STUDIES ON FEATHER KERATIN SYNTHESIS IN VITRO

A thesis submitted by

Geoffrey Arthur Partington, B.Sc.(Hons.),

to the University of Adelaide, South Australia,

for the degree of

Doctor of Philosophy.

DEPARTMENT OF BIOCHEMISTRY
UNIVERSITY OF ADELAIDE
ADELAIDE SOUTH AUSTRALIA

APRIL, 1974.

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SUMMARY

The work of this thesis is concerned with investigating the synthesis of chick embryo feather keratin. Three different approaches were adopted. The first was to investigate protein synthesis in cell-free systems prepared from chick embryo feathers. The second was to isolate the mRNA's coding for chick feather keratin and the third was to investigate the labelling of RNA in feathers incubated *in vitro*. The original findings of this work can be summarized as follows:-

(1) a cell-free system prepared from 14-day feather lysate was extensively investigated and found to be highly active in the incorporation of amino acids into protein. It was shown that the major products of the cell-free system were complete keratin chains, identical with native feather keratin by several criteria. Furthermore, by isolation of labelled keratin N-termini it was shown that about 50% of the chains were synthesised *de novo*. These newly initiated chains were N-acetylated in the system when labelled acetyl-CoA was present. Investigation of the ionic conditions necessary for optimal activity of the system indicated that maximal activity occurred at lower concentrations of K^+ and Mg^{++} than had previously been used in cell-free systems prepared from chick embryo tissues. Under these conditions, using inhibitors of initiation of protein synthesis it was found that greater than 80% of the protein was synthesised *de novo*.

The nature of the protein products synthesised in 12, 13 and 15-day cell-free systems was also investigated. The major

products of the 13 and 15-day cell-free systems were found to be keratins, whereas under most conditions little or no keratin synthesis was detected in the 12-day system.

(2) the mRNA's coding for the embryonic feather keratins were isolated by phenol-extraction of 14-day feather polysomes and purified by two cycles of sucrose-gradient centrifugation. Keratin mRNA activity was found to reside in a 12S RNA by translation in a rabbit reticulocyte lysate cell-free system. Moreover, there was no requirement for any other component from feather tissue. The product of the primed reticulocyte lysate system was found to be identical to authentic keratin by several criteria. The N-termini of these keratin chains were found to be N-acetylated in reticulocyte lysate.

No evidence was obtained to suggest that the keratin chains were synthesised as higher molecular weight precursors. The keratin mRNA was shown to electrophorese as a single band when characterised on polyacrylamide gels under denaturating conditions. The value obtained for the molecular weight under these conditions indicated that the mRNA's must contain large untranslated region(s).

(3) the labelling of RNA was investigated in 12, 13 and 14-day chick embryo feathers incubated <u>in vitro</u> for short periods. The extent of labelling was found to decrease with increasing age. However, an absorbance peak of 12S was found in RNA isolated from 12 and 13-day feathers. This species was not conclusively shown to be identical to the 12S keratin mRNA present in 14-day feather polysomes.

STATEMENT

This thesis contains no material previously submitted for any other degree or diploma in any University. To the best of my knowledge and belief this thesis does not contain any material previously published or written by another person except when due reference is made to such material in the text.

Geoffrey A. Partington

ACKNOWLEDGEMENTS

I wish to thank Professor W.H. Elliott for permission to undertake this project in the Department of Biochemistry, University of Adelaide.

I especially thank my supervisor, Dr. G.E. Rogers, for his enthusiasm and encouragement during the course of this work and for his advice and criticisms during the preparation of this thesis.

I wish to thank Dr. D.J. Kemp of this Department for his collaboration during the course of this work and especially for the work described in Chapter 4 of this thesis, and my thanks are also due to Miss P.Y. Dyer for electron microscopy. I wish to thank in particular Mr. I.D. Walker, Mr. P. Tolstoshev, Dr. R.H. Symons, Dr. J.R.E. Wells and Dr. D.J. Kemp, who, among many others of this Department, have contributed many helpful and stimulating discussions.

I thank also Dr. H.W.J. Harding, Miss C. Hayles and Mr. M. Cichorz for amino acid analyses. I thank Mrs. D.J. Dinan and Miss L. Bissell for technical assistance mainly in the work described in Chapters 6 and 7, and to Miss Bissell my thanks also goes for preparing the diagrams of this thesis. Furthermore, I would like to acknowledge Miss Kim Murray for the typing of this thesis.

The project was carried out while in receipt of a University Research Grant. The work was supported by grants from the Australian Wool Corporation and the Australian Research Grants Committee (to Dr. G.E. Rogers).

ABBREVIATIONS

ATA	aurintricarboxylic acid
ATP	adenosine 5' triphosphate
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
GTP	guanosine 5' triphosphate
HAc	acetic acid
HnRNA	heavy nuclear RNA
HSB	high salt buffer
LSB	low salt buffer
MSB	medium salt buffer
mol.wt.	molecular weight
NaAc	sodium acetate
mRNA	messenger RNA
mRNP	messenger RNA-protein
rRNA	ribosomal RNA
tRNA	transfer RNA
poly(A)	polyadenylic acid
POPOP	1,4-bis(5-phenyloxazolyl)-benzene
PPO	2,5-diphenyloxazole
RNA'se	ribonuclease
RNP	ribonucleic acid-protein
S.Act.	specific activity
SCM	S-carboxymethyl
SDS	sodium dodecyl sulphate (or sodium lauryl sulphate)
TCA	trichloroacetic acid

CHAPTER ONE

INTRODUCTION

INTRODUCTION

A. GENERAL COMMENTS

Feathers are an example of a terminally-differentiating system. The embryonic chick feather grows out from the skin by a process of selective cell division and the cells then fill with an intracellular protein complex, keratin. The eventual aim of the work described in this thesis is to obtain an understanding of the molecular mechanisms involved in the programming of these cells to proceed towards their differentiated state.

The ensuring discussion is a brief description of the morphological development of feathers, and of the factors known to affect this development. Results obtained on other systems relevant to the results presented in the present study are reported in the appropriate chapters.

B.

1. Morphology of the embryonic chick feather

The morphology of embryonic feathers has been cited by numerous authors (for example, Watterson, 1942; Romanoff, 1960; Rawles, 1972), and is illustrated in Figure 1.1. They consist principally of a group of from 10 to 15 barbs, attached to a short calamus at the base. The barbs contain a central core of medulla cells, surrounded by flattened cortical cells. On the basal two-thirds of its length, each barb bears two rows of barbules, composed of strings of single essentially cylindrical, barbule cells, attached end-to-end. All of these

FIGURE 1.1. STRUCTURE OF THE EMBRYONIC CHICK FEATHER.

Figure 1.1a. Feather from newly-hatched chick, after discarding sheath.

Figure 1.1b. Diagram, illustrating details of the structure of barbs and barbules.

B: barbs

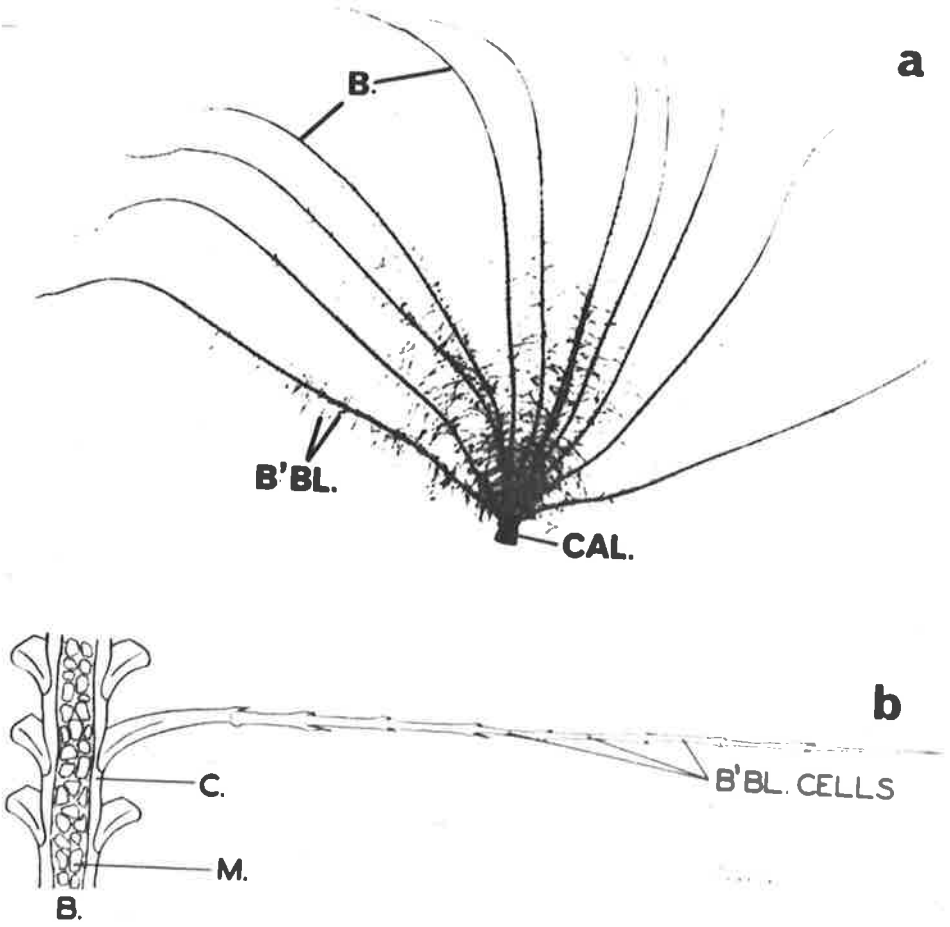
BBL: barbules

CAL: calamus

C: cortex

M: medulla

(from Watterson, 1942).


a
B.
B'BL.
CAL.
b
C.
M.
B.
B'BL. CELLS

cells are dead, dehydrated and filled with keratin.

2. Development of the embryonic chick feather

The development of the embryonic chick feather has been described in detail at the light microscope level (Davies, 1889; Strong, 1902; Watterson, 1942; Wessells, 1965), and at the electron-microscope level (Kischer, 1963; 1968; Kallman et al., 1967; Matulionis, 1970). Reviews on feather development are given by Romanoff (1960), Lillie (1965) and Voitkevich (1966).

Between 5 and 6 days of incubation typical skin (composed of dermis and overlying epidermis) is formed only in the areas corresponding to the future feather-forming areas. Outside these zones, skin differentiates several days later at a much slower rate. However, in the feather-forming areas the dermis is individualised and can be distinguished from the underlying mesenchyme by its high cellular density. At the same time the ectoderm undergoes major transformations in the feather-forming areas. The basal layer finally acquires its permanent columnar epithelial structure. The periderm lying above this basal layer also thickens and takes on the appearance of a pavement epithelium. Thus, the undifferentiated ectoderm becomes transformed into typical embryonic epidermis. Extensive evidence indicates that the dermis plays an important role in theproliferation of the epithelial cells. By about eight days the feather primorida are clearly visible as a series of epidermal thickenings in well-defined tracts. These primordia continue to elongate by cell-division forming

cylindrical epidermal outgrowths with mesodermal cores.

Subsequently, the sinking of the germ within the skin and its progressive growth result in the formation of the feather follicle whose walls are formed by the areas of epidermis directly continuous with the germ envelope. The generative zone of the embryonic feather constitutes the base of this epithelial cylinder and the feather increases in size through the multiplication of these cells. Early on the eleventh day, the rapidly dividing epidermal cells in the feather become organized into a series of discrete sectors, and from this time the presence of several different cell-types all derived from the epidermal cells becomes apparent. However, the bulk of the tissue is accounted for by the barb and barbule cells. The core of the cylinder contains two capillary vessels and a few cells of mesodermal origin.

After 12 days, the feathers rapidly elongate as a result of individual cell elongation and movement, as little division takes place.

Feather keratinization thus begins at the sheath, proceeding inwards and downwards from the apex, and ensures that no active cells are isolated from the two nutrient blood vessels (Voitkevich, 1966). Large scale synthesis of the major species of feather-keratins begins at day thirteen of embryonic life, and eventually the barb and barbule cells fill with keratin, whereas other cell types atrophy and the mesodermal pulp is retracted. By about 17-18 days, development is essentially complete.

At 21 days, the chick hatches and when the feathers

dry the sheaths split open and are discarded as the down feathers open up to take on their typical fluffy appearance.

C. FACTORS AFFECTING KERATINOCYTE DEVELOPMENT

1. The influence of the dermis

A central problem in the biology of skin is concerned with the elucidation of the origin and maintenance of a wide variety of structurally and functionally diverse specifities which the dermis presents. As Grobstein (1967) has pointed out, one of the most fundamental processes in embryonic development is the interaction or intercommunication between cells and tissues of different ontogenetic origins. These 'heterotypic' interactions are responsible for the emergence of a wide range of new differentiated cell and tissue types. It is now generally agreed that the familiar, regionally distinctive differentiation of the epidermis is the result of heterotypic interactions between epithelial and mesenchymal precursors in ontogeny.

In regard to feather development is has been shown that prospective feather dermis caused feathers to form from epidermis destined under normal conditions to form surface epidermis or scale (Rawles, 1963; Wessells, 1965). Furthermore, it has been found that the epidermis in culture failed to keratinize when separated from the dermis and degenerated (McLoughlin, 1961; Wessells, 1962; Dodson, 1963). The presence of living dermal cells is not necessary, dermis killed by repeated freeze-thawing supports the epidermis as well as living dermis (Dodson, 1963). To explain the finding

of 'dermis specificity' the concept of a specific inducer molecule has been proposed. However, Wessells (1968) regards that this hypothesis as being unnecessary to explain the data. He pointed out that this specificity could be the result of such factors as changes in mesodermal cell density, or changes in mesoderm extracellular substances that mediate environmental exchanges of epithelial cells. Another important factor to be kept in mind is that the epidermal cells themselves may be involved in lateral intercellular communication, that is each epidermal cell might influence the development of the surrounding cells.

The mechanism by which the onset of keratin synthesis (cytodifferentiation) is controlled may be to some degree unrelated to the control of morphogenesis. The dermal-epithelial interactions resulting in feather formation begin at about 5 days, as described earlier. However, keratin synthesis does not begin until about 12-13 days, as determined by birefringence and X-ray diffraction (Bell and Thathachari, 1963), electron microscopy (Matulionis, 1970) and by a quantitative assay for the keratin proteins by analysis on polyacrylamide gels (Kemp et al., 1974). By this stage, the embryonic. feather has almost completed its morphological development.

2. Vitamin A

Keratinization can be inhibited in certain epithelia that normally keratinize by a small excess of vitamin A in the culture medium (Fell, 1964). Weiss and James (1955) found that a brief exposure of dissociated 7-8 day chick embryo

skin cells to a high concentration of vitamin A caused the cells to form cysts of actively secreting mucous epithelium; furthermore, feather-germ formation was suppressed. The control cultures developed normally into squamous keratinizing epithelial cells which produced feather germs. Fitton-Jackson and Fell (1963) investigated the changes in fine structure that accompanied the mucous metaplasia induced by vitamin A, using scaly metatarsal skin from 12-day chick embryos. The most interesting observation from these studies was the finding that the basal cells of vitamin A treated skin recovered when returned to a normal medium.

3. Hydrocortisone

The addition of hydrocortisone to the medium hastened keratinization in skin explants from chick embryos, and the feather germs aborted (Fell, 1962). This effect has been verified by a number of workers, for example, Sugimoto and Endo (1969), Stuart *et al*. (1972).

4. Chalones

Bullough and Lawrence (1960) and Bullough (1965) have postulated that epidermal cells secrete some substance, capable of diffusing over short distances, that inhibits and so controls epidermal mitotic activity. In subsequent studies Bullough *et al*., 1967, Bullough and Lawrence, 1968) the substance called epidermal chalone, in the presence of adrenalin and a glucocorticoid hormone, had a marked effect on epidermal mitotic activity, *in vitro* and *in vivo*. The

agent appeared to be highly specific having no influence on cell division in hair follicles or other tissues. Chalones specific for other tissues have been reported (for a review see O'Dell, 1972). No effect has yet been noted on feather follicles, although the epidermal chalone has been reported to inhibit mitosis in chick skin incubated in culture (Rothberg and Ekrel, 1971).

5. Epidermal growth factor (EGF)

The isolation of a protein from the submaxillary gland of rat which stimulated epidermal growth *in vivo* in mouse and in rat has been reported (Cohen, 1962). Furthermore, its effects have been demonstrated in organ cultures of skin (Cohen, 1965). In these cultures EGF stimulated a net accumulation of protein and RNA with no detectable increase of DNA (Hoober and Cohen, 1967). In later work (Cohen and Stastny, 1968) it was reported that one of the initial cellular changes induced by EGF was a conversion of pre-existing monomer ribosomes into the polysomes. Accompanying this alteration there was an increase in protein synthesis and an increase in all classes of cytoplasmic RNA, as assayed by sucrose gradient centrifugation.

6. Pituitary hormones

Yatvin (1966a,b) reported that hypophysectomy of chick embryos at about 36 hours of development did not prevent feather morphogenesis but did prevent the onset of keratinization as assayed by the increase in the level of polysomes

observed to occur concomitantly with the onset of keratin synthesis. In hypophysectomized embryos this change did not occur.

Work in this laboratory (D.J. Kemp - personal communication) using a more specific assay for keratin synthesis, namely, gel electrophoresis of the keratin proteins has indicated that the onset of keratin synthesis is not prevented, but merely delayed as is the development of the whole embryo.

7. Prostaglandins

Kischer has made some studies on the effect of prostaglandins on chick embryo feather development. He has found (Kischer, 1967) that when prostaglandins were applied to the back skin of chick embryo in culture, epidermal proliferation and development were stimulated, whereas feather formation wa suppressed. In a later study (Kischer and Keeter, 1970) it was noted that many breaks or gaps in the dermo-epithelial junction were induced by the in vitro treatment with prostaglandin B_1. Further analysis by electron microscopy (Kischer, 1973) revealed that the epidermal mitochondria underwent a marked alteration in morphology following treatment by prostaglandin B_1. No such changes were observed in the periderm or dermis. When transferred to fresh medium, the feathers again began to develop.

8. Thyroxine

In early studies in vivo it was demonstrated (Bartels, 1943) that precocious stratificiation and keratinization of chick embryo epidermis followed injection of thyroxine into the allantois. Studies in vitro have confirmed this effect. Wessells (1961) reported that thyroxine initiated keratinization of chick embryo skin cultured in a chemically defined medium, and Kitano and Kuroda (1967) reported a similar finding. The role of the thyroid gland in the development of feathers has been extensively reviewed by Voitkevitch (1966). At the time of onset of chick feather keratin synthesis (12-13 days) the thyroid gland has begun to actively secrete thyroxine (Shain et al., 1972). By comparison the thyroid gland of a newly-hatched pigeon is poorly-differentiated. Its biological activity is minimal and it only acquires the typical microscopic structure by the fifth post-embryonic day. Young pigeons are covered with sparse embryonic down during the first three days after hatching. Quills of the contour feathers can be seen through the skin on the fourth and fifth day after hatching. The growth of the feather quills coincides precisely with the early phases of secretion of the thyroid gland. From these results it appears that thyroxine may stimulate feather development but is not essential for the initial formation of the feathers.

From the foregoing discussion it is clear that most of the epidermal effectors have an inhibitory effect on feather development or have not as yet been assigned any clearly defined role in controlling feather development. In this

context, the conclusion of Braverman and Katoh (1971) as a result of their *in vitro* culture experiments using chick embryo lens is particularly noteworthy. They found that if that part of a chick embryo destined to form, the eye, the lens, part of the brain and the surrounding head region, was removed before the lens had begun to develop and placed in culture medium for four days, a structure resembling the lens, synthesised a protein unique and characteristic of the lens.

The lens of the cultured explant developed in an abnormal way but the final result was the same. From this they concluded that at least some for the biochemical and morphogenetic activities of the embryos are rigidly and separately programmed long before their expression and are independent of immediate preceeding events.

D. DNA SYNTHESIS AND MITOSIS

Kischer and Furlong (1967) examined DNA polymerase activity during the development of chick embryo skin and feathers. They reported that DNA polymerase activity was low at early stages, reached maxima at the stages of feather germ outgrowth and elongation, then declined abruptly at the onset of keratinization. Nuclear DNAse activity, in contrast rose slowly to a maximum at the onset of keratinization. Rothberg and Ekrel (1971) found that there was a peak of DNA synthesis in shank skin of chicken embryos just prior to the onset of keratinization, which declined abruptly as keratinization began.

It would appear from these results that DNA and

keratin synthesis are mutually exclusive. Furthermore, the observation that normal differentiation *in vitro* has not been obtained in the absence of mitosis *in vitro* strongly suggests that mitosis is required at a crucial step on the path towards keratinization.

E. THE PATHWAY TO TERMINAL DIFFERENTIATION

All the evidence presented in this discussion for feather differentiation concurs with the model for terminal lens differentiation presented by Modak (1972). In this system the sequence of events leading to acquisition of the terminally differentiated state included, (1) cessation of DNA synthesis and cell division; (2) activation of RNA synthesis; (3) synthesis of lens specific proteins; (4) appearance of strand breaks in the DNA; (5) decrease and halt of RNA synthesis; (6) nuclear degeneration and loss of DNA.

F. FEATHER KERATINS

(a) Keratin structure

The structure of keratin has been extensively reviewed by Fraser *et al*. (1972). Keratins are fibrous, insoluble, intracellular proteins of high cysteine content produced in certain epithelial cells of higher vertebrates.

Electron microscope studies (Filshie and Rogers, 1962; Rogers and Filshie, 1963) have indicated that feather keratin has a filamentous structure, the filaments being some 3 - 3.5 nm in diameter and of indeterminate length. The

proteins are presumably cross-linked *in vivo* by disulphide bonds (Goddard and Michaelis, 1934; Woodin, 1954).

(b) Embryonic chick feather keratin

Kemp (1972) has identified the keratin proteins of embryonic chick feather and developed analytical procedures for their routine identification at different stages of development, based on the finding of Harrap and Woods (1964, a,b) that stable derivatives of the proteins could be prepared by reduction and S-carboxymethylation. Using such procedures, three groups of proteins were apparent, designated the α-, β-, and γ-proteins. The major (β) group appeared to consist of a heterogeneous family of related keratin proteins. The results from N-terminal sequence studies showed that the N-termini of embryonic feather keratin proteins were acetylated. A series of N-terminal peptides of increasing length was prepared from embryonic feather proteins and the homologous N-terminal amino acid sequences Ac-Ser-SCMC-$\frac{\text{Phe}}{\text{Tyr}}$-Asn were established, demonstrating that feather keratins are a family of homologous proteins. Quantititative polyacrylamide gel electrophoretic techniques were applied to the study of the kinetics of keratin synthesis (Kemp *et al.*, 1974). It was shown that the major keratin proteins were detectable by 12 days and attained a maximal rate of synthesis after 13 days. Furthermore, the proteins were synthesised in a coordinated fashion.

Walker (1974) has extended the studies on the heterogeneity of the chick feather keratin chains. The number of discrete keratin chains was investigated by examining fractions of the

the S-carboxymethylated feather protein by polyacrylamide gel electrophoresis at two pH values and at least nineteen prominent proteins were found to comprise the down feather. Each of these proteins was remarkably similar to the others with respect to molecular weight and amino acid composition.

Sequence analysis of the ^{14}C-SCM-tryptic peptides of the down feather indicated that the electrophoretic variants of feather keratin different from one another at only few sites in the primary structure.

CHAPTER TWO

MATERIALS AND GENERAL METHODS

A. MATERIALS

1. Tissue

In all experiments reported in this thesis (unless stated otherwise) the feathers used were from embryos of the domestic white leghorn fowl, Gallus domesticus. Fertilized eggs were obtained from the Parafield Poultry Station of the Department of Agriculture, Parafield, South Australia. The eggs were stored at 10° for no longer than seven days and incubated at 37.8°, 54% humidity in a forced-draught incubator for the required time.

Feathers from eggs which had been incubated a total of, for example, 14 days, were designated '14-day feathers'.

2. Enzymes and proteins

Albumin: Bovine serum, fraction V. Sigma Chemical Co., St. Louis, Missouri, U.S.A.

α-Chymotrypsin: Bovine pancreas. Three times crystallised. Worthington Biochemical Corporation, Freehold, N.J., U.S.A.

Insulin: Crystalline pig. Commonwealth Serum Laboratories, Melbourne, Australia.

3. Radioactive Compounds

Acetyl-1[^{14}C]-Coenzyme A: 57 or 59 mC/mmole. Radiochemical Centre, Amersham, England.

Algal Hydrolysate: [^{14}C] (U) reconstituted protein hydrolysate. Schwarz/Mann, Orangeburg, N.Y., U.S.A.

Adenosine-[2,8-^{3}H]: 10 C/mmole. ICN Chemical and Radioisotopes Division, Irvine, California, U.S.A.

L-Leucine-[^{14}C] (U): 312 mC/mmole. Schwarz/Mann.

Orotic Acid, 5-^{3}H: 15 C/mmole. Schwarz/Mann.

L-Serine-[^{3}H] (G): 1.7 C/mmole. ICN, 3 & 6.3 C/mmole Amersham, 1.2 C/mmole, Schwarz/Mann.

L-Serine-[^{14}C] (U): 312 mC/mmole. Schwarz/Mann.

Uridine-[5,6-^{3}H]: 43 C/mmole. Schwarz/Mann.

4. Fine chemicals for specific procedures

a. Media for culture

Charity Waymouth: MB 752/1, Dried. Difco Laboratories, Detroit, Michigan, U.S.A.

Chick Embryo Extract: Commonwealth Serum Laboratories.

Foetal Calf Serum: Commonwealth Serum Laboratories.

Horse Serum: Commonwealth Serum Laboratories.

Minimal Medium Eagle: Dried. Difco.

b. Extraction, reduction and carboxymethylation of proteins

Ethanolamine: BDH Chemicals Ltd., Poole, England.

Iodoacetic acid: Sigma.

2-Mercaptoethanol: Sigma.

Urea: reagents puro, Carlo Erba, Milan, Italy.

c. Polyacrylamide gel electrophoresis

Acrylamide: Eastman Organic Chemicals, Rochester 3, New York, U.S.A.

Ammonium persulphate: Anala R. BDH.

Bromo-phenol blue: BDH.

Coomassie Brilliant Blue R-250: Mann Research Laboratories ,

Orangeburg, N.Y., U.S.A.

Ethylene Diacrylate: Borden Chemical Co., Philadelphia, Penn., U.S.A.

Formamide: Laboratory reagent. BDH.

N,N'-methylenebisacrylamide: Eastman.

Riboflavin: BDH.

N,N,N',N'-tetramethylethylenediamine: Eastman.

'Stains-all', 1-Ethyl-2-[3-(1-ethylnaptho [1,2d] thiazolin-2-ylidene)-2-methylpropenyl]-naphtho[1,2d] thiazolium bromide: Eastman.

Toluidene blue: George T. Gurr, London, England.

d. Measurement of radioactivity

1,4-bis-[2-[(5-phenyloxazoly)]-benzene: Packard Instruments Co., Inc., La Grange, U.S.A.

2,5-Diphenyloxazole: Scintillation grade: Packard.

Glass-fibre filters: Whatman GF/C. W & R Balston Ltd., England.

NCS Tissue Solubilizer: Amersham/Searle, Arlington Heights, Illinois, U.S.A.

Toluene: reagents puro, Carlo Erba and BDH.

e. Column chromatography

CM-cellullose: Whatman CM-32.

DEAE-cellulose: Whatman DE-11.

Dowex 1: AG 1-X5, minus 400 mesh, Bio-Rad Laboratories Richmond, California, U.S.A.

Dowex 50: AG 50W-X2, 100-200 Mesh, Bio-Rad.

Zeokarb 225: The Permutit Co. Ltd., London, England.

f. Antibiotics

Chloramphenicol: Parke Davis, Sydney, Australia.

Cycloheximide: Sigma.

Cordycepin: Grade III, 3 times crystallized. Sigma.

Pactamycin: Gift from Dr. B. May of this Department. Originally from Dr. H.F. Lodish, M.I.T., U.S.A.

Dactinomycin, Merck, Sharp & Dohme Research Lab., Rahway, N.J., U.S.A.

Penicillin G: sodium salt. Evans Medical Aust. Pty. Ltd., Melbourne, Australia.

5. Miscellaneous chemicals

Adenosine 5'-triphosphate: Crystalline from equine muscle. Sigma.

Aurintricarboxylic acid: Sigma.

L-Amino acids: Mann Research Laboratories.

Creatine phosphate: Sigma.

Creatine phosphokinase: Sigma.

Diethylpyrocarbonate: Fluka AG, Buchs, Switzerland.

N-ethylmorpholine: Eastman. This was distilled under reduced pressure before use and stored under nitrogen at 2 - 4°.

Glutathione: reduced. Sigma.

Guanosine 5'-triphosphate: Trisodium salt, Type I, Sigma.

Heparin: from Hog intestinal mucosa, sodium salt. Sigma.

Phenol: laboratory reagent. BDH.

Phenylhydrazine: Sigma Grade. Sigma.

Sodium lauryl sulphate: Sigma.

Sodium fluoride: Anala R. BDH.

Sucrose: Crystalline, density gradient grade. Schwarz/ Mann.

Tris: Trizma Base. Sigma.

Triton X-100: Rohm and Haas, Philadelphia, Penns., U.S.A.

Urea: reagents pura, Carlo Erba and Analala R, BDH.

6. Miscellaneous materials

Dialysis tubing: Visking. BDH. The tubing was boiled in 1% w/v $NaHCO_3$ before use (Thompson and O'Donnell, 1965).

Homogenisers: Potter-Elvehjem type, 2 ml; chamber bore 7 mm, clearance 0.075 - 0.10 mm. Kenco Scientific, Adelaide, South Australia.

5 ml, and 10 ml: clearance 0.1 mm. Lab Crest. Fischer and Porter Pty. Ltd., Sydney, Australia.

Millipore filters: Type HA, 0.45 µ, Millipore Corporation, Bedford, Massachusetts, U.S.A.

Sterifil Aseptic Filtration System: Millipore Corp.

B. GENERAL METHODS

The techniques that were used routinely throughout this work are described in the present chapter. All other procedures are described in the appropriate chapters.

1. Composition of buffers for the preparation of feather lysates

High salt buffer (HSB).

250 mM KCl

10 mM $MgCl_2$

10 mM Tris-HCl, pH 7.4 (from Heywood et al., 1967).

Medium salt buffer (MSB)

200 mM KCl

5.3 mM $MgCl_2$

10 mM Tris-HCl pH 7.4.

Low salt buffer (LSB)

66.0 mM KCl

2.67mM $MgCl_2$

10 mM Tris-HCl pH 7.4.

2. Preparation of feather lysates

The standard method for the preparation of feather lysates is described below. In some experiments this procedure was varied; details of this aregiven in the appropriate chapters.

It should be noted that in the preparation of both feather and rabbit reticulocyte lysates, rigorous precautions were taken to minimise RNA'se contamination. All glassware was washed with 1 M KOH, rinsed carefully in bi-distilled water and heated at 110° overnight. All solutions were treated with 0.1% diethylpyrocarbonate and allowed to stand overnight before use (Williamson et al., 1971).

Embryos were removed from eggs and washed in Hanks' saline solution (see Paul, 1970). With the embryo immersed

in Hank's solution the body feathers were removed carefully using watch-makers' forceps. The plucked feathers were placed directly into ice-cold MSB. All subsequent procedures were carried out at 4°. The feathers were washed four times by centrifugation at 900 g for 30 sec. The tissue volume was then measured in a graduated centrifuge tube immediately after the feathers had been centrifuged at 4000 g for 5 minutes. The feathers were then resuspended in two volumes of MSB containing 4 mM dithiothreitol and allowed to stand on ice 10 min. For small volumes of feathers a 2 ml Potter-Elvehjem homogeniser was used manually with 5-6 strokes to disrupt the tissue. With larger volumes of feathers a 5 ml or 10 ml homogeniser was used, with 8 - 10 strokes. Cell debris and nuclei were pelleted by centrifugation at 16,000 g for 10 min at 4°. The supernatant was decanted.

3. Incubation of feathers in vitro

All glassware used was washed in 1 M HNO_3, well-rinsed in bi-distilled water and autoclaved before use. All operations were carried out in a sterile tissue culture cabinet. The Charity Waymouth medium containing 1.0 mg/100 ml penicillin and 5% foetal calf serum was sterilized by ultrafiltration using Millipore Sterifil apparatus.

The chicken embryos were rinsed with the medium and placed in a Petri dish also containing the medium. Plucked feathers were collected in a small volume of medium. Samples of the feathers were added carefully to about 2 or 3 ml of the medium contained in 25 ml Delong culture flasks.

The flasks were then gassed for 20 seconds with a 5% CO_2-air mixture and stoppered with serum caps. The flasks were incubated at 37° with shaking at speed setting 4-5 in a Gyrotory Water Bath Shaker Model G76 (New Brunswick Scientific Co. Inc., New Brunswick, N.J., U.S.A.).

After incubation for the required length of time the feathers were chilled by addition of 20 ml of ice-cold MSB, and the flasks placed in ice. The lysates were then prepared as described in the previous section.

4. Preparation of rabbit reticulocyte lysate

(a) Induction of anaemia in rabbits

Reticulocytosis was induced in rabbits by the method of Evans and Lingrel (1968a), by daily subcutaneous injections of 0.3 ml/Kg of 2.5% neutralized phenylhydrazine hydrochloride in 47.5% ethanol (pH 7.0). Generally satisfactory reticulocytosis (i.e., >90% reticulocytes) was produced after 4 - 6 daily injections.

(b) Collection of cells

Blood was collected from a marginal ear vein into tubes containing 2.5 ml of cold reticulocyte saline (0.13 M NaCl, 0.005 M KCl, 0.0074M $MgCl_2$) containing 2 mg heparin. All subsequent operations were performed at 4°. The blood was diluted with the saline to 15 ml and the cells spun down at 4,000 g for 10 minutes. The supernatant and 'buffy' coat of lymphocytes were drawn off using a Pasteur pipette. The cells were washed in the saline solution three times by centrifugation.

Before the final rinse a sample of the cells was collected and incubated at 37° for 15 min in a few drops of stain (1% w/v cresyl blue, 0.6% w/v sodium citrate, 0.68% w/v NaCl). A smear was prepared from these cells to determine the degree of reticulocytosis. Reticulocytes stain dark blue, erythrocytes pale blue.

(c) Preparation of reticulocyte lysate

The washed cells were lysed by addition of 1 volume of 1 mM $MgCl_2$. To aid lysis, the cells were drawn vigorously, several times, into a Pasteur pipette. One volume of buffer was then added (200 mM KCl, 2.67 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.4). The lysed cells were then centrifuged at 16,000 g for 10 minutes to pellet mitochondrial and membrane fragments. The supernatant was collected for *in vitro* studies, the pellet was discarded.

5. Measurement of radioactivity

For kinetic experiments 0.05 ml aliquots were removed from the incubation mix at the stated time intervals and pipetted into 1 ml of cold water. Bovine serum albumin (25 µgm) was added followed by 1 ml of 20% TCA and the protein was allowed to precipitate at 0° for about 30 minutes after all samples had been taken. The protein was then collected by centrifugation at 900 g for 10 minutes at 4°. The pellets were dissolved in 0.4 ml of 0.3 N NaOH to discharge labelled aminoacyl tRNA's and reprecipitated with 20% TCA. The precipitates were collected on Whatman GF/C glass fibre filters. The precipitates were

washed with 10 ml of 5% TCA and finally with ether. The filters were dried in 2 ml vials at 100° for 1 hour.

Fractions from sucrose gradients of feather lysates were each diluted with 1 ml of water and precipitated with 1 ml of 20% TCA. The precipitates were collected on Whatman GF/C filters, and washed as above.

Fractions from sucrose gradients of labelled RNA were diluted with 1 ml of water and precipitated with 20% TCA in the presence of 25 µg of bovine serum albumin as carrier.

Aliquots from fractions were precipitated with 20% TCA without carrier. Precipitation of fraction with and without carrier revealed no difference in the cpm retained on the filters.

All samples were counted in 2 ml vials in a toluene-based scintillation fluid (0.35% w/v 2,5-diphenyloxazole and 0.035% w/v 1,4-bis[2-(5-phenyloxazolyl]-benzene. A Packard Tricarb Liquid Scintillation Counter was used for all radioactivity determinations. Results are generally expressed as the actual counts per minute observed in the aliquot, after subtraction of the background value. The observed counts per minute were recorded usually for 10 min or 20 min. Quenching was monitored using the channel-ratio method, and was found to be constant in each experiment.

6. Densitometry of protein-containing polyacrylamide gels

Gels were stained with Coomassie Brilliant Blue R-250 in 10% TCA (Chrambach et al., 1967).

In early experiments the gels were scanned in a

'Densicord' recording electrophoresis densitometer (Photovolt Corp., New York, U.S.A.). The white light source and red filter (no.610) were used. Normally, the sensitivity setting used was D1. The scanning speed, chart speed ratio setting was 1:2. In a few experiments a Joyce Loebel Chromoscan (Joyce, Loebel & Co. Ltd., Gateshed, U.K.) was used. A white light source and red filter (621) were used. The scanning speed, chart speed ratio setting was 1:1.

In later experiments the gels were scanned at a wavelength of 630 nm using a Gilford linear transport attachment (Gilford Instrument Laboratories, Oberlin, Ohio, U.S.A.) coupled to a W & W Recorder (W & W Electronic Inc., Basle, Switzerland).

Various sensitivity settings were used to optimise the resolution. The scan speed, chart speed ratio was normally 1:1.

7. Solubilization of polyacrylamide gels for radioactivity determinations

The gels were frozen in dry ice and sliced into 1 mm sections using a single blade type gel slicer (Mickle Engineering Co., Gomshall, Surrey, England). Each section was placed in a 2 ml glass vial and solubilized by treatment with 1.0 M NH_4OH overnight at room temperature. The solution was then absorbed onto a GF/C glass fibre filter placed in the vial and dried at 110°. Scintillation fluid was added to the cooled vial, for radioactivity determination.

8. Sucrose gradient centrifugation

In most of the gradient centrifugations reported in this thesis a Beckman SW41 rotor was used in either a Beckman L2-50 or L2-65B ultracentrifuge. Linear sucrose gradients were prepared as described by Noll (1969) with the heavy sucrose solution in the reservoir and light sucrose solution in the mixing chamber, using a two chambered apparatus similar to that described by Britten and Roberts (1960). In a few early experiments the gradients were prepared in a manner similar to that described by Sarma et al. (1969). The gradients contained concentrations of sucrose from 0.3 M (10.2% w/v) to 1.1 M (37.6% w/v) in the appropriate buffer. In later experiments sucrose from 10% w/v to 40% w/v was used, without any change in resolution. The figure legends contain the details of time, temperature and g force used in each particular run.

9. Monitoring of absorbance profiles of sucrose gradients

In early experiments the absorbance profiles of sucrose-gradients was monitored continuously at 260 nm using a modified Optica Densitronic Spectrophotometer (Optica U.K. Ltd., Walthamstow, London) fitted with a 2 mm path-length cell. The profiles were recorded using a TOA Model EPR-2TC recorder (TOA Electronics Ltd., Tokyo, Japan).

In later experiments the absorbance profiles were monitored at 245 nm using an LKB Uvichord II ultraviolet analyser (LKB-Produkter AB, Bromma 1, Sweden) equipped with a

3 mm path length cell and coupled to a Hitachi-QPD-53 recorder (Hitachi Ltd., Tokyo, Japan). Various sensitivity settings were used depending on the concentration of material on the gradient. In both of these analyser systems the gradients were pumped out from the bottom using an LKB perpex peristaltic pump, using a flow rate of 80 ml/hr. In some experiments an Isco model 640 density gradient fractionator (Instrumentation Specialties Co., Lincoln, Nebraska, U.S.A.) was used. The gradients were scanned at A_{254nm} using a 2 mm flow cell.

10. Determination of S values

The S values of the minor RNA species on sucrose gradients were determined by the method of Martin and Ames (1961). In the present work it was observed that the distances that the 18S rRNA and tRNA species sediment from the gradient meniscus are directly proportional to the S value. Thus a plot of S value of the known RNA species versus distance sedimented gave a straight line from which the S values of other RNA species could be determined.

11. Electron microscopy

All specimens were examined in a Siemens Elmiskop 1 electron microscope operated at 80kV with a 50 μ objective aperture. Specimens were negatively-stained on the grid with 2% (w/v) uranyl acetate.

CHAPTER THREE

ISOLATION OF FEATHER POLYSOMES

A. INTRODUCTION

It has long been established that polysomes are the major site of protein synthesis within cells. This was shown in reticulocytes by Warner et al. (1962), Marks et al. (1962), Gierer (1963) and in liver by Wettstein et al. (1963). It is generally accepted that mRNA functions as a 'tape', the amino acid sequence of the protein being encoded in the nucleotide sequence of the mRNA. The ribosomes attach to the mRNA, translate it into an amino acid sequence and detach on completion of the protein. Several ribosomes on a mRNA then constitute a polysome. In support of this hypothesis it was found that polysome breakdown and protein synthesis proceeded synchronously with a step-wise release of monomers, and that the amount of protein synthesised was proportional to the size of the polysome (Noll et al., 1963). Furthermore, it was observed that the size of a polysome was principally determined by the length of its associated mRNA. For example, in reticulocytes, synthesising predominantely haemoglobin, polysomes comprised of 5-6 ribosomes are the major species (Rifkind et al., 1964), and conversely in liver it was found that the length of the mRNA liberated from polysomes of a particular size increased as the ribosome complement of the polysome increased (Staehelin et al., 1964). The tape theory was proven when Evans and Lingrel (1969b) reported that the ratio of 18S rRNA to 9S haemoglobin mRNA liberated from reticulocyte polysomes increased in direct proportion to the number of ribosomes in the polysome.

Isolation of polysomes from epidermal tissues of

mammalian species has met with limited success (for a review see Freedberg, 1972). This has been due to a large extent to the nature of the tissues themselves. Hair follicles for example, are composed predominantly of fibrous protein rendering homogenisation of the cells difficult. In addition large amounts of RNA'se are sometimes present (Wilkinson, 1970a; Steinert and Rogers, 1971a). Polysomes have been isolated from sheep ear epidermis (Priestly and Speakman, 1966), from wool follicles (Wilkinson, 1970a) and from hair follicles (Rogers and Clark, 1965; Clark and Rogers, 1970; Freedberg, 1970; Steinert and Rogers, 1971a). Although large polysomes were evident the majority of ribosomes were present in the monomer fraction. Whether this represents the true in vivo distribution is not clear, but it appears to be more probable that extensive dis-aggregation of polysomes occurs during the prolonged extraction procedures. It has been suggested that during slow cooling of polysomes, initiation is selectively inhibited while chain elongation and termination continue (Das and Goldstein, 1968; Friedman et al., 1969; Morimoto et al., 1972a).

The initial studies on the characterisation of chick embryo feather polysomes at first sight appeared to have great significance. It was demonstrated (Bell et al., 1965; Humphreys et al., 1964) that prior to the onset of keratin synthesis (at 13 days of incubation) many of the ribosomes were found as tetrameric aggregates, having a characteristic 'square planar' configuration, insensitive to ribonuclease, and inactive in protein synthesis. It was proposed that these aggregates were storage forms of ribosomes and the mRNA.

After 13 days the tetramer peak was not longer apparent. Instead polysomes of 5-6 ribosomes, sensitive to disaggregation by low concentrations of RNA'se were predominant. Subsequently, Byers (1966, 1967) reported that ribosome crystallisation could be induced in chick embryo tissues by hypothermia. In a further report Humphreys and Bell (1967) concluded that the tetrameric ribosomal aggregates were induced by the prolonged cooling of feathers, prior to homogenisation, and furthermore, that they did not contain mRNA. Polysomes prepared from 12 day embryo feathers isolated at 37° were characterised by an absence of tetramers and a normal polysome distribution.

As a prerequisite to further studies of protein synthesis in chick embryo feathers the polysome distribution of feathers of several ages was reinvestigated. This chapter details the results obtained.

B. RESULTS

1. Isolation of polysomes from 14- and 15-day feathers using high salt buffer, HSB

Feathers were initially collected by the following method. The chick embryos were washed with Eagle's medium and the plucked feathers collected in a Petri dish of the same medium (all at room temperature). The feathers were then transferred to ice-cold buffer (HSB), which had the same composition as the buffer used by Heywood *et al.* (1967) to prepare polysomes from chick embryo thigh muscle. For the preparation of feather lysates, the procedure of washing the feathers was identical to that described (see Methods 2.B2) but the composition of the high-salt buffer

used in the subsequent homogenisation included the following; 0.1 % w/v; Triton X-100, to aid in the lysis of cells (Borun et al., 1967), 0.2 mM dithiothreitol (DTT) to prevent oxidation and aggregation of the cysteine-rich keratins (Kemp and Rogers, 1972). The use of high salt buffers similar to HSB has been found to maximise the yield of polysomes from wool roots (Wilkinson, 1970a) and from guinea pig hair follicles (Steinert and Rogers, 1971a). Deoxycholate was not used to release membrane-bound ribosomes since the rough endoplasmic reticulum is sparse in developing chick embryo feathers (Matulionis, 1970; Kemp, Dyer and Rogers, 1974). In fact, deoxycholate has been found to be of no advantage in the isolation of polysomes from the wool root follicle (Wilkinson, 1970a). The absorbance profiles of sucrose density gradients of polysomes isolated from 14- and 15-day chick embryo feathers is shown (Fig.3.1 a,b). The profiles for these ages are similar to those described previously (Humphreys et al., 1964; Bell et al., 1965; Yatvin, 1966a) in that the major classes of polysomes are comprised of trimers to pentamers. Polysomes of this size would be expected to have a role in the synthesis of proteins of about the molecular weight of keratin (Heywood and Rich, 1968). Polysomes from 16-day feathers showed a similar size distribution to 14- and 15-day feathers (results not shown), although the yield was greatly reduced; by this stage the feathers are extensively keratinized, rendering homogenisation very difficult.

The yield of polysomes was expressed only on a packed feather volume basis (see lengeds to Figs. 3.1, 3.2) as neither protein nor DNA content remains constant during development

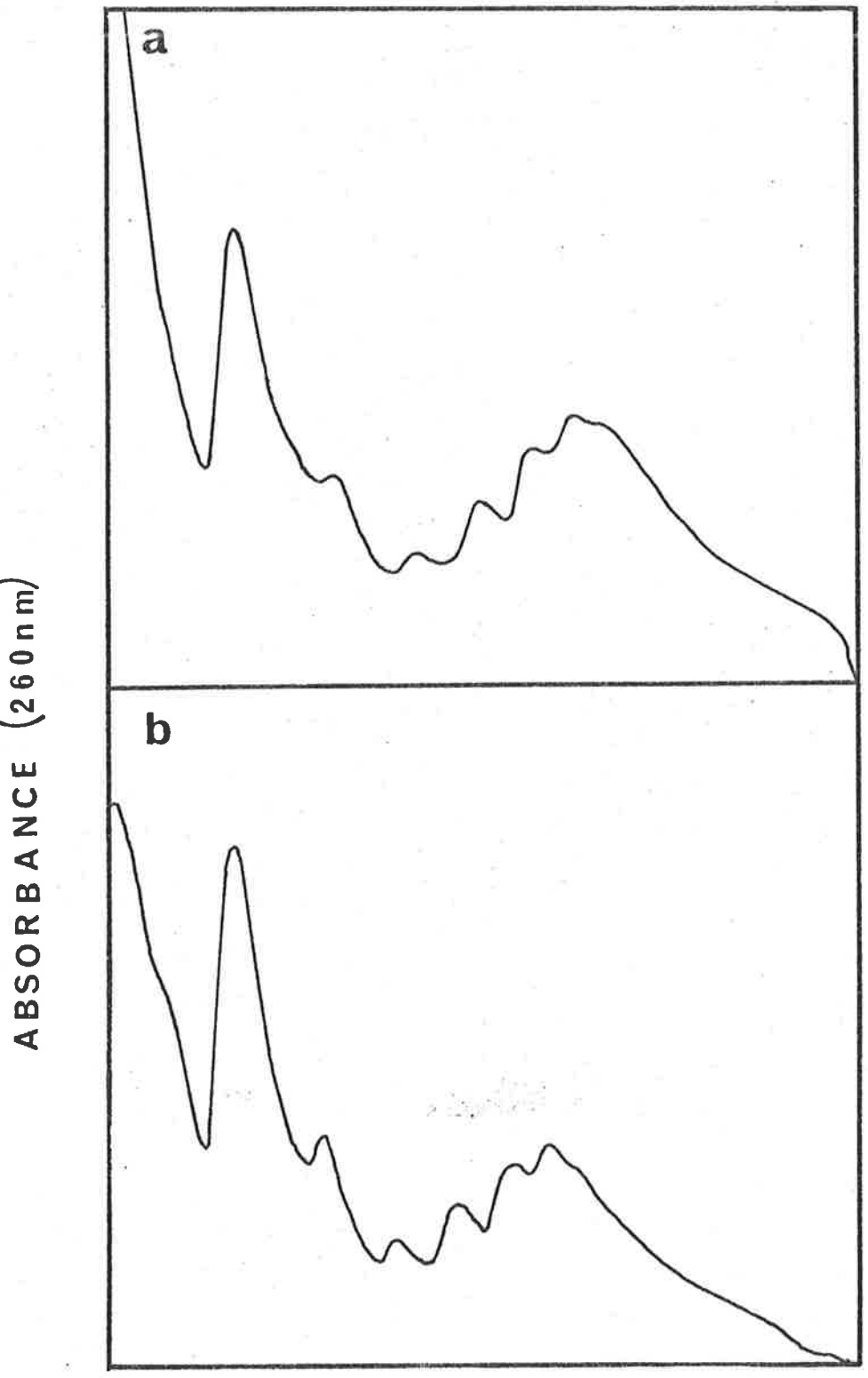
FIGURE 3.1. SUCROSE GRADIENT SEDIMENTATION OF POLYSOMES FROM CHICK EMBRO FEATHERS

Gradients (11.6 ml, 0.3 M - 1.1 M sucrose) were prepared in HSB and centrifuged in an SW41 rotor for 1 hr at 174,000 (a.v.) at 4^{o}.

The absorbance profiles of the gradients were monitored using a modified Optica spectrophotometer (see Methods, 2.B9).

Figure 3.1a. Absorbance profile of polysomes from 14-day feather lysate, prepared in HSB. The material represents the total obtained from 0.125 ml of packed feathers.

Figure 3.1b. Absorbance profile of polysomes from 15-day feather lysate prepared in HSB. The material represents the total obtained from 0.125 ml of packed feathers.


a
b
ABSORBANCE (260nm)
SEDIMENTATION

(Kemp, Dyer and Rogers, 1974; P. Gibbs - personal communication).

2. Isolation of polysomes from 14-, 15- and 16-day feathers using medium salt buffer, MSB

In later experiments the KCl and $MgCl_2$ concentrations in the buffers used for isolation and homogenisation were reduced to 200 mM and 5.3 mM respectively (see Methods 2.B2) in readiness for the use of the lysates for cell-free incubations. Furthermore, the Triton X-100 was omitted as this has been shown to release RNA'se, presumably by the lysis of lysosomes (Gianetto and De Duve, 1955), whereas the concentration of dithiothreitol in the homogensiation buffer was increased to 4 mM; as this has been reported in one instance to be inhibitory to RNA'se at this concentration (Boshes, 1970). The polysomes isolated under these conditions from 14-, 15- and 16-day feathers, using feathers collected as described in the preceding section were identical to those isolated in HSB (results not shown).

Polysome profiles from feathers collected by the original method using Eagle's medium to wash embryos and with feathers collected in the same medium until plucking was complete, were compared with polysome profiles obtained when feathers were plucked from embryos washed in Hank's saline with the feathers collected directly into MSB (see Methods 2.B2). The two methods were compared to determine whether the second method could be routinely used to prepare lysates for *in vitro* protein synthesis studies and for preparation of large amounts of polysomes without undue polysome disaggregation. It can be

seen (Fig. 3.2a,b) that some disaggregation occurred when the polysomes were isolated directly into MSB with a shift to trimers and tetramers. In comparison to this tetramers and hexamers predominated in the polysomes isolated from embryos washed in Eagle's medium.

To determine as closely as possible the true monosome to polysome ratio existing in vivo polysomes were prepared from 14-day feathers in buffers containing 100 µg/ml of cycloheximide to prevent polysome disaggregation during preparation.

When prepared in this manner about 32% of the ribosomes were found in the monomer fraction (profiles not shown) as compared to 37% occurring as monomers when polysomes were prepared in the absence of cycloheximide. It is likely that this represents the true distribution found in vivo. Although this may be true in terms of being an average over the total population of ribosomes of the feather the actual distribution on a per cell basis is unknown, as the feather is more developed towards the tip (Matulionis, 1970; Bell and Merrill, 1967).

3. Comparison of polysome profiles of 14-day feathers prepared in medium salt buffer, MSB, and low salt buffer, LSB

The polysome profiles from 14-day feathers were examined after isolation in MSB (in the presence and absence of 0.1% w/v Triton X-100) and also in a buffer of low ionic strength LSB (66 mM KCl, 2.67mM $MgCl_2$, 10 mM Tris-HCl, pH 7.4) because maximal incorporation of amino acids was obtained in the cell-free system prepared in this buffer (see Chapter 4). No

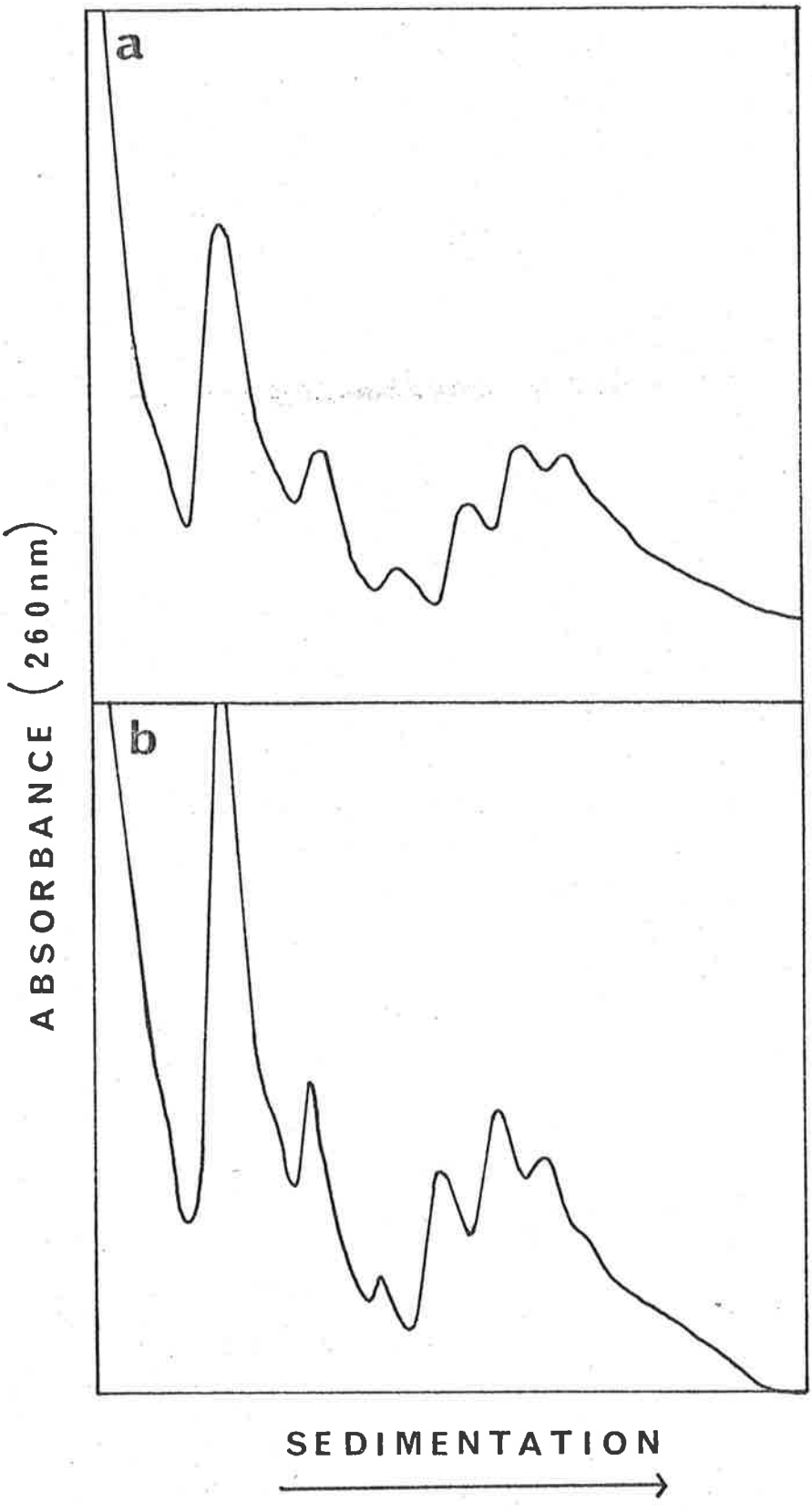
FIGURE 3.2. A COMPARISON OF POLYSOME PROFILES PREPARED USING TWO METHODS FOR COLLECTING THE 14-DAY CHICK EMBRYO FEATHERS.

Other details are as given in Figure 3 .1 except that the gradients were prepared in MSB.

Note that the sensitivity of the spectrophotometer was increased X2 in (b) as compared to (a).

Figure 3.2a. Absorbance profile of polysomes from feathers collected in Eagle's medium at room temperature prior to preparation of the lysate in MSB at 4^{o}.

Figure 3.2b. Absorbance profile of polysomes from feathers collected directly into ice-cold MSB prior to preparation of the lysate in MSB at 4^{o}.


a
b
ABSORBANCE (260nm)
SEDIMENTATION

significant difference was detected in the polysome regions when either buffer was used (result not shown) or whether Triton X-100 was present (Fig. 3.3a,b). However, in the region of the gradients sedimenting more slowly than the monosomes the absorbance profiles are markedly different. The two peaks of absorbance were assumed to be the small (40S) ribosomal subunits and the large (60S) ribosomal subunits respectively (McConkey and Hopkins, 1965; Girard et al., 1965). For the polysomes prepared in MSB there is almost no absorbance in the 60S region and an increased amount in the 40S region as compared to the profile obtained in LSB.

4. Characterisation of polysome profiles from 12- and 13-day feathers

When lysates were prepared from 12- and 13-day feathers using HSB the polysome profiles were characterised by a single peak of absorbance in the polysome region of the gradient (Fig. 3.4a,b). On examination by electron microscopy (Fig. 3.5) square planar ribosomal aggregates (tetramers) described by Humphreys et al. (1964) and Bell et al. (1965) were found in the preparations. This result was obtained even though the feathers were plucked at room temperature in Eagle's medium, conditions under which the above authors had claimed that tetramer formation was prevented.

In later experiments 12- and 13-day feathers were isolated in MSB, and in these preparations a low level of polysomes was observed (Fig. 3.6a,b), and the tetramer peak was absent. However, some tetramers as well as polysomes were seen

FIGURE 3.3. COMPARISON OF 14-DAY CHICK EMBRYO FEATHER POLYSOMES PREPARED IN MSB OR LSB AS ANALYSED BY SUCROSE-GRADIENT SEDIMENTATION.

Gradients (11.6 ml, 10% - 40% w/v sucrose) were prepared in either MSB (Fig. 3.3a) or LSB (Fig. 3.3b). Centrifugation conditions were as given in Figure 3.1. The absorbance profiles of the gradients were monitored using an Isco density gradient fractionator (see Methods 2.B9).

Figure 3.3a. Absorbance profile of polysomes isolated in MSB containing 1% Triton X100. The material represents the total obtained from 0.12 ml of packed feathers.

Figure 3.3b. Absorbance profile of polysomes isolated in LSB containing 1% Triton X100. The material represents the total obtained from 0.10 ml of packed feathers.

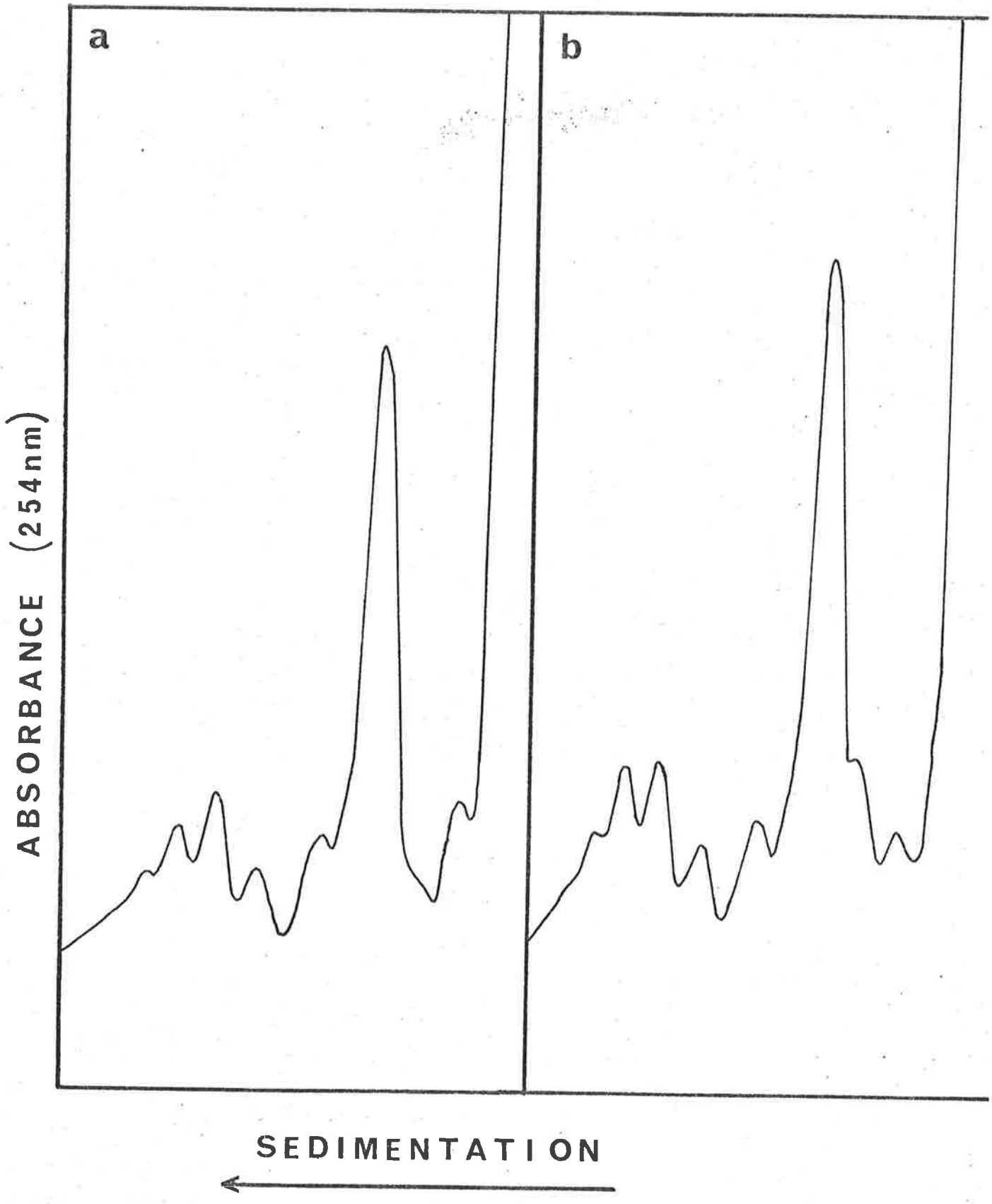

a
b
ABSORBANCE (254nm)
SEDIMENTATION

FIGURE 3.4. CHARACTERISATION OF POLYSOME PROFILES FROM 12 AND 13-DAY FEATHERS AS ANALYSED BY SUCROSE GRADIENT SEDIMENTATION.

The preparation of gradients and the monitoring of absorbance profiles was as given in Figure 3.1.

Note that the sensitivity setting of the spectrophotometer was increased X2 in (b) as compared to (a).

Figure 3.4a. Absorbance profile of polysomes isolated from 0.125 ml of 12-day feathers. The lysate was prepared in HSB.

Figure 3.4b. Absorbance profile of polysomes isolated from 0.125 ml of 13-day feathers. The lysate was prepared in HSB.

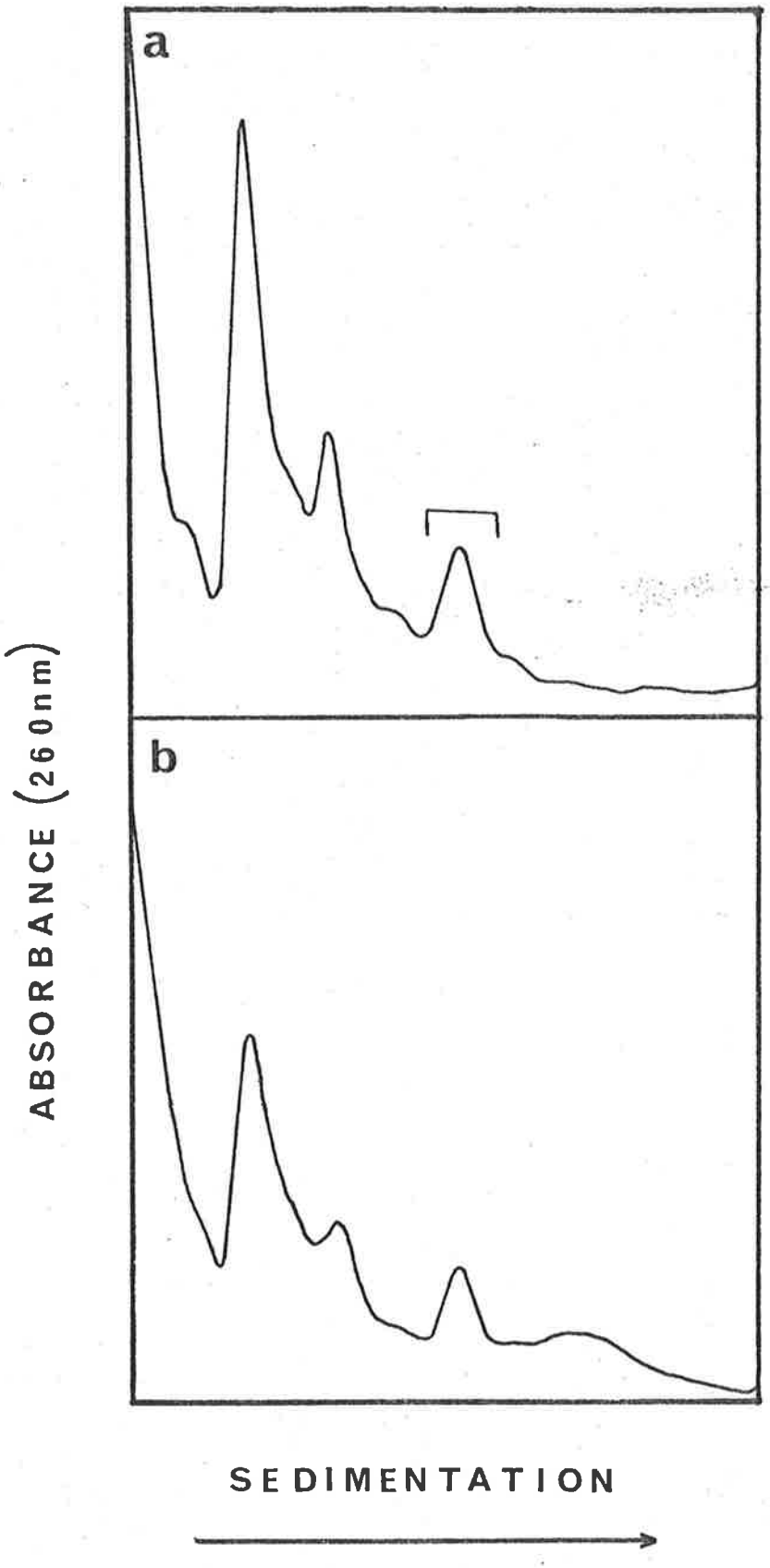

a
b
ABSORBANCE (260nm)
SEDIMENTATION

FIGURE 3.5. ELECTRON-MICROGRAPH OF RIBOSOMAL-TETRAMERIC AGGREGATES OBTAINED FROM THE FRACTION SHOWN BY A BAR IN FIGURE 3.4a.

Negatively stained with uranyl acetate.

Magnification: X100,000.

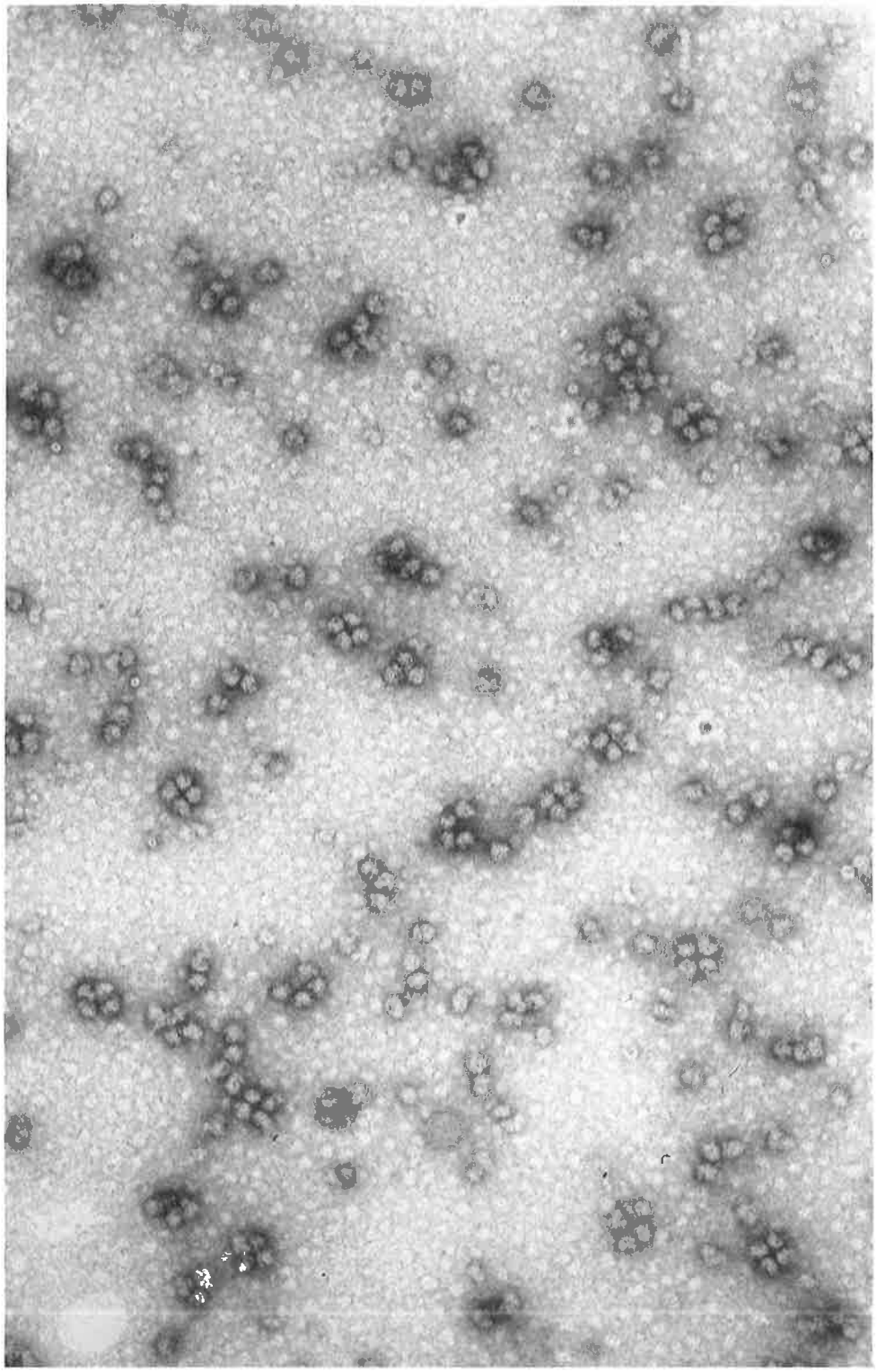
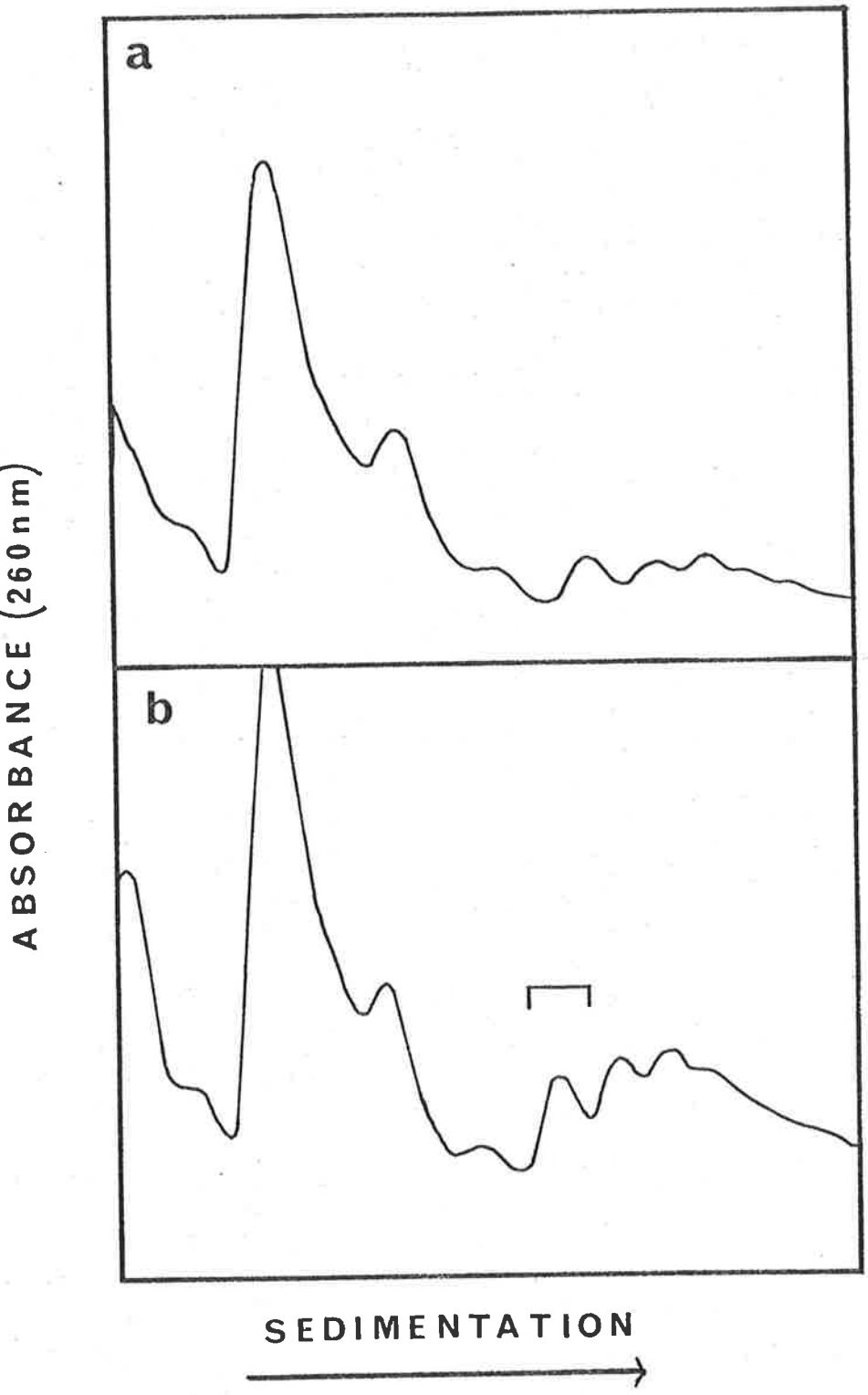


FIGURE 3.6. CHARACTERISATION OF POLYSOME PROFILES FROM 12 AND 13-DAY FEATHERS

Sucrose gradients were prepared and monitored as given in Figure 3.2.

Figure 3.6a. Absorbance profile of polysomes from 12-day feathers collected in Charity Waymouth's medium prior to transfer to ice-cold MSB.
The lysate was prepared in MSB.

Figure 3.6b. Absorbance profile of polysomes from 13-day feathers.
Other details as given in (a).


a
b
ABSORBANCE (260nm)
SEDIMENTATION

when a fraction corresponding to the tetramer region was examined by electron microscopy (Fig. 3.7).

The ratio of monosomes to polysomes in 13-day feathers was found to be somewhat variable. In some preparations a considerable proportion of the ribosomes was present in the polysome fraction, whereas in others very few polysomes were observed.

It was noted, by inspection of the 13-day embryo feathers that the development varied somewhat from one embryo to another. Since the onset of keratin synthesis occurs at 13 days (Bell and Thathachari, 1963; Ben-Or and Bell, 1965; Kemp, Dyer and Rogers, 1974) it is presumed that the variability in the onset of keratin synthesis from one embryo to another was responsible for the parallel changes in the polysome profiles at this age. The absorbance profile of a sucrose density gradient of polysomes from 12-day feathers prepared in low ionic strength buffer (LSB) is shown (Fig. 3.8). The proportion of ribosomes in the monomer fraction was about 65%.

5. Polysomes in cell-debris fraction

The cell-debris fraction from 14-day feathers (see Methods 2.B2) contained about 20% of the total volume of a lysate preparation. When examined by electron microscopy it was found to consist predominantly of fibrous keratin, together with nuclear and mitochondrial material. Hence the debris was washed twice by resuspension and recentrifugation in MSB and the size distribution of the released polysomes was examined by sucrose-gradient centrifugation. It was found

FIGURE 3.7. ELECTRON-MICROGRAPH OF POLYSOMES COLLECTED FROM THE FRACTION SHOWN BY A BAR IN FIGURE 3.6b. Negatively stained with uranyl acetate. Magnification: X90,000.

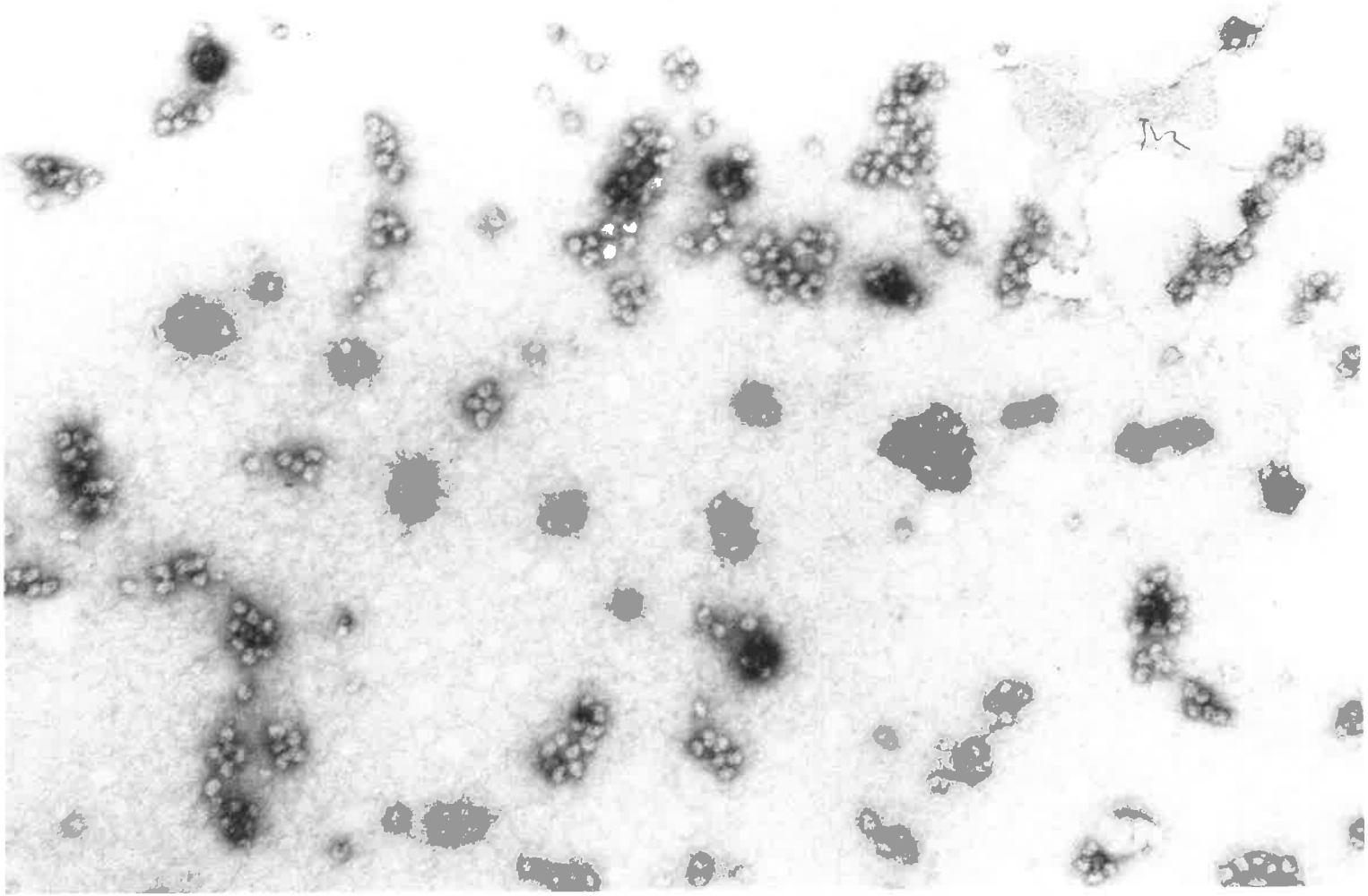
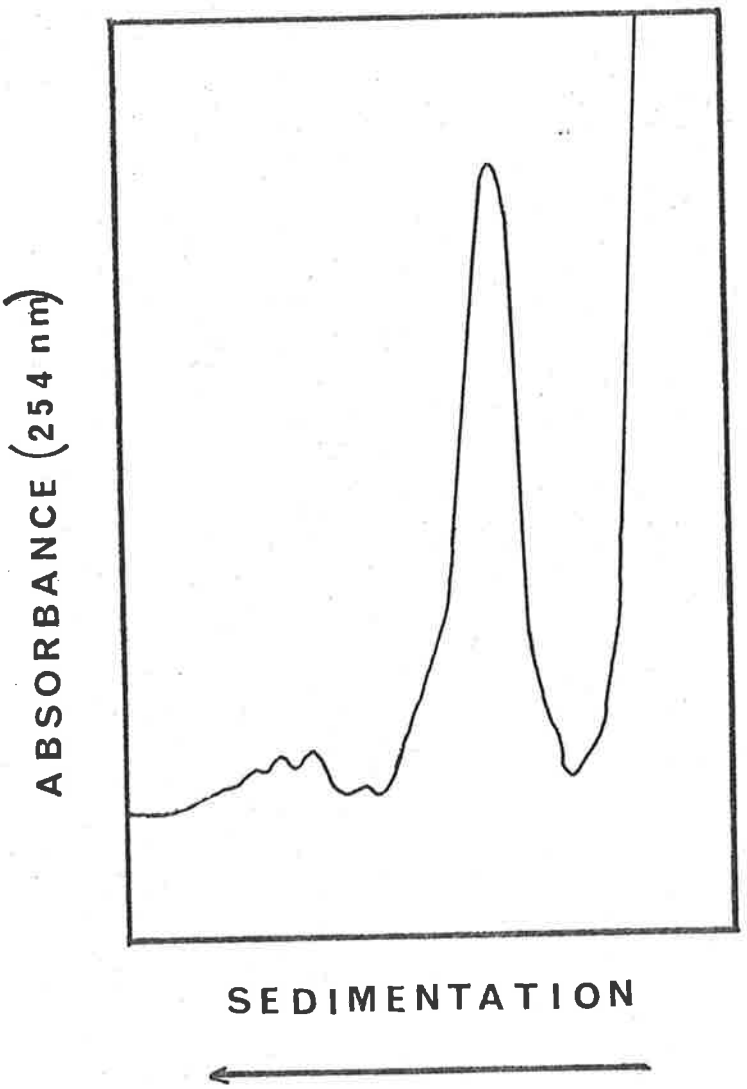


FIGURE 3.8. ABSORBANCE PROFILE OF POLYSOMES PREPARED IN LSB FROM 12-DAY FEATHERS.

The material present was obtained from 0.40 ml of packed feathers. Gradients (11.6 ml, 10% - 40% w/v) were prepared in LSB and centrifuged in an SW41 rotor for 1.5 hr, at 174,000 g (a.v.) at 3^{o}.

The gradient was monitored using an Isco density gradient fractionator.


ABSORBANCE (254 nm)
SEDIMENTATION

that in fact about 15-20% of the total amount of polysomes was present in this debris. Moreover, the size distribution of these polysomes was identical (results not shown) to those found in the post-mitochondrial supernatant prepared by the standard method (see Methods 2.B2).

6. In vitro incubation of whole feathers

Lysates were prepared in MSB buffer from 14-day chick embryo feathers incubated in Charity Waymouths medium (see Methods 2B3) for various times. The polysome profiles from the lysates were analysed in order to establish whether feathers incubated in vitro maintained their protein synthetic activity, unchanged from in ovo. The initial results, however, were not encouraging in that marked disaggregation of the polysomes occurred (as in Fig. 3.9a), and the tetramer peak predominated in the polysome region. It was found that this disaggregation could be prevented when the feathers were incubated in vitro in medium containing a low concentration of cycloheximide (2.5 μg/ml). The polysome content was then restored (Fig. 3.9b) to the level observed to occur in vivo, whereas control feathers in the same experiment incubated without cycloheximide exhibited the marked disaggregation (Fig. 3.9a).

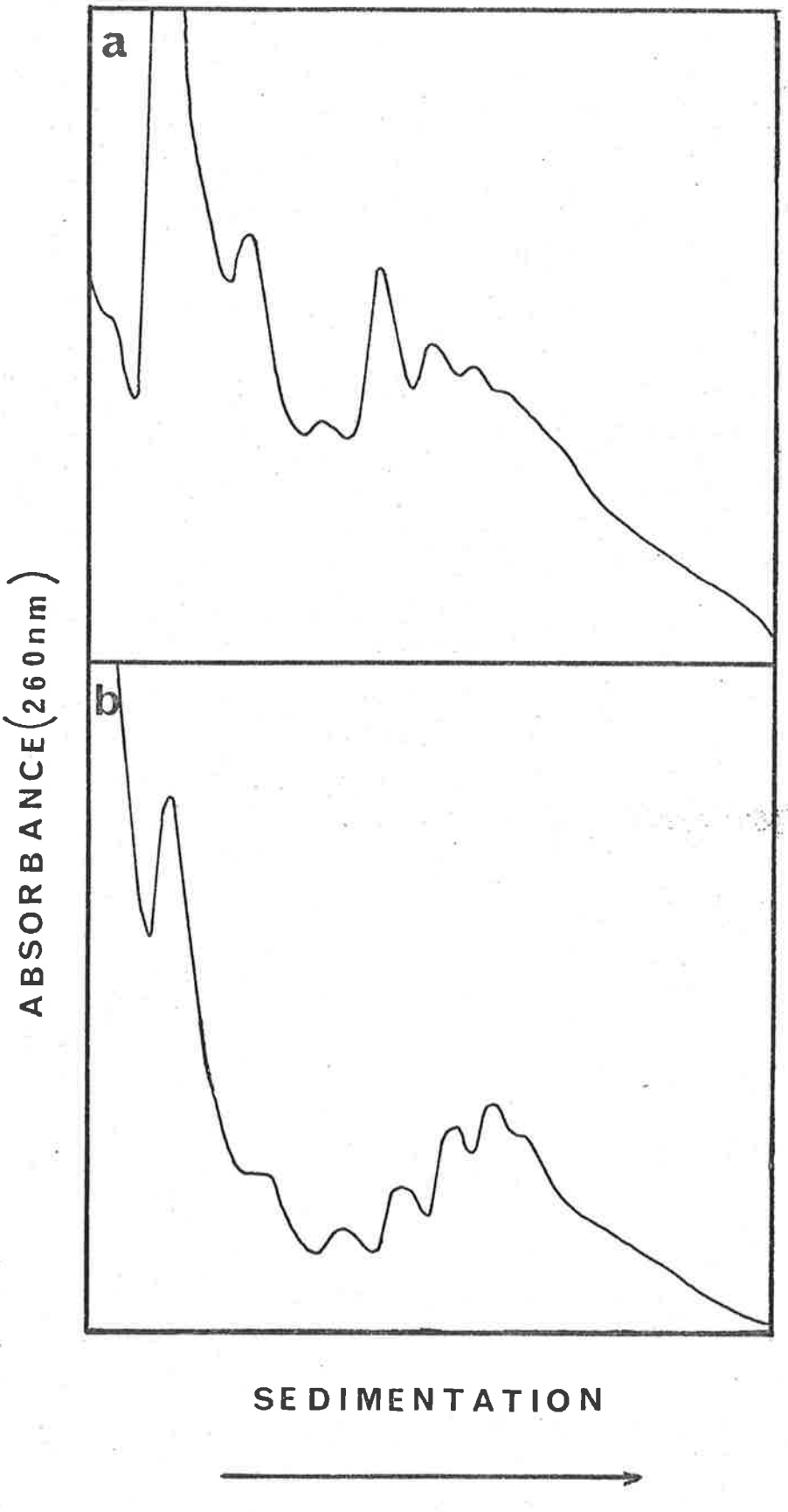
In an attempt to overcome this disaggregation without using a protein synthesis inhibitor, the following were added to separate incubations and the polysome profiles of the feathers analysed - 10% foetal calf serum, 10% horse serum, 5% chick embryo extract or a combination of 10% foetal calf serum and 5% chick embryo extract. However, none of these was

FIGURE 3.9. CHARACTERISATION OF POLYSOME-PROFILES FROM 14-DAY FEATHERS INCUBATED IN VITRO

The sucrose gradients were prepared, centrifuged and monitored as given in Figure 3.1.

Figure 3.9a. Absorbance profile of polysomes isolated from 14-day feathers incubated for 1 hr at 37° in Charity Waymouth's medium. The lysate was prepared in HSB.

Figure 3.9b. Absorbance profile of polysomes isolated from 14-day feathers incubated at 37° in Charity Waymouth's media containing 2.5 µg/ml cycloheximide. Other details are as given in (a).


a
b
ABSORBANCE (260nm)
SEDIMENTATION

effective in preventing the polysome disaggregation (results not shown). In later experiments the concentrations of amino acids were increased to ten times the level normally present in Charity Waymouth medium. In addition, the highly resolving Isco fractionation system was used to monitor the absorbance profiles of the sucrose-gradients in these experiments.

It was found that the polysome disaggregation was not as marked as that noted previously (Fig. 3.10a), and furthermore, the increase in the concentration of amino acids in the medium did not prevent polysome disaggregation (result identical to control). The level of polysomes was again increased in the presence of a low concentration of cycloheximide (Fig. 3.10b). The most interesting observation was that in all three incubation conditions tested, each peak of absorbance in the polysome region, representing each class of polysomes was characteristically a doublet.

In the same experiment, 12-day embryo feathers were incubated in medium in the presence and absence of 10 μg/ml of cycloheximide. The control showed a reduced level of polysomes and the presence of a substantial fraction of the ribosomes in the tetramer peak, whereas in the presence of cycloheximide the polysomes had built up to substantial aggregates with few ribosomes in the monomer fraction (Fig. 3.11b). Again the polysome of each distinct peak appeared to consist of doublets.

When the monosome peak of 14-day feathers (Fig. 3.10a,b) is compared with the monosome peak of 12-day feathers incubated with cycloheximide (Fig. 3.11b) it appears that either the monosome population is not homogeneous with respect to S value

FIGURE 3.10. ANALYSIS OF POLYSOME PROFILES OF 14-DAY FEATHERS INCUBATED IN VITRO.

The sucrose gradients were prepared in MSB and monitored as described in Fig. 3.3.

Figure 3.10a. Absorbance profile from 14-day feathers incubated for 1 hr at 37^{o} in Charity Waymouth's medium. The lysate was prepared in MSB.

Figure 3.10b. As for (a) except that the feathers were incubated in medium containing 2.5 µg/ml of cycloheximide.

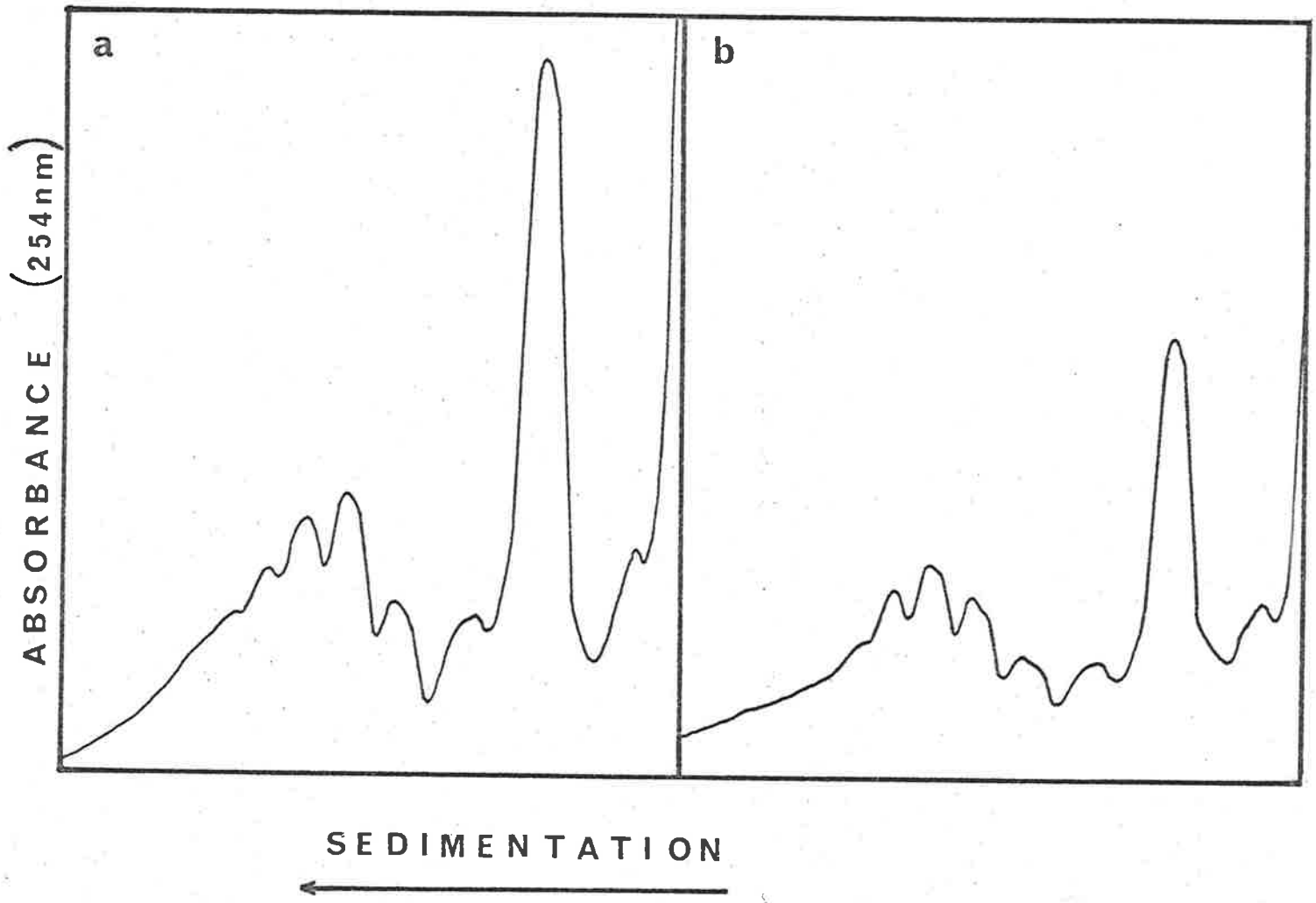
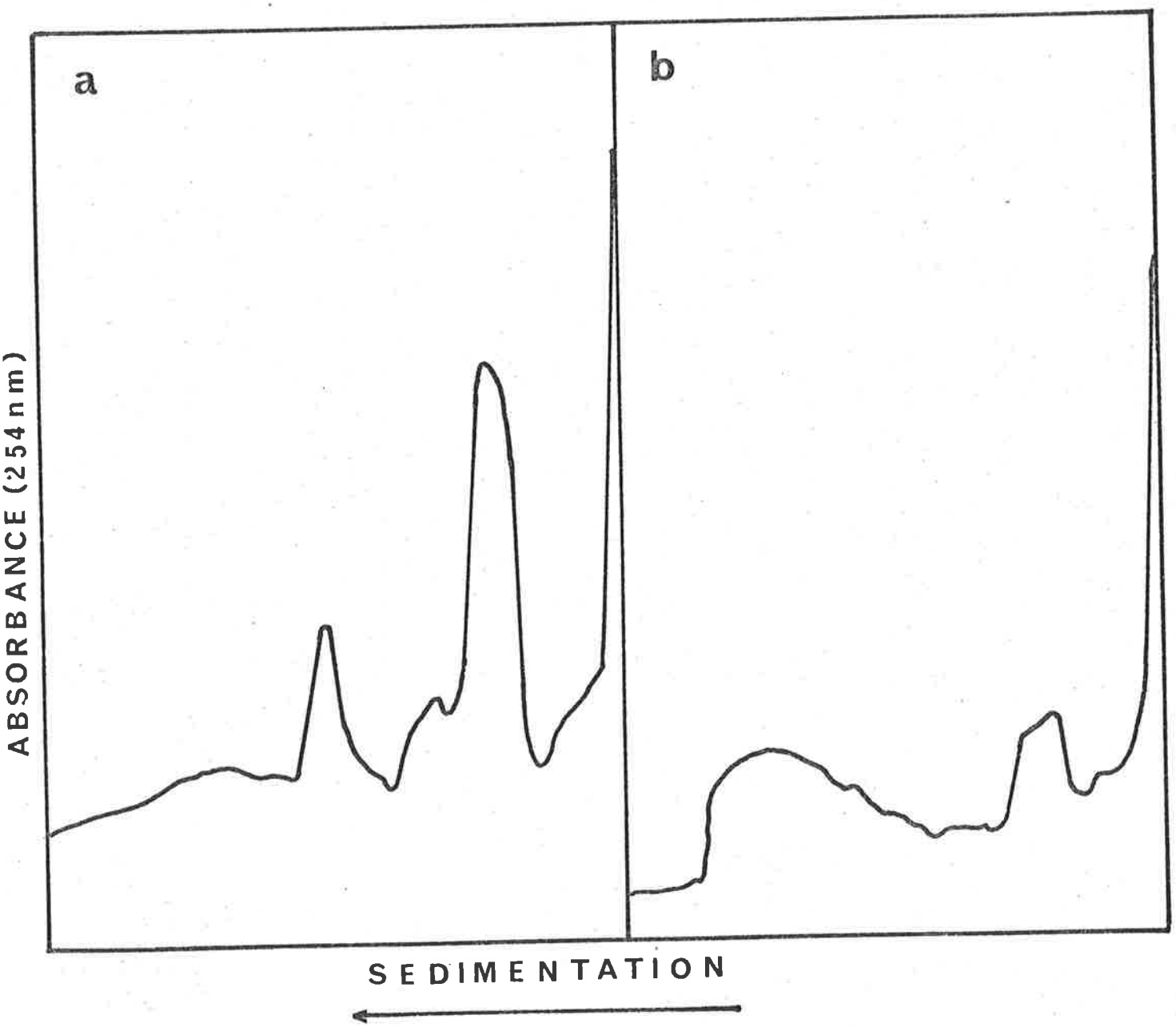

a
b
ABSORBANCE (254nm)
SEDIMENTATION

FIGURE 3.11. ANALYSIS OF POLYSOME PROFILES OF 12-DAY FEATHERS INCUBATED IN VITRO

The sucrose gradients were prepared in MSB, centrifuged and monitored as described in Figure 3.3.

Figure 3.11a. Absorbance profile of 12-day feathers incubated for 1 hr at 37° in Charity Waymouth's medium.
The lysate was prepared in MSB.

Figure 3.11b. Absorbance profile of 12-day feathers incubated for 1 hr at 37° in Charity Waymouth's medium containing 2.5 µg/ml cycloheximide.
Other details as given in (a) above.


a
b
ABSORBANCE (254 nm)
SEDIMENTATION

or more probably that the large ribosomal subunits are in excess of monosomes.

7. Preparation of polysomes from duck feathers

Polysome profiles were examined from feathers of duck embryos of several ages, as a preliminary step to determining some parameters about the development of duck feathers. The intention was to use them to prepare cell-free systems (see Chapter 5). The species investigated was the Khaki Campbell Duck (Anas boschas), which has a 28-day incubation period.

Polysome profiles are shown for feathers from the embryos ranging from 14 to 19 days (Fig. 3.12a-f). There is an increase in polysomes (relative to the monosome peak) at day 15, with the region of trimers to pentamers as the major polysome class size. The ratio of ribosomes in the monomer to the polysome fraction was found to be much higher in the embryonic duck feather than in the chick embryo feathers after the onset of keratinization. This possibly reflects some developmental difference. From the polysome profiles it appeared that the onset of keratinization occurred at about day 15, and this was later confirmed by polyacrylamide gel electrophoresis of the SCM-derivatives of proteins of feathers from day 14 to 17 (see Chapter 5).

FIGURE 3.12. SUCROSE GRADIENT SEDIMENTATION OF POLYSOMES ISOLATED FROM DUCK EMBRYO FEATHERS DURING DEVELOPMENT.

The feather lysates were prepared in MSB (see Fig. 3.3) and centrifuged using the same conditions as are given in Fig. 3.3.

Figure 3.12a. Absorbance profile of polysomes isolated from 14-day duck embryo feathers (0.125 ml).

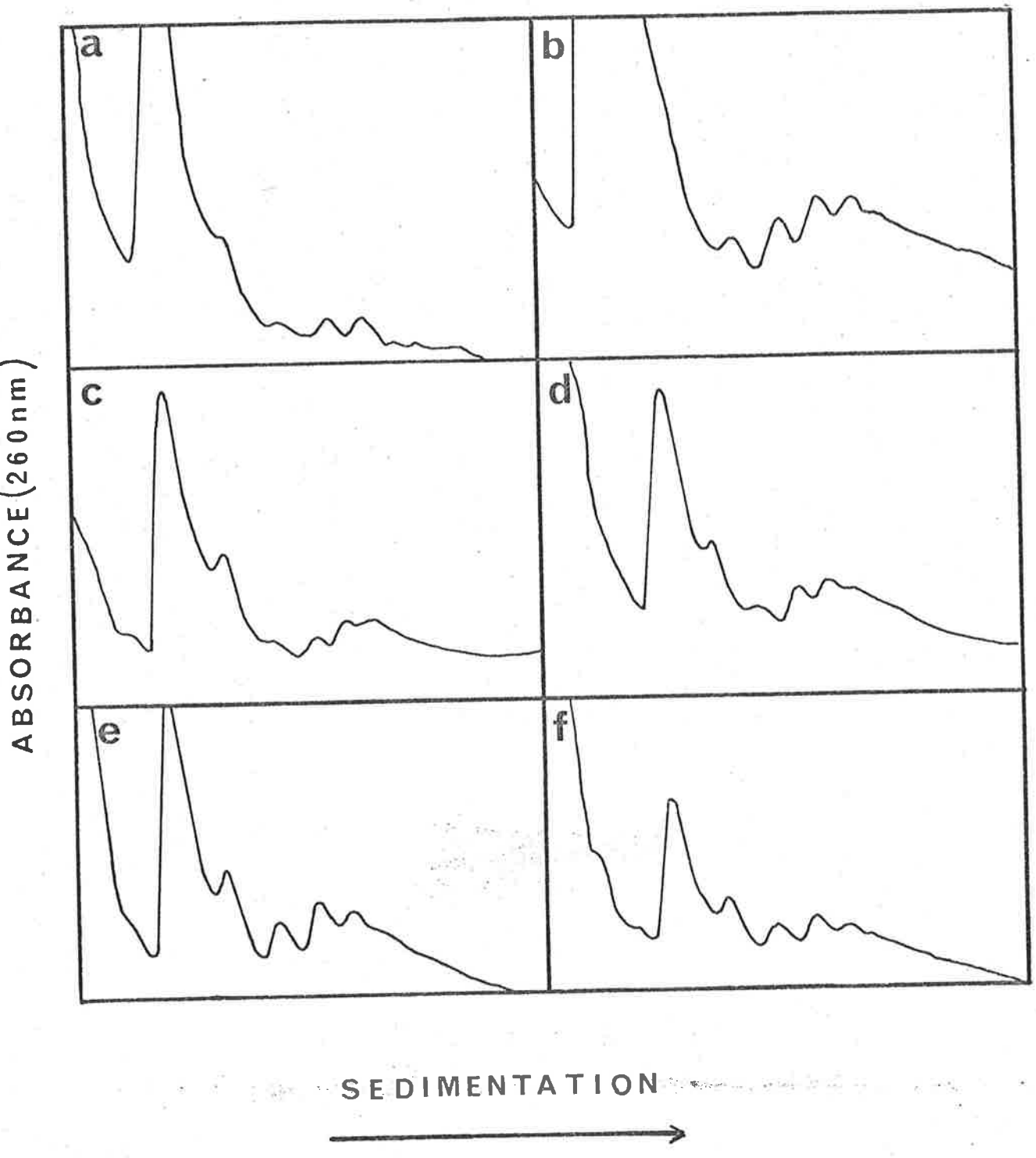
Figure 3.12b. 15-day feathers (0.25 ml).

Figure 3.12c. 16-day feathers (0.125 ml).

Figure 3.12d. 17-day feathers (0.25 ml).

Figure 3.12e. 18-day feathers (0.25 ml).

Figure 3.12f. 19-day feathers (0.25 ml).


a
b
c
d
e
f
ABSORBANCE (260nm)
SEDIMENTATION

C. DISCUSSION

1. General comments

In the present study an increase of polysomes has been observed in the developing chick embryo feathers after the onset of keratin synthesis, in agreement with the observations of Humphreys et al. (1964) and Bell et al. (1965). Yatvin (1966a,b) reported that these molecular events require a functional hypophysis, since surgical removal of the pituitary gland prevented the appearance of the increased polysome levels normally occurring at 13 days. Furthermore, Cohen and Stastny (1968) demonstrated that polysomes were assembled from pre-existing monomers when chick embryo skin was incubated in the presence of epidermal growth factor (EGF).

The assembly of polysomes at the time of onset of the synthesis of a particular protein has been described for a number of differentiating tissues. Examples include, the appearance of a high concentration of disomes in trout testis during the period of rapid protamine synthesis (Ling and Dixon, 1970); changes in polysome levels during the synthesis of lens crystallins (Scott and Bell, 1965); shifts in the polysomes which parallel the changing rates of synthesis of myosin, actin and tropomyosin during chick embryo muscle development (Heywood and Rich, 1968). Similar such effects have been noted in tissues induced by hormones. Palmiter et al. (1970) observed the assembly of polysomes from pre-existing monomers during the secondary stimulation of chick oviduct with either oestrogen or progesterone. Further work by Means et al. (1971) who investigated the primary response of chick oviduct to diethylstilbodiesterol showed that

the increased polysome content resulted from both a conversion of pre-existing monomers and new ribosome synthesis.

It is likely that the polysomesseen to accumulate at 13 days in the present study are assembled from both pre-existing monomers and newly-synthesised ribosomes (see Chapter 7 for further discussion).

2. Effect of composition of isolation buffer on polysome profiles

The polysome profiles from lysates of 14-day feathers prepared in either HSB or MSB were essentially identical. However, when the profiles of polysomes prepared in MSB were compared to those prepared in LSB, marked differences were noted in the subunit region of the gradients. No complete explanation can be given as to whether it was the increased concentration of KCl or $MgCl_2$ or both together in MSB as compared to LSB which caused the change. It seems more likely that the true situation is represented in LSB, since the profile in the subunit region obtained with this buffer more closely resembles the fractionation obtained in other cell types by earlier workers such as McConkey and Hopkins (1965) and Girard et al. (1965). Furthermore, Bryan and Hayashi (1973) reported that Mg^{++} concentration must be kept as low as possible during the isolation of chick embryo cerebral polysomes to prevent the alteration of ribosomal subunits into more slowly sedimenting inactive derivatives. Another independent line of evidence in the present work supports the above contention. Maximal incorporation and reinitiation in a 14-day feather cell-free system was obtained

in LSB (see Results 4.B7).

3. Significance of the ribosomal tetrameric aggregates

Following the observations of Byers (1966, 1967) that the tetrameric aggregates of ribosomes could be induced by hypothermic treatment of chick embryos further work by a number of groups has not, as yet, greatly clarified the position of the aggregates in the ribosomal biogenic and functional cycle.

(a) Studies in vitro

Little accord has been obtained in estimating the S value of the tetrameric aggregates (Humphreys et al., 1964; Bell et al., 1965; Carey, 1970; Morimoto et al., 1972b). It should be noted, however, that the ionic conditions used for preparing the aggregates were different in each case, and furthermore, that the reference S value was the monosome peak, for which a different S value was used in each case. It has been clearly established that the tetramers are comprised of a combination of equal numbers of small and large ribosomal subunits (Morimoto et al., 1972), and that they have no endogenous mRNA activity, but can be primed by poly U in vitro without the need for disaggregating (Byers, 1971; Morimoto et al., 1972a). It is generally agreed that they are dissociated by increasing the concentration of KCl in the medium (Byers, 1971; Carey and Read, 1971; Morimoto et al., 1972a), but Byers (1971) and Carey

and Read (1971) reported that dissociation occurred initially by loss of the small subunits to form tetramers comprised solely of the large ribosomal subunits, whereas Morimoto et al. (1972a) detected dissociation of the tetramers directly to monomers.

(b) Studies in vivo

Crystalline sheets of ribosomes, comprised of the tetrameric aggregates as the basic unit were originally observed by electron microscopy in tissues of chick embryos subjected to hypothermia but not at all in control (uncooled) tissues (Byers, 1966, 1967). Moreover, crystals were not observed by electron microscopy in interphase cells of rapidly-cooled embryos, but were abundant in mitotic cells. Morimoto et al. (1972b) have extended these observations to find that the aggregates can form in interphase cells under conditions which allow polysome disaggregation.

The extensive studies of ribosome crystallization in chick embryos by Barbieri and co-workers, has not as yet shed any significant light on the nature and conditions of formation of the aggregates. Possibly the most significant finding to date is the observation (Maraldi and Barbieri, 1969) that the average size of the aggregates varies in different organs of the hypothermic embryos and is strictly dependent on the degree of differentiation of the particular tissue, crystallization decr easing as the degree of differentiation increases. Similarly, Morimoto et al. (1972b) reported that the proportion of ribosomes involved in tetramer formation decreased with increasing

age of the embryo. Furthermore, other evidence has been obtained showing that ribosome crystals can be induced by hypothermic treatment of adult chicken tissues, either in organs affected by Marek's disease or in rapidly-proliferating cell lines (Simoni et al., 1973). Hence, the crystallization phenomenon is not restricted to embryonic chicken tissues. To date there has been only one report of ribosome crystallization in uncooled tissue (Mottet and Hammar, 1972). The aggregates were observed in degenerating nerve cells from the posterior necrotic zone of developing chick limbs.

Considered overall no consistent pattern emerges from the data to postulate a reasonable theory as to the role of the aggregates in the control of protein synthesis.

Although it has been reported (Morimoto et al., 1972a) that no difference exists in the protein complement of ribosomes isolated from the tetrameric aggregates and ribosomes from polysomes the resolution obtained on the one-dimensional polyacrylamide gel system was not sufficient to draw this conclusion. Further work, possibly using a two-dimensional polyacrylamide gel system, similar to that developed for E. coli ribosomes by Kaltschmidt and Wittman (1970) is required to separate the proteins. It is clear that the present studies do not further clarify the role of the ribosomal aggregates in the control of protein synthesis.

The results described in the present chapter on the presence of the tetrameric ribosomal aggregates in 12 and 13-day feathers prior to the onset of keratinization are in some agreement with result described previously (Humphreys et al.,

1964; Bell et al., 1965). It is presumed that in early experiments of the present study the time taken to pluck the feathers was too prolonged, resuling in polysome disaggregration and the consequent formation of tetramers. The same effect was observed in early experiments on the incubation of 14-day feathers in vitro, where marked polysome disaggregation had occurred, the tetramer peak predominated in the polysome region. Presumably tetramers had formed rapidly when the incubated feathers were chilled prior to homogenisation. Morimoto et al. (1972a) reported that tetramer formation only began to occur after two hours cooling; however, in the experiments described here the time required for the processing of the feathers was considerably less than this.

4. In vitro incubation of 14-day embryo feathers

Pronounced polysome disaggregation occurred when 14-day feathers were incubated in vitro in Charity Waymouth's medium. This disaggregation could only be prevented by addition of a low concentration of cycloheximide to the culture medium. It has been observed in other work that addition of a low concentration of cycloheximide to certain cell lines preferentially inhibits translation while initiation is essentially unaffected. This results in a decrease of ribosomes in the monomer fraction and a concomitant increase in the polysome region (Stanners, 1966; Hogan, 1969; Fan and Penman, 1970; Stanners and Becker, 1971). The results of the present work suggest that the rate of initiation in vitro had decreased relative to the rate in vivo.

It is interesting to compare the effect of cycloheximide on the 12 and 14-day feathers incubated in vitro. In the

presence of cycloheximide the polysome distribution in 14-day feathers was essentially identical to that found in unincubated feathers, whereas cycloheximide caused the accumulation of large polysomes in the incubated 12-day feathers. This result would tend to suggest that 12-day feathers do not contain large amounts of cytoplasmic keratin mRNA which is translated at very low efficiency, because if this was the case the cycloheximide should induce a polysomal distribution identical to that of 14-day feathers.

Increasing the concentrations of amino acids to ten times their normal concentrations was ineffective in preserving the *in vivo* levels of polysomes in feathers incubated *in vitro* (cf. Jefferson and Korner, 1969). However, under all conditions tested in the later experiments polysome peaks were noted to be doublets. This phenomenon was first described by Hoerz and McCarty (1969, 1971) in reticulocyte lysates incubated in the presence of 10 mM NaF and attributed by them to be polysomes each with a 40S subunit attached.

Marked polysome disaggregation of isolated tissues incubated *in vitro* has been described previously. Thus, Jefferson and Korner (1969) reported that the marked polysome disaggregation occurring in perfused liver could be prevented by increasing the amino acid concentration to ten times the normal serum level. Bernelli-Zaggera *et al*. (1972) reported a similar effect in rat liver slices incubated *in vitro*, whereas Ekren *et al*. (1971) showed that both insulin and high amino acids were required to stimulate polysome formation in perfused diabetic rat livers. A similar beneficial effect of high amino acid concentration in reducing polysome disaggregation was found to occur in perfused rat hearts (Morgan *et al*., 1971ab,). Increased toxicity of the medium has also been shown to promote polysome

disaggregation in HeLa cells (Wengler and Wengler, 1972). It is possible that the cause of polysome disaggregation in feathers incubated *in vitro* is simply that the incubation medium was originally formulated for a mammalian cell line (Waymouth, 1959). Another possibility for the decreased polysome content in incubated feathers is that the pH of the medium may not have been optimal. Whatever the cause, the feathers respond to the *in vitro* coniditions with a decreased rate of initiation of translation, resulting in decreased levels of polysomes and this lesion appears to be primarily the joining of the large ribosomal subunit to the initiation complex, as found for other systems both *in vivo* and *in vitro*.

In relation to the appearance of the doublets in the present results, it has been found that the large (60S) ribosomal subunit accumulates in both amino acid-starved liver *in vivo* (Wunner *et al.*, 1966) and in diabetic muscle (Stirewalt *et al.*, 1967). Furthermore, it has been well-established with cell culture lines that polysome disaggregation occurs when amino acids become depleted in the medium and this can be reversed simply by replacing the deficient amino acid(s) (Eliasson *et al.*, 1967; Hogan and Korner, 1968; van Venrooij *et al.*, 1970; Lee *et al.*, 1971a) and in one case this was shown to result directly from a decreased rate of initiation (Vaughan *et al.*, 1971).

CHAPTER FOUR

KINETICS AND CHARACTERISATION OF PRODUCTS OF CELL-FREE SYNTHESIS

A. INTRODUCTION

Recent advances in the understanding of the mechanism and control of protein synthesis in eukaryote cells has largely utilized cell-free systems derived from rabbit reticulocytes. The work of Lamfrom and Knopf (1964) has shown that initiation of globin synthesis proceeds at a high rate in lysate systems in comparison to fractionated cell-free systems (Schweet et al., 1958; Knopf and Dintzis, 1965). Indeed, with some modification, globin synthesis in such systems can proceed for a short time at rates comparable to that in intact cells (Adamson et al., 1968, 1969). Similarly, interest has been revived in the use of the post-mitochondrial supernatant from rat liver cells for studies of protein synthesis (Richardson et al., 1971). These authors have shown this system to be more active, and to have a higher level of de novo protein synthesis than the more fully-fractionated systems previously used.

The synthesis of a number of cellular structural proteins has also been achieved in their respective cell-free systems. Examples include myosin (Heywood and Rich, 1968; Low et al., 1971), crystallins (Strous et al., 1971) and collagen (Lazarides and Lukens, 1971; Kerwar et al., 1972, 1973; Diegelmann et al., 1973). As noted previously, studies of protein synthesis in cells of epithelial origin have lagged behind those of other systems. Recently, a fractionated cell-free system from hair follicles of the guinea pig was prepared (Steinert and Rogers, 1971a). It was reported that the low sulphur keratin proteins (Steinert and Rogers, 1971b) and the high sulphur keratin proteins

(Steinert and Rogers, 1973) were synthesised in this system. However, the de novo synthesis of these proteins was rather low.

This chapter primarily describes studies on cell-free systems prepared from 14-day chick embryo feathers. Preliminary studies on cell-free systems prepared from 12, 13 and 15-day feathers are also reported.

B. METHODS

1. Preparation and incubation of the cell-free system for determination of the kinetics of incorporation

Lysates were prepared from feathers using either MSB (200 mM KCl, 5.3 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.4) or LSB (66 mM KCl, 2.67 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.4). The standard incubation mixture contained in a volume of 0.5 ml, the following components; 0.37 ml lysate (prepared using either LSB or MSB buffer), 0.25 mM GTP, 1.0 mM ATP, 15 mM creatine phosphate, 50 µgm creatine phosphokinase, and 19 amino acids, each at a concentration of 0.01 mM. The concentrations of ATP, GTP, creatine phosphate and creatine phosphokinase were adopted from the method of Adamson et al. (1968). ATP and GTP were prepared as a stock solution, neutralized to pH 7.0 with KOH and stored frozen. Likewise creatine phosphate was prepared as a neutralized stock solution. Creatine phosphokinase was always prepared freshly before use. When a particular labelled amino acid was present the corresponding unlabelled amino-acid was omitted. For lysates prepared in MSB the final composition was 150 mM KCl, 4 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.4. For lysates prepared in

LSB the final composition was 50 mM KCl, 2 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.4. The cell-free systems were incubated at either 25° or 37° for the times indicated (see legends to Figs. 4.1, 2, 3, 4, 6, 10, 12, 15).

2. Preparation of 14-day feather cell-free systems for determination of ionic conditions for optimal activity

Feathers were plucked from two embryos washed in Hanks' saline. Feathers were plucked as rapidly as possible and placed in a Petri dish containing Hanks' saline, at room temperature. When sufficient feathers were collected aliquots were removed and placed in ice-cold LSB or MSB. The lysates were then prepared as described previously (see Methods 2.B.2) except that care was taken to ensure that the packed-feather volumes in the two buffers were identical. Cell-free systems were prepared from the lysates to give final concentrations of 50 mM, 100 mM and 150 mM KCl, and at each of these KCl concentrations the $MgCl_2$ concentration was varied from 2 mM to 8 mM in increments of 2 mM. The cell-free systems were allowed to stand on ice for 30 min prior to incubation at 37° to allow equilibration in the new ionic conditions.

3. Characterisation of proteins synthsised in feather cell-free systems

When characterisation of proteins synthesised in cell-free systems, prepared in either LSB or MSB buffer was required the cell-free systems were usually scaled up. When serine was to be used as the labelled amino acid, the lysate was dialysed

for 2.5 hours against either MSB or LSB buffer but containing only 0.5 mM dithiothreitol, with a buffer change after 1.5 hours. The dithiothreitol concentration was not increased in the incubation mixtures prepared from dialysed lysate. After incubation of these cell-free systems at 37°, the ribosomes were pelleted by centrifuation at 4° for 1 hour at 190,000 (a.v) in a 'Beckman Ti50' rotor or a 10 x10 ml rotor of an 'MSE Super-speed 65' centrifuge.

4. Preparation of reduced and carboxymethyled labelled proteins from post-ribosomal supernatants

To each 2 ml of post-ribosomal supernatant from the cell-free incubation mixture 10 ml of urea/β-mercaptoethanol/ethanolamine solution was added, and 2 cycles of reduction and carboxymethylation were performed as described by Kemp et al. (1974) except that the initial reduction step was for 1 hour, and the centrifugation was omitted.

5. Polyacrylamide gel electrophoresis of labelled proteins synthesised in vitro

Samples containing known amounts of labelled protein were co-electrophoresed with 100 µg of carrier on soluble ethylene diacrylate cross-linked gels at pH 7.5. The carrier protein consisted of reduced and carboxymethylated SCM-feather keratin from newly hatched chicks. This method as well as the subsequent staining, slicing and determination of radioactivity was as described by Kemp et al. (1974). It should be noted however, that several systems were used to measure the absorbance profiles

of the stained keratin bands (see Method 2.B6 and legends to Figs. 4.7, 8, 13, 14, 16 for details).

6. Analysis of polysome profiles in the feather cell-free system by sucrose gradient centrifugation.

Fourteen-day feather lysate was prepared in MSB buffer. The final volume of the incubation mixture was 1.0 ml, 0.75 ml of which was feather lysate. ^{14}C-leucine (5 µc) was added, to label the nascent peptides. At the indicated times of incubation 0.25 ml samples were removed and pipetted into 0.25 ml of buffer containing 100 µg/ml of cycloheximide at 0°. For the control (unincubated lysate) 0.175 ml of lysate was added to 0.325 ml of the buffer containing 100 µg/ml of cycloheximide at 0°.

Each sample was layered over a 10 - 40% sucrose gradient (w/v, prepared in the buffer), and centrifuged for 1 hour at 174,000 g (av.) 3° in a 'Beckman SW41' rotor.

The gradients were monitored for absorbance at 254 nm, using an LKB-Uvicord II ultraviolet analyser coupled to a Hitachi QPD-53 recorder. Thirteen-drop fractions were collected manually for determination of radioactivity.

The fraction of total ribosomes in the monosome and polysome regions respectively was determined by cutting out and weighing the monosome and polysome peaks from the absorbance trace of the sucrose gradient.

7. Deproteinisation of feather lysates

Aliquots of feather-lysates prepared from known values of packed feathers were deproteinised using the method of Stein

and Moore (1954). Briefly, 5.0 ml of a saturated solution of picric acid was added per ml of feather lysate, at room temperature. The insoluble protein-picric acid complex was removed by centrifugation at 3,000 g for 30 min. The excess picric acid was then removed by chromatography on a 1 x 8 cm column of Dowex 2. The eluate collected from the column was taken to dryness by rotary evaporation at 40°.

8. Amino acid analysis

The concentration of free amino acids in the feather lysates was determined by the procedure of Piez and Morris (1960) using a Beckman 120C analyser modified as described by Harding (1971).

C. RESULTS

For initial studies of cell-free protein synthesis, 14-day feathers were chosen as these were known to be highly active in keratin synthesis *in ovo* (Kemp *et al.*, 1974). Lysates were prepared using MSB (see Methods 2.B2). It was shown in the previous chapter (see Results, 3.B2) that polysomes from 14-day feathers could be successfully isolated using this method.

1. Kinetics of incorporation in the 14-day feather cell-free system

The kinetics of incorporation of labelled amino acid into protein were assayed by determining the acid precipitable counts in aliquots taken from a cell-free system at intervals after the beginning of the incubation (see Methods 2.B5). The results of a typical experiment are shown in Fig. 4.1. The

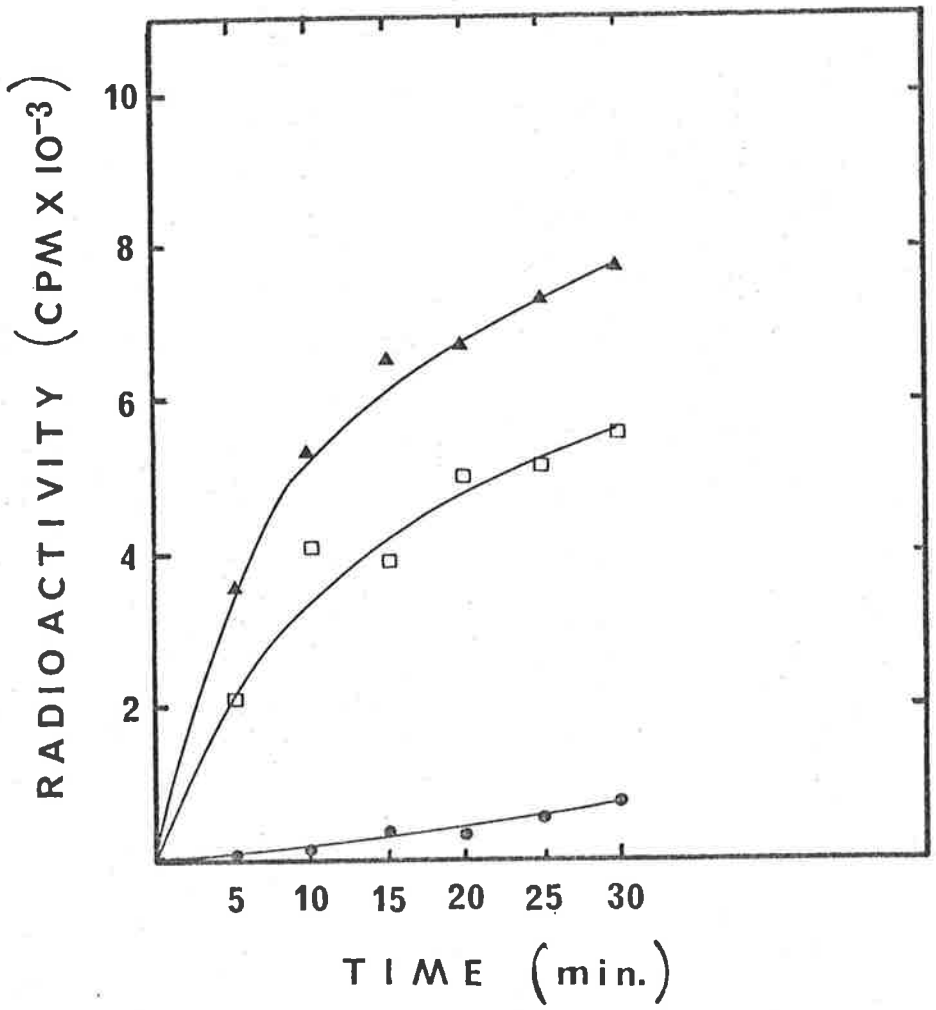
FIGURE 4.1. KINETICS OF AMINO ACID INCORPORATION IN A FEATHER CELL-FREE SYSTEM PREPARED IN MSB.

The cell-free system (0.5 ml) incubated at 37° with 0.5 μC of ^{14}C-leucine (S. Act. 312 mC/mM) as described (see Methods 4.B.1). Aliquots (0.05 ml) were removed at 0, 5, 10, 15, 20, 25, 30 min. for determination of acid precipitable counts incorporated. Radioactivity represents cpm/0.05 ml aliquot.

□—□ control

▲—▲ chloramphenicol (200 μg/ml)

●—● cycloheximide (200 μg/ml)


RADIOACTIVITY (CPM X 10⁻³)
10
8
6
4
2
5
10
15
20
25
30
TIME (min.)

initial rate of incorporation was maintained for only about 5 min and then decreased rapidly. After about 20 min of incubation essentially all activity of the system had ceased. In the presence of a high concentration (200 µg/ml) of cycloheximide, a specific inhibitor of protein synthesis in eukaryotes, protein synthesis was reduced by about 90%. Chloramphenicol (200 µg/ml) however, had essentially no effect. From this result it was concluded that no mitochondrial peptide formation occurred and that bacterial contamination could be excluded. It was found that both the rate of incorporation and the total incorporation values for different lysate preparations under identical conditions were fairly constant. For example, the total incorporation was always within 20% of the maximum. This finding in addition to the fact that the total incorporation was high enabled the system to be investigated more fully without the need for further fractionation.

2. Effect of inhibitors of initiation on cell-free protein synthesis in medium salt buffer, MSB

To determine to what extent, if any, de novo protein synthesis was occurring in the lysate system inhibitors of initiation of protein synthesis namely pactamycin, aurintricarboxylic acid (ATA) and NaF were added to the cell-free system at concentrations which have been shown to preferentially inhibit de novo protein synthesis (Conconi et al., 1966; Marcus et al., 1970; Macdonald and Goldberg, 1970; Stewart et al., 1971; Stewart-Blair et al., 1971; Lodish et al., 1971). In the presence of the inhibitors, protein synthesis was reduced by about 40% (Fig. 4.2).

FIGURE 4.2. EFFECT OF INHIBITORS OF INITIATION ON AMINO ACID INCORPORATION IN A 14-DAY FEATHER CELL-FREE SYSTEM PREPARED IN MSB.

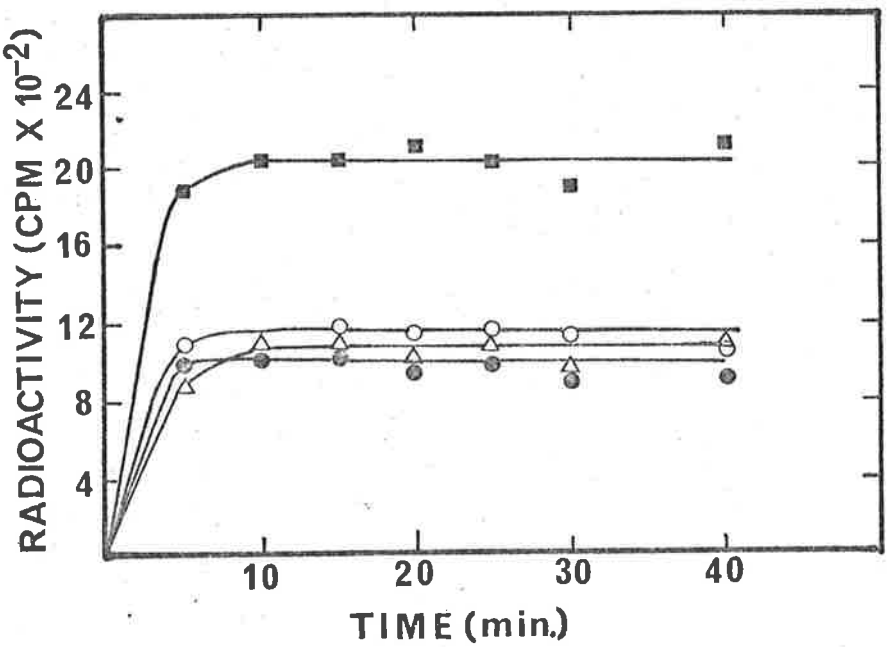
Other details are as given in Figure 4.1.

■—■ control

○—○ ATA (1×10^{-4} M)

△—△ NaF (1×10^{-2} M)

●—● pactamycin (1×10^{-6} M)


RADIOACTIVITY (CPM X 10^{-2})
24
20
16
12
8
4
10
20
30
40
TIME (min.)

Two of the inhibitors ATA and NaF were then tested on a cell-free system prepared from rabbit reticulocyte lysate as described (see Methods 2.B4) to confirm that they were fully active. In this system both ATA and NaF reduced incorporation by about 70% (Fig. 4.3). This result is in good agreement with the published data on the effect of these inhibitors on a reticulocyte cell-free system (e.g., Mathews, 1971; Lodish et al., 1971; Hunt et al., 1972; Shenk and Stollar, 1972).

3. A comparison of incorporation at 25° and 37° of a feather cell-free system prepared in medium salt buffer, MSB

The kinetics of incorporation of a 14-day feather cell-free system incubated at both 25° and 37° is shown in Fig. 4.4. It can be seen that although the rate of incorporation was much slower at 25° than at 37° the maximal incorporation obtained was essentially identical.

4. Relation of polysome profiles to protein synthesis in the buffer of medium ionic strength, MSB

To determine whether the rate of breakdown of polysomes incubated in vitro correlated with the protein synthetic activity, a cell-free system prepared in MSB in the presence of ^{14}C-leucine to label the nascent peptides. Aliquots were removed from the cell-free system for analysis of the polysome distribution by sucrose density gradient centrifugation (Methods 4.B6). The results are shown in Fig. 4.5a-e. In the unincubated sample of lysate (Fig. 4.5a) about 63% of the ribosomes were present in the polysome region. On incubation of the cell-free

FIGURE 4.3. EFFECT OF INHIBITORS OF INITIATION ON AMINO ACID INCORPORATION OF A RABBIT RETICULOCYTE CELL-FREE SYSTEM.

A 0.5 ml reticulocyte lysate cell-free system was prepared (see Methods 2.B.5 and 6.B.4) and incubated at 37^{o} with 1.0 µC of ^{14}C-leucine (S. Act. 316 mC/mM). Other details are as given in Figure 4.1.

□—□ control

▲—▲ NaF (1×10^{-2} M)

●—● ATA (1×10^{-4} M)

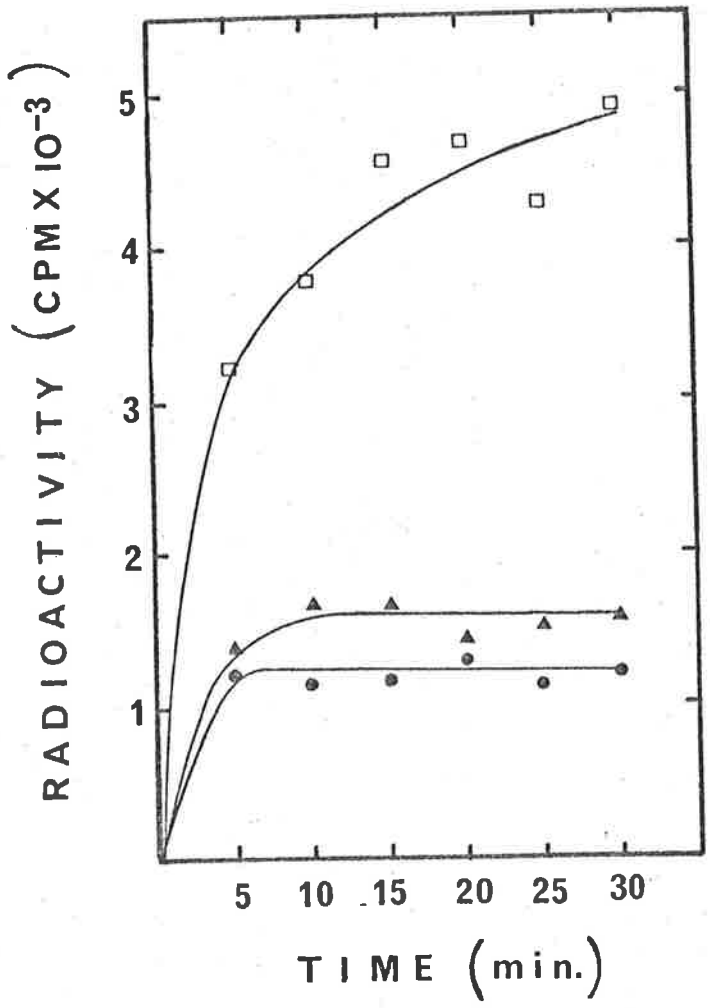

RADIOACTIVITY (CPM X IO⁻³)
5
4
3
2
1
5
10
15
20
25
30
TIME (min.)

FIGURE 4.4. COMPARISON OF THE KINETICS OF AMINO ACID INCORPORATION OF A 14-DAY FEATHER CELL-FREE SYSTEM PREPARED IN MSB AND INCUBATED AT BOTH 25^{o} and 37^{o}.

Other details are as given in Figure 4.1.

○—○ , 25^{o}

●—● , 37^{o}.

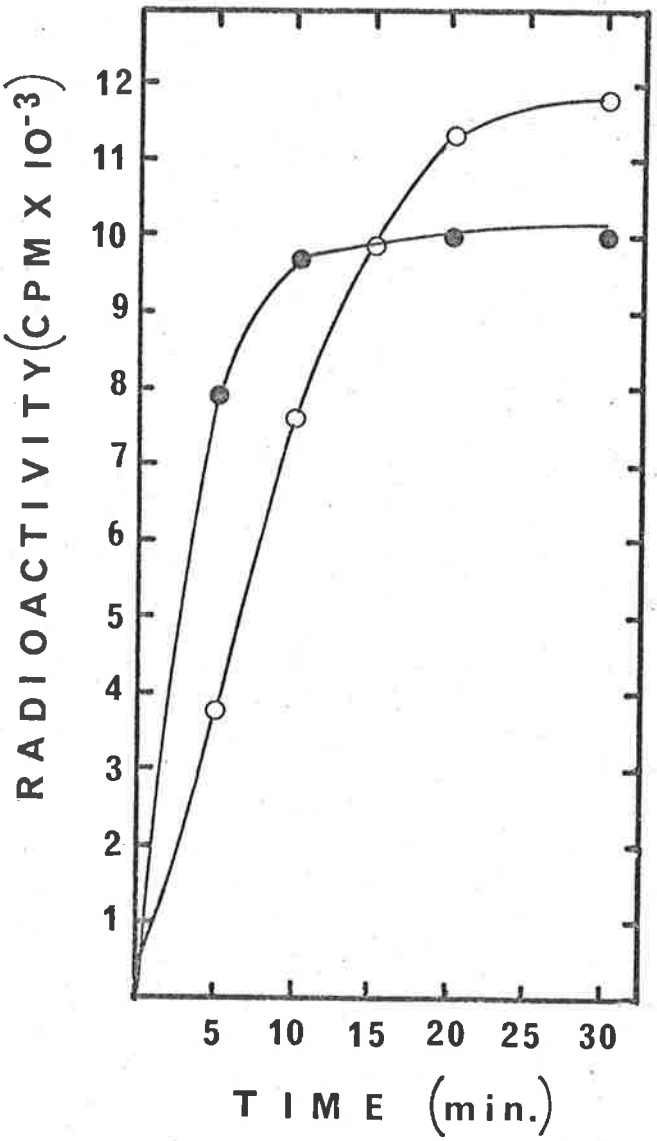

RADIOACTIVITY(CPM X IO-3)
12
11
10
9
8
7
6
5
4
3
2
1
5
10
15
20
25
30
TIME (min.)

FIGURE 4.5. ABSORBANCE PROFILES OF POLYSOMES IN A 14-DAY FEATHER CELL-FREE SYSTEM PREPARED IN MSB AND INCUBATED AT 37^{o}

The cell-free system (1.0 ml) was prepared as described (see Methods 4.B.6) and incubated at 37^{o} with 5 μC of ^{14}C-algal hydrolysate.

Aliquots were removed at various intervals for analysis of polysome and radioactivity profiles by sucrose gradient centrifugation (see Methods 4.B.6).

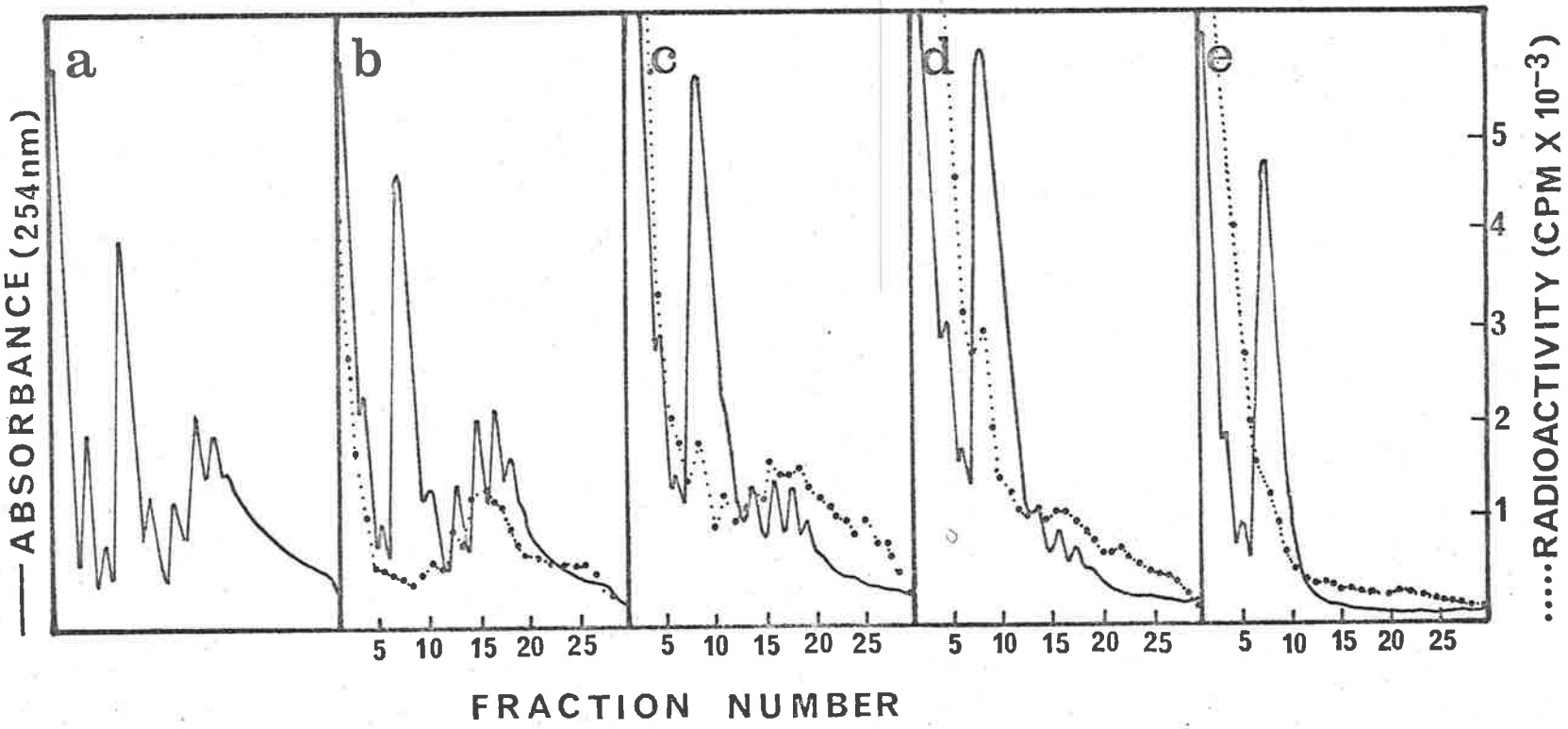
(a) unincubated control

(b) incubated 2 min.

(c) incubated 5 min.

(d) incubated 10 min.

(e) incubated 20 min.


a
b
c
d
e
—ABSORBANCE (254nm)
.....RADIOACTIVITY (CPM X 10⁻³)
1
2
3
4
5
5 10 15 20 25
FRACTION NUMBER

system for 2 minutes at 37°, polysome breakdown was already apparent as a decrease in the polysome region greater than pentamers (Fig. 4.5b). Furthermore, the total amount of ribosomes in polysomes had decreased to 52%. The nascent protein chains were labelled and radioactivity representing completed released chains was present at the top of the gradient. By 5 minutes (Fig. 4.5c) polysome breakdown was well-advanced with only 30% of ribosomes present in the polysome region although nascent chains were maximally labelled at this stage. Few polysomes remained after 10 minutes incubation (Fig. 4.5d) and label on the nascent chains had decreased markedly. In fact, the maximum labelling of nascent chains at this time was over the monosome region of the gradient. By 20 minutes very little absorbance was detectable in the polysome region of the gradient and radioactivity was present only in the monosome region and as released chains at the top of the gradient (Fig. 4.5e).

5. Use of sulphydryl reagents in the preparation of the lysate extraction buffer

Fourteen-day feathers were collected as described (see Methods 4.B2) and equal aliquots were then placed in ice-cold MSB buffer, without sulphydryl reagent, with 4 mM DTT present or with 9 mM glutathione in the buffer. The feather lysate was prepared as described previously (see Methods 2.B2) except that the particular buffer used for the isolation was then used in all subsequent stages of the preparation. The activity of the three lysate preparations was compared in cell-free systems. Aliquots were removed at various time intervals for the determination

of acid precipitable radioactivity. The kinetics of incorporation were similar for all three preparations (Fig. 4.6). However, maximal activity was obtained in the presence of DTT. Least activity was obtained in the system prepared using MSB buffer alone.

To determine whether equal amounts of polysomes were released from the feathers under the three conditions, polysomes were pelleted from the lysate preparations and redissolved in water to determine their absorbance values. It is apparent from Table 4.1 that the amount of polysomes released in the presence of dithiothreitol, was twice that with MSB alone whereas glutathione only marginally increased the yield of polysomes.

It can be seen from a comparison of the absorbance ratios at 260 nm and 280 nm (Table 4.1) that the protein contamination of the polysome pellets was greatest in the preparation containing glutathione and least in the MSB preparation. The values of absorbance ratio obtained are very similar to the value obtained (1.85) for polysomes prepared from chick embryo brain (Bryan and Hayashi, 1973) and from homogenised whole embryos (1.83) by Morimoto et al. (1972a).

6. Nature of the protein products synthesised in the 14-day feather cell-free system

The results presented have indicated that the cell-free system prepared from 14-day feathers was useful for investigating protein synthesis in feathers under cell-free conditions. The major question then to be answered was to determine the nature of the products synthesised in the lysate system. A major part

FIGURE 4.6. THE EFFECT OF SULPHYDRYL REAGENTS ON THE ACTIVITY OF A 14-DAY FEATHER CELL-FREE SYSTEM PREPARED IN MSB.

Cell-free systems were prepared from equal volumes (0.25 ml) of feathers in MSB, MSB containing 4 mM DTT, or MSB containing 9 mM glutathione during the preparation of the lysate. Other details are as described in Figure 4.1.

▲—▲ MSB, 4 mM DTT

●—● MSB, 9 mM glutathione

□—□ MSB.

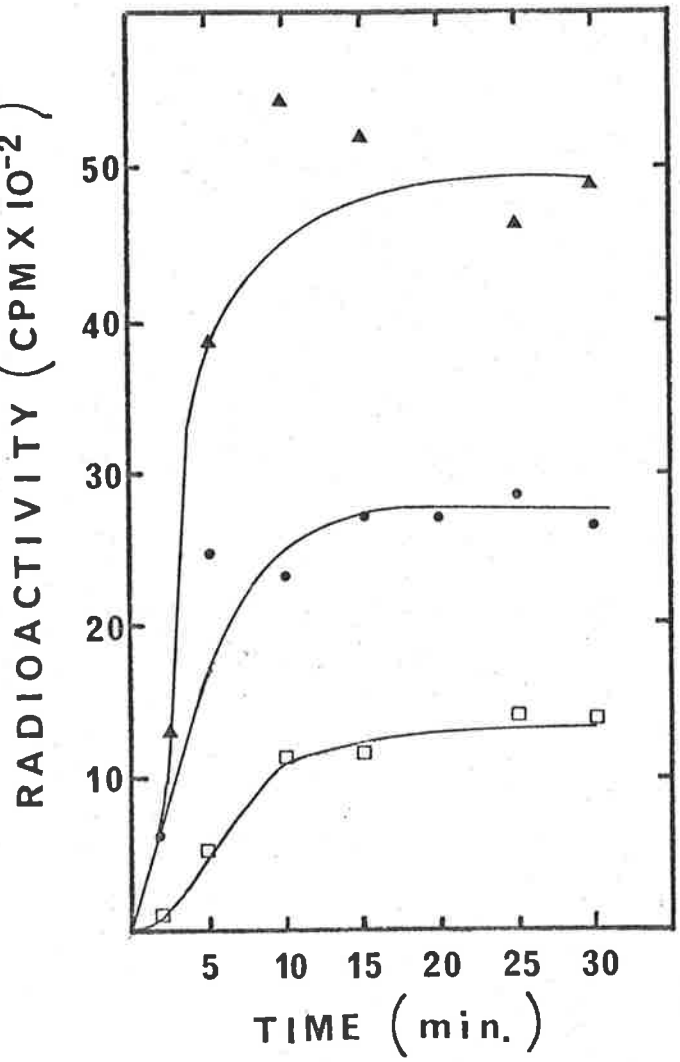

RADIOACTIVITY (CPM X 10⁻²)
50
40
30
20
10
5
10
15
20
25
30
TIME (min.)

TABLE 4.1. EFFECT OF THE PRESENCE OF SULPHYDRYL REAGENTS DURING THE PREPARATION OF FEATHER LYSATE ON THE YIELD OF POLYSOMES OBTAINED.

14-day feather lysate prepared in MSB, in MSB containing 4 mM DTT, or in MSB containing 9 mM glutathione. The polysomes were isolated by centrifugation from equal volumes of each preparation, dissolved in H_2O, and the absorbance at 260 nm and 280 nm recorded.

	A_{260}	A_{280}	A_{260}/A_{280}
MSB	0.309	0.159	1.94
MSB + 9 mM glutathione	0.381	0.215	1.77
MSB + 4 mM DTT	0.626	0.331	1.89

of the characterisation of the protein products was performed by Dr. D.J. Kemp of this Department. The results will be described here only in brief. For a full description, refer to Kemp (1972).

(a) Polyacrylamide gel electrophoresis of in vitro synthesised protein

Cell-free systems were incubated with ^{14}C-leucine, ^{3}H-serine or ^{14}C-algal hydrolysate respectively to label the synthesised products. The post-ribosomal supernatants were reduced and S-carboxymethylated as described (see Methods 4.B4). Aliquots of the freeze-dried preparations were mixed with native SCM-keratin and subjected to electrophoresis on soluble (ethylene diacrylate crosslinked) polyacrylamide gels at pH 7.5. The absorbance profiles and radioactivity profiles are shown (Fig. 4.7a-c). The absorbance in the keratin region of such gels was contributed almost entirely by the added carrier protein, whereas the absorbance in the low mobility regions was contributed by proteins in the lysate samples.

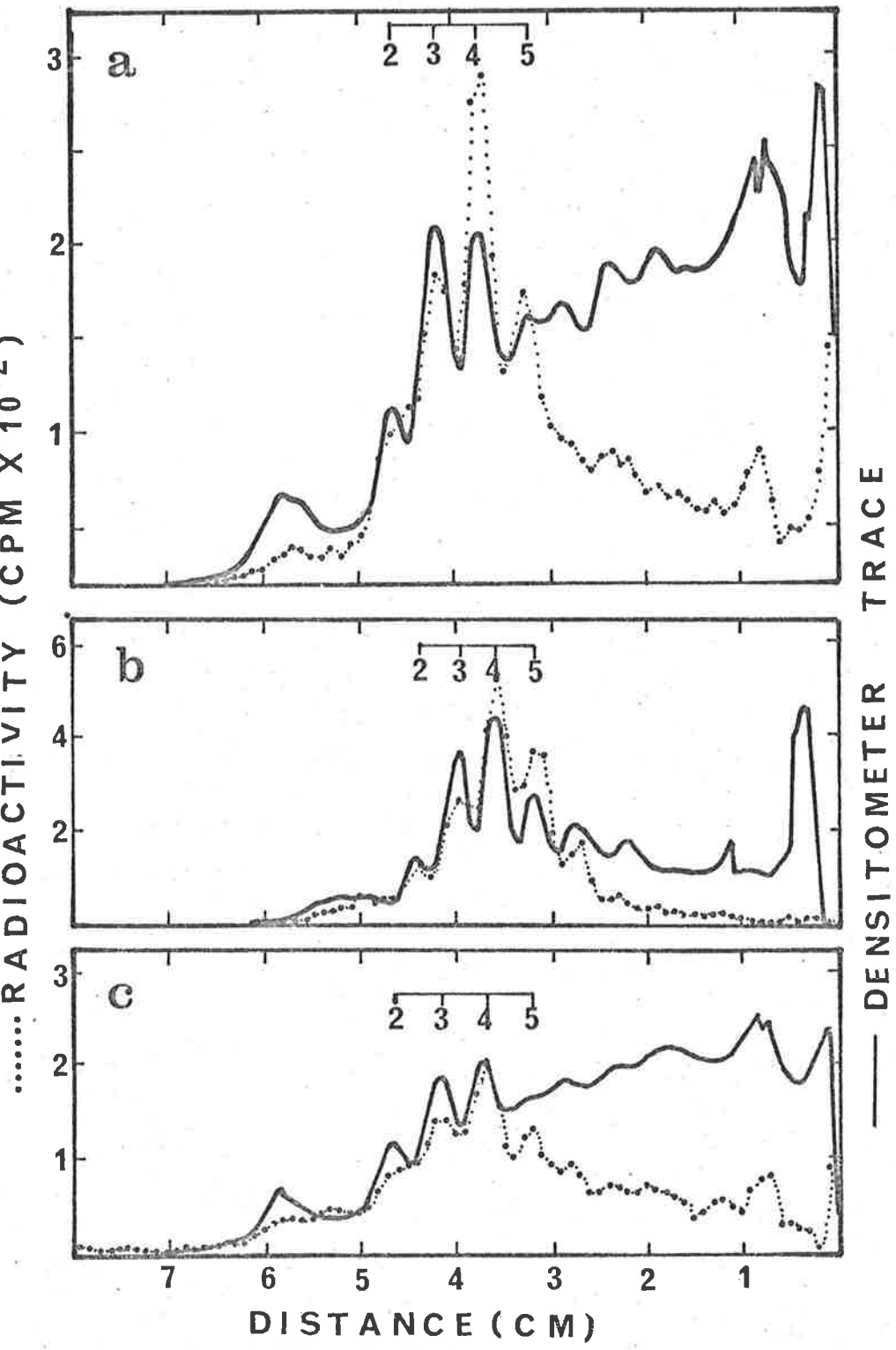
The mobility of the major peaks of radioactivity coincided precisely with the keratin bands β2-β5 (of Kemp and Rogers, 1972) for material labelled with ^{14}C-leucine, ^{3}H-serine or ^{14}C-algal hydrolysate. Some radioactivity co-electrophoresed with the α and γ proteins. The results show that the major protein products synthesised in the cell-free system were the keratin bands β2-β5.

FIGURE 4.7. POLYACRYLAMIDE GEL ELECTROPHORESIS AT pH 7.5 OF LABELLED PROTEINS SYNTHESISED IN A 14-DAY FEATHER CELL-FREE SYSTEM PREPARED IN MSB.

After reduction and carboxymethylation on the polysomal-supernatant fraction an aliquot was mixed with 100 µg of 21-day embryonic feather keratin and subjected to electrophoresis on 8 cm soluble 7.5% polyacrylamide gels in 4 M urea. Electrophoresis was performed at 2 mA/gel until the bromophenol blue tracker dye band just reached the bottom of the gels. Bands were stained with Coomassie Blue, and scanned on a 'Densicord' densitometer. Keratin bands β2 - β5 are indicated.
(a) The cell-free system (1.0 ml) was incubated at 37° for 45 min with 5 µC of ^{14}C-leucine (S. Act. 311 mC/mM). One third of the total product was loaded.

(b) The cell-free system (4.0 ml) was prepared from dialysed lysate and incubated for 1 hr. with 25 µC of ^{3}H-serine (S. Act. 1.2 C/mM). One fifteenth of the total was loaded.

(c) The cell-free system(1.0 ml) was incubated for 1 hour with 10 µC of ^{14}C-algal hydrolysate. One third of the total was loaded.


a
b
c
2 3 4 5
3
2
1
6
4
2
7 6 5 4 3 2 1
DISTANCE (CM)
.....RADIOACTIVITY (CPM X 10^{-2})
—— DENSITOMETER TRACE

(b) Polyacrylamide gel electrophoresis at pH 2.7

Co-electrophoresis of the *in vitro* synthesised proteins labelled with ^{14}C-leucine and embryonic feather keratin on polyacrylamide gels at pH 2.7 is shown in Fig. 4.8. In this system a totally different fractionation of keratin chains is obtained (Kemp and Rogers, 1972). Again the radioactivity profile corresponded closely to the absorbance profile of the unlabelled feather keratin confirming that the major protein products synthesised *in vitro* were keratin chains in bands A and B (of Kemp and Rogers, 1972).

(c) Co-chromatography of *in vitro* synthesised protein with chick embryo feather keratin on DEAE-cellulose and Sephadex G100

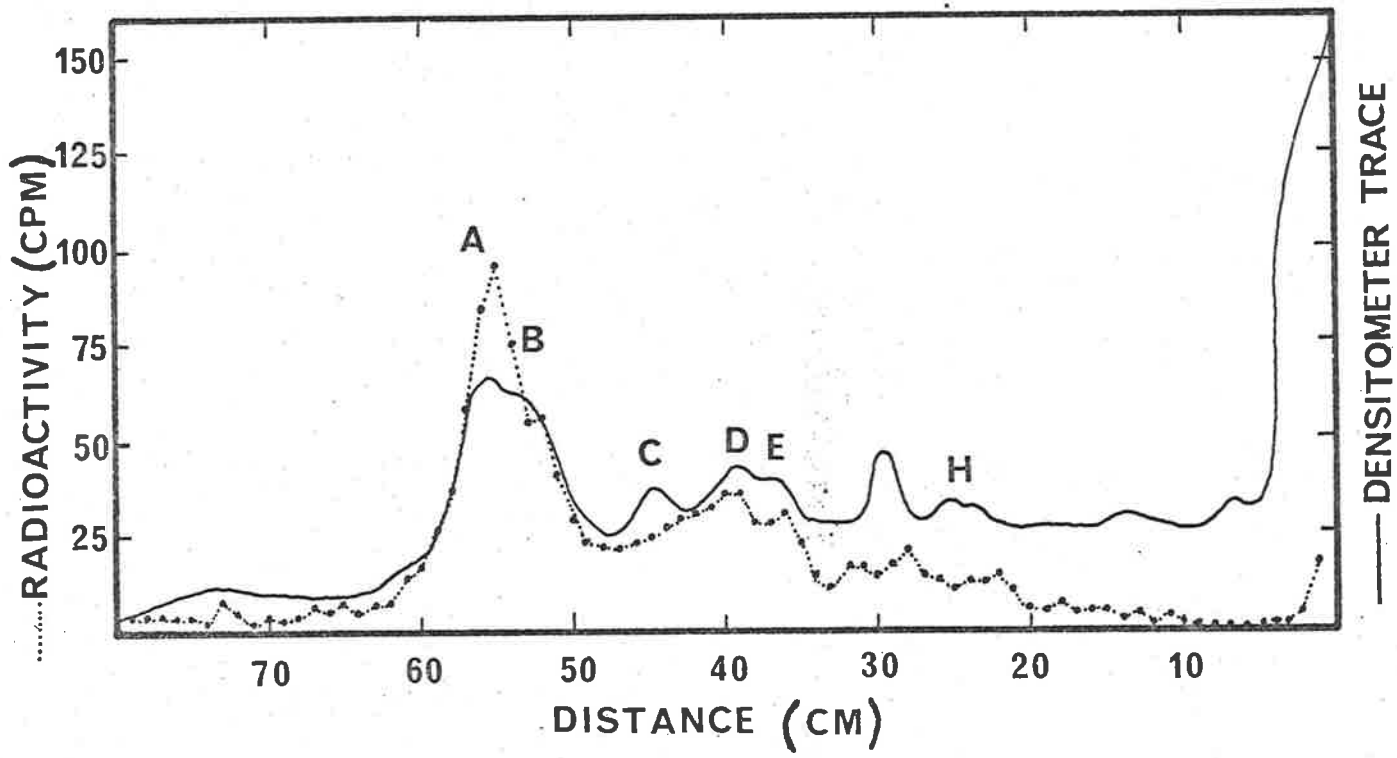
Protein labelled *in vitro* with ^{3}H-serine was co-chromatographed on DEAE-cellulose with 100 mg of embryonic feather keratin. The major peak of radioactivity co-chromatographed with the peak of absorbance contributed by the major keratin proteins. Lower peaks of radioactivity corresponded to absorbance peaks known to be the α and γ keratin proteins (Kemp, 1972; Walker, 1974).

The major peak fractions of ^{3}H-serine labelled protein from the DEAE-cellulose column were pooled, dialysed against distilled water and freeze-dried. A sample (5 mg) of this material was then analysed by chromatography on Sephadex G-100. The major peak of radioactivity precisely co-chromatographed with the peak of absorbance corresponding to the major keratin proteins.

FIGURE 4.8. POLYACRYLAMIDE GEL ELECTROPHORESIS AT pH 2.7 OF LABELLED PROTEINS SYNTHESISED IN A 14-DAY FEATHER CELL-FREE SYSTEM PREPARED IN MSB.

Details of the procedures are as given in Figure 4.7a except that the fractionation was by electrophoresis on a 10% soluble polyacrylamide gel in 2 M urea at pH 2.7.

Keratin bands A-H are indicated.


150
125
100
75
50
25
RADIOACTIVITY (CPM)
A
B
C
D
E
H
DENSITOMETER TRACE
70
60
50
40
30
20
10
DISTANCE (CM)

(d) Isolation of the N-terminal dipeptide from in vitro synthesised keratin

It was suggested by the studies using inhibitors of initiation of proteins synthesis that de novo synthesis of keratin was occurring in feather lysate. To confirm this a cell-free system was labelled with ^{3}H-serine, under which conditions a proportion of the N-terminal serine residues of the in vitro synthesised feather keratin chains should contain labelled serine. To test this prediction the in vitro labelled proteins were mixed with unlabelled feather keratin and digested with trypsin, chymotrypsin and pronase. The N-terminal peptide was then isolated by Dowex-50 chromatography. Fractionation of the Dowex-50 eluate by chromatography on Dowex-1 revealed one major peak of radioactivity, co-eluting with Ac-Ser-SCMC. This dipeptide has been shown to be the N-terminal dipeptide of most or all of the embryonic feather keratins (Kemp,1972).

The percentage of chains initiated in the cell-free system was estimated by determining the specific activity of serine in the undigested protein and in the N-terminal dipeptide. From these values it was calculated that about 50% of the chains were synthesised de novo (Kemp, 1972).

(e) Characterisation of proteins labelled by incubation of the cell-free system in the presence of ^{14}C-acetyl-CoA

Since the mechanism for N-terminal acetylation of keratin was apparently active in the 14-day feather cell-free system some aspects of the mechanism were investigated. It was found

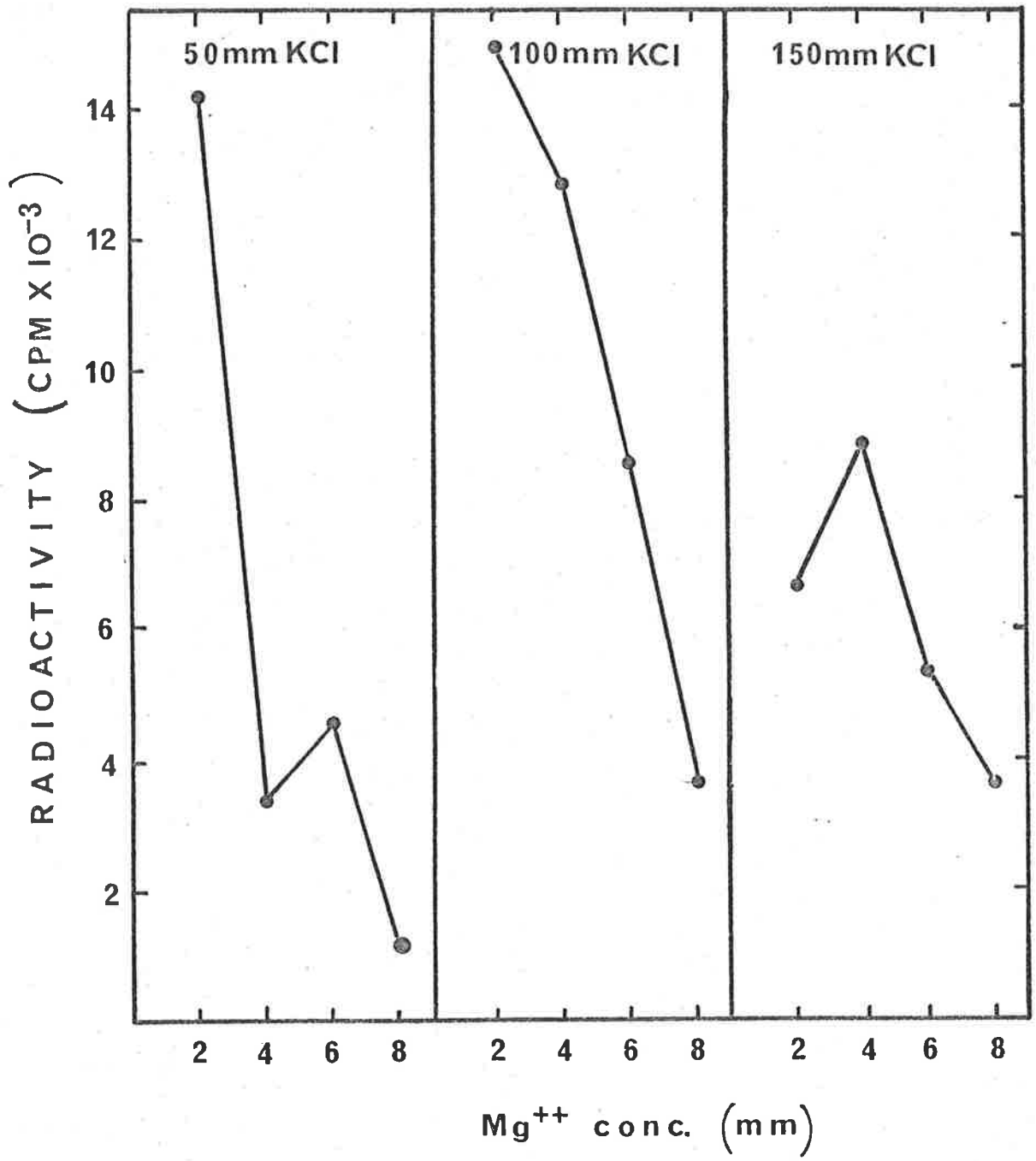
that incubation of a feather cell-free system with ^{14}C-acetyl-CoA resulted in the incorporation of label into acid-precipitable material. Accordingly, the post-ribosomal supernatant from a cell-free system incubated with ^{14}C-acetyl-CoA was prepared for fractionation by polyacrylamide gel electrophoresis at pH 7.5. It was found that the main peaks of radioactivity co-electrophoresed with the carrier keratin bands β2-β5. Much of the label however, was associated with peaks of lower mobility.

7. Determination of the ionic conditions for optimal activity of the cell-free system prepared from 14-day feather lysate

To determine the ionic conditions for optimal activity of the feather lysate cell-free system lysates were prepared in a low salt buffer (LSB) or in MSB. Equal volumes of packed feathers were used for each preparation (Methods 4.B2). Cell-free systems were prepared from the lysates with concentrations of KCl of 50 mM, 100 mM and 150 mM. At each of the three concentrations of KCl, the $MgCl_2$ concentration was varied from 2 mM to 8 mM, in increments of 2 mM. The cell-free systems were then incubated at 37°, and aliquots were removed after 30 min of incubation to assay the cpm incorporated into acid-precipitable protein. The results in Fig. 4.9, clearly show that maximal activity of the system was obtained in buffers of low ionic strength as compared to that of higher ionic strength (> 100 mM KCl). At either 50 mM KCl or 100 mM KCl the activity of the system decreased rapidly when the $MgCl_2$

FIGURE 4.9. DETERMINATION OF THE IONIC CONDITIONS FOR OPTIMAL ACTIVITY OF THE CELL-FREE SYSTEM PREPARED FROM 14-DAY FEATHER LYSATE

Feather lysate was prepared in either LSB or MSB and adjusted to various KCl and $MgCl_2$ concs. Each cell-free system (0.5 ml) was incubated at 37^{o} with 0.5 µC of ^{14}C-leucine. The results shown on the figure represent the total cpm incorporated into a 0.05 ml aliquot after 30 min. Each point represents the average value obtained from three separate experiments.


50mm KCl
100mm KCl
150mm KCl
RADIOACTIVITY (CPM X IO^{-3})
14
12
10
8
6
4
2
2 4 6 8
2 4 6 8
2 4 6 8
Mg^{++} conc. (mm)

concentration was greater than 2 mM. In contrast, maximal activity of the system in the presence of 150 mM KCl was obtained with a $MgCl_2$ concentration of 4 mM. However, it should be noted that the maximal activity obtained at the two lower K^+ concentrations was approximately double the maximal activity obtained in the presence of 150 mM KCl.

8. Effect of inhibitors of initiation on protein synthesis in low ionic strength buffer, LSB

To determine to what extent de novo protein synthesis was occurring in LSB, a cell-free system was incubated at 37° in the presence of the inhibitors of initiation, ATA and NaF, in an analogous manner to that described previously for MSB. The results are shown in Fig. 4.10. In the presence of either of the inhibitors of initiation, the initial rate of incorporation was unaffected, but protein synthesis was reduced by greater than 80%. In fact the total inhibition would be greater than this because the control had not reached maximal incorporation at the time the last sample was taken. Chain initiation is obviously much more efficient under these conditions than in MSB.

9. Relation of polysome profiles to protein synthesis in the low ionic strength buffer, LSB.

To determine whether the rate of polysome breakdown correlated with the enhanced rate of protein synthesis in LSB as compared to MSB an unlabelled feather cell-free system, was incubated at 37° in LSB. Aliquots of the reaction mixture were

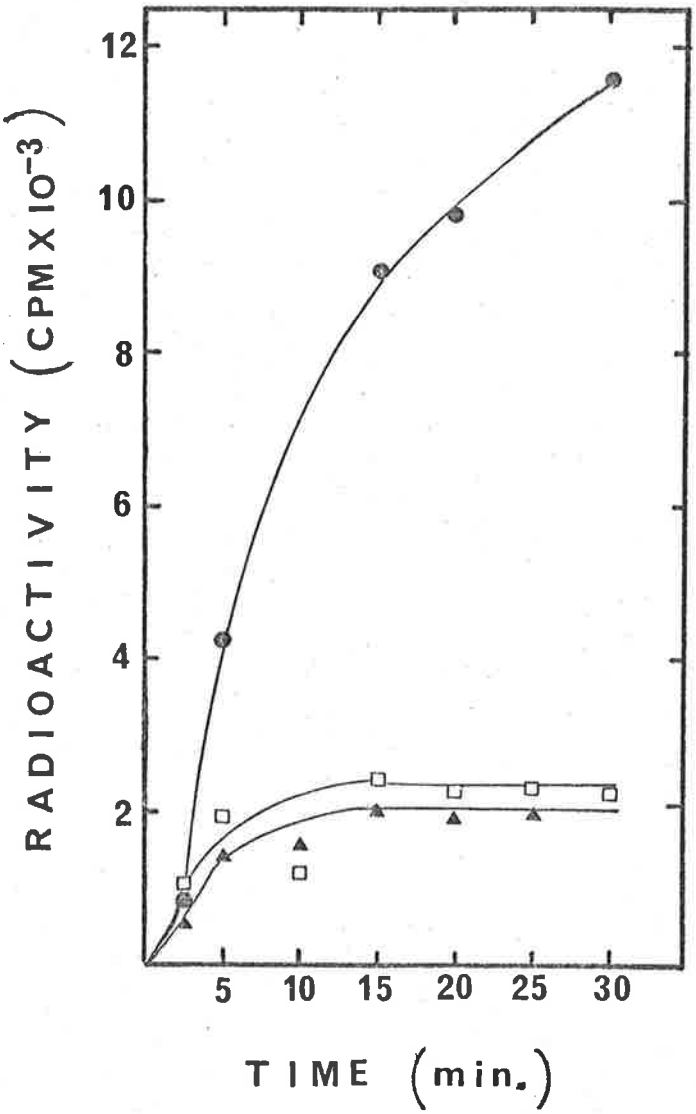
FIGURE 4.10. EFFECT OF INHIBITORS OF INITIATION ON PROTEIN SYNTHESIS IN A 14-DAY CELL-FREE SYSTEM PREPARED IN LSB.

Other details are as given in Figure 4.1.

●—● control

□—□ ATA (1×10^{-4} M)

▲—▲ NaF (1×10^{-2} M).


RADIOACTIVITY (CPM X 10⁻³)
12
10
8
6
4
2
5
10
15
20
25
30
TIME (min.)

removed at various time intervals for analysis of the polysome profiles by sucrose-gradient centrifugation. As expected the rate of polysome disaggregation was much slower under these conditions than in MSB (Fig. 4.11a-e). No significant polysome disaggregation had occurred after 5 minutes incubation, and polysomes consisting of trimers to pentamers were still present after 10 minutes incubation. By 20 minutes the proportion of ribosomes present in polysomal aggregates had decreased, although some large polysomes were still evident.

Aliquots of the same cell-free system were used for measuring the kinetics of incorporation into TCA-precipitable material both at 25° and 37°. The kinetics of incorporation are shown in Fig. 4.12. Two facts should be noted - (a) the rate of incorporation at 37° correlates well with the rate of polysome disaggregation, as shown in Fig. 4.11a-e; (b) the rate of incorporation at 25° was slower than at 37° and the maximal incorporation obtained was somewhat lower.

10. Synthesis of keratin in cell-free systems prepared in low (LSB) and medium salt buffer conditions (MSB)

To determine whether there was any significant difference in the relative amounts of label incorporated into the different keratin bands synthesised in low salt buffer (LSB) and medium salt buffer (MSB), lysates were prepared from equal volumes of 14-day feathers, and the cell-free systems incubated with ^{14}C-leucine to label the *in vitro* synthesised proteins. Aliquots of the reduced and carboxymethylated post-ribosomal supernatants were co-electrophoresed with SCM-feather keratin on polyacrylamide

FIGURE 4.11. ABSORBANCE PROFILES OF POLYSOMES IN A FEATHER CELL-FREE SYSTEM PREPARED IN LSB AND INCUBATED AT 37°.

Details as given (see Methods 4.B.6) except that LSB was used, and no labelled amino acid was added.

(a) unincubated control

(b) incubated 2 min.

(c) incubated 5 min.

(d) incubated 10 min.

(e) incubated 20 min.

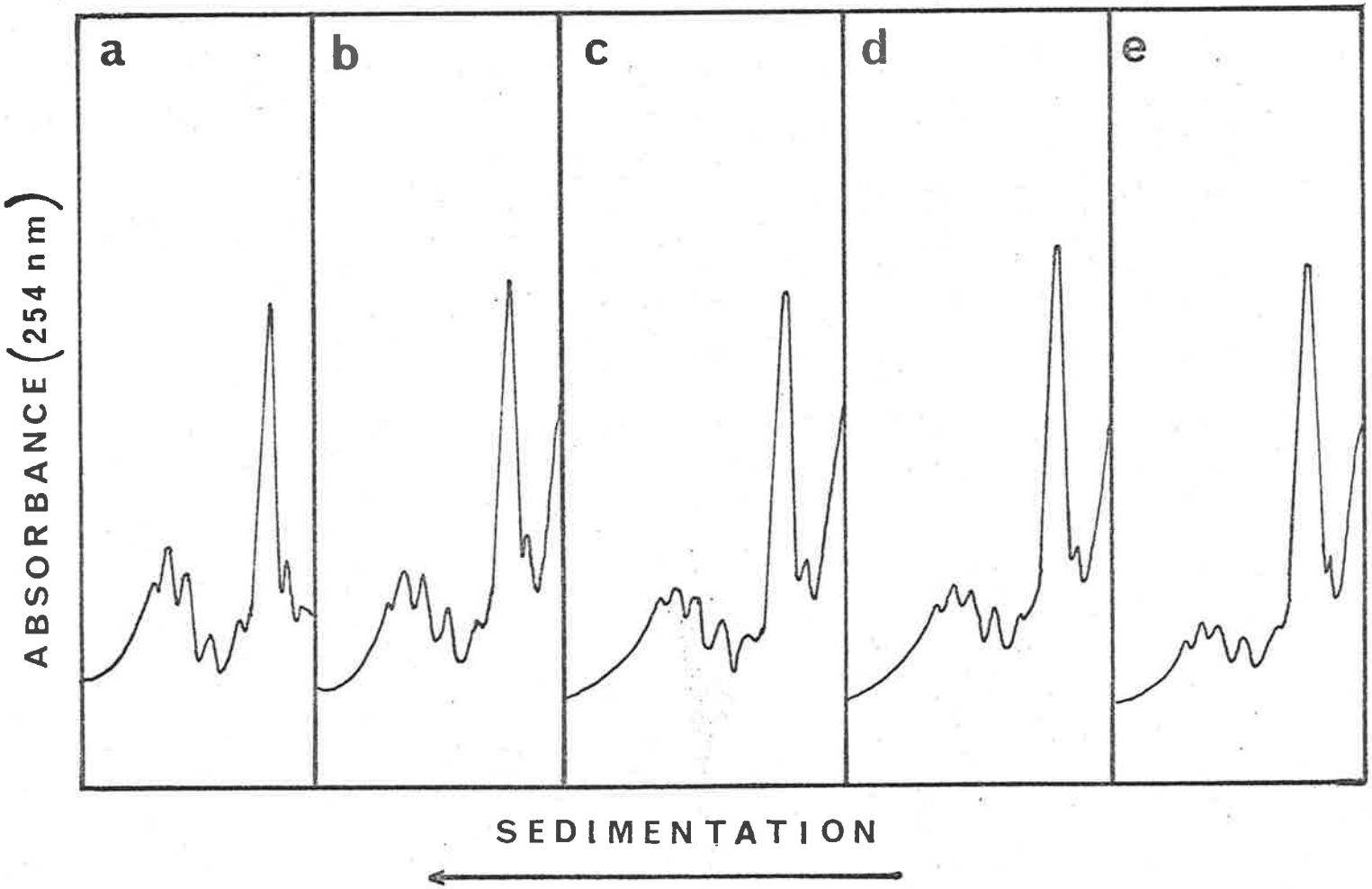
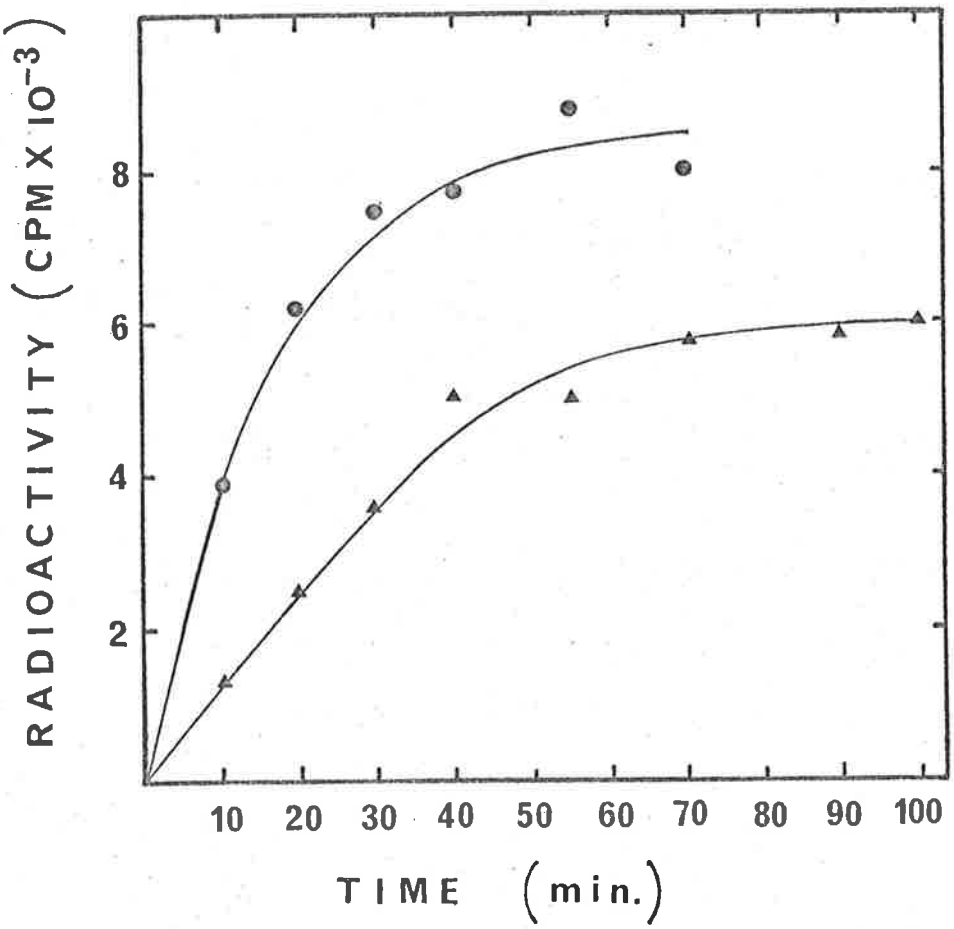

a
b
c
d
e
ABSORBANCE (254 nm)
SEDIMENTATION

FIGURE 4.12. COMPARISON OF KINETICS OF AMINO ACID INCORPORATION OF A 14-DAY FEATHER CELL-FREE SYSTEM PREPARED IN LSB AND INCUBATED AT 25^{o} and 37^{o}.

The cell-free system (0.5 ml) was prepared and incubated at 25^{o} and 37^{o} with 0.5 µC of ^{14}C-leucine (S. Act. 316 mC/mM). Aliquots (0.05 ml) were removed at 0, 10, 20, 30, 40, 55, 70 min. for system incubated at 25^{o} and 37^{o}, but two additional aliquots removed at 90 and 100 min for system incubated at 25^{o}. Radioactivity represents cpm incorporated per 0.05 ml aliquot.

▲—▲ 25^{o}

●—● 37^{o}


RADIOACTIVITY (CPM X IO^{-3})
2
4
6
8
10
20
30
40
50
60
70
80
90
100
TIME (min.)

gels at pH 7.5. The radioactivity profile of the gels are shown in Fig. 4.13a,b. Under both ionic conditions the relative ratios of radioactivity of the keratin bands β2-β5(of Kemp and Rogers, 1972) were very similar.

11. Comparison of protein synthesis activity in cell-free systems prepared from 12, 13 and 15-day feathers with the activity of the 14-day system

(a) Protein synthesis in 15-day feather cell free systems.

When the same volumes of tissue were used in the preparation of 15-day lysates (in MSB) as for 14-day lysates (in MSB also) the maximal activity and kinetics of incorporation were found to be very similar (results not shown). To compare the protein products of the 15-day lysate with those of 14-day lysate a cell-free system was prepared from 15-day feathers and incubated with ^{14}C-leucine as label. The radioactivity and absorbance profiles of a pH 7.5 polyacrylamide gel of the co-electrophoresis of an aliquot of labelled protein from the post-ribosomal supernatant with SCM-keratin is shown (Fig. 4.14). The major product synthesised was the keratin proteins, namely bands β2-β5.

(b) Protein synthesis in 13-day feather cell-free systems

The extent of protein synthesis in terms of the total incorporation of label by the cell-free systems derived from equivalent volumes of embryonic feathers was found to be variable in 13-day feather preparations. Presumably this was

FIGURE 4.13. POLYACRYLAMIDE GEL ELECTROPHORESIS AT pH 7.5 OF LABELLED PROTEINS SYNTHESISED IN 14-DAY FEATHER CELL-FREE SYSTEM PREPARED IN LSB OR MSB.

Other details as given in Figure 4.7, except that the gels were not stained.

FIGURE 4.13a. The cell-free system (0.25 ml) was prepared in LSB and incubated at 37^{o} for 1 hour with 0.5 µC of ^{14}C-leucine (S. Act. 312 mC/mM). One quarter of the total was loaded.

FIGURE 4.13b. As for Figure 4.13a except that the cell-free system was prepared in MSB. One half of the total was loaded.

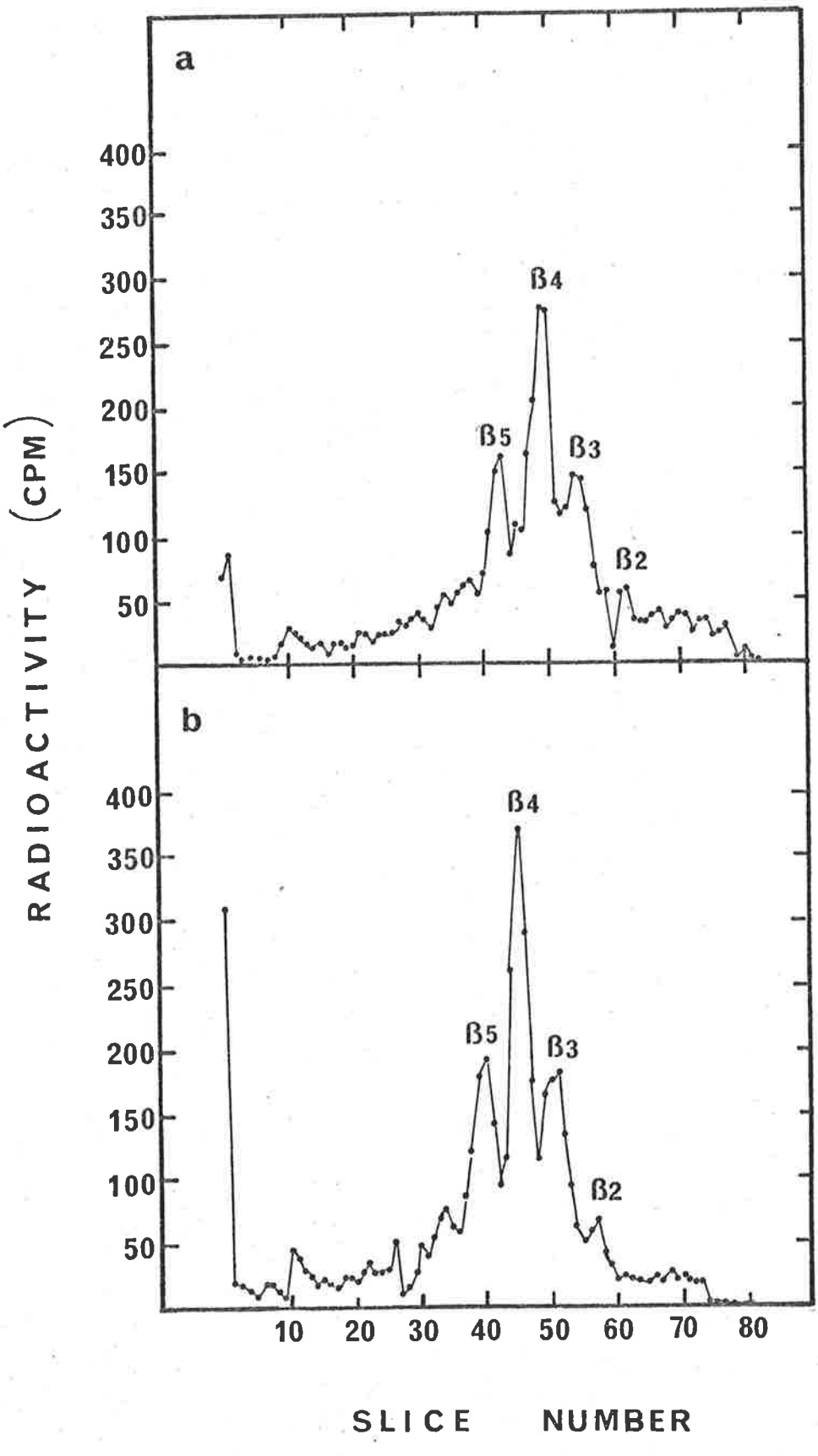
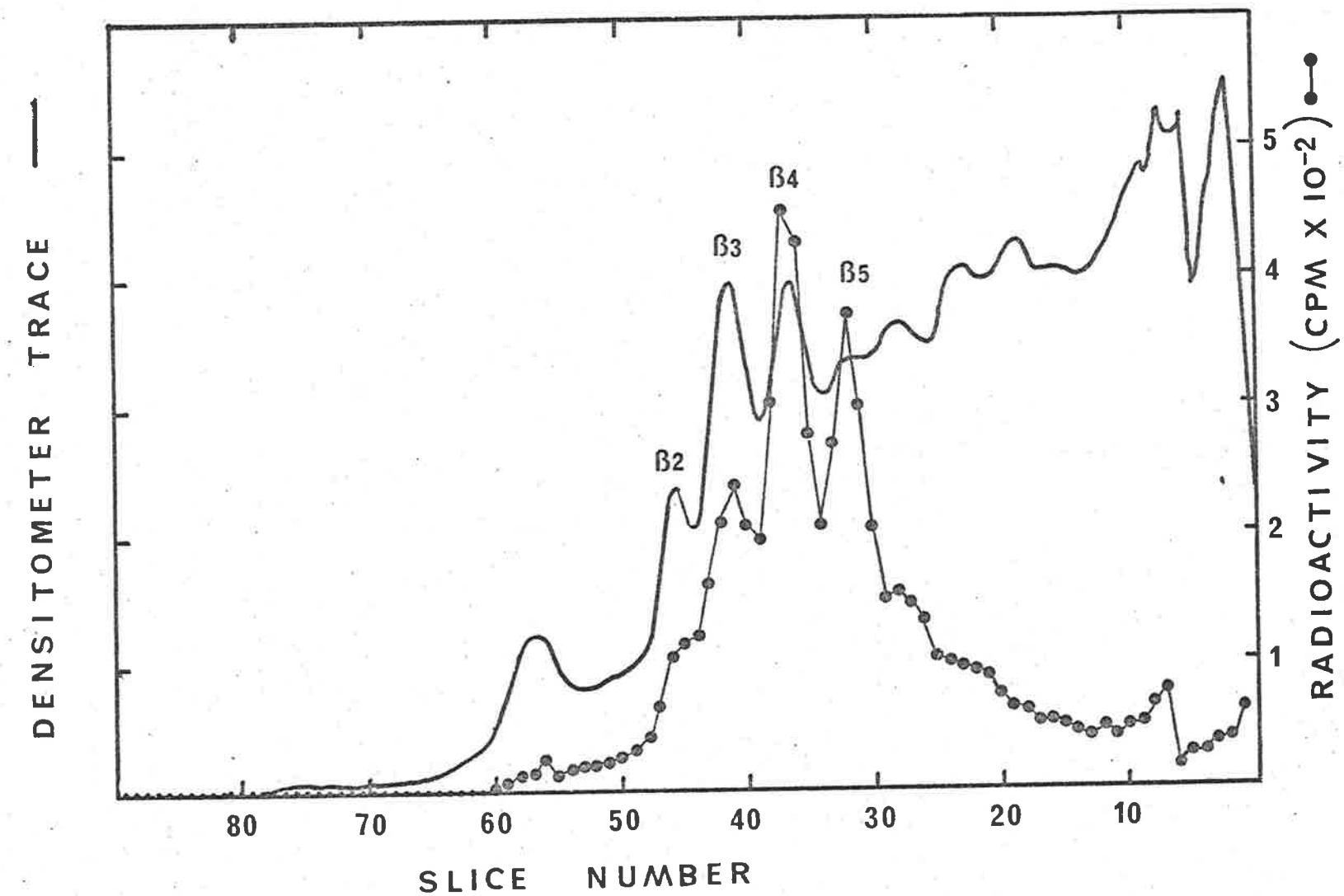

a
b
ß4
ß5
ß3
ß2
400
350
300
250
200
150
100
50
10
20
30
40
50
60
70
80
RADIOACTIVITY (CPM)
SLICE NUMBER

FIGURE 4.14. POLYACRYLAMIDE GEL ELECTROPHORESIS AT pH 7.5 OF LABELLED PROTEINS SYNTHESISED IN A 15-DAY FEATHER CELL-FREE SYSTEM PREPARED IN MSB.

Other details are as given in Figure 4.7. The cell-free system (1.0 ml) was incubated at 37^{o} for 1 hour with 5 µC of ^{14}C-leucine (S. Act. 311 mC/mM). One-third of the total was loaded.


DENSITOMETER TRACE
RADIOACTIVITY (CPM X 10⁻²)
ß2
ß3
ß4
ß5
80
70
60
50
40
30
20
10
SLICE NUMBER
1
2
3
4
5

caused by the variability of embryonic development as discussed previously (see Results 3.B4). However, the kinetics of incorporation and extent of incorporation and extent of inhibition by specific inhibitors of initiation was similar to that found in 14-day lysates. The nature of the protein products synthesised in the 13-day cell-free systems was investigated by analysis on polyacrylamide gels at pH 7.5 as described previously. The radioactivity and absorbance profile of a gel is shown in Fig. 4.15. It can be seen that the major products of the cell-free system were the keratin proteins of bands $\beta 2$-$\beta 5$.

(c) Protein synthesis in 12-day feather cell-free systems

Preliminary experiments using MSB for preparation of the 12-day lysates indicated that on the basis of the same packed feather volume, the capacity for protein synthesis of 12-day feathers was very limited when compared to 14-day feathers. This result was obtained using either ^{14}C-leucine or ^{14}C-serine as label. In an attempt to increase the extent of labelling the 12-day lysate was dialysed, as described for 14-day lysate (see Methods 4.B3) to lower the endogenous amino acid pool. The kinetics of incorporation are shown in Fig. 4.16 and it can be seen that they are very similar to that of 14-day feather lysate. The level of incorporation of ^{14}C-leucine or ^{14}C-serine was increased four-fold after dialysis of the lysate. The activity of both the dialysed and lysates was reduced by about 50% in the presence of NaF, an inhibitor of

FIGURE 4.15. POLYACRYLAMIDE GEL ELECTROPHORESIS AT pH 7.5 OF LABELLED PROTEINS SYNTHESISED IN A 13-DAY FEATHER CELL-FREE SYSTEM PREPARED IN MSB.

Other details as in Figure 4.7. The cell-free system (0.5 ml) was incubated at 37° for 1 hour with 5 µC of ^{14}C-leucine (S. Act. 316 mC/mM). The total product was loaded.

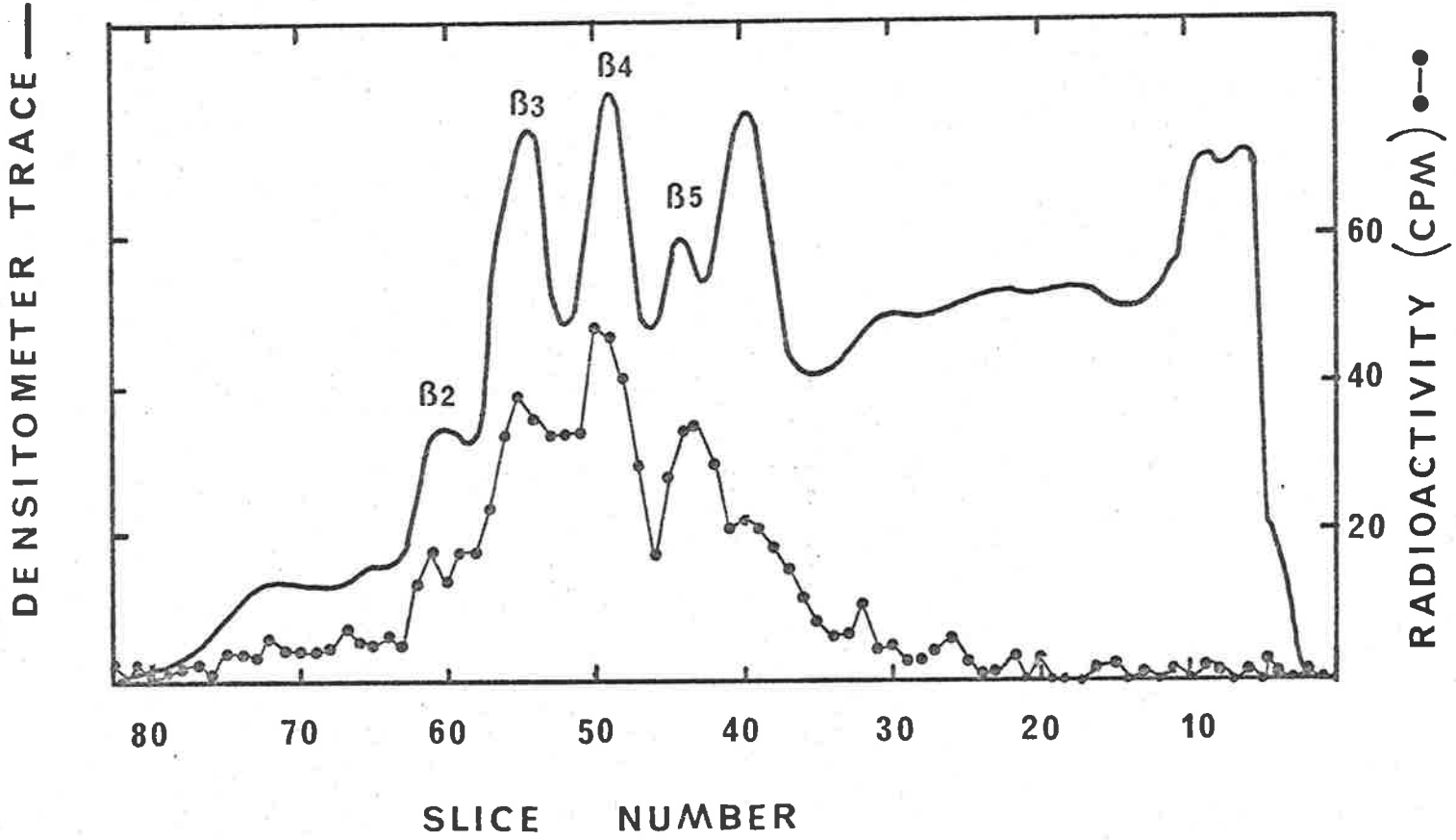

DENSITOMETER TRACE
RADIOACTIVITY (CPM)
ß2
ß3
ß4
ß5
60
40
20
80
70
60
50
40
30
20
10
SLICE NUMBER

FIGURE 4.16. KINETICS OF AMINO ACID INCORPORATION OF DIALYSED AND UNDIALYSED 12-DAY FEATHER CELL-FREE SYSTEM PREPARED IN MSB.

The dialysed and undialysed cell-free systems (0.5 ml) were incubated at 37^{o} in the presence of either 1 μC of ^{14}C-leucine (S. Act. 316 mC/mM) or 1 μC of ^{14}C-ser (S. Act. 316 mC/mM). Aliquots were removed at 0, 5, 10, 15, 20, 25, 30, 45, 60 min, for determination of acid precipitable radioactivity. Radioactivity represents cpm/0.05 ml aliquot.

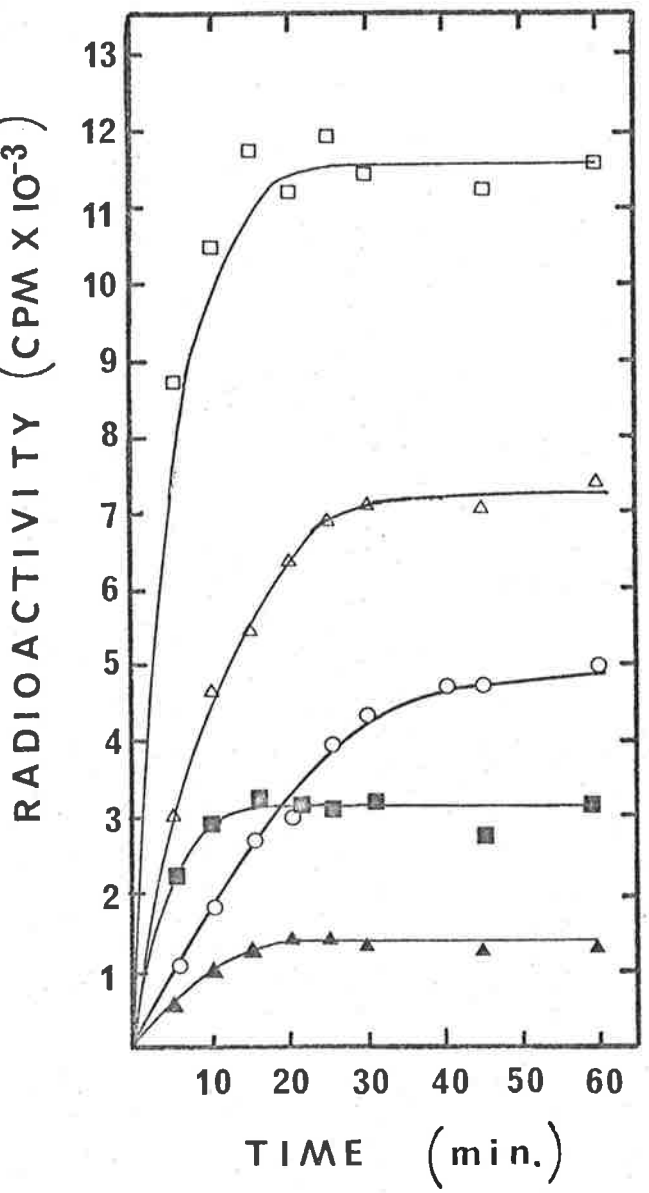
□—□ dialysed + ^{14}C-leucine

○—○ dialysed + ^{14}C-leucine + 10^{-2} M NaF

△—△ dialysed + ^{14}C-serine

■—■ undialysed + ^{14}C-leucine

▲—▲ undialysed + ^{14}C-serine.


RADIOACTIVITY (CPM X IO-3)
13
12
11
10
9
8
7
6
5
4
3
2
1
10
20
30
40
50
60
TIME (min.)

initiation. This value is very similar to the degree of inhibition of protein synthesis observed in undialysed 14-day feather lysate also prepared in MSB. Furthermore, this result indicates that no deleterious effect was produced by dialysing the lysate.

Nature of the proteins synthesised in the 12-day feather cell-free system

To determine whether keratin was synthesised in 12-day lysates, dialysed and undialysed lysates were incubated in two separate experiments with either ^{14}C-serine or ^{14}C-leucine. The post-ribosomal supernatants were prepared as described previously. Aliquots of these supernatants were co-electrophoresed with SCM-feather keratin on pH 7.5 polyacrylamide gels. The radioactivity and absorbance profiles of the gels are shown in Fig. 4.17b,c. Some difficulties were encountered in the running of such gels in that a large amount of unlabelled protein was present in the lysate. Consequently, comparison were made with a control gel run separately with carrier SCM-keratin alone. Indications of some keratin synthesis were observed when ^{14}C-serine was used as label but only in the dialysed lysate whereas no keratin synthesis was detectable when ^{14}C-leucine was used, even in a lysate dialysed to reduce the free amino acid pool. When ^{14}C-serine was used in the dialysed lysate the relative ratios of label incorporated into the keratin bands β2-β5 appeared to be very similar to that obtained for keratin synthesised by the dialysed 14-day feather lysate in the presence of ^{14}C-serine. Some extra bands of

FIGURE 4.17. POLYACRYLAMIDE GEL ELECTROPHORESIS AT pH 7.5 OF LABELLED PROTEINS SYNTHESISED IN DIALYSED AND UNDIALYSED 12-DAY FEATHER LYSATE PREPARED IN MSB.

Other details as given in Figure 4.7, except that the gels were scanned for absorbance at 630 nm using a Gilford linear transporter (see Methods 2.B6).

FIGURE 4.17a. Absorbance profile of 100 µg of 21-day embryonic chick feather keratin.

Keratin bands β2 - β5 are indicated.

FIGURE 4.17b. The cell-free system (0.5 ml) was prepared from either dialysed or undialysed lysate and incubated at 37^{o} for 1 hour with 0.5 µC of ^{14}C-serine (S. Act. 120 mC/mM). The total was loaded.

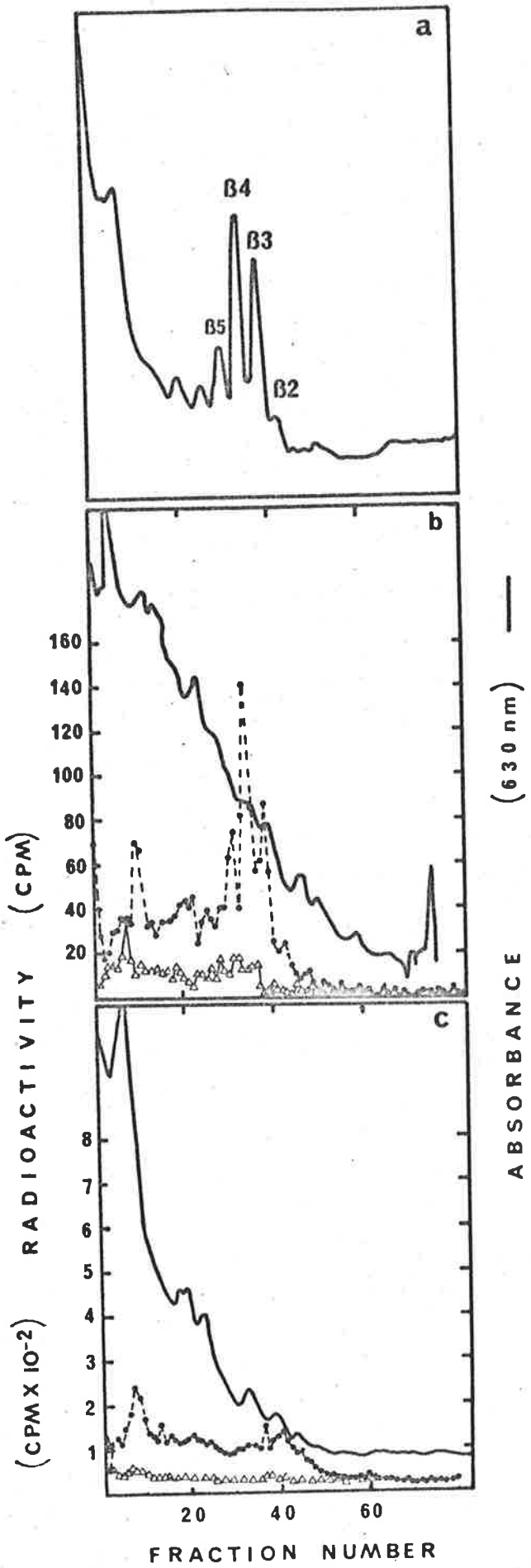
●—● dialysed

△—△ undialysed

FIGURE 4.17c. The cell-free system (0.5 ml) was prepared from either dialysed or undialysed lysate and incubated at 37^{o} with 0.5 µC of ^{14}C-leucine (S. Act. 316 mC/mM). The total was loaded.

●—● dialysed

△—△ undialysed


a
β4
β3
β5
β2
b
160
140
120
100
80
60
40
20
RADIOACTIVITY (CPM)
c
8
7
6
5
4
3
2
1
(CPM X 10⁻²)
20
40
60
FRACTION NUMBER
ABSORBANCE (630 nm)

labelled protein were detected in the lower mobility regions of the gel. The results demonstrate that the major protein products of the 12-day cell-free system are non-keratin proteins, with only traces of keratin being synthesised. It is clear that keratin synthesis can be detected provided that ^{14}C-serine is used in a dialysed lysate and not ^{14}C-leucine presumably because the content of this amino acid is higher than leucine in embryonic feather keratin (Kemp and Rogers, 1972).

The free amino acid concentration was determined (see Methods 4.B7,8) on equal volumes (0.5 ml) of dialysed and undialysed lysate. It can be seen (Table 4.2) that there was approximately a two-fold decrease in the concentration of serine during dialysis. Moreover, the pool of free leucine was negligible even in the undialysed lysate.

D. DISCUSSION

1. Kinetics of incorporation of the feather cell-free system prepared in MSB buffer

The kinetics of incorporation in the feather cell-free system, prepared in MSB buffer were found to be very similar to the kinetics of incorporation of rabbit reticulocyte lysate incubated at 37°, without the addition of exogenous haemin Furthermore, results obtained in the present studies were identical to those reported for reticulocyte lysate for example (Lamfrom and Knopf, 1964; Adamson *et al.*, 1968; Maxwell *et al.*, 1971; Balkow and Korner, 1971; Hunt *et al.*, 1972). The rate of breakdown of the polysomes incubated *in vitro* was found to

TABLE 4.2. FREE AMINO ACID CONCENTRATION IN DIALYSED AND UNDIALYSED 12-DAY FEATHER LYSATE.

The amino acid concentration was determined as described (see Methods 4.B.7 and 8) on equal volumes (0.5 ml) of dialysed and undialysed lysate.

	Dialysed	Undialysed
	conc. µm/per ml	
Aspartic acid	0.130	0.210
Serine	0.106	0.175
Glutamic acid	0.212	0.415
Proline	0.060	0.065
Glycine	0.164	0.245
Alanine	0.086	0.135

Other amino acids were only present in trace amounts.

correlate well with the protein synthetic activity of the system. Moreover, the complete disaggregation of the polysomes in an orderly manner suggested that all were active in protein synthesis. It should be noted, however, that there was no increase of ribosomes in the polysome fraction apparent at any stage during the incubation *in vitro*. This is to be contrasted to the results of Lamfrom and Knopf (1964) who observed an increase, during the eary stages of the incubation in the polysome fraction of rabbit reticulocyte lysate incubated at 37°. Nevertheless, the inhibition of incorporation by the inhibitors of initiation of protein synthesis together with the isolation of the labelled N-terminal dipeptide (N-acetyl-ser-SCMC) clearly demonstrated that initiation was occurring in the feather lysate. However, the extent of inhibition of protein synthesis in the feather lysate by inhibitors of initiation was far less than that obtained using the inhibitors in a rabbit reticulocyte cell-free system. *A priori*, there appeared to be no reason for the lower efficiency of initiation in the feather cell-free system as compared to that of the rabbit reticulocyte system, unless the *in vitro* conditions were not optimal and hence these were investigated.

2. Determination of the ionic conditions for optimal activity of the cell-free system prepared from 14-day feathers

The possibility that the concentrations of K^+ and Mg^{++} were not optimal were the first parameters to be investigated, as the concentrations of all other components necessary to support protein synthesis were identical to those in the lysates prepared from rabbit reticulocytes. It was found that the incorporation of labelled amino acid was increased by two-fold in the lower ionic strength buffers (i.e., 50 or 100 mM KCl and 2 mM $MgCl_2$) as compared to the incorporation obtained using MSB.

Furthermore, the extent of de novo protein synthesis in the lower ionic strength (LSB) conditions was equal to that observed in the reticulocyte cell-free system, as measured by the effect of the inhibitors of initiation on the level of incorporation.

The rate of polysome breakdown was assayed in a cell-free system under the same conditions, and again the rate of polysome breakdown was much slower than in the cell-free system prepared using MSB. In addition, some increase was apparent in the proportion of polysomes present during the incubation. That is, the rate of initiation must have exceeded the rate of translation resulting in an increase in the size of the polysomes (Lamfrom and Knopf, 1964).

The finding that the low ionic strength buffers were optimal for protein syntehsis in the feather cell-free system was somewhat surprising in view of the fact that buffers of moderately high ionic concentration have been routinely used in the preparation of cell-free systems from both adult and embryonic avian systems. For example, it was reported

(Jernigan et al., 1972; 1973) that the polysome yield from adult chicken liver was greater in a buffer of high ionic strength as compared to low ionic strength buffer. Likewise Lazarides and Lukens (1971) reported that concentrations of 150 mM KCl and 7.5 mM $MgCl_2$ were optimal in a reconstituted cell-free system prepared from chick embryo wings and legs. It should be noted that the concentrations of KCl and $MgCl_2$ chosen originally for the feather cell-free system were used in a chick embryo muscle cell-free system (Heywood adn Nwagwu, 1969) and determined to be optimal in a reconstituted muscle cell-free system prepared from salt-washed ribosomal subunits (Rourke and Heywood, 1972). Moreover, another line of evidence indicated that high concentrations of KCl should be optimal for cell-free systems. It has been shown (Valberg et.al. 1966; Lubin, 1967; Quissell and Suttie, 1973) for reticulocytes and a number of cell-lines that the intracellular K^+ concentration lies in the range 120 - 150 mM. Contrary to these lines of evidence the feather cell-free system was found to have optimal activity when prepared in buffers of low ionic strength. Likewise, Sarkar (1973) has reported that the highest amino acid incorporating activity was found when chick embryo muscle was homogenised in 80 mM KCl and not in the 250 mM KCl medium of Heywood et al. (1967). The results obtained in the present study may be explained by the finding (see Results 3.B3) that the ribosomal subunits behaved anomalously in MSB, as compared to LSB. In the presence of low concentrations of KCl the concentration of Mg^{++} for optimal activity of the system was found to be 2 mM. In contrast the optimal $MgCl_2$ at 150 mM KCl was 4 mM. This

finding can be related to the studies by Hultin et al., 1973, on the stabilisation of ribosomes by Mg^{++}. These authors found that at low concentrations of K^{+}, concentrations of Mg^{++} above 1 - 1.5 mM markedly reduced the structural and functional stability of the ribosomes. With increasing K^{+} concentration the Mg^{++} concentration needed to stabilise the ribosomes increased also.

In the same context, Manchester (1970) reported that the free concentration of Mg^{++} in solution is far below the total Mg^{++} concentration when the binding by nucleotides and binding by Cl^{-} is taken into account. The binding by Cl^{-} becomes significant when the solution is of the order of 100 mM, and greater with respect to KCl concentration.

3. Use of sulphydryl reagents in the preparation of feather lysate

It has been standard practice to include a reducing agent, usually 2-mercaptoethanol in the preparation of cell-free systems. It has been less common, however, to include a sulphydryl reagent in the buffer used to prepare the lysate. It was recognised that it could be of advantage to include a sulphydryl reagent in the preparation of lysate from chick feathers to prevent aggregation of the cysteine-rich keratins (Kemp and Rogers, 1972). Furthermore, it had been shown (Steinert and Rogers, 1971a) that addition of dithiothreitol (DTT) maximised the yield of polysomes extracted from wool follicles. It should be noted that use of 2-mercaptoethanol was avoided in the present work because recent reports indicate that it is

inhibitory to cell-free protein synthesis (Maxwell et al., 1971; Adamson et al., 1971; Hunt et al., 1972).

The rates of incorporation in a feather cell-free system prepared in MSB containing DTT was greater (measured on a packed feather volume basis) than a system prepared with either glutathione or with MSB alone. However, when the yield of polysomes, determined as mg per ml of packed feathers was determined it was clear that while less polysomes were liberated in the presence of glutathione their activity was greater than those released in the presence of DTT. A similar finding was reported by Huston et al. (1970) for the preparation of lysates from rat liver. These authors found that the presence of glutathione in the extraction buffer enhanced the activity of the cell-free system whereas DTT had essentially no effect.

4. A comparison of incorporation in feather cell-free systems incubated in either MSB or LSB at 25° and 37°

No stimulation of incorporation was observed in a cell-free system incubated at 25°, in the presence of MSB or LSB, as compared to the incorporation at 37°. As expected, the rate of incorporation at 25° was somewhat lower than that at 37°. This is to be contrasted with the results obtained in the reticulocyte cell-free system, incubated without exogenous haemin. In this system it has been reported that the total incorporation at 25° is significantly greater than at 37°. (Adamson et al., 1968; Maxwell et al., 1971; Gross and Rabinovitz, 1973; Hunt et al., 1972). This effect has been attributed (Gross and Rabinowitz, 1973) to the rapid formation of an

inhibitor of chain initiation at 34°, but which forms slowly at 25°. It has been postulated that there is a temperature dependent conversion of a proinhibitor to an irreversible inhibitor. This conversion is slow at 25° but occurs rapidly at 34°. The proinhibitor is inactive at 25° but not at 34°. Furthermore, the proinhibitor can be inactivated and its conversion to irreversible inhibitor delayed by addition of exogenous haemin to a cell-free system incubated at 34°.

The role of haemin in stimulating protein synthesis is at present unresolved. Several reports have indicated that haemin not only stimulates the synthesis of globin in reticulocyte lysate but also the translation of exogenous mRNA's (Lodish and Desalu, 1973; Mathews et al., 1973). Furthermore, Beuzard et al. (1973) reported that haemin markedly enhanced protein synthesis in ascites cells and lysates prepared from the cells. These findings cannot be reconciled with the observation of Giglioni et al. (1973) that the simultaneous injection into Xenopus oocytes of exogenous haemin togehter with the α and β globin mRNA's, results in the selective stimulation of α globin chain synthesis only. Hence the answer to the question as to whether haemin is a control element in the translation of proteins generally must await the results of future work.

5. Nature of the protein products synthesised in 14-day feather cell-free system

The results, from polyacrylamide gel electrophoresis at two different pH values, DEAE-cellulose and Sephadex G100 chromatography demonstrated that the major products of the

cell-free system were identical by these criteria, to embryonic feather keratins. All radioactive protein species had absorbance counterparts, as determined by each analytical technique. The observation that keratin was the major product of the 14-day cell-free system is consistent with the observation that the synthesis of keratin accounts for the major part of the protein synthetic activity *in ovo* in 14-day chick embryo feathers (Kemp *et al.*, 1974).

Isolation of the labelled N-terminal dipeptide of the *in vitro* synthesised keratin confirmed thatinitiation was occurring in a cell-free system prepared in MSB. Furthermore, the incorporation of ^{14}C-acetate, derived from ^{14}C-acetyl-CoA into the N-terminal confirmed this result, implicating acetyl-CoA as the *in vivo* acetyl donor. Similarly, Shih and Kaesburg (1973) found that acetyl-CoA could act as the N-terminal acetyl donor to in vitro synthesised viral coat protein.

No significant difference was detected in the relative ratios of ^{14}C-leucine incorporated into the keratin bands β_2-β_5 synthesised in feather lysate in high salt (MSB) and low salt (LSB) conditions. This is to be contrasted with the findings of Lodish and Nathan (1972) for the reticulocyte system. These authors reported that increasing the Mg^{++} concentration in a reticulocyte cell-free system, by a factor of two caused the selective inhibition of β globin chain synthesis, resulting in a two-fold excess of α to β chains being synthesised. The present results would tend to indicate that no translational control of this type is operative in the feather system, although it must be borne in mind that small changes in the

relative ratios of the labelled keratin bands may not be detected as each band consists of several discrete keratin chains (Walker, 1974).

6. Nature of the protein products synthesised in cell-free systems from 12, 13 and 15-day feathers

(a) Thirteen and 15-day feathers

The spectrum of proteins synthesised in 13 and 15-day feather cell-free systems was very similar to those of the 14-day system. The major protein products in both systems were the keratin proteins. Furthermore, there was little difference detected in the relative ratios of label incorporated into the keratin bands β2 -β5 as determined by polyacrylamide gel electrophoresis at pH 7.5. Moreover, the results indicate that after the onset of feather keratin synthesis at 13 days of development of the embryo the translation of all the keratin mRNA's is coordinated and that there is no sequential switch-on of the separate keratin genes. This result is in agreement with the findings of Kemp (1972) who estimated that the relative amounts of each keratin band of protein synthesised *in vivo* remained approximately constant after 13 days of development. There is however, no definite evidence to indicate whether all keratin genes are expressed in each feather cell type.

(b) Twelve-day feathers

The level of incorporation of labelled amino acids was very low in cell-free systems prepared from 12-day feathers as compared to that of cell-free systems prepared from older

ages. This was not unexpected in view of the earlier findings (see Results 3.B4) that very few polysomes were present at this stage.

After dialysis of the lysate the maximal incorporation was increased by four fold, and equal to the level of incorporation obtained in undialysed 14-day cell-free systems. This would be presumed to result from the removal of free amino acids and hence an increase in the specific activity of the labelled amino acids in the system. The fact that the level of initiation was not increased by dialysis confirms this view. Nevertheless, the change in the amino acid pool was found to be only a two-fold decrease after dialysis. One explanation of this paradox may be that the charged-tRNA's present in the lysate became deacylated during dialysis and hence more effectively charged with labelled amino acids when incubated in the cell-free system.

No keratin synthesis was detected in undialysed 12-day lysate. However, after dialysis some keratin synthesis could be detected using ^{14}C-serine as label but not ^{14}C-leucine and the relative ratios of label incorporated into the keratin bands β2 -β5 was very similar to those observed in the other ages tested.

CHAPTER FIVE

INITIAL ATTEMPTS TO ISOLATE FEATHER KERATIN mRNA

A. INTRODUCTION

Feathers of the embryonic chick are ideally suited to the isolation of keratin mRNA since at 14-15 days of development of the embryo, keratin synthesis accounts for the major part of the protein synthetic activity of the feather cells (Kemp et al., 1974). Definitive evidence for the isolation of the mRNA which codes for a particular protein is the translation of that mRNA and identification of the protein product in a system which does not normally synthesise the protein under investigation. This is true for all but very special cases. Silk fibroin consists of alternating glycine and alanine residues and the mRNA has a very high G + C content which enabled identification by partial sequence analysis (Suzuki and Brown, 1972).

When this work was begun a 9S RNA species from mouse reticulocytes had been demonstrated to direct the synthesis of mouse β globin chains when added to a rabbit reticulocyte lysate (Lockard and Lingrel, 1969). This was in fact the first definitive demonstration of the translation of the mRNA from one mammalian species in a cell-free system from another. An earlier report (Laycock and Hunt, 1969) describing the synthesis of rabbit α and β globin chains in a cell-free system from E. coli primed with the 9S RNA from rabbit reticulocytes has been found to be unrepeatable (Noll et al., 1972; Gielkens et al., 1972). Closely fcllowing this work, Heywood (1969, 1970) reported that myosin mRNA isolated from chick embryo thigh

muscle could only be translated by ribosomes derived from chicken reticulocytes if the homologous (those derived from muscle) but not if the heterologous initiation factors were present. The overall evidence at that time favoured the view that initiation factors for translating various mRNA's were not species specific but were specific for mRNA's within a particular differentiated cell line.

The original method used by Marbaix and Burny (1964) to demonstrate the presence in reticulocyte polysomes of a minor RNA species which sedimented at 9S, and had the expected properties of globin mRNA (for a review see Chantrenne et al., 1967) was to dissociate the reticulocyte polysomes using SDS and to separate the RNA species by sucrose gradient centrifugation. This 9S RNA was shown unequivocally to contain globin mRNA by Lockard and Lingrel (1969) using the same method to isolate the mRNA.

The 9S globin mRNA has also been released as 14S ribonucleoprotein complex (RNP) using EDTA to dissociate the reticulocyte polysomes (Huez et al., 1967; Lebleu et al., 1971).

This chapter describes the initial attempts to isolate the chick embryo feather mRNA from 14-day feathers using both of the methods used to prepare globin mRNA.

B. METHODS

1. Dissociation of feather polysomes by SDS treatment

Feather polysomes at a concentration of 5-10 mg/ml were resuspended in buffer (20mM sodium acetate, 5 mM EDTA, 0.5% w/v,

SDS, 40 mM Tris-HCl, pH 7.4). The polysomal suspensions were kept on ice, then incubated at 37° for 5 min, cooled and immediately layered over the sucrose gradients.

2. Dissociation of feather polysomes by EDTA treatment

Feather polysomes at a concentration of 5-10 mg/ml were resuspended in buffer (150 mM KCl, 30 mM EDTA, 20 mM Tris-HCl, pH 7.4). In most experiments the polysomal suspensions were kept on ice for 30 min, incubated at 37° for 5 min, cooled and immediately layered over the sucrose gradients. In some experiments, however, the procedure was varied in that the composition of the resuspension buffer was altered and the polysomal suspensions were not incubated at 37° prior to loading onto the sucrose gradients. Refer to text for details.

3. Identification of products synthesised in feather cell-free systems

(a) Preparation of rabbit globin

Rabbit erythrocytes were collected and washed as described for the collection of reticulocytes (see Methods 2.B.4b) The erythrocytes were lysed by the addition of 1 volume of H_2O. The lysed cells were then centrifuged at 38,000 g for 30 min to pellet the cell debris. The supernatant was decanted and treated to remove haemin using the method of Schapira et al. (1968) as modified by Lane et al. (1971). The globin was then exhaustively dialysed against H_2O and freeze-dried.

(b) Fractionation of feather post-ribosomal supernatants by CM-cellulose chromatography

The method of fractionation of the rabbit α and β globin chains was essentially as described by Rabinovitz and Fisher (1964). Globin (10 mg) was dissolved in 1.0 ml of the column loading buffer (0.04 M pyridine, 0.4 M formic acid). This solution was then added to the post-ribosomal supernatant (see Methods 4.B3) from the feather cell-free system. The mixture was then treated to remove any traces of haemin as described in section (a) above. The protein pellet obtained was redissolved in 2 ml of the loading buffer and dialysed against 1 litre of the same buffer for 2 hr, with a buffer change after 1 hr. The keratin which was insoluble was removed by centrifugation for 1 hour at 38,000 g at 4°. The supernatant was decanted and fractionated on a 1 x 8 cm column of Whatman CM 32 cellulose. The gradient used for elution of the globin chains was prepared in a Technicon varigrad (9 chambers). Each of six consecutive chambers of the varigrad contained equal volumes of the loading buffer in the following multiples of concentration (1,3,5,7,1,11). A total gradient of 300 ml per column was used and 6 ml fractions were collected using a flow rate of 15 ml/hr.

C. RESULTS

1. Liberation of mRNA from 14-day chick feather polysomes using SDS

The 14-day chick feather polysomes were prepared in MSB by methods previously described (see Methods 2B2 and 4B6). The pelleted polysomes were rinsed with MSB and resuspended in a buffer containing 0.5% SDS (see Methods.5B2). The absorbance profile (A_{260}) of the RNA liberated from the polysomes, as analysed by sucrose gradient centrifugation, is shown in Fig. 5.1. The major peaks of absorbance, are the tRNA, sedimenting near the top of the gradient, the 18S rRNA and the 28S rRNA sedimenting near the bottom of the gradient. There is clearly no minor peak of RNA sedimenting in the 5-17S region, although there is some evidence of a shoulder sedimenting on the trailing edge of the 18S rRNA peak. The experiment was repeated essentially as described, with a minor modification in that aliquots of the SDS-dissociated polysomes were layered over 10% - 40% w/v sucrose gradients prepared in the same buffer but without SDS. The gradients were centrifuged in a Beckman 'SW41' rotor (refer to legend to Fig. 5.2 for details) as it was found that this system had superior resolving power, to the 'SW25' rotor previously used.

In this experiment two minor RNA species were detected sedimenting in the 5-17S region, that is, between the tRNA and 18S rRNA peaks. The absorbance profile of one such gradient is shown in Fig.5.2. The sedimentation coefficients were determined using the method of Martin and Ames (1961) as described (see

FIGURE 5.1. ABSORBANCE PROFILE OF RNA LIBERATED FROM 14-DAY CHICK FEATHER POLYSOMES BY TREATMENT WITH SDS.

The polysomes were dissociated as described (see Methods 5.B.1).

1.0 ml of the polysomal suspension (5 mg) was layered over a gradient (28 ml, 15% - 30% w/v, sucrose) prepared in the same buffer (see Methods 5.B.1) and centrifuged in an SW25 rotor at 64,700 g (a.v.) for 16 hr. at 4^{o}.

The absorbance profile of the gradient was monitored using an Optica Spectrophotometer.

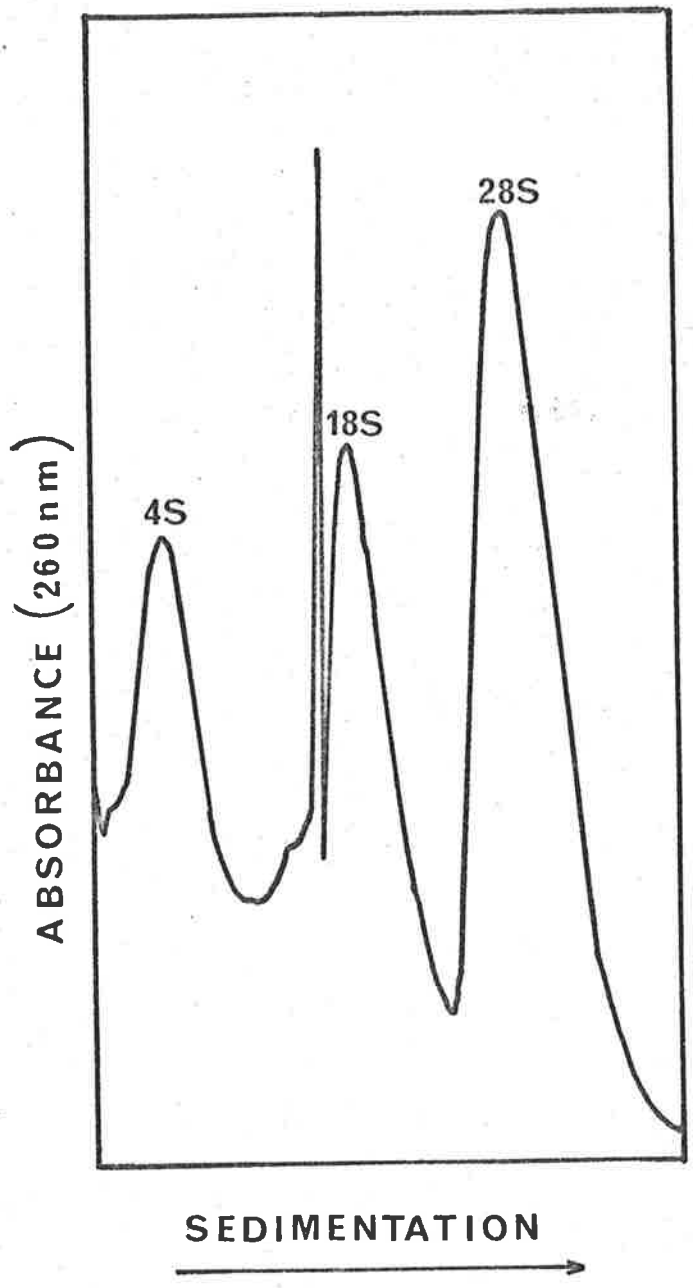
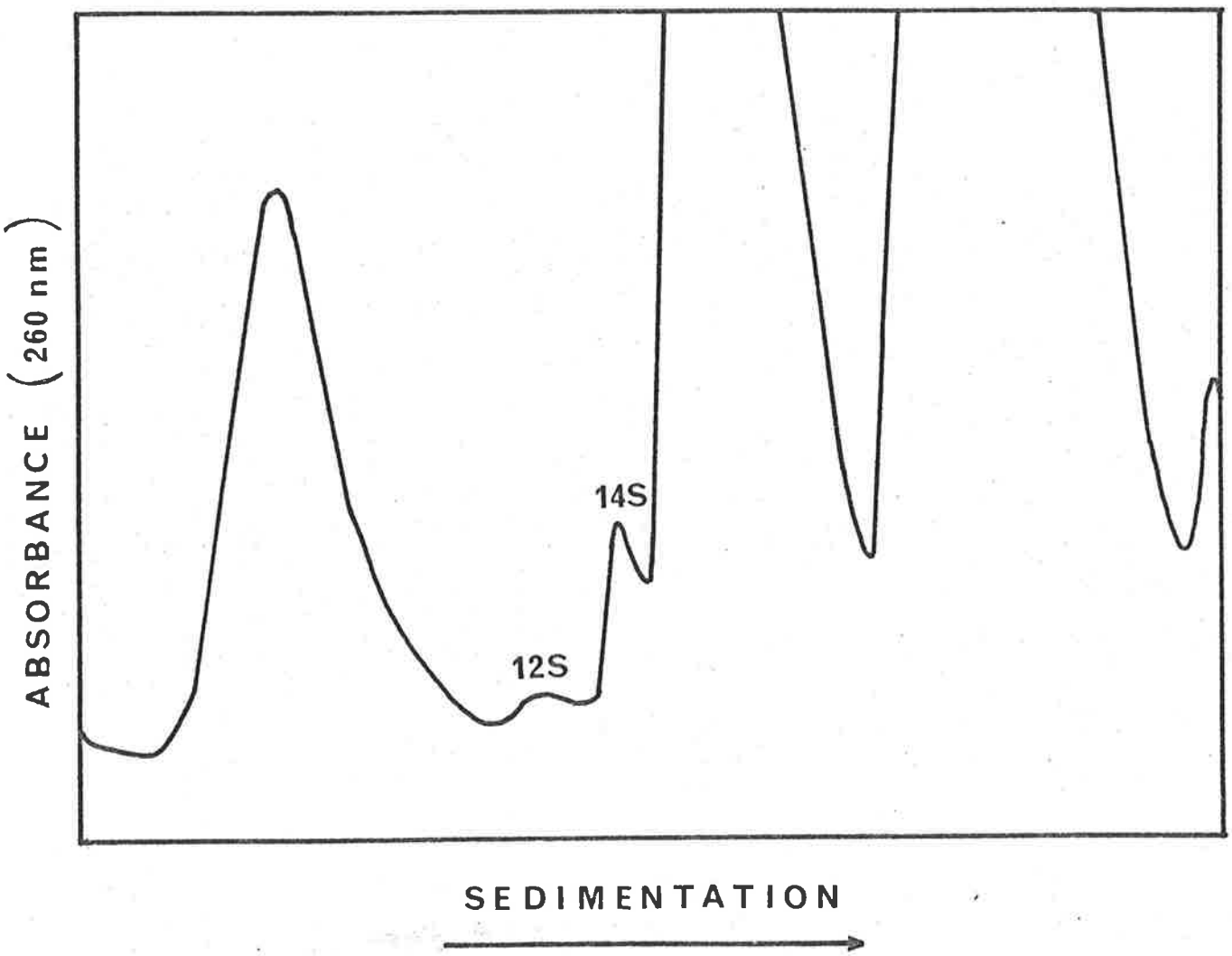

28S
18S
4S
ABSORBANCE (260 nm)
SEDIMENTATION

FIGURE 5.2. ABSORBANCE PROFILE OF RNA LIBERATED FROM 14-DAY CHICK FEATHER POLYSOMES BY TREATMENT WITH SDS

Other details as given in Figure 5.1 and in text.

An aliquot (0.75 ml) of dissociated feather polysomes was layered over a gradient (11.6 ml) and centrifuged in an SW41 rotor at 147,640 g (a.v.) for 16 hr at 4^{o}.


ABSORBANCE (260 nm)
14S
12S
SEDIMENTATION

Methods, 2.B10) and found to be 12S and 14S respectively. However, these two species could not be detected reproducibly in a series of experiments, even though the standard conditions for preparation and dissociation of the polysomes were maintained.

2. Liberation of mRNA from rabbit reticulocyte polysomes by dissociation with SDS

Polysomes were prepared (see Methods 2.B4) from rabbit reticulocytes. The reticulocyte polysomes were resuspended in buffer containing 0.5% SDS using identical conditions to those described for dissociation of the feather polysomes, and analysed by sucrose gradient centrifugation. The result is shown in Fig. 5.3. In this case, in addition to the major peaks of absorbance of the tRNA, 18S rRNA and 28S rRNA species, a minor but significant component was present sedimenting in the 7-17S region. The S value of this peak was calculated to be about 9. In a series of experiments, using reticulocytes from several rabbits the results were entirely reproducible in that the minor RNA peak sedimenting at about 9S was always detected.

3. Attempted release of keratin mRNP from feather polysomes by EDTA treatment

The polysomes prepared in MSB buffer were resuspended in a buffer containing 30 mM EDTA as described (see Methods 5B.2). The sucrose gradient centrifugation profile is shown in Fig. 5.4.

FIGURE 5.3. ABSORBANCE PROFILE OF RNA LIBERATED FROM RABBIT RETICULOCYTE POLYSOMES BY TREATMENT WITH SDS.

The rabbit reticulocyte polysomes (3 mg) were dissociated with SDS as described for that of feather polysomes (see Methods 5.B.1). The polysomal suspension (0.75 ml) was centrifuged using identical conditions to those given in Figure 5.2.

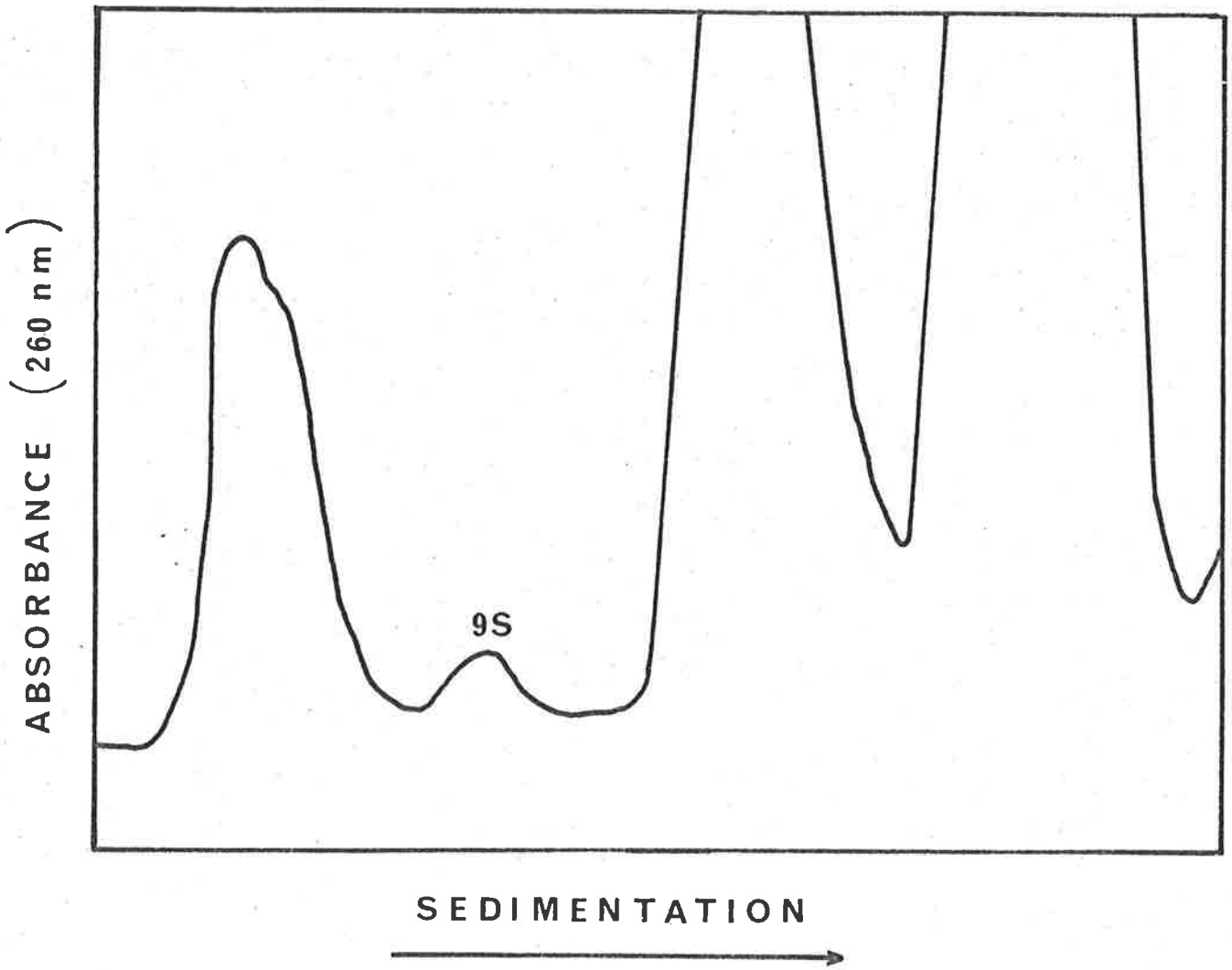
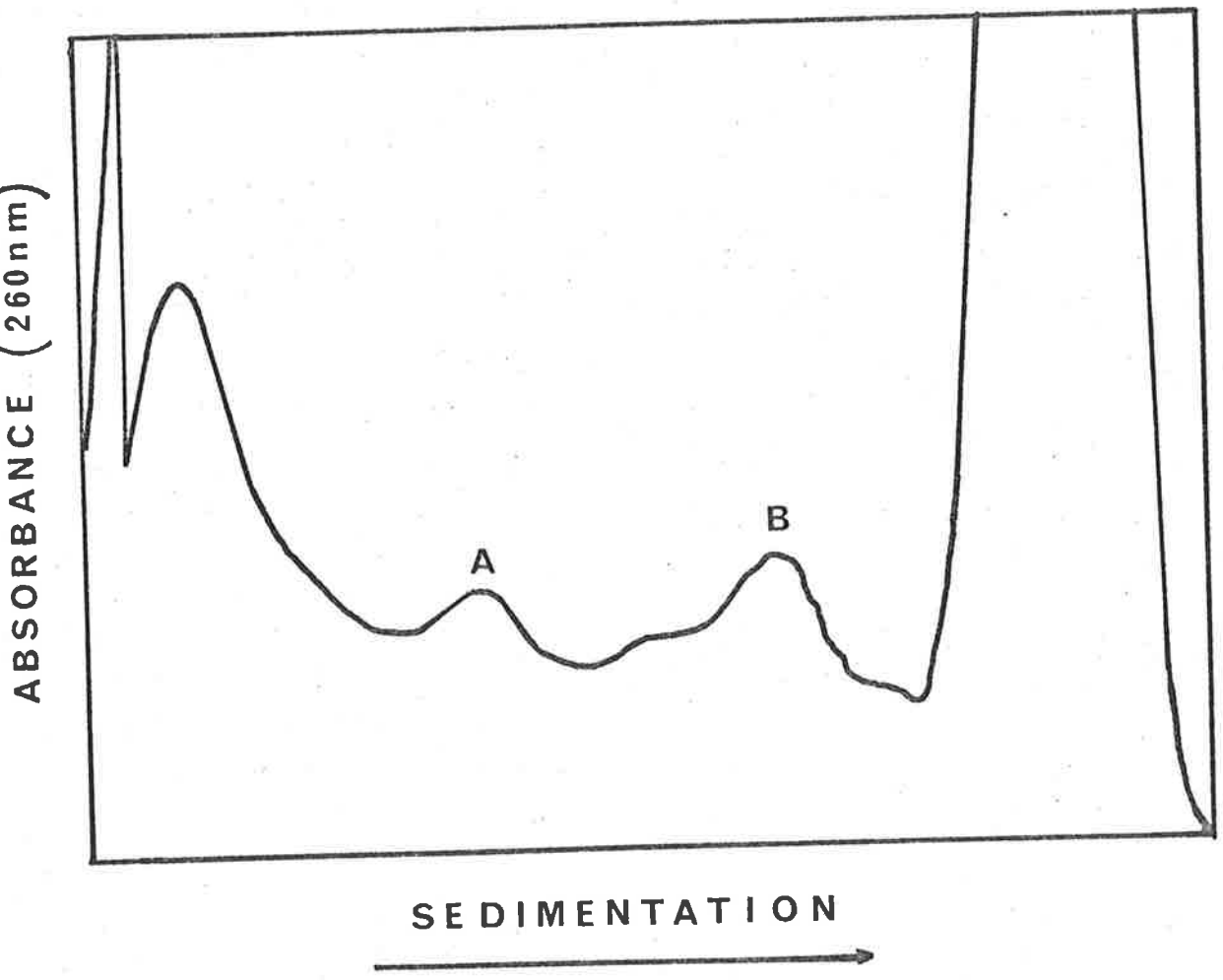

ABSORBANCE (260 nm)
9S
SEDIMENTATION

FIGURE 5.4. ABSORBANCE PROFILE OF 14-DAY FEATHER POLYSOMES DISSOCIATED WITH EDTA.

Feather polysomes were dissociated by treatment with EDTA as described (see Methods 5.B.2).

An aliquot (0.75 ml) of the polysomal suspension was layered over a gradient (11.6 ml, 10% - 40% w/v sucrose) prepared in the same buffer and centrifuged in an SW41 rotor at 201,125 g (a.v) for 16 hr at 4^{o}.


ABSORBANCE (260nm)
A
B
SEDIMENTATION

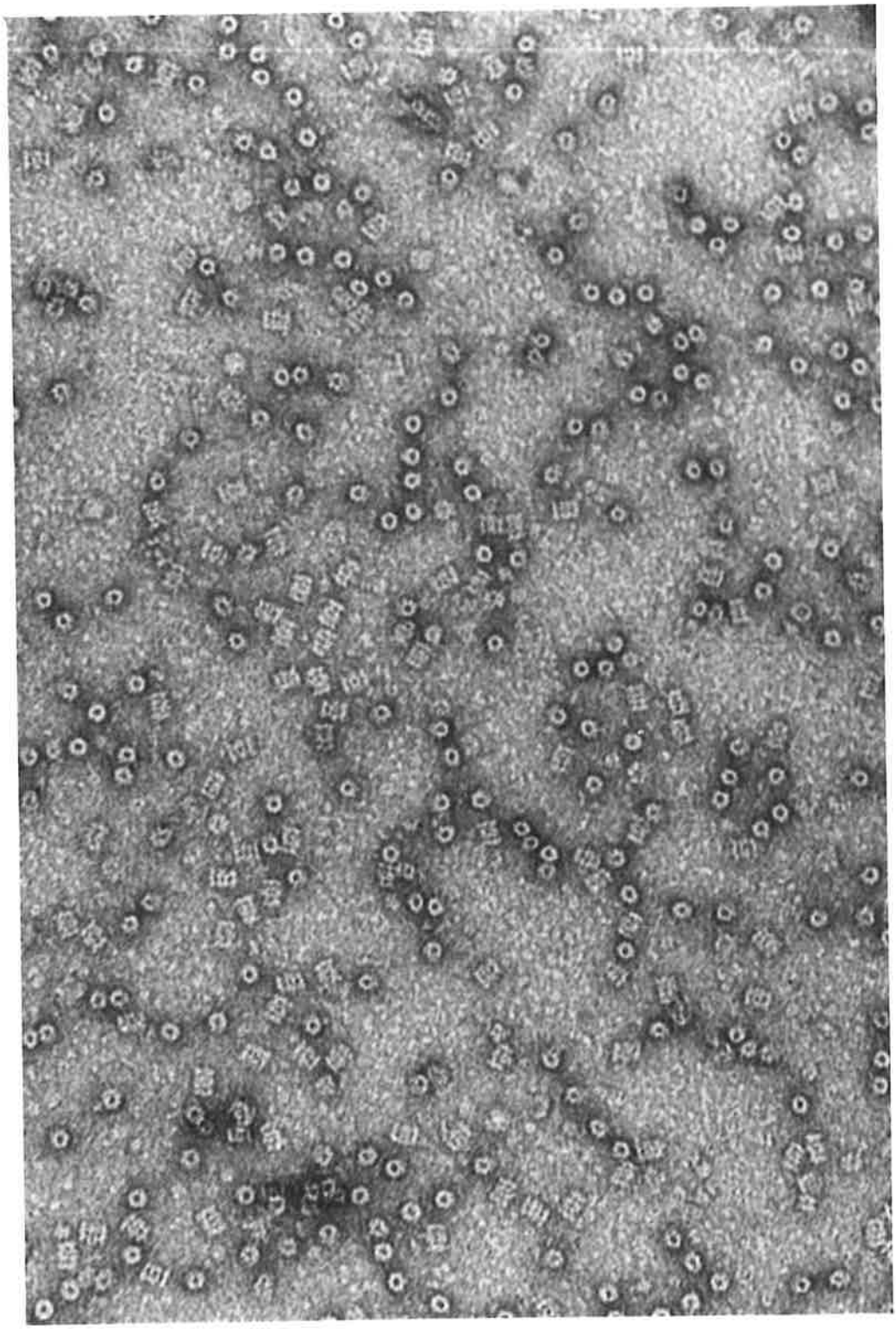
Two minor peaks (designated A and B on the figure), were detected, sedimenting between the tRNA peak and the small ribosomal subunit which had sedimented almost to the bottom of the gradient under the conditions used for centrifugation. The fraction of the gradient containing peak B was collected and precipitated by the addition of two volumes of ethanol (see Methods 6.B2). However, resuspension of the pellet obtained from this fraction proved to be very difficult. The pellet was almost insoluble in the EDTA buffer used to dissociate the polysomes, and dissolved only slowly when incubated at 37° in the presence of 0.5% SDS. When the pellet fraction was partially dissociated in the EDTA-buffer and examined by electron microscopy, using negative staining (see Methods 2.B11), it was shown to consist predominantly of ring-shaped particles, about 9 nm in diameter, and with a central core about 3 nm in diameter (Fig. 5.5). The rings were observed to be stacked together in cyclindrical aggregates comprised of four rings. Examination of fraction B from sucrose gradients before ethanol precipitation also revealed the presence of the rings (result not shown). No particles of this nature were detected in the fraction corresponding to the peak A region of the sucrose gradients.

4. Reproducibility of feather polysome dissociation by EDTA treatment

In two further experiments, results identical to that shown in Fig. 5.4 were obtained. In later preparations however, the original results could not be repeated. That is very little

FIGURE 5.5. ELECTRON MICROGRAPH OF MATERIAL ISOLATED FROM THE PEAK B REGION OF A SUCROSE GRADIENT SIMILAR TO THAT SHOWN IN FIG. 5.4.

Negatively stained with uranyl acetate. Magnification: X240,000.



absorbance was detected in the region of peak B of the earlier gradients (Fig. 5.6). In comparison the amount of material sedimenting in the region of peak A was greatly increased (Fig. 5.6). The partially dissociated small ribosomal subunits from one such experiment were collected from four gradients, dialysed against MSB buffer and redissolved in buffer (20 mM sodium acetate, 5 mM EDTA, 40 mM Tris-HCl, pH 7.4). The solution was then made 0.5% with respect to SDS to dissociate the subunits. The sucrose-gradient profile of the RNA liberated from the subunits is shown in Fig. 5.7. In addition to the major peaks of absorbance of the tRNA, 18S rRNA and a smaller 28S rRNA peak two minor peaks sedimenting in the 7 - 17S region were evident. The S value of these two species was calculated to be 12S and 14S respectively.

In an effort to obtain more reproducible dissociation of the feather polysomes by EDTA treatment, variations in the conditions of release were investigated. For example, the feather polysomes were resuspended in a modified buffer in which the concentration of KCl was reduced from 150 mM to 25 mM and the pH was increased from 7.4 to 7.8. One aliquot of the polysomes was left on ice prior to loading on the sucrose gradients, whereas, the other was incubated at 37° for 5 min. and cooled prior to loading on the gradients. However, the original result still could not be obtained; no peak was observed in the region of the gradient where peak B was originally detected (results not shown).

FIGURE 5.6. ABSORBANCE PROFILE OF 14-DAY FEATHER POLYSOMES DISSOCIATED WITH EDTA.

Other details are as given in Fig. 5.4.

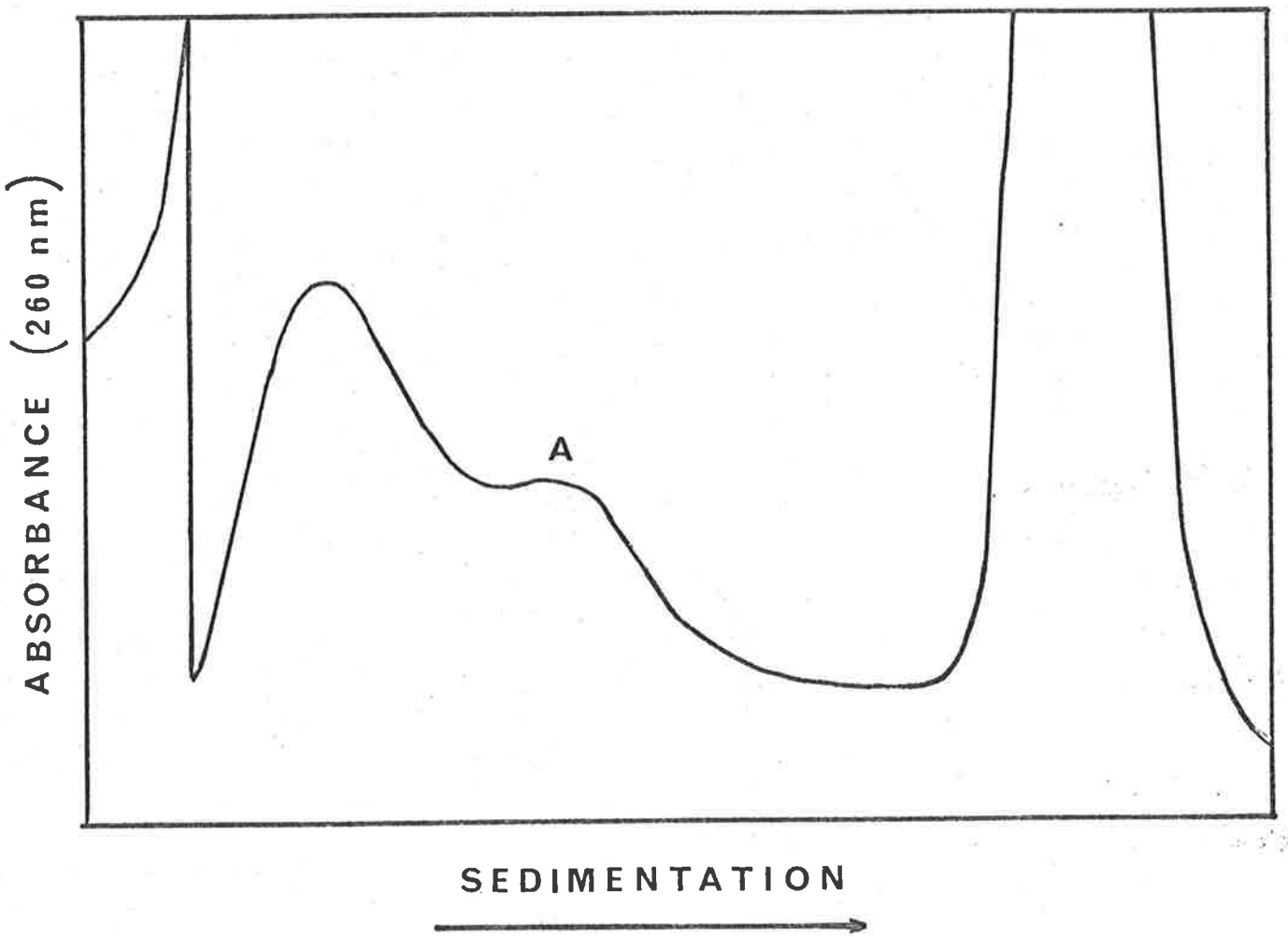
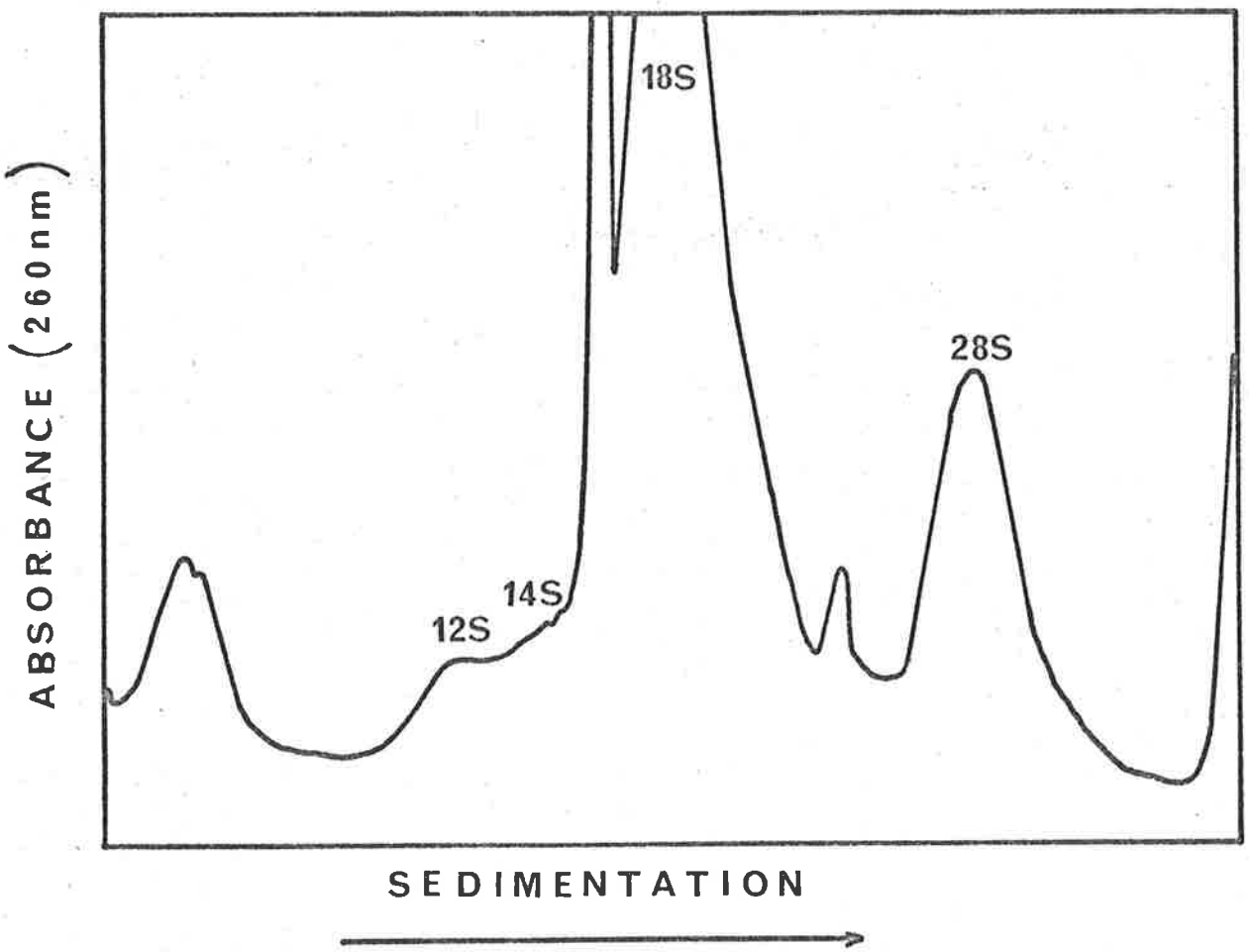

ABSORBANCE (260 nm)
A
SEDIMENTATION

FIGURE 5.7. ABSORBANCE PROFILE OF RNA LIBERATED BY SDS DISSOCIATION OF THE SMALL RIBOSOMAL SUBUNITS FROM 14-DAY FEATHER POLYSOMES.

The small ribosomal subunits isolated from four gradients similar to that shown in Fig. 5.6 were dissociated by treatment with SDS and analysed by sucrose-gradient centrifugation as described in Fig. 5.2, except that the centrifugation was at 201, 125 g (a.v.).


18S
28S
14S
12S
ABSORBANCE (260nm)
SEDIMENTATION

5. Release of mRNP from rabbit reticulocyte polysomes by EDTA treatment

Rabbit reticulocyte polysomes were resuspended in the original EDTA buffer (150 mM KCl, 30 mM EDTA, 20 mM Tris-HCl, pH 7.4) at a concentration of 5 - 10 mg/ml. The method used was identical to that used initially to dissociate the feather polysomes. The absorbance profile of the RNP's released is shown in Fig. 5.8. This profile is similar to that obtained in the initial preparations of RNP's from feather polysomes. Two additional peaks of absorbance (designated A and B in Fig. 5.8) were again present in the region of the gradient between the tRNA peak and the small ribosomal subunit. In a number of preparations the results were completely repeatable, both under the original conditions of dissociation as well as when the modified buffer was used with a decreased concentration of KCl at pH 7.8.

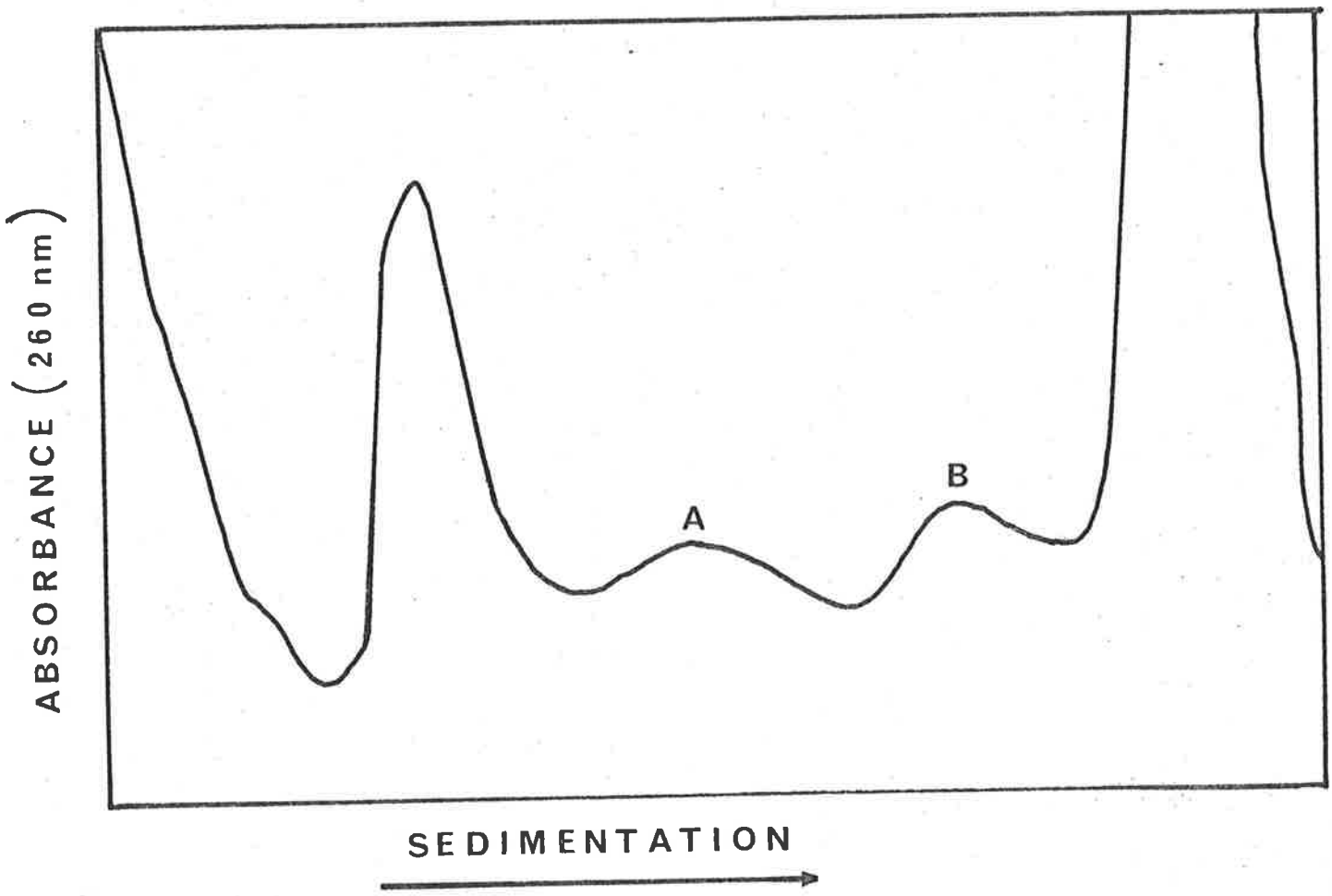
The fraction of the gradient containing peak B was examined by electron microscopy before ethanol precipitation and found to contain a few of the rings. However, these rings were 13 nm in diameter, which was considerably different from the size of the rings found to be associated with the feather polysomes. Furthermore, they were never observed in great abundance.

6. Translation of the reticulocyte 9S globin mRNA and globin mRNP particles in the 14-day chick feather cell-free system

A cell-free system was prepared from 14-day feather

FIGURE 5.8. ABSORBANCE PROFILE OF RABBIT RETICULOCYTE POLYSOMES DISSOCIATED BY TREATMENT WITH SDS.

Other details are as given in Fig. 5.2 except that the centrifugation was at 201, 125 g (a.v.).


ABSORBANCE (260 nm)
A
B
SEDIMENTATION

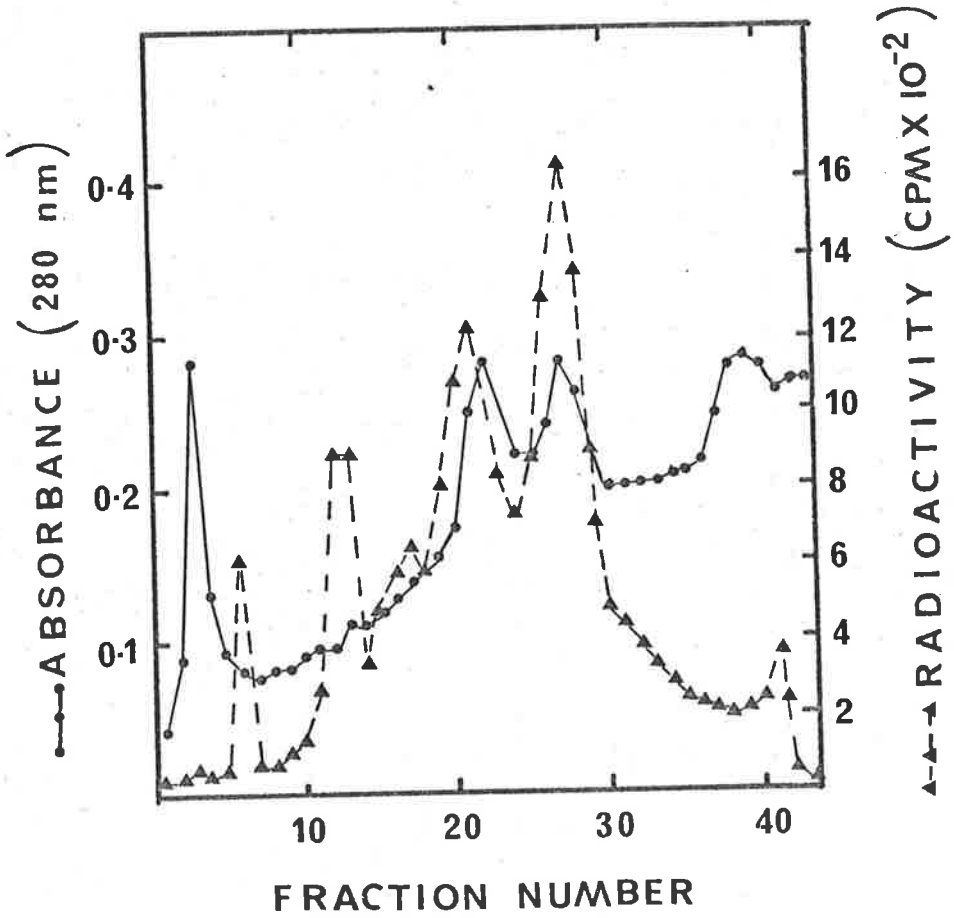
lysate using MSB (see Methods 4B.1). Reticulocyte RNP particles (fraction B of gradients identical to that shown in Fig. 5.8), was dialysed against MSB (see Methods 4B.3) to remove sucrose and EDTA.

The post-ribosomal supernatant was prepared from the incubation mixture and treated for the removal of traces of haemin that might be present from contaminating erythrocyte in the feather lysate (see Methods 5B.3). The product so obtained was co-chromatographed with 10 mg of carrier globin on a column of CM-32 cellulose (see Methods 5.B4).

The absorbance and radioactivity profile obtained is shown in Fig. 5.9. The peak of unlabelled protein eluting near the gradient front was previously shown to be keratin. The main peaks of radioactivity co-chromatographed with the unlabelled carrier α and β globin chains. It should be noted, however, that the peak of radioactivity associated with the unlabelled α chain did not precisely align with it; rather the peak of protein-bound radioactivity chromatographed one fraction ahead of the peak of absorbance of the unlabelled carrier α chain. The nature of the labelled protein which eluted just ahead of the α and β globin peaks is unknown. The total radio-activity present in the α chain peak was 2818 cpm whereas it was 3171 cpm in the β chain. The α and β chains of rabbit globin are known to contain 17 and 18 leucine residues respectively (Dayhoff, 1969). Hence the ratio of α to β chains that were synthesised was almost unity. This experiment was repeated several times using both the globin mRNP complex and the 9S globin mRNA. In all of these however, it was found that the

FIGURE 5.9. TRANSLATION OF RABBIT RETICULOCYTE mRNP IN A 14-DAY FEATHER CELL-FREE SYSTEM AS ASSAYED BY CHROMATOGRAPHY ON CM-CELLULOSE

A feather-cell free system (2.0 ml) was incubated with 2.5 μC of ^{14}C-leucine (S.Act. 312 mC/mM) for 1 hr at 37° in the presence of ~1 A_{260} units of reticulocyte RNP. The post-ribosomal supernatant was treated to remove traces of haemin and chromatographed with 10 mg of carrier globin on a column of CM-32 cellulose as described (see Methods 5.B3). Each fraction (6 ml) was precipitated with TCA for determination of radioactivity.


ABSORBANCE (280 nm)
0·4
0·3
0·2
0·1
RADIOACTIVITY (CPM X 10⁻²)
16
14
12
10
8
6
4
2
10
20
30
40
FRACTION NUMBER

stimulation of synthesis of the α chain of globin was greatly enhanced compared to β chain synthesis (Fig. 5.10). The total incorporation of radioactivity in the feather cell-free system was either unaffected, or slightly decreased by the presence of the exogenous globin mRNA, whereas the addition of the mRNP complex invariably lowered the total incorporation 10-15 fold as compared to the control.

7. Translation of phenol-extracted total reticulocyte polysomal RNA

In one experiment about 100 µg of phenol-extracted reticulocyte polysomal RNA (see Methods 6.B1) was added to a cell-free system and incubated at 25° for 2.5 hr with ^{14}C algal hydrolysate. The post-ribosomal supernatant was co-chromatographed with 10 mg of carrier globin as in Fig. 5.9. The absorbance and radioactivity profiles are shown (Fig. 5.11a). In this experiment the ratio of the in vitro synthesised α and β chains was about unity. To completely confirm that the in vitro synthesised α and β chains were released into the supernatant fraction the polysome pellet was treated in a similar fashion to the supernatant fraction and co-chromatographed with carrier globin. It is clearly evident from the profile of absorbance and radioactivity that very little completed chains remained on the polysomes after the incubations of the cell-free system (Fig. 5.11b).

8. The proteins of embryonic duck feather

For reasons discussed in the Introduction to this chapter it was considered that a cell-free system prepared from duck

FIGURE 5.10. TRANSLATION OF RABBIT RETICULOCYTE GLOBIN mRNA IN A 14-DAY FEATHER CELL-FREE SYSTEM AS ASSAYED BY CHROMATOGRAPHY ON CM-CELLULOSE

Details as given in Fig. 5.8 except that the volume of the cell-free system was 2.0 ml to which 35 µg of rabbit reticulocyte mRNA was added.

Aliquots of the column fractions (2 ml/6 ml) were precipitated for the determination of radioactivity.

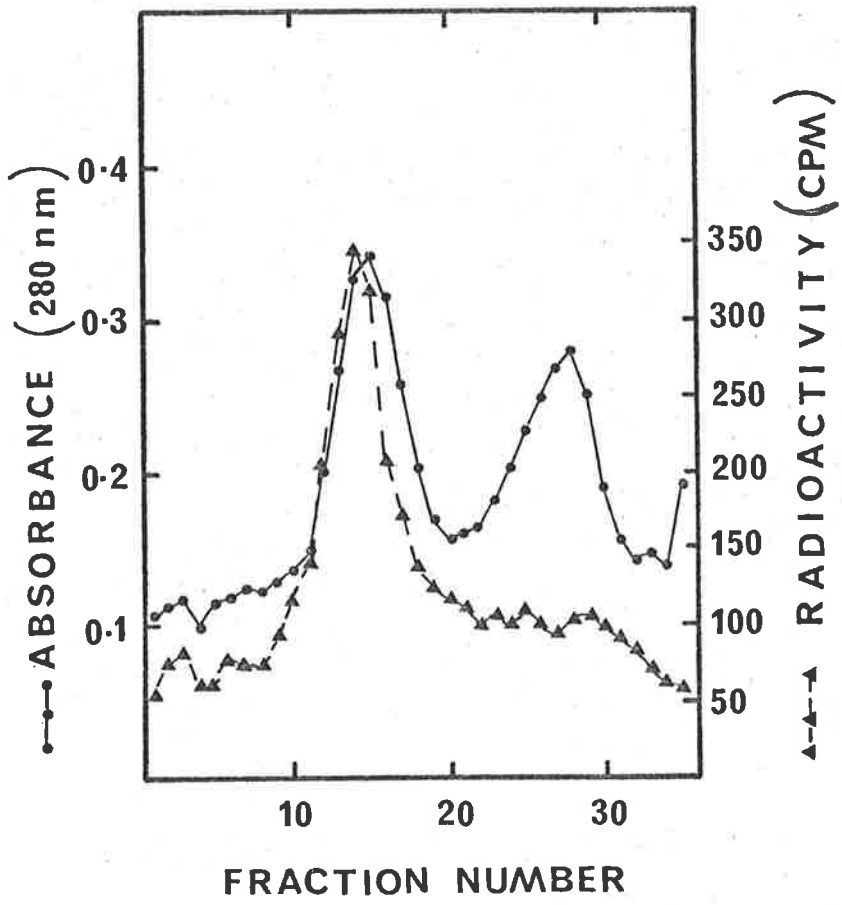
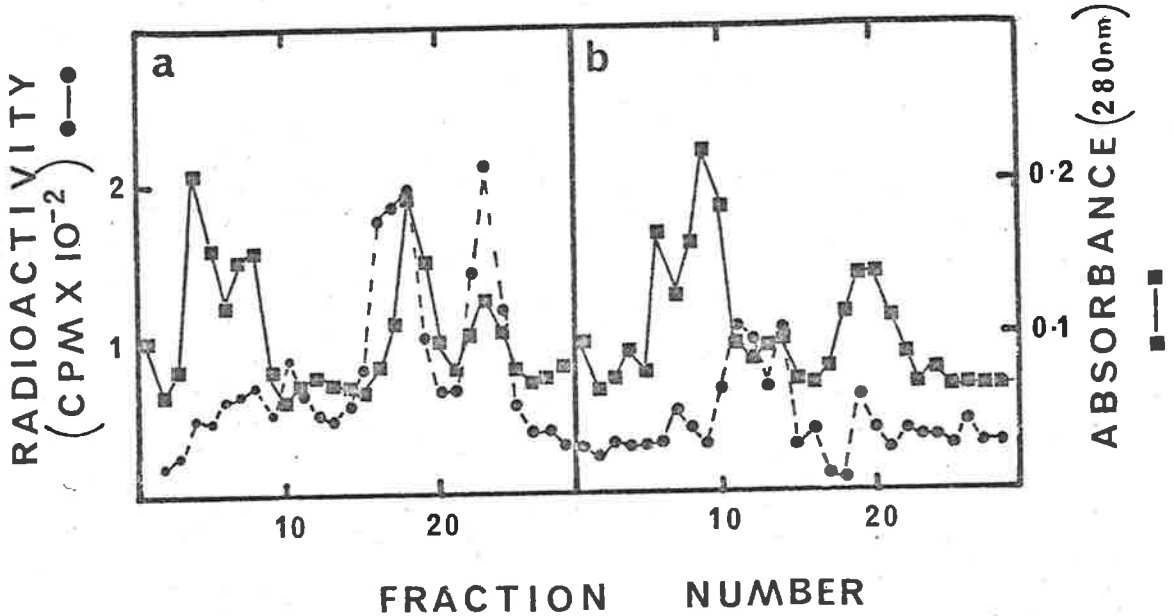

ABSORBANCE (280 nm)
0·4
0·3
0·2
0·1
RADIOACTIVITY (CPM)
350
300
250
200
150
100
50
10
20
30
FRACTION NUMBER

FIGURE 5.11. TRANSLATION OF PHENOL-EXTRACTED TOTAL RETICULOCYTE POLYSOMAL RNA IN A 14-DAY FEATHER CELL-FREE SYSTEM AS ASSAYED BY CM-CELLULOSE CHROMATOGRAPHY

100 µg of reticulocyte polysomal RNA was incubated in a 2.0 ml 14-day feather cell-free system with 2.5 µC of ^{14}C-algal hydrolysate. The system was incubated at 25^{o} for 2.75 hr. The lysate was then fractionated into the ribosomal and post-ribosomal supernatant fraction. Each of these fractions was then chromatographed on CM-32 cellulose. Aliquots of the column fractions (2 ml/6 ml) were then precipitated with TCA for determination of radioactivity.

Figure 5.10a. Chromatography of the post-ribosomal supernatant.

Figure 5.10b. Chromatography of the ribosomal pellet fraction.


a
b
RADIOACTIVITY (CPM X 10⁻²) ●—●
ABSORBANCE (280nm) ■—■
2
1
0·2
0·1
10
20
10
20
FRACTION NUMBER

embryo feathers could be particularly useful for assaying RNA obtained from chick embryo feather polysomes for mRNA activity. The spectrum of proteins synthesised in duck feathers was investigated from embryos ranging in age from 14 to 17 days to determine, (a) the time of onset of keratin synthesis; (b) whether these proteins were similar to those of chick embryo feathers. The method adopted was identical to that described by Kemp et al. (1974) for determining the onset of keratin synthesis in chick embryo feathers. Briefly, feathers plucked from 14, to 17-day duck embryos were reduced, carboxymethylated, centrifuged to remove insoluble debris, dialysed and freeze-dried. Equal quantities of protein (100 µg) from each age were fractionated by polyacrylamide gel electrophoresis at pH 7.5. The gel of 14-day feather protein showed no clearly defined bands, the major peak of absorbance was present in the low-mobility region of the gel (Fig. 5.12). There was, however, a rapid increase after this time in several bands of similar mobility to the major bands (β2 -β5) of chick embryo feather keratin shown for comparison. It should be noted that this result is in agreement with the finding that the polysome content increased in duck feathers after 14 days of incubation (see Results 3.B.7).

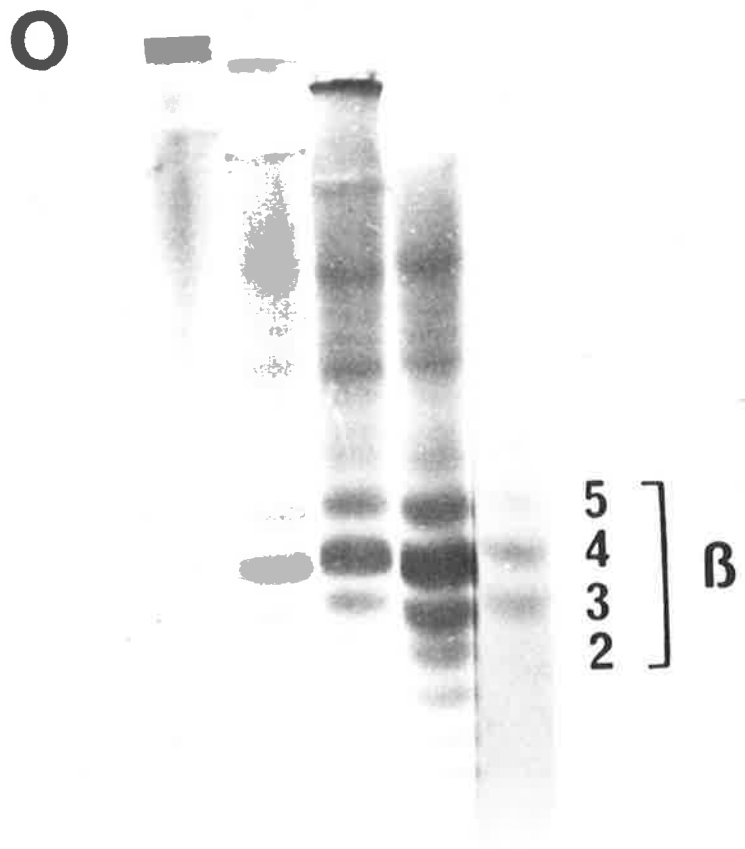
The proteins of 16-day feathers were also characterised by polyacrylamide gel electrophoresis at pH 2.7. Comparison of the electrophoresis of the SCM-derivatives of chick feather keratin (Fig. 5.13a) with those of duck feather (Fig. 5.13b) indicates that the major protein band from duck feathers was found to be almost coincident with band B (of Kemp and Rogers, 1972) of chick embryo feathers; furthermore, the leading edge of

FIGURE 5.12. POLYACRYLAMIDE GEL ELECTROPHORESIS OF PROTEINS FROM DUCK EMBRYO FEATHERS DURING DEVELOPMENT

Samples (100 µg/gel) of reduced and carboxymethylated protein preparations from duck embryo feathers at 14-17 days of development were subject to polyacrylamide gel electrophoresis at pH 7.5 and stained with Coomasie Blue (see legend to Fig. 4.7).

CF: for comparison the electrophoresis of chick feather keratin is included. The chick keratin bands β2 - β5 are indicated.

O: origin + : anode.



+ 14 15 16 17 CF

Days

FIGURE 5.13. COMPARISON OF THE PROTEINS OF DUCK EMBRYO FEATHER AND CHICK FEATHER BY ELECTROPHORESIS ON POLYACRYLAMIDE GELS AT pH 2.7.

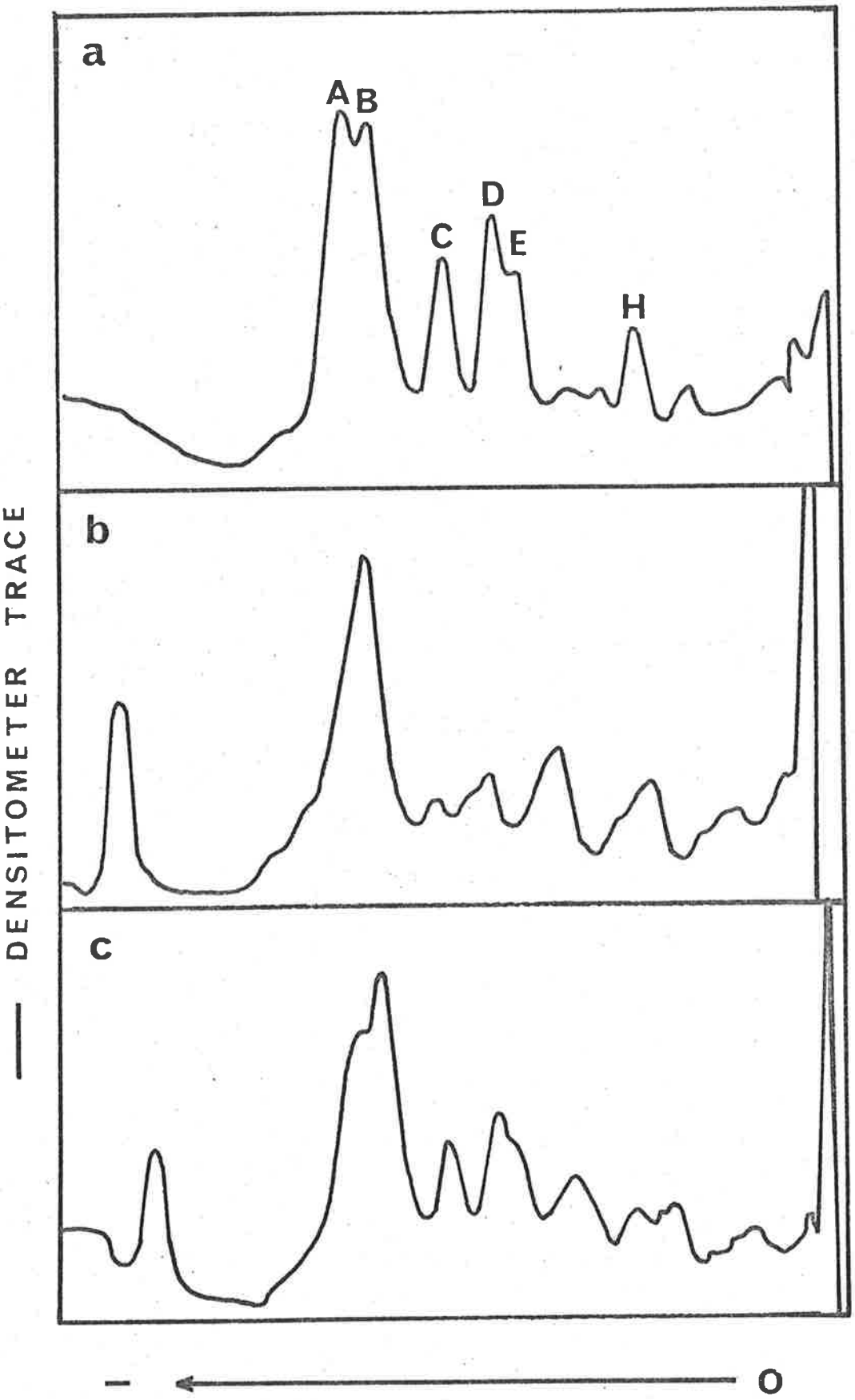
Samples (100 µg/gel) of reduced and carboxymethylated proteins from 16-day duck feathers and 21-day chick feathers were subject ed to polyacrylamide gel electrophoresis at pH 2.7 as described (see Fig. 4.8).

O: origin - : cathode.

Figure 5.13a. Electrophoresis of 100 µg of chick embryo feather keratin.
Keratin bands A - H are indicated.

Figure 5.13b. Electrophoresis of 100 µg of 16 day duck feather keratin.

Figure 5.13c. Co-electrophoresis of 100 µg of chick feather keratin and 100 µg of duck feather keratin.


a
A
B
C
D
E
H
b
c
DENSITOMETER TRACE
O

this band coincides with band A of chick keratin. Several minor protein bands of duck keratin were observed to be almost coincident with the minor bands C - E of chick keratin. Co-electrophoresis of duck and chick embryo keratin (Fig. 5.13c) confirmed that the protein species present from each feather type did not resolve into individual bands.

It was clear from this result that this system was inadequate for assaying for chick feather keratin mRNA activity as the products could not be distinguished from those of the homologous mRNA's.

D. DISCUSSION

1. Comments on the dissociation of polysomes by SDS or EDTA treatment

The method of Marbaix and Burny (1964) was adopted in initial attempts to release keratin mRNA from chick embryo feather polysomes. However, no minor species of RNA could reproducibly be released with a sedimentation coefficient expected for a mRNA coding for protein of molecular weight about 10,000 (Walker, 1974). Furthermore, it was known that the keratin protein chains were heterogenous (Walker, 1974) and it seemed possible that this heterogeneity could be reflected in a similar heterogeneity in the size of the mRNA's coding for the keratin chains. As a consequence, it would have been impossible to detect the low concentrations of each mRNA species from the absorbance profile of sucrose-gradients of feather-polysomal RNA.

The dissociation of feather polysomes with SDS was

variable whereas the dissociation of rabbit reticulocyte polysomes was completely repeatable. A species of RNA, sedimenting at about 9S was detected in all reticulocyte preparations. This RNA has been shown to code for both the α and β globin chains (Housman et al., 1971; Lane et al. 1971; Mathews, 1972; Metafora et al., 1972; Sampson et al., 1972; Lockard and Lingrel, 1973).

The initial results were encouraging when an attempt was made to release the keratin mRNA as a mRNP complex using the method of Huez et al. (1967) and Lebleu et al. (1971). Two peaks of absorbance were detected sedimenting between the tRNA and the partially dissociated small ribosomal subunit. Both Huez et al. and Lebleu et al. described an RNP complex (designated peak A on Fig. 5.4) sedimenting at about 9S which contained the 5S RNA-protein moiety dissociated from the large ribosomal subunit. Furthermore, the same authors had shown that a larger RNP complex contained the 9S globin mRNA. Hence by analogy again it was expected that peaks A and B (Fig. 5.4) isolated from the EDTA-dissociated feather polysome would contain respectively the 5S RNA-protein complex and the mRNA species coding for the keratin chains. However, after ethanol precipitation of the peak B fraction of the sucrose gradient, the pellet so obtained was found to be virtually insoluble even in the presence of 0.5% SDS. Examination of the resuspended pellet fraction revealed the presence of large numbers of rings about 10 nm in diameter. In subsequent experiments no peak of absorbance was detected in this region, whereas the absorbance in the peak A

region of the gradient was considerably more prominent. Complete dissociation of the small ribosomal subunits from several such gradients revealed, in addition to the major RNA species two minor species of RNA with sedimentation coefficients of 12S and 14S respectively. It should be noted by way of comparison that the dissociation of rabbit reticulocyte polysomes by EDTA treatment was always completely reproducible. Furthermore, the results were identical to those obtained by Huez et al. (1967) and Lebleu et al. (1971).

In retrospect it is clear that neither SDS dissociation nor EDTA treatment of polysomes was reproducibly releasing the keratin mRNA. In some experiments minor species of RNA sedimenting at 12S and 14S respectively were detected. In the following chapter the 12S RNA species is shown to contain the mRNA coding for chick feather keratin. It is interesting to note that Kennedy (1972) reported that Semliki Forrest Virus RNA could not be released from infected chick embryo cells using either SDS or EDTA treatment of the isolated polysomes. Pulse-labelled mRNP's have been prepared by EDTA treatment of polysomes of both chick embryo retinal tissue (Sarkar and Moscona, 1971), and chick embryo cerebral polysomes (Bryan and Hayashi, 1973). The mRNP's released were heterogenous in S value when fractionated by sucrose gradient centrifugation. In neither case however, was it proven that any of these RNP particles contained a translatable mRNA.

2. Nature of the rings

During the course of the present work similar findings

of ring structures were reported by Narayan and Rounds (1973) in the media they used to culture cells of malignant origin. These particles were reported to be composed of protein, but also contained small quantities of RNA and DNA. The particles were found, after cell fractionation to be associated predominantly with the polysomes. It is interesting to note that similar particles were independently detected as components of polysomes from HeLa cells by Spohr et al. (1970). In a further report Narayan et al. (1973) have reported that the particles could be detected in the media used to culture a large number of human tissue samples obtained from pathological and non-pathological conditions. However, very few of the particles were detected in media used to culture non-malignant tissue samples. Particles of similar size and topology have also been detected in cells infected with mouse leukemia virus (Schafer et al., 1972) and from avian myeloblastosis virus (Bolognesi et al., 1972). In these reports the particles were reported to be composed of protein only, although no evidence was presented confirming the absence of RNA or DNA.

In the present study, small number of the rings were seen in the sucrose gradient fractions containing the mRNP complex released by EDTA treatment of rabbit reticulocyte polysomes. They were not nearly as abundant as those released from the feather polysomes by EDTA treatment. Moreover, their size was significantly greater than those seen in feather polysome preparations. Harris (1970, 1971) has observed rings of a similar size in reticulocytes to those reported in the present study.

The rings could be seen by electron microscopy loosely attached to the membranes of human and bovine erythrocyte ghosts. Furthermore, Harris suggested that the rings he observed may be absorbed cytoplasmic contaminants.

3. A note on the significance of infection of experimental material with virus

The adult bird population from which the fertilised eggs were obtained for the present work were known to have been infected by several viral diseases (Bray - personal communication). The initial preparations of polysomes for release of RNP's were made during the winter months when the adult birds were known to have been infected by the following viral diseases, Marek's disease (a tumourogenic Herpe's virus), avian leukosis, avian bronchitis, and avian encephalomyelitis (Bray). Marek's disease is not thought to be transmitted vertically, but some doubt still remains on this point (Purchase et al., 1971). However, it may be significant that the only known site where the complete virus can be detected in adult birds is in the feather follicle epithelium (Calnek et al., 1970).

The origin of the ring-like particles observed to be associated with the chick feather polysomes must remain highly speculative. It remains to be established as to whether the occurrence of the particles is associated with a pathological condition. Nevertheless, it does emphasise that caution must be used in studies on chick embryos unless adequate

controls are maintained to exclude the possibility of viral infection.

4. Translation of rabbit globin 9S mRNA and mRNP complex in 14-day chick feather lysate

Rabbit globin mRNA and the mRNP complex were translated in a feather cell-free system. Nevertheless, the ability of the globin mRNA or mRNP to compete with the endogenous mRNA was rather low as shown by the low efficiency of the incorporation. The protein products of the system were characterised by the CM-cellulose chromatography. Further, confirmation that authentic rabbit globin was synthesised in the lysate is desirable. However, analysis of the product by tryptic digestion or by co-electrophoresis of the in vitro synthesis product with carrier globin on SDS-polyacrylamide gels was precluded by the low specific activity of the in vitro synthesised protein. Furthermore, the in vitro synthesised α chain did not precisely co-chromatograph with the unlabelled α chain carrier. Such a finding is not without precedent. Nascent α chains isolated from reticulocyte polysomes were found to chromatograph one fraction ahead of the carrier α chain isolated from the supernatant fraction (Baglioni and Campana, 1966). A similar effect was noted by Schreier and Staehelin (1973). The α chain of globin synthesised in a homologous reconstituted cell-free system, primed with rabbit globin mRNA, was found to be displaced slightly from the in vivo synthesised α chain when assayed by chromatography on CM cellulose. It is possible that the

low rate of globin synthesis in feather lysate could reflect the need for tissue specific initiation factors not present in feather lysate (cf. Heywood, 1970; 1971; Rourke and Heywood, 1972). However, a more probable explanation is that the *in vitro* conditions were not optimal for globin mRNA translation. The feather cell-free system for these experiments was prepared in MSB. Under these conditions the capacity of the ribosomes for *de novo* synthesis of protein is rather low. The reason for the enhanced degree of translation of the α chain mRNA in comparison to the β chain mRNA remains obscure. It has been shown (Lodish and Nathan, 1972) that increasing the Mg^{++} concentration in reticulocyte lysate by two-fold caused the selective inhibition of β globin synthesis. However, variable ratios of α to β globin chain synthesis has been observed in heterologous cell-free systems primed with globin mRNA. For example, the ascites system generally synthesises an excess of β-globin (Mathews, 1972; Mathews *et al.*, 1972; Housman *et al.*, 1971; Sampson *et al.*, 1972) even though the globin mRNA derived from reticulocytes is known to contain more mRNA for α-globin than for β-globin (Lodish, 1971). In a cell-free system from liver, however, α-globin chains are produced in excess (Sampson *et al.*, 1972). The globin mRNP complex was translated in the feather cell-free system with a similar efficiency to the 9S globin mRNA itself. Similarly, the finding that globin mRNP can be translated in several cell-free systems as efficiently as free 9S mRNA has been reported by several workers (Lingrel *et al.*, 1971; Sampson *et al.*, 1972; Olsen *et al.*, 1972).

There is now general agreement as to the number and molecular weight of the proteins bound to several mRNA's (Blobel, 1972; 1973; Lebleu et al., 1971; Morel et al., 1971, 1973; Bryan and Hayashi, 1973). Nevertheless, some controversy remains as to whether the variable amounts of minor protein bands present in the mRNP are contaminants or fulfill some functional role (for a review see Williamson, 1973). Moroever, the proteins of the mRNP's do not appear to be the ribosomal initiation factors (Nudel et al., 1973).

It is obvious that before any final conclusions can be drawn about the ability of the 14-day feather lysate to translate globin mRNA and mRNP further studies must be undertaken. The variability of response of the system to the added mRNA and mRNP and the variable ratios of α and β globin chains synthesised cannot be reconciled on the present data. The titration of the globin mRNA and mRNP should be done in the feather cell-free system using a range of KCl and $MgCl_2$ concentrations. This will provide information as to the optimum concentrations of mRNA to prime the system and furthermore, the optimal conditions of the system for its translation. When these conditions have been determined it will be possible to conclude whether or not the apparent preferential synthesis of α globin chains is in fact real.

CHAPTER SIX

ISOLATION OF FEATHER KERATIN mRNA AND ITS TRANSLATION IN A RABBIT RETICULOCYTE CELL-FREE SYSTEM

A. INTRODUCTION

The approach to the problem of the isolation of feather keratin mRNA was altered following the unsuccessful attempts to obtain reproducible results in isolating it either by SDS treatment of polysomes (to release the free mRNA) or as an RNP complex by treating polysomes with EDTA. During the period of this earlier work Berns et al. (1972a) had reported that the mRNA coding for the lens crystallin protein αA_2 (mol. wt. 19,000) had an S value of 14. When this was compared with globin mRNA which was 9S and coded for a protein of 16,000 mol. wt. it was obvious that keratin mRNA could be larger than the 6-7S expected for a mRNA coding for a protein of mol. wt. 10,000. The presence of a sequence of adenylic acid residues (poly A) associated with vaccinia virus mRNA was reported by Kates (1970). Further work indicated that this could be true of most eukaryotic mRNA species (Lim and Canellakis, 1970; Edmonds et al., 1971; Darnell et al., 1971; Sheldon et al., 1971). Moreover, Lee et al. (1971b) and Brawermann et al. (1972) reported that polyA-containing RNA species were lost into the phenol phase, during the phenol extraction of polysomes if high concentrations of salt were present in the aqueous phase. The modified approach used was phenol-extraction of the 14 day feather polysomes (Lee et al., 1971b) followed by assaying this unfractionated RNA in a rabbit reticulocyte lysate cell-free system. It was becoming increasingly clear at this time that the reticulocyte lysate system was capable of translating mRNA species isolated from cells of non-erythroid origin. Examples of such mRNA species

translated include those of immunoglobulin light chain (Stavnezer and Huang, 1971); ovalbumin (Rhoads et al., 1971; Rosenfeld et al., 1972a,b); histones (Gallwitz and Briendl, 1972); crystallins (Berns et al., 1972a).

The initial aim was to establish that keratin mRNA was present in an undegraded form in total polysomal RNA and could be translated in reticulocyte lysate without the addition of any other component from feathers. The mRNA species itself could then be isolated by assaying in the reticulocyte cell-free system, fractions obtained by sucrose gradient centrifugation of the polysomal RNA. The present chapter describes the successful isolation of feather keratin mRNA using this approach.

B. METHODS

1. Precautions taken to minimise RNAse contamination

Rigorous precautions were taken to minimise RNAse contamination at all stages during the preparation and translation of the mRNA. All glassware was washed with 1 M KOH before use, rinsed thoroughly in bi-distilled water and dried at 100°. All solutions were prepared in alkali washed glassware and treated with 0.1% diethylpyrocarbonate overnight before use (Williamson et al., 1971). Pipettes were given a final rinse in bi-distilled water containing 0.1% diethylpyrocarbonate and dried at 110° overnight before use. Dialysis tubing used to dialyse rabbit reticulocyte lysate was allowed to reach boiling point in bi-distilled water containing 0.1% diethylpyrocarbonate and 1% $NaHCO_3$ and then rinsed twice in bi-distilled water.

Plastic disposable gloves were worn when preparing the lysate for dialysis. Ethanol and phenol were re-distilled before use in alkali washed apparatus which had been dried at 110° overnight before use.

2. Phenol extraction of polysomal RNA from chick embryo feathers and rabbit reticulocytes

The method of Lee et al. (1971b) was used except that extraction was carried out at room temperature. Centrifugation to separate the phenol and aqueous phases was at 5000 g for 10 minutes at 15°. The combined aqueous phase were pooled and precipitated overnight at -15° by the addition of 2.5 volumes of redistilled ethanol and 0.1 volume of 1 M NaCl. The RNA was collected by centrifugation at 20,000 g for 30 minutes at -10°. The supernatant was decanted and the RNA dried either using a stream of dry N_2 or in later preparations, in a vacuum desiccator over fused $CaCl_2$. For assaying in the cell-free system, RNA samples were dissolved in 10 mM Tris-HCl, pH 7.4 and aliquots were taken to determine the concentration. The absorbance of unity in a 1 cm cell at 260 nm (A_{260} = 1) was assumed to be a concentration of RNA of 40 µg/ml (Rosenfeld et al., 1972a).

3. Preparation of rabbit reticulocyte lysate

Lysate was prepared as described (see Chapter 2.B.4). In initial experiments lysate was freshly prepared before use, for later experiments larger batches were prepared and stored as 1 ml aliquots in liquid N_2. No loss of activity was found

on storage for three months.

4. Dialysis of reticulocyte lysate

Lysate was dialysed at 1° for 3 hours in 500 ml of the buffer used for dilution except that the DTT concentration was lowered to 0.5 mM. A buffer change was made after 1.5 hours.

5. Composition of cell-free system

The reaction mixture contained, in a total volume of 0.5 ml: 0.37 ml of lysate, 1 mM ATP, 0.2 mM GTP, 15 mM creatine phosphate, 50 µg creatine phosphokinase, 10 nmoles of each of the 19 amino acids (minus either serine, or leucine whichever was used as the label), 10 mM tris-HCl pH 7.4. The concentrations of KCl and $MgCl_2$ were adjusted to 125 mM and 3.8 mM respectively by adding aliquots of 1 M KCl and 100 mM $MgCl_2$. In some experiments the lysate volumes were altered (refer to figure captions for details).

The reticulocyte cell free systems were incubated at 25°, to overcome the need for adding exogenous haemin (Adamson et al., 1968; Hunt et al., 1972; Gross and Rabinovitz, 1973).

6. Identification of products synthesised in rabbit reticulocyte lysate

The post-ribosomal supernatants were prepared by centrifugation of incubation mixtures at 190,000 g for 1 hour (4°C) in a 'Beckman Ti 50' rotor using either an 'L-50' or 'L2-50' ultracentrifuge. The supernatants were treated to remove haem using the method of Schapira et al. (1968) as modified by

Lane et al. (1971).

7. Preparation and S-carboxymethylation of haem-free post-ribosomal supernatants of incubation mixtures

The method was essentially that described by Harrap and Woods (1964ab) as modified by Kemp et al. (1974). The centrifugation step was omitted. The S-carboxymethylated proteins were dialysed exhaustively against distilled water and then freeze-dried.

8. DEAE-cellulose chromatography of SCM-proteins from reticulocyte lysate supernatants

The freeze-dried preparations were each dissolved in 2 ml of buffer (8 M urea, 1 mM EDTA and 10 mM Tris-HCl, pH 7.0) and fractionated on 1 x 8 cm columns of DEAE-cellulose. Elution was with a linear gradient of KCl (200 ml, 0-0.15 M) dissolved in the loading buffer. Flow rate was 10 ml/hour and 6.0 ml fractions were collected.

Aliquots from fractions were treated with 20% TCA for the determination of acid insoluble radioactivity.

9. Polyacrylamide gel electrophoresis of radioactive proteins

Samples containing known amounts of protein were subjected to polyacrylamide gel electrophoresis at pH 2.7, using ethylene diacrylate as the cross-linking agent as described by Kemp et al. (1974).

10. Electrophoresis of radioactive proteins on 8M urea/ SDS gels

Known aliquots of radioactive proteins were co-electrophoresed with SCM keratin on the SDS/8M urea gel system devised by Swank and Munkres (1971). After staining and scanning the gels at 515 nm the gels were sliced into 1 mm sections and counted in the NCS-based toluene scintillation fluid of Ward et al. (1970) as modified by Hopgood (personal communication). This scintillation fluid was prepared using 0.3 ml NCS and 0.025 ml 8 N NH_4OH for 2.2 ml of toluene, POPOP/PPO. Aliquots of 2 ml dispensed into 2.5 ml scintillation vials. The gels were serially sliced into 1 mm sections and each section was placed directly into the scintillation cocktail. The vials were capped and allowed to stand at room temperature for 24 hours, before counting. A check revealed that there was no further increase in radioactivity released from the gel slice after this time.

11. Immunoprecipitation of keratin synthesised in rabbit reticulocyte lysate

Antisera against native adult feather keratin was raised in a goat by Dr. D.J. Kemp.

Generally, two equal aliquots were taken from post-ribosomal supernatants of the incubation mixtures and to each was added 10 µl of goat anti-keratin serum or 10 µl of normal serum. The mixtures were then incubated at 37°C for 1 hour. Rabbit-anti goat serum (50 µl) was added to each tube to precipitate the goat serum protein. The mixtures were allowed to stand at 4° to complete the

precipitation. The protein was pelleted by centrifugation at 3,000 g for 10 min. The supernatants were decanted and the pellets resuspended in 1 ml of polysome buffer to remove non-specifically adsorbed material. The protein was allowed to settle by standing the tubes on ice for 15 min. and recentrifuged. The protein pellet was resuspended in the buffer, collected on GF/C discs and dried at 110° for determination of radioactivity.

It should be noted that the overall efficiency of the two step immunoprecipitation procedure in precipitating labelled keratin from the cell-free system was only about 50% (Kemp, personal communication).

12. Isolation of acetylated N-terminal peptides from keratin synthesised in the lysate system

Labelled keratin synthesised in vitro was mixed with 40 mg of SCM-feather keratin and dissolved in 4 ml of 0.2 M N-ethylmorpholine acetate, pH 8.3 and incubated with 1.0 mg of chymotrypsin, for 24 hours at 37°. The enzymic digest was adjusted to pH 5.2 by the addition of acetic acid and immediately fractionated by chromatography on Dowex AG 50W-X8, 100-200 mesh (H^+ form), (Narita, 1958; O'Donnell, 1971).

The bound fraction was eluted from the column with 70 ml of 1 N NH_4OH. Both fractions were taken to dryness by rotary evaporation at 40°. The residue of the non-bound fraction was dissolved in 1 ml of water and applied to a column of Dowex 1x8, -400 mesh (formate form) and eluted with a non-linear formic acid gradient (270 ml, 0.1-0.5 M)(Kemp, 1972).

The eluate was automatically monitored by the ninhydrin reaction after alkaline hydrolysis using a Technicon autoanalyser and 3 ml fractions were collected. Aliquots (1 ml) were dried at 100°C and counted in NCS-based toluene scintillation fluid (see Methods 6.10)

13. Polyacrylamide gel electrophoresis of RNA samples

Electrophoresis of RNA in 98% formamide was based on the system devised by Staynov et al. (1972) with the modification suggested by Gould and Hamlyn (1973), except that Zeokarb 225 was used instead of Amberlite to deionize the formamide. Acrylamide and bis-acrylamide were recrystallised before use (Loening, 1967). Cylindrical gels (8 cm x 6 mm diam.) were prepared in Perspex tubes and overlayed with water to give level gel surfaces during polymerisation. The gels were allowed to polymerise overnight at 37° and pre-electrophoresed for 1 hr at 1 mA/gel before use. The RNA samples were dissolved in 50 µl of loading buffer (Gould and Hamlyn, 1973) and shaken at 37° for 15 min in a water bath before loading. Electrophoresis was carried out at 1.0-1.5 mA/gel until the tracker dye band of bromophenol blue just reached the bottom of the gels (about 24 hr). In initial experiments the gels were soaked for 3 hr in 10% HAc, and stained in 0.5% toluidene blue (dissolved in 55 mM NaAc, 0.1 mM EDTA, pH 5.5). The gels were destained overnight in distilled water. In later experiments the gels were stained using 'stains all' (Dahlberg et al., 1969). The gels were scanned in a Gilford linear transport attachment coupled to a W & W recorder. The distance that the RNA bands

migrated from the origin was then accurately plotted.

C. RESULTS.

1. Characterisation of proteins synthesised in a reticulocyte cell-free system in response to added 14-day feather polysomal RNA

Fig. 6.1 shows the radioactivity profiles obtained by chromatographic separation on DEAE-cellulose of carboxymethylated reticulocyte cell-free system post-ribosomal supernatants after incubation of about 75 µg of phenol-extracted 14-day feather polysomal RNA. The main peak of radioactivity eluting at the gradient front was the *in vitro* synthesised globin. This had a much higher specific activity with ^{14}C-algal hydrolysate than when ^{3}H-serine was used. In contrast, the peak eluting after globin, in the region expected for keratin was labelled to a greater extent relative to globin when ^{14}C-serine was used as the label. To determine whether the radioactivity in the latter fraction was synthesised keratin the fractions indicated were pooled, exhaustively dialysed against distilled water and freeze-dried. Aliquots were co-electrophoresed with unlabelled keratin on soluble polyacrylamide gels at pH 7.5. The gels were prepared for counting, as described, (see Methods 2.B6,7).

Fig. 6.2a-c shows the results obtained. The major peaks of radioactivity precisely coincided with the major keratin bands β3 and β4. The absorbance in the keratin region of the gel was almost entirely due to added carrier protein, whereas the absorbance in the lower mobility region of the gel was due to protein from the lysate co-chromatographing with

FIGURE 6.1. CHARACTERISATION BY DEAE CELLULOSE CHROMATOGRAPHY OF PROTEINS SYNTHESISED IN A RETICULOCYTE CELL-FREE SYSTEM IN RESPONSE TO ADDED FEATHER POLYSOMAL RNA

Figure 6.1a. A reticulocyte cell-free system prepared as described (see Methods 6.B5) was incubated with 2.5 μC of ^{14}C-algal hydrolysate at 25^{o} for 3 hr in the presence of 75 μg of phenol extracted 14 day feather polysomal RNA. The post-ribosomal supernatant was prepared as described (see Methods. 6.B.6 & 7) and fractionated on a column of DEAE-cellulose (see Methods 6.B.8). Aliquots (1 ml/6 ml) of the column fractions were precipitated with TCA for determination of radioactivity.

Figure 6.1b. As for (a) except that the cell-free system was incubated with ^{3}H-serine (25 μC, S.Act. 1.2 C/mM).

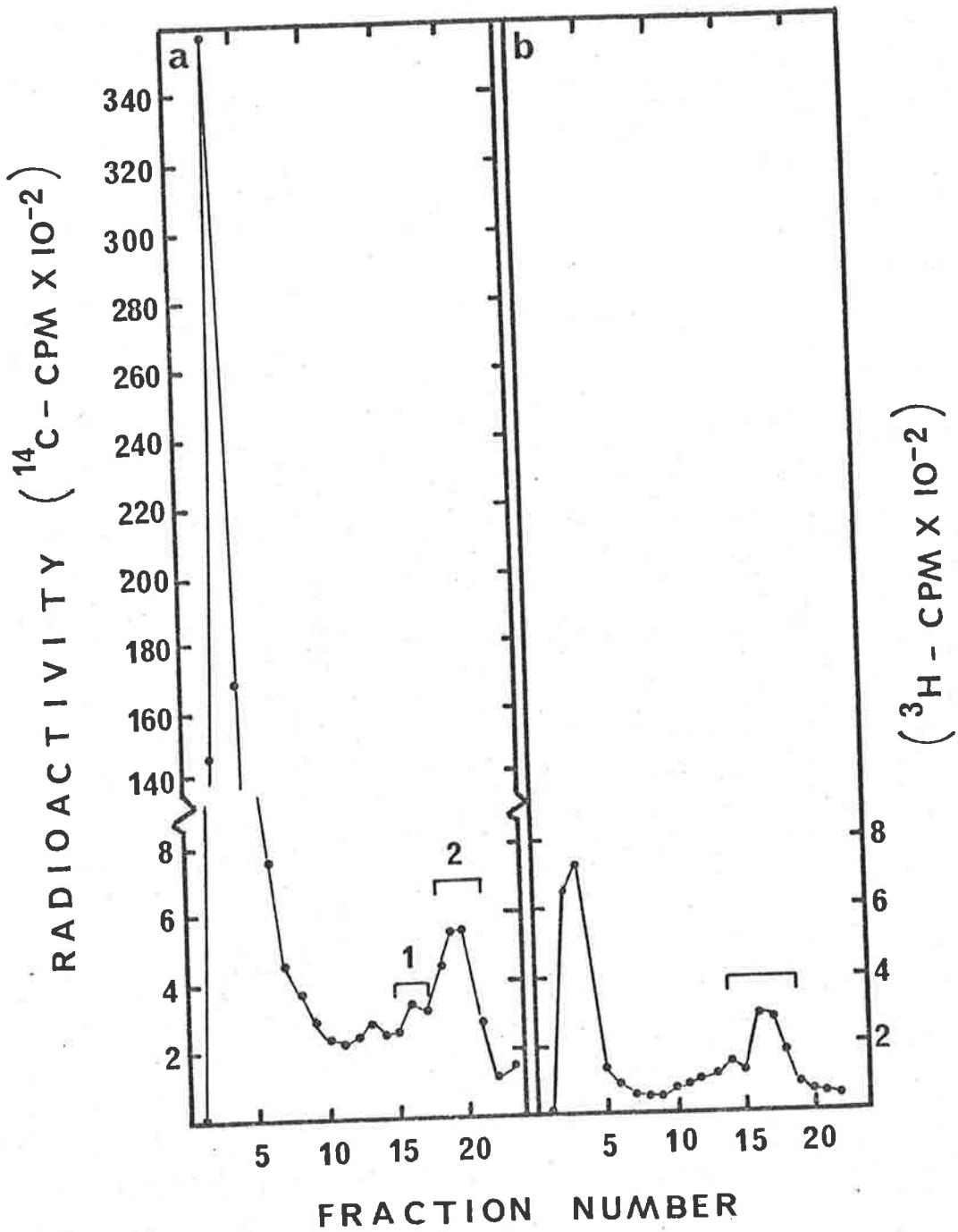

a
b
340
320
300
280
260
240
220
200
180
160
140
8
6
4
2
1
2
5
10
15
20
5
10
15
20
8
6
4
2
RADIOACTIVITY (^{14}C - CPM X 10^{-2})
(^{3}H - CPM X 10^{-2})
FRACTION NUMBER

FIGURE 6.2. POLYACRYLAMIDE GEL ELECTROPHORESIS AT pH 7.5 of IN VITRO SYNTHESISED PROTEIN.

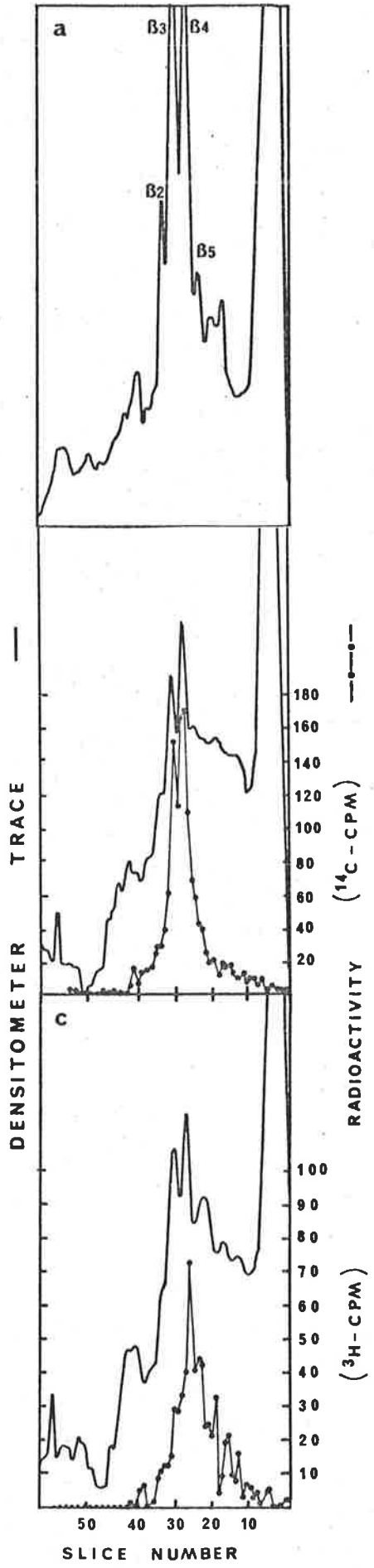
The soluble gels were prepared and stained as described in Figure 4.7.

The gels were scanned using a Joyce Loebel Chromoscan (see Methods 2.B.6) and sliced (see Methods 2.B.7) for determination of radioactivity.

Figure 6.2a. Electrophoresis of 100 µg of embryonic chick feather keratin.
Keratin bands β2 - β5 are indicated.

Figure 6.2b. Co-electrophoresis of 100 µg of feather keratin with the material, isolated from pooled fractions (2) as shown in Fig. 6.1a.

Figure 6.2c. As for (b) except that the material was isolated from the pooled fractions as shown in Fig. 6.1b.


a
ß3
ß4
ß2
ß5
DENSITOMETER TRACE
180
160
140
120
100
80
60
40
20
(14C - CPM)
RADIOACTIVITY
c
100
90
80
70
60
50
40
30
20
10
(3H - CPM)
50 40 30 20 10
SLICE NUMBER

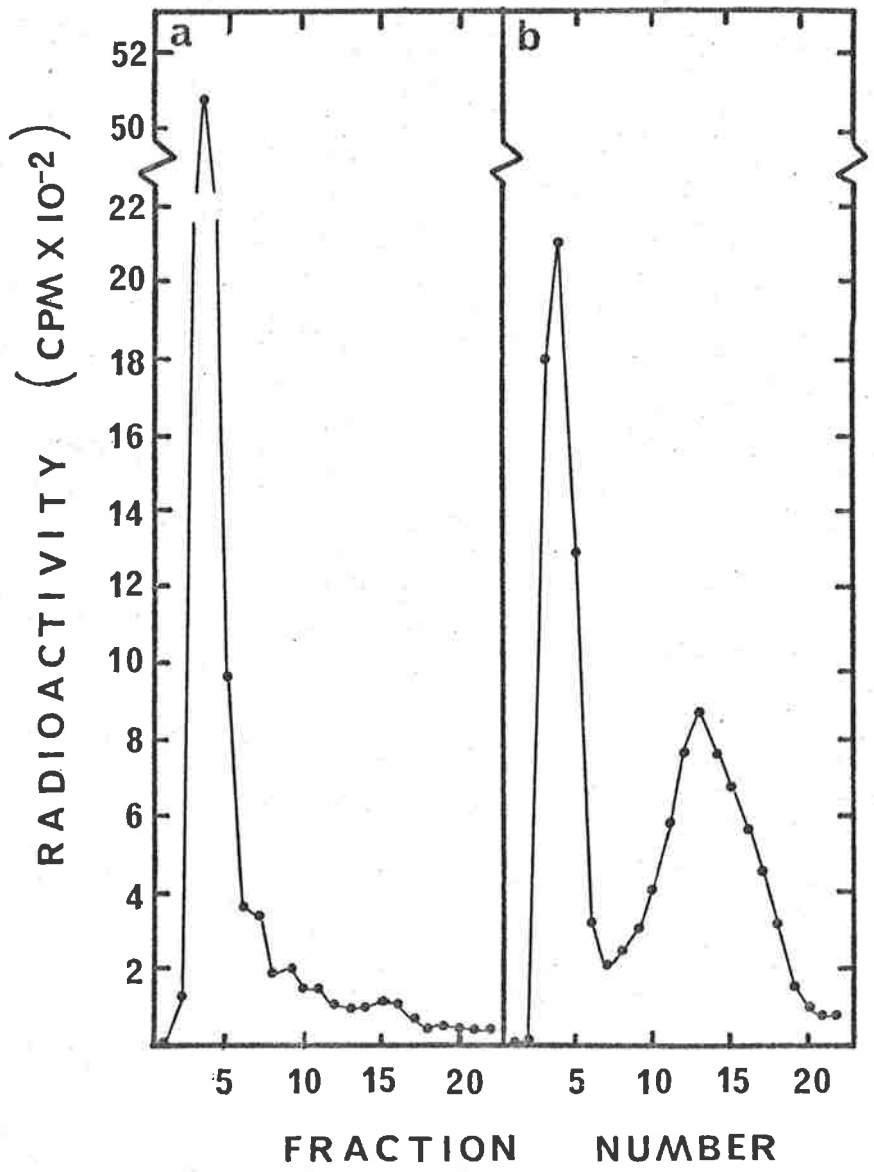
keratin. It was not present in the gel of unlabelled keratin alone (Fig. 6.2a). Pooled fraction one, from the ^{14}C-algal hydrolysate labelled preparation contained no radioactivity on the gel (result not shown) and was presumed to be endogenous protein synthesis of the reticulocyte cell-free system. From this result it was concluded that keratin was synthesised in reticulocyte lysate in response to the added feather polysomal RNA. It appeared that it was advantageous to use radioactive serine for following keratin synthesis presumably as the content of this amino acid was 50% higher in keratin at 12 moles per cent (Kemp and Rogers, 1972) than in globin which has 8 moles per cent (Dayhoff, 1969).

The experiment was repeated using dialysed reticulocyte lysate to lower the endogenous serine pool and hence increase the specific activity of *in vitro* synthesised product. It is known that the level of free serine is very high in reticulocytes (P. Tolstoshev - personal communication). Fig. 6.3a,b shows the radioactivity profiles obtained by DEAE-cellulose chromatography of the products synthesised *in vitro* in response to the addition of about 200 µg of feather polysomal RNA and control lysate, that is, lysate incubated without exogenous RNA. As before, addition of the feather RNA resulted in a peak of radioactivity chromatographing in the region expected for keratin whereas this peak was essentially absent in the control. Confirmation of the nature of the product was obtained by co-electrophoresis of the pooled fractions with carrier keratin (result not shown).

FIGURE 6.3. CHARACTERISATION BY DEAE CELLULOSE CHROMATOGRAPHY OF PROTEINS SYNTHESISED IN A RETICULOCYTE CELL-FREE SYSTEM IN THE PRESENCE AND ABSENCE OF FEATHER POLYSOMAL RNA.

Figure 6.3a. Details as given in Fig. 6.1a except that dialysed reticulocyte lysate (see Methods 6.B.4) was used.
No exogenous RNA was added and the cell-free system was incubated with ^{3}H-serine (10 μC, S.Act. 1.2 C/mM).

Figure 6.3b. As for (a) except that approximately 200 μg of 14-day feather polysomal RNA was added to the cell-free system.


a
b
52
50
22
20
18
16
14
12
10
8
6
4
2
RADIOACTIVITY (CPM X IO-2)
5
10
15
20
5
10
15
20
FRACTION NUMBER

2. Sucrose gradient sedimentation of 14-day feather polysomal RNA

Fig. 6.4a shows the absorbance profile of total feather polysomal RNA fractionated by sucrose gradient centrifugation. In addition to the major peaks of RNA, the 18S, 28S rRNA's and tRNA peak (which also contains the 5S rRNA from the 60S subunit (Comb and Zehavi-Willner, 1967; Knight and Darnell, 1967) two minor peaks of RNA sedimenting at 12S and 14S respectively were apparent. The S values of these minor RNA species were estimated (Fig. 6.5a) using the method of Martin and Ames (1961) as described in Methods 6B.11. When the centrifugation conditions were altered (see Fig. legend for details) the 14S RNA was unresolved from the 18S and RNA and a further RNA species sedimenting at 9S was observed (Fig. 6.4b). This result should be compared with the absorbance profile of total phenol-extracted. rabbit reticulocyte RNA shown in Fig.3b.4c. The result is in accord with the published data (for example, Williamson et al., 1971; Lingrel et al., 1971) in that there are three minor RNA species sedimenting in the 7 - 17S region, with S values of 9, 12, and 15 respectively (see Fig. 6.5b). The 9S RNA isolated from rabbit reticulocyte polysomes codes for the α and β globin chains (Housman et al., 1971; Lane et al., 1971; Mathews, 1972; Metafora et al., 1972; Sampson et al., 1972; Lockard and Lingrel, 1973). The 12S and 15S RNA species are of uncertain origin (Williamson and Morrison, 1971).

FIGURE 6.4. SUCROSE GRADIENT SEDIMENTATION OF POLYSOMAL RNA

Figure 6.4a. About 25 A_{260} units of 14-day feather polysomal RNA were dissolved in 0.5 ml of buffer (30 mM NaAc, 20 mM EDTA, 40 mM Tris-HCl, pH 7.5, 0.5% SDS) and layered over a 10-40% w/v sucrose gradient prepared in the same buffer, but without SDS.
Centrifugation was for 15 hr at 147,638 g (a.v.) at 4^{o}. The absorbance was monitored using an LKB ultraviolet analyser (see Methods 2.B.9).

Figure 6.4b. Details as for (a) except that the centrifugation was for 16 hr at 201, 125 g (a.v.) at 4^{o}.

Figure 6.4c. About 50 A_{260} units of phenol-extracted rabbit reticulocyte polysomal RNA was analysed in a similar manner to that given in (a). Centrifugation conditions were as given in (b).

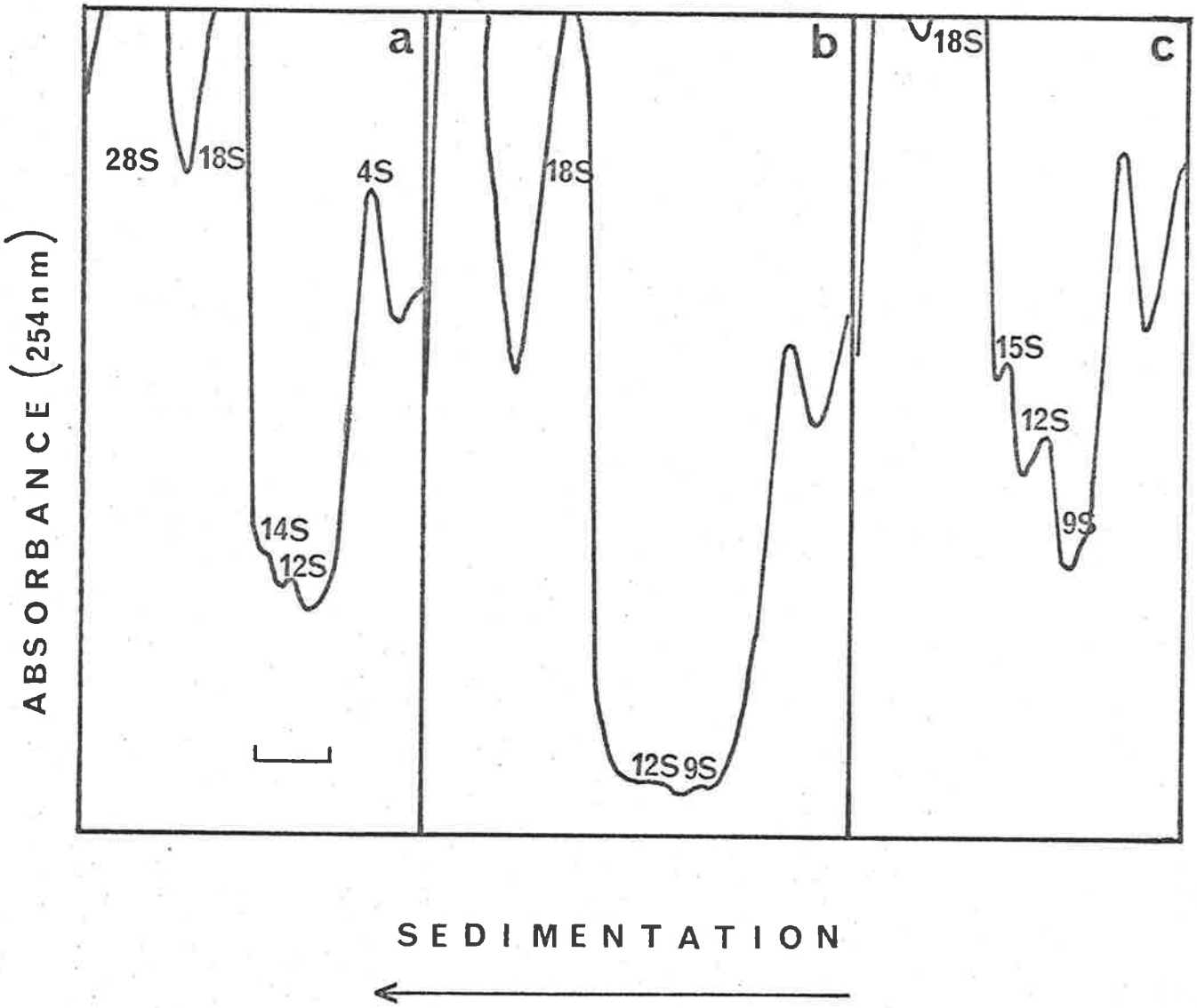
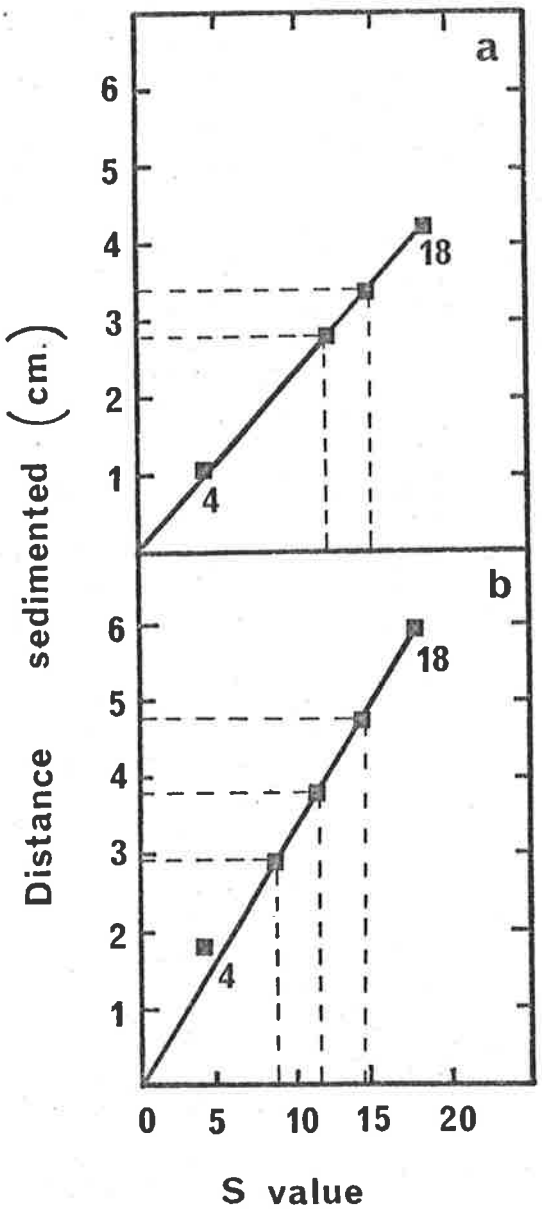

a
b
c
28S
18S
4S
14S
12S
18S
12S 9S
18S
15S
12S
9S
ABSORBANCE (254nm)
SEDIMENTATION

FIGURE 6.5. ESTIMATION OF S VALUE OF RNA SPECIES DETECTED BY SUCROSE GRADIENT SEDIMENTATION

Figure 6.5a. The tRNA (4S) and the 18S rRNA were used as standards (see Methods 2.B.10) to determine the S value of the two minor peaks of RNA shown in Fig. 6.4a.

Figure 6.5b. The S value of the three minor peaks of RNA shown in Fig. 6.4c were determined by the method described in (a).


a
b
18
4
18
4
6
5
4
3
2
1
0
5
10
15
20
Distance sedimented (cm.)
S value

3. Experiments to determine the location of keratin mRNA activity in the sucrose-gradient fractionated feather polysomal RNA

A total of approximately 100 A_{260} units of polysomal RNA was centrifuged on 4 gradients.

The absorbance profiles of all 4 gradients were identical to that shown in Fig. 6.4a. The gradients were pooled as shown into the 28S, 18S, 7-17S and tRNA regions. The RNA in these fractions was ethanol precipitated, and dried as described (see Methods 6.B2). The fractions (half of the total 7-17S region RNA only) were incubated individually in 0.5 ml reticulocyte cell-free systems using ^{14}C-leucine to label the *in vitro* synthesised products. Figure 6.6a-e shows the radioactivity profiles of the chromatographic separation on DEAE-cellulose of SCM-derivatives of the products in the post-ribosomal supernatants prepared as described previously. In this experiment unlabelled SCM-keratin was chromatographed to determine precisely in which fractions the keratin eluted. Fig. 6.6f shows the absorbance profile (A_{280}) of the unlabelled keratin. The greatest amount of labelled amino acid incorporated in the keratin region was obtained with the 7-17S RNA (Fig. 6.6c). A lesser degree of incorporation was obtained with the added tRNA and 18S RNA species (Fig. 6.6b,d). The 28S RNA significantly lowered the total incorporation and no labelling in the keratin region was apparent (Fig. 6.6d). To completely substantiate that the labelled material was keratin the indicated fractions were pooled, dialysed and freeze-dried. Aliquots were co-electrophoresed on soluble polyacrylamide gels at pH 2.7 (see Methods 6.B9) with

FIGURE 6.6. CHARACTERISATION BY DEAE-CELLULOSE CHROMATOGRAPHY OF PROTEINS SYNTHESISED IN A RETICULOCYTE CELL-FREE SYSTEM IN RESPONSE TO ADDED FEATHER POLYSOMAL RNA FRACTIONATED BY SUCROSE GRADIENT SEDIMENTATION.

Figure 6.6a. A dialysed reticulocyte cell-free system (0.5 ml) was incubated with 1.5 μC of ^{14}C-leucine (S. Act. 312 mC/mM) for 3 hr at 25°.
No exogenous RNA added.
Other details as given in Fig. 6.1a.

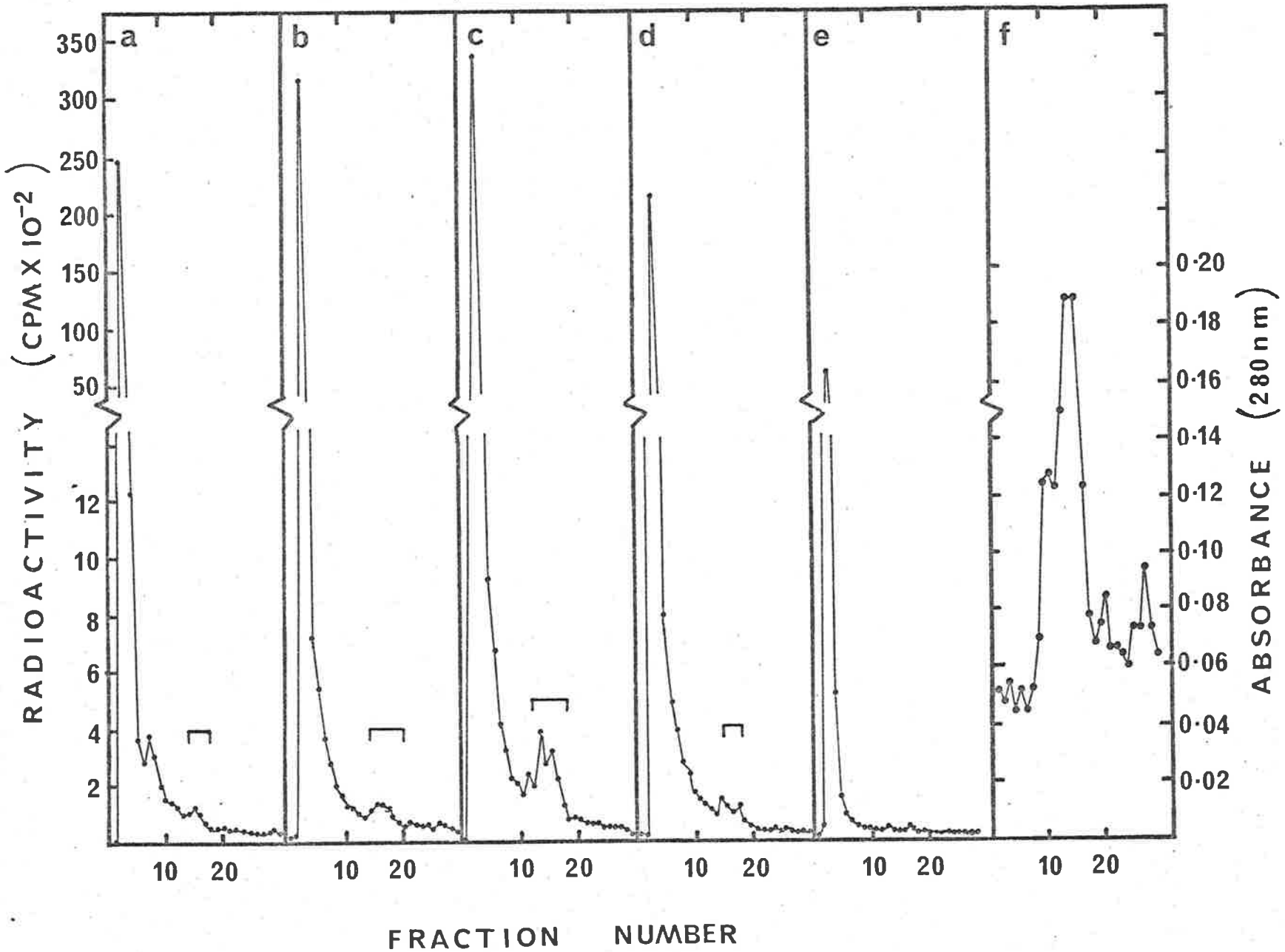
Figure 6.6b. As for (a) except that the tRNA fraction was isolated from 14-day feather polysomal RNA and incubated in the cell-free system.

Figure 6.6c. Addition of 7-17S region RNA.

Figure 6.6d. Addition of 18S rRNA fraction.

Figure 6.6e. Addition of 28S rRNA fraction.

Figure 6.6f. Chromatography of 10 mg of chick feather keratin.


a
b
c
d
e
f
350
300
250
200
150
100
50
12
10
8
6
4
2
RADIOACTIVITY (CPM X 10⁻²)
0·20
0·18
0·16
0·14
0·12
0·10
0·08
0·06
0·04
0·02
ABSORBANCE (280 nm)
10
20
FRACTION NUMBER

labelled SCM-feather keratin carrier.

The results are shown in Fig. 6.7a-d. Keratin bands A-E of Kemp and Rogers (1972) are indicated. Clearly the maximum stimulation of radioactivity incorporation which is co-incident with the absorbance profile of keratin occurred with the added 7-17S RNA fraction, with lesser stimulation of incorporation by the 18S RNA. No counts above background were present in either the control lysate (i.e., without added RNA) or with the tRNA fraction (Fig. 6.7a,b). The results from both the DEAE-column chromatography and polyacrylamide gel electrophoresis at pH 2.7 demonstrated that the 8-17S RNA fraction was the one most highly enriched in keratin mRNA activity.

The experiment was repeated exactly as has been described except that ^{14}C-serine was used to label the *in vitro* synthesised protein. The DEAE-column profiles obtained are shown in Fig. 6.8a-d. In agreement with the previous result the main stimulation of keratin synthesis was promoted by the addition of the 7-17S RNA fraction (Fig. 6.8b), with progressively less stimulation by the 18S and 28S RNA species (Fig. 6.8c and d). The tRNA fraction gave little or no stimulation of keratin synthesis. The fractions from the DEAE-cellulose columns were pooled as shown and prepared for polyacrylamide gel electrophoresis. Fig. 6.9a-c shows only the radioactivity profiles of the samples electrophoresed on soluble polyacrylamide gels at pH 7.5. It should be noted that for samples 6.9b and c the total material obtained was electrophoresed whereas for 6.9a only one quarter of the total material obtained was loaded onto the gel.

FIGURE 6.7. POLYACRYLAMIDE GEL ELECTROPHORESIS AT pH 2.7 OF IN VITRO SYNTHESISED PROTEIN

The pH 2.7 gels were prepared as given in Figure 4.8. Keratins bands A - E are indicated.

Figue 6.7a. Co-electrophoresis of the material isolated from pooled fractions showed in Fig. 6.6a, with 100 µg of chick embryo keratin.

Figure 6.7b. As for (a) except that material was isolated as shown in Fig. 6.7b.

Figure 6.7c. As for (a) except that one-half of the material isolated as shown in Figure 6.7c was electrophoresed.

Figure 6.7d. As for (a) except the material was isolated as shown in Fig. 6.6d.

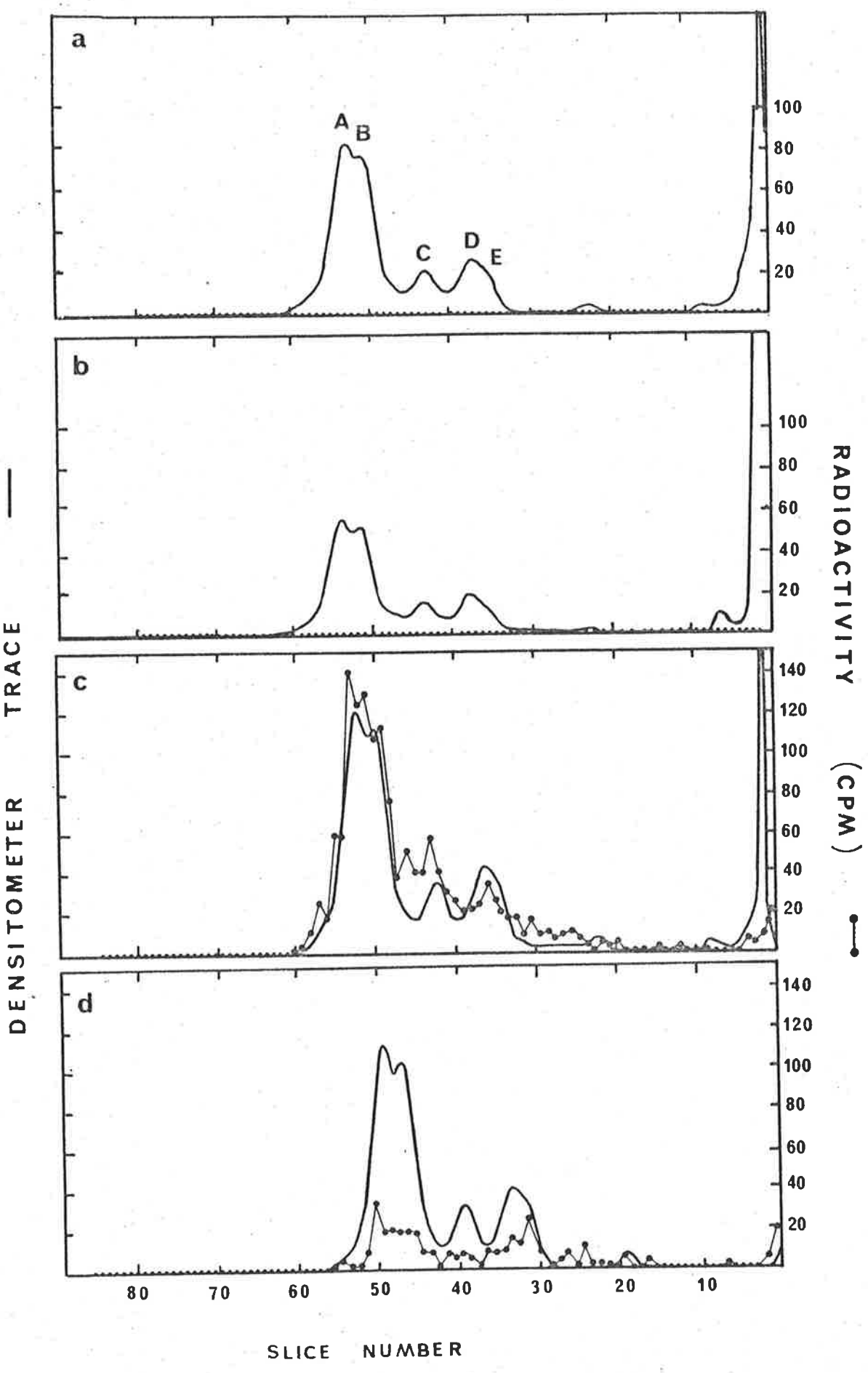

a
A
B
C
D
E
100
80
60
40
20
b
100
80
60
40
20
c
140
120
100
80
60
40
20
d
140
120
100
80
60
40
20
80
70
60
50
40
30
20
10
SLICE NUMBER
DENSITOMETER TRACE
RADIOACTIVITY (CPM)

FIGURE 6.8. CHARACTERISATION BY DEAE-CELLULOSE CHROMATOGRAPHY OF PROTEINS SYNTHESISED IN A RETICULOCYTE CELL-FREE SYSTEM IN RESPONSE TO ADDED FEATHER POLYSOMAL RNA FRACTIONATED BY SUCROSE GRADIENT SEDIMENTATION.

Details as given in Fig. 6.6 except that the cell-free system (2.0 ml) was incubated with ^{3}H-serine (10 µC, S.Act. 1.2 C/mM).

Figure 6.8a. Chromatographic separation of <u>in vitro</u> products synthesised when feather tRNA added.

Figure 6.8b. Addition of 7-17S region RNA.

Figure 6.8c. Addition of 18S rRNA.

Figure 6.8d. Addition of 28S rRNA.

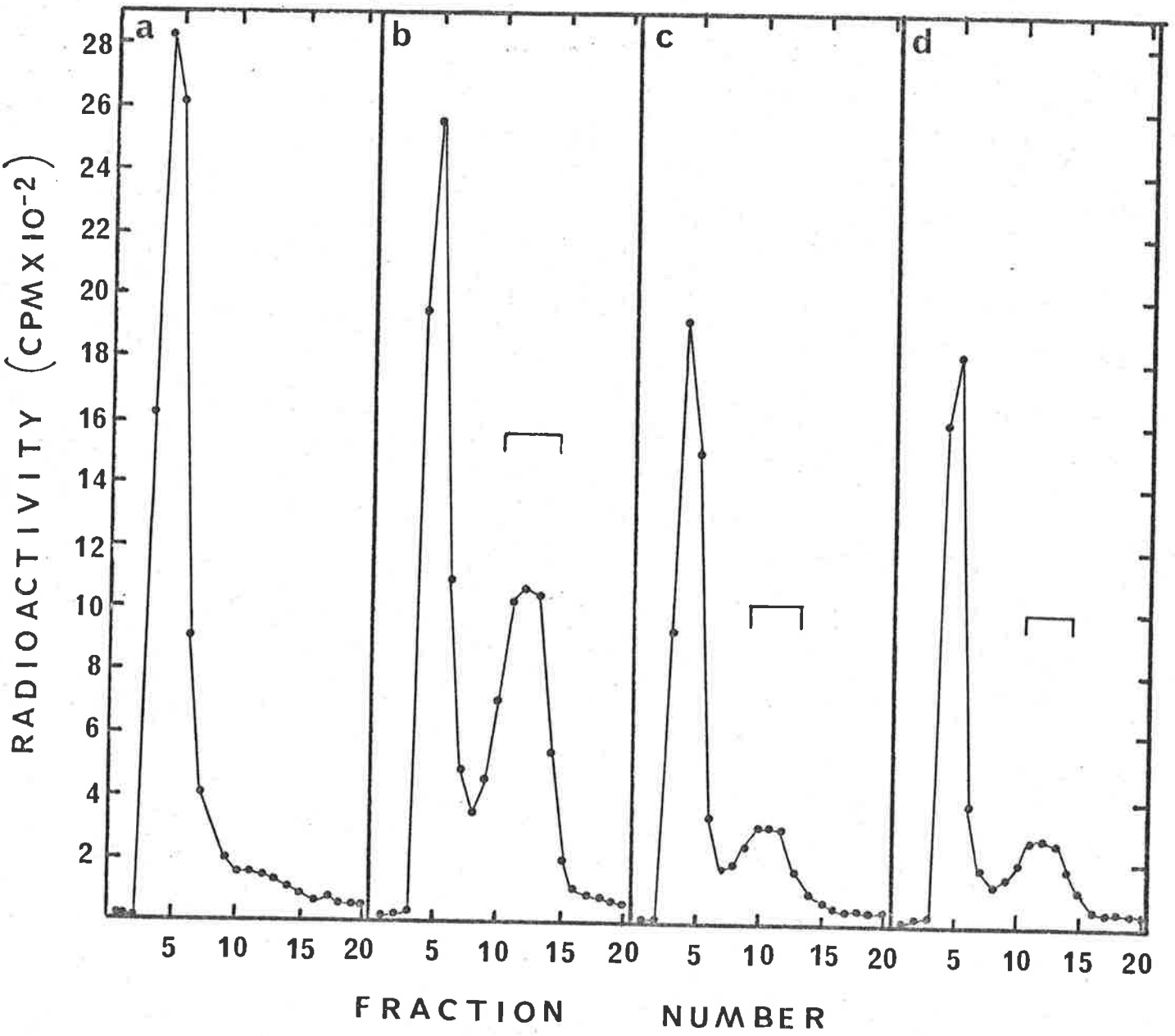

a
b
c
d
RADIOACTIVITY (CPM X IO-2)
28
26
24
22
20
18
16
14
12
10
8
6
4
2
5
10
15
20
FRACTION NUMBER

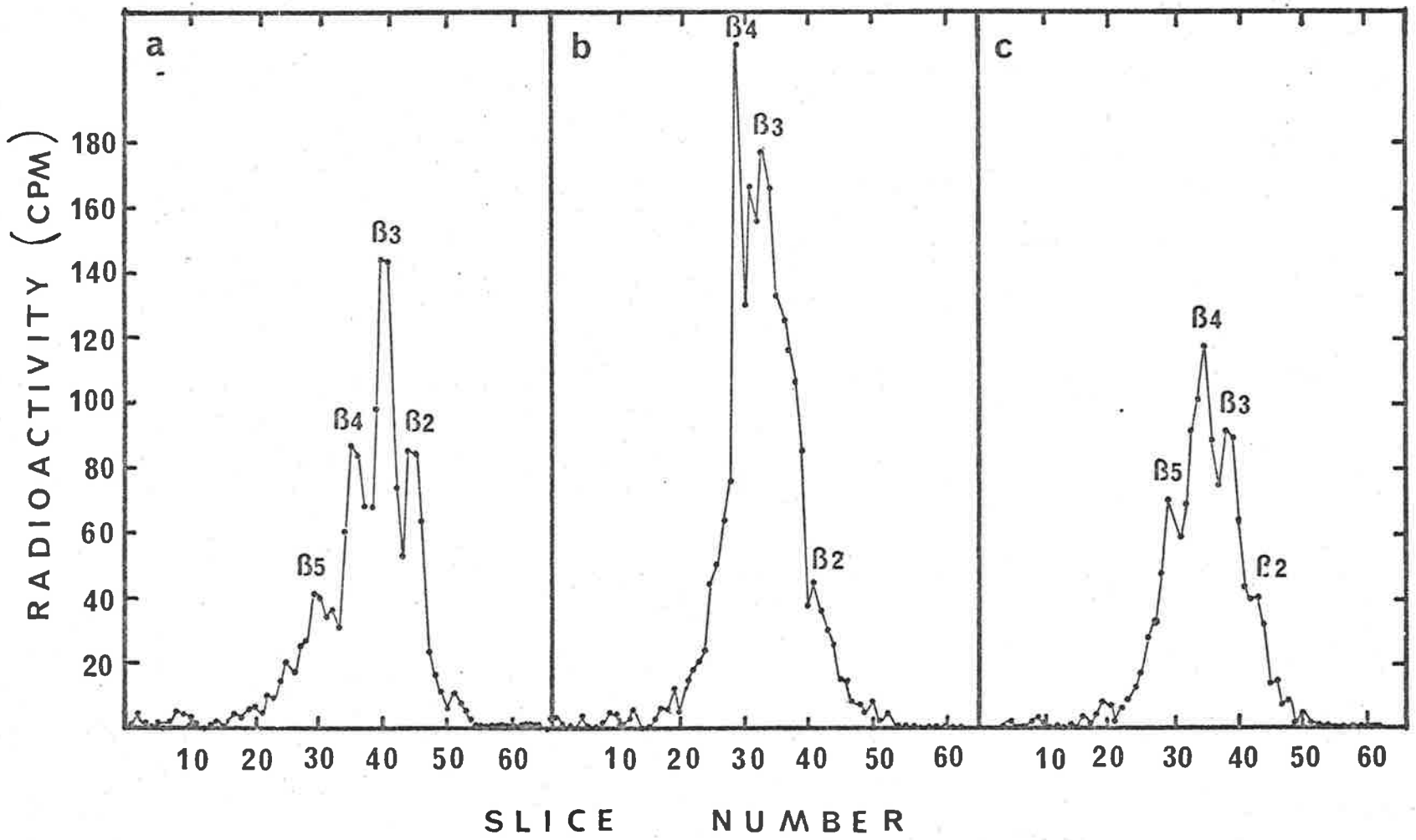
FIGURE 6.9. POLYACRYLAMIDE GEL ELECTROPHORESIS AT pH 7.5 OF IN VITRO SYNTHESISED PROTEIN

The pH 7.5 gels were prepared as described in Figure 5.7a, except that no carrier keratin was added.

Figure 6.9a. Electrophoresis of one half of the material isolated as shown in Fig. 6.8b.

Figure 6.9b. Electrophoresis of material isolated as shown in Fig. 6.8c.

Figure 6.9c. Electrophoresis of material isolated as shown in Fig. 6.8d.


a
b
c
ß5
ß4
ß3
ß2
RADIOACTIVITY (CPM)
20
40
60
80
100
120
140
160
180
10 20 30 40 50 60
SLICE NUMBER

4. Purification of the 8-17S region RNA species by a second cycle of sucrose gradient centrifugation

To determine which species of RNA sedimenting in the 8-17S region coded for the feather keratin the RNA from this region of 4 gradients similar to that shown in Fig. 6.4a were pooled, reprecipitated and recentrifuged on a sucrose gradient (see legend to Fig. 6.7a). Fig. 6.10a shows the absorbance profile obtained. Clearly the major species present (other than the tRNA and 18S rRNA species) are the 14S and 12S species with the 9S RNA sedimenting as a shoulder on the 12S RNA peak.

For comparison Fig. 6.10b shows the profile obtained on recentrifugation of the 7-17S pooled RNA fractions from rabbit reticulocyte polysomal RNA. A plot of S value, taking the 4S tRNA and 18S rRNA as standards, indicated that the S values of the three peaks were 9, 12 and 15 respectively. That is, the same species of RNA were resolved in the recentrifugation as in the first purification run (see Fig. 6.5b).

5.a. Assay for keratin mRNA activity in purified feather polysomal RNA fractions

The 12S, 14S and 18S peaks of RNA isolated from feather polysomes isolated from a second cycle of sucrose density centrifugation were isolated as shown (see Fig. 6.10a). These fractions were tested for keratin mRNA activity in a reticulocyte cell-free system. Incubation mixtures of 0.5 ml, each containing 5 μC of ^{14}C-serine to label the products were used.

For comparison, total 7-17S region RNA was added to one incubation mix. Fig. 6.11a-e shows the DEAE-chromatography profile

FIGURE 6.10. PURIFICATION OF 7-17S REGION RNA SPECIES BY A SECOND-CYCLE OF SUCROSE-GRADIENT CENTRIFUGATION

Figure 6.10a. The 7-17S RNA from four gradients of feather polysomal RNA was pooled as shown in Fig. 6.4a and rerun on a gradient prepared as described in Fig. 6.4. Centrifugation was for 16 hr at 201, 125 g (a.v.) at 3^{o}.

Figure 6.10b. Recentrifugation of 7-17S RNA from two gradients of rabbit reticulocyte polysomal RNA pooled as shown in Figure 6.4c. Other details as in (a).

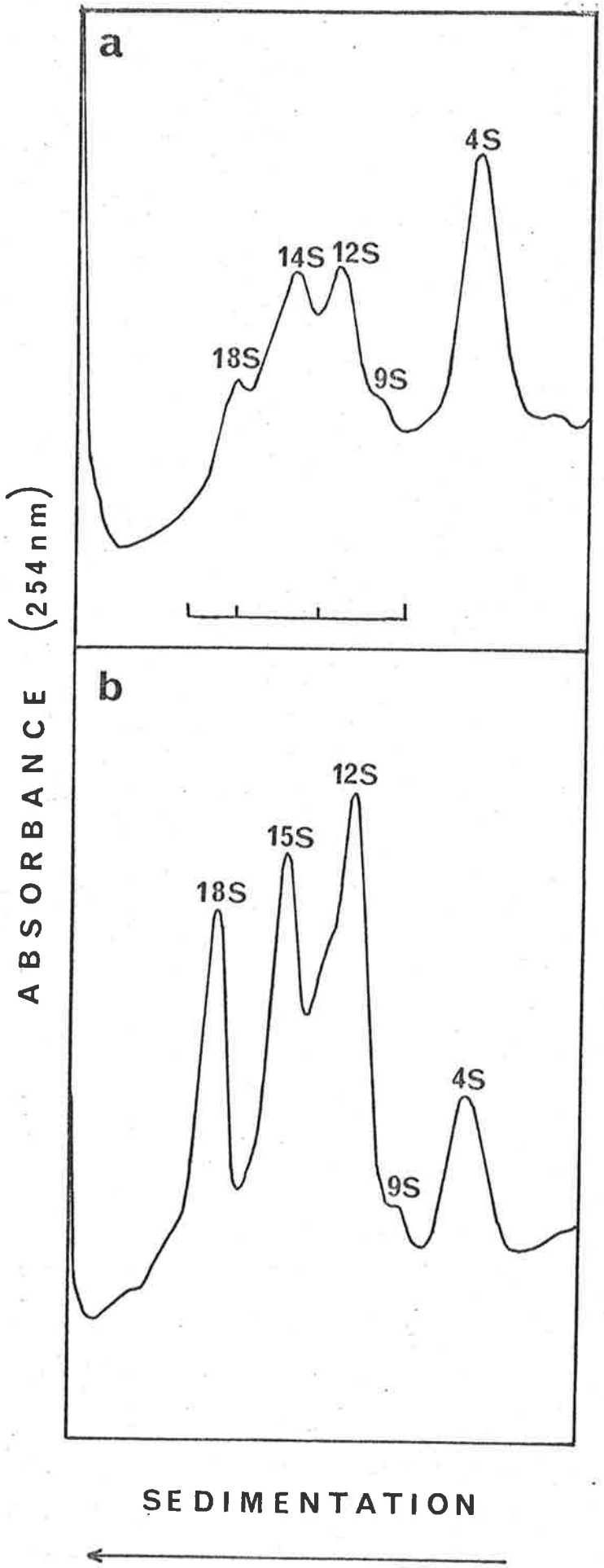

a
4S
14S
12S
18S
9S
b
12S
15S
18S
4S
9S
ABSORBANCE (254nm)
SEDIMENTATION

FIGURE 6.11. KERATIN mRNA ACTIVITY IN PURIFIED FEATHER POLYSOMAL RNA FRACTIONS AS ASSAYED BY DEAE-CELLULOSE CHROMATOGRAPHY OF IN VITRO SYNTHESISED PRODUCTS.

Details of chromatography of post-ribosomal supernatants as given in Fig. 6.1.

Figure 6.11a. Radioactivity profile of fractions eluted from the column when no exogenous RNA added to cell-free system.

Figure 6.11b. Crude 7-17S feather polysomal RNA added (40 µg).

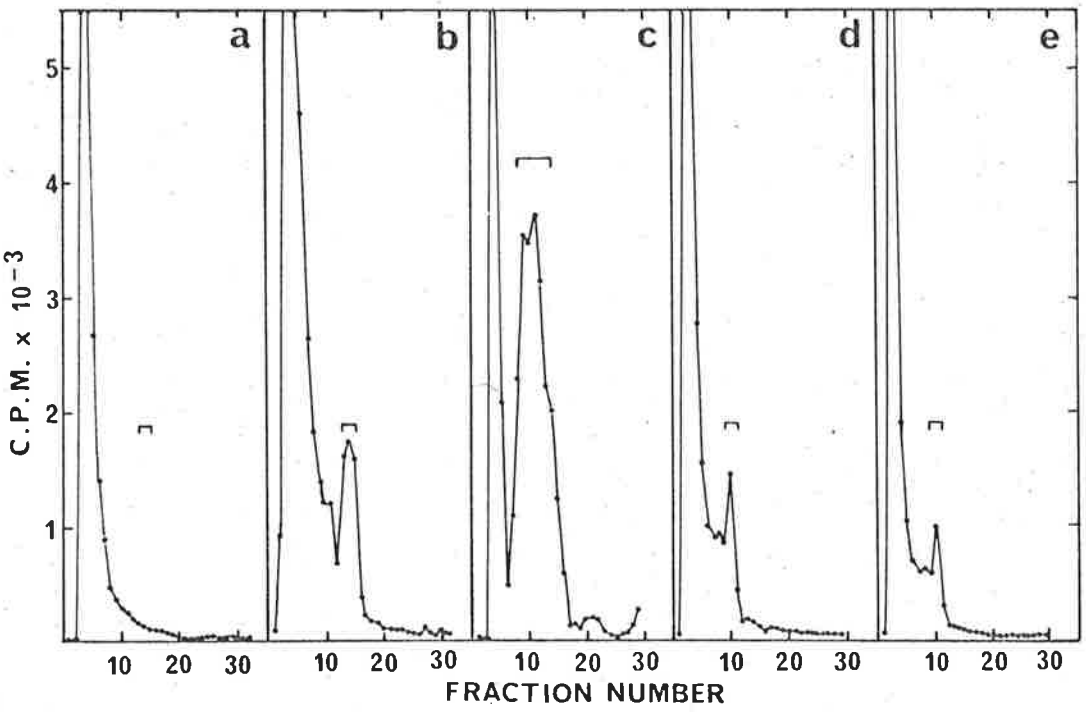
Figure 6.11c. 12S RNA (25 µg) added.

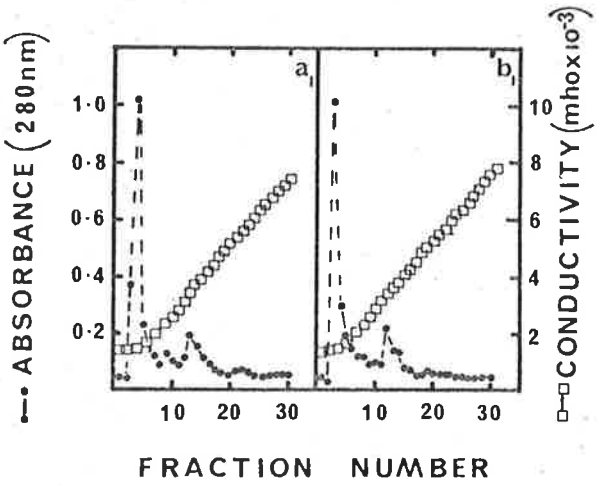
Figure 6.11d. 14S RNA (22 µg) added.

Figure 6.11e. 18S RNA (45 µg) added.

Figure 6.11a'. Absorbance profile (A_{280}) of unlabelled protein eluted from the column run shown in (a).

Figure 6.11b'. Absorbance profile (A_{280}) of (b).


a
b
c
d
e
C.P.M. x 10⁻³
FRACTION NUMBER


a1
b1
ABSORBANCE (280nm)
CONDUCTIVITY (mhox10⁻³)
FRACTION NUMBER

of the post-ribosomal supernatants (prepared as described previously). For the control reticulocyte cell-free system and the 8-17S RNA the absorbance profile (A_{280}) of the fractions and conductivity are shown as well as the radioactivity profile.

b. Analysis of in vitro synthesised products by polyacrylamide gel electrophoresis at pH 7.5.

The results of polyacrylamide gel electrophoresis at pH 7.5 of the cell-free products synthesised in the lysate in response to the addition of the 12S, 14S and 18S RNA fractions are shown in Fig. 6.12a-c. These results confirm that the main stimulation of keratin synthesis in the cell-free system was obtained from the 12S RNA species, and to progressively lesser extents, from the 14 and 18S RNA fractions.

c. Analysis of in vitro synthesised products on SDS/8M urea polyacrylamide gels

In addition to establishing that the keratin synthesised in the cell-free system co-electrophoresed with unlabelled SCM-keratin at both pH 7.5 and pH 2.7, the in vitro synthesised product was also characterised by co-electrophoresis with SCM-feather keratin using the SDS polyacrylamide gel system devised by Swank and Munkres (1971). The result is shown in Fig. 6.13a-c. Unlabelled SCM-keratin is seen to run as predominantly one major band (Fig. 6.13a), with minor peaks even though (as has been discussed in the Introduction) the chick feather keratins have been shown to consist of a family of about 25 homologous proteins, all of about 10,000 daltons. Comparison of this absorbance profile

FIGURE 6.12. POLYACRYLAMIDE GEL ELECTROPHORESIS AT pH 7.5 OF CELL-FREE PRODUCTS SYNTHESISED IN RESPONSE TO THE ADDITION OF 12, 14, and 18S RNA SPECIES.

The electrophoresis was carried out as described in Fig. 6.2.

Figure 6.12a. One quarter of the material isolated as shown in Fig. 6.11c was electrophoresed.

Figure 6.12b. One half of the material isolated as shown in Fig. 6.11d was electrophoresed.

Figure 6.12c. Electrophoresis of one-half of the material isolated as shown in Fig. 6.11e.

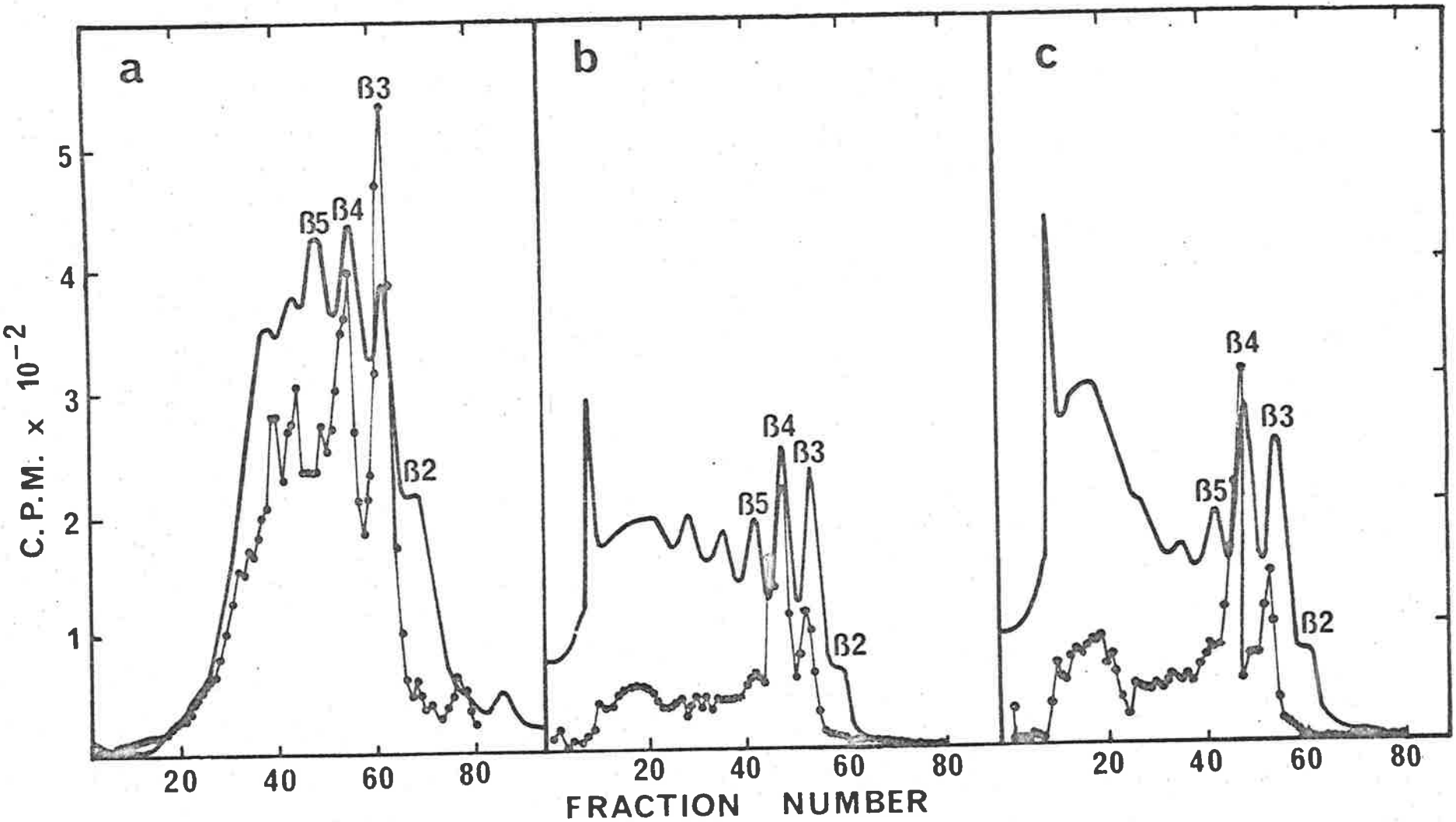

a
b
c
ß5
ß4
ß3
ß2
C.P.M. x 10^{-2}
1
2
3
4
5
20
40
60
80
FRACTION NUMBER

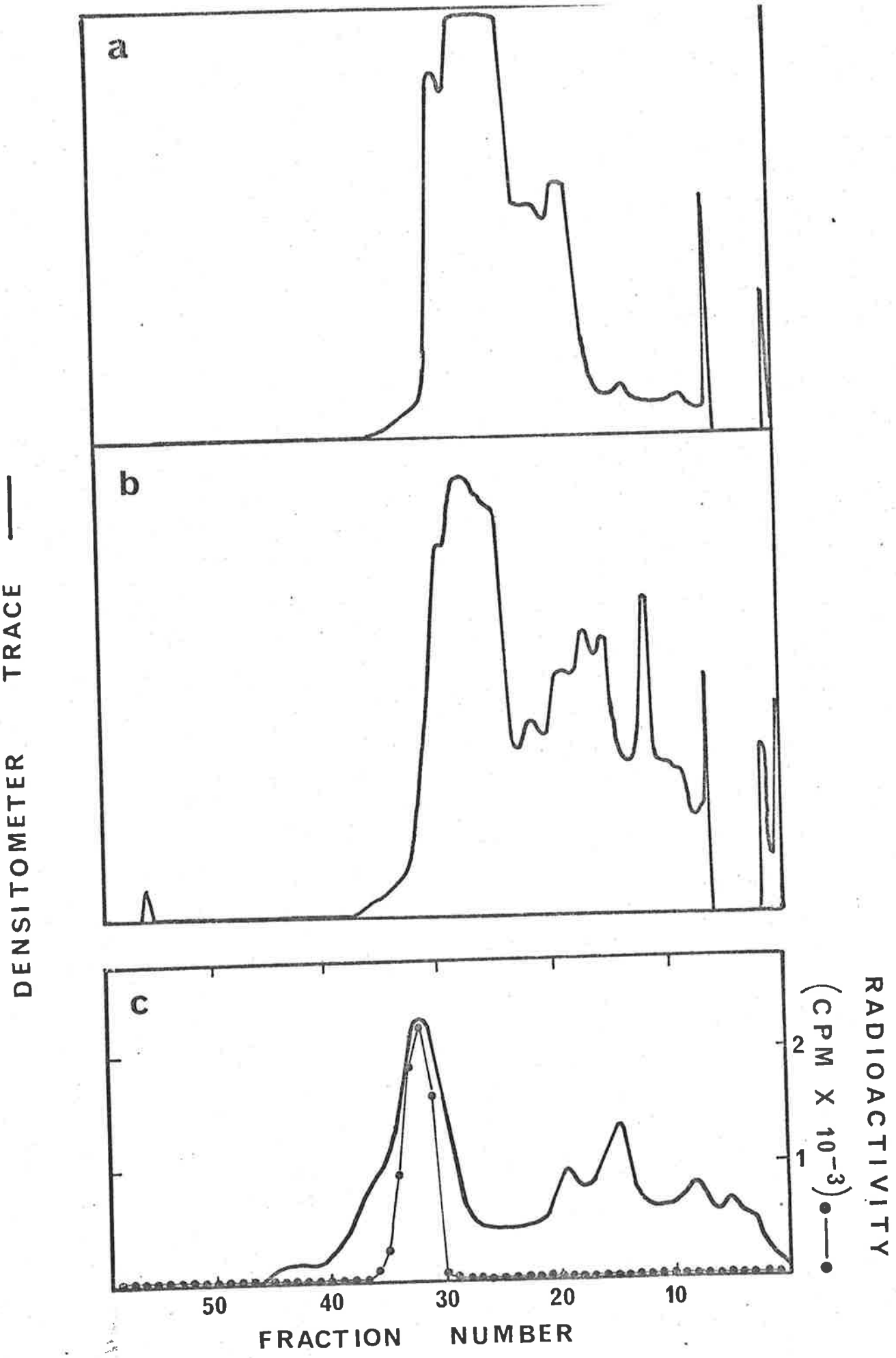
FIGURE 6.13. ANALYSIS OF IN VITRO SYNTHESISED PRODUCTS ON SDS/8M UREA POLYACRYLAMIDE GELS.

Electrophoresis staining and determination of radioactivity was carried out as described (see Methods 6.B.10). Densitometry was as given in Fig. 4.7.

Figure 6.13a. Electrophoresis of 100 µg of unlabelled feather keratin.

Figure 6.13b. Co-electrophoresis of 100 µg of feather keratin with one half of the material isolated as shown in Fig. 6.11a.

Figure 6.13c. As for (b) except that one half of the material isolated as shown in Fig. 6.11b was electrophoresed.


a
b
c
DENSITOMETER TRACE
RADIOACTIVITY (CPM X 10^{-3}) •—•
2
1
50
40
30
20
10
FRACTION NUMBER

of unlabelled SCM-keratin and those of the co-electrophoresis analysis of the products (material obtained from pooled fractions shown in Fig.6.11a obtained from a control reticulocyte cell-free system (no exogenous RNA added) shows that some proteins contributing to the absorbance profile were derived from the reticulocyte lysate. Absorbance and radioactivity profile of the SDS polyacrylamide gel in which unlabelled SCM-keratin was co-electrophoresed with labelled protein synthesised in the cell-free system in response to the added 8-17S RNA are shown in Fig. 6.13c. No cpm above background was detectable in any region of the gel other than the keratin band.

(d) Immunoprecipitation of the cell-free product

Specific immunoprecipitation (see Methods 6.B11) of the in vitro synthesised product from the post-ribosomal supernatants of the reticulocyte lysate to which 8-17S feather polysomal RNA had been added provided further evidence that keratin synthesis was promoted by the exogenous RNA. Table 6.1 shows that about 17.6% of the radioactivity in the lysate precipitated by goat-anti-keratin serum whereas only about 0.84% of the radioactivity was precipitated in the control incubation.

If allowance is made for the finding that about 50% of the labelled keratin in the post-ribosomal supernatant was precipitated by the anti-serum (see Methods 6.11) it follows that about 35% of the label in the supernatnat protein represents keratin.

TABLE 6.1. SPECIFIC IMMUNOPRECIPITATION OF CELL-FREE PRODUCT.

RNA	Total cpm. incorporated	Anti-keratin serum cpm	normal serum	% of total cpm ppt. by anti-keratin serum
None	20,057	169	195	0.8
7-17S	18,311	3,228	-	17.6

Equal aliquots (0.2 ml) of the post-ribosomal supernatant of a reticulocyte cell-free system (0.5 ml) incubated in the presence and absence of 7-17S feather polysomal RNA were precipitated with either normal serum or anti-keratin serum as described (see Methods 6.B.11). The radioactivity is expressed as per 0.2 ml aliquot.

(e) Acetylation of the N-terminal serine residues of keratin synthesised in vitro

The keratin synthesised in a reticulocyte cell-free system was tested for the presence of acetyl-blocked N-termini. An aliquot of ^{14}C-serine labelled cell-free product, isolated by DEAE-cellulose chromatography (Fig. 6.11c) was digested with chymotrypsin and the blocked N-terminal peptides isolated on Dowex 50 (see Methods 6.B12). The total radioactivity in the blocked peptides eluted from the column was 4484 cpm compared to 59958 cpm in the bound peptides, giving a ratio of about 1:12. Since keratin synthesis must have been completely de novo, the specific activity of the N-terminal serine residues should be identical to the specific activity of each internal serine residue. It has been established that there are about 12 serine residues per mole in embryonic feather keratin (Kemp and Rogers, 1972) and thus the result obtained agrees well with the expected value.

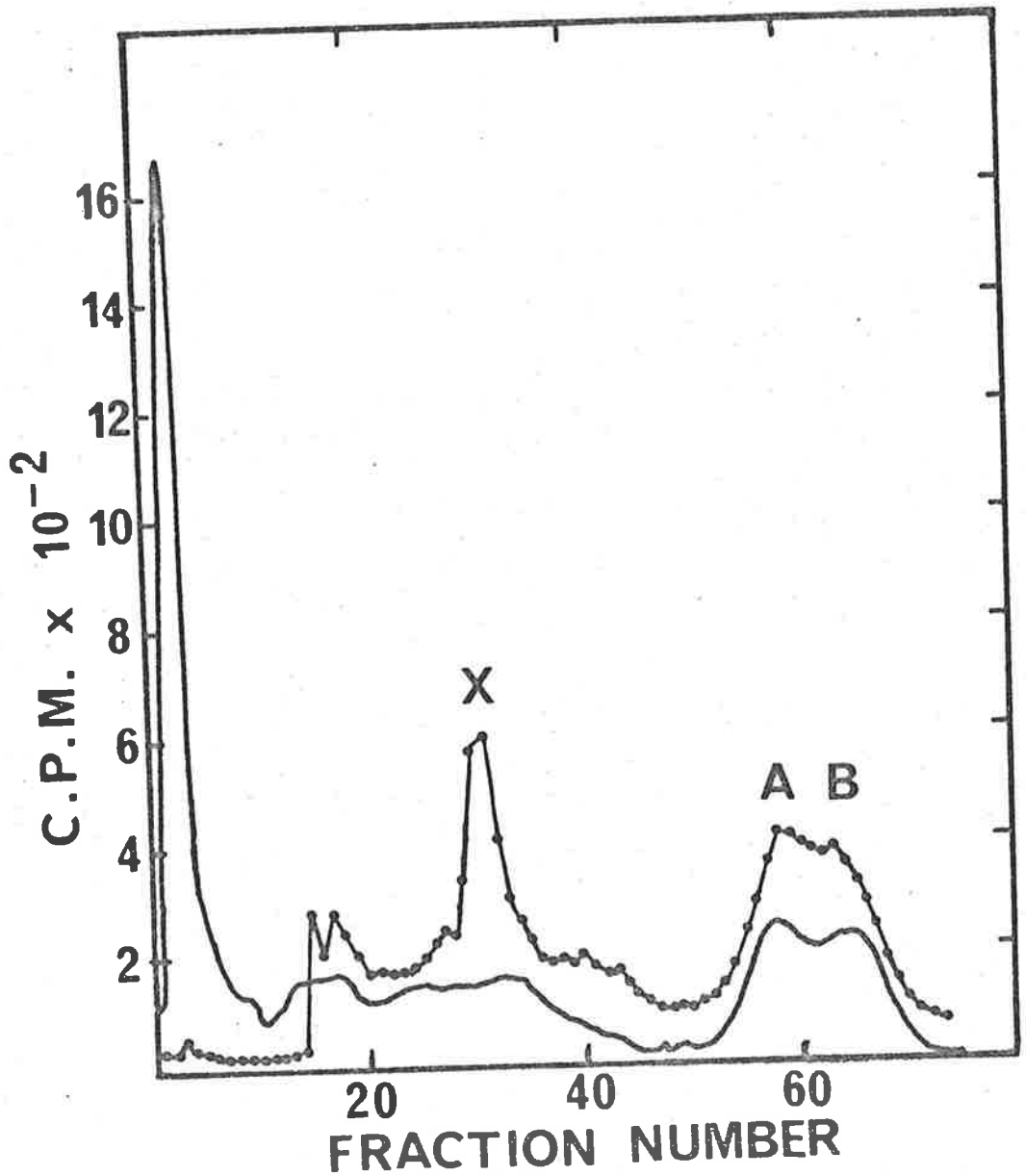
Fig. 6.14 shows the absorbance and radioactivity profile obtained after fractionation of the N-terminal peptides on Dowex 1 (see Methods 6.B12). The peaks of absorbance marked A and B have beenshown to be Ac-Ser-SCM-Phe and Ac-Ser-SCM-tyr respectively (Walker, 1974). Label was coincident with both of these peaks indicating that both of the major N-terminal peptides were acetylated in the cell-free system. It is uncertain whether peptide X is an N-terminal peptide (Walker, personal communication).

FIGURE 6.14. ISOLATION OF ACETYLATED N-TERMINAL PEPTIDES FROM KERATIN SYNTHESISED IN VITRO

Approximately three quarters of the cell-free product isolated as shown in Fig. 6.11c was used. The chromatographic separation of the blocked N-terminal peptides on Dowex-50 is shown.

—— A_{570}

●—● cpm.


C.P.M. x 10^{-2}
16
14
12
10
8
6
4
2
X
A B
20
40
60
FRACTION NUMBER

6. Effect of addition of exogenous RNA on endogenous incorporation by the reticulocyte cell-free system

Table 6.2 shows the total incorporation obtained in several experiments in the presence and absence of various feather RNA fractions isolated by sucrose gradient centrifugation. It is apparent that the addition of tRNA, 18S rRNA or 28S rRNA to the cell-free system markedly reduced the total incorporation, when either serine or leucine was used as label. The degree of inhibition by a particular RNA fraction varied markedly and the effect of these RNA species was not constant relative to one another.

In most experiments the addition of this 7-17S RNA fraction slightly loweredthe total incorporation. However, in two experiments its presence stimulated the total incorporation as compared to the control. Moreover, it should be noted that the addition of 7-17S RNA (40 µg) stimulated the reticulocyte system to a greater extent than 12S RNA (25 µg), purified by two cycles of centrifugation when the effect of the two RNA fractions was compared in the same experiment. However, it is clear from the DEAE-column chromatography of the *in vitro* synthesised products (see Fig. 6.11b,c) that the 12S RNA stimulated a greater amount of keratin synthesis than did the 7-17S RNA.

7. Estimation of molecular weight of keratin mRNA on polyacrylamide gels containing formamide

Keratin mRNA prepared by two successive cycles of centrifugation was electrophoresed alone and with RNA

TABLE 6.2. EFFECT OF ADDITION OF EXOGENOUS RNA ON ENDOGENOUS INCORPORATION OF THE RETICULOCYTE CELL-FREE SYSTEM.

Aliquots (0.02 ml) were removed for determination of acid-precipitable radioactivity from cell-free systems incubated in the presence of various feather RNA fractions. The radioactivity is expressed as cpm incorporated per aliquot of 1 ml of incubation mix.

It should be noted that different batches of reticulocyte lysate were used in each experiment.

RNA added	^{3}H-serine 10µC/ml (S.Act. 1.2C/ mM)	^{3}H-serine 10µC/ml (S.Act. 1.2C/ mM)	^{14}C-leucine 3µC/ml (S.Act. 312mC/ mM
None	-	1979	17941
tRNA	792	987	16878
7-17S	808	1589	24636
18S	422	-	14718
28S	513	1118	6132

Total incorporation of experiment described in Results 6.C.5. Label used was 4 µ C/ml ^{3}H serine (S. Act. 1.2 C/mM).

RNA added	cpm incorporated in (0.2 ml) per 1 ml incubation mix
None	9526
7-17S	34704
12S	18568
14S	21874
18S	16900

markers in gels containing formamide. An absorbance scan of the mRNA co-electrophoresed with markers is shown in Fig. 6.15. An estimate of the mol. wt. of the keratin mRNA was obtained by plotting the distance migrated by the markers against the log of mol. wt. Using this method a value of 230,000 was obtained for the mol. wt. of the keratin mRNA.

Rabbit globin mRNA and keratin mRNA were also prepared by EDTA release of the mRNP complex from the polysomes, followed by sucrose gradient centrifugation for purification of the mRNA liberated from the isolated mRNP complexes by SDS (prepared by D.J. Kemp). It can be seen (Fig. 6.16) that keratin mRNA prepared by this method electrophoresed as a single sharp band, whereas the globin mRNA split into two discrete bands. Keratin mRNA prepared by the same method was co-electrophoresed with RNA markers (Fig. 6.17b). In this particular run the mol. wt. was estimated to be 250,000. At the same time an aliquot of the same keratin mRNA preparation was co-electrophoresed with globin mRNA (also prepared from the mRNP complex) and with markers (Fig. 6.17a). Comparison of Fig. 6.17a and b clearly shows that keratin mRNA did not migrate as far as globin mRNA and must therefore be of higher mol. wt. The mol. wt. of globin mRNA in this run was estimated to be about 230,000.

It should be noted that the keratin mRNA prepared by first isolating the mRNP complex electrophoresed on gels as a single sharp band (Fig. 6.16) whereas by comparison the

FIGURE 6.15. ESTIMATION OF MOL. WT. OF KERATIN mRNA BY ELECTROPHORESIS ON POLYACRYLAMIDE GELS CONTAINING FORMAMIDE.

Details of the gel electrophoresis are given in Methods (6.B.13). Gels were stained with toluidene blue.

O: origin +: anode

K: 12S keratin mRNA (1 µg)

I: 16S *E. coli* rRNA (2 µg)

2: 23S *E. coli* rRNA (2 µg)

3: 28S reticulocyte rRNA (2 µg)

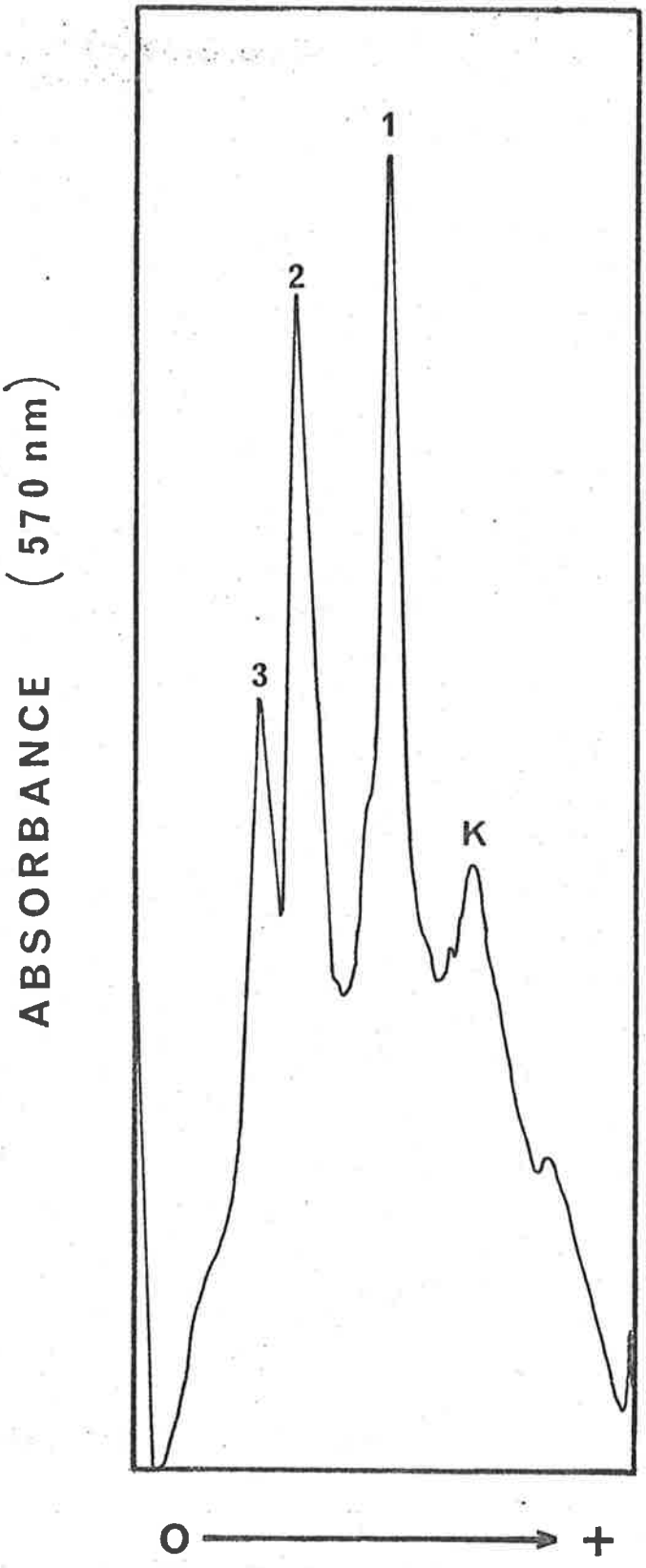

ABSORBANCE (570 nm)
1
2
3
K
O
+

FIGURE 6.16. ELECTROPHORESIS OF KERATIN mRNA AND GLOBIN mRNA ON POLYACRYLAMIDE GELS CONTAINING FORMAMIDE.

O: origin +: anode

Details as given in Fig. 6.15, except that gels were stained with 'stains-all'.

G: globin mRNA (2 µg)

K: keratin mRNA (4 µg).

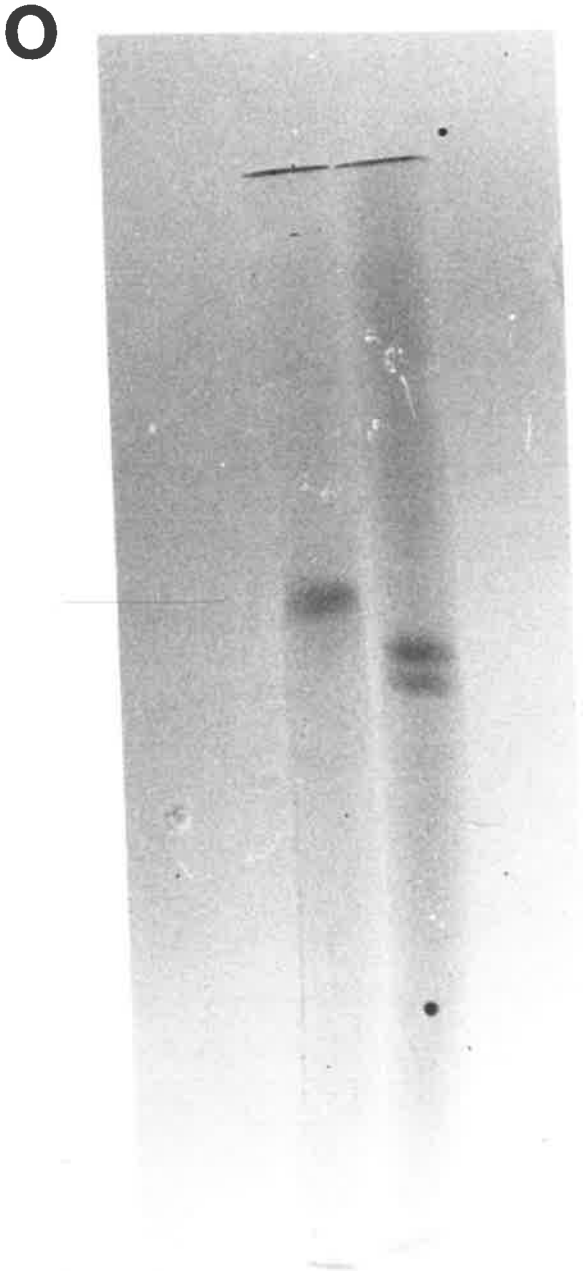
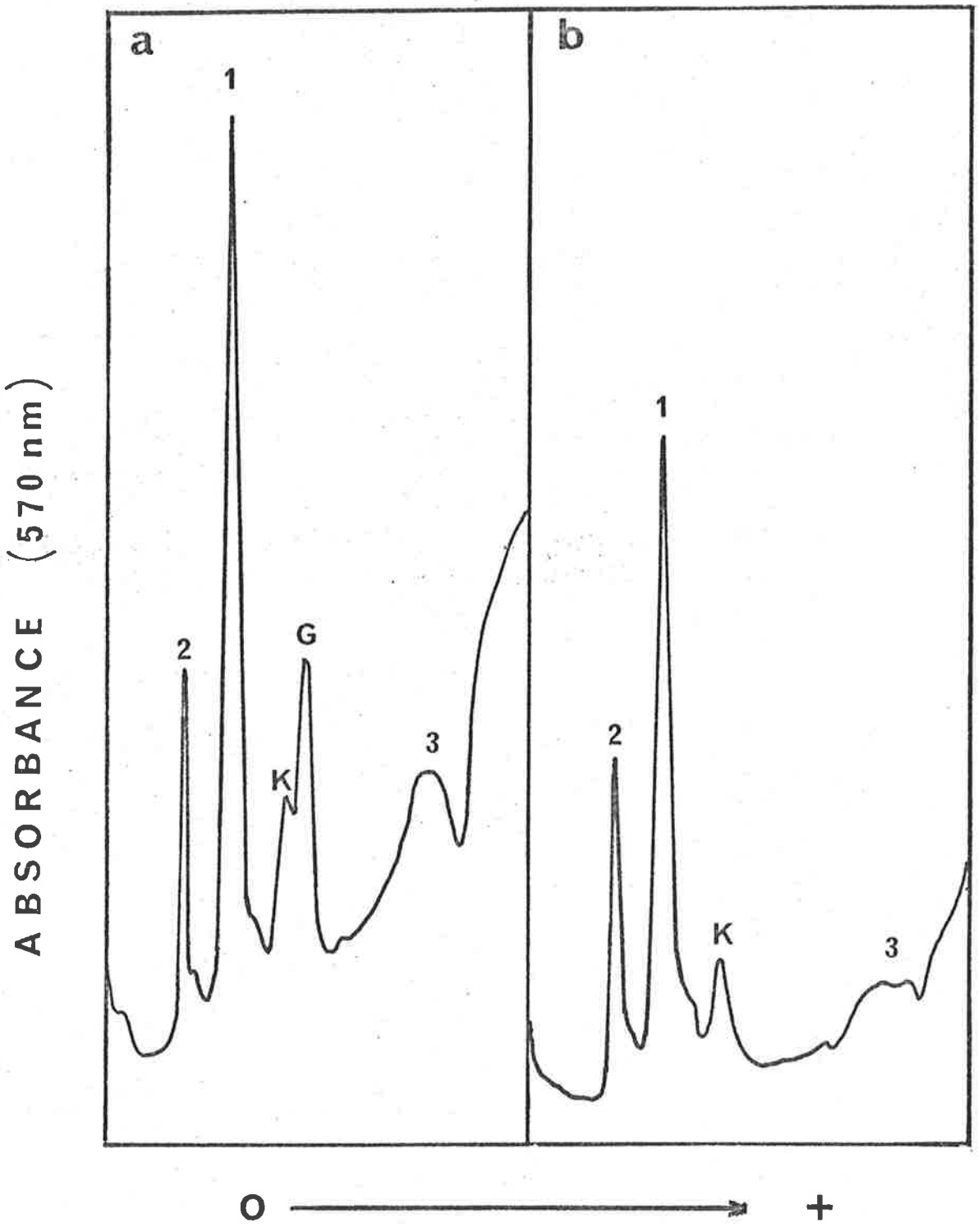

O
+
K
G

FIGURE 6.17. ESTIMATION OF MOL. WT. OF KERATIN mRNA BY ELECTROPHORESIS ON POLYACRYLAMIDE GELS CONTAINING FORMAMIDE.

Figure 6.17a. 1. 18S reticulocyte rRNA
2. 28S reticulocyte rRNA
3. feather tRNA
K. keratin mRNA
G. globin mRNA.

Figure 6.17b. As for (a), no globin mRNA present.


a
b
1
2
G
K
3
1
2
K
3
ABSORBANCE (570 nm)
O
+

mRNA prepared by phenol-extraction of the polysomes was a more diffuse band (Fig. 6.15) and was obviously impure.

D. DISCUSSION

1. Comments on the translation of keratin mRNA in a reticulocyte cell-free system

The experimental results presented in this chapter show that chick embryo feather keratin can be synthesised in a cell-free system prepared from rabbit reticulocyte lysate in response to an added 12S RNA species isolated from chick embryo feather polysomes. Furthermore, there was no requirement for specific initiation factors or any other component from feather tissue. The keratin synthesised in the reticulocyte cell-free system was found to be identical to native chick embryo feather keratin by a number of criteria. However, evidence that any amino acid substitutions had occurred could only come from peptide analysis of the *in vitro* synthesised products.

No evidence was obtained to suggest that higher molecular weight precursor chains to keratin were synthesised in the reticulocyte cell-free system. A mechanism for specific cleavage of a keratin-precursor protein would not be expected to be operative in reticulocyte lysate in view of the finding (Stavnezer and Huang, 1971; Mach *et al.*, 1973) that the 14S mRNA coding for the immunoglobin light-chain directs the synthesis in reticulocyte lysate of a precursor protein containing about 20 extra amino acid residues.

It is noteworthy that the relative ratios of label

incorporated into the electrophoretic bands of keratin β2 - β5 synthesised in both the reticulocyte cell-free system and the 14-day feather cell-free system were similar when ^{14}C-serine was used as label. Hence it could be concluded that the various mRNA species were translated at the same relative rates in both the homologous and heterologous systems. This should not, however, be taken as unequivocal. It has been shown that each of the major keratin bands on high pH polyacrylamide gels can be fractionated further into separate proteins (Walker, 1974). Some minor changes in the relative rates of synthesis could occur but not be detected using this fractionation system.

2. Acetylation of the N-terminal serine residues of keratin synthesised in reticulocyte lysate

Since the keratin chains synthesised in a reticulocyte cell-free system behaved in an identical manner to native feather keratin by several criteria the N-terminal sequences were isolated and shown to be identical to the acetylated N-terminal sequences isolated from native feather keratin.

An analogous situation has previously been reported by Berns et al. (1972a); a main structural protein of lens, the native αA_2-crystallin has an N-terminal acetylated methionine and this acetylation was found to occur when the lens crystallin mRNA was translated in either rabbit reticulocyte lysate or on injection of the mRNA into Xenopus oocytes (Berns et al., 1972b). Hence the present finding strengthens the view of Strous et al. (1972) that the acetylation mechanism

is universal and recognises either a particular amino acid sequence of the protein chain or some feature of its secondary structure.

3. Efficiency of keratin mRNA translation

In the present study no detailed attempt was made to determine the exact saturating value of exogenous RNA in the system, for the following reasons: (a) the 12S keratin mRNA was detected and purified by two cycles of sucrose gradient centrifugation but it was obviously impure as shown by electrophoresis on formamide-containing polyacrylamide gels. A possible contaminant was the 12S mitochondrial rRNA, reported to be a contaminant of the mRNA coding for the immunoglobulin light chain mRNA (Brownlee et al., 1973); (b) it was observed that the activity of the reticulocyte cell-free system and its stimulation by exogenous RNA varied from one preparation to another. Palmiter (1973) has reported a similar finding; (c) it has been reported that the stimulation of a cell-free system by an exogenous mRNA is markedly influenced by the ionic conditions of the cell-free system (Metafora et al. 1972; Sampson et al., 1972; Palmiter, 1973; Hall and Arnstein, 1973).

Heterologous protein-synthesising systems have been used extensively for the assay of specific mRNA's but there have been few determinations made on the efficiency of the exogenous mRNA translation relative to endogenous mRNA. When the keratin synthesised in the reticulocyte cell-free system was precipitated using the keratin anti-serum (see

Table 6.I). about 35% of the total label incorporated was precipitated, when allowance was made for the finding that the immunoprecipitation was only 50% efficient. This value is very high when compared with values reported by others for the total incorporation into protein coded by exogenous mRNA's. For example, it was reported (Rosenfeld et al., 1972a) that when a saturating concentration of hen oviduct RNA added to a reticulocyte lysate only about 3% of total radioactivity was precipitable with antiovalbumin serum. However, as discussed previously, it was found in thepresent study that the use of ^{14}C-serine in the reticulocyte lysate favoured the labelling of keratin, as the number of serine residues per mole is greater for keratin than for globin. When ^{14}C-leucine was used as label the ratio of cpm incorporated into keratin to those incorporated into globin was not nearly so favourable. For example, for the experiment described in Results (6.C3) summation of the cpm incorporated into globin as compared to cpm incorporated into keratin as assayed by the DEAE-cellulose chromatography of the in vitro synthesised (see Fig. 6.6c), protein indicated that only 4 % of the radioactivity was incorporated into completed keratin chains.

From these results it is clear that it is preferable to use ^{14}C-serine in the reticulocyte cell-free system when assaying for keratin mRNA activity because it gives an apparent enhanced incorporation of label into keratin.

Although the main activity of keratin synthesis was initially determined to be promoted in the reticulocyte

cell-free system when the 7-17S RNA fraction was added, and finally located to a 12S RNA species, some keratin synthesis activity was noted in the higher S value fractions. A probable cause was the aggregation of the mRNA with the 18S rRNA species. This effect was observed some time ago (Staehelin et al., 1964). More recently, the aggregation of mRNA's during isolation has been noted (Palmiter, 1973; Schechter, 1973; Skoultchi and Gross, 1973).

It should be noted that the 12S RNA fraction isolated from chick feather polysomes by two successive cycles of centrifugation was contaminated by a minor RNA species sedimenting at 9S. At this stage of the work it could not be ruled out that the 9S species was not the keratin mRNA. However, when the present study was nearing completion, feather polysomal RNA was purified by cellulose-chromatography. The 12S was purified from the contaminating 9S species and shown to code for keratin in a reticulocyte cell-free system. The 9S RNA did not stimulate keratin synthesis (Kemp - personal communication).

4. Inhibition of endogenous-protein synthesis by added RNA

In most experiments the addition of exogenous RNA to the reticulocyte cell-free system depressed the incorporating activity of the system to some extent. This depression was particularly marked when the tRNA, 18S rRNA or 28S rRNA fractions were added. Moreover, in most experiments addition of the 7-17S RNA containing the keratin

mRNA depressed the incorporation, although not as markedly as theother RNA fractions tested. It is important to point out that in these experiments the RNA was not quantitatively assayed and thus all that can be said is that there was a marked inhibition of incorporation by the exogenous RNA. This observation appears to be universal for all mRNA preparations tested in the reticulocyte cell-free system (Lockard and Lingrel, 1969; Stavnezer and Huang, 1971; Gallwitz and Briendl, 1972; Rhoads et al., 1973; Stewart et al., 1973). This inhibition has been examined in detail (Rhoads et al., 1973; Stewart et al., 1973) and has found to result from inhibition of de novo protein synthesis by high concentrations of exogenous mRNA, tRNA or rRNA's. As a result, increasing concentrations of added RNA frequently produce a non-linear response if the concentrations are high enough. The most satisfactory solution then to this problem is to work at the highest specific activity of labelled amino acid so that very low levels of exogenous RNA can be assayed, thus perturbing the system as little as possible (Rhoads et al., 1973).

5. Estimation of the mol. wt. of keratin mRNA by polyacrylamide gel electrophoresis

When it was established that a 12S RNA species isolated from the chick feather polysomes coded for the keratin proteins it was important to know whether the mRNA was as large as the S value indicated or whether the value was anomously high as a result of conformational effects.

Hence the mRNA was analysed by polyacrylamide gel electrophoresis using formamide to denature the RNA, a system devised by Staynov et al. (1972) for removing conformational effects due to base stacking and pairing.

By co-electrophoresis of keratin mRNA with rabbit globin mRNA and RNA markers it was found that the keratin mRNA had a higher mol. wt. than the globin mRNA. It is noteworthy that in some runs the globin mRNA prepared from mRNP was resolved into 2 bands on the formamide gels. This result is in agreement with those reported by Gould and Hamlyn (1973).

However, in the co-electrophoresis run with keratin mRNA only one band was observed, in agreement with the results obtained by Lanyon et al. (1973) for the 9S mouse globin mRNA isolated as an mRNP complex. Furthermore, these authors reported that electrophoresis of the mouse globin mRNA on 6% gels in aqueous buffer showed only one band. Kazazian et al. (1973) have also reported that rabbit globin mRNA (isolated from an mRNP complex) yielded only one band when electrophoresed on 6% gels in aqueous buffer.

As reported by Gould and Hamlyn (1973) the mol. wt. estimate of globin mRNA varied from 219,000 - 236,000 for the slower moving component of the two bands, and from 198,000 - 213,000 for the faster moving component. From this result and from the fact that the mol. wt. values of the standards were originally determined in aqueous buffered gel systems (see Staynov et al., 1972) it is clear that the estimates of the mol. wt. of the keratin mRNA could have a considerable

degree of error. Nevertheless, it has been clearly established that the keratin mRNA has a lower mobility and hence higher mol. wt. than globin and yet it codes for protein chains with mol. wts. considerably lower than those of globin.

Embryonic feather keratin is comprised of at least 19 homologous peptide chains all with a mol. wt. of about 10,000 (Walker, 1974). Only one band was obtained when the keratin mRNA was electrophoresed on formamide-containing polyacrylamide gels and thus all of the mRNA's coding for these proteins must be of almost identical mol. wt. Active keratin mRNA has been shown to bind selectively to cellulose during chromatography (Kemp - personal communication), and presumably therefore contains a poly(A) sequence (Schutz et al., 1972; Delarco et al., 1973). An mRNA of 250,000 mol.wt. would contain about 800 bases, of which only about 300 would be required to code for an individual keratin chain. The length of the untranslated sequence(s) is greater than that of any poly(A) sequence reported and may represent additional sequence(s) required for translational control.

CHAPTER SEVEN

RADIOACTIVE LABELLING OF FEATHER RNA *IN VITRO*

A. INTRODUCTION

1. The synthesis of mRNA in Chordates

One of the central problems of eukaryotic cell biology concerns the elucidation of the origin and fate of mRNA. Among the primary products of transcription in the nuclei of animal cells is a population of high molecular weight RNA molecules (HnRNA), with a DNA-like base composition. This HnRNA is extremely heterodisperse, with sedimentation coefficients ranging between 30S and 100S. Most of this newly synthesised RNA is quickly degraded within the nucleus, so that only a small fraction is transported to the cytoplasm. It has been suggested that the HnRNA may be a precursor to cytoplasmic mRNA (for references see review by Darnell, 1968). Several theories of gene activity regulation in animal cells postulate that mRNA sequences are interspersed between longer regulatory DNA sequences which may be transcribed (Georgiev, 1969; Britten and Davidson, 1969; Crick, 1971; Paul, 1972). This would account for the transcription of very large HnRNA precursors to mRNA.

The precursor-product relationship of HnRNA and mRNA is supported by several ~~by several~~ lines of evidence. The recent finding that some HnRNA and most mRNA species contain large covalently attached sequences of poly(A), at the 3'-OH terminus, and which appear to be synthesised in a post-transcriptional step is consistent with a product-precursor relationship. The exploration of the physiological role of poly(A) has been facilitated by the use of an antimetabolite, cordycepin (3'-deoxyadenosine). This drug which terminates

RNA chains prematurely stopped the synthesis of some RNA molecules but not others. For example, in cells treated with cordycepin, rRNA synthesis was quickly halted, but incorporation of labelled precursor into HnRNA was not affected. Nevertheless, mRNA failed to appear in the cytoplasmic polysomes, although some RNA containing attenuated poly(A) sequences did appear in the cytoplasm. (for references see review by Darnell et al., 1973).

Melli and Pemberton (1972) have shown that HnRNA from duck reticulocytes hybridised with RNA strands complementary to the 9S duck globin mRNA. In an analogous manner Imaizumi et al. (1973) established that anti messenger DNA prepared from the duck globin mRNA by the RNA-directed DNA polymerase hybridized with HnRNA molecules of various sizes. Evidence of a more direct nature of the precursor-product relationship of HnRNA and mRNA has been obtained by demonstrating that HnRNA of high molecular weight from embryonic mouse erythroblasts coded for the synthesis of the α and β globin chains when injected into Xenopus oocytes (Williamson et al. 1973). Similarly, Stevens and Williamson (1972) have reported the translation of poly(A) containing HnRNA from murine plasmacytoma cells into the heavy and light immunoglobulin chains; and by use of the ingenious finding that the immunoglobulin protein bound to the heavy chain mRNA went on to isolate and purify the HnRNA precursor to the mRNA, coding for the immunoglobulin heavy chain (Stevens and Williamson, 1973).

It should be noted that there must be some reservation

to these results, as some controversy still remains as to whether HnRNA is actually of very high molecular weight or is simply aggregated lower mol. wt. RNA. Bramwell (1972) has presented strong evidence to support his contention that this is the case, by showing that HnRNA had a much lower S value when run in strongly denaturing conditions. Equally, Holmes and Bonner (1973) have shown that under strongly denaturing conditions HnRNA was not disaggregated to lower mol. wt. species. To add further doubt about understanding the mechanism for processing of HnRNA Kumar and Lindberg (1972) have shown that under conditions designed to rigorously prevent both aggregation of RNA and contamination by RNA'se the cytoplasmic mRNA's were of the same order of size as the HnRNA, thus disputing the need for extensive cleavage of HnRNA. Recently, Reuiz-Carrillo et al. (1973) presented strong evidence that HnRNA of duck reticulocytes directed the synthesis of duck globins in the Krebs ascites cell-free system. Rigorous precautions were taken to ensure that aggregation of the mRNA was prevented. Furthermore, to rule out the possibility that 9S duck globin mRNA cosedimented with the HnRNA rabbit globin mRNA was added as an internal control. No rabbit globin mRNA activity was detected in RNA fractions sedimenting at greater than 45S.

It has been observed that all mRNA's examined to date contain poly(A), with the one notable exception, the mRNA's which direct the synthesis of histones (Adesnik and Darnell, 1972; Schochetman and Perry, 1972). Moreover, histone

mRNA appears in the cytoplasm without any appreciable lag after exposure of the cells to radioactive RNA precursor, whereas there is a lag of about 20 min before poly(A) containing mRNA becomes associated with the polysomes (Adesnik and Darnell, 1972; Schochetman and Perry, 1972; Greenberg and Perry, 1972).

2 mRNA transport in eukaryotes

Early studies on the transport of mRNA in eukaryotes indicated that newly synthesised mRNA was transported to the cytoplasm complexed with the small ribosomal subunit, for example, McConkey and Hopkins, 1965; Girard et al., 1965. Later evidence, based on the buoyant density analysis of the native 40S ribosomal subunit fraction, suggested the existence of mRNP particles distinct from the native 40S subunits, these particles were called informosomes (for a review see Spirin, 1969). Some doubt was thrown on to this theory when it was reported that non-specific RNA-protein interactions could occur in cytoplasmic extracts of animal cells (Girard and Baltimore, 1966; Baltimore and Huang, 1970). Extensive studies by Scherrer and co-workers (Spohr et al., 1970; Spohr et. al., 1972; Morel et al., 1973) have shown that the mRNP complexes are stable in solutions of high ionic strength and are composed of specific proteins complexed to the mRNA. Furthermore, there is now general concurrence with the results of Morel et al. (1973), on the number and mol. wt. of the proteins bound to mRNA (Blobel, 1973; Bryan and Hayashi, 1973). Gander et al. (1973)

have isolated a 20S RNP particle from the postribosomal supernatant fraction of duck erythroblasts. This particle contains the 9S mRNA's of all duck globins. Significantly, the protein complement of this complex is completely different from that found on the mRNP released from polysomes, suggesting that this may be a form of translational control. The controversy as to whether newly synthesised mRNA can be detected bound to the 40S subunits has been revived by Zehavi-Willner et al. (1973). These authors studied the distribution of RNA in rabbit reticulocytes by exposure of anaemic rabbits to ^{32}P for 2 hr prior to sacrifice. They found that most of the radioactivity associated with the 40S subunit appeared in the 9S RNA peak. Buoyant density analysis revealed two forms of 40S subunits, one which they proposed was the initiation complex contained the 9S globin mRNA the other did not. No reconciliation can be made at present with these findings and those reported by Legon et al. (1973) who detected no 9S globin mRNA associated with the 40S subunits from rabbit reticulocytes.

3. A note on the stability of mRNA in eukaryote cells

Most studies in the past decade on the half-life of mRNA in mammalian cells have utilised actinomycin D to inhibit further transcription. The assumption has always been that actinomycin D did not greatly perturb any other process within the cells. It was of course recognised that actinomycin D was cytotoxic if used for prolonged periods. The stability of many mRNA species in rapidly growing tissues were estimated using this method to be about 3-4 hr (for example, Penman et al.,

1963; Craig et al., 1971). Nevertheless, in some differentiating cell lines there were always clear examples of mRNA's whose translation was insensitive to actinomycin D. Examples of these include lens crystallin mRNA's (Stewart and Papaconstantinou, 1967); cocoonase mRNA (Kafatos and Reich, 1968); trout testis protamine mRNA (Ling and Dixon, 1970). In relevance to the present study is the finding reported by Bell and Merrill (1967) that protein synthesis in 12-day chick embryo feathers was insensitive to actinomycin D treatment indicating that keratin was synthesised on a stable template.

In all studies involving the use of actinomycin D to measure the half-life of mRNA the assumption has been that the decay of protein synthesis observed in the presence of the drug resulted from the decay of the mRNA coding for the particular protein. Singer and Penman (1972) have recently proven this assumption to be incorrect, and thus have thrown doubt on the validity of all experiments where the drug was used to measure the half-life of mRNA. They have shown that the lesion resides in the capacity of the ribosomes to reinitiate on the mRNA, that is to say, the availability of the mRNA is not the rate limiting factor to protein synthesis. These findings have been independently verified by Craig (1973). Furthermore, the life-time of mRNA of rapidly growing cells has now been shown to be of the order of one to one and one-half cell-generations (Greenberg, 1972; Singer and Penman, 1972; Murphy and Attardi, 1973; Craig, 1973). It follows then that the half-life of mRNA species of specialised proteins of terminally differentiating

cells is probably not significantly greater than that of the bulk of the mRNA species of rapidly growing cells. The situation is still complicated by the finding that in differentiating cells the synthesis of the specialised proteins was not inhibited by the drug. Moreover, in the case of lens crystallin mRNA, Stewart and Papaconstantinou (1967) reported that while the translation of the mRNA was sensitive to actinomycin in young calf epithelial cells, the translation was insensitive in adult epithelial cells. A similar stabilisation of template activity in the yolk sac erythroid cells from foetal mice has been reported by Djaldetti et al. (1970). These workers found that at 12 days of gestation addition of actinomycin D to the cells resulted in a decline in haemoglobin synthesis, while at 15 days the cells were insensitive to the drug in this respect.

4. The synthesis of rabbit globin mRNA

It has been emphasised in other sections of this work that reticulocytes have been widely used to study many aspects of protein synthesis, and this is also true of RNA synthesis. Some time ago Marbaix and Burny (1964) observed that a 9S RNA isolated from rabbit reticulocyte polysomes was labelled with ^{32}P to a much higher specific activity than either the rRNA's or tRNA when rabbits were injected with ^{32}P prior to isolation of the cells. This RNA had many of the properties expected for haemoglobin mRNA (for a review see Chantrenne et al., 1967). Much later Evans and Lingrel (1969b) compared the rates of synthesis of rRNA and the 9S RNA in mouse reticulocytes, by

collecting cells at various times after injection of ^{32}P into anaemic mice. At short times of labelling only the reticulocytes from the more mature erythroid cells present in the circulation were labelled. At longer labelling times, labelled reticulocytes from the early erythroid cells appeared in the circulation. It was found that in the more mature cells the specific activity of the 9S RNA and hence its synthesis was greater than either of the rRNA's whereas in younger cells somewhat less 9S RNA was synthesised. From this study it was concluded that rRNA synthesis was maximal at early stages of development, while 9S RNA synthesis was maximal in more developed cells. This 9S RNA was subsequently shown to be the globin mRNA (Lockard and Lingrel, 1969).

The isolation of a 12S RNA species from 14-day chick feather polysomes, coding for the keratin proteins was reported in the preceding chapter. In the present chapter results are reported on the *in vitro* labelling of 12, 13 and 14-day feathers with RNA precursors in an attempt to determine the temporal relationship of keratin mRNA synthesis to the onset of keratin synthesis in an analogous manner to that described for the synthesis of globin mRNA.

B. METHODS

1. Cellulose chromatography of RNA

RNA was fractionated on a column (1.2 x 15 cm) of Whatman standard-grade cellulose as described by Schutz *et al.* (1972). The fines were removed from the cellulose which was then treated with 0.1% diethylpyrocarbonate and stored in the

presence of 0.1% sodium azide.

The RNA which bound to the cellulose and eluted with water was collected and recovered by ethanol precipitation. The non-bound fraction was collected and treated separately.

C. RESULTS

1. A comparison of RNA profiles from 12, 13 and 14-day feathers

When polysomes from 13-day feathers and total post-mitochondrial lysate from 12-day feathers were phenol extracted by the method of Lee et al. (1971b) and analysed by sucrose gradient centrifugation the absorbance profiles were found to be essentially identical to that obtained from 14-day phenol-extracted feather polysomes in that two minor species of RNA with sedimentation coefficients of 12 and 14 respectively were observed in addition to the rRNA peaks (results not shown). Since the same peaks of absorbance were obtained in the 12, 13 and 14-day feathers some aspects of the labelling of these species was investigated in these three ages.

2. RNA labelling of incubated feathers

Feathers were cultured at 37^{o} in Charity Waymouth's medium containing 10% foetal calf serum as described (see Methods 2.B3). In the initial attempts to label the RNA, ^{3}H-uridine was added to the incubations at a concentration of 50 μC/ml. Lysates from the feathers was prepared in MSB (see Methods 2.B2) and the post-mitochondrial supernatant

was then fractionated into the polysomal and post-polysomal supernatant. The RNA was phenol-extracted from each fraction by the method of Lee et al. (1971b), and analysed by sucrose gradient centrifugation (see Methods 5.B.1). Fractions were collected from the gradients for the determination of acid-insoluble radioactivity (see Methods 2.B.5). Table 7.1 shows the total incorporation of ^{3}H-uridine into the post-mitochondrial supernatant fraction of 12, 13 and 14-day feathers incubated for various times. It is clear that the incorporation per ml of packed feathers rapidly fell with increasing age of the feathers. Furthermore, it was observed that the labelling of the rRNA and tRNA species was appreciable but relatively little labelling was detected in the 7-17S region of the gradients. The results are not shown here as they are essentially identical to the later results shown.

To determine whether another nucleic acid precursor might give enhanced labelling ^{3}H-adenosine was used to label 12, 13 and 14-day feathers. The results of the total label incorporated into the post-mitochondrial supernatants are shown in Table 7.2. Clearly using similar concentrations of ^{3}H-adenosine as were used for uridine labelling the total amount of label incorporated was markedly increased. However, the labelling again decreased with increasing age of feathers. The phenol-extracted RNA from the polysomal and post-polysomal supernatants were analysed by sucrose gradient centrifugation. The results, however, were essentially identical to those obtained with ^{3}H-uridine labelling except that the specific activity of the tRNA and rRNA peaks was enhanced.

TABLE 7.1. RELATIVE RATES OF INCORPORATION OF RADIOACTIVE URIDINE INTO THE CYTOPLASMIC RNA FRACTION OF FEATHERS OF VARIOUS AGES INCUBATED IN VITRO

Feathers were incubated in vitro for various times as described (see Methods 2.B.3) and aliquots of the lysate were acid-precipitated for the determination of radioactivity.

Experiment 1.

Feathers were incubated in vitro for 5 hr at 37° with 10 μC/ml of 5 ^{3}H-uridine (S. Act. 20 C/mM).

Day	cpm incorporated/ml feathers
13	80,000
14	16,885

Experiment 2.

Feathers were incubated in vitro for 4 hr with 20 C/ml of 5,6 ^{3}H-uridine (S. Act. 43 C/mM).

Day	cpm incorporated/ml feathers
12	151,350
13	112,700

TABLE 7.2. RELATIVE RATES OF INCORPORATION OF RADIOACTIVE ADENOSINE INTO THE CYTOPLASMIC RNA FRACTION OF FEATHERS OF VARIOUS AGES INCUBATED IN VITRO.

Other details are as given in Table 7.1.

Experiment 1.

Feathers were incubated in vitro for 3 hr at 37° with 20 µC/ of 2,8-^{3}H adenosine (S. Act. 10 C/mM).

Day	Incorporated/ml feathers
12	920,000
13	471,420
14	24,960

Experiment 2.

Feathers were incubated in vitro at 37° for 4 hr with 20 µC/ml of 8, ^{3}H-adenosine (S. Act. 24.5 C/mM).

Day	Incorporated/ml feathers
12	415,625
13	248,000

The labelling of 13-day feathers was also attempted using ^{3}H-orotic acid by incubating the feathers in Charity Waymouth's medium for 4 hr. The lysate was prepared in MSB and fractionated into the polysomal and post-polysomal supernatant fractions as described previously. The absorbance and radioactivity profiles are shown in Fig. 7.1.a,b. As mentioned previously the absorbance profile of 13-day polysomal RNA was found to be essentially identical to that shown for 14-day polysomal RNA in that again there were two minor peaks of RNA of 12S and 14S respectively in addition to the rRNA's. The main peaks of radioactivity coincided with the 18S and 28S rRNA's, although a peak of very high specific activity was repeatedly detected sedimenting at about 20S. Some label was detected, coincident with the 12S and 14S species, the specific activity of these species being greater than the rRNA species. There was little labelling detected in the tRNA region. In the supernatant fraction the main peak of label coincided with the tRNA fraction. It should be noted, that there is no absorbance discernible in the region where the rRNA's would be expected to sediment. The conditions used for separating the two fractions (1 hr, 190,000 g, see Methods 4.B.3) had been calculated to completely sediment the monosomes (Spinco publication DS-327) however, it is clear that under these conditions the ribosomal subunits had also been completely pelleted. In an effort to determine whether longer labelling times would enhance the labelling of the presumptive 12S keratin mRNA, 13-day feathers were incubated overnight with the highest specific activity ^{3}H-uridine available.

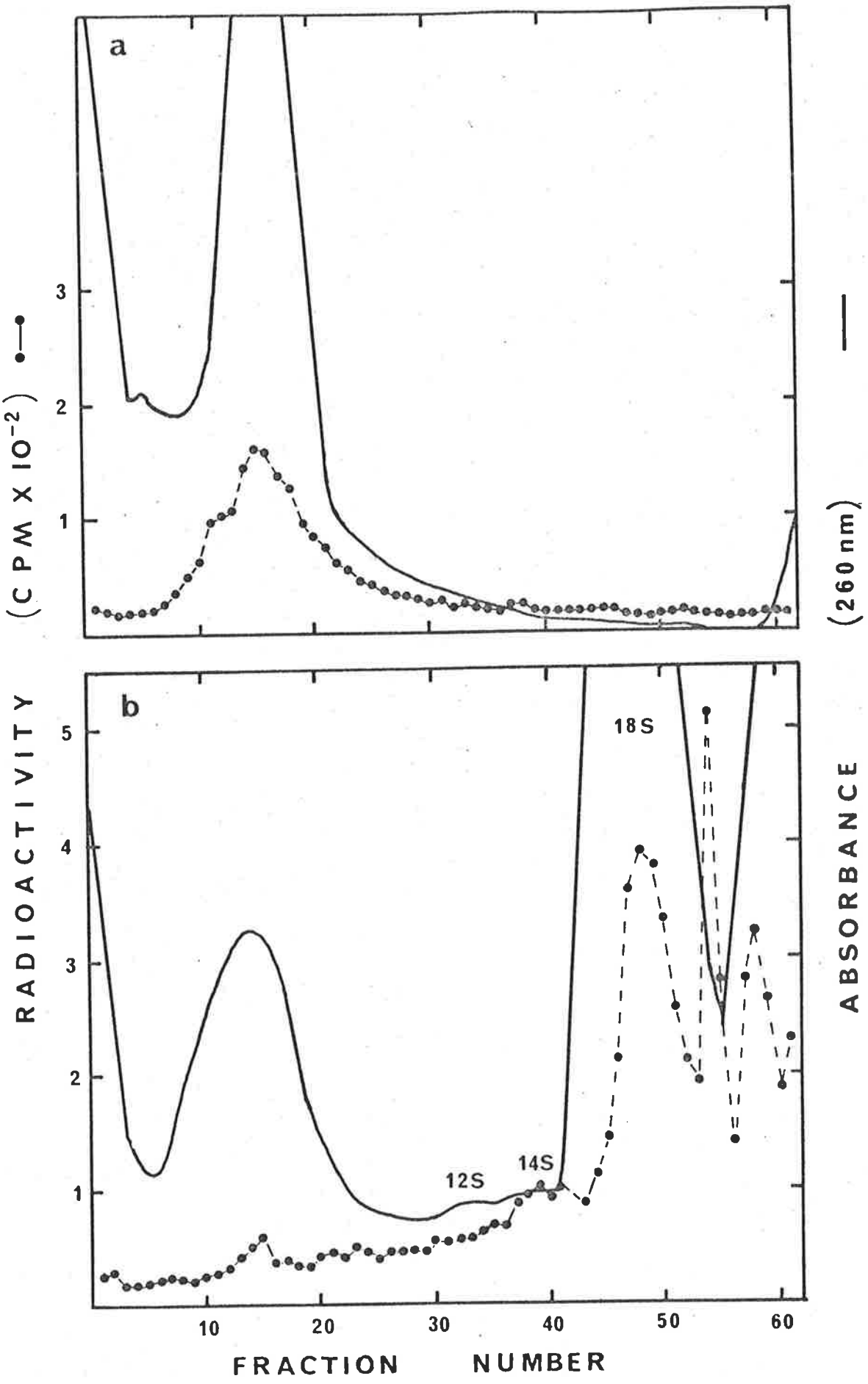
FIGURE 7.1. SUCROSE GRADIENT SEDIMENTATION OF LABELLED RNA ISOLATED FROM 13-DAY FEATHERS INCUBATED IN VITRO.

13-day feathers were incubated in vitro for 4 hr (see Methods 2.B.3) with 20 μC/ml of ^{3}H-orotic acid (S. Act. 15 mC/mM).

The lysate was prepared in MSB and fractionated into the polysomal and post-polysomal supernatants. The RNA from each was phenol-extracted and analysed by sucrose-gradient centrifugation (see Fig. 6.4).

Figure 7.1a. Absorbance and radioactivity profile of RNA isolated from supernatant fraction.

Figure 7.1b. As for (a), RNA from polysomal fraction.


a
(CPM X 10⁻²)
(260nm)
b
RADIOACTIVITY
ABSORBANCE
18S
14S
12S
FRACTION NUMBER

The absorbance and radioactivity profiles of the polysomal and post-polysomal supernatant RNA fractions are shown in Fig. 7.2.b and a respectively. It can be seen that the specific activity of the 12S and 14S RNA species was very high relative to the specific activities of the rRNA species. Furthermore, there was no peak of radioactivity discernible in the 20S region gradient as was found in the short term labelling with ^{3}H-orotic acid. For the supernatant fraction (see Fig. 7.2b) the major peak of absorbance was found to be in the tRNA region and the main peak of radioactivity coincided with this fraction. Moreover, there was neither significant absorbance nor radioactivity present in the 7-17S region of the gradient. It is possible however, that a minor peak of RNA could have been occluded by the large amount of tRNA present.

3. Comparison of 12SRNA species by cellulose chromatography

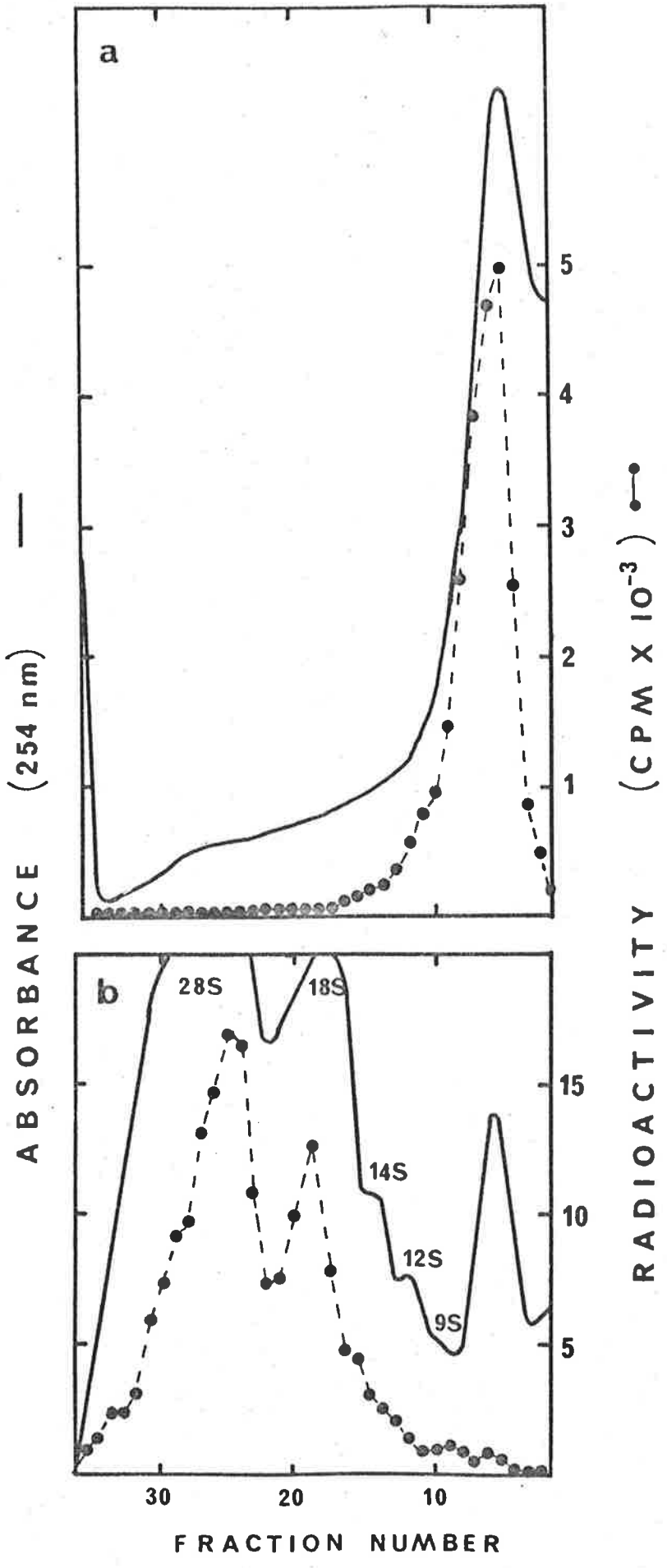
To investigate the relationship of the 12S RNA species observed in the 12-day feather lysate to the 12S keratin mRNA isolated from 14-day feather polysomes a comparison was made of the binding of the two 12S species to cellulose. Equal quantities of both phenol-extracted 12 and 14-day feather polysomal RNA were fractionated by chromatography on cellulose using the method of Schutz et al. (1972) (see Methods 7.B.1). The bound and non-bound fractions from each age were collected and ethanol precipitated. It was found that about 5.7% of the 14-day RNA was cellulose-bound. By comparison,

FIGURE 7.2. SUCROSE GRADIENT SEDIMENTATION OF LABELLED RNA ISOLATED FROM 13-DAY FEATHERS INCUBATED IN VITRO.

Details are as given in Fig. 7.1 except that feathers were incubated for 16 hr with 20 μC/ml of 5, 6-^{3}H-uridine (S.Act. 43 C/mM).

Figure 7.2a. Absorbance and radioactivity profile of RNA from polysomal-supernatant fraction.

Figure 7.2b. As for (a), RNA from polysomal fraction.


a
b
28S
18S
14S
12S
9S
5
4
3
2
1
15
10
5
30
20
10
FRACTION NUMBER
ABSORBANCE (254 nm) —
RADIOACTIVITY (CPM X IO^{-3}) ●—

4.7% of the 12-day RNA was bound to cellulose. The bound and unbound fractions were then analysed by sucrose gradient centrifugation. The results are shown in Fig. 7.3a-d. It can be seen (Fig. 7.3b) that a signficant proportion of the 12S RNA from 14-day feathers was bound to cellulose, together with a small amount of the rRNA species. By comparison (Fig. 7.3d) a minor amount of the 12S RNA from 12-day feathers was bound to cellulose, most was present in the non-bound fraction (Fig. 7.3c). It should be noted that the most of the 14S RNA's isolated from either age was not bound to cellulose.

4. Fraction of reticulocyte polysomal RNA by cellulose chromatography

Rabbit reticulocyte polysomal RNA has been fractionated by cellulose chromatography resulting in the retention and enrichment of an RNA fraction with messenger properties (Schutz et al., 1972). To ensure that the cellulose-chromatography was satisfactory rabbit reticulocyte polysomal RNA was chromatographed on cellulose using identical conditions to that described for feather RNA. The bound and non-bound fractions were analysed by sucrose-gradient centrifugation. The results are shown in Fig. 7.4a,b. It can be seen that the bound fraction (Fig. 7.4b) was enriched in the 10S globin mRNA whereas the unbound fraction contained only a small quantity of 10S RNA, but did contain most of the rRNA. The result is very similar to that obtained by Brawerman et al. (1971) using nitrocellulose filters to selectively retain the mRNA and the result obtained by Aviv

FIGURE 7.3. SUCROSE GRADIENT SEDIMENTATION OF RNA FRACTIONATED BY CELLULOSE-CHROMATOGRAPHY ISOLATED FROM 12 AND 14-DAY FEATHERS.

Sixty-five A_{260} units of RNA from 12 or 14-day feathers was fractionated separately by chromatography on cellulose (see Methods 7.B.1). The bound and unbound fractions were collected separately and redissolved in formamide (75 µl), heated 10 min at 37°. Each sample was then diluted to 0.50 ml with 0.1 M Tris-HCl, pH 9.0, and was loaded onto a sucrose gradient (11.6 ml, 10% - 40% w/v sucrose, prepared in 10 mM KCl, 2 mM EDTA, 20 mM Tris-HCl, pH 7.6) and centrifuged in an SW41 rotor for 16 hr at 201,125 g (a.v.).

Figure 7.3a. 14-day RNA, cellulose non-bound.

Figure 7.3b. 14-day RNA, cellulose bound.

Figure 7.3 12-day RNA, cellulose non-bound.

Figure 7.3d. 12-day RNA, cellulose bound.

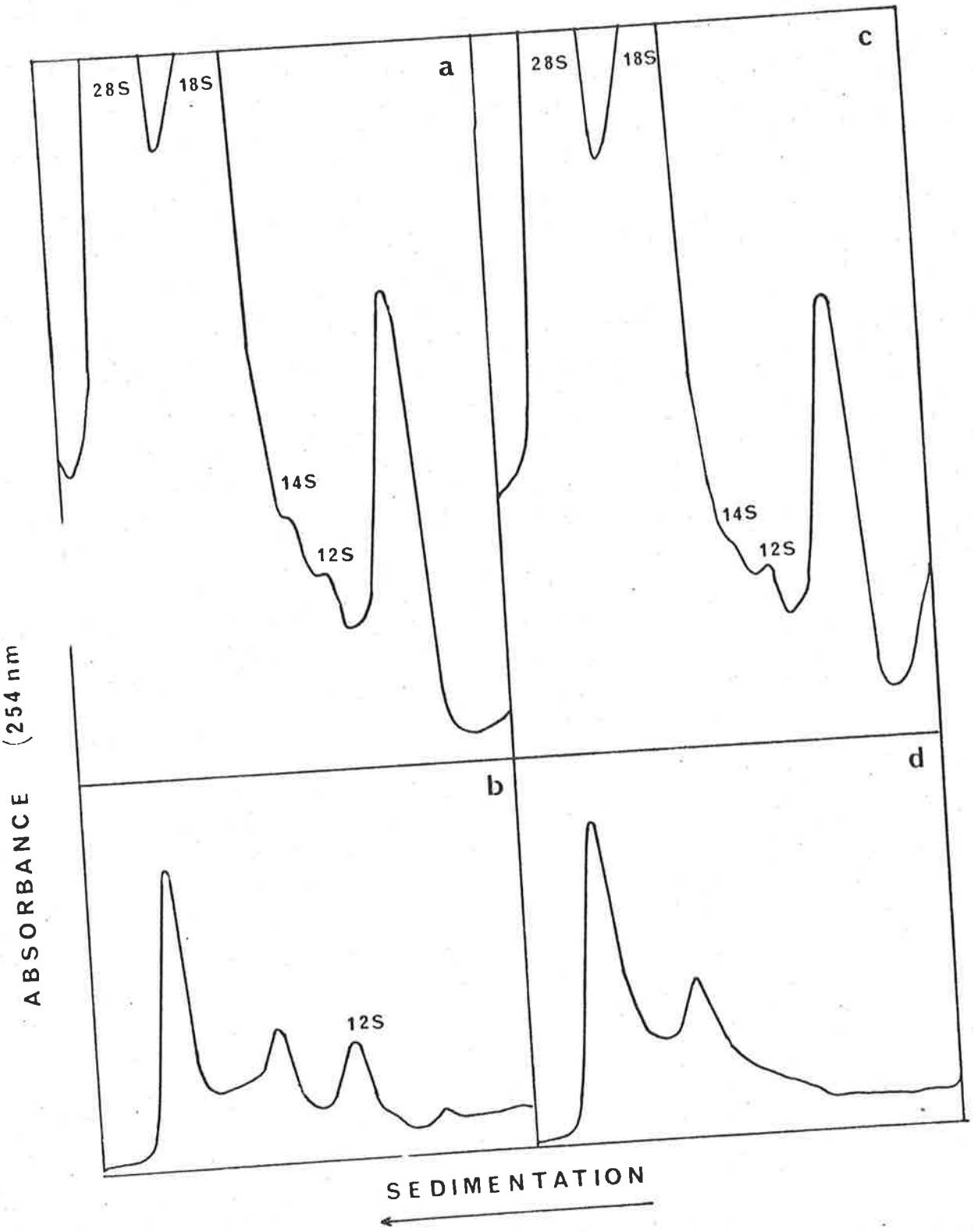
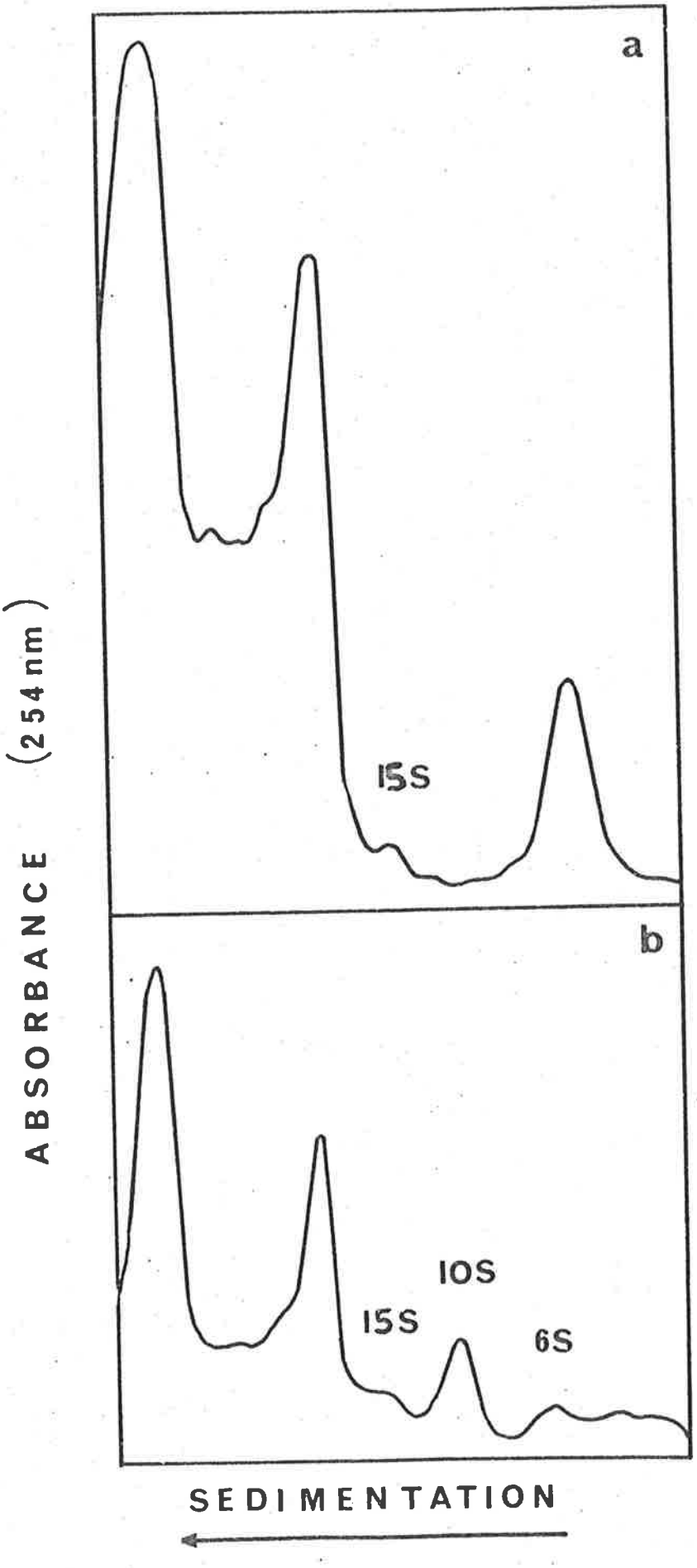

a
28S
18S
14S
12S
c
28S
18S
14S
12S
b
12S
d
ABSORBANCE (254 nm
SEDIMENTATION

FIGURE 7.4. SUCROSE GRADIENT SEDIMENTATION OF RNA (FRACTIONATED BY CELLULOSE CHROMATOGRAPHY) ISOLATED FROM RABBIT RETICULOCYTE POLYSOMES.

20 µg of phenol-extracted rabbit reticulocyte polysomal RNA was fractionated by cellulose chromatography (see Fig. 7.3). Centrifugation conditions as for Fig. 7.3. Time of run 12 hr.

Figure 7.4a. Cellulose non-bound RNA. One-quarter only loaded.

Figure 7.4b. Cellulose bound RNA.


a
15S
b
10S
15S
6S
ABSORBANCE (254 nm)
SEDIMENTATION

and Leder (1972) who used oligodeoxythymidine covalently bound to cellulose to fractionate reticulocyte polysomal RNA.

5. A comparison of the binding to cellulose of labelled RNA from 12- and 14-day feathers

Feathers isolated from 12 day embryos were labelled with ^{3}H-adenosine in Charity Waymouth's medium as described (see Methods 2.B.3). The post-mitochondrial supernatant was precipitated overnight by the addition of ethanol and collected by centrifugation (see Methods 6.B.2). The total cytoplasmic RNA was phenol-extracted using the method of Lee et al. (1971b) as described in Methods 6.B.2. Similarly, 14-day feathers were labelled in vitro with ^{3}H-adenosine and the polysomal RNA was then phenol-extracted. The RNA preparations from both ages chromatographed on cellulose as described previously. The RNA from the bound and unbound fractions of both ages was analysed by sucrose-gradient centrifugation. The absorbance and radioactivity profiles are shown in Fig. 7.5a - d. It can be seen that no significant amount of labelled 12S and 14S RNA species from the 12-day preparation was bound to cellulose (Fig. 7.5a). Furthermore, the major fraction of this RNA, as judged by the absorbance profile was found in the unbound RNA (Fig. 7.5b). In contrast much of the labelled RNA in the 7-17S region from 14-day feather RNA was cellulose bound (Fig. 7.5c); moreover, the peaks of radio-activity had absorbance counterparts in this region, indicating that the bulk of the 12S keratin mRNA at this age

FIGURE 7.5. SUCROSE GRADIENT SEDIMENTATION OF LABELLED RNA (FRACTIONATED BY CELLULOSE CHROMATO GRAPHY), ISOLATED FROM 12 OR 14-DAY FEATHERS INCUBATED IN VITRO.

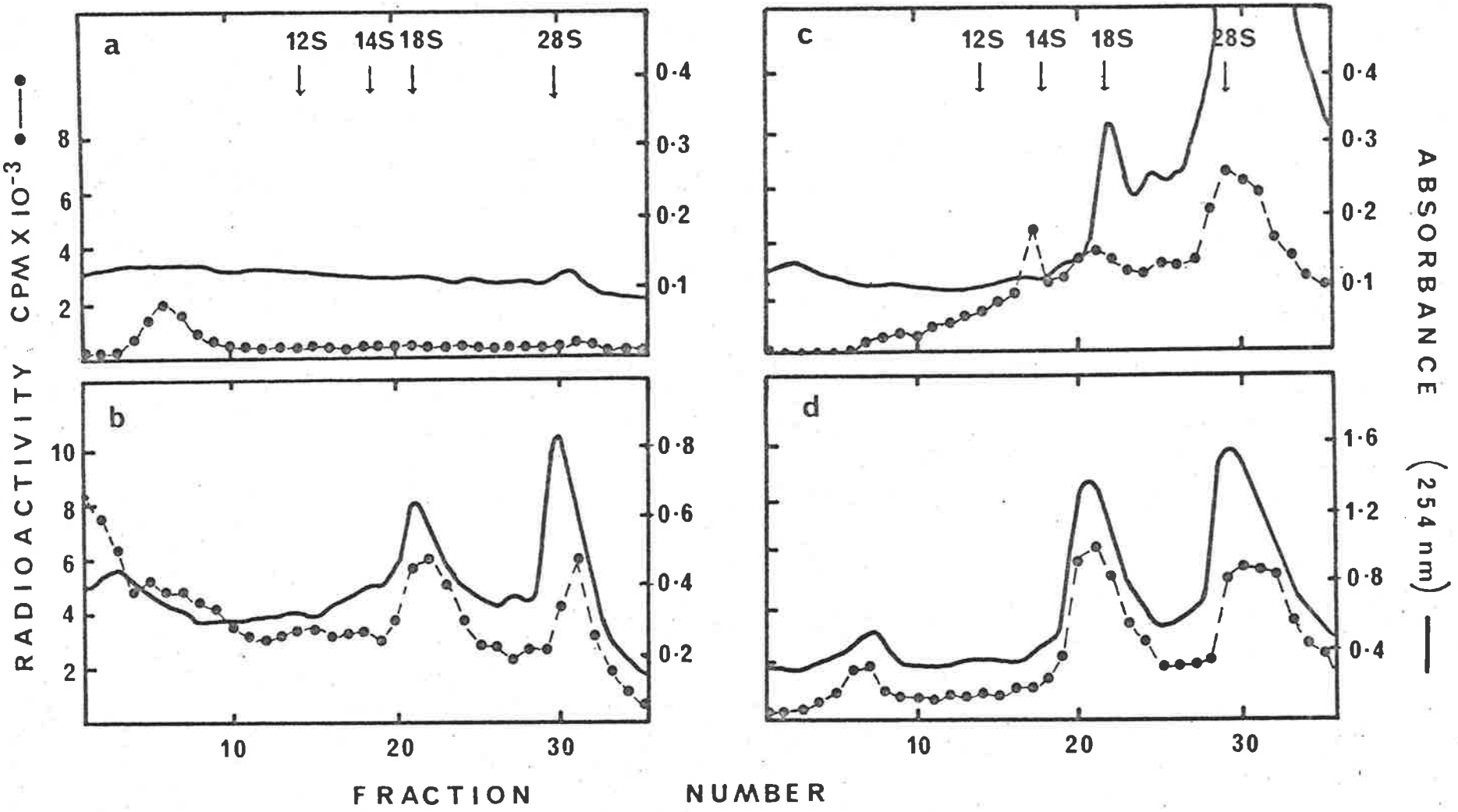
12 and 14-day feathers were incubated separately for 3 hr at 37° in vitro (see Fig. 7.1), with 20 μC/ml of 2,8 ^{3}H-adenosine (S. Act. 10 C/mM). The total cytoplasmic RNA from 12-day feathers and the polysomal RNA from 14-day feathers were each phenol-extracted and fractionated by cellulose-chromatography (see Fig. 7.3). Each fraction was then analysed by sucrose-gradient sedimentation (as given in Fig. 7.1) except that duration of run was 12 hr, at 201,125 g (a.v.).

Figure 7.5a. Cellulose bound 12-day RNA.

Figure 7.5b. Cellulose non-bound 12-day RNA.

Figure 7.5c. Cellulose bound 14-day RNA.

Figure 7.5d. One-quarter of cellulose non-bound 14-day RNA.


a
12S
14S
18S
28S
b
c
d
RADIOACTIVITY CPM X 10⁻³ ●——●
ABSORBANCE (254 nm) ——
FRACTION NUMBER
10
20
30

was cellulose-bound.

6. The effect of cordycepin on RNA synthesis in 12- and 13-day feathers

Thirteen-day feathers were incubated in two flasks containing Charity Waymouth's medium and in addition 0.05 µg/ml of actinomcyin D to suppress the labelling of rRNA (Perry, 1963; Roberts and Newmann, 1966; Penman et al., 1968). After a 25 min preincubation ^{3}H-uridine was added to one flask and 25 µg/ml of cordycepin to the other, which was then incubated a further 10 min before the addition of the label (Penman et al., 1970). Both preparations were incubated for 4 hr, before the feathers were chilled by addition of cold MSB (see Methods 2.B.3). Lysates prepared from both preparations were then fractionated into polysomal and post-polysomal supernatant fractions respectively. The RNA species present in each fraction was isolated and then analysed by sucrose-gradient centrifugation. The absorbance profile and radioactivity profile of the supernatant RNA fractions and the polysomal RNA fractions are shown in Fig. 7.6a-d. It can be seen that the actinomycin D had very little effect on the labelling of the feather rRNA. By comparison the supernatant fraction of RNA from the cordycepin-treated feathers had a number of radioactive peaks in the 7 - 17S region, with absorbance counterparts (Fig. 7.6c). These peaks were absent from the polysomal fraction (Fig. 7.6d), which had a similar level of radio-activity coincident with the 18S and 28S rRNA as did the

FIGURE 7.6. SUCROSE GRADIENT SEDIMENTATION OF LABELLED RNA ISOLATED FROM 13-DAY FEATHER INCUBATED IN VITRO IN THE PRESENCE OF INHIBITORS OF RNA SYNTHESIS.

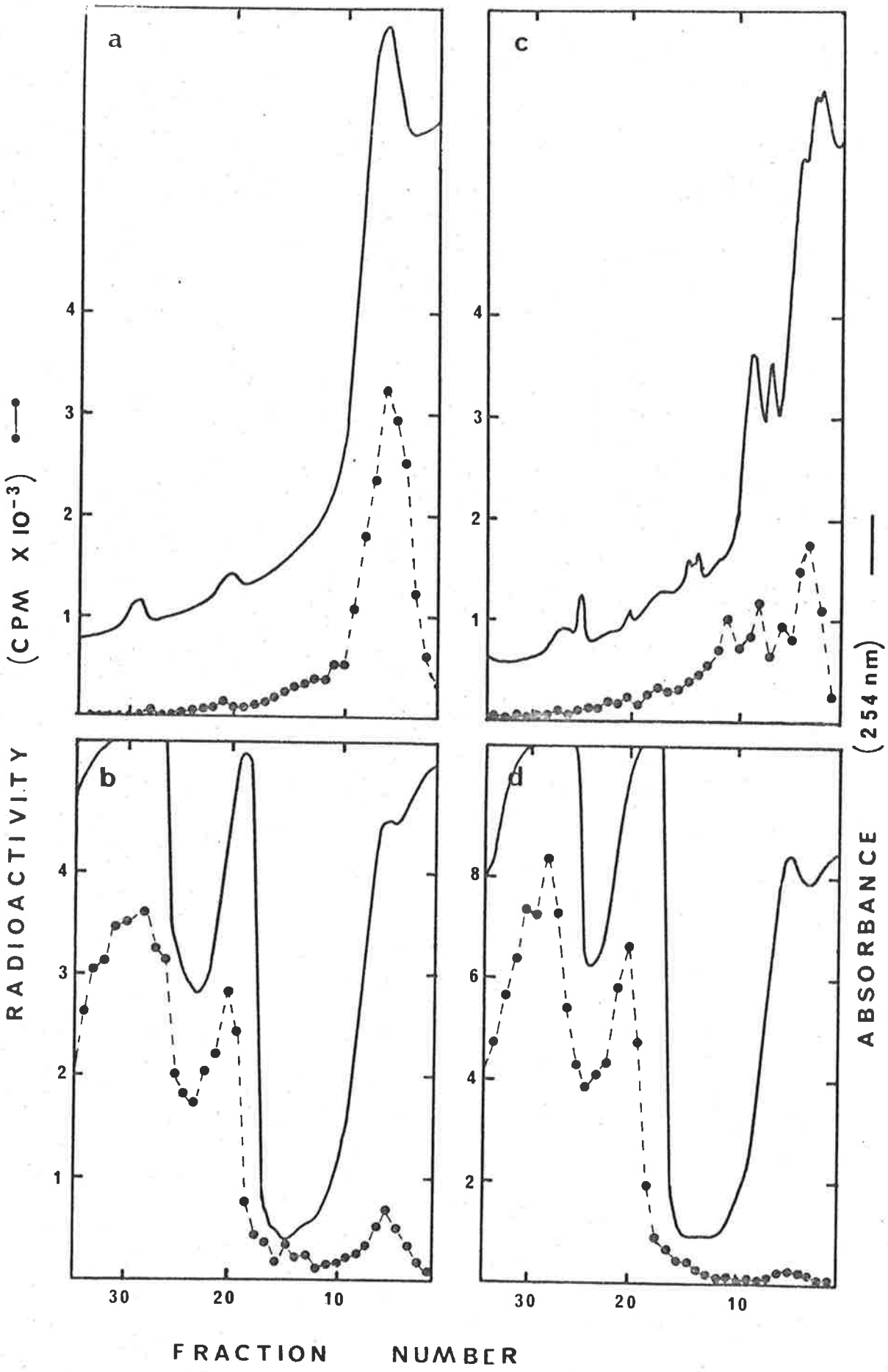
13-day feathers were incubated for 4 hr in vitro (see Fig. 7.1) with 50 μC/ml of 5,6-^{3}H-uridine (S. Act. 43 C/mM) present and with either 0.05 μg/ml actinomycin D or actinomycin D + 25 μg/ml cordycepin. The RNA was isolated from the polysomal and post-polysomal supernatant fractions and fractionated by sucrose-gradient sedimentation (see legend to Fig. 7.1 for method). Time of run 12 hr, at 3°.

Figure 7.6a. Supernatant RNA + actinomycin D.

Figure 7.6b. Polysomal RNA + actinomycin D.

Figure 7.6c. Supernatant RNA + actinomycin D + cordycepin.

Figure 7.6d. Polysomal RNA + actinomycin D + cordycepin.


a
c
b
d
(CPM X 10⁻³)
RADIOACTIVITY
ABSORBANCE (254 nm)
FRACTION NUMBER
30
20
10

incubation with actinomycin D alone. Nevertheless, there was no absorbance peak of 12S RNA in the cordycepin treated preparation, whereas it was clearly evident in the preparation treated only with actinomycin D (Fig. 7.6b).

Twelve-day feathers were labelled for 3 hr with ^{3}H-adenosine as a control and in the presence of a low concentration of actinomycin D (0.05 µg/ml) together with cordycepin (25 µg/ml). Lysates were prepared in MSB from both incubations (see Methods 2.B.2) and centrifuged to maximise the resolution on the sucrose gradients of the ribosomal subunits from the monosomes (see legend to Fig. 7.7 for details). Fractions were collected from the gradients to determine the acid-precipitable radioactivity. The major peak of radioactivity in the control incubation (Fig. 7.7) coincident with the small ribosomal subunit peak, with progressively lesser amounts over the large subunit region and the monosomes. Presumably the labelled peak at the top of the gradient was tRNA. Between the tRNA and the small ribosomal subunit there was a low level of heterodisperse labelling. The cordycepin treated preparation showed markedly reduced labelling over the ribosomal subunit region (Fig. 7.7). Furthermore, the labelling in the region just greater than tRNA was enhanced, a peak of labelled RNA ofabout 12S became more prominent.

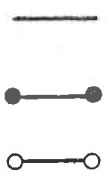
D. DISCUSSION

1. General comments

The resultsof RNA labelling *in vitro* indicated that whatever RNA precursor was used for labelling the level of

FIGURE 7.7. SUCROSE GRADIENT SEDIMENTATION OF LYSATE PREPARED FROM 12-DAY FEATHERS INCUBATED IN VITRO IN THE PRESENCE AND ABSENCE OF CORDYCEPIN AND ACTINOMYCIN D.

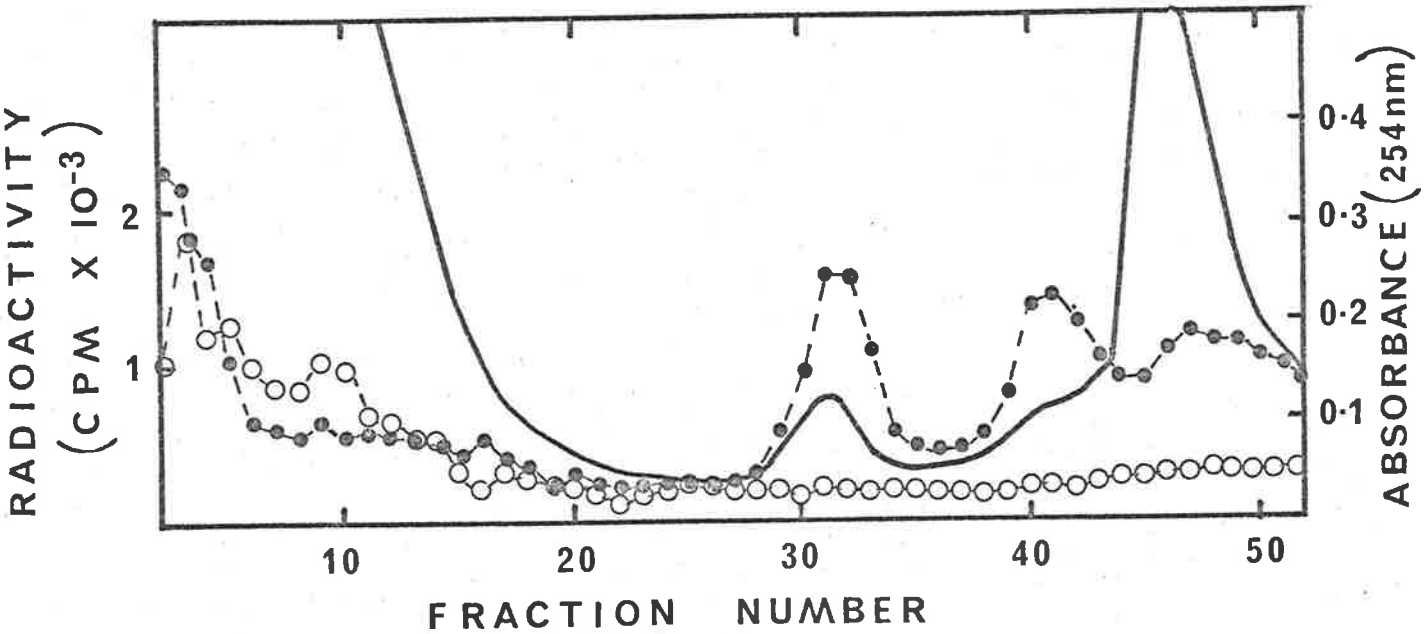
12-day feathers were incubated in vitro for 4 hr with 50 μC/ml of 2,8 ^{3}H-adenosine (S. Act. 10 C/mM) in the presence and absence of actinomycin D (0.05 μg/ml) plus cordycepin (25 μg/ml). Lysate was prepared in MSB from each of the feather preparations. Each preparation was centrifuged separately on a sucrose gradient (28 ml, 10 - 30 w/v sucrose) prepared in MSB. Centrifugation was for 16 hr at 52,635 g (a.v.) at 3^{o} in an SW25 rotor.



$A_{(254)}$

control

+ actinomycin D and cordycepin


RADIOACTIVITY
(CPM X 10-3)
2
1
0·4
0·3
0·2
0·1
ABSORBANCE (254nm)
10
20
30
40
50
FRACTION NUMBER

label incorporated into the RNA was low. Furthermore, the absorbance profiles of phenol-extracted RNA were very similar from 12-, 13- and 14-day feathers, in that two minor species of RNA of 12S and 14S respectively were detected in all three ages. It could be significant that under the conditions of labelling used it was shown (see results 3.B.6) that some polysome disaggregation occurred in 14-day feathers and extensive disaggregation was apparent in 12-day feathers. However, it should be noted that labelling of RNA *in ovo* with either ^{3}H-uridine or ^{3}H-adenosine resulted in much lower levels of label being incorporated into RNA, nevertheless, the spectrum of RNA synthesised was essentially identical to that found *in vitro* (Adams - personal communication).

Studies on the labelling of RNA in cultured 12- and 13-day feathers have previously been reported by Bell and Merrill (1967). This work is difficult to compare with the present studies as little detail of their methodology was published. From their data it can be seen that the peaks of radioactivity in lysates of 12- and 13-day feathers was mainly coincident with the ribosomal subunit region but with a second peak of high specific activity sedimenting at about 85S - 95S. While label was evident in these higher S value regions there was little absorbance contributed by polysomes. Some evidence was presented in Bell and Merrill's results on RNA labelling to show that the highest specific activity RNA sedimented in the 7-17S region when labelling times were short. However, the labelling was very heterodisperse, and not localised over any particular absorbance peak. There were

no absorbance peaks which corresponded to the 14S RNA nor the 12S keratin mRNA. It may be signficant that the transport of labelled heterogenous RNA was more rapid than was observed in the present study and may represent release of some nuclear RNA into the cytoplasmic fraction. Careful studies by many workers (for example, Perry and Kelley, 1968; Plagemann, 1969) have cautioned that nuclear disruption during preparation released into the cytoplasm highly labelled RNP's which cosedimented with the polysomes. That this may have occurred in the study described here must be borne in mind.

Studies on the labelling of 7, 9 and 11 day chick embryo skin in organ culture have been reported by Soriano and Desveux-Chabrol (1971). In this work it was found that the specific activity of RNA extracted from 7 and 9-day skin was higher than that extracted from 11-day skin. Furthermore, the total amount of label incorporated was of a similar order to that found in the present study. With long times of labelling two species of RNA sedimenting at 6S and 25S, respectively, and with a base composition similar to that of DNA were observed in the polysome fraction. The resolution of the sucrose gradients was not adequate to resolve these species with any measure of precision.

Wilkinson (1970b) reported <u>in vivo</u> labelling studies on wool follicles. However, using either ^{32}P or ^{3}H-uridine the only cytoplasmic species labelled in periods up to 4 hr were the rRNA and tRNA species.

Studies on the synthesis of specific mRNA's in terminally-differentiating cells have not yielded well

defined answers concerning the nature of the temporal relationship of mRNA synthesis to the onset of its translation. The study by Evans and Lingrel (1969b) referred to earlier has indicated that the synthesis of globin mRNA lagged to some extent the synthesis of rRNA, although in older cells the synthesis was concomitant, with the rRNA synthesis declining prior to that of mRNA synthesis. Nevertheless, it is not clear from that study, whether the synthesis of the mRNA preceeded its translation, that is whether the mRNA is stored prior to translation. No clear picture has emerged from the labelling studies of embryonic erythropoietic cells. Terada et al. (1971) investigated RNA synthesis in the yolk sac erythroid cells from 11- and 13-day foetal mice by incubating the cells in culture medium with RNA precursors. While two species of RNA of 9S and 14S respectively were observed to be labelled, in addition to the rRNA and tRNA species, neither had the properties expected for globin mRNA. The 9S species did not co-electrophorese with authentic globin mRNA and furthermore, its synthesis was inhibited by hydroxyurea (an inhibitor of DNA synthesis), indicating that it was probably histone mRNA, since histone synthesis appears to be coupled to the G1 phase of the cell cycle. By comparison, Weintraub et al. (1972) reported that erythroblasts from four-day chick embryos synthesised a labelled 10S RNA species when incubated in vitro. The synthesis of this RNA species was insensitive to cytosine arabinoside, an inhibitor of DNA synthesis.

The synthesis of polyribosomal RNA in duck erythroid cells has been investigated by many workers but the results

obtained by Pemberton and Baglioni (1973) are the most relevant to the present studies. Very immature erythrocytes were obtained from anaemic ducks and incubated *in vitro* for various times with ^{3}H-uridine. It was found after 1 hour of incubation that the amount of label in the polysomes was very low and furthermore, very heterogeneous.

It was only after 4 hr of incubation that clearly discernible labelling of the rRNA's and tRNA became apparent. Moreover, the amount of label incorporated after a 24 hr incubation was not significantly greater than that incorporated after 7 hr of labelling. The specific activity of a 10S RNA, which was shown to be globin mRNA was significantly higher than that of the rRNA species at all times after 4 hr of incubation. The results obtained in the present study were very similar to those described above in that little labelling of polysomal RNA was detected in labelling periods of less than 3 hr.

In the present study it was noted that a 20S RNA species was labelled during *in vitro* incubations. It could be argued that this may have been a cytoplasmic precursor to the 12S keratin mRNA. Some evidence was presented by Marou n *et al*. (1971) purporting to show that there was a 16S cytoplasmic precursor to globin mRNA. However, in the study by Pemberton and Baglioni (1973) mentioned previously no polysomal precursor to 10S globin mRNA was detected by the very sensitive technique of hybridization of RNA to DNA complementary to globin mRNA. In the present study very little keratin mRNA activity was found in the 18-28S regions of sucrose-

gradient fractionated 14-day feather polysomal RNA (see Results 6.C.3) and in fact, none was seen if the RNA was disaggregated with formamide prior to analysis (Kemp - personal communication).

2. Cellulose-chromatography of 12- and 14-day feather RNA

Eukaryotic mRNA's bind to cellulose during chromatography on this material in high salt buffers (Kitos et al., 1972; Schutz et al., 1972; Delarco and Guroff, 1973). In the present study it was found that the major part of the 12S keratin mRNA isolated from 14-day feather polysomes was cellulose bound, and therefore presumably contained poly(A) sequence(s). Furthermore, the bound fraction of this 12S has been shown to be enriched in keratin mRNA activity (Kemp - personal communication). It is obvious that evidence must be obtained to show definitively that the 12S RNA isolated from 12-day feathers is keratin mRNA lacking poly(A). It is interesting to note that maternal mRNA of sea urchin eggs becomes polyadenylated following fertilization (Slater et al., 1972, 1973; Wilt, 1973).

It is possible that the onset of keratin synthesis is preceded by polyadenylation of pre-existing keratin mRNA in and analogous manner to that described for the polyadenylation of pre-existing sea urchin mRNA after fertilization. Furthermore, it has been found in this same system (Skoultchi and Gross, 1973; Gross et al., 1973) that the maternal histone mRNA is located in the subribosomal fraction before fertilization. It would be of extreme interest to show that the mechanism

of mRNA storage in the cytoplasm prior to translation is a mechanism general to all eukaryotic cells.

3. The effect of cordycepin on the labelling pattern of 12- and 13-day feathers

It was observed that the low level of actinomycin D used did not supress rRNA labelling to any great extent in 13-day feathers. The reason for this is not clear but possibly it was the result of keratinized cells of the feather-sheath being relatively impermeable to the drug in the case of the feather. This also may explain the observation that there was progressively less labelling in 13- and 14-day feathers in comparison to 12-day feathers. Furthermore, the presence of cordycepin did not suppress the labelling of the rRNA species, although very little label was incorporated into these species in 12-day feathers in the presence of actinomycin D and cordycepin. The most interesting effect of cordycepin on 13-day feathers was the observation of several peaks of absorbance with radioactivity counterparts in the supernatant fraction; this was not observed in the presence of actinomycin D alone. It is of interest to compare these findings with those of Lavers et al. (1974), in which the effect of cordycepin on the 14S mRNA coding for lens α-crystallin was investigated. These authors found that when lens tissue was incubated with cordycepin the pre-existing lens α-crystallin mRNA lost its template activity for reverse transcriptase. This enzyme requires a poly(A) sequence as primer. Moreover, most of the

radioactivity over the 14S mRNA peak was no longer present. In the same context it has recently been reported (Nature New Biology News and Views, 245, 161, 1973) that there is a non-mitochondrial poly(A) activity in the cytoplasm. Since poly(A) length may correlate with the lifetime of mRNA (Darnell et al., 1973) such an activity could be involved in a translational control phenomenon.

4. Comments on future prospects

The studies reported in this chapter are of a preliminary nature but are reported in detail and dicussed in relation to the pertinent and extensive work of many authors. This has been done because it is an area of great importance in the overall understanding of the events involved in the terminal differentiation of keratinocytes, and the experiments performed to date, indicate the general direction in which the work should proceed.

The results of the labelling studies indicate that labelling of feathers in vitro is not likely to provide much definitive evidence for the existence of keratin mRNA stored in the cytoplasm prior to the onset of keratinization. Labelling studies could only provide definitive evidence for this if it could be shown that ten- day embryo feathers contained a 12S RNA species which could be proven to be keratin mRNA. Furthermore, these studies are beset by a problem hitherto unmentioned in the context of RNA labelling, namely that a gradient of differentiation exists in the feather,

with the most differentiated cells being at the tip of the feathers (Matulionis, 1970; Kemp et al., 1974).

This in effect means that some cells of 14-day feathers are no further developed than most of the cells of 12-day feathers, making interpretations of results exceedingly difficult.

The most interesting facts obtained in the present study relate to the finding that a 12S RNA species, presumably not containing poly(A) was present in large amounts in 12-day feathers. If it can be shown that this is keratin mRNA, by hybridization to DNA complementary to keratin mRNA it will be a very significant result in the understanding of the control of mRNA activity within developing embryo feathers and possibly in other differentiating tissues. Furthermore, it should be possible to show that prior to keratinization this 12S species is located in the post-polysomal fraction.

CHAPTER EIGHT

CONCLUDING DISCUSSION

The aims of the work reported in this thesis were directed towards the eventual understanding of the mechanisms operating at the molecular level to control the onset of keratinization in the developing chick embryo feather. Although some achievements have been made towards this aim the results obtained have mainly left more intriguing questions to be answered.

The results described in Chapter 4 demonstrated that a cell-free system prepared from 14-day feathers was active in the de novo incorporation of amino acids into protein. Furthermore, the major product of the system was feather keratins, which had, as far as the assay system was able to resolve, the same relative rates of synthesis as occurs in vivo. The most significant finding was that the system had an efficiency for de novo protein synthesis greater than that of reticulocyte lysate when the ionic conditions were optimized. It should be of general use for investigating the mechanisms of protein synthesis and possibly for the translation of exogenous mRNA's.

Some experiments were conducted to compare the kinetics of incorporation in 12, 13 and 15-day feather cell-free systems. While it was observed that the kinetics and the nature of the products of the 13 and 15 day systems were similar to the 14-day system it appeared that the 12-day system may warrant further investigation. Although the experiments on the 12-day feather cell-free system were done in the non-optimal MSB conditions, preliminary experiments (not reported

in this thesis) have indicated this system, when prepared in LSB has the same relative rate of de novo incorporation as has the 14-day system prepared under the same conditions. The total incorporation, however, was some four-fold lower.

It should now be possible to determine whether some form of translational control is operative at the time of onset of keratinization. If keratin mRNA is stored in the cytoplasm prior to the onset of keratinization there would appear to be three main possibilities for controlling this,

(a) there may be a ribosome bound inhibitor of protein synthesis which is removed at the onset of keratinization. A similar situation has been reported to exist in sea-urchin eggs prior to fertilization (Metafora et al. 1971).

(b) an inactive mRNP complex could undergo some modification, for example, by loss or exchange of certain proteins of the mRNP complex to allow ribosome binding to occur. Such an event has been suggested to explain the difference in the protein complement of free globin chain mRNP in comparison to polysome-bound globin-mRNP of duck reticulocytes (Gander et al., 1973).

(c) the mRNA itself could undero some modification. Preliminary work described in Chapter 7 indicated that there was a 12S RNA species in 12-day feathers which lacked poly(A). Poly-adenylation of this RNA could precede the onset of keratin synthesis. This mechanism is another described

to explain the increase in protein-synthetic activity following the fertilization of sea-urchin eggs (Slater et al., 1972, 1973; Wilt, 1973).

(d) there may be a specific initiation factor or tRNA requirement for keratin mRNA, with either one of these being the controlling element in the 'switch-on' of keratin synthesis. This would appear to be rather unlikely as the keratin mRNA can be translated in an heterologous system without the need for any other components of feather tissue.

The preparation of the highly active cell-free system will allow these possibilities to be investigated by careful fractionation and recombination of various components from 12 and 14-day feather cell-free systems.

Unquestionably, the most significant finding described in this thesis was the isolation of a 12S RNA species from 14-day feather polysomes, which coded for, as far as it could be discerned, the complete complement of feather keratin chains, identical to native feather keratin. This mRNA was translated in a rabbit reticulocyte cell-free system without any requirement for other component(s) from feather tissue. This system has been somewhat superseded in usefulness by the finding (Kemp and Schwinghamer - personal communication) that the keratin mRNA can be translated in the wheat embryo system of Shih and Kaesburg (1973). This system has an important advantage in that the endogenous protein synthetic activity is very low,

allowing for easy identification of the protein products of the exogenous mRNA.

The results of attempts to isolate keratin mRNA as an mRNP complex were described in Chapter 5. However, it has been found (Kemp - personal communication) that by treatment of polysomes with EDTA at pH 9.0 (Zehavi-Willner, 1970) the mRNP can be reproducibly released. The mRNA from this mRNP runs as a single band when electrophoresed under strongly denaturing conditions. Furthermore, this result confirmed that the mRNA's coding for the keratins all had essentially the same molecular weight, indicating that large untranslated sequences must have been rigorously conserved during the evolution of these separate species.

The purified mRNA has now been used as a template to direct the RNA-dependent DNA polymerase of avian myeloblastosis virus, to produce a DNA copy of the mRNA. This can be used to determine the reiteration frequency of the keratin genes in the chick genome and to determine if specific amplification of the keratin genes occurs in the feather tissue. It has been suggested (Kemp, 1972) that the large number of keratin chains present may simply reflect a need for a high rate of mRNA production at the time of onset of keratinization. However, this would seem to be an unlikely possibility for the following reason. The reiteration frequency of globin genes in the erythpoietic series of mouse (Harrison et al., 1972) and duck (Bishop and Rosbash, 1973) has been determined. Even though these cells produce predominantly haemoglobin only 1-3 globin genes were found to be present. Suzuki et al. (1972)

reported a similar finding for the reiteration frequency of the genes coding for silk fibroin in the silk glands in Bombyx mori. Furthermore, they presented calculations to show that transcription of each fibroin gene was sufficient to account for the large amount of stable mRNA present. No amplification of the genes coding for the immunoglobulin light chain was detected in myeloma cells (Deltovich and Baglioni, 1973). From these results it would appear that neither gene reiteration nor amplification is necessary for the production of large amounts of mRNA in cells committed mainly to the synthesis of a particular protein.

Using the DNA copy of keratin mRNA it should be possible to determine the nature of the nuclear RNA precursor to keratin mRNA. This may produce significant results, as the keratin genes may be closely linked on the chromosome giving rise to the possibility that the gene-battery may have only one controlling element so that all the keratin genes may be transcribed as a single HnRNA species which is subsequently cleaved, to produce the discrete keratin mRNA's. It should be noted, however, that as yet no precedent has been reported in eukaryotic cells for this postulate.

As discussed in Chapter 7 the main disadvantage of the feather system as an example of a terminally differentiating system appears to be that the feather cells are not phased in development, the onset of keratinization occurs at the feather tip, and the feather sheath then spreading inwards and downwards (Bell and Merrill, 1967; Matulionis, 1970). Such a situation could conceivably cause erroneous conclusions to be made on

protein synthetic activity of a 14-day feather cell-free system as compared to a 12-day system when in fact both would contain a mixture of cytoplasmic components from both completely differentiated and undifferentiated cells, with the only variable being the ratios of the two cell types in each preparation.

Furthermore, it would be of advantage to obtain culture conditions in which the onset of keratinization could be studied in a chemically defined medium, to enable investigations to be made into the effect on keratinization of hormones, vitamins and antimetabolites. To date this has not been achieved but *a priori* there appears to be no reason why this cannot be done. Piatagorsky *et al*. (1973) have reported that explanted lens epithelia undergo many changes characteristic of maturing fibres. A similar result was noted by Braverman and Katoh (1971).

Despite this shortcoming the progress made on the understanding of the events involved in the terminal differentiation of the chick embryo feather justifies further investigation and it would seem that the most exciting phase of this research lies in the near future.

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APPENDIX - PUBLICATIONS

1. PAPERS PUBLISHED*

Isolation of Feather Keratin mRNA and its Translation in a Rabbit Reticulocyte Cell-free System.
(with D.J. Kemp and G.E. Rogers)
Nature New Biology, 246, 33-36 (1973).

2. PAPERS PRESENTED AT MEETINGS

Differentiation in the Developing Chick Feather III. In vitro Studies of Protein Synthesis.
(with D.J. Kemp and G.E. Rogers)
Proc. Aust. Biochem. Soc. 4, 33 (1971).

3. PAPERS IN PREPARATION

Isolation and Molecular Weight of Pure Feather Keratin mRNA.
(with D.J. Kemp and G.E. Rogers)
Submitted to Biochem. Biophys. Res. Commun.

Keratin Synthesis in a Cell-free System from the Embryonic Chick Feather.
(with D.J. Kemp and G.E. Rogers)

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*Reprint bound at back of thesis.

Partington, G. A., Kemp, D. J. & Rogers, G. E. (1973). Isolation of feather keratin mRNA and its translation in a rabbit reticulocyte cell-free system. *Nature New Biology*, *246*(150), 33-36.