rightarrow$
$\leftarrow$-C14-----$\rightarrow$-$\leftarrow$----C15--------------$\rightarrow$ $\leftarrow$-------C16------$\rightarrow$
                        $\rightarrow$ $\rightarrow$ $\rightarrow$ $\rightarrow$ $\rightarrow$ $\rightarrow$ $\rightarrow$ $\rightarrow$ $\rightarrow$ $\rightarrow$
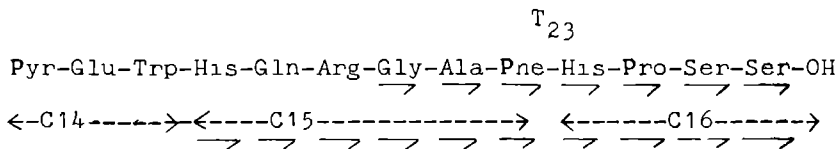
6.2.6.Overlap between CB_2 and CB_3.

The allignment of CB_2-CB_3 has been confirmed by the isolation of the tryptic peptide T_{22} from the peptide map of total βB_p (see fig.6.12).The amino acid composition and amino acid sequence are shown in table 6.VI and fig.6.11,res-pectively.The amino acid sequence of the 83 residues from the C-terminal side of βB_p is shown in fig.6.14.

Fig.6.14.Proposed amino acid sequence of the 83 residues from the C-terminal side of βB_p

T_{14} 10

Gln-Val-Ile-Asp-Asp-Asp-Val-Pro-Ser-Phe-His-Ala-His-Gly-Tyr-Glu-Gln-Lys-

20   T_{15} 30 T_{16}

Val-Ser-Ser-Val-Arg-Val-Gln-Ser-Gly-Thr-Trp-Val-Gly-Tyr-Gln-Tyr-Pro-Gly-

40   T_{17} T_{18} 50

Tyr-Arg-Gly-Leu-Gln-Tyr-Leu-Leu-Glu-Lys-Gly-Asp-Tyr-Lys-Asp-Ser-Gly-Asp-

T_{19} 60 T_{20} T_{21} 70

Phe-Gly-Ala-Pro-Gln-Pro-Gln-Val-Gln-Ser-Val-Arg-Arg-Ile-Arg-Asn-Met-Glu-

T_{22} 80 T_{23}

Trp-His-Gln-Arg-Gly-Ala-Phe-His-Pro-Ser-Ser-OH.

6.3.DISCUSSION

From the results presented in this chapter the amino acid
sequence of the C-terminal part (83 residues) of βB_p has
been established.The sequence of the N-terminal part of βB_p
(the CB_1 fragment) has not yet been completed.Only partial
sequences were obtained.

From the results of amino acid analysis of βB_p,CB_1,CB_2
and CB_3 it appeared that there is a discrepancy between the
value found for Glu in βB_p and the sum of the values found for
Glu in CB_1,CB_2 and CB_3.The reason for this discrepancy is that
when amino acid analysis is performed under standard conditions,
homoserine is eluted under the glutamic acid peak yielding a
too high value for glutamic acid.

Since not all of the tryptic peptides of AE-CB_1 could
be isolated by peptide mapping it is not completely certain
whether T_1 really represents the N-terminal tryptic peptide.
Further,since aminoethylcysteine is eluted at the same position
as lysine during amino acid analysis,T_{11} might contain amino-
ethylcysteine.All the other tryptic peptides have also been
isolated from unmodified βB_p.

Most of the cleavages obtained by digestion with chy-
motrypsin,thermolysin and S.aureus protease were in agreement
with the specificities of these enzymes.However,some anomalous
cleavages have also occurred.Chymotrypsin,apparently,has cleft
next to glutamine in C3,C12 and C13.In C5 chymotrypsin has

cleft next to histidine instead of phenylalanine.Another un-
usual cleavage of chymotrypsin can be found in C14,where chy-
motrypsin cleft next to asparagine.These cleavage sides,how-
ever,though less favorable to chymotryptic cleavage,have also
been reported by others (Needleman,1975).

Thermolysin cleaved specifically at the N-terminal side
of valine,isoleucine,leucine,phenylalanine,tyrosine and alanine.
An unusual cleavage side was found in T_{16} (Th5),where ther-
molysin cleaved at the N-terminal side of threonine.From the
amino acid composition of Th8 cleavage at the N-terminal side
of aspartic acid might be concluded.However,from the electro-
phoretic mobility on the peptide map a charge of minus two
could be calculated.The only possibility would be the presence
of the aspartic acid residu at position 54 in the thermolytic
fragment.Possibly abnormal loss of aspartic acid during acid
hydrolysis has occurred.

In one case it was found that S.aureus protease had cleft
at the C-terminal side of glutamine (position 63 in CB_2).

Finally,it should be noted that the C-terminal amino
acid sequence of the βB_p chain (Pro-Ser-Ser) is the same
as found for the αA_2 chain (Van der Ouderaa,1974).However,
no further homology could be detected between βB_p and either
the αA_2 or αB_2 chain of α-crystallin.

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In this thesis a study of the polypeptide chain composition of bovine β crystallin,a structural protein from the eye lens,is presented.

In the first chapter a survey of the present knowledge of β crystallin is given.

In the second chapter the isolation and partial characterization of β crystallin is described.By gel filtration on Sephadex G-200 β crystallin can be separated into two fractions, β_H (high molecular weight β) and β_L (low molecular weight β). β_H and β_L,with a molecular weight of approximately 210,000 and 52,000 respectively,showed a great similarity in their amino acid composition.Alkaline polyacrylamide gel electrophoresis in the presence of 6 M urea revealed at least nine polypeptide chains for β_H and eight for β_L.As in the case of α-crystallin,the corresponding chains of β crystallin have been divided into an A (acidic) and a B (basic) group.A number of β chains migrate with identical mobility in β_H and β_L. Moreover,both fractions have one predominant band in common, for which the nomenclature βB_p was proposed,where B stands for basic and p for principal.The main difference in polypeptide chain composition between β_H and β_L is the occurrence of two basic chains and an acidic chain in β_H and two chains in the

neutral region in β_L.Sodium dodecylsulphate gel electrophoresis of β_H and β_L showed the presence of five,respectively four classes of polypeptide chains of different size.Furthermore this electrophoretic analysis showed that β_H and β_L share a number of polypeptide chairs of identical molecular weight.

In chapter 3 the isolation and partial characterization of six polypeptide chains of β crystallin is described.Four polypeptide chains,isolated from β_H,with different electro- phoretic mobilities appeared to have the same molecular weight, amino acid composition and tryptic peptide map as the four chains with corresponding electrophoretic mobility,isolated from β_L.A close relationship between a number of chains with different electrophoretic mobility(βB_p and βB_4; βB_2, βB_{2-5} and βB_5) is suggested by a striking similarity in amino acid composition,molecular weight and tryptic peptide map.From these results it was assumed that the polypeptide chains of β crys- tallin,like in the case of α crystallin,are subjected to de- amidation processes or other post-translational modifications. The βB_4 chain,therefore,would originate from the βB_p chain by a single deamidation step,whilst the βB_5 chain would arise from the βB_2 chain by two-step deamidation,with the βB_{2-5} chain as intermediate.

The βB_p chain,as well as the βB_4 chain,turned out to have a molecular weight of approximately 24,000.The two highly basic chains found by alkaline urea gel electrophoresis of β_H, are identical to the two " high " molecular weight chains.

The βA chain has a molecular weight of approximately 21,000. After sodium dodecylsulphate gel electrophoresis with and without 6 M urea different molecular weights were estimated for the βB_2, βB_{2-5} and βB_5 chain (respectively **22.500** in the presence of 6 M urea and 26,000 in the absence of 6 M urea).From the results described in chapter 3 it seems likely that only a limited number of β polypeptide chains are direct translational products.

In chapter 4 the results of a study on the biosynthesis of β crystallin chains in vitro is presented.Of the β crystallins mainly β_L is synthesized in the cell-free system.Sodium dodecylsulphate gel electrophoresis suggested that β_H de novo synthesized is built up by the βB_{1a}, βB_2 and βA chain.From re-aggregation experiments using [^{35}S]-labeled β crystallin chains,it appeared that a number of labeled β_L chains are incorporated in β_H after reaggregation.

In chapter 5 a new method for the coupling to aminated supports of proteins and peptides containing lysine,aminoethyl-cysteine or ornithine is described.The method is based on the use of the bifunctional reagent isothiocyanatobenzoylhomoserine-lactone (IBHL),containing two functional groups of different reactivity.Using this reagent the risk of cross-linking of pep-tides encountered with bifunctional reagents ,bearing two groups of equal reactivity,is greatly diminished.This renders appli-cation of a large excess of the bifunctional reagent unnessary.

In chapter 6 the partial elucidation of the primary

structure of the βB_p chain is presented.By cleavage with cyanogen bromide three fragments were obtained and purified.The amino acid sequence of the 83 C-terminal amino acids (comprising the cyanogen bromide fragments CB_2 and CB_3) has been established.The C-terminal amiro acid sequence of the βB_p chain is the same as the C-terminal sequence of the αA_2 chain (Pro-Ser-Ser-OH).However,so far,no further homology between the βB_p chain and α crystallin chains could be detected.

In dit proefschrift worden de resultaten van een onder-
zoek naar de polypeptideketensamenstelling van het ooglens-
eiwit β crystalline van het kalf beschreven.

In het eerste hoofdstuk is een overzicht gegeven van
het werk dat tot nu toe aan β crystalline is verricht.

Het tweede hoofdstuk beschrijft de zuivering en gedeel-
telijke karakterisering van β crystalline.Door middel van gel-
filtratie over Sephadex G-200 kan β crystalline gescheiden
worden in twee fracties, β_H (hoog moleculair β) en β_L (laag
moleculair β),met een moleculair gewicht van ongeveer 210,000,
respectievelijk 52,000. β_H en β_L vertonen grote overeenkomst
wat betreft hun aminozuursamenstelling.Met behulp van basische
polyacrylamide gel electrophorese in aanwezigheid van 6 M ureum
werden minimaal negen polypeptideketens voor β_H en acht voor
β_L aangetoond.Evenals bij α crystalline werden de polypeptide-
ketens van β crystalline onderverdeeld in een zure (A) groep
en een basische (B) groep.Een aantal ketens van β_H en β_L
hebben eenzelfde electrophoretische mobiliteit. β_H en β_L hebben
de voornaamste band, βB_p genaamd,gemeenschappelijk,waarbij B
staat voor "basic" en p voor "principal".De belangrijkste
verschillen in polypeptideketensamenstelling zijn twee basische
en een zure band in β_H en twee banden in β_L in het neutrale

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gebied.SDS-gelelectrophorese gaf 5 banden voor β_H en 4 banden voor β_L.Een aantal polypeptideketens van β_H en β_L blijkt eenzelfde molecuulgewicht te hebben.

In hoofdstuk 3 wordt de zuivering en gedeeltelijke kaakterisering van zes polypeptideketens van β crystalline beschreven.Vier polypeptideketens met verschillende electrophore ische mobiliteit,gezuiverd uit β_H,bleken hetzelfde molecuulgewicht,dezelfde aminozuursamenstelling en dezelfde tryptische peptide-kaart te hebben als de overeenkomstige ketens uit β_L.Op grond van deze gegevens werd geconcludeerd dat β_H en β_L deze ketens gemeenschappelijk hebben.Een aantal ketens met verschillende electrophoretische mobiliteit (βB_p en βB_4; βB_2,βB_{2-5} en βB_5)bleek een grote overeenkomst te vertonen wat betreft aminozuursamenstelling,molecuulgewicht en tryptische peptide-kaart.Op grond van deze resultaten lijkt het waarschijnlijk dat de polypeptideketens van β crystalline, evenals die van α crystalline,onderhevig zijn aan deamidatie (of een andere modificatie na de biosynthese).Derhalve zou de βB_4 keten uit βB_p kunnen ontstaan door een enkelvoudige deamidatie,terwijl de βB_5 keten het resultaat zou kunnen zijn van een tweevoudige deamidatie van βB_2,waarbij βB_{2-5} als intermediair zou optreden.

Het molecuulgewicht van βB_p en βB_4,bepaald door middel van SDS-gelelectrophorese,bleek ongeveer 24.000 dalton te zijn. Detwee basische banden van β_H bleken overeen te komen met de "hoog" moleculaire banden met een molecuulgewicht van ongeveer

30.000.Voor de βA keten werd een molecuulgewicht van ongeveer 21.00 dalton gevonden.De molecuulgewichten van βB_2,βB_{2-5} en βB_5 ,bepaald door middel van SDS-gelelectrophorese met en zonder 6 M ureum,waren verschillend (respectievelijk **22.500** en 26.000).Op grond van de resultaten van hoofdstuk 3 lijkt het waarschijnlijk dat slechts een beperkt aantal β ketens direkte translatieproducten zijn.

In hoofdstuk 4 worden de resultaten van een onderzoek naar de biosynthese van β crystalline in vitro besproken.Van de β crystallines wordt voornamelijk β_L gesynthetiseerd in het celvrije systeem.Op grond van SDS-gelelectrophorese lijkt de novo gesynthetiseerd β_H opgebouwd te zijn uit de βB_{1a},βB_2 en βA keten.Uit reaggregatieproeven met [^{35}S]-gelabelde β crystalline-ketens bleek dat een aantal gelabelde β_L-ketens na reaggregatie geïncorporeerd was in β_{II}.

In hoofdstuk 5 wordt een nieuwe methode voor het koppelen aan vaste dragers van eiwitten en peptiden,die lysine, aminoethylcysteine of ornithine bevatten,besproken.De methode is gebaseerd op het gebruik van isothiocyanatobenzoylhomoserine-lactone (IBHL) dat twee functionele groepen met verschillerde reactiviteit bevat.Bij gebruik van dit reagens wordt het risico van "cross-linking" van peptiden,zoals dat optreedt bij een bifunctioneel reagens met twee functionele groepen van gelijke reactiviteit,sterk verminderd.Dit maakt gebruik van een grote overmaat van dit reagens overbodig.

In hoofdstuk 6 wordt de gedeeltelijke opheldering van

de primaire structuur van de βB_p-keten besproken.Door split-
sing met cyanogeenbromide werden drie fragmenten verkregen en
vervolgens gezuiverd.De aminozuurvolgorde van 83 aminozuren,
gerekend vanaf de C-terminus (omvattend de cyanogeenbromide-
fragmenten CB_2 en CB_3) is opgehelderd.De volgorde van de 4
C-terminale aminozuren van de βB_p-keten is gelijk aan die van
de αA_2-keten (Pro-Ser-Ser-OH).Verdere homologie tussen βB_p
en α-ketens kon tot nu toe echter niet worden aangetoond.

De schrijver van dit proefschrift werd op 12 januari 1946 geboren te Pendleton (G.B.).Na het behalen van het diploma gymnasium β aan het Sint Janslyceum te 's-Hertogenbosch in 1965 werd een aanvang gemaakt met de chemie-studie aan de Katholieke Universiteit te Nijmegen.Het kandidaatsexamen (letter S_1) werd behaald in januari 1969.Het doctoraalexamen (hoofdvak biochemie,bijvak organische chemie) werd behaald in mei 1972.Van 1 augustus 1971 tot 1 juni 1976 was hij als wetenschappelijk medewerker verbonden aan het laboratorium voor biochemie van de Katholieke Universiteit te Nijmegen.Sinds 1 juni 1976 is hij werkzaam op de afdeling Pathologie van het Akademisch ziekenhuis te Leiden.

STELLINGEN

1. De conclusie van Huebner et al. dat expressie van endo-
   gene virussen significant onderdrukt kan worden door
   middel van specifieke immuunrespons wordt niet gestaafd
   door hun experimenten.

   Huebner,R.J.,Gilden.R.V.,Lane,W.T.,Toni,R.,Trimmer,R.W.
   en Hill,P.R. (1976) Proc.Natl.Acad.Sci.USA $\underline{73}$,620.

2. De door Gorelic gekozen condities voor het induceren van
   RNA-eiwit-cross-links door middel van ultra-violet licht
   kunnen leiden tot een foutief beeld van de topografie van
   het ribosoom.

   Gorelic,L. (1975) Biochemistry $\underline{14}$,4627.

3. De bewering van Sundkvist en Staehelin dat in hun prepa-
   raten natieve 40 S subunits met een dichtheid in CsCl van
   1,49 g/cm^3 niet aanwezig zouden zijn is waarschijnlijk
   onjuist.

   Sundkvist,I.C. en Staehelin,T. (1975) J.Mol.Biol.$\underline{99}$,401.

4. Uit de experimenten van Hung en Lee kan niet worden af-
   geleid dat de waargenomen denaturende effecten van de
   door hen geisoleerde eiwitfraktie uitsluitend een gevolg
   zijn van zogenaamde " unwinding" enzymaktiviteit.

   Hung,P.P. en Lee,S.G. (1976) Nature $\underline{259}$,499.

5. De experimenten van Shoyab et al. rechtvaardigen niet de
   conclusie dat AMV-DNA in tandem wordt geïntegreerd aan
   het endogene provirus DNA.

   Shoyab,M.,Dastoor,M.N. en Baluda,M.A. (1976)
   Proc.Natl.Acad.Sci.USA 73,1749.

6. De door Laursen et al. gekozen pH voor het koppelen van
   peptiden en eiwitten via zijketen aminogroepen aan vaste
   dragers is niet optimaal.

   Laursen,R.A.,Horn,M.J. en Bonner,A.G. (1972)
   FEBS-letters 21,67.
   Dit proefschrift,hoofdstuk V.

7. Ondanks dat in dit proefschrift de thans veelal in zwang
   zijnde aanduiding dalton om een molecuulgewicht aan te
   geven is gebruikt,moet zij als zinloos worden beschouwd
   aangezien het molecuulgewicht gedefinieerd is als een
   verhoudingsgetal.

8. Het onderbrengen van oudere mensen in zogenaamde bejaar-
   dentehuizen is een intolerante cultuurtrek.

P.Herbrink
30 september 1976