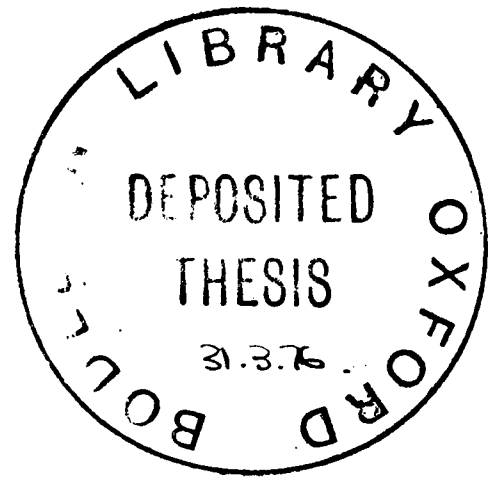
THE RIBOSOMAL PROTEINS OF EUKARYOTIC CELLS

A thesis presented for the
Degree of
Doctor of Philosophy



David J. Kuter
Sir W^m Dunn School of Pathology
South Parks Road
Oxford

Magdalen College, Oxford
Michaelmas Term
1975

TABLE OF CONTENTS

		Page
LIST OF FIGURES		iv
LIST OF TABLES		vi
LIST OF ABBREVIATIONS		vii
ACKNOWLEDGEMENTS		viii
CHAPTER 1	INTRODUCTION	1
CHAPTER 2	MATERIALS AND METHODS	6
2.000	Chemicals	6
2.010	Cell culture	6
2.011	Media and solutions	6
2.012	Description of cell lines, tissues and transplantable tumors	8
2.013	Bulk cell culture	10
2.014	Radioactive labelling of cells	11
2.015	Freezing and storing cell lines	11
2.016	Cloning	11
2.020	Production of hybrid cells	11
2.030	Chromosome technique	12
2.031	Preparation for staining	12
2.032	Chromosome stain	13
2.033	C-banding	13
2.034	Chromosome counting	13
2.040	Isolation of ribosomes	14
2.050	Extraction of ribosomal protein	15
2.060	Subunit preparation	16
2.070	Two-dimensional gel electrophoresis	17
2.071	Standard method	17
2.072	Micro 2-D method	18
2.073	Drying gels	19
2.074	Counting radioactive gels	20
2.075	Recovery of protein from stained gel spots	20
2.080	Extraction and electrophoresis of RNA	20
2.081	Hot chloroform-phenol method	20
2.082	Cold chloroform-phenol extraction	21
2.083	2.7% polyacrylamide gels	21
2.084	10% polyacrylamide gels	22
2.090	5S rRNA-protein preparation	23
2.100	SDS polyacrylamide gel electrophoresis	24
2.110	pH 4.5 gels	24
2.120	Preparation of ribosomal precursor particles	25
2.130	Immunology	27
2.131	Solublization of ribosomal proteins	27
2.132	Immunization	27
2.133	Double diffusion analysis	28
2.134	Haemagglutination	28
2.140	Analytical ultracentrifugation	29
2.150	Determination of protein concentration	29
2.160	Ribosome dimers	29
2.170	CsCl gradients	30

2.180	Dialysis tubing	31
CHAPTER 3	COMPARISONS OF RIBOSOMAL PROTEIN PATTERNS BETWEEN SPECIES -- CATIONS	32
3.000	Introduction	32
3.010	Purity and characterization of isolated ribosomes	37
3.020	Extraction of ribosomal proteins	39
3.030	Interpreting 2-D gels	41
3.040	Chick embryo ribosomes	45
3.050	Ribosomes of mouse cell lines	46
3.060	Rat tissues and lines	48
3.070	Human ribosomes -- Hela	50
3.080	Antibodies to chick and mouse ribosomes	51
3.090	Discussion	52
3.100	Summary	62
CHAPTER 4	COMPARISONS OF RIBOSOMAL PROTEIN PATTERNS BETWEEN SPECIES -- ANIONS	63
4.000	Introduction	63
4.010	Interpretation of gels	65
4.020	Standard method anion patterns	66
4.030	Microgel analysis of anionic ribosomal proteins	67
4.040	One-dimensional comparisons	67
4.050	Discussion	68
4.060	Summary	71
CHAPTER 5	THE 5S rRNA BINDING PROTEIN	72
5.000	Introduction	72
5.010	Release of the complex by EDTA	75
5.020	RNA gel electrophoresis of 5S rRNA	77
5.030	Is the 5S rRNA-protein complex a true complex?	80
5.040	Molecular weight of the 5S rRNA binding protein	81
5.050	2-D gels of 5S rRNA binding protein	82
5.060	Discussion	83
5.070	Summary	87
CHAPTER 6	THE PROTEINS OF HELA RIBOSOMAL SUBUNITS	88
6.000	Introduction	88
6.010	RNA extraction and electrophoresis	91
6.020	Subunit preparation	93
6.030	Subunit purity	96
	6.031 40S subunits	96
	6.032 60S subunits	96
	6.033 60SP subunits	97
	6.034 60' particles	97
	6.035 Criteria of purity	97
6.040	Two-dimensional gels of isolated subunits	98
6.050	Discussion	101
6.060	Summary	103
CHAPTER 7	THE PROTEIN COMPOSITION OF HELA NUCLEOLAR RIBOSOMAL PRECURSOR PARTICLES	104
7.000	Introduction	104
7.010	Precursor isolation and purity	112

7.020	2-D gel electrophoresis of isolated precursors	113
7.030	Discussion	115
7.040	Summary	120
CHAPTER 8	THE SYNTHESIS OF RIBOSOMAL PROTEINS AND rRNA IN HYBRID CELLS	121
8.000	Introduction	121
8.010	Selection and karyotype analysis of hybrids	131
8.020	Ribosomal RNA in A9HT-rat leukemia hybrid cells	135
8.030	Ribosomal proteins in hybrid cells	136
	8.031 A9HT/rat leukemia hybrid	136
	8.032 Mouse-chick hybrid cells	138
8.040	Do hybrid cells have hybrid ribosomes?	138
8.050	Discussion	139
8.060	Summary	143
CHAPTER 9	FURTHER DIRECTIONS	144
REFERENCES		150
ABSTRACT		163

LIST OF FIGURES

		Follows Page
Fig 1	Structure and processing of Hela precursor RNA.	2
Fig 2	Sedimentation profile of Hela ribosomes.	37
Fig 3	Absorbance profile of Hela ribosomes.	38
Fig 4	Scans of 10% RNA gels of rat liver polysomes.	39
Fig 5	10% RNA gels of rat liver ribosomes.	40
Fig 6	Chick embryo ribosomal proteins.	41
Fig 7	Chick embryo cationic ribosomal proteins.	41
Fig 8	Schematic diagram of chick embryo ribosomal proteins.	42
Fig 9	Schematic diagram of mouse ribosomal proteins.	46
Fig 10	Two-dimensional gel pattern of A9HT ribosomal proteins.	47
Fig 11	Two-dimensional pattern of A9HT cationic ribosomal proteins.	47
Fig 12	Two-dimensional gel pattern of Ehrlich ascites ribosomal proteins.	47
Fig 13	Two-dimensional pattern of Ehrlich ascites cationic ribosomal proteins.	47
Fig 14	Two-dimensional gel pattern of L929 ribosomal proteins.	48
Fig 15	Two-dimensional gel pattern of A9(L) ribosomal proteins.	48
Fig 16	Two-dimensional pattern of A9(L) cationic ribosomal proteins.	48
Fig 17	Two-dimensional pattern of A9(H) ribosomal proteins.	48
Fig 18	Two-dimensional pattern of rat liver cationic ribosomal proteins.	49
Fig 19	Two-dimensional pattern of rat leukemia cationic ribosomal proteins.	49
Fig 20	Two-dimensional pattern of Novikoff hepatoma ribosomal proteins.	49
Fig 21	Two-dimensional pattern of Novikoff hepatoma ribosomal proteins.	49
Fig 22	Schematic diagram of rat ribosomal proteins.	49
Fig 23	Two-dimensional pattern of Hela cationic ribosomal proteins.	50
Fig 24	Schematic diagram of Hela ribosomal proteins.	50
Fig 25	Double diffusion analysis of antisera to ribosomes and ribosomal proteins.	51
Fig 26	One-dimensional electrophoresis of anionic proteins of extracted and unextracted ribosomes.	66
Fig 27	Two-dimensional pattern of rat liver anionic ribosomal proteins.	67
Fig 28	Two-dimensional pattern of Hela anionic ribosomal proteins.	67
Fig 29	Two-dimensional pattern of rat liver anionic ribosomal proteins.	67
Fig 30	Two-dimensional pattern of Hela anionic ribosomal proteins.	67
Fig 31	Schematic diagram of anionic ribosomal proteins.	67

Fig 32	Separation of anionic ribosomal proteins at pH 8.6.	68
Fig 33	Sedimentation profile of EDTA-treated rat liver polysomes.	75
Fig 34	Sedimentation profile of EDTA-treated rat liver ribosomes.	75
Fig 35	Sedimentation profiles of 5S rRNA-protein complex at varying EDTA concentrations.	76
Fig 36	Scans of 10% RNA gels of 5S rRNA-protein complex.	78
Fig 37	Stained 10% RNA gels of 5S rRNA-protein complex.	78
Fig 38	Scans of 10% RNA gels of EDTA-treated ribosomes.	79
Fig 39	Stained 10% RNA gels of EDTA-treated ribosomes.	79
Fig 40	Stained 10% RNA gels of EDTA-treated rat liver ribosomes.	80
Fig 41	Release of 5S rRNA-protein complex from ribosomes; gel scans.	80
Fig 42	Release of 5S rRNA-protein complex from ribosomes; stained gels.	80
Fig 43	Molecular weight standard curve.	81
Fig 44	SDS polyacrylamide gel electrophoresis of 5S rRNA binding protein and molecular weight standards.	81
Fig 45	SDS polyacrylamide gels of molecular weight standards and 5S rRNA-protein complex.	81
Fig 46	SDS polyacrylamide gel of 4S and 5S RNA complexes.	82
Fig 47	Two-dimensional cation pattern of EDTA-treated rat liver ribosomes.	83
Fig 48	Two-dimensional cationic pattern of rat liver ribosomes not treated with EDTA.	83
Fig 49	Scans of 2.7% polyacrylamide gels of rRNA.	92
Fig 50	Scans of 2.7% RNA gels run with rRNA prepared by different methods.	93
Fig 51	Sedimentation profiles of puromycin-treated Hela polysomes.	94
Fig 52	Sedimentation profiles of Hela subunit preparations.	94
Fig 53	Scans of 2.7% RNA gels of isolated Hela subunits.	96
Fig 54	Sedimentation profiles of isolated Hela subunits.	96
Fig 55	Two-dimensional pattern of Hela 40S ribosomal subunit cationic proteins.	99
Fig 56	Two-dimensional pattern of Hela 60S ribosomal subunit cationic proteins.	99
Fig 57	Two-dimensional pattern of the cationic proteins from a mixture of Hela 40S and 60S ribosomal subunits.	99
Fig 58	Schematic diagram of caticnic ribosomal proteins of Hela subunits.	99
Fig 59	Sedimentation profiles of Hela nucleolar precursor particles.	112
Fig 60	Two-dimensional pattern of anionic proteins of Hela 55S precursor particles.	113
Fig 61	Two-dimensional pattern of anionic proteins of Hela 80S precursor particles.	114
Fig 62	Two-dimensional pattern of cationic proteins of Hela 55S precursor particles.	114
Fig 63	Two-dimensional pattern of cationic proteins of Hela 80S precursor particles.	114
Fig 64	Schematic diagram of the proteins of Hela precursor particles.	114

Fig 65	Karyotype analysis of A9HT (unbanded).	132
Fig 66	Karyotype analysis of A9HT (C-banded).	132
Fig 67	Karyotype analysis of rat leukemia (C-banded).	132
Fig 68	Karyotype analysis of rat embryo fibroblasts (unbanded).	133
Fig 69	Karyotype analysis of rat embryo fibroblasts (C-banded).	133
Fig 70	Karyctype analysis of A9HT/rat leukemia (unbanded).	133
Fig 71	Karyotype analysis of A9HT/rat leukemia (C-banded).	133
Fig 72	Sedimentation profiles of ribosome dimers.	135
Fig 73	Two-dimensional patterns of cationic proteins of hybrid ribosomes.	137
Fig 74	Two-dimensional pattern of the proteins of A9/ chick ribosomes.	138
Fig 75	The presence of hybrid ribosomes in hybrid cells.	139

LIST OF TABLES

		Follows Page
Table I	Summary of ribosomal proteins in different species.	52
Table II	Summary of C-banded chromosomes of A9HT-rat leukemia and its parents.	134

LIST OF ABBREVIATIONS

ATP	adenosine triphosphate.
C-band	centromere (centromeric) band.
DMEM	Minimal Essential Medium -- Dulbecco's Modification (2.011).
DNA	deoxyribonucleic acid.
DNase	deoxyribonuclease.
DOC	deoxycholate.
EA	Ehrlich ascites (2.012).
EMC	Encephalomyocarditis virus.
EDTA	ethylenediamine tetraacetic acid.
g_{ave}	relative centrifugal force (average).
GTP	guanosine triphosphate.
GuHCl	guanidine hydrochloride.
HAT	hypoxanthine, aminopterin, thymidine (2.011).
HAU	haemagglutinating units.
HCOH	formaldehyde.
HGPRT	hypoxanthine, guanine phosphoribosyl transferase.
HnRNA	heterogeneous, nuclear ribonucleic acid.
HSB	high salt buffer (2.120).
IMPase	inosinic acid pyrophosphorylase.
ma	milliampere.
MEM	Minimal Essential Medium (2.011).
mRNA	messenger ribonucleic acid.
MW	molecular weight.
$\overline{M}w$	weight-average molecular weight.
NHAC	Novikoff hepatoma ascites cells (2.012).
NKS	neomycin, kanomycin, streptomycin (2.011).
ODU	optical density unit.
1-D	one-dimensional.
PBS	phosphate buffered saline (2.011).
psi	pounds per square inch.
RBC	red blood cell(s).
rDNA	ribosomal DNA.
RNA	ribonucleic acid.
RNase	ribonuclease.
RNP	ribonucleoprotein.
rpm	revolutions per minute.
rRNA	ribosomal RNA.
RSB	reticulocyte standard buffer.
s.c.	subcutaneous.
SDS	sodium dodecyl sulfate.
TCA	tri-chloroacetic acid.
TEMED	N,N,N',N'-tetramethylethylenediamine.
tRNA	transfer (soluble) RNA.
2-D	two-dimensional.
u.v.	ultraviolet.
V	volts.
vs..	versus.
wt	weight.

ACKNOWLEDGEMENTS

There are many to whom I am grateful for making my stay in Oxford interesting, happy and thought-provoking. Writing this thesis after a year's absence and in less amenable surroundings, the memories become more acute and pleasant. I am fortunate to have had Dr. A. Rodgers suggest this project to me and serve as my supervisor. His kind instruction (to an often difficult student) and friendship have made these studies progress without undue difficulty. It has been a pleasure to have been closely associated with him for three years and his ideas and conversation I have come to miss.

The more routine burdens of this project have been reduced by the help of Mrs. M. Ramage and Mr. A. J. Parkes who gladly helped make ribosomes, culture cells and turn off centrifuges at odd hours of the day or night. To numerous others in the School of Pathology I am grateful for tips on experimental technique and for the gifts of reagents and equipment. I thank Mr. James Kent for his tireless devotion and willing provision of the animals used in these experiments. Most important, I am grateful for Mr. Kent's friendship and conversation that made the long nights at the lab most pleasurable.

Ms. R. Herbert and Mr. S. Buckingham were always willing to photograph my experimental results and are responsible for the excellent photos that improved my often mediocre gels. I also thank Mr. H. Stroud & Co. for the many pieces of equipment built and repaired for me. Without their assistance little could have been accomplished.

Drs. Heatley, Gladstone and Webb have been very kind in expressing their interest in this work and providing me with that sense of history which is associated with the School of Pathology.

My fellow students, Alan Jones, Steven Goss, Ted Duvall, Ann Wakefield and Dennis Parker, have made these several years pleasant and rewarding. My wife and fellow student, Irene Kuter, has been a valuable source of ideas and encouragement during these experiments. Her patience has made the writing of this thesis go more swiftly.

I thank Prof. H. Harris for providing a space for me in his lab. The Rhodes Trust has partially supported me during this work.

CHAPTER 1

INTRODUCTION

The 80S* eukaryotic ribosome is the site of protein synthesis and is a complex organelle constructed from interacting RNA and protein molecules. In certain physiological states and upon dissociation by artificial means, two different subunits are found, the large (60S*) and the small (40S*) (113,132,365,366). The 60S subunit consists of a number of proteins and 28S rRNA while the 40S subunit also consists of a number of proteins but with 18S rRNA. In addition to the 18S and 28S rRNA, two small molecular weight ribosomal RNAs are found in the 80S ribosome, the 5S rRNA (29,130, 164) and the 5.8S rRNA [previously called the 7S rRNA (276,397)]. Both appear to be associated with the larger subunit. The eukaryotic ribosome is thus analogous to the much more intensely studied prokaryotic ribosome (for reviews see refs. 113, 259,261,418) which also is found to have two different subunits, one of 50S (containing 34 proteins and 23S rRNA) and another of 30S (containing 21 proteins and 16S rRNA).

The eukaryotic ribosome is formed by a complex process which is only partly understood. Ribosomal proteins are made on cytoplasmic ribosomes (58,59,150,225,327,379), probably on free ribosomes (266), and certain of these migrate to the nucleolus (185) where they bind to nascent rRNA to form a precursor complex (23,196,212, 305,380,393).

The structure of the rDNA and the alterations of its rRNA transcript in the nucleolus have been the subject of the most intense study.

*These sedimentation coefficients for the monomeric ribosome and its subunits are not necessarily exact for the conditions described but represent those values determined under standard sedimentation conditions. Such standard values have customarily been used to refer to ribosomes and their subunits even under altered buffer conditions.

The rDNA has been found to be localized to certain chromosome areas, such as 2° constrictions in human chromosomes (271), and to be repetitive. Xenopus is a well studied system [serving as a model for other species (42,44)] in which the rDNA sequence alternates with a non-transcribed spacer. The 45S rRNA transcript of Hela rDNA has been mapped (211, 249,405,406) and found to consist of both the 18S and 28S rRNA as well as some non-conserved sequences (Fig 1). At the 5' end is located the 28S rRNA separated from the 18S rRNA by a non-conserved spacer while on the 3' side of the 18S sequence is another non-conserved area. In Hela cells the 45S precursor is cleaved to a 41S precursor followed by an endonucleolytic cleavage into a 20S rRNA (the precursor to the 18S rRNA) and a 32S rRNA (the 28S rRNA precursor). Further cleavages of both these molecules yield the mature rRNA molecules. The cleavage maps of Xenopus, L cell, and Hela have all been compared and the order of conserved and non-conserved rRNA and spacer sequences is the same though the size and order of cleavage varies.

Nazar et al (249) have recently shown that the 5.8S rRNA molecule is a part of the 32S rRNA precursor and becomes non-covalently associated with the 28S rRNA after its cleavage. 5S rRNA is found even at the earliest stages in the maturation of the 45S precursor (393) though recent evidence calls this into question (7,340). Precursors such as those defined above also seem to be present in prokaryotes (88,89,117,243,255) but their presence is only fleeting.

Though varying considerably in the amounts and sizes of ribosomal proteins (124) and rRNAs (220), both eukaryotic and prokaryotic ribosomes seem to carry out the same chemical reaction in almost exactly the same way. It is only in initiation that the

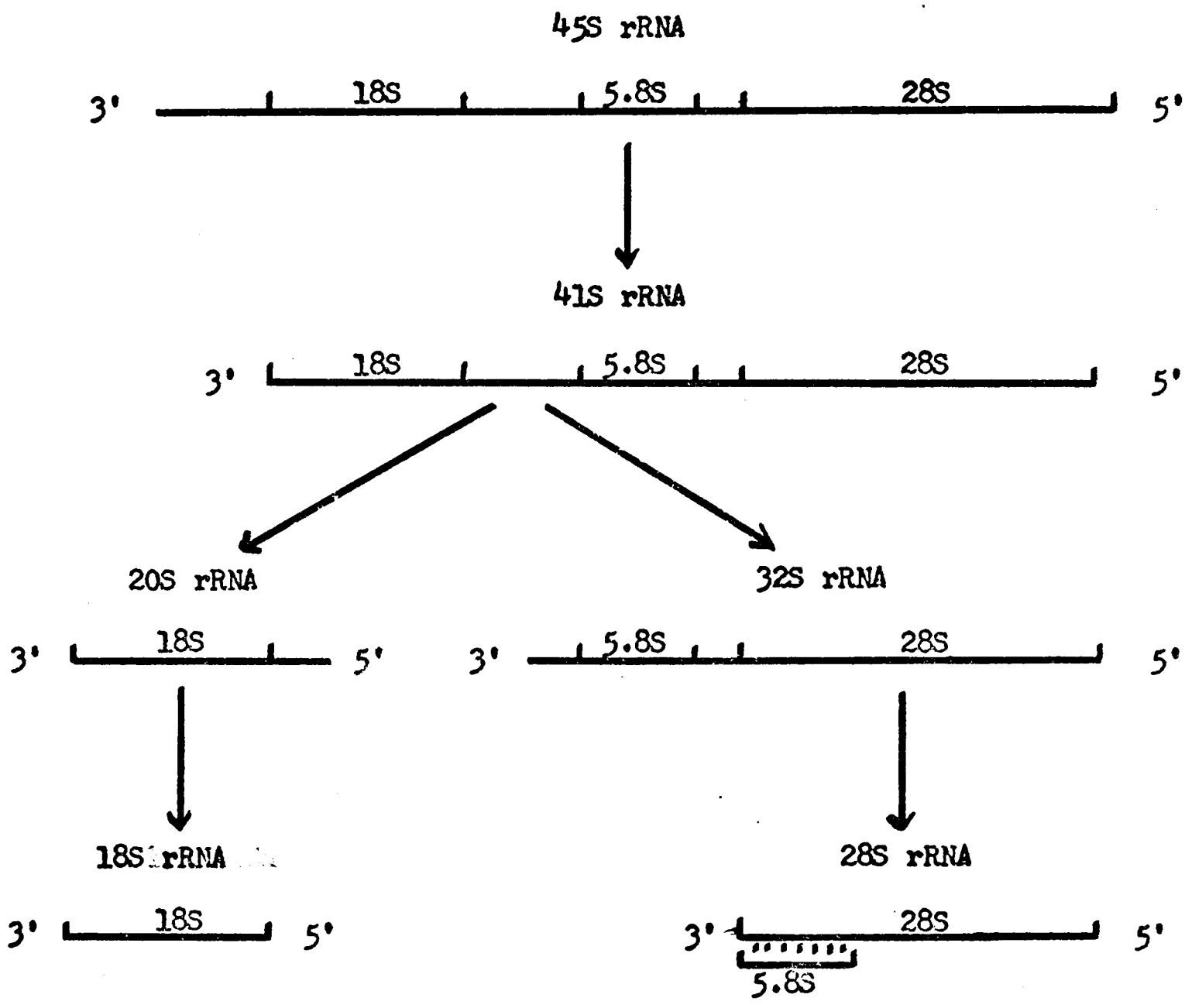


Figure 1
Structure and processing of Hela precursor rRNA.

Drawn after Lewin (211).

eukaryotic protein synthesis has been found to differ from that in prokaryotes (62,148,329). Since other significant differences have not been found, speculation has arisen that the extra ribosomal proteins and rRNA in the eukaryotic ribosome might be used for translational control mechanisms. Levisohn and Thompson (210) have suggested that induction of tyrosine amino transferase is concerned with translation. This suggestion becomes all the more feasible when it is considered that in the eukaryote considerable post-transcriptional regulation occurs (122). Also, some hormones (148,226,247,410) and interferon (187) seem to alter the translational specificity of the eukaryotic ribosome just as interference factor, i (ribosomal protein S1), alters the specificity of translation in QB-infected E. coli (61,128,129,166,387). The presence of numerous initiation and other factors in washes of ribosomal subunits (15,108,388) support these ideas.

Though they are present in some bacteria such as Azotobacter vinelandii (31), the role of membrane-bound ribosomes is much more apparent in eukaryotic cells. Those ribosomes bound to the endoplasmic reticulum appear to be involved in secretion (31,319) while those free in the cytoplasm make proteins for endogenous use. This again implies a possible role for the increased complexity of the mammalian ribosome.

It is thus of importance to analyze the protein composition of these eukaryotic ribosomes to establish which proteins are normally present and what their physiological function(s) are. At the start of the experiments described in the following chapters little work had been done to determine these ribosomal protein compositions (for review of earlier work, see ref. 376). Workers had concentrated on the preparation of subunits by numerous methods

(32,101,232,290,365) and the enumeration of ribosomal proteins on 1-D polyacrylamide gels in SDS or urea (16,161,182,189). The general conclusions from all these studies were that eukaryotic ribosomal proteins:

a. Are much more basic than those of E. coli (124);
b. Are present in greater numbers and of a higher molecular weight than those of E. coli (16,124);
c. Consist of 85 ± 17 proteins in the 80S ribosome, 49 ± 12 in the large subunit and 35 ± 5 in the small subunit (124);
d. Vary little between different species and tissues but show some limited variation (161);
e. Are possibly phosphorylated in some proteins (176,177);
f. Are not all present in molar amounts.

The obvious difficulty with most of these experiments was that the one-dimensional gel methods failed to yield sufficient separation of the 70 or so proteins. In addition, the conditions of electrophoresis were not optimal for the separation of potential variants of ribosomal proteins which possessed identical molecular weight but varied in charge.

The development of the two-dimensional gel electrophoresis system by Kaltschmidt and Wittmann (179) and its success in analyzing the ribosomal protein patterns of bacterial ribosomes (180) suggested a different approach to the analysis of the eukaryotic ribosome. Since these gels separated ribosomal proteins on the basis of both size and charge, it was felt that these would optimize the separation of ribosomal proteins and also allow the detection of subtle charge differences between possible mutational variants. This technique was thus extensively employed to analyze the ribosomal proteins of the eukaryotic ribosome. The purposes were several:

a. To map the protein components of ribosomes from various tissues, species and physiological states with the intent of cataloging any differences. Such a catalogue of ribosomal proteins appears in Chapters 3 and 4 where the cationic and anionic ribosomal proteins are determined and discussed.

b. To employ any species difference in ribosomal protein patterns as markers in cell fusion experiments. Such markers could be used to map the chromosomal locations of the eukaryotic ribosomal proteins as well as allow a genetic analysis of the regulation of ribosome biosynthesis. Chapter 8 discusses the results of such experiments.

c. To analyze the ribosomal proteins of one type, Hela, in depth and determine the subunit composition (Chapter 6) and its pattern of biosynthesis in the nucleolus (Chapter 7).

d. To correlate ribosomal function with particular ribosomal protein(s). Since the 5S rRNA binding protein is perhaps the only eukaryotic ribosomal protein that can be isolated with little difficulty, it is this protein that is analyzed (Chapter 5).

The approach to all these experiments is to take one technique -- two-dimensional gel electrophoresis -- and extend it to analyze the structure, biosynthesis and genetics of ribosomes. Others have also pursued similar lines of experimentation during this period of research and their results are discussed in parallel.

CHAPTER 2

MATERIALS AND METHODS

2.000 Chemicals.

Unless otherwise stated, all reagents and chemicals were supplied by British Drug Houses (BDH) and were of Anal&R quality. All sucrose was ribonuclease-free (Cambrian Chemical Co., Croyden, Surrey).

2.010 Cell culture.

2.011 Media and solutions.

Phosphate buffered saline (PBS, pH 7.3, ref. 87) was prepared by dissolving buffer tablets (Oxoid Ltd., London) in distilled water.

Neomycin, kanomycin, streptomycin (NKS) stock solution contained 1 g streptomycin base [Streptomycin sulfate B.P. (Glaxo Laboratories Ltd., Greenford)], 0.15 g kanomycin base [145,000 international units Kanomycin Sulfate (Winthrop Laboratories, Surrey)] and 105,000 international units of neomycin sulfate [Mycifradin Sulfate (Upjohn Ltd., Crawley, Surrey)] in 5 ml of sterile PBS. NKS solutions were stored at 4° C. Cells were not routinely cultivated in media containing NKS to permit early detection of contamination. If necessary, media was supplemented with 1 ml NKS per liter of media.

Hypoxanthine, aminopterin, thymidine (HAT, ref. 217) was made as a 100 times concentrated stock solution. Hypoxanthine (0.1360 g) was dissolved in 50 ml of distilled water by dropwise addition of about 1 ml of 10 N NaOH. Aminopterin (0.0019 g), thymidine (0.0387 g), glycine (0.0022 g) and sodium pyruvate (1.1005 g) were then added. After increasing the volume to 100 ml with distilled water, solutions were sterilized by membrane filtration. HAT solutions were stored at 4° C and were stable for a month.

Trypsin-ethylenediamine tetraacetic acid (EDTA) solution.

Trypsin [2.5 g Difco trypsin 1:250 (Difco Laboratories, Detroit)] was dissolved in 100 ml of distilled water, filtered through paper and sterilized by membrane filtration. This trypsin solution was diluted by addition of 1900 ml of a solution (previously sterilized by autoclaving) containing 20 PBS pH 7.3 tablets (Oxoid) and 0.4 g EDTA. This final solution was stored at 4° C or frozen at -20° C.

Minimal Essential Medium, MEM (90), was prepared from 200 ml of a 10 times concentrate (Flow Laboratories) to which was added 1730 ml sterile distilled water, 50 ml sterile sodium bicarbonate (3.4 g $NaHCO_3$) and 20 ml L-glutamine (0.58 g). Each 10 volumes of this medium was then supplemented by the addition of 1 volume of foetal calf serum (Flow Laboratories). Use of calf serum instead was not satisfactory especially with roller cultures for it seemed to decrease cell adhesion and growth rate.

MEM-HAT medium was derived by adding one volume of HAT solution to 100 volumes of MEM.

Minimal Essential Medium -- Dulbecco's Modification, DMEM (86), was prepared from a 10 times concentrated solution without sodium bicarbonate (Flow Laboratories). One volume of the concentrate was diluted by addition of 7 volumes of distilled water, sodium bicarbonate and glutamine followed by two volumes of foetal calf serum. The final concentrations of sodium bicarbonate and glutamine were 3.4 g/l and 0.3 g/l, respectively. One ml of NKS was added per liter.

Hela Spinner Medium was essentially a supplemented Earle's salts solution (91). It was prepared from the following stock solutions:

Earle A (per liter)	NaCl	170 g
	KCl	10 g
	$MgSO_4.7H_2O$	5 g
	Phenol red	0.5 g
Earle B (per liter)	Glucose	50 g
	$NaH_2PO_4.2H_2O$	3.5 g

Earle D (per liter) $NaHCO_3$ 44 g

The medium (per liter) contained: Solution A, 32 ml; Solution B, 32 ml; Solution D, 40 ml; yeast extract, 2.5 g; nutrient broth, 2.5 g; penicillin, 50,000 units; and streptomycin, 50 mg base. The medium was supplemented with heat-inactivated (30 minutes at 56°C) swine serum (Flow Laboratories) to a concentration of 5%.

2.012 Description of cell lines, tissues and transplantable tumors.

Tissues: Chick embryos were removed from 11-day old fertilized eggs, decapitated, and rinsed in ice-cold PBS. Rat livers were excised from 250 g Wistar rats that had been starved (water only) for 18-24 hours. The livers were rinsed in ice-cold PBS. It was essential to starve rats before isolating livers otherwise the ribosomal pellets were heavily contaminated with glycogen. After rinsing, all tissues were used immediately or frozen at -70° C.

Transplantable tumors:

Ehrlich ascites (EA) was that line previously described (144) and was grown by passage in the peritoneal cavities of adult outbred mice. Cells were harvested from the peritoneal fluid by centrifugation.

Novikoff Hepatoma Ascites Cells (NHAC) were a line obtained from the Chester Beatty Cell Bank (Novikoff B/P6, #10078/18-7-72). Cells were grown intraperitoneally in adult Wistar rats by injection of 1×10^7 cells/rat on Day 1 and harvested from the peritoneal fluid on Day 6-7. Prepared in this way, the NHAC cells were contaminated by variable amounts of red blood cells (RBC) which were removed by several cycles of differential centrifugation in PBS.

Rat leukemia was an experimentally derived line obtained after intrasplenic injection of ^{185}W (315). Cells were propagated by biweekly passage in 150 g PGV/c hooded rats and were kindly supplied

by Dr. M. Muirhead, Dept. of Biochemistry, Oxford. Leukemia cells were obtained from dissected lymph nodes of leukemic rats, freed from surrounding nodal tissue by gentle pipetting with PBS and pelleted by centrifugation.

Tissue culture lines and strains:

L929 (322) is a derivative of L (91), is highly malignant (267) and was obtained from Flow Laboratories.

A9(L) was received from Dr. J. Littlefield (216) in April, 1972 and is a derivative of L cells selected for resistance to azaguanine and is deficient in inosinic acid pyrophosphorylase (IMP-).

A9(H) is a descendant of an earlier A9(L) line from Littlefield and is a clone of those (supposedly non-malignant) A9 cells used in the early work of Harris (145). This clone is, however, now found to be malignant.

A9HT is a malignant derivative of the earlier A9(H) line obtained from a malignant tumor after injection of the (supposedly non-malignant) A9(H) cells into C3H mice (412).

L929, A9(L), A9(H), and A9HT were all grown in MEM in 20 oz. glass medical flats or in Falcon flasks (75 cm^2 surface area, Becton Dickinson Co., USA) and gassed with a mixture of 5% CO_2, 95% air before sealing the top. Cells were removed at confluence (every 2-3 days) with trypsin-EDTA and subcultured by plating at a lower density. Cells not removed by this treatment were removed after incubation of the trypsin-EDTA solution with the cells for a few minutes at 37° C.

Hela cells were obtained from Dr. M. Fenwick and grown in spinner pots in spinner medium in equilibration with the air. Cells were maintained at a density above 0.8 x 10^6/ml (150 ml total

volume per pot). Typically, cell cultures at 1.6 x 10^6/ml were diluted by half daily. In this way bulk preparations of Hela cells (16 ml packed cells) were obtained.

Fibroblasts. Ten-day old rat embryos or 11-day old chick embryos were decapitated, rinsed in sterile PBS and mashed with a sterile garlic press. The resulting mash was treated with trypsin-EDTA for 10 minutes at room temperature with pipetting and large bits of tissues removed by letting them settle. The supernatant was pelleted by centrifugation and plated in Falcon bottles containing MEM (+ NKS). Cells were subcultured as described above for the L cell derivatives.

Hybrid lines:

A9/RBC was a hybrid line prepared by the fusion of A9(H) with chick red blood cells (331) and obtained from P. R. Cook. This line has the normal complement of A9 chromosomes but no detectable chick chromosomes.

A9HT/rat leukemia was obtained from Prof. H. Harris as an uncloned fusion population between A9HT and rat leukemia. Clones were isolated from this population as described below (2.016).

All hybrids were grown in MEM-HAT in 20 oz. glass medical flats or in Falcon flasks.

Cells harvested from all such transplantable tumors, tissue culture lines and strains were washed three times in ice-cold PBS by centrifugation. The final pellet was either frozen at -70° C or used immediately.

2.013 Bulk cell culture.

For large scale preparation of cells normally grown on glass or plastic surfaces, roller cultures were employed. Inocula of 1.5 x 10^7 cells were added to 100 - 150 ml of MEM (+ NKS) and grew to

confluence in 2-5 days. Cells were harvested as usual.

2.014 Radioactive labelling of cells.

Cells added to roller bottles in 100 ml MEM-NKS were allowed to attach for 12 hours. Then 50-100 uCi of ^{14}C algal protein hydrolysate (50 uCi/ml, Amersham) were added and the cells grown to confluence.

2.015 Freezing and storing cell lines.

Cells were removed by trypsin-EDTA, rinsed several times in sterile PBS, pelleted, and the pellet resuspended in freezing-down solution (MEM without foetal calf serum made instead 10% with respect to dimethyl sulfoxide). Once placed in 1 ml ampoules containing 2-10 x 10^6 cells, the automatic freezing routine was immediately begun. Frozen ampoules were stored under liquid N_2.

2.016 Cloning.

Cells were plated into small Falcon bottles (100-200 cells per bottle) and cultivated in MEM-HAT (+NKS) with media changes every 4 days. In two weeks clones of $\frac{1}{4}$ - $\frac{1}{2}$ cm in diameter appeared and were removed after mild trypsinization in cloning rings. The trypsinate was rinsed with an excess of media and plated into small Falcons.

2.020 Production of hybrid cells.

Fusion partners were selected so that one parent was deficient in inosinic acid pyrophosphorylase and the other lacked thymidine kinase; or one partner had either of the enzyme deficiencies and the other did not adhere to (or grow on) glass. The parental cells were rinsed several times in MEM to counteract any remaining trypsin and then several times in PBS to remove adhering serum. After the final wash, cells were resuspended in MEM (without foetal calf serum) to a density of approximately 10^7 cells/ml. Aliquots (0.45 ml) of

each parent were mixed with 3000 HAU (75,000 HAU/ml) of u.v.-inactivated Sendai virus (147) and incubated on ice for 20 minutes. The clumped suspension was then transferred to a 37° water bath for ½ hour and then to Falcon bottles containing MEM-HAT. Clones appeared in 10 days to 2 weeks and were picked as in 2.016. Isolated clones were maintained in MEM-HAT and a sample frozen and stored.

The amounts of cells and virus are empirically determined. Also, proper controls are necessary (i.e., mock fusion without addition of virus as well as both parental lines plated separately on MEM-HAT). This is especially true for those fusions where one parent is selected against simply on the basis of its failure to adhere to glass or plastic; in some cases (NHAC x A9HT) the presence of the adhering cell (A9HT) is sufficient to cause the non-adhering parent (NHAC) to adhere.

2.030 Chromosome technique.

2.031 Preparation for staining.

The media in a 20 oz. flat of near-confluent cells was replaced by 16 ml of warm (37° C) MEM (without serum) containing Colcemid at a final concentration of 1 ug/ml and incubated for 1 - 1½ hour at 37° C. The cells were removed by trypsin-EDTA and rinsed in PBS in a conical tube. After centrifugation the supernatant was removed and the pellet resuspended in a drop of the remaining supernatant. Ten to 15 ml of hypotonic (75 mM KCl) were added and the suspension incubated at room temperature. Timing of the hypotonic is a critical parameter that varies from one cell type to another -- A9 requires 8 minutes while hybrids and rat leukemia need 12 minutes. After pelleting by centrifugation and resuspension in the remaining drop of hypotonic, 15 ml of

fixative [acetic acid : methanol (1:3)] were added and incubation continued for 15 minutes. The fixation step was repeated, the cells pelleted, resuspended in a drop of fixative and drops placed on clean, dry slides. Slides were air-dried as described by Rothfels and Siminovitch (316).

2.032 Chromosome stain.

Ten ml of Giemsa (G. T. Gurr) diluted with 90 ml of pH 6.8 PBS (Gurr) were used to stain all chromosomes whether treated for C-bands or not. This dye varies from batch to batch and each new bottle needs to be tested.

2.033 C-banding.

After "ageing" for 5-7 days in a dark drawer at 20° C, slides were banded for centromeric heterochromatin (C-banding) as described by Allderdice et al (4)[as adapted from Dev et al (82)] with the slight modifications suggested by Marshall (229). One hundred ml of formamide were brought to pH 7.0 with concentrated HCl using a pH meter fitted with non-aqueous electrodes. With the neutralized formamide, a solution of 95% formamide in SSC (150 mM NaCl, 15 mM trisodium citrate pH 7.0) was prepared, incubated at room temperature for 15 minutes and then warmed to 65° C in a water bath. The slides were then incubated for 7-8 minutes, washed rapidly in cold, running tap water, stained with 10% Giemsa for 40-50 minutes and rinsed with deionized water.

2.034 Chromosome counting.

Slides were examined with a Leitz Orthoplan microscope incorporating the Leitz side-arm tracing device. Chromosomes were divided into three categories: biarmed, acrocentric/telocentric, and "dot." In this context, dot chromosomes refer to those very short chromosomes (presumably acrocentric) that are found in mouse,

chicken and other lines (331). All calculations of the modal chromosome number were based on 50-60 metaphase spreads.

2.040 Isolation of ribosomes.

The method used for all these experiments was adapted from that of Falvey and Staehelin (101). Stock solutions were as follows:

	Buffer A	0.2 M Sucrose	0.7 M Sucrose	2 M Sucrose
NH_4Cl (100 mM)	2.68 g	2.68 g	2.68 g	2.68 g
MgAcetate (5 mM)	0.54 g	0.54 g	0.54 g	0.54 g
Tris (20 mM, pH 7.5)	1.21 g	1.21 g	1.21 g	1.21 g
2-mercaptoethanol (1 mM)	40 ul	40 ul	40 ul	40 ul
Sucrose (RNase-free)		34.2 g	120 g	342 g
Final volume	500 ml	500 ml	500 ml	500 ml

Two ml of ice-cold 0.2 M sucrose were added to each 1 ml (packed volume) of cells and the mixture homogenized with 10 strokes of a mechanically-powered, rotary, Teflon homogenizer. Care was taken to avoid heating the mixture. The homogenate was centrifuged at 12,000 rpm at 4° C in a Type 65 rotor for 12 minutes, the upper two-thirds of the supernatant removed and stored on ice. For samples in limited supply, a volume of 0.2 M sucrose equal to that removed was added to the pellet and the homogenization repeated. To the removed supernatant fraction(s) was added sufficient 13% deoxycholate (DOC) to make the final concentration 1.3%.

Discontinuous sucrose gradients were prepared for the Type 65 (or Type 50Ti) rotor containing 2.5 ml of 2M sucrose overlaid with 2.5 ml of 0.7 M sucrose. The detergent-treated, post-mitochondrial supernatant was carefully placed on top and centrifugation carried out at 4° C for 4½ hours at 45,000 rpm. After centrifugation, the supernatant was pipetted off, the walls of the tube wiped dry and the bottom 3 cm of the tube rinsed gently with distilled water. One to 2 mg of polysomes were usually recovered

per ml (packed volume) of cells.

Pellets were clear and colourless and were stable for months when stored at -70° C. When resuspended in buffer (10 mM Tris pH 7.4, 3 mM Mg Acetate) the pellets were almost totally solublized and remained stable at 4° C for a week or for months at -70° C.

Salt-washed ribosomes were prepared by resuspending the ribosome pellet in Buffer A and increasing the concentration of KCl to 500 mM. A drop of 2M sucrose was placed in the bottom of a centrifuge tube and the ribosomes laid over it. Centrifugation was for 2 hours at 45,000 rpm at 4° C.

When required, ribonuclease treatment of polysomes was at room temperature at a concentration of 6 ug RNase (4 times re-crystallized, protease-free from bovine pancreas)/15 ODU_{260}.

All ribosome preparations were scanned with the Unicam Recording Spectrophotometer to assess purity and determine concentration.

2.050 Extraction of ribosomal protein.

Ribosomes were resuspended in Buffer A (2.040) or, most often, 10 mM Tris pH 7.4, 3 mM Mg Acetate and any aggregated material removed by centrifugation at 10,000 rpm for 10 minutes in the Type 65 rotor. The supernatant was decanted and made 100 mM in $MgCl_2$ with a stock solution of 1 M $MgCl_2$ (139). Upon addition of 2 volumes of glacial acetic acid, the sample was incubated on ice for 45 minutes with occasional stirring. The precipitating RNA was pelleted by centrifugation at 10,000 rpm for 10 minutes in the Type 65 rotor and the supernatant removed. The RNA pellet was resuspended in ½ the original volume of 67% acetic acid, 33 mM Mg Acetate and centrifuged as before. The combined supernatants

were diluted with 3 or 4 volumes of distilled water, the solution frozen in dry ice-acetone and lyophilized. The dried sample was white and fluffy if sufficient protein was present; however, with minute samples, a thick, stick residue was produced.

2.060 Subunit preparation.

This was done exactly as described by Sabatini and Blobel (32). Ribosome pellets were resuspended to a concentration of 50 ODU_{260}/ml in dissociation buffer [500 mM KCl, 5 mM Mg Acetate, 50 mM triethanolamine pH 7.5 (20° C)] made 1 mM dithiothreitol, 1 mM puromycin and incubated at 37° C for 10 minutes. During this time the initially turbid solution became transparent. Samples containing approximately 7-9 ODU_{260} were placed on top of 11.8 ml, 5-20% sucrose gradients* made up with the dissociation buffer and centrifuged at 35,000 rpm for 160 minutes at 20° C using an SW 41 rotor. After fractionation with an ISCO gradient fractionator (Instrumentation Specialty Co., Lincoln, Nebraska), peak fractions were pooled, made 20 mM in Mg Acetate, and pelleted at 35,000 rpm at 4° C using a Type 40 rotor. The surfaces of the subunit pellets were gently rinsed with distilled water. The 60S subunit pellets were repurified by repeating the above dissociation step. Purified subunits were stored as a pellet at -70° C and their concentration determined from the A_{260}.

For analysis of the various sedimentation classes of ribosomes several different gradient systems were used. For polysomes, 11.8 ml, 10-40% sucrose gradients made with the dissociation buffer were centrifuged at 35,000 rpm at 4° C for 125 minutes in a SW 41 rotor. Monosomes and subunits were best separated on 5-20% gradients

*The gradients employed in all experiments were linear throughout the tube and all percentages of sucrose in gradients are based on wt/vol.

in the same buffer as for polysomes but centrifuged at 35,000 rpm at 20° C for 120 minutes on the SW 41 rotor.

2.070 Two-dimensional gel electrophoresis.

2.071 Standard method.

This was done exactly as originally described (179) except the pH of several of the standard solutions so described were not those obtained with the reagents used here. The solutions and pHs employed here were the following:

DIMENSION I, pH 8.6

	Separation Gel	Sample Gel	Running Buffer
urea	54.0 g	48.0 g	
acrylamide	6.0	4.0	
bisacrylamide	0.2	0.2	
Na_2EDTA	1.2	0.85	10.8 g
boric acid	4.8	0.32	43.2
Tris	7.3	0.49	65.48
TEMED	0.45 ml	0.06 ml	
Final volume	148.5 ml	99 ml	1000 ml
pH	8.60	9.00	8.05

All acrylamide solutions were filtered and stored at 4° C. The running buffer was diluted to 4.5 l just prior to use.

DIMENSION II, pH 4.6

	Separation Gel	Dialysis Buffer	Running Buffer
urea	432 g	480 g	
acrylamide	216		
bisacrylamide	6		
glacial HAc	62.8 ml	0.74 ml	15.0 ml
5 N KOH	11.52	2.4	
TEMED	6.96		
glycine			140 g
Final volume	1160 ml	1000 ml	1000 ml
pH	4.60	6.20	4.00

All urea solutions were filtered and kept at 4° C. The running buffer was diluted to 10 l before use.

With these solutions, gels were prepared exactly as Kaltschmidt and Wittmann describe (179). Gels were usually run for 26 hours in

the first dimension at 100 V (constant voltage), 20 ma (starting current), 26 ma (final current). Gels were dialyzed for 3 hours before layering on the second dimension. The second dimension was run for 40-46 hours at 100 V (constant voltage), 500 ma (initial current), 100 ma (final current).

2.072 Micro 2-D method.

This was a modification of the original method suggested by Howard and Traut (163). First dimension glass tubes [0.46 cm (internal diameter) x 12 cm] were sealed at one end with Parafilm and separation gel [19.8 ml gel + 0.2 ml of 70 mg/ml ammonium persulfate (added immediately before pouring)] poured to the 9 cm level. Gels were carefully overlaid with distilled water using a fine syringe needle and allowed to sit for one hour.

Lyophilized ribosomal proteins were solubilized in sample gel at a concentration of 10 mg/ml and 20 ul samples (200 ug) applied to the tops of gels which had excess water removed with a bit of filter paper. Using a long, slender needle, the running buffer was applied over the samples. Gels were usually run for 30 minutes at 3 ma/tube (constant current, 80-85 V) and then for 4 hours at 6 ma/tube (constant current, 160-180 V).

Since the sample was not polymerized in the middle of the first dimension gel but placed on the top, separate gel runs were needed to analyze both cationic and anionic ribosomal proteins. Usually only the cations were analyzed and hence the cathode was placed at the bottom of the gel. To analyze anions, the potentials were reversed. After a cation gel run, the pH of the upper reservoir buffer was 8.10 while that of the bottom was 8.30.

Gels were removed from the tubes with gentle rinsing with a long (6 inch) needle and dialyzed for 1½ hours with two changes of

dialysis buffer.

The second dimension gel slabs were identical with those in 2.071 but only 1.6 mm wide. (The 2-D gel apparatus was modified to allow spacers of any thickness to be inserted. Those of 1.6 mm width were found the most manageable though spacers of 0.5 - 1.0 mm were still practical.) The bottom of each chamber was sealed with adhesive tape and 10 ml of the second dimension separation gel [48.35 ml gel + 1.65 ml of ammonium persulfate (2 gm + 20 ml H_2O)] added and allowed to polymerize. The dialyzed 1st dimension gels were rinsed in 50 ml of ice-cold separation gel. To 350 ml of ice-cold separation gel were added 12.1 ml of ammonium persulfate solution, the slabs poured till overflowing and all bubbles removed. The 1st dimension gels were then quickly placed in the V-shaped area on top of each slab and polymerization completed in 15-20 minutes under N_2. After cooling for another one hour, running buffer was added and electrophoresis allowed to proceed for one hour at 40 V (constant voltage), 80 ma with the cathode on the bottom. Then the voltage was increased to 100 V, 200 ma and usually continued for 30 hours. After 30 hours the current had decreased to 50 ma and the pH of the upper reservoir was 3.60 while that of the bottom was 4.50.

Gels were stained for 4-12 hours with 0.1% Coomassie Brilliant Blue (Mann Chemical Co.) in 7.5% acetic acid, 50% methanol. Destaining was completed in 4 hours with several changes of 7.5% acetic acid, 50% methanol. Destained gels were then photographed, stored at 4° C in plastic bags, or dried (2.073).

2.073 Drying gels.

Two-dimensional gels were applied to several thicknesses of filter paper, placed on a porous support, covered with plastic

wrap and dried under vacuum in 4 hours (163).

2.074 Counting radioactive gels.

Radioactive ribosomal protein samples were always mixed with sufficient unlabeled protein to yield spots upon staining. Stained spots were cut out of the gels with a fine scalpel, placed into scintillation vials and dried overnight at 80° C. Each vial then received 0.2 ml of 100% H_2O_2 and was incubated with the cap on tightly for two days at 50° C. Addition of 0.5 ml NCS (Amersham) and incubation for 2-3 hours at 37° C was followed by the addition of 10 ml of Bray's (39) scintillation fluid (60 g napthalene, 4 g PPO, 0.2 g POPOP, 100 ml methanol, 20 ml ethylene glycol, and dioxane to a final volume of 1 liter).

2.075 Recovery of protein from stained gel spots.

Electrophoretic elution was done according to Feit et al (104). Stained spots were cut out and incubated for two hours in SDS running buffer (2.100). Dialysis tubing (2.180) was incubated in the same buffer and then placed over the bottom of a glass tube forming a small pouch held to the tube by rubber bands. The gel sample was lodged near the base of the tube and then electrophoresed in the buffer at 100 V for one hour. After electrophoresis, all stain had been removed from the gel slice and was in the dialysis bag. This solution was then concentrated by freeze-drying or vacuum dialysis.

2.080 Extraction and electrophoresis of RNA.

2.081 Hot chloroform-phenol method.

Extraction was performed as described by Penman (277). Samples were suspended in 4 ml of Extraction Buffer (100 mM NaCl, 0.5% SDS, 1 mM EDTA, 10 mM Tris, pH 7.4) in a 15 ml conical centrifuge tube and two ml of phenol (saturated with Extraction Buffer made without

SDS) were added along with two ml of chloroform. The mixture was vigorously vortexed (10 sec), placed in a 55° C water bath for one minute, vortexed again (10 sec) and centrifuged on a bench centrifuge to separate the layers [5 minutes at medium speed (2000 rpm)] . This yields a clear top layer, a turbid bottom layer (due to SDS coming out of solution at the lower temperature -- reheating makes it clear again), and a fluffy interface. Taking care to avoid aspirating the interface, the bottom layer was removed, more phenol and chloroform (2 ml each) added and the above steps repeated three times or until the interface was clear.

To remove phenol saturating the aqueous phase, the organic phase was removed, four ml of chloroform added, the solution vortexed and centrifuged. After removing the organic phase, these steps were repeated twice more. At the end of the last step, both aqueous and organic phases were clear and there was no whiteness at the flat interface. The aqueous phase was then removed, two volumes of 100% ethanol added and then incubated for 20 hours at -20° C. The flocculant precipitate was collected by centrifugation, dried with a gentle breeze of N_2 and stored at -70° C. The A_{260}/A_{235} ratio of extracted RNA was always greater than 2.00.

2.082 Cold chloroform-phenol extraction.

Identical to that in 2.081 except performed at room temperature.

2.083 2.7% polyacrylamide gels.

These were prepared exactly as described by Weinberg and Penman (398). Gel Buffer (2 times concentrated) contained 80 mM Tris, 40 mM sodium acetate, 4 mM EDTA and was brought to pH 7.4 with acetic acid. Stock 15% acrylamide solution contains 15 g acrylamide and 1.40 ml ethylene diacrylate (Eastman) in 100 ml of water. Both these stock solutions were stable at 4° C for months. After degassing

under water vacuum a mixture consisting of 5 ml stock 15% acrylamide, 13.90 ml Gel Buffer and 8.625 ml distilled water, 25 ul TEMED and 0.25 ml ammonium persulfate (70 mg/ml) were added. The solution was poured into gel tubes, overlaid with water and allowed to sit for one hour.

Running buffer was made from a 1-1 dilution of Gel Buffer that was made 0.2% in SDS by addition of a 20% solution. Gels were pre-run for one hour at 4 ma/gel (constant current), 50 V. Samples (0.3-0.5 ODU_{260}) were solublized in Sample Buffer (50 ml Gel Buffer, 10 ml glycerol, 1 ml 20% SDS made up to 100 ml) and applied to the gels. Electrophoresis was carried out at 4 ma/tube (constant current), 50 V for 4-5 hours. During the early stages of electrophoresis, refractile bands of RNA were visible moving through the gel.

After electrophoresis, gels were removed from the tubes and incubated (with stirring) in the running buffer for 2-3 hours to lower the background absorbance (221). Gels were then scanned for u.v.-absorbing material on a Joyce-Loebl gel scanner.

2.084 <u>10% polyacrylamide gels</u> (397).

Gels were prepared from 20% acrylamide solution (20 gm acrylamide + ½ ml ethylene diacrylate made up to 100 ml) and Gel Buffer (2.083). After degassing a mixture of 13.90 ml 20% acrylamide and 13.90 ml Gel Buffer, gels were polymerized and poured as in 2.083. Pre-electrophoresis and sample application were as in 2.083 but the gels were run at 4 ma/tube for 6 hours. After 2 hours washing with running buffer, gels were either scanned with the Joyce-Loebl gel scanner or stained with Coomassie Brilliant Blue [1.25 g in a mixture of 454 ml of 50% methanol and 46 ml of glacial acetic acid (94)] . Gels were destained electrophoretically

with 7.5% acetic acid solution made 5% in methanol.

Purified tRNA (Boehringer-Mannheim) served as a mobility marker.

2.090 5S rRNA-protein preparation.

As modified from Blobel (29), ribosome pellets were resuspended in ice-cold 10 mM Triethanolamine, pH 7.5, 10 mM KCl to a density of 100 ODU_{260}/ml. Any debris was removed by centrifugation at 10,000 rpm for 10 minutes. EDTA (usually 1 µM/10 ODU_{260}) was added from a stock solution of 0.2 M EDTA, pH 7.0. Approximately 25 ODU_{260} of ribosomes were carefully layered onto 11.8 ml, 5-20% sucrose gradients (made in 10 mM KCl, 10 mM Triethanolamine, pH 7.5) and centrifuged at 39,000 rpm at 4° C for 24 hours using the SW 41 rotor. If polysomes were to be analyzed, they were centrifuged for 2 hours at 39,000 rpm at 4° C in the SW 41 rotor in 10-40% sucrose gradients made up in the same buffer.

Gradients were fractionated with the ISCO gradient fractionator, 4S and 7S fractions collected, optical density measured, and pelleted at 45,000 rpm at 4° C for 40 hours. The 7S fraction (the 5S rRNA-protein complex) formed a small, clear pellet but the 4S fraction failed to form a pellet. Pellets were carefully rinsed with distilled water and frozen at -70° C.

Isolated gradient fractions were also concentrated by freeze-drying following 24 hour dialysis against several changes of ammonium formate, pH 6.30. This allowed both the 4S and 7S fractions to be concentrated.

The release of 4S and 7S fractions from ribosomes was also demonstrated by using 5% polyacrylamide gels (29). All solutions (running buffer, sample buffer, and the gels themselves) contained

the same pH 8.3 Buffer (Tris, 10.8 g; boric acid, 5.5 g; KCl, 3.73 g; Mg Acetate, 0.428 g per liter). Ribosomes were resuspended in sample buffer (pH 8.3 Buffer made 10% glycerol) and EDTA added as above. Two to six ODU_{260} of the sample were electrophoresed for 10 hours at 4 ma/gel, 50 V and then stained for protein or scanned for RNA.

2.100 SDS polyacrylamide gel electrophoresis.

Gels were prepared and run as described by Weber and Kuter (395). All samples were heated in boiling water for 1 minute immediately after addition of SDS dialysis buffer and then dialyzed for 4-6 hours vs. dialysis buffer before applying to gels.

Protein standards for preparing the molecular weight reference curve were from the following sources: horse heart cytochrome C (MW 13,500), bovine pancreas chymotrypsinogen (MW 25,000), hen albumin (MW 45,000), bovine albumin (MW 67,000), rabbit muscle aldolase (MW 40,000), bovine liver catalase (MW 60,000), glyceraldehyde phosphate dehydrogenase (MW 36,000), and ferritin [MW 18,600 (26,120)] from Boehringer-Mannheim; bovine pancreas deoxyribonuclease I (MW 31,000, ribonuclease-free) from Worthington; soybean trypsin inhibitor (MW 21,500) and egg albumin (MW 43,500) from Sigma; bacterial α-amylase (MW 48,960) and trypsin (MW 23,800) from BDH.

2.110 pH 4.5 gels.

A solution identical to the pH 4.6 Separation Gel (2.071) was made but with half the amount of acrylamide and bisacrylamide. To 29 ml of this gel solution was added 0.5 ml of ammonium persulfate (1 g/10 ml) and the gels poured (3 ml/tube) and overlaid with distilled water. Samples were resuspended in Dialysis Buffer

(2.071) made 10% in glycerol and run with the second dimension running buffer (2.071) for 5 hours at 4 ma/tube. On one gel 10 ul of 0.05% bromophenol blue was added as a marker. Staining of the gels was for 10 hours with 0.25% Coomassie Blue (2.100) and destaining was done electrophoretically.

2.120 Preparation of ribosomal precursor particles.

The original method of Penman (277) for preparing nuclei was scaled up to accomodate larger amounts of Hela cells. A precision, steel ball, Dounce-type homogenizer with a clearance of 0.003 ± 0.0001 inch was employed. As suggested by Shepherd and Maden (333), this worked more efficiently than one with 0.002 inch clearance.

Hela cells were harvested from medium by centrifugation at 2000 rpm for 4 minutes and immediately resuspended in ice-cold PBS. Cells were washed twice with PBS and pelleted into conical 15 ml tubes each containing 2 x 10^8 cells. After resuspending the contents of each tube in 10 ml of RSB (0.01 M NaCl, 1.5 mM Mg Acetate, 0.01 M Tris, pH 7.4) and allowing to swell for 10 minutes, cells were broken with 10 strokes of the Dounce. Breakage was monitored microscopically. The homogenate was centrifuged in a 15 ml conical tube for 2 minutes at 2000 rpm in a clinical centrifuge and the nuclei resuspended in 10 ml of RSB by vigorous pipetting followed by a second centrifugation at 2000 rpm. Upon resuspension in another 10 ml of RSB, at most 5% of the cells were unbroken with 90-95% recovery of the initial number of nuclei. If the percentage of unbroken cells exceeded this, a second homogenization (5 strokes) was carried out followed by the two washes in RSB.

To lyse the remaining intact cells and remove contaminating cytoplasm, 1.5 ml of a mixture of deoxycholate and Tween [(one

part 10% (w/w) deoxycholate + two parts 10% (w/w) Tween 40] were added and the conical tube vortexed for 10 seconds. After centrifugation at 2000 rpm for 2 minutes, the supernatant was removed and the walls of the tube and the surface of the nuclear pellet rinsed with 1.5 - 4 ml of RSB to remove excess detergent (273). At this stage, contaminating intact cells were usually fewer than one per several hundred nuclei, the nuclear pellet had a gelatinous consistency and the nuclei could not be resuspended with ease. On occasion the pellet did not form a gel-like pellet and the contaminating intact cells had not been lysed. Such preparations were subjected to a second cycle of detergent treatment to yield a proper nuclear pellet.

The nuclear pellet from 2 x 10^8 cells was resuspended in ten ml of ice-cold HSB (500 mM NaCl, 50 mM Mg Acetate, 10 mM Tris pH 7.4) followed by the addition of 200 ul (0.4 mg) of a 2 mg/ml solution of DNase I. With continuous pipetting with a Pasteur pipette at room temperature the viscosity of the solution decreased in about 10 minutes. When all visible strands and debris were gone, the turbid solution was centrifuged for 10 minutes at 10,000 rpm at 4° C in the SW 41 rotor. Supernatant was then discarded, the walls of the tube wiped dry and the pellets rinsed gently with several ml of NEC (same as NEB but made 10 mM in dithiothreitol).

Nucleolar precursors were extracted according to Warner and Soeiro (393). The pelleted nucleoli were resuspended in 9 ml NEC (3 ml for nucleoli from each 4 x 10^8 cells), brought to room temperature and stirred continuously for 15 minutes. After centrifugation at 15,000 rpm for 10 minutes at 4° C in the SW 41 rotor, the supernatant was placed atop three 28 ml, 15-30% gradients

in NEB (10 mM NaCl, 10 mM EDTA, 10 mM Tris pH 7.4) and centrifuged at 22,000 rpm for 13 hours at 4° C in the SW 25.1 rotor.

After fractionation with the ISCO gradient fractionator, fractions were pooled and pelleted at 45,000 rpm for 19 hours at 4° C in the Type 65 rotor. Pellets were rinsed and stored at -70° C.

An alternate method for the extraction of precursors employing pyrophosphate rather than EDTA (340) was also tried. Nucleoli were prepared as above but extracted with NCPOP (10 mM NaCl, 10 mM dithiothreitol, 10 mM $Na_4P_2O_7$, pH 7.4) instead of NEC.

2.130 Immunology.

2.131 Solublization of ribosomal proteins.

Lyophilized ribosomal proteins (2.050) are highly insoluble in non-denaturing buffers but are fairly soluble in 8 M urea or 6 M guanidine hydrochloride (GuHCl). Since 6 M GuHCl is the most effective, ribosomal proteins (20 mg) were dissolved in approximately 2 ml of it. Insoluble debris formed a layer at the top upon centrifugation and was removed. The now clear solution was diluted dropwise at room temperature into 9 volumes of 140 mM NaCl, 10 mM phosphate, pH 7.4. Any turbidity was removed by centrifugation at 10,000 rpm for 10 minutes before concentration by ultrafiltration at 4° C with the Amicon UM 10 membrane (using 50 psi N_2). By this method 50-75% of starting protein was lost.

2.132 Immunization.

Solublized ribosomal proteins (10 mg) in 2 ml of buffer were added to two ml of Freund's complete adjuvant (Difco) and emulsified. Multi-site injections were made s.c. into the footpads of rabbits or into adult hens on Days 1 and 30. Sera were harvested on Day 40.

Whole ribosomes (25-60 mg) in a volume of 3 ml were emulsified in an equal volume of Freund's complete adjuvant and injected s.c. into rabbits or hens at multiple sites on Days 1 and 14 (or 28). Sera were harvested on Day 21 (or 35).

2.133 Double diffusion analysis.

Ouchterlony gels were made according to Wikman (413). One hundred ml of buffer (50 mM Tris, pH 7.4, 25 mM KCl, 1 mM EDTA, 0.01% 2-mercaptoethanol, 0.01% merthiolate) were brought to boiling while one g of agarose was added and the solution kept at 100° C for 40 minutes. The solution was then cooled in a water bath to 42° C and brought to the initial volume by the addition of distilled water. For each 10 ml of this solution, 0.8 ml of 13% deoxycholate was added (at 42° C) and the gel poured onto clean slides at room temperature. When cooled to 4° C, holes were cut and the slides kept at 4° C in a humid Petri dish. Antisera and antigen were placed in the proper wells and incubated at 6° or 37° C (in humid Petri dishes) for 1-6 days. Precipitin lines were photographed under indirect light.

Plates prepared using NaCl (0.57 g), barbitone (0.49 g) pH 7.4, glycine (1.5 g), merthiolate (7 mg), distilled water (100 ml) and agarose (1 g) were not effective.

2.134 Haemagglutination.

Passive haemagglutination has been shown to be a sensitive method for assaying antibody titre to ribosomes (257). Sheep RBC (3 ml packed volume) were washed three times with PBS by pelleting for 10 minutes at 1500 rpm in a 15 ml conical tube in a bench centrifuge. To each of two capped 20 ml bottles was added 0.6 ml of washed RBC, 9.4 ml of PBS and 10 ml of tannic acid (20 mg + 200 ml PBS). After incubation at 37° C for 15 minutes, RBC were

pelleted and washed once with PBS. Both bottles of pelleted RBC were resuspended in 20 ml of PBS. To one was added 2.4 mg of ribosomes and both bottles were then incubated at room temperature for ½ hour and then overnight at 4° C.

The RBC in both bottles were then washed three times with 20 ml of PBS containing bovine serum albumin (1 mg/ml). After washing, all samples were resuspended to a volume 100 times greater than the final RBC packed volume. The RBC not exposed to ribosomes served as the control.

All sera were preincubated with an equal volume of control RBC. Tests were set up using a microtiter tray with each well receiving 0.1 ml of sensitized RBC and 0.1 ml of diluted sera. Incubation was for 2 hours at 37° C.

2.140 Analytical ultracentrifugation.

All runs were done with a Spinco Model E Ultracentrifuge equipped with schlieren optics. A cell with 30 mm path length was employed in an An-D rotor at a speed of 44,777 rpm at 20° C. Photographic plates were read with a micro-comparator to calculate accurate sedimentation coefficients. Ribosome concentrations varied from 4 mg/ml to 1 mg/ml and buffers ranged from dissociating (EDTA) buffers to those with 0.8 M KCl. Most runs were in a buffer consisted of 3 mM Mg Acetate, 300 mM KCl, 20 mM Tris pH 7.5.

2.150 Determination of protein concentration.

A modified version (8) of the Lowry (222) technique was employed which used sodium citrate in Reagent B instead of sodium tartrate. This yields more stable stock solutions.

2.160 Ribosome dimers.

About 10^7 cells were harvested and washed three times at room temperature with PBS and then prepared as previously described (350). The pelleted, washed cells (0.1 ml packed volume) were resuspended to a volume of 0.9 ml with ice-cold NaH buffer [100 mM NaCl, 20 mM $MgCl_2$, 10 mM Tris, pH 7.4 (20° C)], incubated for 30 minutes at 4° C, made 0.5% (w/v) in Lubrol W and homogenized with 15 strokes of a small, pre-cooled, loose-fitting, glass Dounce homogenizer (Kontes Glass Co.). The homogenate was centrifuged at 15,000 g_{ave} for 15 minutes and the supernatant then placed on top of 15-30% sucrose gradients prepared in NaH buffer. Gradients were centrifuged for 190 minutes at 35,000 rpm at 4° C in the SW 41 rotor and then fractionated. Maintenance of the temperature below 10° C throughout these steps was critical; any increase over this prevented dimer formation.

Large scale dimer preparations were as above except 0.8 ml (packed cell volume) was employed and centrifugation was for 10 hours at 20,000 rpm using three 10-30% gradients in the SW 25.1 rotor.

2.170 CsCl gradients.

Since eukaryotic ribosomes are degraded by CsCl, the formaldehyde fixation procedure of Spirin (347) was used. 4% formaldehyde was buffered to pH 7.2 in 0.01 M phosphate. Ribosomes in 10 mM $MgCl_2$, 10 mM phosphate, pH 7.2 were diluted with an equal volume of 4% (neutralized) HCOH and allowed to sit at 4° C overnight. Analytical ultracentrifugation showed no alteration of the sedimentation pattern of fixed ribosomes compared with unfixed ribosomes. However, recent evidence by McConkey (237) suggests that even HCOH does not prevent some degradation of

ribosomes by CsCl.

Gradients were prepared in 2% neutralized formaldehyde containing CsCl (0.742 gm/ml) and centrifuged for 48 hours at 35,000 rpm at 25° C in the SW 50.1 rotor.

2.180 Dialysis tubing.

Tubing (Gallenkamp) was boiled three times in 0.1% SDS followed by three cycles of boiling in distilled water. Tubing was stored in the cold in 10 mM EDTA and well rinsed with distilled water before use.

CHAPTER 3

COMPARISONS OF RIBOSOMAL PROTEIN PATTERNS BETWEEN SPECIES -- CATIONS

3.000 Introduction.

Since it may be analyzed by a number of different techniques, the definition of what constitutes the ribosome is often slanted toward the technique used. Hence the electron microscopist sees small, regularly arranged polygons on the endoplasmic reticulum or free in the cytoplasm and so defines the ribosome. The nucleic acid chemist isolates structures that contain equimolar amounts of 18S, 28S and 5S rRNA and these comprise the ribosome. Likewise the enzymologist isolates ribosomes which when combined with various factors result in incorporation of labelled amino acids into TCA-precipitable material. None of these approaches ignores the others but all tend to confer upon the ribosome certain attributes of size, composition and function.

When analyzing the protein composition of these particles one is confronted by the uncertainty of what proteins are truely ribosomal and which fit into the realm of "associated" factors. This distinction is necessarily imposed by the conditions used for isolation of the particles and hence the division into a ribosomal pellet and a supernatant fraction is somewhat arbitrary. Indeed, in E. coli the ribosomal protein, S1, may be associated with either the small subunit or the supernatant (61) and acts as a translational control factor. Until a functional or synthetic approach [such as the reassembly of small (152) and large (251) ribosomal subunits from E. coli ribosomal protein and rRNA] becomes available for eukaryotic ribosomal protein, any determination by 2-D gels of whether a protein is ribosomal or not must remain

tentative.

At present the standard methods of preparing ribosomes often employ a 500 mM KCl wash [analogous to the 1 M NH_4Cl wash for E. coli ribosomes (357)] to remove the associated factors. Such ribosomes (101,232) are unable to synthesize protein without added factors but retain their typical appearance in the electron microscope. Whether all such factors are removed and/or true ribosomal proteins removed must await further studies. For the purposes of these experiments, the ribosome is empirically defined as that particle which invariably is isolated by the technique employed and yields consistent 2-D gel patterns. Which of these are truly ribosomal proteins will be determined once functions have been assigned to these specific proteins.

Until recently the analysis of the ribosomal proteins of eukaryotic cells has been overshadowed by the successes of analyzing those of prokaryotes. Many E. coli ribosomal proteins have been purified, sequenced and assigned a function in the ribosome. For example, ribosomal proteins L7 and L12 have an identical sequence except that L7 has its N-terminal serine acetylated (40,295,368) and is involved in EF-G and EF-T dependent GTP hydrolysis (198, 369). Using 2-D gel electrophoresis, even allelic variations of ribosomal protein S12 (responsible in part for streptomycin resistance) have been detected (110). L16 is identified as the chloramphenicol binding protein in E. coli (250,302,418) while L25 (99,112,125) and L18 (99,125) interact with 5S rRNA.

At the start of the studies described in this chapter, only a few studies on eukaryotic ribosomal proteins had been attempted. It had been established that Hela cells contained large, balanced pools of ribosomal protein for when cycloheximide was added to

growing cells, complete ribosomes continued to be made for a considerable time (390,392). Ribosomes formed under these conditions or without cycloheximide were found to have approximately equal amounts of RNA and protein (134) while the large subunit was 40% protein and the small subunit 50% protein (189). Estimates of the molecular weight of the small and large subunits were 1.18 x 10^6 and 3.12 x 10^6, respectively (134). Given an estimate of 0.6 x 10^6 and 1.7 x 10^6 daltons for the rRNA of the two subunits (292), calculations of the molecular weight of the protein associated with the subunits were made; the small subunit having 0.6 x 10^6 daltons and the large subunit with 1.4 x 10^6 daltons.

Coupled with these determinations of the total number of daltons of protein on the ribosomes were one-dimensional urea (133,181,400,402,408) and SDS (124,189,391,400) gels to determine the number of protein species, their molecular weights and their molar amounts on the ribosome. The most successful of these studies were those that ran isolated subunit ribosomal proteins on SDS gels and then used the intensity of staining to estimate molar amounts (16,189,391). Using yeast ribosomes, Warner (391) found 79 ribosomal proteins on the 80S with 50 on the large and 29 on the small subunit. For the rabbit reticulocyte ribosome (189), 49 $\pm$ 12 ribosomal proteins were estimated to compose the large subunit while 34 $\pm$ 5 made the small. The weight-average molecular weight ($\bar{M}w$) of the large subunit proteins was 25,000 while that for the small was 21,000 (135). For the mouse ribosomes, Bickel and Traut (16) found 18-27 proteins on the small ($\bar{M}w$ = 34,000) and 42-52 ($\bar{M}w$ = 32,000) on the large; the 80S particle therefor containing 60-79 proteins in comparison to the 45-56 associated with the E. coli 70S determined under the same conditions. In all

of these studies most of the ribosomal proteins were interpreted as being present in molar amounts with at least one protein present in submolar quantities. But the differences in the estimates for the number of proteins reflects the inherent weakness of this approach.

During this same period, a number of experiments had suggested little or no tissue or species specificity in the translation of several purified messenger RNAs. Using injection of purified mRNA into oocytes, Lane et al (203) found no species barrier. Likewise in in vitro systems (235,236,275,351) mRNA from one species was efficiently translated by ribosomes of another species. Given this seeming universality of the translational machinery (and hence of the ribosome itself), the initial experiments of Houssais (161) seemed at odds for they showed bands on 1-D gels indicative of species and tissue specific ribosomal proteins. Bielka and Welfle (18) had also found dissimilarity in the protein pattern on 1-D gels of ribosomes from different species though no tissue specificity had been found. Rat liver, yeast (S. cerevisia) and plant ribosomes were similarly found to have unique ribosomal proteins (323). Indeed the first 2-D gels published on eukaryotic ribosomes by Huynh-van-tan et al (165) showed that the proteins of rat and rabbit ribosomes differed as did those between rabbit liver and rabbit RBC. These experiments thus pointed the way for experiments to define more explicitly any such differences in ribosomal proteins between species and between different organs of the same species.

Further evidence of species differences in ribosomal proteins came from testing for cross-reacting antibodies to the ribosomal proteins of one species with sera prepared against another species' ribosomes or ribosomal proteins. Using such sera, Noll and Bielka

(256) were able to demonstrate that antisera to rat liver ribosomes cross-reacted poorly with mouse ribosomes and not at all with rabbit or bovine ribosomes. Rothschild et al (317) found no cross-reaction of antisera against N. crassa ribosomal proteins with Xenopus laevis or sheep ribosomes. Sarker and Som (323) compared plants, yeast and rat liver ribosomes and found no cross-reaction. But distinct cross-reaction was found between N. crassa ribosomes and antisera to rabbit 80S ribosomes (1) while sera from patients suffering from systemic lupus erythematosus (SLE) cross-reacted with several mammalian ribosome species (330). The conflicting results of all these experiments also suggested the need to compare the ribosomal protein patterns of different species. A possible rationalization for these conflicting antibody experiments is given below (3.090).

The possibility that there was some specificity in the ribosome structure that altered their translational properties also concerned an older idea that perhaps neoplastic tissue so differed from normal tissue. Using antisera perpared against rat liver or NHAC ribosomes (48,49), Wikman-Coffelt et al (414) have reported two antigenic differences between proteins of the 60S ribosomes of hepatoma cells and those of normal liver. Rodgers (311) has described a unique acidic ribosomal protein of MW 58,000 not found in normal tissue but present in ribosomes from neoplastic mouse cells. Most recently, Subramanian et al (359) found four obvious differences between ribosomes of rat forebrain and a mouse neuroblastoma. The problem with all three of these studies is that they are comparing not just neoplastic vs. non-neoplastic cells but growing vs. non-growing tissues. The difference so described may thus be a function not of neoplasia but of growth. As described in this chapter, when an

attempt is made to eliminate the difference in growth kinetics, no cationic ribosomal protein differences are found.

It was in light of these questions about ribosome structure that the present studies, applying the technique of 2-D gel electrophoresis to eukaryotic ribosomes, were begun. The specific intent was to find species-specific marker ribosomal proteins for cell fusion experiments. However, answers to some of the other questions about ribosome structure became apparent during the course of this study.

3.010 Purity and characterization of isolated ribosomes.

As prepared by the methods of Falvey and Staehelin (2.040) the ribosomal pellets consisted basically of polysomes and monosomes though most monosomes and subunits were supposed to be held back by the 2M sucrose (411). Though not tested here, these preparations are thought to be active in protein synthesis (101). Fig 2a shows a typical polysome profile though the classes below 80S were not resolved. Approximately 45% of the total optical density is in the polysome peaks. Others have found that usually about 50% of the ribosomal optical density is greater than 80S (334,364). When centrifuged slightly longer (Fig 2b) the 80S, 60S and 40S classes are resolved. It should be noted that the 80S peak is barely present due to the fact that under these conditions of high salt (500 mM KCl) polysomes and messenger-bound monosomes remain stable whereas free 80S ribosomes dissociate into subunits (101). This suggests that there has been little degradation of the polysomes during preparation and that the majority of ribosomes not in polysomes are free ribosomes. The presence of an 80S peak at high salt thus serves as a reliable indicator of ribonuclease

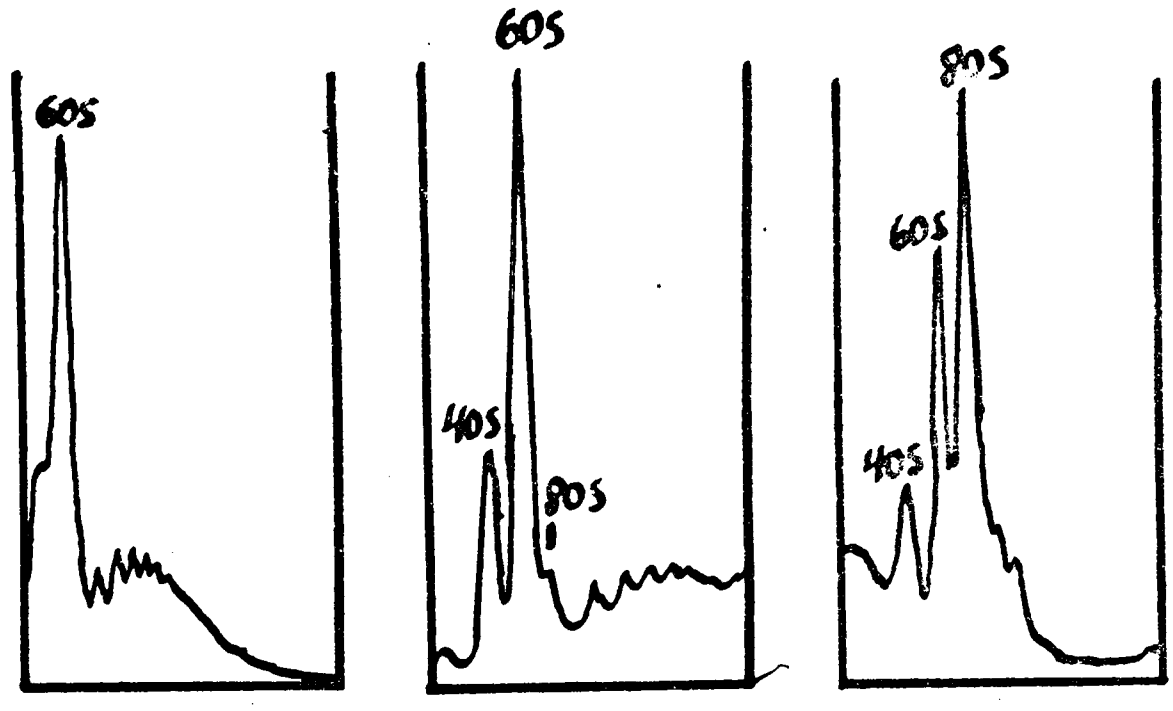


Figure 2
Sedimentation profile of Hela ribosomes.

Abscissa: fraction number.
Ordinate: A_{254}.
Hela ribosomes (15 ODU_{260}) were resuspended in 1 ml of dissociation buffer (2.060) without puromycin, incubated with or without RNase (6 ug/15 ODU_{260}) for 10 minutes at 37° and then centrifuged (1.2 ODU_{260}/ gradient) for varying times on 10 - 40% sucrose gradients (2.060) at 35,000 rpm at 4° C. (a) Without RNase, 100 minutes; (b) without RNase, 130 minutes; (c) with RNase, 130 minutes.

degradation of polysomes (Fig 2c).

For each ribosome preparation one tube of pelleted ribosomes was routinely resuspended and scanned with a spectrophotometer (2.040) to assay for purity and determine protein concentration (Fig 3). The ratio A_{260}/A_{235} was usually between 1.45 and 1.75. But this is a relatively insensitive method for assessing purity since no difference in 2-D gel pattern was observed between preparations from the same source with values ranging between those two above. Samples with ratios less than 1.40 or greater than 1.80 were discarded for these indeed showed abnormal gel patterns. From such a u.v. scan the amount of ribosomes present was estimated using the relation that 1 mg ribosomes = 15 ODU_{260} (A. Rodgers, personal communication). This relation has been used by a number of other investigators but recent evidence (337) suggests the value is more like 1 mg = 10 ODU_{260}. Since the 80S ribosome is approximately 50% protein (337), it was calculated that there was 1 mg of ribosomal protein per 30 ODU_{260} of intact ribosomes.

Analytical centrifugation with the Model E was initially employed to check ribosomal preparations. Under these conditions (a total concentration of 1 mg/ml in 3 mM Mg, 300 mM KCl, 20 mM Tris pH 7.4) the polysomes rapidly sedimented but the sedimentation coefficients so determined were 37.6S for the small subunit, 53.5 S for the large and 71.2 S for the monosome. These values showed a definite concentration dependence upon centrifugation as suggested by Sherman and Petermann (334) with the small subunit showing the least variation with respect to concentration and the monosome the most. No attempt has been made to determine the exact regression coefficients for these particles nor to correct for the KCl concentration though this too alters the sedimentation coefficient (334).

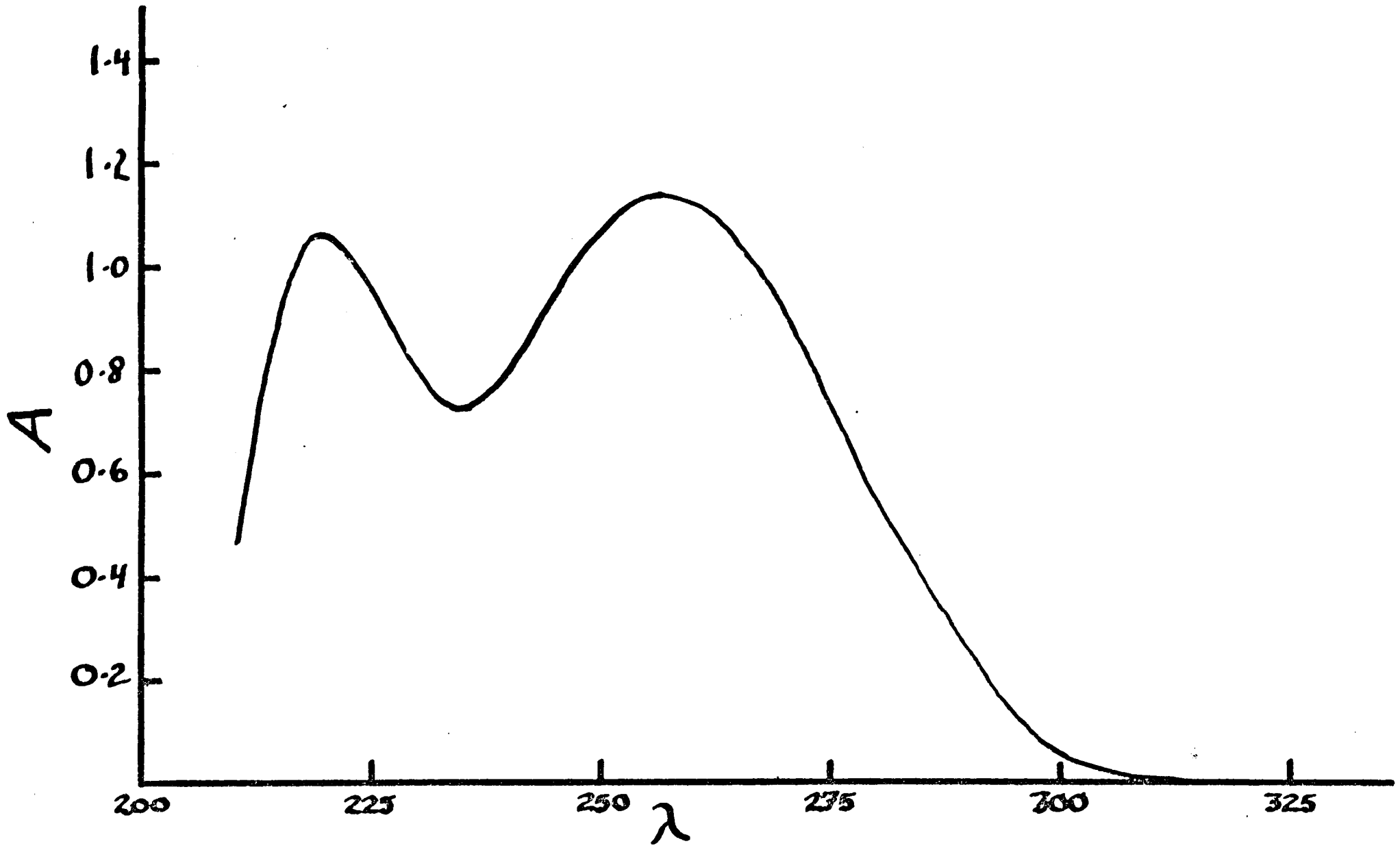


Figure 3
Absorbance profile of Hela ribosomes.

Abscissa: wavelength (nm).
Ordinate: absorbance.

These values for the 80S particle may be compared with that of 81S at infinite dilution in lower ionic strength buffer (334) or 78S run at increased Mg++ concentration (364).

In addition, analytical centrifugation and sucrose gradient centrifugation have not detected any difference in sedimentation coefficient for ribosomes isolated from any of the different species discussed later in this chapter. Nor has CsCl centrifugation of HCOH-fixed ribosomes revealed any differences in density between these different ribosome species.

3.020 Extraction of ribosomal proteins.

The method of Hardy and Kurland (139) gives very reproducible results on 2-D gels and is applicable to small and large amounts of sample. The use of Mg++ is critical to the removal of the RNA (337). Sherton and Wool (337) have estimated that 80-90% of ribosomal protein is extracted and 80-90% of the total protein is recovered. Though the extraction appears nearly complete, experiments were conducted to assure the complete removal of RNA and qualitative isolation of ribosomal proteins.

Using 10% RNA gels (2.084) samples of intact rat liver polysomes and ribosomal protein previously extracted from rat liver polysomes were electrophoresed and then scanned for u.v. absorbing peaks. Both 4S and 5S rRNA were well resolved by this method and were seen in the profile of the intact ribosomes (Fig 4a). But the extracted ribosomal protein showed neither RNA peak even when gels were vastly overloaded (Fig 4b). Since this technique allows one to detect 5S rRNA down to a level 1/100 of that normally found in intact ribosomes, it was concluded that there is insignificant 5S contamination of the extracted protein. When extracted ribosomal proteins were run on 2.7% gels (2.083), no larger

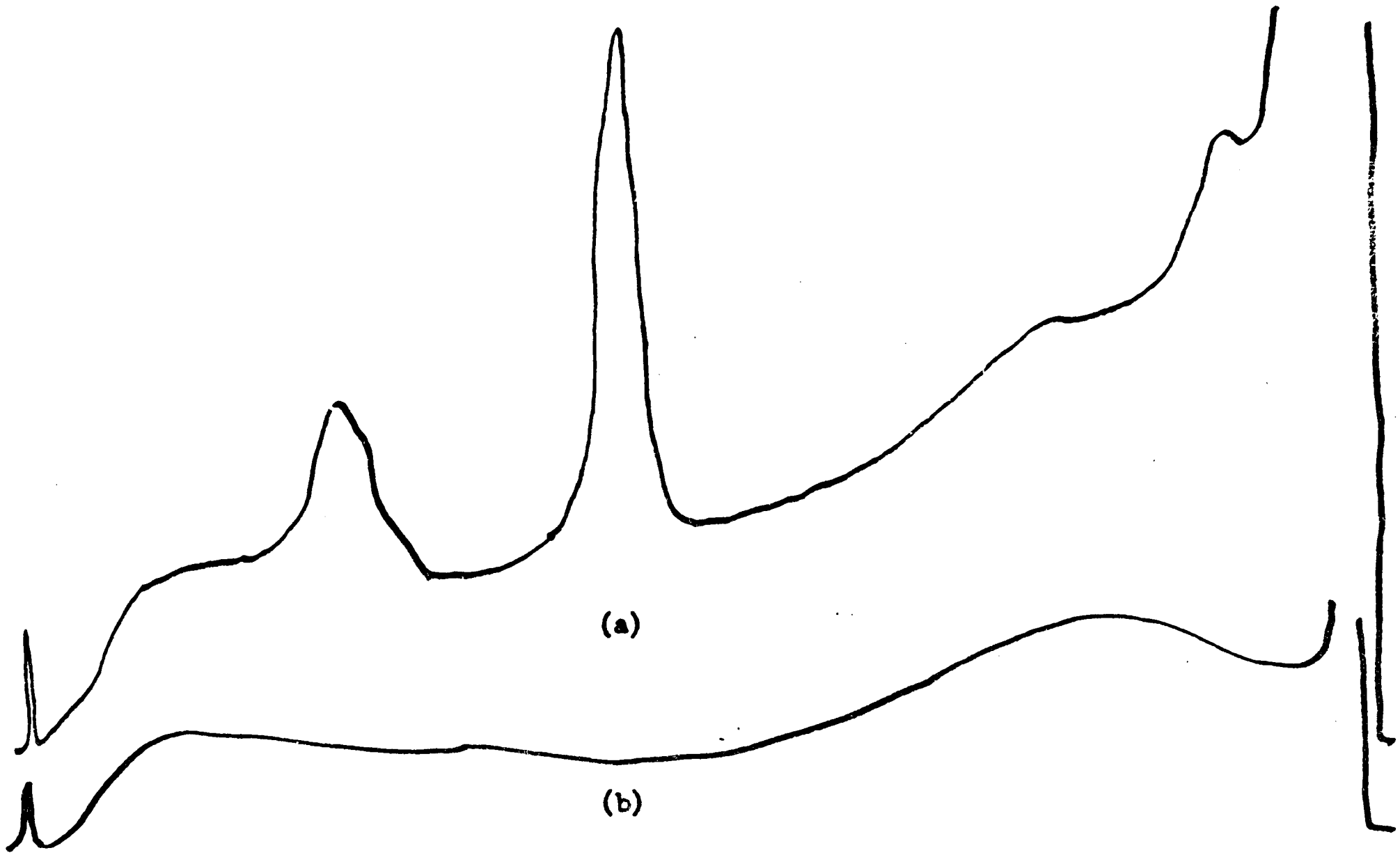


Figure 4
Scans of 10% RNA gels of rat liver polysomes.

Abscissa: distance along gel; top of gel to right.
Ordinate: A_{260} (arbitrary scale).
Gels were run as in 2.084. Rat liver ribosome samples consisted of either 2.1 ODU_{260} of polysomes (a) or the acetic acid extracted ribosomal protein from 3.0 ODU_{260} of polysomes.(b).

contaminating rRNA species were revealed either. Though suggesting complete removal of rRNA, these experiments do not exclude the presence of small oligomers. However, significant levels of these would yield some A_{260} absorbance in the purified ribosomal proteins and such was not found.

Upon staining the 10% RNA gels for protein, some differences were noted between the protein bands from the intact polysomes and the acetic acid extracted proteins. The extracted ribosomes (Fig 5, compare f,g,h with a) showed a decrease in band 1 and complete loss of bands 4 and 5. One of the lost ribosomal proteins is an acidic protein (4.010). It thus seems that there is some specific loss of two low molecular weight proteins and a reduction in one of high molecular weight upon extraction with acetic acid while the rest of the ribosomal protein bands appear identical in position and in relative intensity. These losses upon extraction are minor with respect to the presence of the majority of the other ribosomal proteins but should be remembered when interpreting the 2-D gels. Since the number of distinct molecular weight species on SDS gels is considerably below the number of ribosomal proteins seen on 2-D gels it is possible that other proteins may also be lost and yet not revealed by these SDS gels.

On a number of occasions, especially when dealing with small amounts of samples, the ribosomal protein extract turned into a gelatinous residue after freeze-drying. Though less easy to solublize than the fluffy powder obtained from larger amounts of the same protein, the 2-D gel patterns appeared unaffected except for the presence of larger amounts of very high molecular weight species which were already known to be artifacts of the gel process (see 3.030).

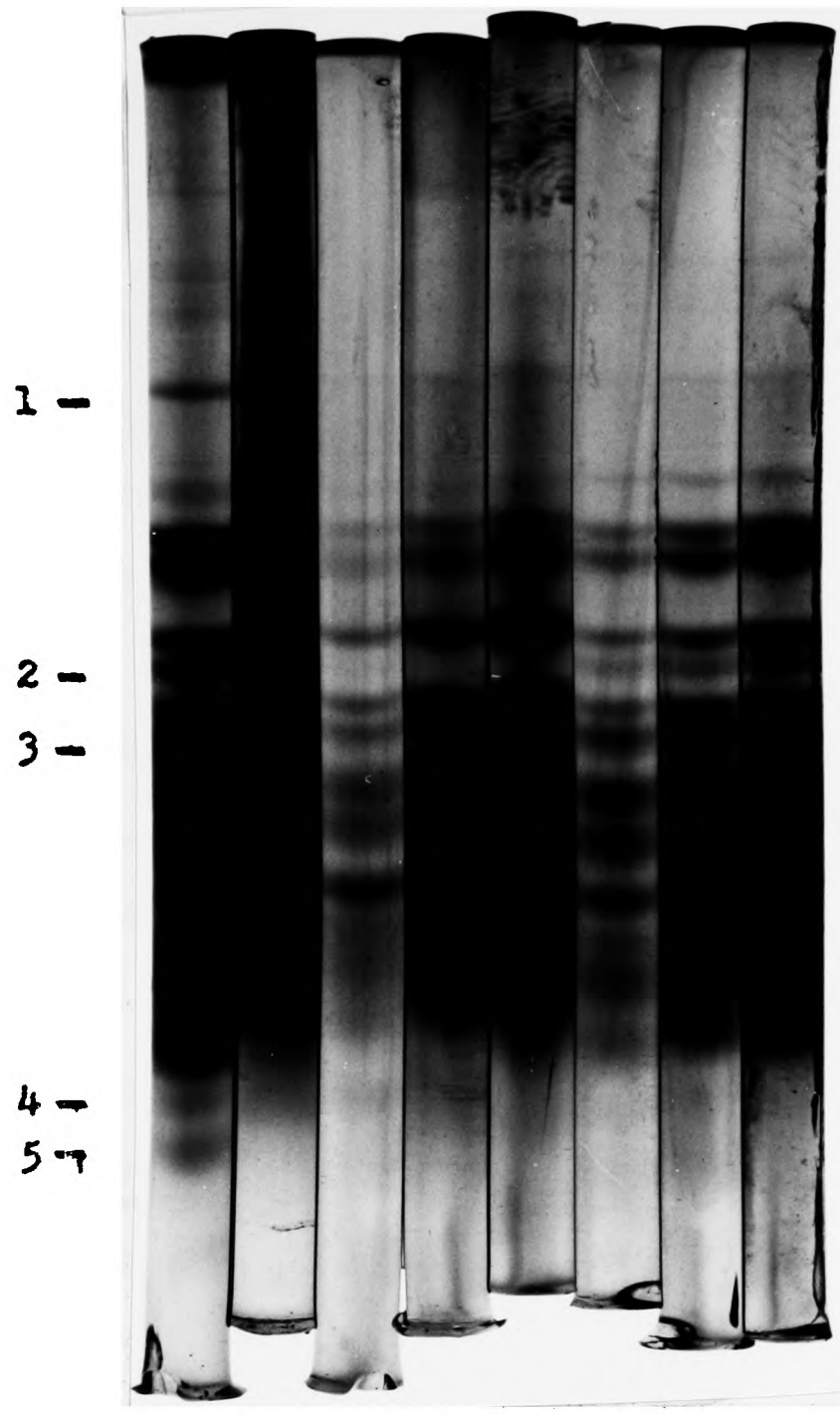


Figure 5
10% RNA gels of rat liver ribosomes.

Gels are identical to those in Figure 4 but are stained for protein as in 2.084. Samples consisted of: intact rat liver polysomes (a, 1.5 ODU_{260}; b, 2.1 ODU_{260}); acetic acid extracted ribosomal proteins of EDTA-treated (1uM/10 ODU_{260}) ribosomes as in 2.090 (c, 1.0 ODU_{260}; d, 2.0 ODU_{260}; e, 5.0 ODU_{260}); acetic acid extracted ribosomal proteins of polysomes (f, 1.0 ODU_{260}; g, 2.0 ODU_{260}; h, 3.0 ODU_{260}) not extracted with EDTA.
Band 1 = that protein greatly reduced after acetic acid extraction.
Band 2 = the 5S rRNA binding protein.
Band 3 = approximate position of 5S rRNA.
Bands 4 & 5 = those ribosomal proteins lost after acetic acid extraction.

3.030 Interpreting 2-D gels.

Two-dimensional gel patterns allow greater resolution of the ribosomal protein components than was possible with single dimensional gel electrophoresis (compare Figs 5 and 6). Separation is basically by charge in the first dimension and by size in the second. On a gel pattern seventy or more protein spots may easily be discerned and each runs distinct from the prokaryotic ribosomal proteins. Unlike prokaryotic ribosomes, most eukaryotic ribosomal proteins are cations as was easily seen with the sample polymerized toward the middle of the 1st dimension gel (Fig 6). Cationic ribosomal proteins moved to the right and anionic ribosomal proteins to the left at pH 8.6. At pH 4.0 in the second dimension, all proteins moved as cations. Experiments have been conducted to determine whether any anions were present at this pH; upon reversal of the potential of the 2nd dimension, no such spots were found. Not all anions are seen in Fig 6 since several show high mobility in the 1st dimension and have run off the end of this gel. Experiments reported in Chapter 4 discuss these.

A number of cations rapidly migrating in the 2nd dimension do not appear in Fig 6 but can be seen in Fig 7. It was thus important to vary the times of electrophoresis to assure that no ribosomal proteins were undetected. In addition to varying the duration of electrophoresis to prevent loss of proteins off the ends of the gels, varying the duration of electrophoresis in either or both directions (Fig 73a) enhanced the resolution of closely lying spots. Such resolution was also a function of the sample size.

Gel patterns were exactly reproducible from run to run using the same sample. However, slight variability occured between

+ 2-D

+ 1-D ORIGIN - 1-



- 2-D

Figure 6
Chick embryo ribosomal proteins.

Standard gel method (2.071). 3.3 mg protein was electrophoresed for 24 hours in the first dimension and 40 hours in the second dimension. Arrow points to the "tail" of a ribosomal protein.

+ 2-D

ORIGIN

+ 1-D

- 1-D



- 2-D

Figure 7

Chick embryo cationic ribosomal proteins.

Two-dimensional microgel electrophoresis of ribosomal proteins (200ug) run for cations only as in 2.072.

different samples and always involved only certain areas of the gel patterns (Fig 8): spots 13 and 14, 22 (a + b), 28 and 29. The published gel patterns of others also show such inconsistency (311). That this reflects an intrinsic property of the ribosomes is doubted; rather such was probably due to oxidation of the proteins during polymerization of the sample gel since application of the samples without polymerization reduced this variability. The extraction and freeze-drying steps might also contribute since artefactual spots such as these, resulting in the occurrence of a second neighboring spot, have been reported with E. coli ribosomal proteins (151,265). With the exception of these few variable regions, the rest of the protein pattern was perfectly constant between different runs and samples. The introduction of the microgel technique has also greatly served to eliminate these artefacts.

The interpretation of any 2-D gel pattern thus must be a composite of gels run with different samples, for varying lengths of time and with different protein loads. One gel rarely reveals the complete picture though it always presents the great majority of proteins.

The intensity of staining of protein spots often varied with the standard method but not with the micro method. Presumably this reflected the loss of ribosomal protein in the polymerization of the sample gel. It was thus not possible to draw conclusions from staining intensities in the standard method (2.071) gels presented here. In addition, some of the spots destained faster than others. For example, protein 48 (Fig 8) was particularly rapidly destained and hence often looked faint. The more acidic proteins also stain poorly. That this is due to the intrinsic charges of these proteins as some suggest (189) is doubted and most probably reflects their

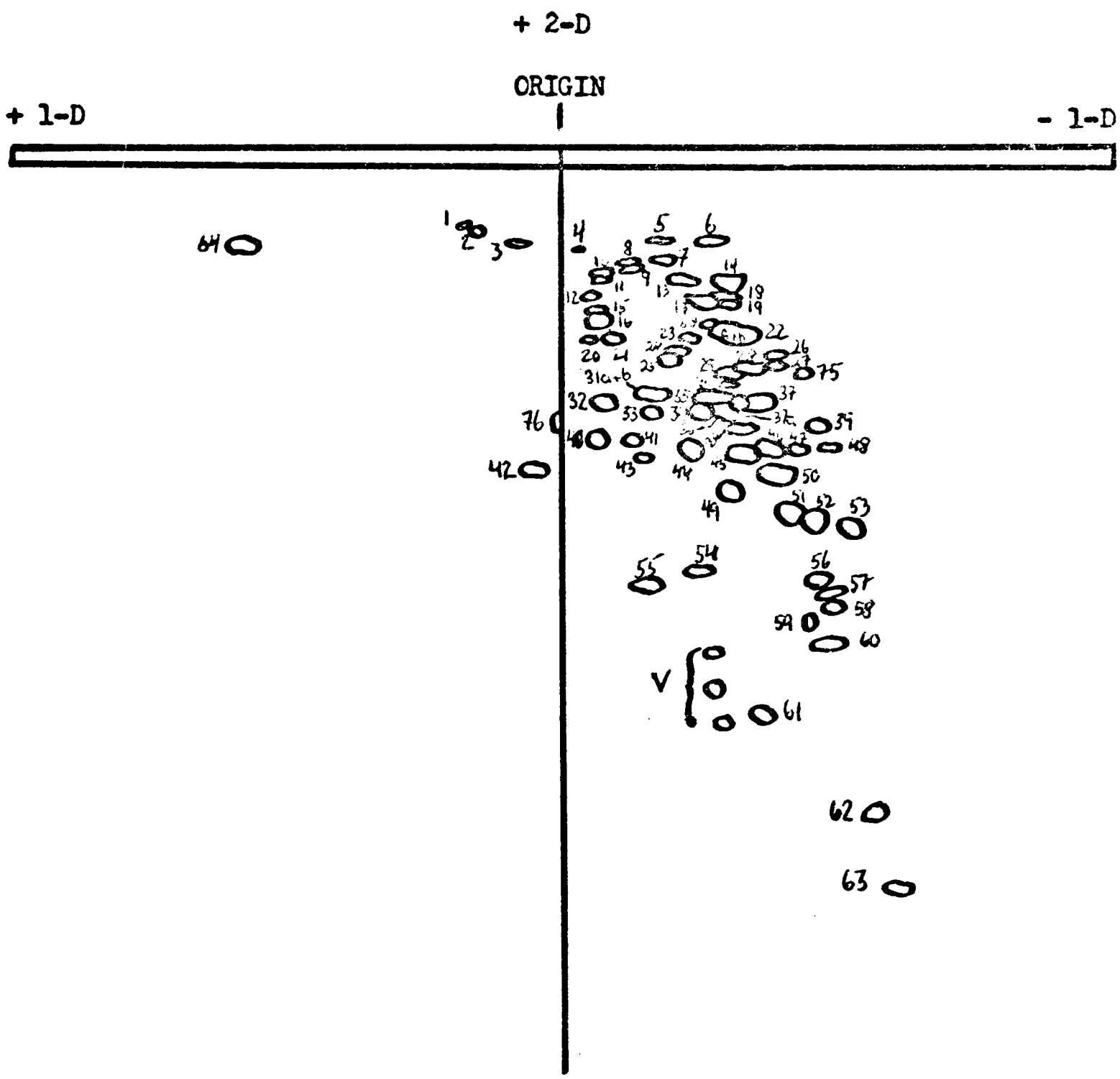


Figure 8

Schematic diagram of chick embryo ribosomal proteins.

Data from 38 different preparations of standard method and microgels with varying sample size and duration of electrophoresis. "V" denotes derivative spots frequently found.

presence in lesser amounts (337). That the relative staining intensities of these spots on microgels accurately reflect the amount of protein present has not been tested here but has been assumed to be true.

Gels often show a shadowy streak spreading diagonally from the origin on the cationic side (Fig 6) or numerous small spots of high molecular weight (Fig 7) in that same area. These are due to aggregates of ribosomal proteins linked by disulfide bonds as has been reported by others (335). Kaltschmidt and Wittmann (180) have shown that such streaks were eliminated when proteins were oxidized with performic acid or reduced and alkylated with iodoacetamide. Since these spots or shadows only affect the very high molecular weight ranges of these gels, they do not alter the interpretation of the gel patterns and hence ribosomal proteins were not routinely reduced before electrophoresis.

As seen in Fig 6, a broad, dark vertical streak also emanated from the origin. This is a constant feature of all 2-D gels run here and by others (180,335). They are interpreted as being ribosomal proteins that did not enter the 1st dimension gel from the sample gel at pH 8.6 and represent not a special population of proteins but a fraction of the total ribosomal protein that is not solublized at pH 8.6. At the pH of the second dimension they are much more soluble and run downward (180,335). Indeed, the intensity of staining in this vertical strip varies as does the number of cationic proteins at the same second dimension mobility, supporting the non-specific insolubility of these species.

A third sort of smearing/streaking is also illustrated in Fig 6 (arrow). Usually in the direction of the 1st dimension (but

also occasionally observed in the direction of the 2nd dimension) a single ribosomal protein spot will show a long tail. These were prominent on overloaded gels and suggested that the protein was either coming out of (or going into) solution as it electrophoresed. Polymerization in the sample gel also seemed to increase the frequency of these tails.

The standard gel method was found to be awkward. In addition to the artefacts caused by the polymerization of the sample in the sample gel (such as increased streaking and trapping of variable amounts of protein in the sample gel) the sample gel proved to be difficult to polymerize and often resulted in losses of 50% of the sample. This method also required considerable amounts of sample (2-4 mg protein) to yield good gels. Coupled with the long times required to run and destain (5-6 days), ways were sought to modify the standard method.

The use of the microgel method (2.072) was very advantageous. Samples of 100-200 ug could now be used to give patterns of higher quality than the standard method (Fig 7). This was due to the use of a liquid sample layer that did not trap or precipitate the protein, smaller gel size, and a more sensitive stain [Coomassie Blue (6,102)] . The limit of detection here is about 1-1½ ug/spot. In addition, it was possible to run and destain a gel in 24 hours (though longer times were later made "standard"). As shown in Fig 7, all of the cationic pattern fits into one gel and reduces the need for other runs. When compared to the identical sample run by the standard method (Fig 6) no new spots were found. On the microgels, spots were more compact, less smeared, less variable and more intense. The only drawback to this method was the necessity to run a second

gel to analyze the anionic proteins. But since there are so few anions, this was not a great objection.

Proteins of gel patterns are numbered arbitrarily but consistently throughout this chapter.

3.040 Chick embryo ribosomes (Figs 6,7,8)

The 2-D gel pattern of chick embryo ribosomes contains 71 distinct spots of which 6 are anionic proteins (Fig 6). Additional anions are present but are either poorly stained or run off the gel under these conditions (as does protein 64 in Fig 6); these are discussed further in Chapter 4. Spot 22 occasionally runs as two separate spots and is considered to contain two proteins. Since spots 36 and 45 are also each composed of two proteins (Chapter 6), the chick embryo ribosome comprises at least 73 proteins. The faint spots designated "V" in Figure 8 are often present in these gels and those of Sherton and Wool (335). They most likely are derivatives of spots of some other proteins and are not taken into account here.

Several other spots appear as doublets (8 and 9, 10 and 11) or triplets (56,57,58). Whether they are unique protein species or just alternate forms of each other cannot be determined. Since they are always found in these gels they are each tentatively identified as different species of ribosomal protein.

Before the introduction of the microgel technique, several proteins showed ambiguous mobilities after tens of runs. As seen in Fig 6, spot 13 often ran with 14. Also, though 28,29 and 30 were always constant, spots 26 and 27 (Fig 6) migrated with several different patterns. A faint spot, 75, was also frequently observed. The microgels (Fig 7) agreed with the majority of the

standard method patterns and allowed the schematic presentation shown in Fig 8. With the exception of 75, 48 and the several derivative spots, most of the proteins are of similar staining intensity and suggest that they are present in approximately equal amounts. When compared with the proteins of other species below, the chick ribosomal protein pattern is strikingly similar to them. It is, however, distinguished by several unique features: 1) It possesses anionic proteins 2 and 3 that the other species lack. 2) It is missing 69 and 70 that are distinct proteins present in the other species. 3) It has a unique ribosomal protein, 45, that seems to replace 45b and 45c in mouse. This protein is only slightly less basic in the 2nd dimension and hence may be closely related to 45b and 45c. 4) Protein 48 moves slightly differently in the 2nd dimension (seen more clearly upon coelectrophoresis) than proteins of similar mobility in the other species examined. 5) Proteins 26 and 27 move differently from those of other species but not so distinctly that coelectrophoresis can resolve them.

3.050 Ribosomes of mouse cell lines.

The L cell derivatives [L929, A9(H), A9(L), A9HT] along with EA are commonly used as parents in cell fusion experiments. Because of this and the uncertainty that ribosomal protein patterns for a species were altered upon cell culture, numerous 2-D gels were run to find any slight mobility changes. Since the ribosomal protein patterns of various mouse organs had already been completed (311), a ready comparison was possible.

With the exception of only 1 or 2 spots, the patterns of all these cell lines are identical and are schematized in Fig 9. The 72 spots on the gel pattern include 4 anions one of which, 64, has

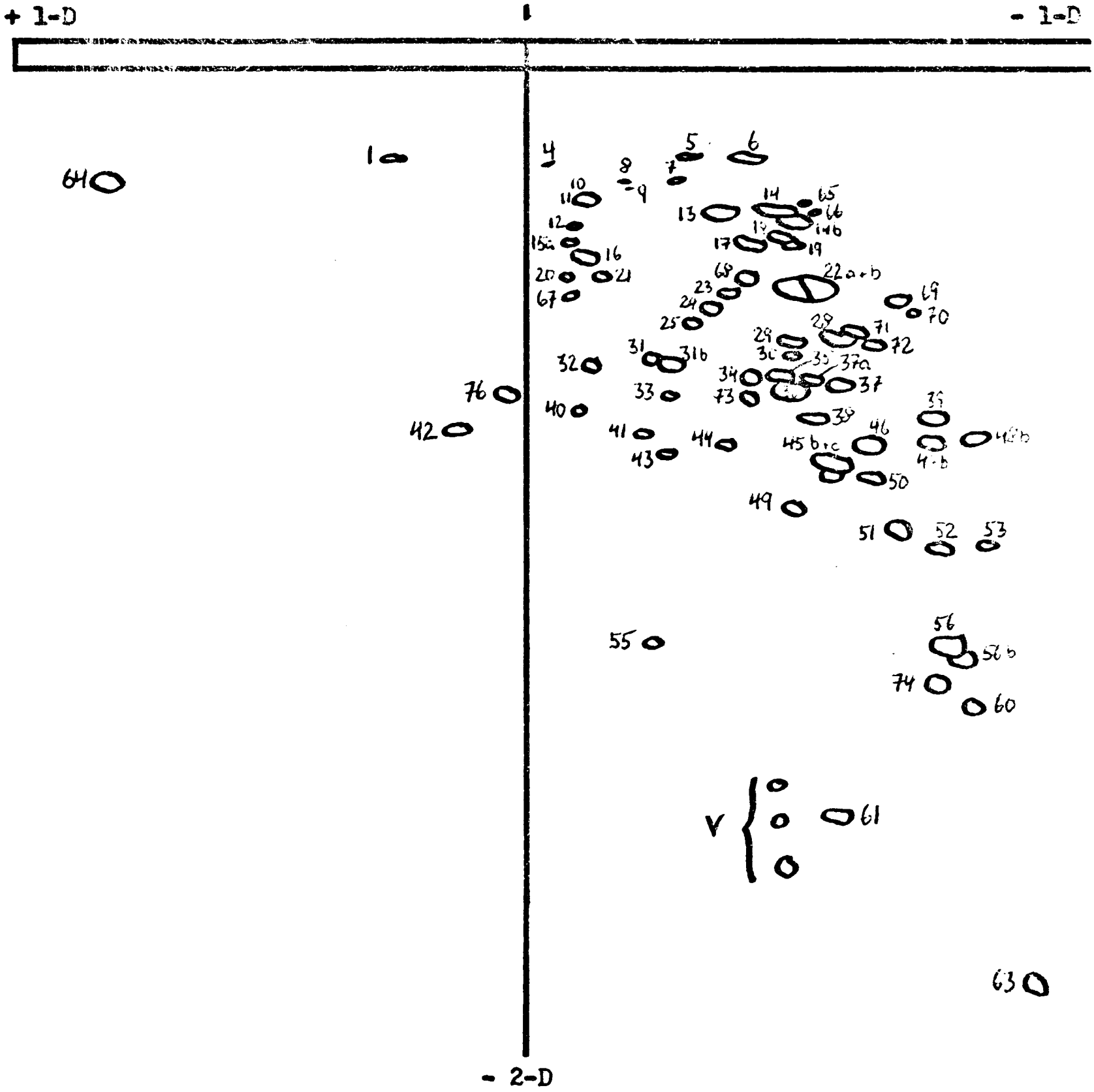


Figure 9
Schematic diagram of mouse ribosomal proteins.

Compiled from the ribosomal protein patterns of over 100 different preparations of EA, A9(HT), A9(L), A9(H), and L929 run on standard method gels and microgels. "V" denotes derivative spots frequently found.

run off the end of the gel in the 1st dimension in Figs 10,12,14, 15,17. Again, 3 of these spots [22,36,45 (b + c)] contain two proteins, yielding a total of 73 proteins composing these ribosomes. Before discussing the fine features of the patterns of each of these lines, the common characteristics of them all may be presented and a comparison made with the chick patterns previously discussed.

Most of the ribosomal proteins are identical with those of chick. Spot 22 seems to consist of 2 closely migrating proteins while spots 8 and 9, 10 and 11 appear as doublets and may be only one protein though counted as two here. As in chick patterns the protein derivatives, V, are present. The differences between mouse and chick concern the following:

a) Though 28,29,30 appear in mouse as in chick, 26 and 27 are absent and replaced with two other spots of slightly different mobility, 71 and 72. These are distinct differences but are difficult to resolve upon coelectrophoresis of chick and mouse.
b) Mouse patterns lack 45 but have two nearby proteins unique to mouse, 45b and 45c.
c) Mouse possess 69 and 70 (which may be derivatives of each other). Chick patterns lack these.
d) Mouse patterns show protein 48b which differs from 48 in chick by running slightly less in the second dimension. Proteins 65 and 66 are present here but lacking in chick.
e) Protein 56b and 74 may be derivatives of 56 but move with only slightly altered mobility from 57 and 59 in chick.
f) Protein 15 of chick is replaced by 15a of mouse.
g) 47b replaces 47 of chick.
h) Mouse patterns possess anionic protein 64 but lack anions 2 and 3 as well as the following cationic proteins that have not yet been discussed: 12*, 54, 58, 62.

Turning to the specific patterns of each mouse line, the following exceptions to the general pattern (Fig 9) may be summarized:

a) A9HT (Figs 10,11) -- pattern is identical to that in Fig 9 but lacks 12 and shows 23 only faintly.
b) EA (Figs 12,13) -- identical to Fig 9 but 40% of preparations lack protein 12.

*Present in some EA ribosomal protein patterns.



Figure 10
Two-dimensional gel pattern of A9HT ribosomal proteins.

Standard method 2-D electrophoresis (2.071) of 3.5 mg ribosomal proteins. Electrophoresis was for 24 hours at 100 V in the first dimension and 41 hours at 100 V in the second dimension.

+ 2-D

ORIGIN

+ 1-D

- 1-D



- 2-D

Figure 11
Two-dimensional pattern of A9HT cationic ribosomal proteins.

Microgel electrophoresis (2.072) of 220 ug ribosomal protein. Electrophoresis was for 1 hour at 3 ma/tube and then 5 hours at 6 ma/tube for the first dimension followed by ½ hour at 40 V and 19 hours at 150 V in the second dimension.



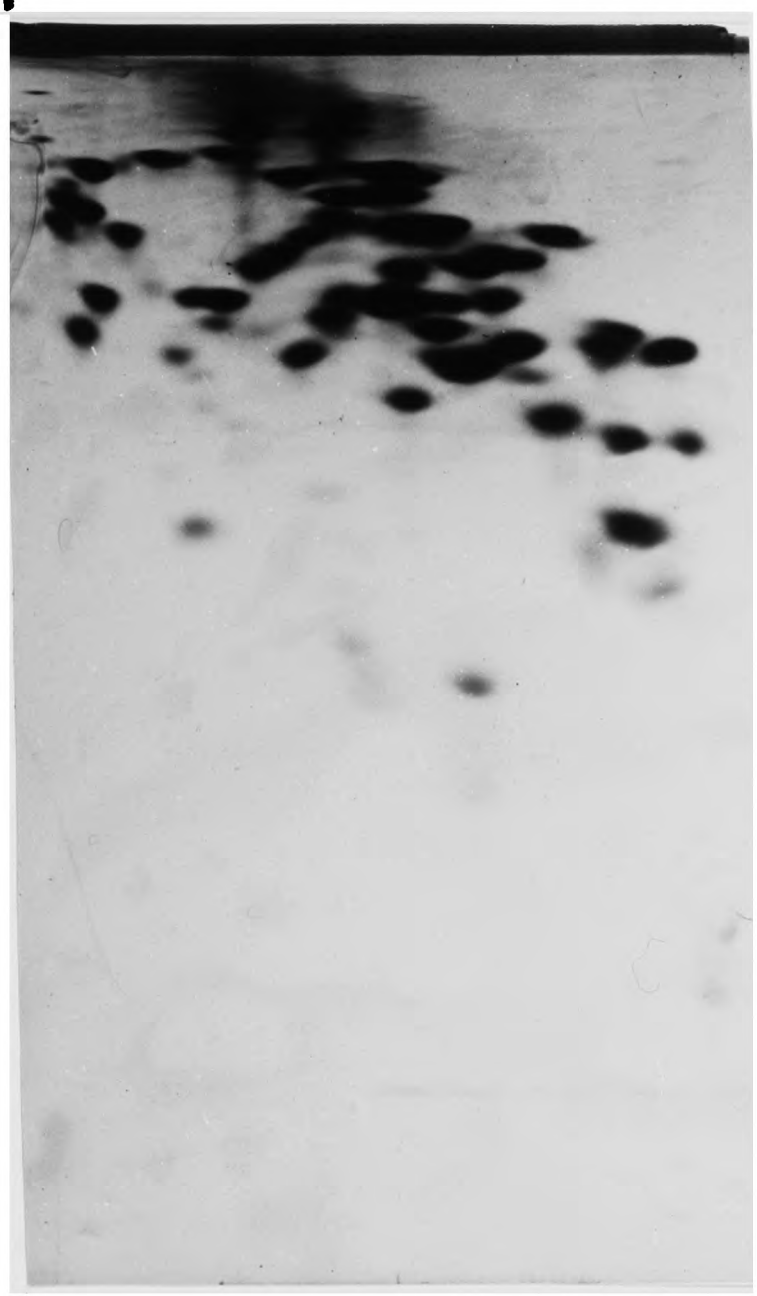
Figure 12
Two-dimensional gel pattern of Ehrlich ascites ribosomal proteins.

Standard method gel of 3.5 mg protein. Conditions of electrophoresis identical to those in Figure 10.

+ 2-D

ORIGIN

+ 1-D



- 1-D

- 2-D

Figure 13
Two-dimensional pattern of Ehrlich ascites cationic ribosomal proteins.

Microgel electrophoresis of EA ribosomal proteins (200 ug). Electrophoresis was for ½ hour at 3 ma/tube and then 5 hours at 6 ma/tube in the first dimension followed by ½ hour at 40 V and then 20 hours at 100 V in the second dimension.

c) L929 (Fig 14) -- though all the proteins of the general pattern are not shown in this gel, other gels have shown that only protein 12 is consistently missing.
d) A9(L) (Figs 15,16) -- only protein 25 is missing.
e) A9(H) (Fig 17) -- though this particular gel does not show proteins 28 and 72 as well, other gels have confirmed that only 25 and 1 are consistently missing.

It thus appears that all these mouse lines vary only slightly from each other and that these differences concern only proteins 1, 12, 23, 25 but are reproducible. Whether this is due to problems of extraction or is of functional importance cannot be determined here.

The patterns presented here are in close agreement with those of Rodgers (311) which show all mouse tissues and cell lines to have protein 12, a faintly staining spot. Rodgers' EA pattern was found to lack 65,66,67, and 50 unlike that in Figs 12 and 13 while the A9(L) pattern was identical to that in Figs 15 and 16. However the patterns from the normal tissues possessed a variation of protein 50 that was more basic in the second dimension and also showed the chick protein 45, not 45b and 45c as in the other mouse patterns.

3.060 Rat tissues and lines (Figs 18,19,20,21,22).

Just as the several mouse lines and tissues were almost identical except for a few proteins, so too were the patterns for the rat lines (NHAC, rat leukemia) and tissue (liver) -- the differences between the rat types being even fewer. Of the 74 spots (75 ribosomal proteins) including 4 anionic proteins, only protein 23 and/or 24 showed any variability in appearance. Rat liver frequently lacked 23 (Fig 18) while rat leukemia (Fig 19) often showed an absence of 24. However from a number of preparations, both 23 and 24 were present in all three cell types. A characteristic of adult rat liver (Fig 18) is the greatly reduced amount

+ 2-D

ORIGIN

+ 1-D

- 1-D



- 2-D

Figure 14
Two-dimensional gel pattern of L929 ribosomal proteins.

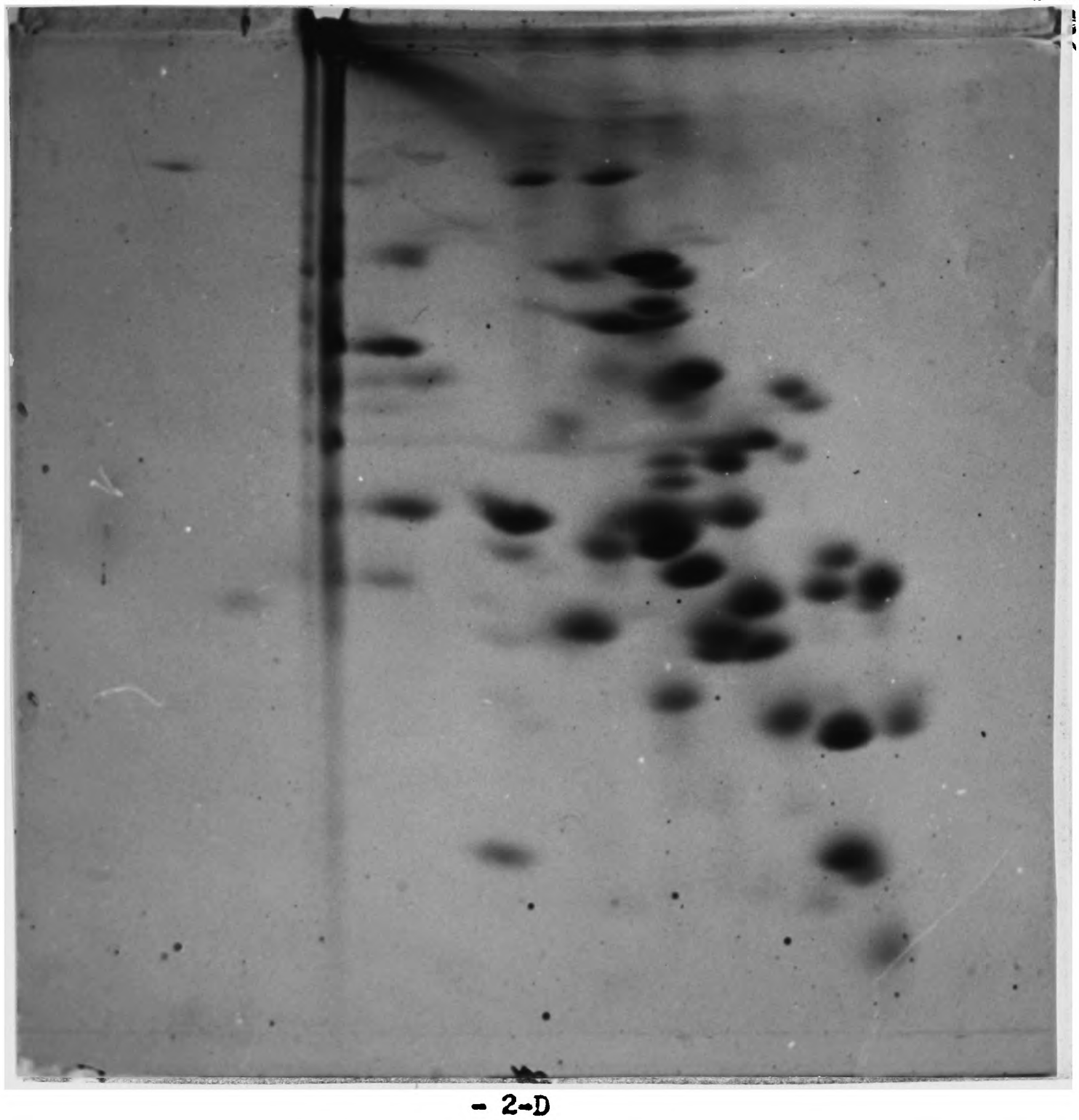
Standard method electrophoresis (2.071) of 2.6 mg ribosomal proteins. Conditions of electrophoresis were 100 V for 26 hours in the first dimension and 100 V for 45 hours in the second dimension.

+ 2-D

ORIGIN

+ 1-D

- 1-D



- 2-D

Figure 15
Two-dimensional gel pattern of A9(L) ribosomal proteins.

Standard method (2.071) electrophoresis of 3.4 mg protein for 26 hours in the first dimension and 46 hours in the second dimension.



Figure 16
Two-dimensional pattern of A9(L)
cationic ribosomal proteins.

Microgel electrophoresis of 200 ug of ribosomal protein was done under conditions identical to those in 2.072.

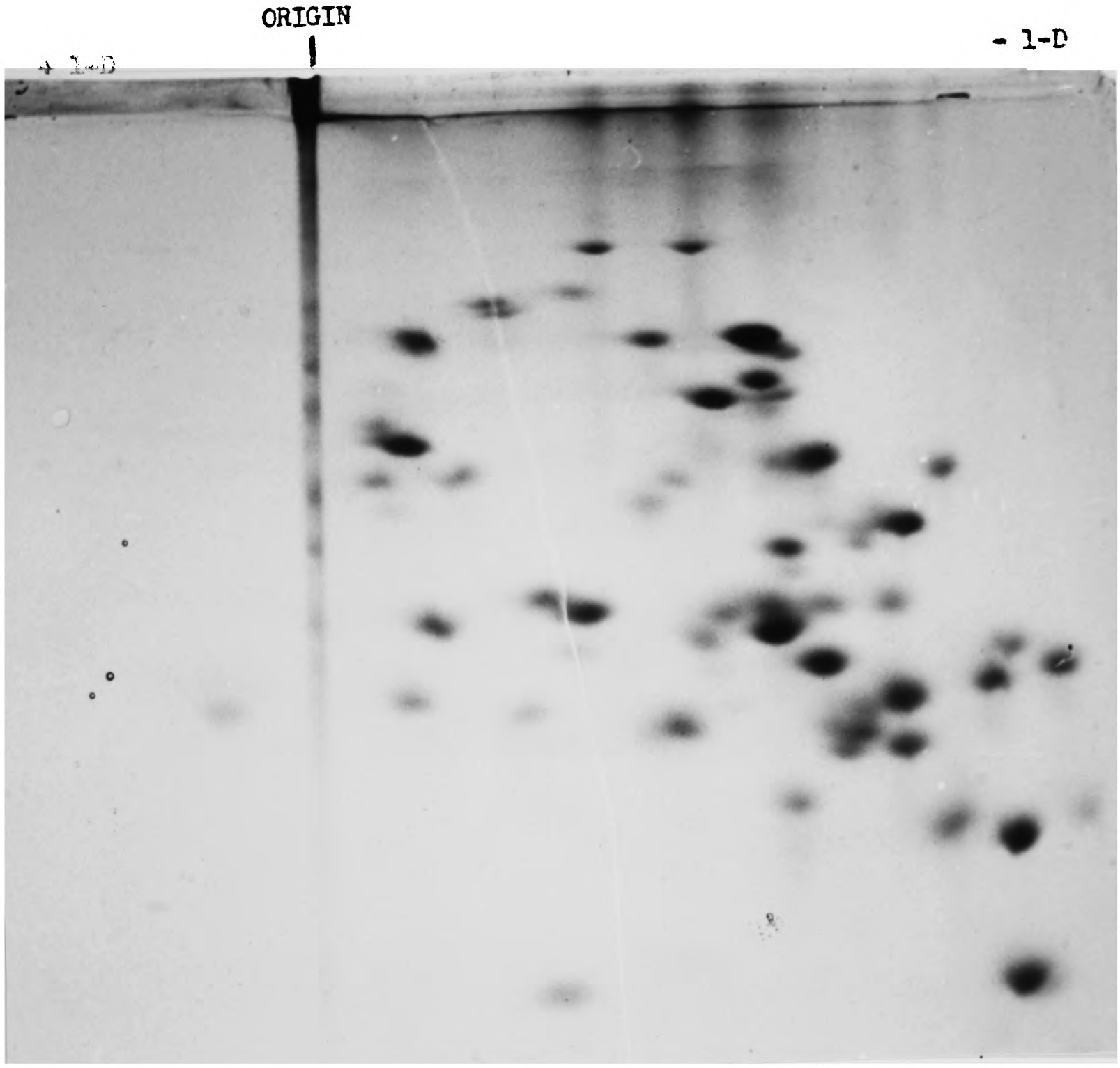


Figure 17
Two-dimensional pattern of A9(H) ribosomal proteins.

Standard method electrophoresis of 3.2 mg of protein for 24 hours at 100 V in the first dimension and 36 hours at 100 V in the second dimension.

of 53 with respect to the other proteins. In ribosomes from young rat livers, 53 is present.

Two faint ribosomal proteins were found to divide these rat preparations into two classes. Rat leukemia showed 78 but not 79 while both rat liver and NHAC had 79 but not 78. Both these proteins are present in amounts much less than the other ribosomal proteins and hence it is uncertain if these are indeed ribosomal proteins. In any event, it shows a subtle difference between ribosomes of myeloid and those of liver origin.

These ribosomal proteins are schematized in Fig 22 and allow for several comparisons to be made of the rat ribosomal protein pattern with those of chick and mouse already discussed:

a) Aside from proteins 78 and 79 which are protein species not yet encountered (and are of uncertain origin), rat ribosome patterns show two characteristic species-specific markers, 48c and 77. These are discussed more extensively in 8.031. Protein 77 runs close to where 12 is found in other species and may be a variant of it while 48c is another variation of the protein 48 where chick has 48, mouse 48b and rat 48c.
b) Unlike chick and mouse, rat patterns lack 12.
c) In every other way, the rat pattern is identical to the mouse.

The rat ribosomal protein patterns presented here are identical to those pictures published by others; any differences seem to exist in the interpretations of these gels. With the exception of Sherton and Wool (336), others have failed to look for proteins that migrate off the bottom of the 2nd dimension gel (such as 61 and 63). Most have also found no acidic proteins. The reasons for this relate to the relative staining intensities of these ribosomal proteins and are discussed in Chapter 4. Delaunay and Schapira (76) found 60 cationic protein spots identical to those here except their rat tissue must have been derived from old rats



Figure 18
Two-dimensional pattern of rat liver cationic ribosomal proteins.

Microgel electrophoresis of 300 ug of protein done exactly as described in 2.072.

+ 2-D

ORIGIN

+ 1-D



- 1-D

- 2-D

Figure 19
Two-dimensional pattern of rat leukemia cationic ribosomal proteins.

Microgel electrophoresis of 180 ug protein run exactly as described in 2.072.



Figure 20
Two-dimensional pattern of Novikoff hepatoma ribosomal proteins.

Standard method electrophoresis of 3.2 mg protein was for 26 hours at 100 V in the first dimension and 45 hours at 100 V in the second dimension.



Figure 21
Two-dimensional pattern of Novikoff hepatoma ribosomal proteins.

Microgel electrophoresis of 200 ug protein for ½ hour at 3 ma/tube and then 6 ma/tube for 4 hours in the first dimension followed by 1 hour at 40 V and then 20 hours at 100 V in the second dimension.

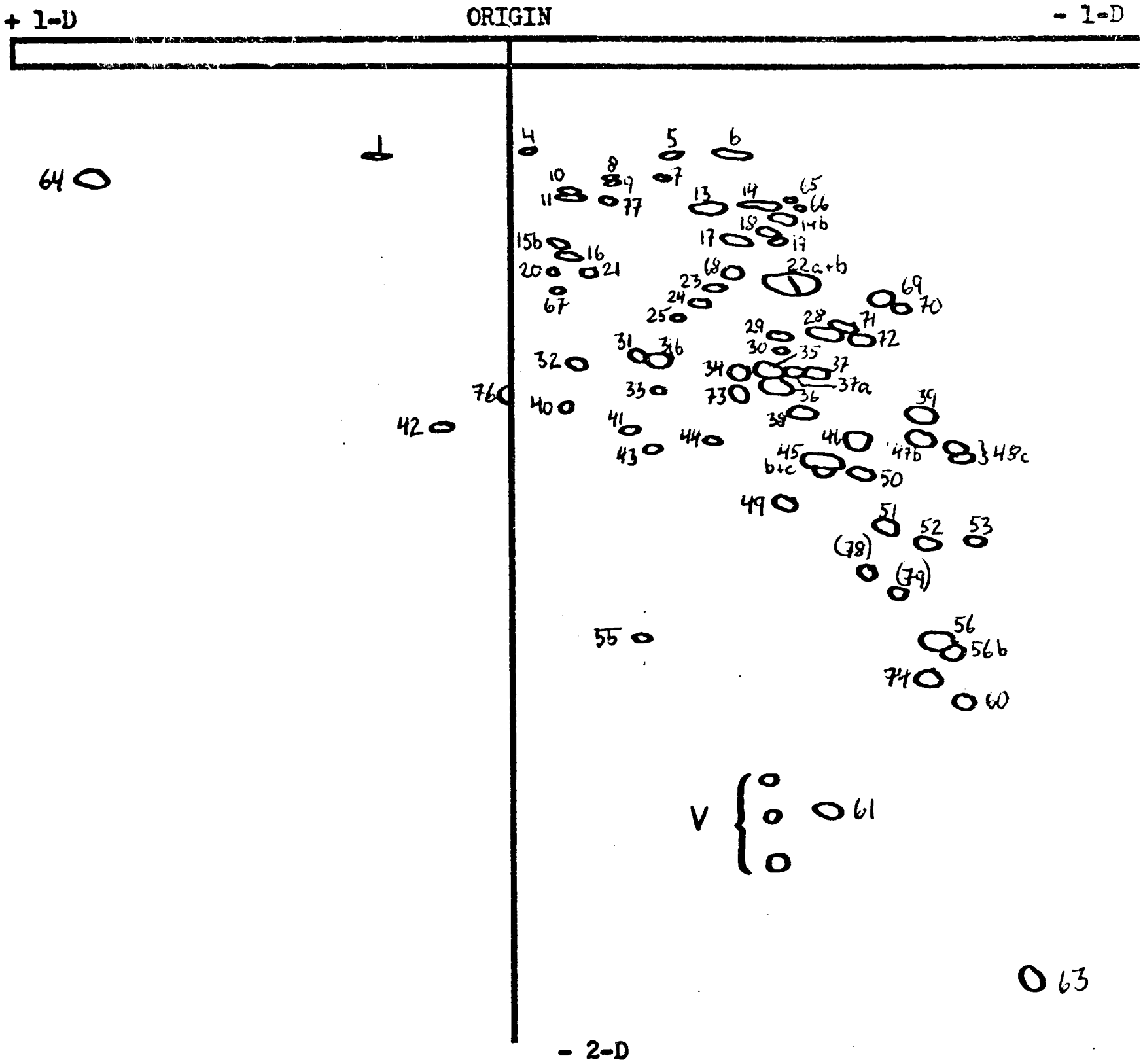


Figure 22
Schematic diagram of rat ribosomal proteins.

Drawing summarizes the ribosomal proteins of over 80 different preparations of rat liver, rat leukemia and NHAC after two-dimensinnal electrophoresis by either the standard method or microgel method. Numbers in parentheses indicate those proteins not always present in patterns. V denotes those derivative spots occasionally present.

since their protein 53 was only faintly seen, though clearly present in a rat tumor line. Those patterns of Welfle et al (404) are perfectly superimposable with those in Fig 18. In the most careful study on rat ribosomes, Sherton and Wool (335,336) present gels differing from those here in several minor ways; they suggest that each pair of spots 8 and 9, 10 and 11, 56 and 56b, and 69 and 70 represent only one ribosomal protein.

3.070 Human ribosomes -- Hela.

The Hela ribosome pattern (Figs 23,24) shows the vast majority of its proteins to be common to those species already discussed. Of the 76 spots (77 proteins) there are 8 anionic proteins, 4 of which were previously not seen. (These anions are discussed in detail in Chapter 4.) It possesses protein 12 (missing in the rat) and has protein 48 as in the chick. Though 28 and 29 move more closely together here than in other species, it has not been possible to determine if this is significant. Proteins 78 and 79, 43 and 34 are missing while 25a, 66b and 66c are added. Any relationship between these changed proteins has not been detected. Otherwise all other proteins are the same as those in the other species examined.

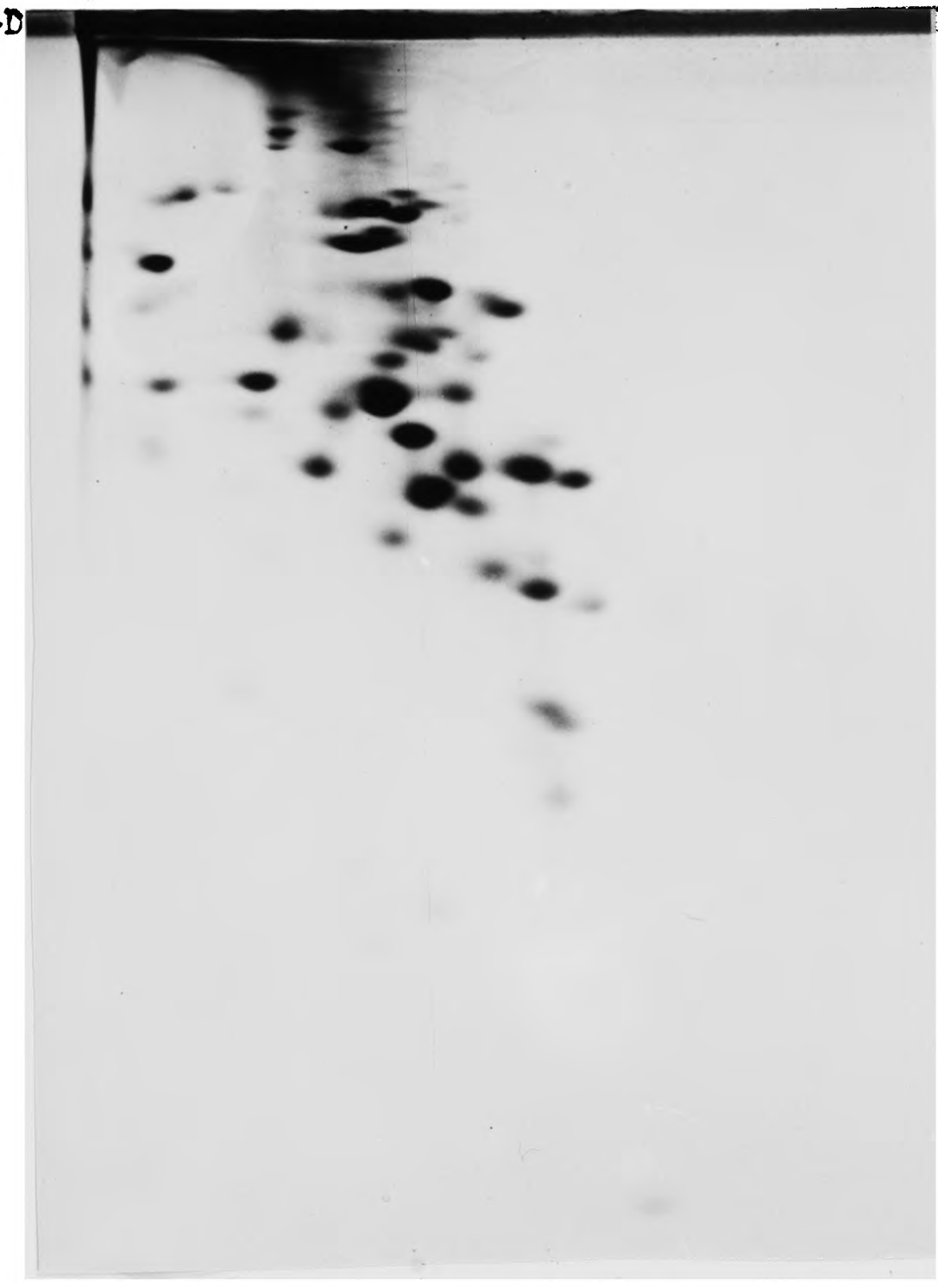
A previous report of Hela gel patterns has suggested the absence of protein 48 (199) however it is present in these experiments though easily destained and hence of variable intensity. Its absence may also be due to the use of standard method gels in this earlier report. The gel patterns presented here compare favorably with the two other published studies on human ribosomal proteins. In one, tonsillar ribosomes were electrophoresed (381) and produced a pattern identical to that in Figure 23 except

+ 2-D

ORIGIN

+ 1-D

- 1-D



- 2-D

Figure 23

Two-dimensional pattern of Hela cationic ribosomal proteins.

Microgel electrophoresis of 200 ug of protein was done as in 2.072.

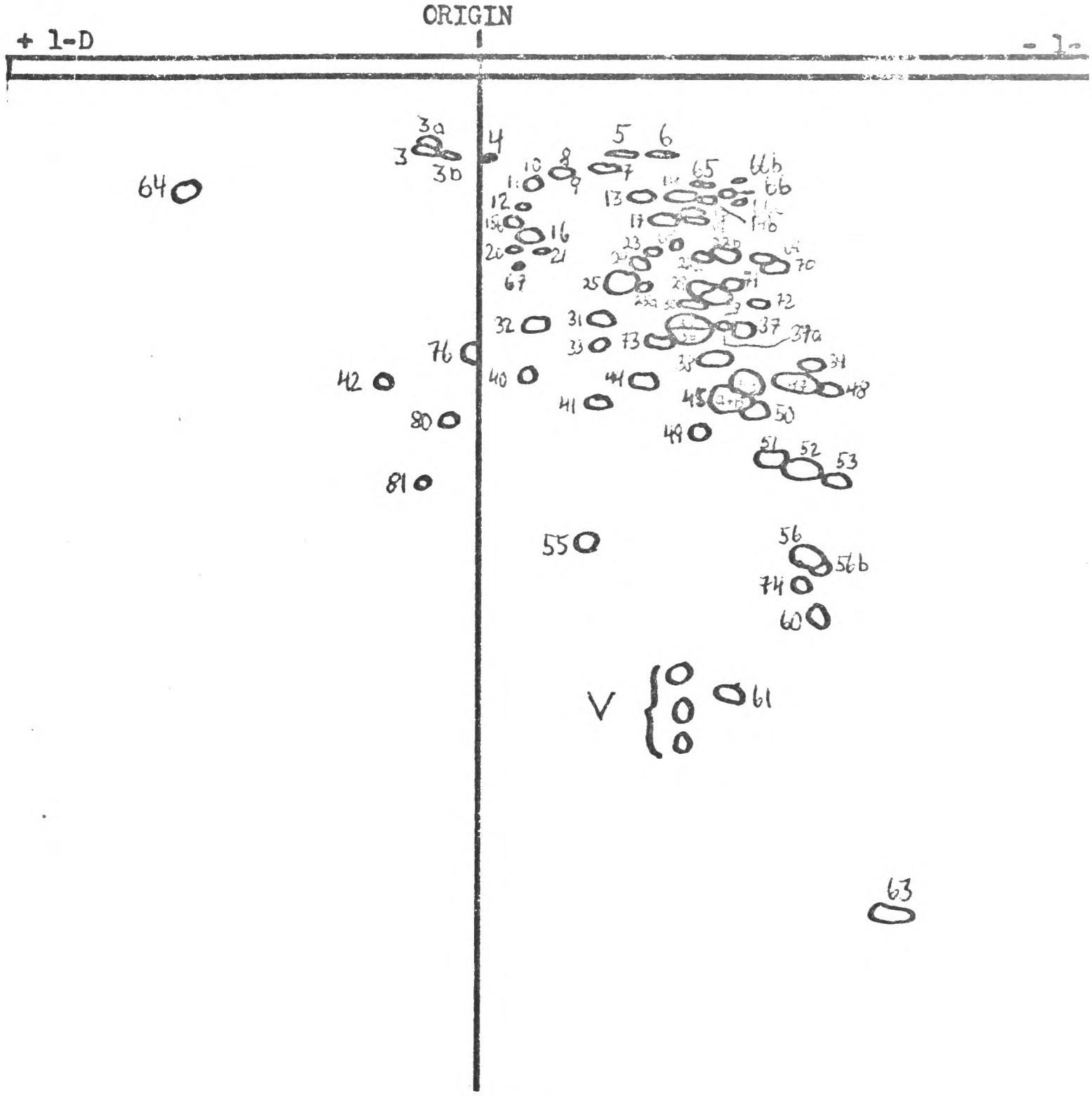


Figure 24

Schematic diagram of Hela ribosomal proteins.

Composite drawing summarizes the proteins of Hela ribosomes from over 40 different preparations. V denotes those derivative proteins often present.

several faint smudges were also present and interpreted as being ribosomal proteins. It is felt that these smudges may be artefacts due to failure to adequately wash the ribosome preparations or due to the LiCl extraction procedure. In the other study, placental ribosomes (274) were electrophoresed and 66 spots seen (including 2 anions) which were identical with the darker spots seen here. However, several of the faint ribosomal proteins such as 41 and 48 were not observed.

3.080 Antibodies to chick and mouse ribosomes.

Antisera were prepared either to intact ribosomes or solublized ribosomal protein from chick embryo and EA ribosomes (2.132). After a number of attempts, a few sera showed positive reactions with intact ribosomes upon Ouchterlony analysis. A single precipitin arc was visible for antisera prepared against both ribosomal proteins (EA) and intact ribosomes (EA and chick) when tested against intact chick (or mouse) ribosomes (Fig 25). The results were the same when tested against chick (or mouse) ribosomal proteins. The precipitin arc for antisera to EA ribosomal proteins is not well seen in Fig 25 but was faintly present in other preparations. In general, reactive antisera could be readily obtained only against intact ribosomes. Since there was no spur formation between wells 3 and 4 in Fig 25, it seems that the reaction was against the same antigen, most likely the intact ribosome. However, only one band (no spur) was also seen when reacted against ribosomal proteins.

However when tested by haemagglutination (2.134), no specific activity was detected and this suggests that the precipitin reactions observed above might have been non-specific. It was also not possible to remove cross-reacting antibodies by incubation with

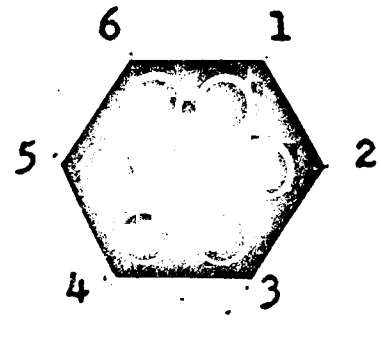


Figure 25
Double diffusion analysis of antisera to ribosomes and ribosomal proteins.

Diffusion analysis was performed as in 2.133. Center well contains 10 ul of 0.6 mg/ml chick embryo ribosomes in buffer (50 mM Tris, pH 7.4, 200 mM NH_4Cl, 150 mM KCl, 1 mM EDTA). Rabbit antisera were prepared as in 2.132 and applied (10 ul at 80 - 90 mg protein / ml) as follows:

well 1 - antisera to extracted protein of EA ribosomes;
well 2 - antisera to proteins extracted from chick embryo ribosomes;
well 3 - antisera to intact chick embryo polysomes;
well 4 - antisera to intact EA polysomes;
well 5 - sera from unimmunized rabbits;
well 6 - buffer only.

ribosomes of the other species to prepare species-specific sera. No further attempts were made to characterize these sera.

3.090 Discussion.

Table I summarizes the data from all the gel patterns compared. Ninety-seven different ribosomal protein spots have been localized on these gel patterns. Of these 97 ribosomal proteins the individual patterns contain 71-76. These numbers are very close to those of others (162,335) who find the total to be between 68 and 75. The values presented here are slightly higher due to the inclusion in the totals those doublet spots that usually are interpreted by others as being only one protein.

By comparing these ribosomal protein patterns (Table I) it is possible to identify that set of proteins common to the species examined. If just rat and mouse are compared, 70 of the proteins are held in common; if Hela is included, the common set of proteins is 65; and if chick is included too, the common set is 51. It thus appears that these different species of ribosomes show considerable conservation in their ribosomal protein composition (at least with respect to size and charge) just as their rRNAs show little variation (218). This correlates well with the evolutionary studies of Delaunay and Shapiro (72,73) who found that the more distant the species compared, the fewer common ribosomal proteins in the pattern. Hence distant species such as chick and Hela have only 55 common proteins while evolutionary less distant species (mouse and rat) show virtual identity (70 common proteins).

Far more extensive comparisons have been made of E. coli ribosomal proteins with those of other species in the family Enterobacteriaceae by 2-D gels and by immunological methods (116).

	1	2	3	3a	3b	4	5	6	7	8	9	10	11	12	13
Chick (C)	+	+	+			+	+	+	+	+	+	+	+	+	+
Mouse (M)	+*					+	+	+	+	+	+	+	+	+*	+
Rat (R)	+					+	+	+	+	+	+	+	+		+
Hela (H)			+	+	+	+	+	+	+	+	+	+	+	+	+

	14	14b	15	15a	16	17	18	19	20	21	22a	22b	23	24	25	25b	26	27	28
C	+		+		+	+	+	+	+	+	+	+	+	+	+		+	+	+
M	+	+		+	+	+	+	+	+	+	+	+	+	+	+*				+
R	+	+		+	+	+	+	+	+	+	+	+	+	+	+				+
H	+	+		+	+	+	+	+	+	+	+	+	+	+	+	+			+

	29	30	31	31b	32	33	34	35	36	37	37a	38	39	40	41	42	43	44	45
C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
M	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
R	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
H	+	+	+		+	+		+	+	+	+	+	+	+	+	+		+	

	45b	45c	46	47	47b	48	48b	48c	49	50	51	52	53	54	55	56	56b	57	58
C			+	+		+			+	+	+	+	+	+	+	+		+	+
M	+	+	+		+		+		+	+	+	+	+		+	+	+		
R	+	+	+		+			+	+	+	+	+	+		+	+	+		
H	+	+	+	+		+			+	+	+	+	+		+	+	+		

	59	60	61	62	63	64	65	66	66b	66c	67	68	69	70	71	72	73	74	75
C	+	+	+	+	+	+						+							+
M		+	+		+	+	+	+			+	+	+	+	+	+	+	+	
R		+	+		+	+	+	+			+	+	+	+	+	+	+	+	
H		+	+		+	+	+	+	+	+	+	+	+	+	+	+	+	+	

	76	77	78	79	80	81	Total protein spots	Total ribosomal proteins**
C	+						71	73
M	+						72	73
R	+	+	+*	+*			74	75
H	+				+	+	76	77

* Only some members of this species contain this ribosomal protein.

** Calculated by taking into account those spots which are composed of two ribosomal proteins.

TABLE I

Summary of ribosomal proteins present in different species.

These investigations gave several results (418):

a) Some ribosomal proteins are more conserved than others and this reflects the evolutionary pressure on different protein structures.
b) All Enterobacteriaceae ribosomes have S7 similar to that of E. coli B and distinct from K strains.
c) Ribosomal proteins from species belonging to different genera (E. coli vs. Shigella dispar) can be more similar than those of the same genus (E. coli vs. E. freundii).

Comparisons of ribosomal proteins from the family Bacillaceae (115) show much less resemblance among ribosomes from its different species than was seen for Enterobacteriaceae. But B. stearothermophilus and E. coli are very close (418) and the ribosomal proteins at the sequence level are 30-70% identical, suggestive of strong conservation of 1° structure.

Nevertheless, conservation of size and charge, as identified by identical 2-D gel electrophoresis patterns is no guarantee of sequence homology (256). Indeed the regions (spots 36 and 45) where proteins of both subunits run together (Chapter 6) suggests this. Similarly, ribosomal proteins running separately from each other may be of virtually identical sequence, such as L7/L12 in bacteria which differ only by an acetylated serine. These possibilities must always be held in mind. Thus the fact that two ribosomal protein populations show identical 2-D gel patterns only implies conservation of size and charge -- it says nothing about sequence conservation. Considering the complexity of the ionic interactions between RNA and protein and between the proteins themselves that build up the ribosome, there would appear to be great selective advantage toward the conservation of size and charge. But some amino acid substitutions that preserve the size and charge may be evolutionarily permissible. Only those alterations of size and/or charge compatible with normal ribosome function would be

detected by 2-D gels.

Indeed very recent experiments suggest the existence of this microheterogeneity. When antibodies to ribosomal proteins are used to assay for species differences, none of the ribosomal proteins of the two species cross-react. Theise et al (370) have found that the 60S subunits of rat, ox, hen and trout ribosomes all show virtually identical 2-D patterns but antisera to rat liver 60S (258) does not cross-react with that of the other species. These experiments are similar to earlier work by Wikman-Coffelt et al (48,49,414) in which antisera to NHAC 60S subunits showed two different ribosomal proteins present not found in the rat liver 60S. Though 2-D gels were not done in these experiments, the results presented in this chapter show no such differences observable with the 2-D gel patterns and suggest differences not resolvable by this method.

However, these studies need to be interpreted with caution. In none of these experiments was the specificity of the antisera ever rigorously defined. The antisera may be only reactive against one or a few ribosomal proteins in which case the success or failure of cross-reaction is testing only a small proportion of the total proteins. More importantly, the antigen-antibody reactions themselves seem at doubt especially in the case of Theise et al (258,370) for here the antisera to the intact ribosomes does not react with the isolated ribosomal proteins of those same ribosomes. Unless entirely different and exclusive antigen populations are being exposed in these experiments, one must question what reaction is going on.

In the case of the Wikman-Coffelt et al (414) results, the serum was said to precipitate only two ribosomal proteins of the

NHAC large subunit -- however when run on SDS gels numerous other bands were also seen,suggesting a less specific antisera. Indeed, it is known that antisera bind whole ribosomes non-specifically via their Fc regions (77,157,244). Perhaps those two ribosomal proteins that were thought to comprise the majority of the immune precipitate are either adhering to the IgG or are proteins containing the Fc receptors.(which if polyvalent would also cause an immune precipitate). Finally, it is important to show that antisera are not reacting with the rRNA -- to date the only evidence to the contrary consists of showing that purified rRNA added to the antisera gives no immune precipitate. But given the levels of RNase in sera such would hardly be likely. The failure of attempts here (3.080) to make species-specific sera against ribosomes might be due either to the fact that the antigen was the RNA or that the immune precipitate was a non-specific one.

These criticisms are not intended to belittle the attempts of these workers but to suggest the pitfalls of such experiments. Given the demonstrated conservation of size and charge of ribosomal proteins it is important to know if sequence heterogeneity still does exist. Next to the isolation and sequencing of ribosomal proteins, antisera are a convenient tool for determining this (355). If such heterogeneity exists within any one class of proteins (as defined by size and charge), one might envision their participation in altering the translational properties of the ribosome. Such arguments, however, must remain hypothetical. Until such microheterogeneity has been proven, there is no reason not to assume that the conservation in size and charge does imply sequence conservation as well.

During the course of these experiments, numerous applications of the 2-D gel method to the eukaryotic ribosome have appeared and all have noted the great conservation of proteins from species to species and tissue to tissue. However, little has been said about specific differences between species. Delaunay et al (72) have so sought to stress this resemblance that they have ignored several differences revealed in their own earlier work (75). Since the experiments in this chapter sought to find ribosomal protein markers for cell fusion experiments, attention was focused on such. In brief, it was found that:

a) Few ribosomal proteins were unique to a species though usually 1 or 2 species-specific markers were found.
b) Such markers were found in only certain areas of the gel pattern.
c) Some proteins formed a set of proteins that were not unique to any one species but also did not appear in all species.

Species-specific marker ribosomal proteins (Table I) for the chick are 2,15,26,27,45,54,57,58,59,62 and 75; for mouse: only 48b; for rat: 48c, 77,78,79; for Hela: 25b, 80,66b,66c; for Syrian hamster: 48d [53a in (165)] ; for Chinese hamster: none, though it lacks entirely protein 48 (199). Many of these appear to be only slightly different in mobility from ribosomal proteins found in other species (such as 15 and 15a). But others (such as chick 45,54,58; mouse 48b; rat 48c,77; and Syrain hamster 48d) move sufficiently differently from other proteins to be detected readily upon coelectrophoresis and hence be of use in cell fusion experiments (Chapter 8).

What is of interest is that these differences appear to be clustered in certain areas. Certain ribosomal proteins in the gel pattern are consistently more variable than others, reminiscent

of "hot spots" in phage systems (12). The ribosomal proteins designated 48,48b,48c,48d form such a set. Whether they indeed are sequence variants is not known but they all cluster about the same area and are virtually unchanged in the rest of their pattern. Other areas, such as proteins 31,32,40,41,44 or 36,38,46 never show any alterations. Other areas showing increased variability are those formed by proteins 26,27,75 or 71,72,69,70 or 45,45b,45c. The acidic ribosomal proteins show no such localized areas of increased variability (Chapter 4).

Such areas of increased variability may reflect regions of the ribosome where size and charge are not so essential to overall structure or function and hence not so strongly conserved. Alternatively, they may be due to post-translational modifications; in interpreting these variations it is important to remember that altered mobilities on 2-D gels may correspond to modification of existing ribosomal proteins and not necessarily a new polypeptide species. The example of E. coli L7/L12 where L7 varies from L12 in being acetylated has already been mentioned. Similar modifications may occur on mammalian ribosomes. Such potential modifications would include: acetylation, methylation, acylation by amino acids, glycosylation, and phosphorylation.

Aside from the acetylation of L7/L12, ribosomal protein S1 in E. coli changes its function upon acetylation (227). Lieu and Gornall (214) have found some acetylated ribosomal proteins in rat liver incubated with radioactive acetate but these workers failed to demonstrate that the labelled proteins were indeed ribosomal. Ribosomal proteins of rat liver have also been acetylated in vitro by an acetyl transferase described by Pestana and Pitot (289) but such does not occur in vivo. If acetylation

does occur *in vivo* it might be expected to cause mobility changes.

Numerous nuclear proteins are affected by methylation and ethylation (123) but there is to date no evidence for such in ribosomes.

Soffer (342,343,344,345) has isolated a group of amino acid-tRNA transferases that catalyze the transfer of amino acids to protein, including *E. coli* ribosomes (208). Other work also shows it to be effective in eukaryotes as well. But all these systems are descriptive of *in vitro* modification -- no experiments have shown such to be the case *in vivo*.

As of yet no documentation of glycosylation of ribosomal proteins has been presented. It is suggested here simply because some of the ribosomal proteins observed in these 2-D gels react with the stain by altering the stain color (from blue to pink) and retain it poorly. Brief neuraminidase treatment has also been found to alter ribosomal protein mobilities though this was not investigated further. If these observations are confirmed, such may account for the varied 2-D gel mobility.

Only the phosphorylation of ribosomal proteins has received much attention do far. Numerous experiments have shown that a number of different protein kinases can phosphorylate ribosomal proteins *in vitro* (74,93,234,349). Seven to 14 proteins were found to be so phosphorylated however such phosphorylated ribosomes do not appear to be functionally altered with respect to their *in vitro* translational capacity when measured with EMC virus or poly(U)(64). But these tests are not sufficiently rigorous to detect a variation with respect to real messages. Furthermore, these experiments are misleading in that they were done *in vitro*, any physiologically important phosphorylation having occured previously.

Only a few experiments have observed the relevant *in vivo* phosphorylation (5,127,268,296,307,348,349) of defined ribosomal proteins. These reports have all found considerably fewer ribosomal proteins phosphorylated *in vivo* than *in vitro*. In the most definitive of these experiments, Gressner and Wool (127) found only one protein phosphorylated in the rat liver, a small subunit ribosomal protein, S6 (17 in the nomenclature of this chapter). They have shown that when phosphorylated to different degrees, it has different mobilities. In normal liver, *in vivo* phosphorylation affects mainly S6 and produces a small "tail" on autoradiograms but not on stained gels. However in autoradiograms of regenerating liver, the tail labelled most and S6 was little labelled; stained gels also showed this tail. This suggests that the one ribosomal protein that is phosphorylated is phosphorylated to different extents in growing cells and shows this altered mobility as a tail. In the experiments reported in this chapter, protein 17 frequently showed such a tail, especially in preparations from rapidly growing cells (Fig 16).

Whether any of these modifications are responsible for the characteristic clustering of ribosomal protein variants or the species-specific ribosomal proteins has yet to be determined. But the fact that marker proteins of different species both appear in interspecific hybrid cells (8.031) argues against attributing these differences to modifying enzymes.

The experiments in this chapter have suggested that there is no difference by these methods between the ribosomal protein patterns of malignant and non-malignant lines. Two such sets of comparisons have been made. The first is between L929, A9HT and A9(L). According to Harris (412) A9HT is highly malignant [having been selected as

a malignant derivative of A9(L)] while A9(L) is of low malignancy when both are tested in compatible C3H mice. L929 is a highly malignant (267) derivative of L which itself is a 20-methyl cholanthrene-induced mouse line. But there is only one difference between the ribosomes of the high malignancy (A9HT, L929) lines and those of low malignancy [A9(L)] . The highly malignant lines have ribosomal protein 25 but lack 12; the non-malignant line lacks 25 but has 12. However this loss of protein 12 does not seem to be a significant difference with respect to malignancy because another malignant line, EA, only lacks 12 some of the time (3.050). Since normal mouse liver lacks 25 some times (311), this rules out 25 as relating to malignancy.

But the A9HT/A9 system is not an ideal comparison for both were derived from the malignant parent, L. They may only vary in the degree of malignancy. A better comparison is that between rat liver taken from a young rat and a rat hepatoma (NHAC). A young liver is necessary to insure a population of growing cells; adult liver is in a stationary growth phase and the ribosomal protein pattern from it differs from those of growing cells in that spot 53 is only shown faintly. Comparisons of the NHAC and rat liver patterns (3.060) show them to be identical. This again suggests that there is no ribosomal protein difference between malignant and non-malignant cells. Bielka has also observed this to be true in other comparisons of sarcomas and hepatocarcinomas to rat liver ribosomes (17). One exception to this is, as discussed above, the existence of any heterogeneity that is not picked up by these 2-D gels. Whether the presence of any anionic ribosomal proteins correlates with malignancy is discussed in Chapter 4.

The ribosome preparations used in these experiments have

included both free and bound polysomes. No attempt has been made here to distinguish between the two. Whether there is any such difference is yet unclear (314). In several bacterial systems [Azotobacter vinelandii (324) and Streptococcus fecalis (45)] a protein was found to be associated with ribosomes derived from the membrane fraction. In both cases, the protein was removed from intact ribosomes by 1 M NH_4Cl. However, in Bacillus licheniformis preparations (41) any such differences have been found to be due to adventitiously adhering cytoplasmic proteins. In eukaryotic systems a difference of one ribosomal protein has been found between free and bound ribosomes in some cases [chick embryo (109), rat liver (296)] while others (136,137,314) have not found any such differences for rat liver. This conflict in results seems to be due to whether both free and bound ribosomes were treated with deoxycholate, for free ribosomes have a protein that is removed by deoxycholate. Though a number of differences between free and bound ribosomes were suggested by Fehlmann et al (103), these several spots were also seen infrequently in some of the gels described in this chapter but have not been reproducible. It is thus felt that few if any differences are demonstrable between free and bound ribosomes when purified by these techniques.

The ribosomal protein patterns presented here are those of cells dividing at different rates from tissue culture, embryos or young liver. No difference in ribosomal protein composition has been found with respect to the rate of growth -- the pattern of adult, non-dividing rat liver ribosomes is identical with that of the rapidly growing NHAC with the possible exception of a decrease in the amount of protein 53. Thus the control of the amount of translational activity seems to lie with factors outside the ribosome

(as defined here). Likewise the great conservation of ribosomal proteins in different species and organs argues against much specificity in the ribosomal proteins in regulating translation.

3.100 Summary.

Eukaryotic ribosomal proteins from a variety of different species have been compared by 2-D gel electrophoresis. Seventy-one to 76 proteins (4-8 anions and the rest cations) were found associated with each ribosome. Though two-thirds of the proteins were common to all the different species, most species possessed one or two species-specific marker ribosomal proteins. In general, the more evolutionary distant the species, the fewer proteins held in common.

The species-specific markers in a number of cases were associated with only certain areas of the gel pattern. It was suggested that these areas might be those proteins less rigorously conserved and hence these species variants were sequence variations of the same primitive ribosomal protein.

The few variations seen in these gels could not be correlated with differences in growth rate, state of differentiation or malignancy.

CHAPTER 4

COMPARISONS OF RIBOSOMAL PROTEIN PATTERNS BETWEEN SPECIES -- ANIONS

4.000 Introduction.

One major difference between the ribosomes of eukaryotes and prokaryotes is the lack of ribosomal protein spots on the anionic side of 2-D gels. In E. coli B, four small subunit proteins (S1,S2,S6,S10) and seven large subunit proteins (L4,L7,L8,L9,L10, L12,L21) are anions at pH 8.6 (178) while few such ribosomal proteins have been demonstrated for eukaryotes. Early experiments with 2-D gels by Huynh-van-tan et al (165) and Welfle et al (403) reported no anionic proteins in rat liver. However, later reports suggested the presence of a few anions. Welfle et al (404) always found 2 faintly staining spots in gels of the 80S ribosome but only infrequently in gels of the 60S subunit. Neither was phosphorylated in vitro (349). Though their existence was ignored, the gel patterns of the rabbit caecum, adult liver, and foetal liver 80S ribosomes by Delaunay et al (75) also showed three anions. Finally, in detailed studies of the rat liver ribosome, Sherton and Wool (335) located one acidic protein on the 40S subunit and two on the 60S. These latter two proteins stained well in 80S patterns but poorly in the 60S subunit patterns.

All of these reports showed different mobilities for these anions but all were unanimous in calling attention to their low intensity of staining with respect to the cationic ribosomal proteins. In many cases (335) they only appeared when gels were overloaded with protein. Whether this was due to their inability to take up stain due to their amino acid composition, or modifications, or due to the low amounts of protein present was not known. However

three factors weighed against interpreting these spots as simply being non-specific cytoplasmic contamination.

The first was that in E. coli a number of the acidic proteins seemed to serve regulatory and not just structural roles. Protein S1 had been found to be associated with certain functional states of the ribosome and not with others (128,129,387), and seemed to exert some control over translation. Proteins L7/L12 had been associated with GTPase activity (40,198,295,368) and possibly involved in translocation (369). L7/L12 were present in three copies per ribosome and their ratio varied with the physiological state of the cell. Since L7/L12 often stained poorly (336,356) or were extracted less well (180), this suggested a possible significance for these faint eukaryotic ribosomal proteins as well as potential functions.

More importantly, early experiments of Rodgers (311) suggested some functional aspects to the presence (and absence) of these anionic ribosomal proteins. When the gel patterns of a number of slowly proliferating mouse tissues (brain, kidney, liver and intestine) were compared with those of rapidly proliferating mouse lines (EA, A9, B82), only one major difference was detected -- the rapidly growing tissues contained an acidic ribosomal protein (MW = 58,000) not found in the stable tissues. Both the growing and stable tissues also possessed three other faint anionic proteins in common.

Lastly, the initial experiments described in Chapter 3 showed very consistent though faint anionic ribosomal protein patterns with the standard 2-D gel method. In addition, early experiments also suggested one of the anions was the 5S rRNA binding protein (see Chapter 5). When the microgel system was begun, these anionic

proteins became more distinct and intense. Evidently the polymerization of the sample gel in the standard method may have been partly responsible for their indistinct appearance.

With these insights into the existence and relevance of these anionic proteins, experiments were begun to analyze them in greater depth. This line of experimentation was also prompted by the original inability of different workers to show consistent 2-D gel patterns which suggested some potential species specificity in these anions. Though the results of these experiments have not elucidated the function of these proteins, the gel patterns of these anions have been just as extensively investigated as the cations and warrent separate mention here.

4.010 Interpretation of gels.

The data presented here for the anionic ribosomal proteins has been accumulated from both standard and microgels. In both cases, gels of different duration and loadings were used. A particular problem with the standard method was that a fast running spot, 64, usually electrophoresed off the end of the first dimension gel. This spot was first seen by Rodgers (311) and later by Sherton and Wool (336). It was preserved using the microgels. No other spots aside from those presented below have been discovered when the duration of electrophoresis was altered, nor do any of these proteins move as anions in the second dimension. Washing these ribosomes with 500 mM KCl (2.040) did not alter the anion pattern.

It has previously been demonstrated that not all ribosomal proteins were extracted from ribosomes by the acetic acid technique (3.020), a few protein bands appearing upon SDS gel electrophoresis of unextracted ribosomes that were not seen when acetic acid extracted

ribosomal proteins were electrophoresed. As shown in Fig 26, at least one of these proteins lost upon extraction is an acidic protein. These gels are identical to those of the first dimension in the microgels but contain double the amount of acrylamides, thus allowing greater resolution. Acetic acid extracted rat liver ribosomes were electrophoresed on gel (a) while intact rat liver ribosomes were run on gel (b). The only discernible difference was that the unextracted preparation shows a band migrating more rapidly than any other band on the gel of the extracted proteins. Any further identification of this band has not been attempted except to show that it labels with ^{14}C amino acids and is thus most likely a protein.

4.020 Standard method anion patterns.

In Chapter 3 the several anionic proteins found with the standard 2-D gel method were mentioned. It is the intent of this section to summarize and compare this data.

All the ribosomal protein patterns (Figs 8,9,22,24) have several anionic ribosomal proteins in common, 64,76 and 42. Protein 76 usually does not move far into the gel in the first dimension as has also been observed by Sherton and Wool (336), whose patterns are close to these. The rest of the anion pattern for the several species and cell lines examined shows characteristic variations, all of which seem to occupy the same relative area. In chick (Figs 6,8) spots 1,2,3 are in this area while in the mouse (Fig 9) and rat (Fig 22) only one ribosomal protein (and several satellites only infrequently found) are seen. Hela possesses another set of variations in this area, 3,3a, and 3b. All of these spots, 1,2,3,3a,3b have been shown to be distinct by coelectrophoresis. Whether they indicate different ribosomal

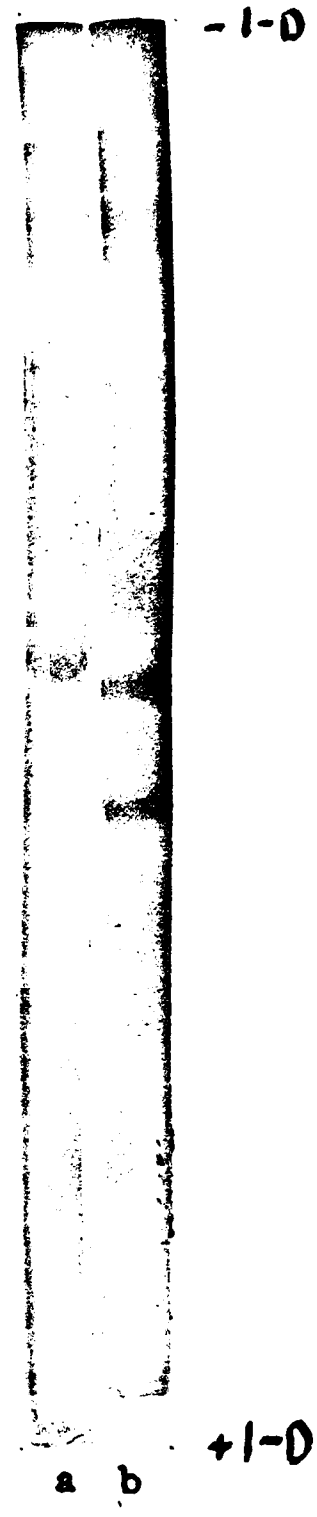


Figure 26
One-dimensional electrophoresis of anionic proteins of extracted and unextracted ribosomes.

Gels were prepared and run exactly as for the first dimension of the microgels (2.072) except that the gel was prepared with twice the amount of both acrylamides. Samples were (a) 280 ug acetic acid extracted rat liver ribosomal proteins, and (b) an equal amount (7.8 ODU_{260}) of unextracted rat liver polysomes.

proteins is not certain. But not all of these spots were seen on the microgels and hence the accuracy of this data was questionable. So, all comparisons were repeated with microgels.

4.030 Microgel analysis of anionic ribosomal proteins.

These gels allowed a more detailed analysis of the anions and were used to compare Hela, rat liver, rat leukemia and NHAC ribosomes. Since mouse anions had already been extensively investigated (311) they were not included here.

In general, all these gels show a uniform pattern with only a few individual variations. All have proteins 76,80,81,42 and 64 (see Fig 31 for numbering). Proteins 42,81 and 80 are most clearly seen in Figs 29 (rat) and 30 (Hela) where the gels have only been run for 1/3 the time in the second dimension. Not all these spots appear to be present in the same intensities -- the intensity seems to depend on the growth rate; slowly growing tissue (rat liver) showing greater levels of 76,80, and 42 than the faster growers.

However Hela, NHAC and rat leukemia also possess a set of high molecular weight proteins 3,3a and 3b (Figs 28,30) which are only faintly present in the adult rat liver (Figs 27,29). Though only the pattern for Hela ribosomal proteins is presented here, those for NHAC and rat leukemia were identical to it. Thus the difference here is a quantitative one; the more rapidly growing tissues possess these high molecular weight proteins while the unproliferating tissue does not.

4.040 One-dimensional comparisons.

A more convenient way to demonstrate this quantitative difference between the rapidly growing cells and the stable

+ 2-D

ORIGIN

- 1-D



+ 1-D

- 2-D

Figure 27
Two-dimensional pattern of rat liver anionic ribosomal proteins.

Microgel electrophoresis of 408 ug of protein was carried out exactly as in 2.072.

+ 2-D

ORIGIN

- 1-D



+ 2-D

- 2-D

Figure 28
Two-dimensional pattern of Hela anionic ribosomal proteins.

Microgel electrophoresis of 200 ug of ribosomal protein was carried out exactly as in 2.072.

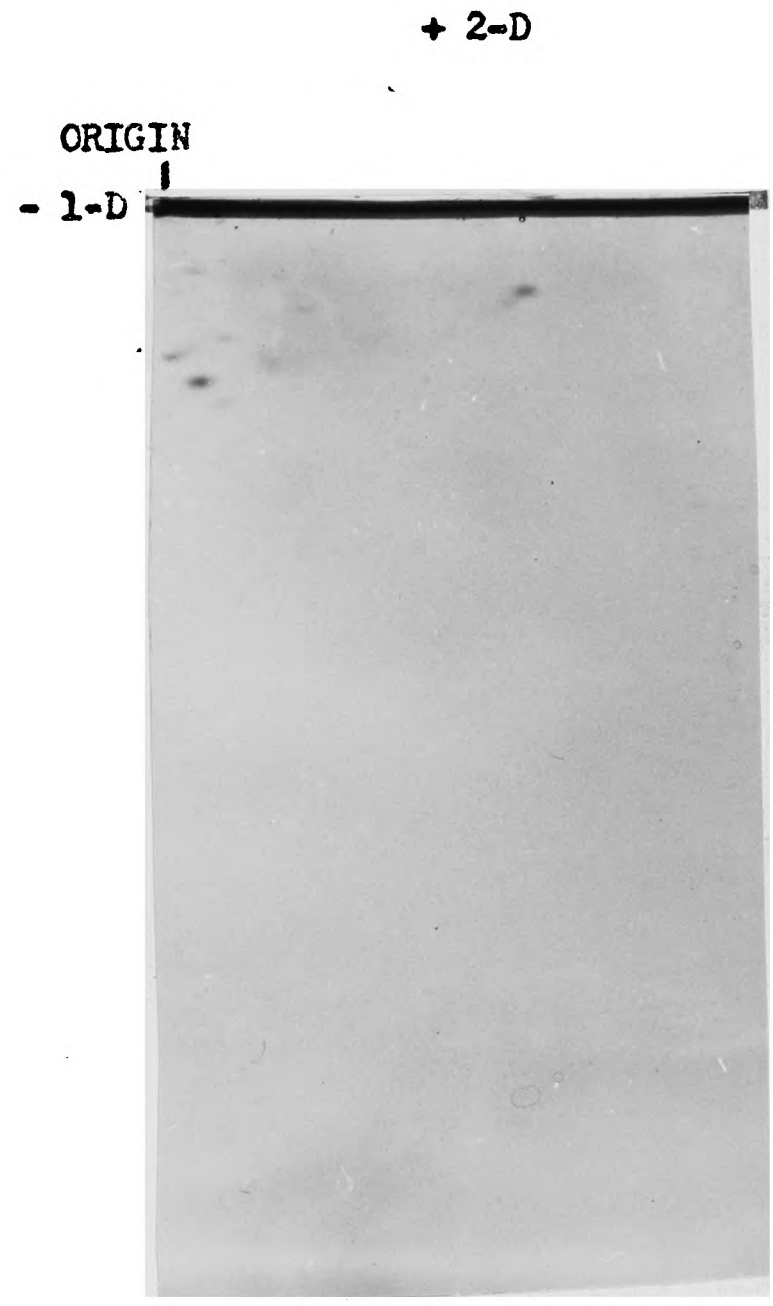


Figure 29
Two-dimensional pattern of rat liver anionic ribosomal proteins.

Microgel electrophoresis of 408 ug of protein run under conditions identical with those in 2.072 but run for only 1 hour at 40 V followed by 10 hours at 100 V in the second dimension.

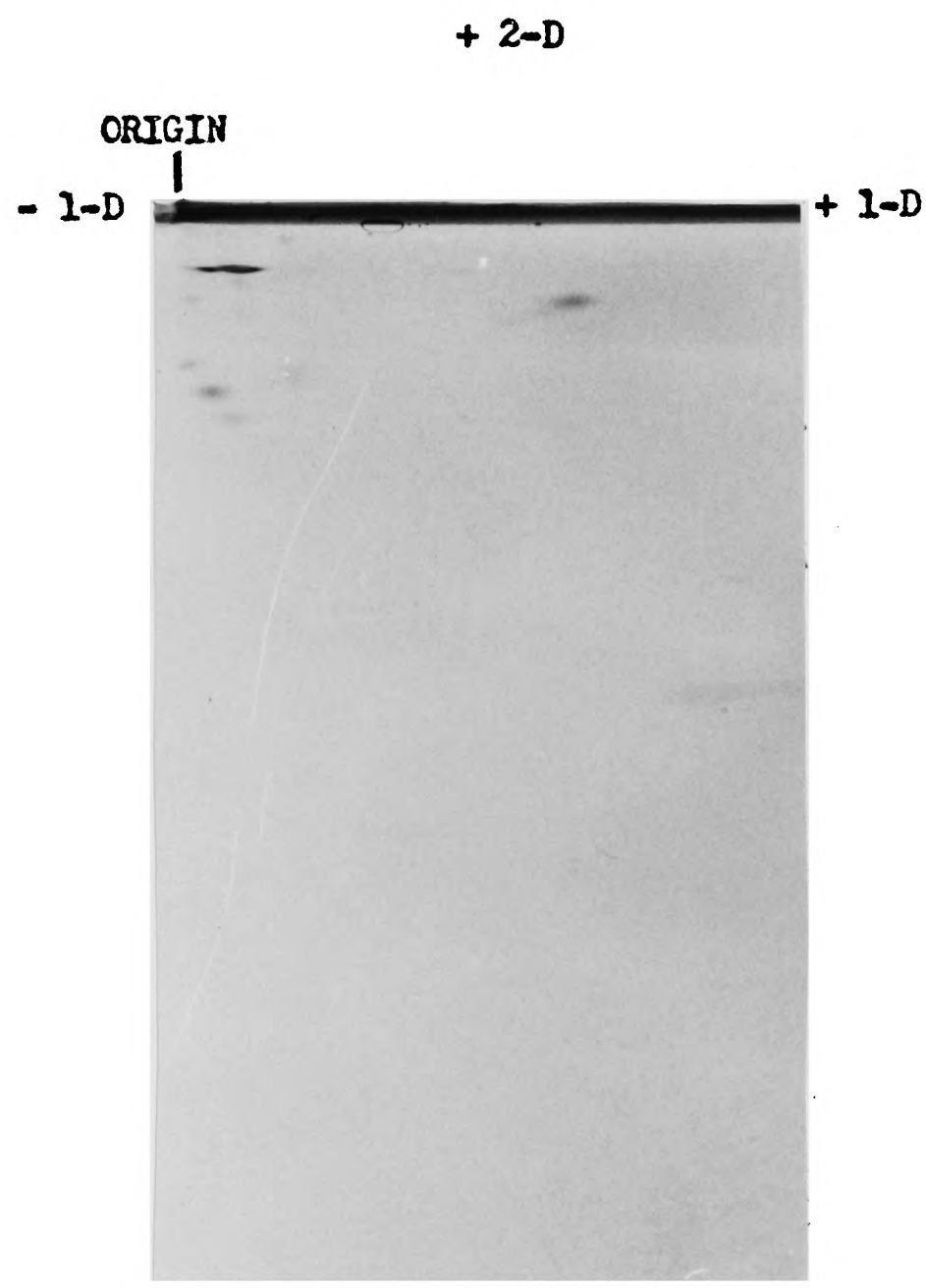


Figure 30
Two-dimensional pattern of Hela anionic ribosomal proteins.

Microgel electrophoresis of 300 ug of protein run under conditions identical to those in 2.072 but for only 1 hour at 40 V followed by 10 hours at 100 V in the second dimension.

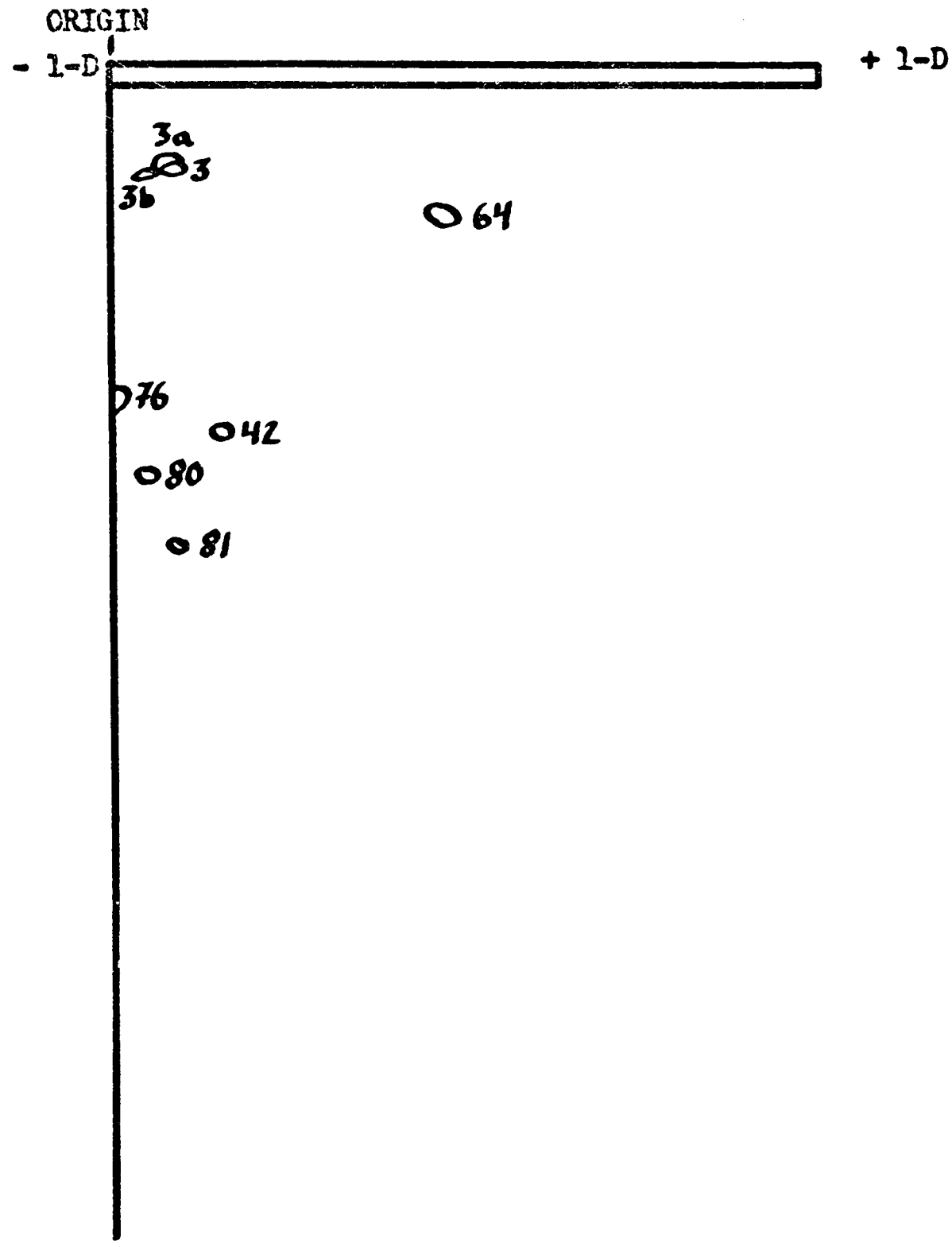


Figure 31
Schematic diagram of anionic ribosomal proteins.

Composite drawing of anionic proteins from rat, Hela and mouse ribosomes on a scale equal to that when run on microgels under standard conditions (2.072).

tissues was to run the anionic ribosomal proteins only on the first dimension gels (2.072) and then stain. Four major regions are easily distinguished (Fig 32) and may be partly correlated with the spots on the 2-D gels. The movement of band d is variable on all these gels and correlates with protein 64 on the 2-D gels. The reason for its diffuse staining in some of the gels is not known but it is interesting to note that the band moves to different lengths down the 1-D gel. Upon careful measurements of the 2-D gels, protein 64 also was found to vary in its mobility in the first dimension.

Band c does not vary between the different species and tissues and corresponds to protein 42 in the second dimension. Bands a and b seem to vary with the growth rate just as does the set of spots 3,3a and 3b. Rapidly dividing tissues (Hela, rat leukemia and NHAC) show an intensely staining band b (Fig 32) while non-dividing tissue shows only a faint band. But the rat liver shows a strong band a while the others show a weak band a. Hence the presence of an intensely staining band a correlates with greatly reduced amounts of ribosomal proteins 3,3a and 3b but increased amounts of 76,80 and 42; an intensely staining band b correlates with the reverse.

4.050 Discussion.

These experiments have accurately mapped the anionic proteins in the eukaryotic ribosome and found them to compose a set of spots 42,76,80,81,64,3,3a,3b. Among the species examined here there were no unique anionic ribosomal proteins such as those variants found for the cations. Earlier standard method 2-D gels suggested some species-specific anionic ribosomal proteins (Chapter 3) but the microgels have not upheld these conclusions -- all species

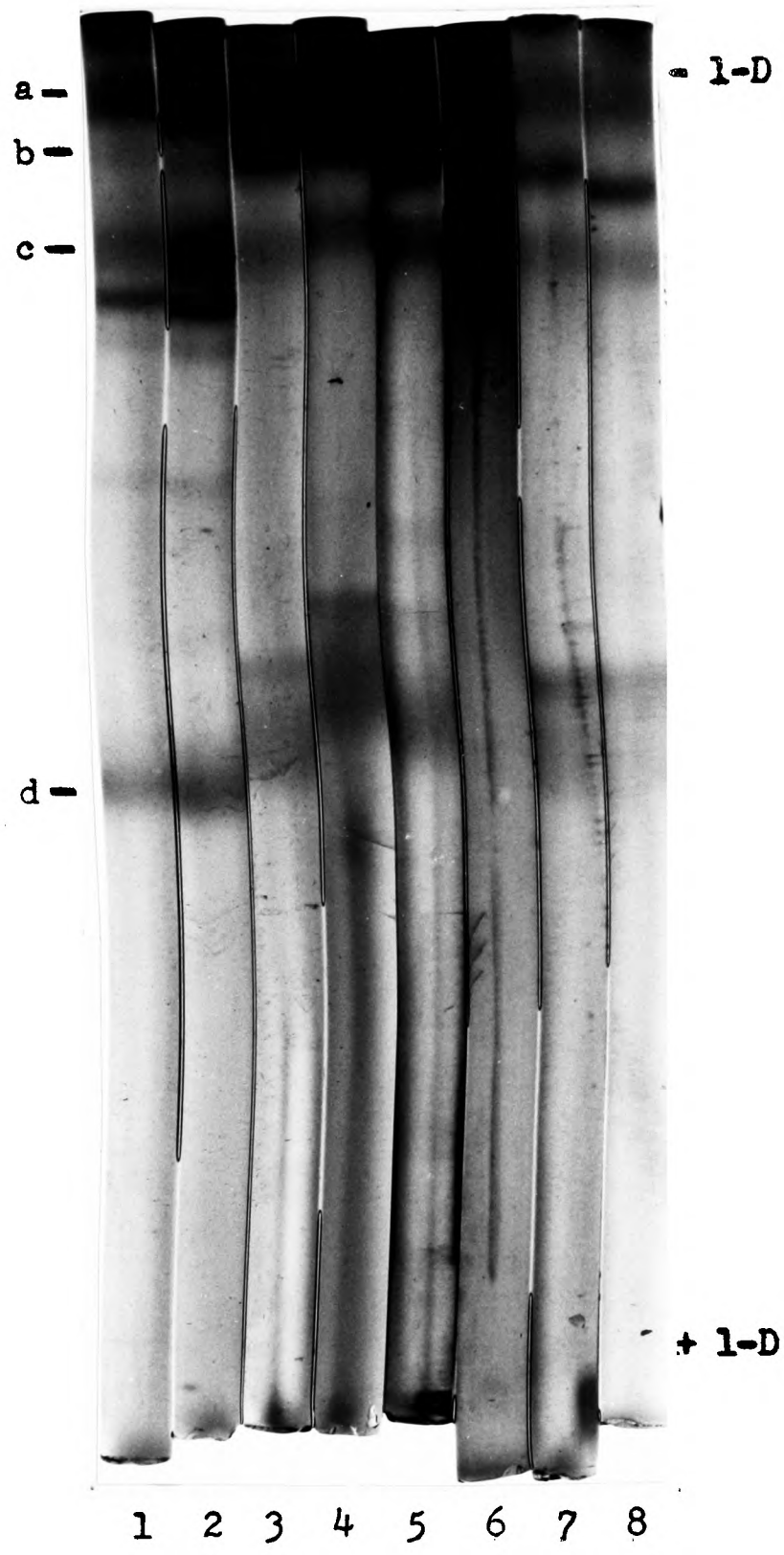


Figure 32

Separation of anionic ribosomal proteins at pH 8.6.

Gels and samples were prepared and run exactly as in the first dimension of the microgel 2-D method when analyzing anionic proteins. Rat liver ribosomal proteins were run on gels 1 (300 ug) and 2 (400 ug); NHAC ribosomal proteins on gels 3 (300 ug) and 4 (400 ug); Hela ribosomal proteins on gel 5 (300 ug); and rat leukemia ribosomal proteins on gels 7 (300 ug) and 8 (400 ug). Gel 6 was left blank. Arrows are described in the text.

examined showed the same anionic ribosomal proteins but in different amounts.

The relative amounts of these different proteins seems related to the rate of growth of these tissues; fast growing tissues showing greater amounts of the high molecular weight ribosomal proteins seen only faintly in slowly growing tissues. There is no relation of these proteins to the malignancy of the different lines since both A9HT (high malignancy) and A9 (low malignancy) have the same anion pattern. This is analogous to those results of Rodgers (311) with the exception that the differences seen here were quantitative not qualitative. This difference in interpretation is most likely due to the greater sensitivity of the microgel technique. What the functional importance of these ribosomal proteins can be is not known. They may simply be translational factors that vary with the degree of protein synthesis and aren't washed off by the 500 mM KCl wash. But with respect to the empirical definition of what constitutes a ribosomal protein (3.000), these are considered ribosomal proteins.

During the progress of these experiments others have also repeatedly found ribosomal proteins moving as those described here. Howard et al (162) have found a number of anionic spots on gel patterns from rabbit reticulocyte ribosomes. Two of these correspond to the protein 64 here and are from the large subunit while the third is from the small subunit. In addition to the spot designated 64 here, Sherton and Wool (336) also found proteins 80,42,3,3a and 3b to be present in rat muscle and liver ribosomes though it is a bit difficult to compare results. They have also found a number of other spots clustering about the 3,3a,3b region but these were found only in their monosomes.

As is shown in Chapter 6, no anionic ribosomal proteins have been found upon 2-D gel electrophoresis of isolated ribosomal subunits. Though ribosomal proteins corresponding to protein 64 here have definitely been found on 60S subunits of rat liver ribosomes they seem to be susceptible to loss upon subunit preparation (336). The other protein spots seen here also show such loose attachment to isolated subunits and suggests that they may be only associated with the 80S monosome and serve to carry out its protein synthetic function.

That these ribosomal protein spots may indeed be true ribosomal proteins and not just tightly adhering translational factors is suggested by very recent results of Wool (336,356) where it has been shown that ribosomal proteins L40 and L41 (corresponding to 64 here) are eukaryotic analogues of the bacterial ribosomal proteins L7/L12. Antisera to L7/L12 was found to cross-react with L40/L41 though other sera showed them not totally identical. Functionally, L40/L41 could also replace L7/L12 in reconstituting bacterial ribosomes. Whether L40/L41 will also be seen to vary from each other by only an acetyl group has yet to be determined. The slightly altered mobilities for spot 64 described here and that seen when comparing rat muscle and liver ribosomal proteins (336) suggests some such functional alteration in these eukaryotic ribosomes.

The fact that some of these anionic ribosomal proteins do have a ribosomal function helps allay the suspicion, expressed above, that these anionic proteins are only contaminants. It remains to be shown that other anions have a role in translation or its regulation. Chapter 5 presents evidence that one of these other anions may be the 5S rRNA binding protein.

4.060 Summary.

All eukaryotic ribosomes examined carry the same complement of 8 anionic ribosomal proteins when analyzed by 2-D gels. No species-specific differences were found but differences in the intensities of staining for certain of these anions varied between rapidly growing and non-growing cells. Several high molecular weight ribosomal proteins appeared only faintly in non-growing cells while actively growing cells showed large amounts of them. This difference was also demonstrable with another gel system. One of the ribosomal protein spots, 64, appears to be identical to a ribosomal protein described by others as a eukaryotic version of the E. coli ribosomal protein L7/L12.

CHAPTER 5

THE 5S rRNA BINDING PROTEIN

5.000 Introduction.

Early attempts to prepare eukaryotic ribosomal subunits using EDTA to disassemble the ribosome yielded particles that were inactive in protein synthesis *in vitro* (32,189), ie. they did not support polyphenylalanine synthesis in a poly(U)-directed system. These EDTA subunit particles differed considerably from the native subunits in that they assumed a more open (and hence less rapidly sedimenting) conformation (293, 364,365,366). In addition to the loss of mRNA, these EDTA subunits had lost 4S and 5S RNA (293). What other structural changes had occured upon EDTA treatment were not apparent until the other products of the dissociation mixture had been analyzed. These were found to sediment even more slowly than the EDTA subunits and to be of two types: a mRNA-protein complex (164, 206) and a 5S rRNA-protein complex (132,164,206).

Several different approaches have been used to analyze the protein composition of these 5S rRNA-protein complexes. Lebleu *et al* (206) found that the complex consisted of only 5S rRNA and a protein of molecular weight 45,000. More extensive studies by Blobel (29) showed that only above a certain EDTA concentration was a RNA-protein complex released that sedimented at 7S and consisted of 5S rRNA in association with a ribosomal protein of molecular weight 35,000. Analysis of the protein of the EDTA-treated 60S subunit showed no other ribosomal protein to be lost. It was thus suggested that the loss of activity upon EDTA treatment was due to the loss of 5S rRNA and its associated protein from the 60S subunit. Attempts to restore the binding of the complex to

the derivative large subunit were not successful.

The results of these experiments were only in partial agreement with those of King et al (189) who also found that EDTA removed a ribosomal protein (MW = 37,000) from the large subunit during subunit preparation. Though this protein was found only on 60S subunits prepared by non-chelating agents, after EDTA dissociation it was removed from the 60S subunit and found associated with the small subunit. The interpretation of these experiments was that either the ribosomal protein had been transferred from the large to the small subunit during EDTA-promoted dissociation or EDTA partitions the ribosome differently from the other methods.

Petermann et al (291) have reported that treatment of ribosomes with formamide or urea also releases a 5S rRNA-protein complex with sedimentation coefficient of 6.8S. Formamide was found to yield more of the somewhat unstable complex than the EDTA method. Equilibration sedimentation studies on HCOH-fixed complex and SDS gel electrophoresis both yielded a molecular weight of 41,000 for the 5S rRNA binding protein. No other ribosomal structural proteins were released, only a 40,000 MW band was gone.

These isolated 5S rRNA-protein complexes have recently been shown to possess both GTPase and ATPase activity (130,160). The GTPase activity was also found to vary depending on whether the protein component of the complex was free or bound to the 5S rRNA. The protein showing these activities, however, had a molecular weight of 32,000 unlike that isolated from the same tissue by Blobel (29).

These studies on the eukaryotic 5S rRNA-protein complex parallel (and were most likely prompted by) studies of a similar complex from the bacterial ribosome. Treatment of bacterial

ribosomes with chelating agents also released 5S rRNA (246) from the larger subunit yielding biologically inactive subunits; addition of 5S rRNA to the reconstitution mix of B. stearothermophilus 50S ribosomal subunits was necessary for the recovery of biological activity (98). Upon centrifugation of a mixture of 5S rRNA and isolated E. coli 50S ribosomal proteins, a complex containing mostly L18 (MW 14,300) and L25 (MW 12,000) was found (125). For B. stearothermophilus, the 5S rRNA was not precipitated by LiCl (100) and the 5S rRNA-protein complex was isolated simply by centrifugation of the LiCl extracted protein. Two ribosomal proteins, B-L5 and B-L22 were found attached to it (99). Since the complex from B. stearothermophilus is more stable, most studies have involved it.

Horne and Erdmann (160) have shown that the bacterial 5S rRNA-protein complex also shows both ATPase and GTPase activity. Since prokaryotic ribosomes possess a certain GTPase that is needed for translocation, this may be the function of the protein(s) bound to the 5S rRNA. In addition to the two proteins associated with it, the 5S rRNA itself binds to 23S rRNA (156) and may play a role in the complex functions of protein synthesis.

With the possible exception of the L7/L12-like activities of one of the anionic ribosomal proteins (4.050), only the eukaryotic 5S rRNA binding protein has been shown to possess a definite activity. It was thus the object of the experiments described in this chapter to correlate one of the spots on the 2-D gel pattern with this protein. However, it was first necessary to define rigorously the 5S rRNA-protein complex given the conflicting results of previous workers. In particular, attention was given to obtaining an accurate molecular weight of the protein and

determining whether the protein did transfer from one subunit to another during EDTA treatment.

5.010 Release of the complex by EDTA.

In all of the experiments presented here, rat liver polysomes were used though Hela and NHAC polysomes showed identical behavior. It was found to be unnecessary to use separated subunits. Rat liver polysomes present a typical sedimentation profile as seen in Fig 33a. Most of the optical density sediments greater than 80S. Since these gradients were run at low salt, the 80S peak is intact though at higher salt (500 mM KCl) it dissociates completely into subunits, as was seen for Hela ribosomes in 3.010. Upon addition of EDTA to a concentration of 0.3 uM/10 ODU_{260} all the polysomes are reduced to monosomes and subunits (Fig 33b). The subunits here are mostly native 40S and 60S subunits (and cosediment with isolated 40S and 60S subunits) but the broad peaks here suggest that they are in transition (or mixed with) the more slowly sedimenting EDTA subunits*. At levels of EDTA equal to 0.5 uM/10 ODU_{260} or greater, all sedimenting particles are the EDTA-derived subunits 40' and 60' (Fig 33c).

This EDTA-induced transition of more rapidly sedimenting particles into less rapidly migrating ones can be more clearly seen when those particles sedimenting below 80S are better resolved (Fig 34). As in Fig 33a, the sedimentation profile of polysomes before EDTA treatment (Fig 34a) consists of monosomes and a few 60S subunits, most polysomes having pelleted to the bottom of the tube. Upon addition of EDTA to a level of 0.2 uM/10 ODU_{260} (Fig 34b)

*In this and further discussions, the native ribosomal subunits are designated 40S and 60S while those subunits obtained from EDTA dissociation are denoted 40' and 60'.

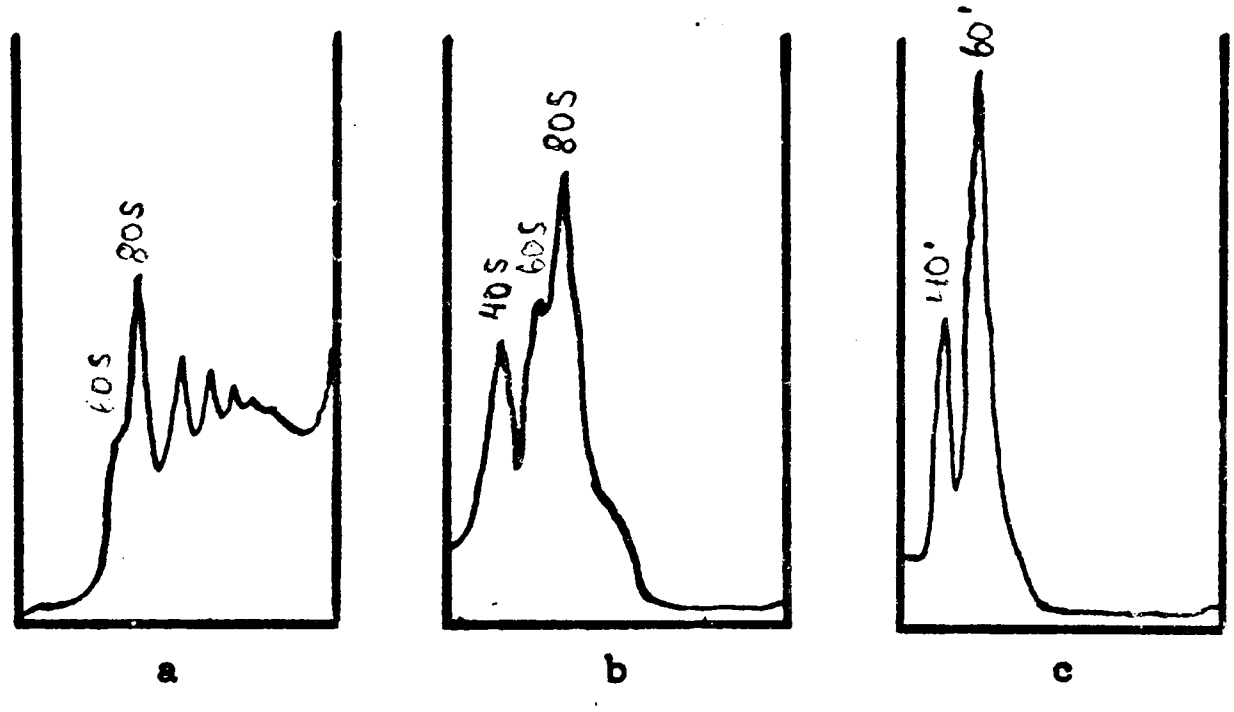


Figure 33

Sedimentation profile of EDTA-treated rat liver polysomes.

Abscissa: distance along gradient - top of gradient to the left.
Ordinate: A_{254}.

Rat liver ribosomes were treated with varying amounts of EDTA (2.090) and then sedimented through 11.8 ml, 10 - 40% sucrose gradients (in 10 mM KCl, 10 mM triethanolamine pH 7.5) at 39,000 rpm at 4° C for 2 hours. Each gradient received 4 ODU_{260} of polysomes that had been treated with (a) no EDTA, (b) 0.3 uM EDTA/ 10 ODU_{260}, (c) 0.5, 1.0, 5.0, or 10.0 uM EDTA/ 10 ODU_{260}.

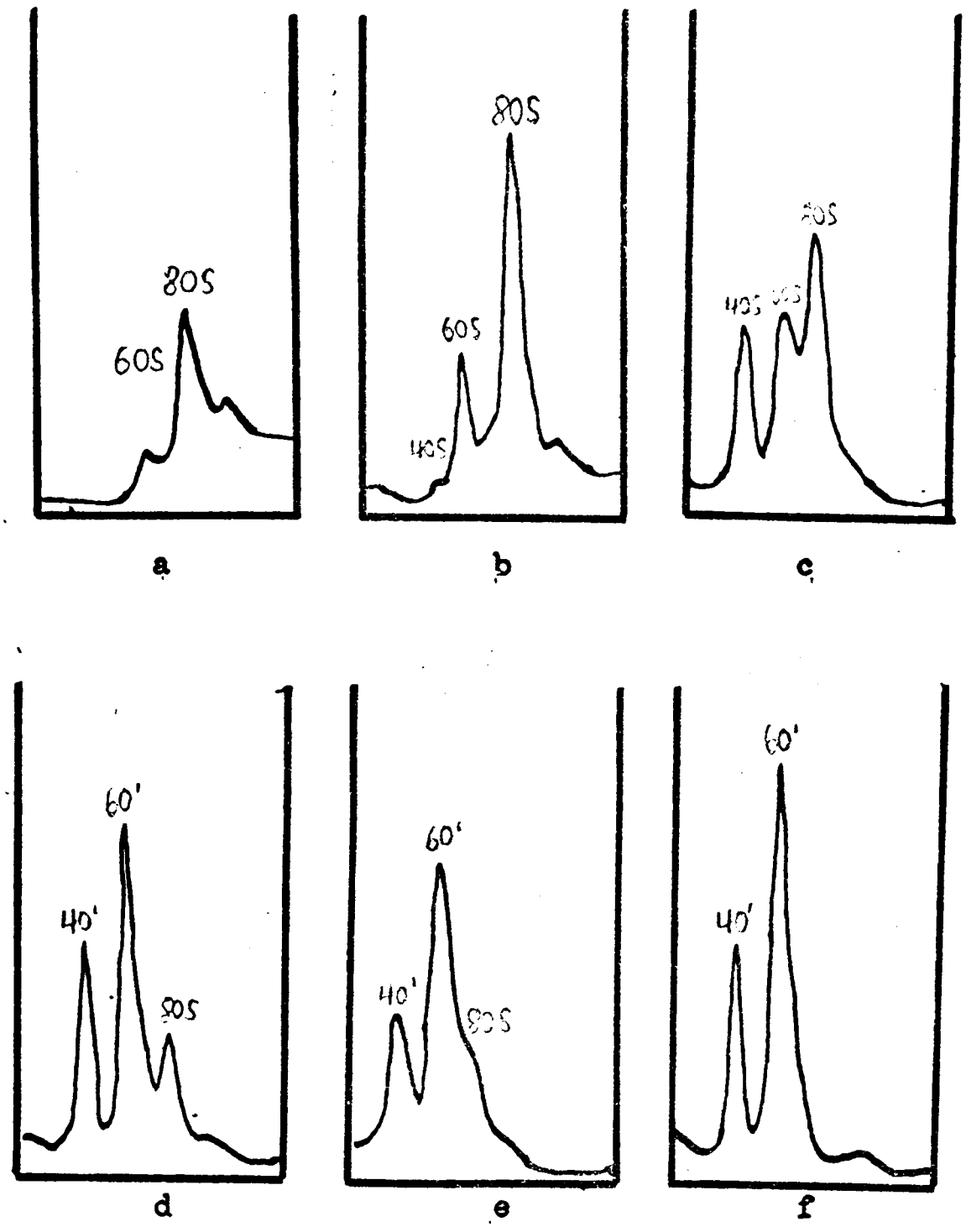


Figure 34

Sedimentation profile of EDTA-treated rat liver ribosomes.

Abscissa: distance along gradient - top of gradient to left.
Ordinate: A_{254}.
Rat liver ribosomes were treated with various amounts of EDTA (2.090) and then sedimented through 11.8 ml, 5 - 20% sucrose gradients (in 10 mM KCl, 10 mM triethanolamine, pH 7.5) at 39,000 rpm for 2.6 hours. Each gradient received 4 ODU_{260} of polysomes that had been treated with (a) no EDTA, (b) 0.2 uM EDTA/ 10 ODU_{260}, (c) 0.3 uM EDTA/10 ODU_{260}, (d) 0.4 uM EDTA/ 10 ODU_{260}, (e) 0.5 uM EDTA/ 10 ODU_{260}, (f) 0.6, 0.8, or 1.0 uM EDTA/ 10 ODU_{260}.

most of the polysomes are reduced to monosomes and some particles cosedimenting with native subunits are found. At 0.3 uM/10 ODU_{260} (Fig 34c) more subunits are being formed while the monosome peak (originating from the polysomes) is decreasing. In Figs 34b and c, the subunit peaks are broad and may contain both native and derived subunits or some transitional forms. By an EDTA concentration of 0.4 uM/10 ODU_{260} the subunits are of the EDTA-derived sort (Fig 34d) and by 0.6 uM/10 ODU_{260} (Fig 34f) all the 80S particles have been converted to subunits 40' and 60'.

The release of 5S rRNA-protein complex (which runs at approximately 7S) at several EDTA levels is shown in Fig 35. Without any addition of EDTA, little optical density is seen at the position where 4S RNA and the 5S rRNA-protein complex run (Fig 35a). At 0.2 uM EDTA/10 ODU_{260} much of the 4S RNA, but little of the 5S rRNA-protein complex, is released (Fig 35b). Up to 0.5 uM EDTA/ 10 ODU_{260} mostly 4S RNA is recovered in these gradients (Figs 35 c,d). But though additional 4S RNA is recovered at levels of EDTA 0.5 uM/10 ODU_{260} and greater (Figs 35e,f,g,h) most of the increase seems to be of the 5S rRNA-protein complex. Above 1 uM EDTA/10 ODU_{260} nc further increases in recovery of 4S RNA and 5S rRNA complex are seen.

When the data from all three sets of gradients (Figs 33,34, 35) are compared, several steps in the dissociation process by EDTA may be discerned. With no addition of EDTA, the polysomes are stable and little or no 4S RNA and 5S rRNA complex are released. At low concentrations of EDTA (0.3 uM/10 ODU_{260}) the polysomes are completely reduced to monosomes and subunits; most of the 4S RNA but little 5S complex is removed. The subunits formed at these levels of EDTA are either a mixture of native and EDTA-derived or

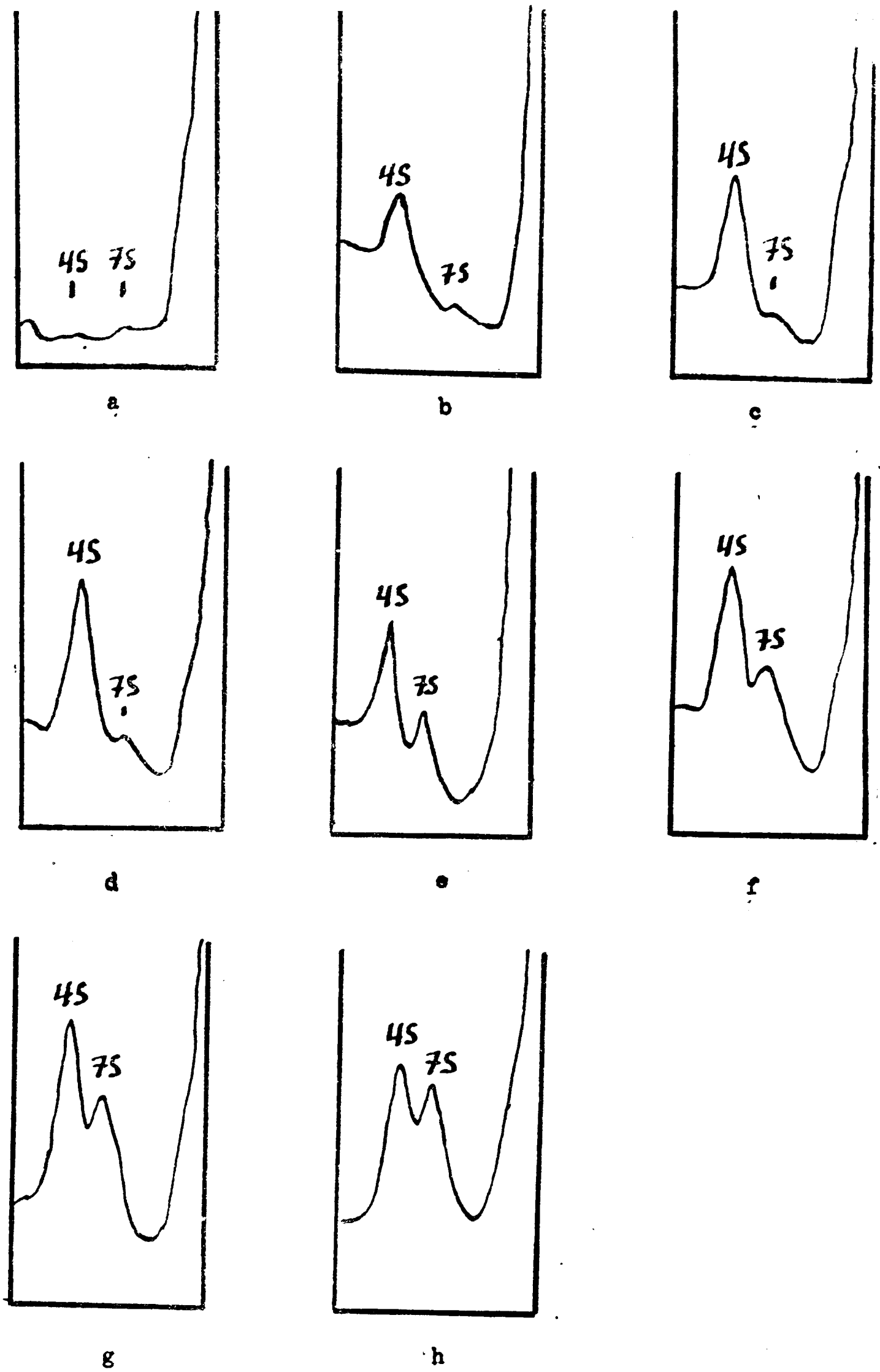


Figure 35
Sedimentation profiles of 5S rRNA - protein complex at varying EDTA concentrations.

Abscissa: distance along gradient - top of gradient to left.
Ordinate: A_{254}.
Rat liver ribosomes were treated with varying amounts of EDTA and then centrifuged to isolate 5S rRNA complex as in 2.090. Each gradient received 10 ODU_{260} of ribosomes that had been treated with the following amounts of EDTA: (a) none, (b) 0.2 uM, (c) 0.3 uM, (d) 0.4 uM, (e) 0.5 uM, (f) 0.6 uM, (g) 0.8 uM, (h) 1.0, 5.0, or 10.0 uM.

are in some transitional states. Between 0.4 uM and 0.5 uM/10 ODU_{260} the remaining monosomes are transformed into 40' and 60' particles. But only at EDTA levels of 0.5 uM are significant amounts of 5S rRNA-protein complex detected and not till 1.0 uM/ 10 ODU_{260} is the maximum amount of the complex recovered. It thus seems that the detection of complex does not correlate exactly with the formation of the EDTA subunits 40' and 60'; by 0.5 uM EDTA/10 ODU_{260} all of the ribosomes exist as the EDTA subunits (Fig 33c) but only about 1/3 the maximum amount of complex is recovered (Fig 35e).

To maximize the yield of the 5S rRNA-protein complex and presumably to ensure its complete removal, all further preparations of this complex were done at EDTA levels of 1 uM/10 ODU_{260} (Fig 35h). The EDTA subunits formed (Fig 34f) have also been analyzed with the Model E and found to have sedimentation coefficients of 20.5S and 38.5S at a concentration of 3.3 mg/ml in 3 mM Mg, 20 mM Tris, 300 mM KCl, pH 7.5 at 20° C. No corrections have been made for concentration dependence or buffer.

5.020 RNA gel electrophoresis of 5S rRNA.

Small molecular weight RNA species (4S and 5S) were easily analyzed by electrophoresis on 10% polyacrylamide gels run in SDS-containing buffers (2.084). There was no need to use phenol-chloroform extracted RNA since gels run instead with whole ribosomes solublized in the SDS sample buffer produced identical RNA scans and had the further advantage of showing protein bands upon staining for protein.

When peak fractions of the 4S and 5S rRNA-protein complex were collected and pelleted as in 2.090, only the 5S rRNA-protein

complex produced a pellet. When analyzed on 10% RNA gels (Fig 36) the isolated 5S rRNA-protein complex showed only a 5S rRNA peak (Fig 36a). The minor, more rapidly migrating peak associated with the major peak is usually present to varying degrees. It has been analyzed by Weinberg and Penman (397) who found it to represent a reversibly denatured form of the 5S rRNA that was generated by hot phenol extraction. The conformer peak has never been found in gels of intact ribosomes and it is assumed that its presence here reflects some aspects of the isolation procedure and perhaps the stability of the 5S rRNA-protein complex itself.

No 4S RNA was found after pelleting even when the bottom of the tube containing the 4S fraction was rinsed and this sample electrophoresed (Fig 36b). The 4S and 5S RNA of whole ribosomes served as mobility markers for these runs (Fig 36c). To further identify these RNA peaks, isolated soluble RNA was electrophoresed and found to run with the 4S peak.

When these same gels were stained with Coomassie Blue, no protein bands were found on the gel with the 4S sample but the 5S rRNA-protein complex showed a single strong band (Fig 37b). On occasion, some stain was also seen in a faint band migrating slightly faster than the main band (Fig 37d). When this gel was scanned for optical density (Fig 36d) the faint band was seen to comigrate with the 5S rRNA band. This staining of the 5S rRNA was due to some contaminant of the stain for when a different batch of stain was used to stain a gel run with the identical sample, the band no longer appeared and only the main band was visible (Fig 37b). From these gels it was thus seen that the 5S rRNA-protein complex contained only one protein which electrophoresed only slightly slower than the 5S rRNA.

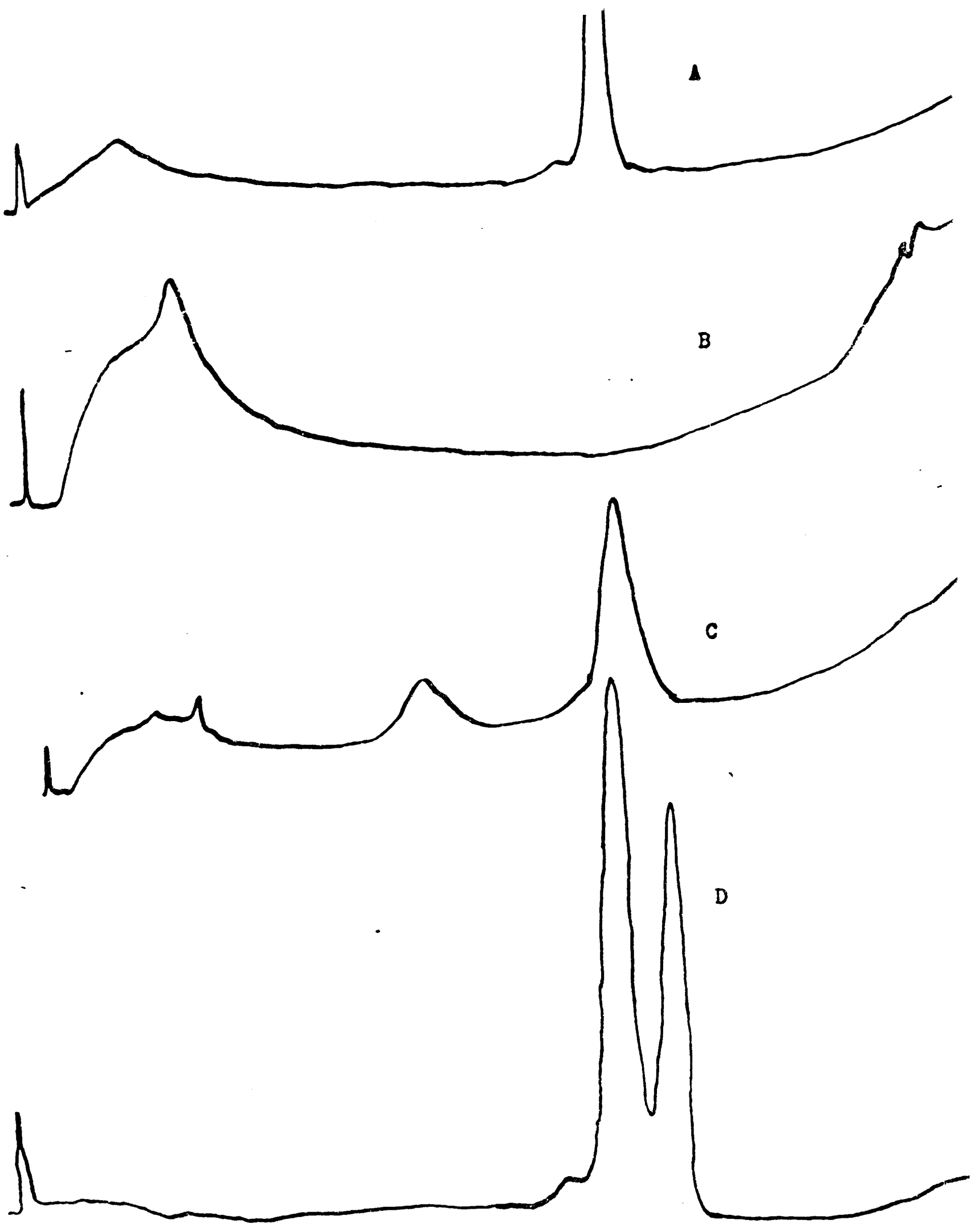


Figure 36

Scans of 10% RNA gels of 5S rRNA-protein complex.
Abscissa: distance along gel - only bottom 60% is shown - origin to right.
Ordinate: A_{260}. Scale is same for all but (B) which is magnified 3-fold.
Gels were prepared and run as in 2.084. Samples were (A) 0.3 ODU_{260} of 5S rRNA-protein complex (the amount recovered from 14 ODU of polysomes), (B) rinse of tube of pelleted (?) 4S rRNA, (C) 2.5 ODU_{260} of rat liver polysomes + 0.2 ODU 5S rRNA complex, (D) gel (A) after Coomassie Blue stain.



a b c d e f

Figure 37
Stained 10% RNA gels of 5S rRNA-protein complex.

Gels were prepared and run as in 2.084. Samples a,b and c were run at one time; (a) 2.5 ODU_{260} of rat liver polysomes; (b) 0.3 ODU_{260} 5S rRNA-protein complex (Preparation # 2); (c) as (b) but 0.2 ODU_{260}. Samples d,e and f were run at another time; (d) 0.3 ODU_{260} 5S complex (Preparation # 2) and same gel as in Figs 36 a,d; (e) 0.08 ODU_{260} of 5S complex (Preparation # 3); (f) identical to (e) but with 0.16 ODU_{260}.

Under the standard conditions for the isolation of the 5S rRNA-protein complex, the EDTA subunits have pelleted to the bottom of the centrifuge tube (Fig 35h). To determine whether all 5S rRNA-protein complex had been released, these pelleted, EDTA-treated subunits were electrophoresed on 10% polyacrylamide gels and their RNA and protein composition compared with identical preparations that had not been treated with EDTA. When these gels were scanned for RNA those samples treated with EDTA showed no detectable 5S or 4S RNA (Fig 38a) while those that had not been so treated showed a strong 5S rRNA peak and a little 4S RNA (Fig 38b). When stained for protein these gels showed one striking difference between the two preparations; the EDTA-treated ribosomes had lost a protein band (Figs 39a,c) that was present on ribosomes that had not been treated with EDTA (Fig 39b,d). This missing band ran in a position identical to that protein from the 5S rRNA-protein complex (Fig 37b). This band is somewhat blurred on these gels but is seen more accurately if the EDTA-treated and untreated pellets have had their ribosomal proteins extracted with acetic acid and are then run on the same gels (Fig 5); one band is clearly lost from those ribosomes exposed to EDTA (compare gels c,d,e with gels f,g,h in Fig 5).

These experiments have thus shown that at an EDTA concentration of 1 uM/10 ODU_{260} of polysomes, a 5S rRNA-protein complex has been released completely from the polysomes: the treated ribosomes have lost 5S rRNA and one ribosomal protein and these are isolated together upon centrifugation. However, at lower concentrations of EDTA some of the 5S rRNA is released from polysomes but most of the protein part of the complex remains attached to the ribosomes. This was observed when a sample of rat liver polysomes was divided

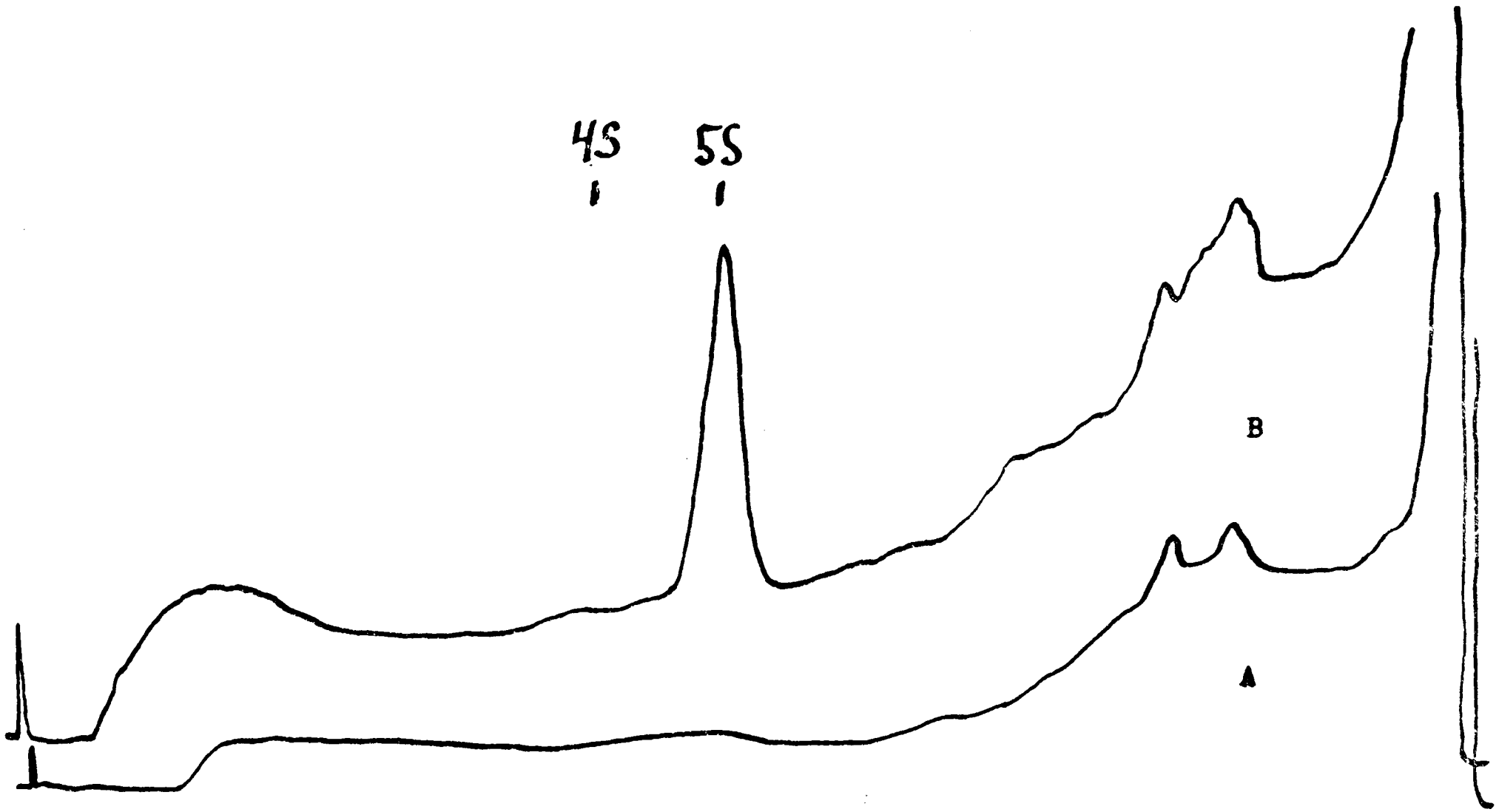
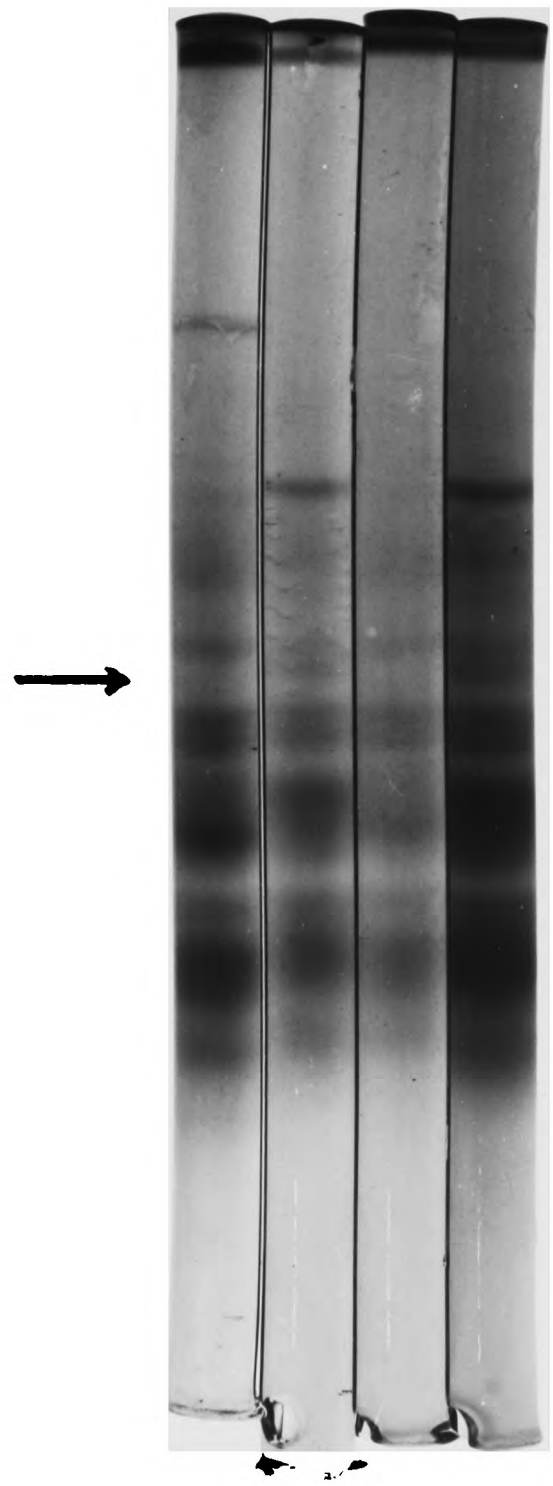


Figure 38
Scans of 10% RNA gels of EDTA-treated ribosomes.

Abscissa: distance along gel - origin to right.
Ordinate: A_{260}.
Gels were prepared and run as in 2.084. Samples consisted of ribosomes that had been previously treated with (1 uM/ 10 ODU_{260}) or without EDTA and then centrifuged as if preparing 5S complex (2.090). The pelleted ribosomes were then electrophoresed. (A) 3.0 ODU_{260} of EDTA-treated ribosomes.



a b c d

Figure 39
Stained 10% RNA gels of EDTA-treated ribosomes.

Gels were prepared and run as in 2.084. Samples consisted of ribosomes that had been previously treated with (1 ul/ 10 ODU_{260}) or without EDTA and then centrifuged as if preparing 5S complex (2.090). The pelleted EDTA-treated ribosomes were then run on gels a (1.92 ODU_{260}) and c (1.28 ODU_{260}) while ribosomes not treated with EDTA were run on gels b (1.0 ODU_{260}) and d (2.0 ODU_{260}). The arrow indicates the position of the 5S rRNA binding protein.

into several aliquots which received varying amounts of EDTA and then were centrifuged as when isolating the complex. The pelleted ribosomes were then analyzed for the presence of 5S rRNA and the binding protein. When identical amounts of the pelleted ribosomes were run on 10% RNA gels, the sample not treated with EDTA showed the normal amount of 5S rRNA while that sample treated with 0.5 uM EDTA/10 ODU_{260} lost about $\frac{1}{2}$ of its 5S rRNA. When stained for protein, however, both samples showed the same amount of the binding protein (Fig 40) and virtually no binding protein was found in the 7S fraction. Only at 1 uM EDTA/10 ODU_{260} was complete removal of 5S rRNA accompanied by removal of most of the binding protein. These experiments thus suggest that the extraction and formation of a 5S rRNA-protein complex proceeds in two stages: the release of 5S rRNA at lower EDTA concentrations followed by the binding protein at higher concentrations.

5.030 Is the 5S rRNA-protein complex a true complex?

Under the standard conditions of isolation described here, it was possible that the 5S rRNA and its associated protein did not form a complex but were just cosedimenting. It was also uncertain what amount of the optical density sedimenting in the 5S rRNA-protein peak was free 5S rRNA (since the gradients were unable to resolve completely the complex from 5S rRNA) and what was complexed with protein. To test these possibilities, gels were run under conditions (2.090) such that free and complexed 5S rRNA could be identified. Intact polysomes were placed on top of these gels (with or without EDTA additions at 5 uM/10 CDU_{260}) and electrophoresed. Upon scanning for RNA bands, only those treated with EDTA showed significant RNA peaks (Fig 41) and these were in

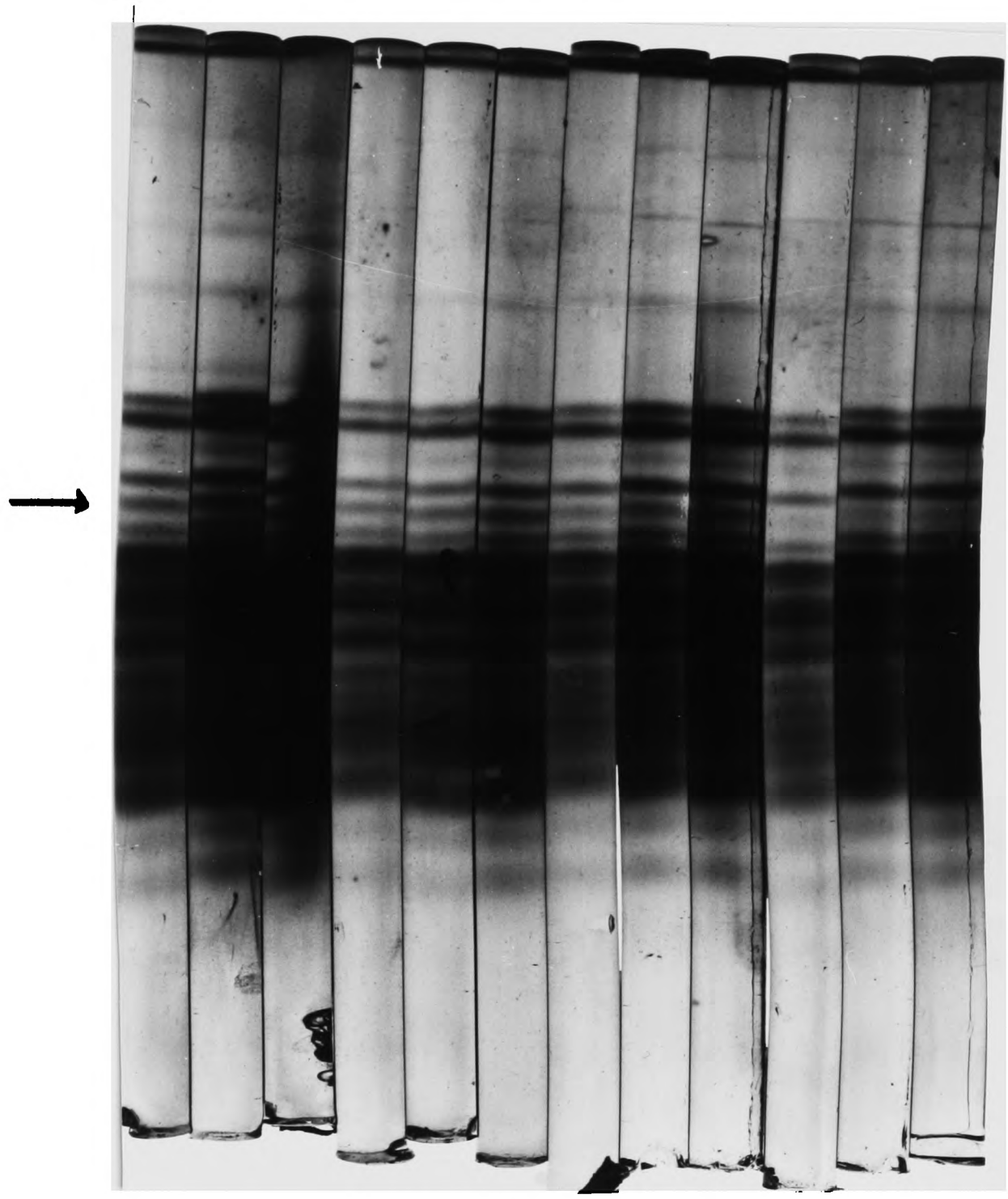


Figure 40

Stained 10% RNA gels of EDTA-treated rat liver ribosomes.

Samples consisted of ribosomes that had been previously treated with varying amounts of EDTA and then centrifuged as if preparing 5S complex (2.090). The pelleted ribosomes were then electrophoresed on gels prepared and run as in 2.084. Ribosomes that had been treated with neither EDTA nor been pelleted were run on gels a (0.92 ODU_{260}), b (1.84 ODU_{260}) and c (2.76 ODU_{260}). Ribosomes that had not been treated with EDTA but were pelleted in preparing 5S complex were run on gels d (1.20 ODU_{260}), e (2.00 ODU_{260}) and f (3.30 ODU_{260}). Ribosomes that had been exposed to EDTA (0.5 uM/10 ODU_{260}) before pelleting were run on gels g (1.26 ODU_{260}), h (2.20 ODU_{260}) and i (3.15 ODU_{260}). Ribosomes that had been treated as in (g), (h) and (i) but with 1 uM EDTA/10 ODU_{260} were run on gels j (1.32 ODU_{260}), k (2.31 ODU_{260}) and l (3.30 ODU_{260}). The arrow indicates the position of the 5S rRNA binding protein.

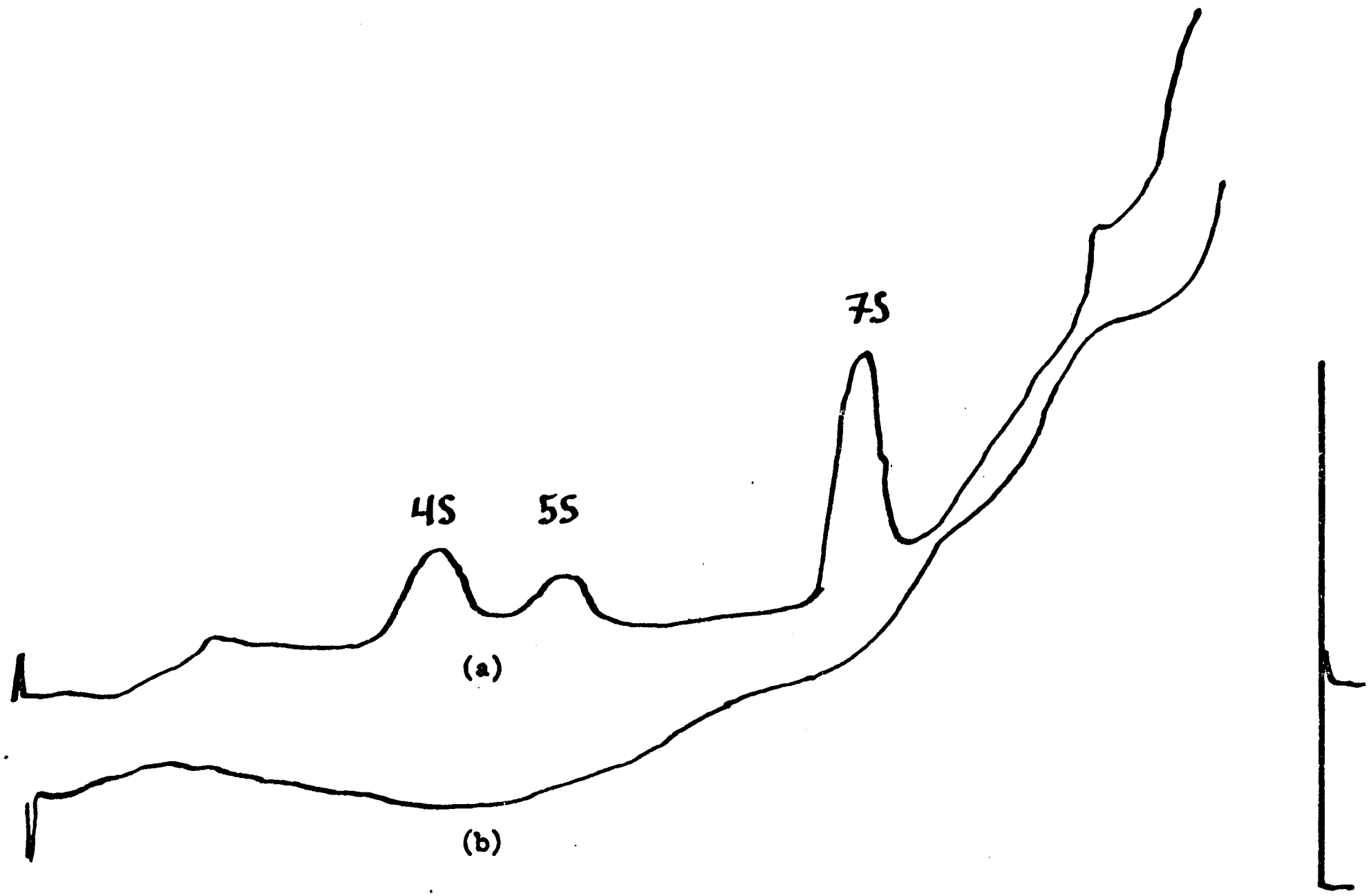


Figure 41
Release of 5S rRNA-protein complex from ribosomes; gel scans.

Abscissa: distance along gel - top of gel to right.
Ordinate: A_{260}.
Gels were prepared and run as in 2.090. Rat liver polysomes (4.0 ODU_{260}) were treated with EDTA (1 uM/ 10 ODU_{260}) and then electrophoresed (a). Identical samples not treated with EDTA were also run (b).

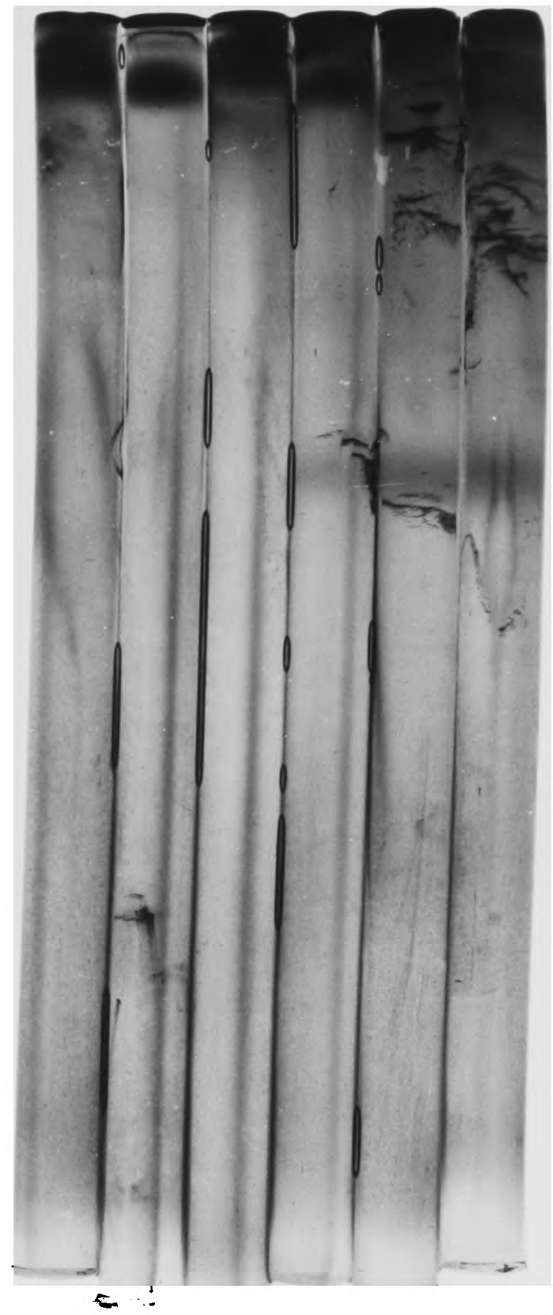


Figure 42
Release of 5S rRNA-protein complex from ribosomes; stained gels.

Gels were the same as those in Figure 41 but stained with Coomassie Blue. Samples not treated with EDTA were run on gels a (2.0 ODU_{260}), b (4 ODU_{260}) and c (7 ODU_{260}). Those treated with EDTA were run on gels d (2.0 ODU_{260}), e (4.0 ODU_{260}) and f (7 ODU_{260}). The position of the 5S rRNA-protein complex is indicated. Positions of 4S and 5S RNA were determined by scanning these gels (Fig 41).

positions corresponding to 4S RNA, 5S rRNA and a more slowly migrating species (7S). Upon staining for protein, the protein band (present only in the EDTA-treated sample) ran in a position identical with the more slowly migrating (7S) RNA species (Fig 42) and confirms that RNA and protein form a complex under these conditions. In these gels, all 5S rRNA is released from the EDTA-treated sample and about 25% of it is not associated with protein.

5.040 Molecular weight of the 5S rRNA binding protein.

Polyacrylamide gel electrophoresis in the presence of SDS-containing buffers (2.100) has proven to be a convenient and accurate method for the determination of protein molecular weight (332, 396). Subsequent to determination of a standard molecular weight curve (Fig 43), pelleted 5S rRNA-protein complexes were electrophoresed. Fig 44 shows the result of such a molecular weight determination including the standards. From seven independent preparations and molecular weight determinations, a molecular weight of 40,000 ± 500 was determined for the 5S rRNA b nding protein.

In a number of preparations there were a number of minor bands including one of molecular weight 18,400. These minor contaminants were seen more clearly when a gel was overloaded with the complex (Fig 45f). When roughly estimated by comparing the intensity of staining, these contaminants compose only about 3-5% of the total protein.

During the course of these experiments another method of concentrating fractions isolated after EDTA dissociation came into use: gradient fractions were dialyzed and lyophilized (2.090). This allowed more quantitative recovery of both 4S and 7S fractions.

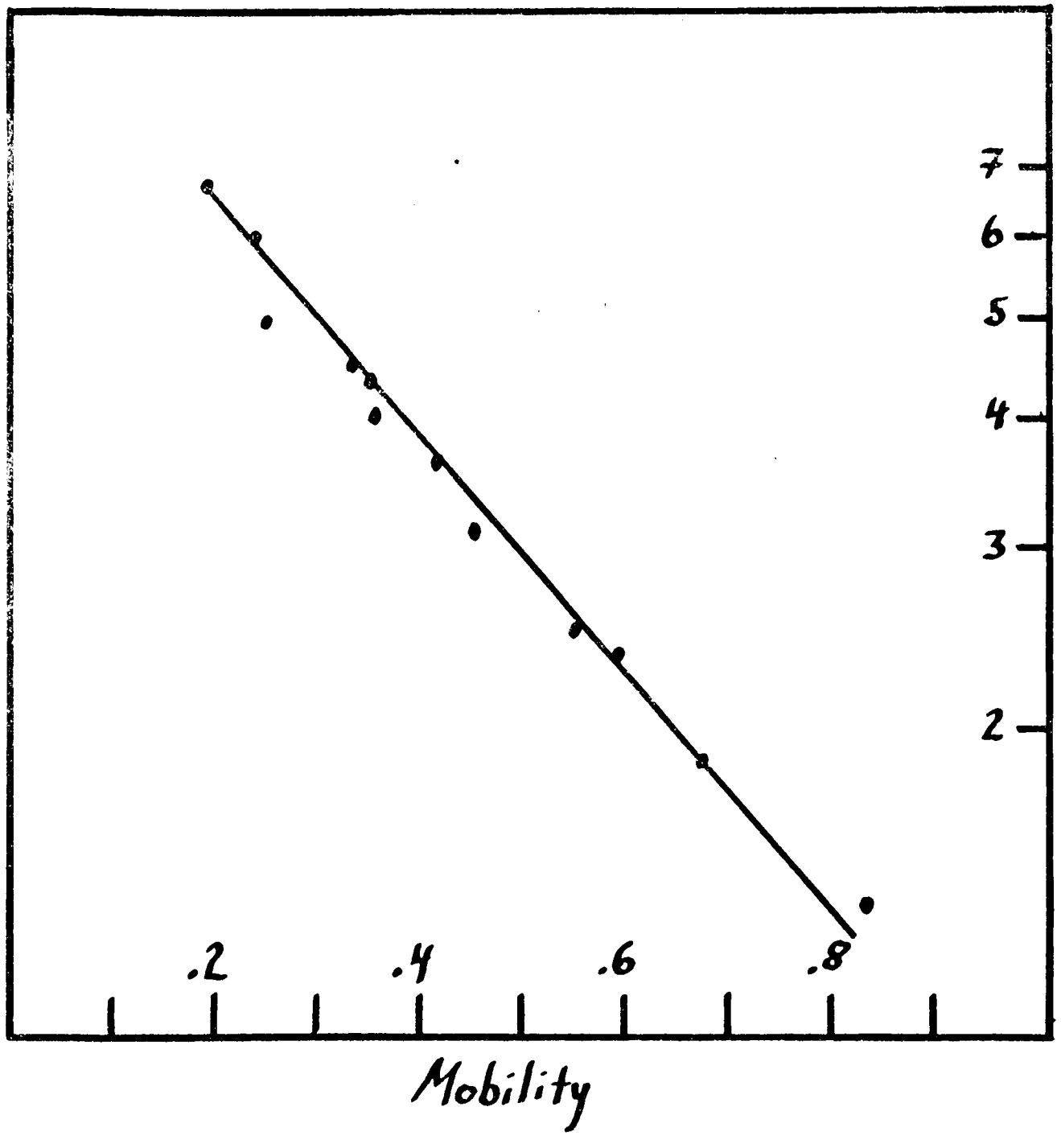
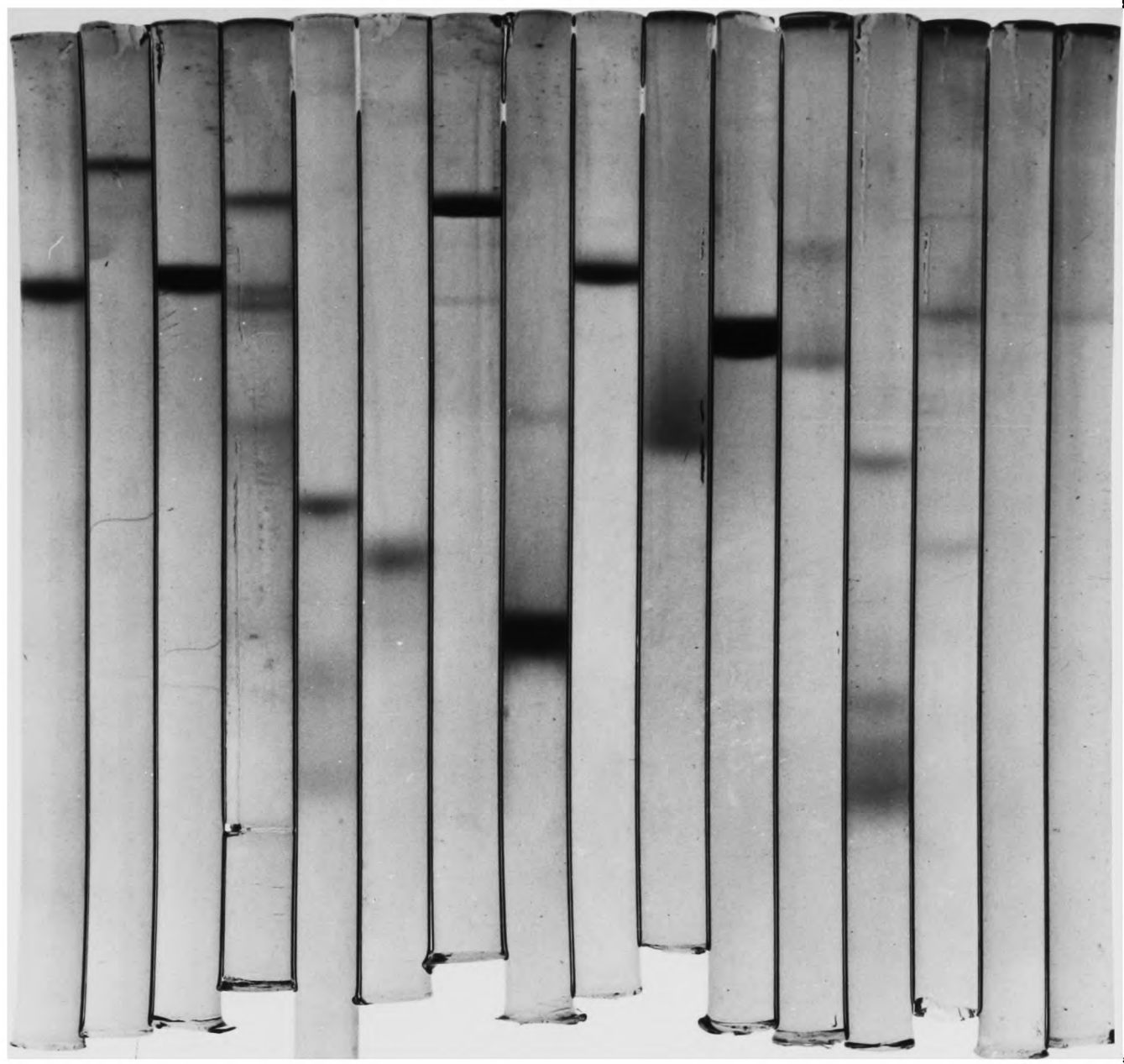


Figure 43
Molecular weight standard curve.

Relative mobility values of proteins of known molecular weight were determined with respect to Bromophenol Blue marker

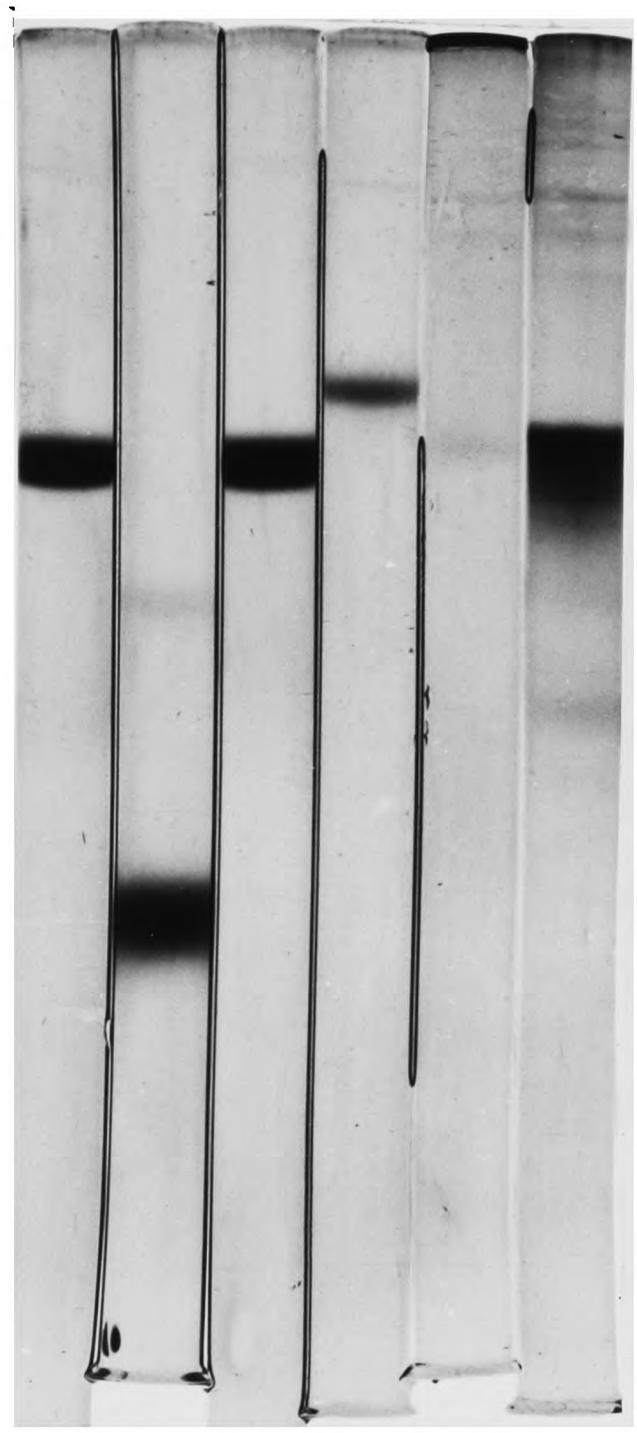


a b c d e f g h i j k l m n o p

Figure 44

SDS polyacrylamide gel electrophoresis of 5S rRNA binding protein and molecular weight standards.

Gels were prepared and run exactly as described in 2.100. Sample loading of standard proteins varied from 5 - 30 ug but exact loadings were empirically determined. Samples were: (a) hen albumin, (b) bovine albumin, (c) aldolase, (d) catalase, (e) ferritin, (f) soybean trypsin inhibitor, (g) α amylase, (h) cytochrome C, (i) egg albumin, (j) chymotrypsinogen, (k) glyceraldehyde phosphate dehydrogenase, (l) deoxyribonuclease, (m) trypsin, (n) 0.55 ODU_{260} 5S rRNA-protein complex (Preparation # 3), (o) 0.13 ODU_{260} complex (Preparation # 4), (p) 0.2 ODU_{260} complex (Preparation #2).



a b c d e f

Figure 45
SDS polyacrylamide gels of molecular weight standards and 5S rRNA-protein complex.

Gels were prepared exactly as in Fig 44. Samples were: (a) hen albumin, (b) cytochrome C, (c) glyceraldehyde phosphate dehydrogenase, (d) egg albumin, (e) 0.15 ODU_{260} of 5S rRNA complex (Preparation # 2), (f) 0.7 ODU_{260} of complex (Preparation # 1).

When electrophoresed on SDS gels (2.100) and scanned, the fractions contained the expected 4S or 5S RNA and there was no detectable cross-contamination of one fraction by the RNA of the other. Upon staining the gel run with the 5S complex, the 5S binding protein (Fig 46b,c) was easily distinguished but other contaminating bands were also seen. The 4S fraction (Fig 46a) showed some contamination with the 5S rRNA binding protein but also showed two high molecular weight bands of 69,000 and 65,000. Though the 69,000 molecular weight band was associated in similar amounts with both the 4S and 5S RNA, the 65,000 molecular weight band was associated mostly with the 4S RNA. These two proteins were also seen in gels of the entire ribosome and were the two proteins of highest molecular weight in the ribosome.

5.050 2-D gels of 5S rRNA binding protein.

When the 5S rRNA-protein complex or 4S fractions were run on 2-D gels, no protein spots (anion or cation) were visible even when amounts of the complex sufficient to overload the SDS gel (100 ug) were run. Since these microgels can detect 1-2 ug of protein, the reason for this failure to detect any ribosomal protein is not obvious. Even when run only on gels of the first dimension, no anionic or cationic protein bands could be detected upon staining though the RNA peak could be seen to enter the gel when scanned for u.v. absorbing material. When run on pH 4.5 gels no protein band could be detected; only on SDS gels was the isolated protein visible.

Since neither the 5S binding protein nor the 4S proteins could be directly observed on the 2-D gels, attempts were made to correlate the loss of one of the 2-D gel spots of polysomes with the EDTA treatment. Already 1-D gels had shown that one of the

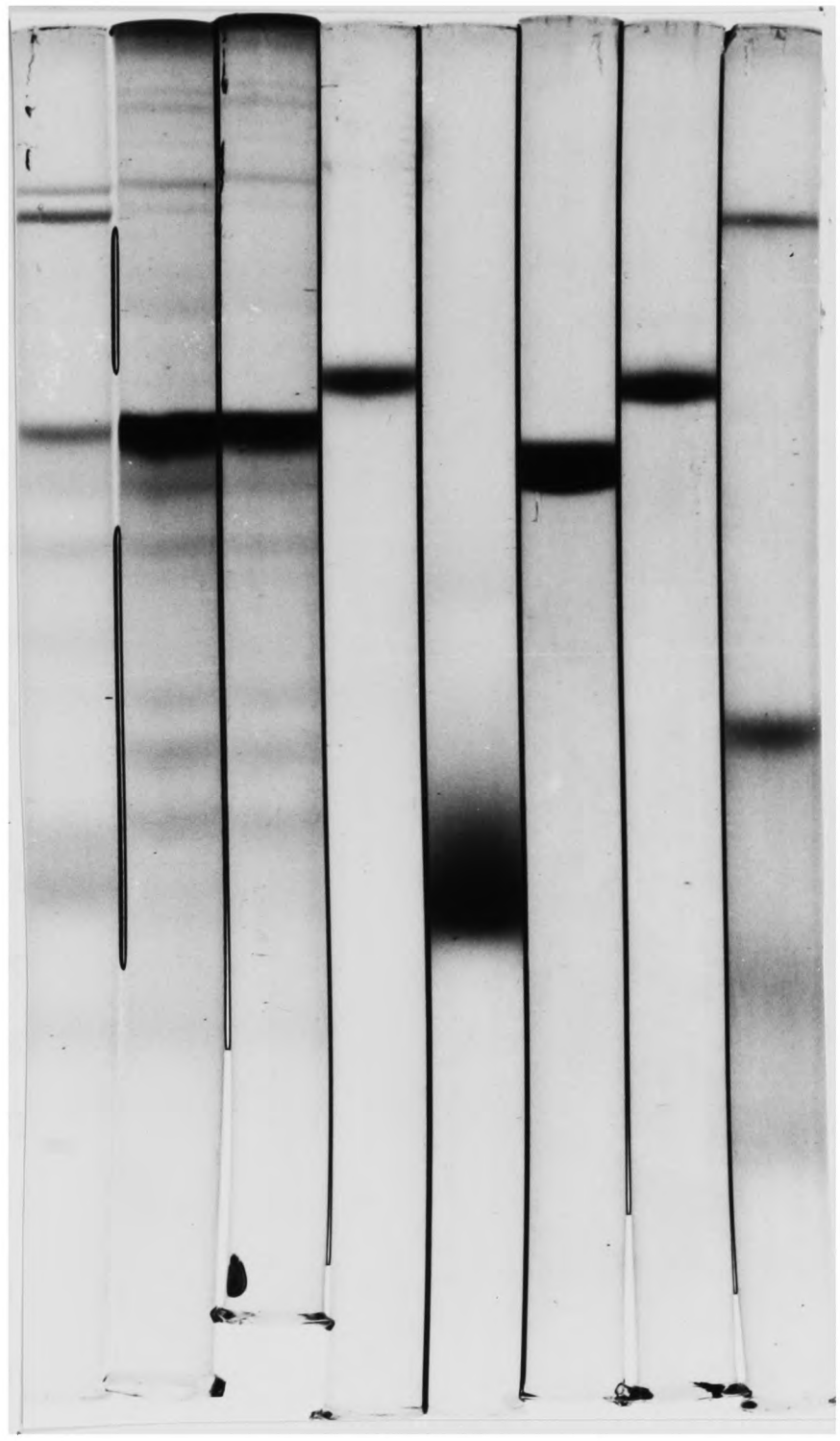


Figure 46

SDS polyacrylamide gel of 4S and 5S RNA complexes.

Gels were prepared and run as in 2.100. Molecular weight standards were hen albumin (d), cytochrome C (e), glyceraldehyde phosphate dehydrogenase (f), egg albumin (g), bovine albumin (h), and ferritin (h). The 4S and 7S fractions from gradients prepared as in 2.090 were dialyzed, lyophilized and electrophoresed. (a) 0.5 ODU_{260} of 4S associated proteins (the amount obtained from 25 ODU_{260} of polysomes). (b) 0.25 ODU_{260} of 5S rRNA complex (the amount obtained from 12 ODU_{260} of polysomes. (c) identical to (b) but with 0.30 ODU_{260} of complex.

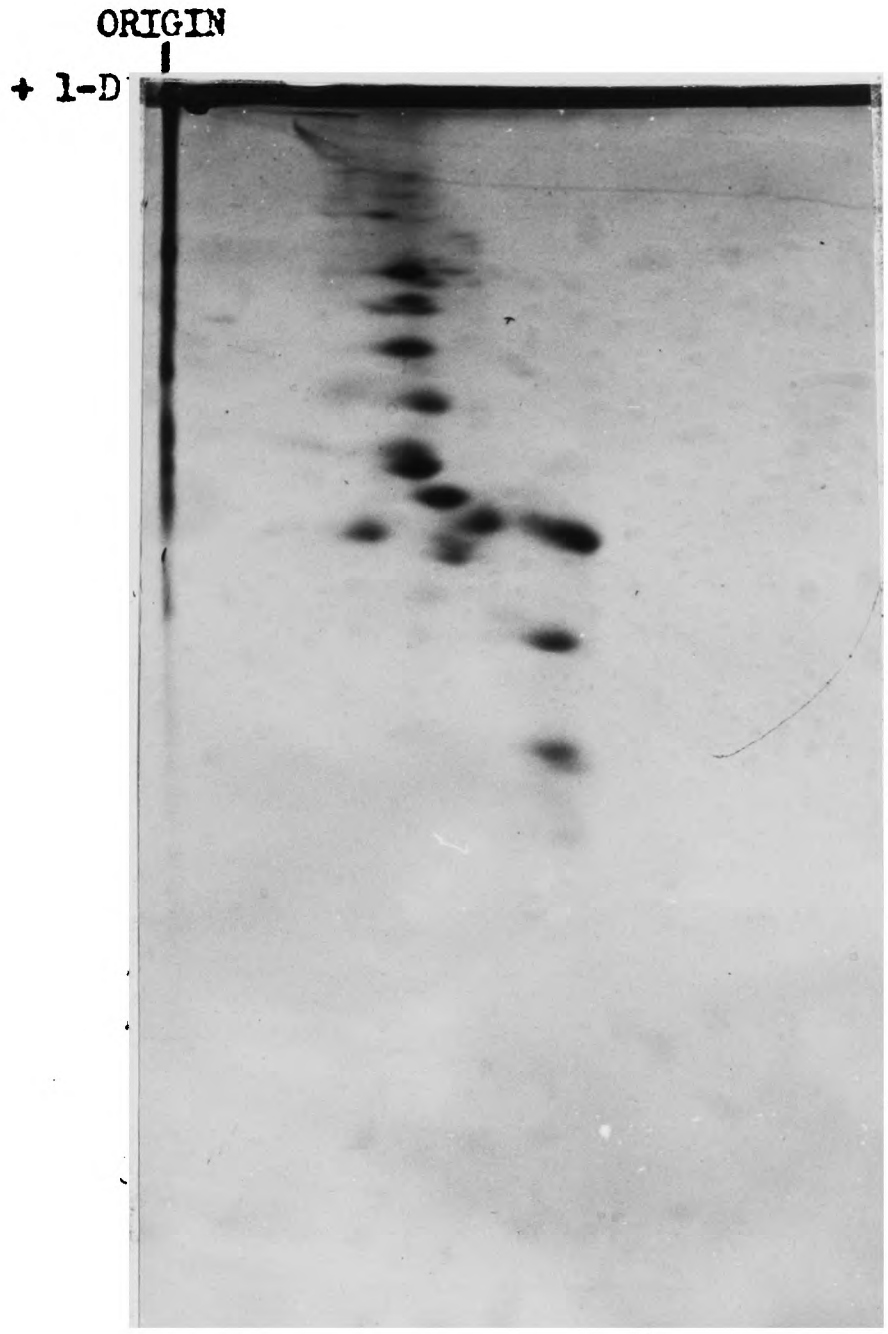
proteins was missing from acetic acid extracted ribosomes that had been previously treated with EDTA (5.020). When 2-D gels of such ribosomal proteins (Fig 47) were compared with those not so treated with EDTA (Fig 48), the loss of a number of cationic proteins was observed. It should be noted that these same samples, when run on the 1-D 10% polyacrylamide gels had shown the loss of only one protein (see Fig 5), illustrating how inadequate such 1-D methods can be. In particular, ribosomal proteins 32,33,40,31/31b,69/70,37, and 10/11 were removed and did not appear even when larger samples were applied. When similar gels were run for anionic ribosomal proteins, a single protein, 3, was lost.

These results alone were not sufficient to suggest which protein corresponded to the binding protein. But with the determination of the molecular weights of each of the 2-D gel proteins (162,215), it was seen that only two of these missing ribosomal proteins had a molecular weight of 40,000: 10/11 and 3. Since Petermann et al (291) have stated that the ribosomal protein in question has an isoelectric point of 7.0, it appears that the binding protein is the anionic protein, 3.

5.060 Discussion.

These experiments have only partly satisfied their goal of identifying which ribosomal protein on the 2-D pattern binds the 5S rRNA. Among a number of proteins lost upon EDTA treatment, only two were found to be of the same molecular weight as the isolated protein. Until it can also be demonstrated that the isolated protein shows mobility identical to that of protein 3 upon 2-D gel electrophoresis, this identification must remain tentative.

+ 2-D



- 2-D

Figure 47
Two-dimensional cation pattern of EDTA-treated rat liver ribosomes.

Rat liver polysomes were treated with EDTA and sedimented exactly as in isolating 5S rRNA complex (2.090). The pelleted ribosomes were extracted with acetic acid (2.050) and electrophoresed (200 ug protein) exactly as in 2.072.



Figure 48
Two-dimensional cationic pattern of rat liver ribosomes not treated with EDTA.

Sample was prepared exactly as in Figure 47 but without addition of EDTA. Microgel electrophoresis was as described in 2.072.

There are several possible reasons why samples of the isolated complex failed to reveal a protein band on all the gel systems tried except when run on SDS gels. One possibility is that the complex was applied directly to the gels in 8M urea sample gel rather than first having the RNA extracted with acetic acid. But since others have shown the complex to be unstable (291) this was felt unnecessary, though in light of the present difficulty this ought to be tried. A more favorable hypothesis is that this protein is insoluble even in 8M urea and in no case ever enters a gel unless SDS is present. What makes this a likely contention is the observation that in all the gel systems that failed to show a protein band for the 5S complex, a large Coomassie Blue staining mass remained at the top of the gels. In this case it is also to be expected that protein 3 is not the binding protein for the true binding protein would not have been soluble in the sample gel; it would suggest that an important ribosomal protein exists that is not seen on these 2-D gels. Until further studies are made with more isolated protein, this must remain a distinct possibility.

During the course of these experiments, two other studies have made reference to the location of the 5S rRNA binding protein on 2-D gels. Prestayko et al (305) have found that one of the 2-D gel spots obtained from polyvinyl sulfate prepared nucleolar precursors was missing from precursors prepared by an EDTA method. A similar ribosomal protein of 40,000 molecular weight was found in the supernatant of EDTA-treated ribosomes. However, no evidence was presented for this claim nor was there proof that this protein bound to 5S rRNA. Godwin et al (121) have shown that EDTA-treated large subunits lack two ribosomal proteins of about MW 32,000 but the molecular weight of these proteins suggests that they are

different from that protein described in this chapter.

The 5S binding proteins isolated by different workers vary considerably in their estimated molecular weight, from 32,000 (225) to 45,000 (206). Since a majority of the ribosomal proteins lie between these two limits (162,215), it is of importance to know whether these different molecular weights reflect different proteins or are simply due to errors in determination.

The fact that several different experiments (29,291,305), including that presented in this chapter, all used the same isolation technique and the same tissue (rat liver) but obtained different molecular weights suggests that it is the molecular weight determinations that are giving the ambiguous results. Hence particular attention was paid to the molecular weight determination presented in this chapter and the value of 40,000 presented here agrees closely with that from equilibrium sedimentation (291). Whether there is any species or tissue difference in this protein is still a possibility but experiments with Hela binding protein (data not presented here) show it also to have a molecular weight of 40,000.

The experiments presented in this chapter have not confirmed the observation of King et al (189) that the 5S rRNA binding protein is released from the large subunit and then attaches to the small. As shown here, the 5S rRNA and binding protein are both totally removed at 1 uM EDTA/10 ODU_{260}. Godwin et al (121) and Prestayko et al (305) also show it to be removed completely. Why then is there this difference with the results of King et al? The explanation may be that these workers were the only ones not to separate their EDTA dissociation mixture on gradients containing KCl or NaCl; the low ionic strength of their gradients may favor

the association of the complex with the small subunit.

It has been suggested that the isolation of this 5S rRNA-protein complex might allow the reconstitution of active 60S subunits from the complex and the 60' subunits. Attempts at this have not met with success (29,291). However in neither case was it rigorously proven that the 60' subunits had not been altered in other ways. In both instances 1-D gels of the EDTA-treated ribosomes were used to show the loss of only one protein band. However, such gels are not sufficiently accurate to determine if other ribosomal proteins are also lost or not. As shown here (Fig 5) though 1-D gels show only one protein lost upon EDTA treatment sufficient to remove all of the 5S rRNA complex, other ribosomal proteins are found to be reduced in quantity upon 2-D gel electrophoresis (5.050). Future reconstitution studies need to bear this in mind and perhaps employ alternative preparation measures to overcome this problem.

These experiments in no way prove the assumption that this binding protein is bound to 5S rRNA in the intact ribosome. It is only by the analogy with the bacterial systems that this has been thought to be the case. What has been shown in the studies here is that as a function of the EDTA concentrations:

a) the formation of the 40' and 60' subunits do not correlate with the loss of 5S rRNA (5.010).
b) the release of the 5S rRNA seems to preceed (with respect to increasing EDTA levels) the release of the 5S binding protein.

It thus seems that the conversion to the 60' and 40' forms is very sensitive to the EDTA concentration while the release of 5S rRNA followed by the binding protein are the less sensitive steps, respectively. The formation of the complex thus occurs only above certain EDTA levels. Whether this complex is only a coincidental

association of RNA and protein under these circumstances remains to be proved, as does their interaction within the intact ribosome.

5.070 Summary.

Upon exposure of intact polysomes to EDTA, subunit-like particles are formed accompanied by the release of a 5S rRNA-protein complex. By analyzing the release of this complex at several concentrations of EDTA it has been shown that the release of 5S rRNA does not solely account for the formation of these EDTA subunits nor does the release of 5S rRNA occur at the same EDTA concentration as the release of the 5S rRNA binding protein. The binding protein was observed to possess a molecular weight of 40,000 ± 500 and to be associated with most of the 5S rRNA released under the conditions employed. Two-dimensional gel electrophoresis of treated ribosomes showed them to be deficient in approximately 8 different ribosomal proteins of which only one, 3, was of the appropriate molecular weight and charge to be identified tentatively as the binding protein. Attempts to run the isolated 5S rRNA complex on 2-D gels, however, failed to show any protein spot.

CHAPTER 6

THE PROTEINS OF HELA RIBOSOMAL SUBUNITS

6.000 Introduction.

With no known exceptions, all living organisms possess a protein synthesizing machinery that centers on the ribosome as the site of peptide bond formation (54). Characteristic of all types of ribosomes is that they consist of two subunits which freely exist in certain physiological states or are created by various methods *in vitro*. According to Spirin (346), "the first and main principle of the structural organization of the ribosome" is that it "is always constructed from two unequal subparticles." Why there should be this need for two subparticles is really not yet apparent however some have suggested (346) that it may provide some mechanical advantage with respect to movement during translation.

Though the relative amounts of free subunits, monosomes and polysomes vary with the physiological state and stresses imposed on the cell, the basic "cycle" of the ribosome begins with the free subunits and has been worked out in detail for both eukaryotes and prokaryotes (for a detailed review see ref 148). In brief, the smaller subunit appears to be responsible for recognition and binding to the mRNA and forming an initiation complex with it, f met-tRNA* and various initiation factors. This is followed by the binding of the larger subunit and the release of factors. The resulting 70S . fmet . mRNA complex then goes on to form peptide bonds using the peptidyl transferase enzymes contained in the large subunit (252). Upon completion of the peptide chain, the protein

*Eukaryotic initiation uses unformylated tRNA exclusively due to the absence of formyl donors.

is released and the ribosome disaggregated to its subunits via a set of well-described (though still controversial) steps.

These observations that initiation is primarily a small subunit function have given a convenient shorthand for characterizing each of these ribosomal subunits (346). But given the complex interrelationships possible in the ribosome it is an oversimplification to segregate the various activities of the ribosome; enzyme activities correlated with one particular ribosomal protein on one of the subunits will undoubtedly be proven to be affected (or regulated) by other ribosomal proteins on the same or other subunit. It is thus of importance that individual ribosomal proteins be analyzed both in the context of the subunit but also in terms of the whole ribosome. Given the present rate of progress on the E. coli ribosome, these complex interactions may soon be open to study. Most recent studies, however, have concerned the characterization of which ribosomal proteins compose each subunit and what possible functions they represent.

The relative ease with which the bacterial ribosomal subunits may be prepared has allowed their very detailed description. Upon lowering the Mg++ concentration, bacterial ribosomes readily dissociate into subunits (374). Two-dimensional gel electrophoresis of the small subunit showed it to possess 21 ribosomal proteins while the large subunit had 34 (180), none of which overlapped on 2-D gels.

Similar attempts to dissociate mammalian polysomes with low Mg++ concentration only resulted in the reduction in the amount of polysomes and the formation of a complex of particles with sedimentation coefficient of 50-70S (364). Only when Mg++ concentration was actively reduced with a chelating agent (EDTA, pyrophosphate)

did complete dissociation occur (364). However these EDTA-derived subunits did not recombine to form monosomes nor were they active in protein synthesis (189,231). This later was seen to be due to the loss of 5S rRNA (294) and certain proteins (Chapter 5).

However, recently several techniques have become available for the isolation of eukaryotic ribosomal subunits that do reform biologically active 80S monosomes and polysomes. These methods involve the dissociation of ribosomes by high salt (101,232), urea (290) or puromycin-salt (32). Using such techniques, a wide variety of cytoplasmic ribosomes have been analyzed with 2-D gel electrophoresis and the protein composition of their subunits determined (13,60,138,162,182,209,233,274,304,305,335, 336,367,371,380,381,404). The results of these experiments may be summarized as follows:

a) Of the 70-75 ribosomal proteins found on 2-D gels of the 80S monosome of higher eukaryotes, most workers reported 31 $\pm$ 3 proteins associated with the small subunit and 39 $\pm$ 4 with the large. The assignments of proteins to one or the other subunit usually showed agreement among the several published reports, any variation seeming to reflect the preparative methods (336).
b) Only 3 spots on the 80S proved to be due to overlapping proteins of both subunits.
c) One or 2 acidic proteins were associated with the small subunit and 2-4 with the large.
d) Several high molecular weight anionic spots seen on the 80S pattern were not found to run on gels of either subunit and were felt to be protein synthesis factors and/or tightly adhering contaminants.
e) To the extent that the 80S gel patterns themselves were similar (as described in Chapter 3), the assignment of proteins to each subunit showed no species or tissue variation.

In addition to analyzing the subunits of cytoplasmic ribosomes with 2-D gels, a number of investigations have also included the proteins of the subunits of chloroplast and mitochondrial ribosomes. Chloroplast ribosomes were found to have 22 proteins on the small

subunit and 26 on the large (36,138) with few proteins overlapping with the cytoplasmic ribosomal proteins. Though supposedly of bacterial origin, these chloroplast ribosomal proteins seem to be fewer than those of E. coli but this may reflect only the difficulty in their preparation. The ribosomal proteins of mitochondrial subunits have been less clearly determined; one study (204) found 23 proteins on the small and 30 on the large subunit while another (209) showed 44 on the small and 40 on the large. However, the poor 2-D gels of the latter workers made this interpretation difficult to accept.

Given the results of the above experiments assigning the various cytoplasmic ribosomal proteins to one or the other subunit, it was possible to predict which protein spot on the 80S pattern of a closely related species will be found on which subunit. Hence the known subunit distribution of the rat ribosomal proteins could be used to predict accurately that of the mouse. But the application of this reasoning to more distantly related species (like human ribosomes) was uncertain, no human patterns having been published at the start of the studies described in this chapter. These studies were thus begun to determine the ribosomal protein composition of Hela ribosomal subunits. Such an accurate assignment to each subunit was essential for interpreting the composition of nucleolar ribosomal precursor particles (Chapter 7) as well as allowing a more detailed comparison at the subunit level of those species-specific protein differences previously presented (Chapter 3).

6.010 RNA extraction and electrophoresis.

Electrophoresis on 2.7% polyacrylamide gels (2.083) accurately resolves the 18S and 28S rRNA species as well as the several

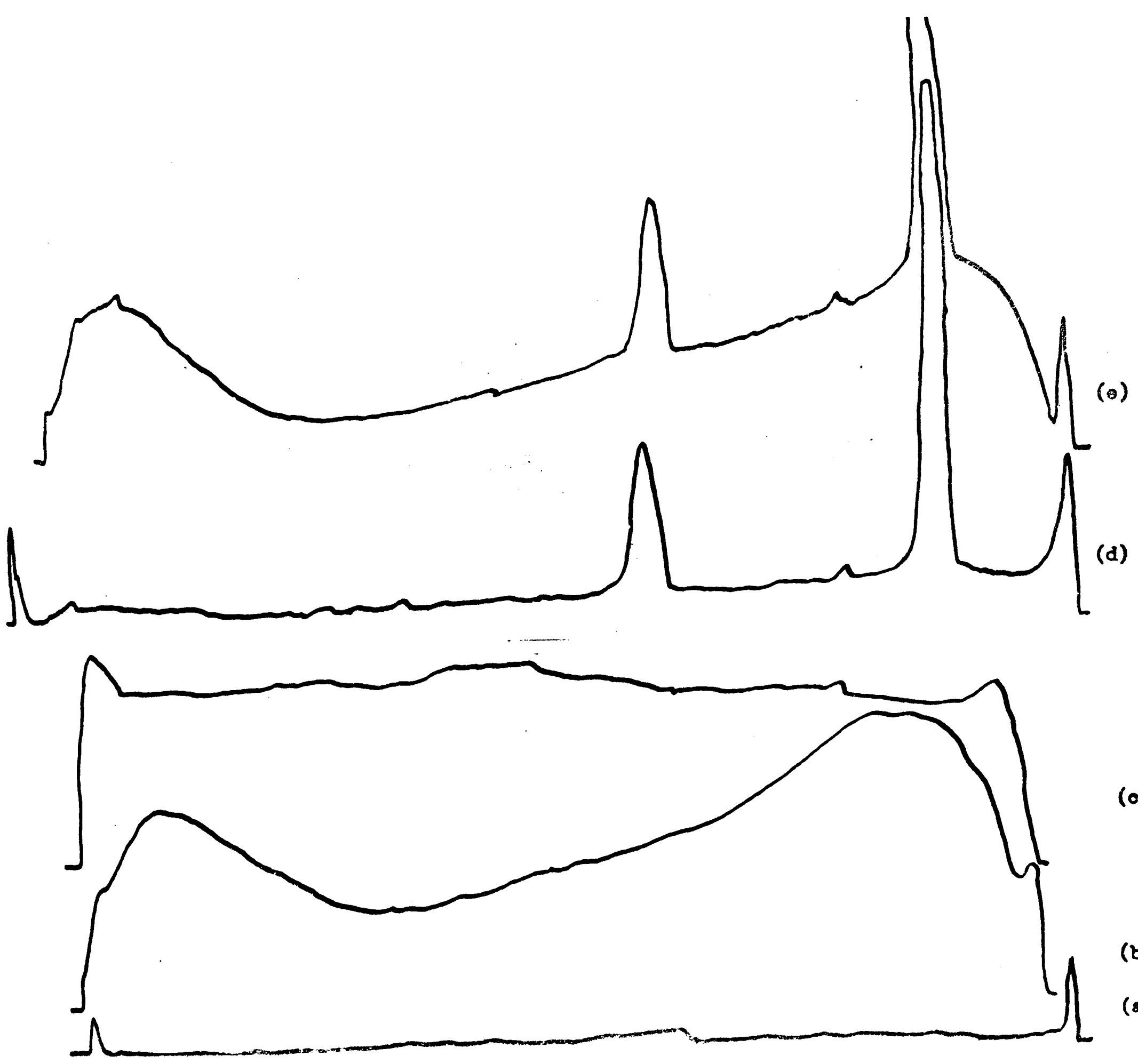
nucleolar precursors. However, a number of very common experimental problems deserved some attention to prevent ambiguous or misleading interpretations of these gels. One such problem was a result of the relatively large amounts of RNA available from pelleted ribosomes. When ribosomes were extracted by the hot phenol-chloroform method (2.081) at concentrations greater than 1 mg/ml (15 ODU_{260}/ml) at 55° C, a number of additional peaks were observed between the 18S and 28S peaks and at molecular weights close to and greater than the 28S peak. These aggregates of the rRNA were found to be solely the result of the elevated RNA concentration and were eliminated by working at lower concentration (5 ODU_{260}/ml). This phenomenon also has been described by Wagner et al (386).

A second practical matter in running these gels was to ensure a flat top to optimize band separation. Under the conditions described here (2.083) a perfectly even surface was maintained thoughout the gel run only if the gels were polymerized without containing SDS and then the SDS electrophoresed into the gel. Inclusion of SDS in the gels when polymerizing or use of SDS concentrations of 0.5% in the running buffer always resulted in the rounding up of gel tops and the distortion of RNA bands.

Since these gels were analyzed solely by scanning for u.v. absorbing peaks, any non-uniform background absorbance could be misinterpreted. In particular, it was found that during electrophoresis some material within the gel was redistributed such as to give increased absorbance at both ends of the gel (Fig 49b,c). Since these occured in the region where the 28S rRNA and its nucleolar precursors were found to run, such absorbance was of concern. However, it was found that if gels were washed for 2½-

Figure 49
Scans of 2.7% polyacrylamide gels of HeLa rRNA.

Abscissa: distance along gel - origin to right.
Ordinate: A_{260}.
Gels were prepared and run as in 2.083.
(a) Same as (b) but washed for 3 hours vs. running buffer.
(b) Freshly polymerized, electrophoresed gel. Not dialyzed.
(c) Freshly polymerized, unelectrophoresed gel.
(d) Same gel as (e) but after 3 hours washing.
(e) 0.19 ODU_{260} of cold phenol-chloroform extracted Hela rRNA; before washing.



3 hours in the running buffer before scanning, a perfectly flat background could be obtained (Fig 49a) without altering the RNA bands by diffusion (Fig 49d,e). The gels were only slightly increased in length during dialysis. Loening (221) had previously described such a method.

Finally, it was found that the different methods of extraction of rRNA yielded different RNA gel profiles. When Hela ribosomes were extracted with the hot phenol-chloroform technique (2.081), a number of RNA bands migrating faster than the 18S and between the 18S and 28S bands were observed (Fig 50a). Though these extra peaks were not very noticeable with Hela ribosomes, the RNA extracted from the ribosomes of other tissues (such as rat liver) which have high endogenous RNase levels was almost totally degraded into these extra peaks. The reason for these new peaks thus seems to be due to the separation of the parts of ribonuclease "nicked" RNA when the temperature reduces the non-covalent interactions still holding them together. Cold phenol-chloroform extraction (2.082) does not cause this phenomenon (Fig 50b). However, it was found unnecessary even to extract the rRNA for electrophoresis on these gels. Simply dissolving the ribosomes or nucleolar precursor particles in the sample buffer (2.083) yielded patterns identical to those of the cold extracted RNA (Fig 50c). In fact, unlike the usual losses of 25% involved in either cold or hot phenol-chloroform extraction, this method allowed electrophoresis of the entire sample. All RNA gels of intact ribosomes, subunits and nucleolar particles were run by this method.

6.020 Subunit preparation.

The procedure of Sabatini and Blobel (2.060) was employed to

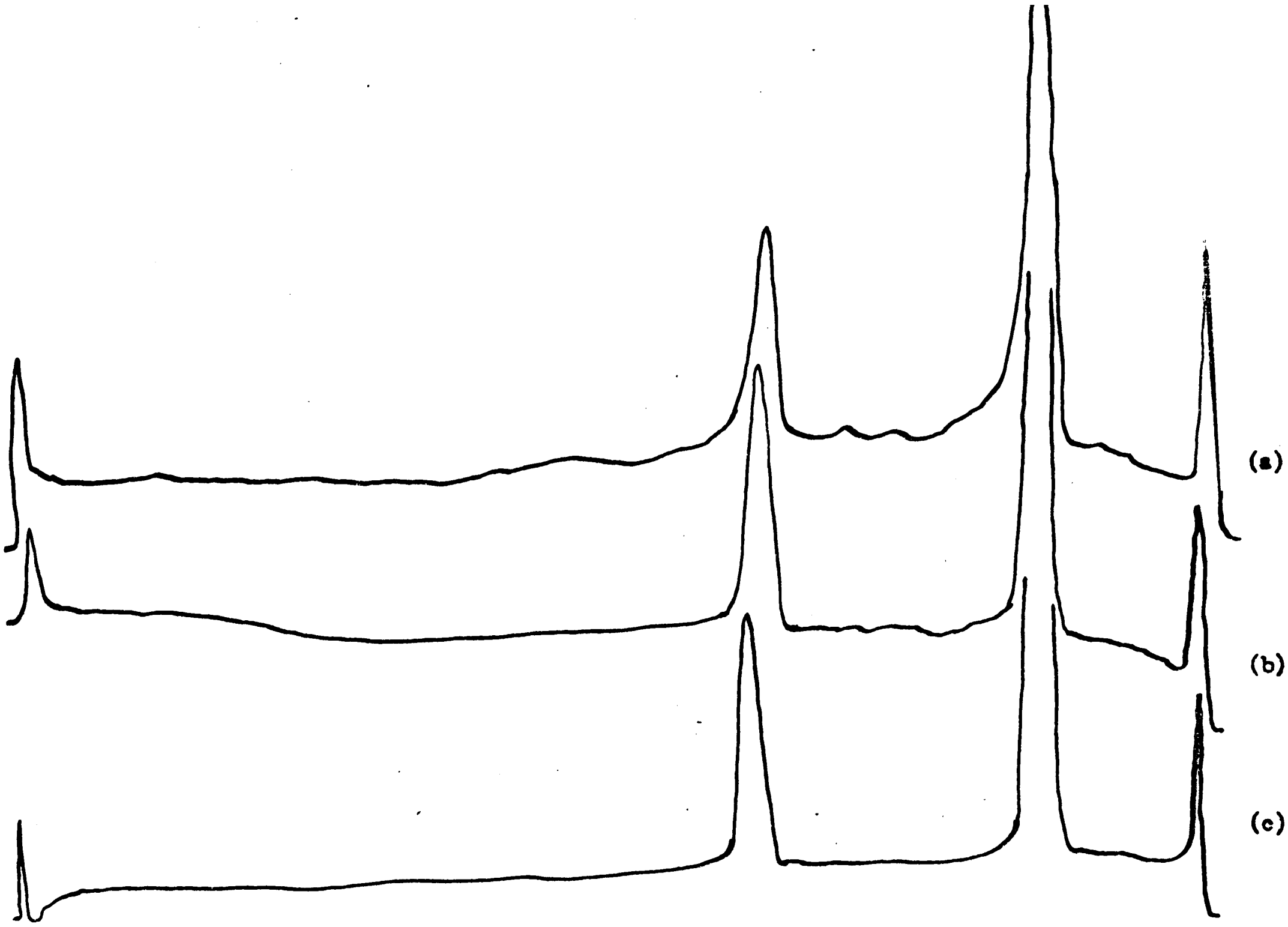


Figure 50

Scans of 2.7% RNA gels run with rRNA prepared by different methods.

Abscissa: distance along gel - top to right.

Ordinate: A260.

Gels were prepared and run as in 2.083 and washed before scanning. rRNA samples had been extracted from Hela ribosomes by (a) the hot phenol-chloroform (2.081) method (0.24 ODU_{260}); (b) the cold phenol-chloroform (2.082) method (0.18 ODU_{260}). Unextracted Hela ribosomes (0.41 ODU260) were run on gel (c).

prepare from Hela polysomes subunits that have been shown to be biologically active. This method is based upon the peculiar kinetics of the peptidyl transferase using puromycin as a donor. At high ionic strength (500 mM KCl) and 0° C, peptidylpuromycin compounds are formed and released from the polysome. The release of nascent chains is complete though solely a function of the KCl concentration but yet does not cause the concurrent release of the tRNA moiety of the peptidyl-tRNA or mRNA from the polysomes. These are released only upon elevation of the temperature to 37° C accompanied by apparently complete dissociation of these polysomes into subunits.

That this method works efficiently for Hela ribosomes under the conditions stated in 2.060 is illustrated in Fig 51. Without addition of puromycin (Figs 51a,d) the Hela polysomes present a sedimentation profile exactly as described before (3.010). Only a small 80S peak is present due to the dissociation in high salt of 80S ribosomes not bound to mRNA. That this is the 80S peak is shown in Figs 51c,f where ribonuclease has been added with a concomitant increase in monosomes and shorter polysomes (or aggregates of monosomes). Upon addition of puromycin, there is a total dissociation of the polysomes and monosomes into subunits (Fig 51b,e). The slight shoulder on the side of the 60S peak (Fig 51e) has been shown by RNA gel electrophoresis to contain only 28S rRNA. This conversion to subunits was thus complete and showed a recovery of 90-95% of the initial optical density.

Preparative amounts of ribosomal subunits were made as in 2.060. A typical preparation started with 50 ODU_{260} of dissociated Hela polysomes distributed over 6 gradients in the SW 41 rotor and yielded the separation shown in Fig 52a. With sample loadings above 8 ODU_{260}/gradient there is decreased resolution of the subunits.

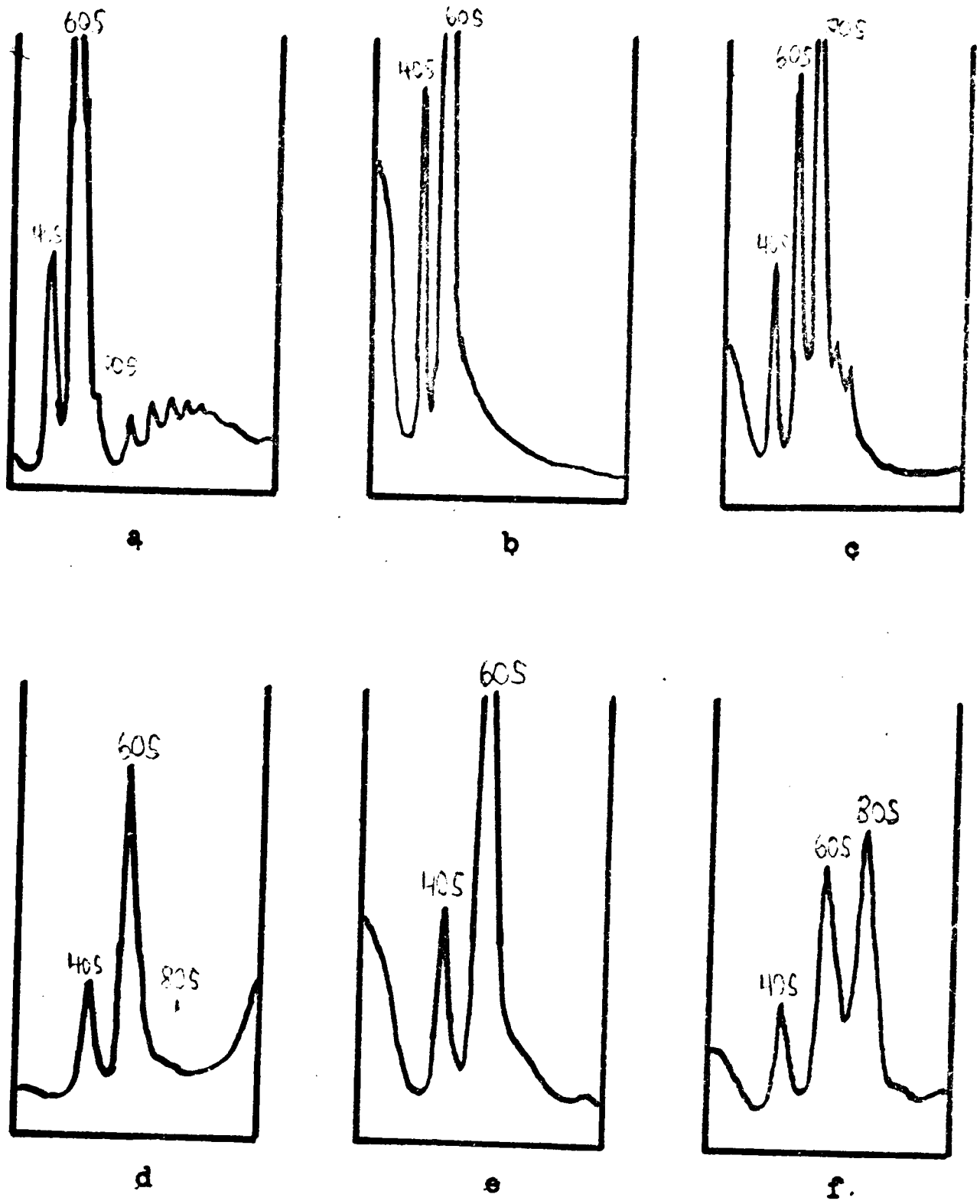
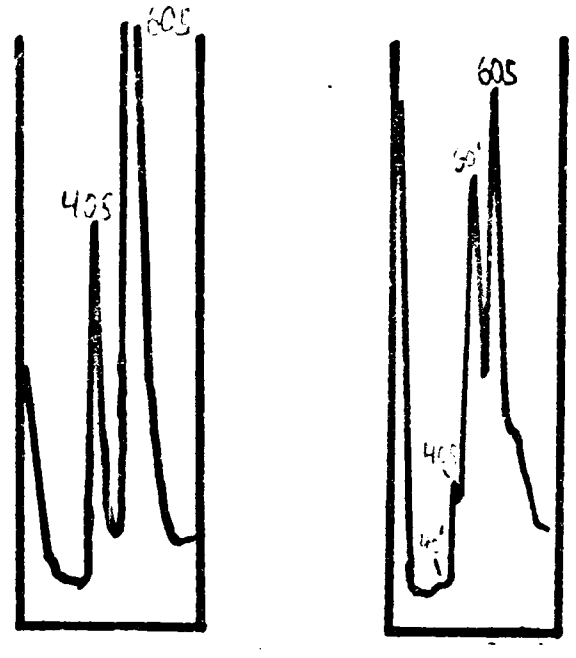


Figure 51
Sedimentation profiles of puromycin-treated Hela polysomes.

Abscissa: distance along gradient - top is to left.
Ordinate: A_{254}.
Hela ribosomes were resuspended with dissociation buffer with or without puromycin and/or RNase and then then treated exactly as during dissociation into subunits. Samples (2.4 ODU_{260}) were analyzed on 10 - 40% sucrose gradients (a,b,c) or 5 - 20% sucrose gradients (d,e,f) as described in 2.060. (a) and (d), without puromycin and RNase; (b) and (e), with puromycin only; (c) and (f), with RNase (1 ug/4.8 ODU_{260}) only.



a b

Figure 52

Sedimentation profiles of Hela subunit preparations.

Abscissa: distance along gradient - top is to left.
Ordinate: A_{254}.
Hela ribosomes were dissociated as in 2.060 and 8.0 ODU_{260} centrifuged on 5 - 20% gradients (a). 5 ODU_{260} of 60SP particles were treated with puromycin and centrifuged under identical conditions (b).

This trade-off between sample loading and resolution seems to be a general rule when preparing eukaryotic subunits (unlike their prokaryotic counterparts) as has been noted by others (192).

Peak subunit fractions were collected from these gradients and yielded approximately 5.5 ODU_{260} of 40S and 21.6 ODU_{260} of 60SP* subunits for every 50 ODU_{260} of Hela polysomes. When the isolated subunits were concentrated by centrifugation it was found that an elevated Mg++ concentration (20mM) was necessary for efficient recovery. Recovery of the 60SP fraction was 94-98% while that of the 40S subunit was 80-90%, the more efficient recoveries associated with those preparations containing higher subunit concentration.

As described below, the 60SP preparation was moderately contaminated with 40S subunits and hence was given a second puromycin-salt dissociation. Others (335) have also experienced this difficulty and found it necessary to resort to a second purification cycle. Usually 25 ODU_{260} of the 60SP pellets were resuspended in 1 ml of the dissociation buffer and the procedure of 2.060 repeated. Optimal separation was attained by centrifuging 5 ODU_{260}/gradient which yielded the profiles seen in Fig 51b. Typically, four peaks were seen:

40', a derivative of the 40S subunit that was assumed to be the same as that seen upon EDTA treatment of intact ribosomes (5.010);
40S, the native small subunit;
60', a derivative of the 60S subunit that was assumed to be the same as that seen upon EDTA treatment of intact ribosomes (5.010);
60S, the native large subunit.

The 60S fraction was carefully collected to minimize any 60' contamination. From 25 ODU_{260} of 60SP subunits, 10 ODU_{260} of 60S

*60SP is used here to distinguish this subunit fraction from the uncontaminated 60S subunits obtained after the second dissociation step.

subunits were obtained with 95% recovery upon pelleting by centrifugation. Overall, then, from 50 ODU_{260} of Hela polysomes, 5.5 ODU_{260} of 40S and 8.6 ODU_{260} of 60S subunits were obtained.

6.030 Subunit purity.

The purity of isolated subunits was assessed by analyzing their RNA composition and sedimentation profile. Integration of the area under the different RNA peaks seen after electrophoresis on 2.7% RNA gels allowed an approximation of the relative amounts (wt/wt) of 18S and 28S rRNA present in each preparation. After puromycin-salt treatment (as in the original dissociation reaction) of isolated subunit fractions, the relative amounts of the different sedimentation classes could also be calculated. As will become apparent, this method is less reliable than RNA gel electrophoresis for estimating contamination of one subunit by the other.

6.031 40S subunits.

Electrophoresis of the RNA isolated from 40S subunits (Fig 53e) showed only a minor 28S peak (more visible when scanned at a greater scale amplification) that usually accounted for a 1-2% contamination of 18S rRNA by the 28S rRNA. Re-centrifugation of isolated 40S subunits (Fig 54b) showed a major 40S peak and two minor peaks. One of the minor peaks sedimented with the 40' particle and was presumed to be due to degradation of the 40S during subunit preparation while the other represented the 60S subunit contamination, present maximally in amounts equal to 2% (wt/wt,RNA) of the 40' and 40S components combined.

6.032 60S subunits.

RNA gel electrophoresis (Fig 53c) shows no detectable contamination by 18S rRNA nor any degradation products of the 28S

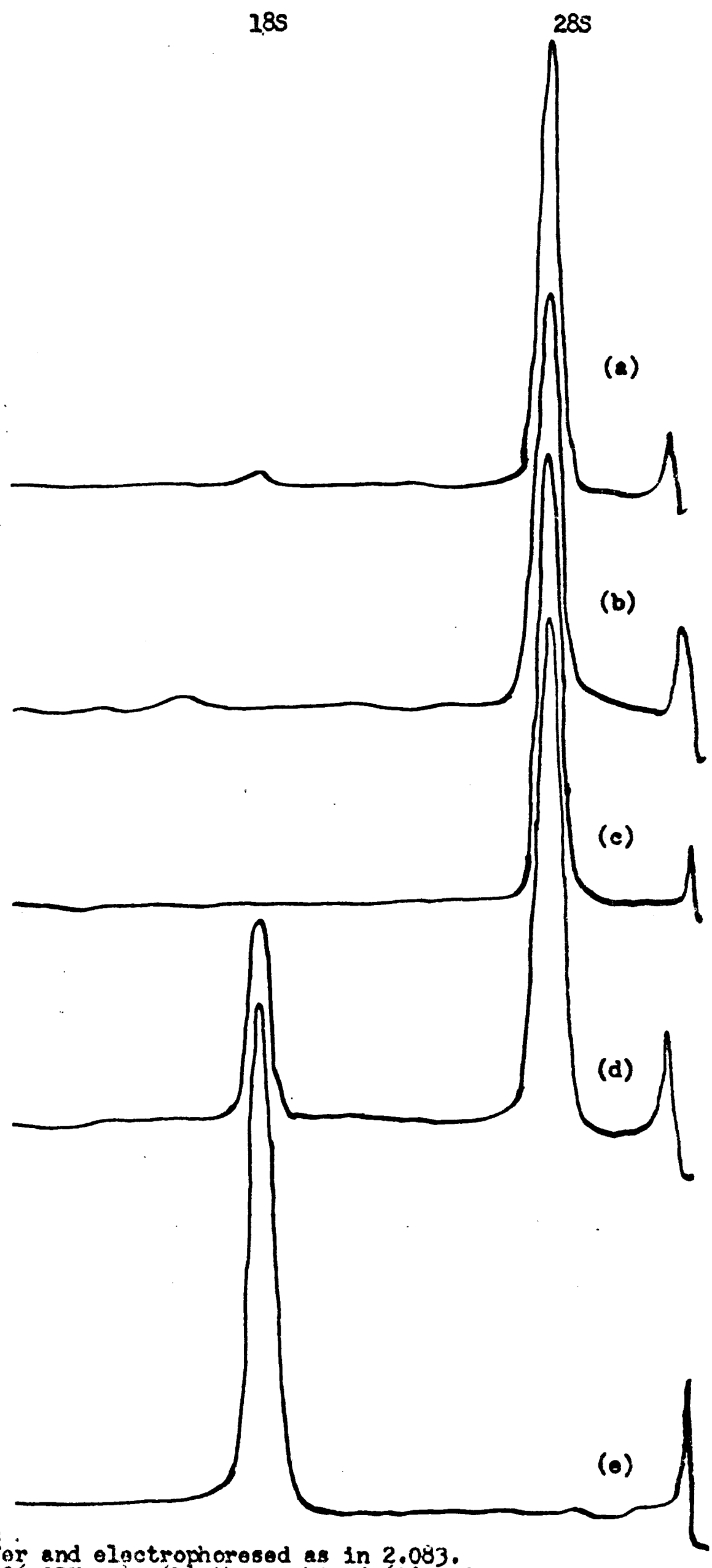


Figure 53

Scans of 2.7% RNA gels of isolated Hela subunits.

Abscissa: distance along gel - top is to right - only upper ½ of gel is pictured.
Ordinate: A_{260}.
Pelleted subunit fractions were dissolved in SDS buffer and electrophoresed as in 2.083. Samples were: (a) 60SP (0.36 ODU_{260}), (b) the combined 60° and 40S fractions (0.52 ODU_{260}) after the dissociation of the 60SP particles (see Fig 52b), (c) 60S (0.57 ODU_{260}), (d) cold phenol-chloroform extracted Hela polysomes (0.36 ODU_{260}), (e) 40S (0.55 ODU_{260}).

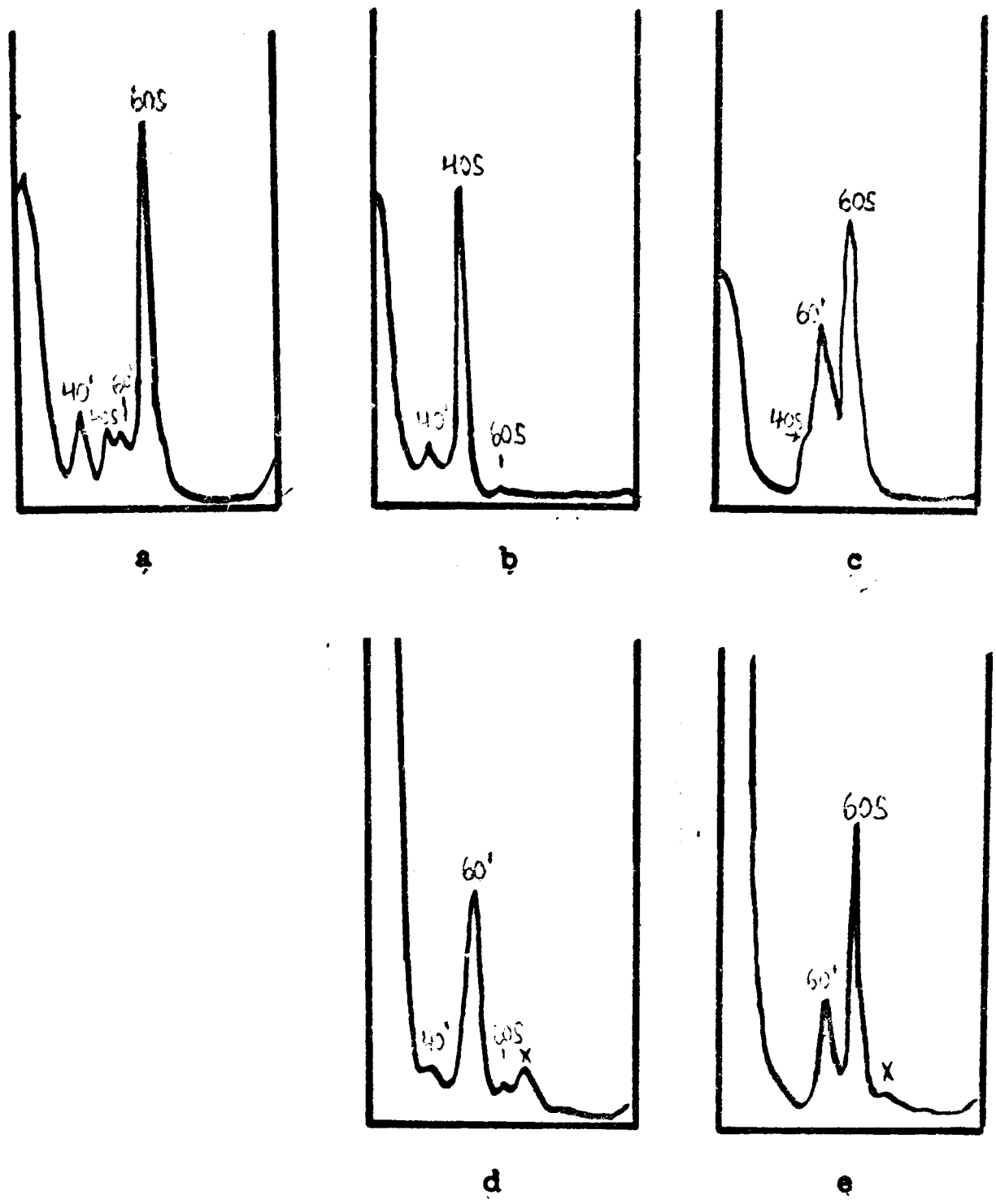


Figure 54
Sedimentation profiles of isolated Hela subunits.

Abscissa: distance along gradient - top is to left.
Ordinate: A_{254}. Full scale varies: 0-2 ODU for (b) and (c); 0-1 ODU for (a); and 0-0.5 ODU for (d) and (e). Pelleted subunit fractions were resuspended in dissociation buffer with puromycin, redissociated and centrifuged as in 2.060. Samples consisted of (a) Hela polysomes (1 ODU_{260}) that had been freeze-thawed in the dissociation buffer-puromycin before incubation at 37° C, (b) 40S (0.45 ODU_{260}), (c) 60SP (0.6 ODU_{260}), (d) the combined 60' and 40S fractions (0.26 ODU_{260}) collected after dissociation of the 60SP particles (see Fig 52b), (e) 60S (0.5 ODU_{260}). X denotes the position of dimers of the large subunit.

rRNA. In some preparations, however, some RNA was found migrating at the 18S position and suggested that the maximal contamination was 1%. Only two sedimentation classes were visible upon sucrose gradients (Fig 54e), the 60S subunit and its 60' derivative. However, due to the degradation products at the top of the gradient, no minor 40' or 40S peaks could be defined and the degree of impurity assessed.

6.033 60SP subunits.

This fraction was contaminated with 40S subunits to the extent of 5% when analyzed on RNA gels (Fig 53a) and was due either to incomplete dissociation or the dimerization of 40S (or 40') subunits. It may also represent an intermediate dissociation state when the 80S runs at about 60S as seen in EDTA dissociation (364). Sedimentation analysis of these subunits showed (Fig 54c) that about equal amounts of 60S and a mixture of 40S and 60' particles were present. Since the 40S and 60' particles cannot be completely separated, it was not possible to assess the purity by this method.

6.034 60' particles.

After dissociation of the 60SP fraction, the 60' peak and its 40S shoulder were collected together and found to be contaminated by 18S rRNA (Fig 53b). Recentrifuged upon sucrose gradients, such a preparation consisted mostly of 60' particles and 40' contaminants (Fig 54d).

6.035 Criteria of purity.

The meaning of purity as defined by these RNA gels and gradients is somewhat misleading. Sedimentation profiles do not allow adequate resolution between 60' and 40S particles and they are of little value in assessing purity. Nonetheless they have been commonly used by others for this purpose; it is apparent from

previous reports that "pure" 40S subunits that give a single gradient peak may actually be contaminated with 60' particles that have not been resolved adequately. It is for this reason that contamination should be assessed from RNA gels.

The RNA gel profiles yield a ratio of 18S to 28S rRNA based on the absorbance for each peak. But since the molar absorbance of 28S rRNA is about twice that of 18S rRNA, these calculations incorrectly estimate the subunit cross-contamination. Since it is the contamination of the ribosomal proteins, not rRNA, of one subunit by those of another which is relevant, this correction is important. Thus the contamination of the 40S by the 60S was 2% when based on the ratio of 28S to 18S rRNA but is actually half that, or 1%, with respect to the amounts of subunits (and ribosomal proteins) themselves. The 1% contamination of the 60S by 40S subunits calculated on the basis of the 18S to 28S rRNA is double that, or 2%, with respect to the subunits themselves. Similarly, the previously estimated 5% contamination of the 60SP (6.033) is actually 10% with respect to molar amounts of subunits. Since any cross-contamination of the subunits above 10% was clearly visible on the microgels, this explains why it was necessary for the 60SP subunits to undergo a second puromycin-salt dissociation.

6.040 Two-dimensional gels of isolated subunits.

The gel patterns of these subunits have not been labelled so as to correspond with any previously published gels or with those in previous chapters. Nor has there been much of an attempt to interpret each protein spot as more than one ribosomal protein unless the spot shape or coloring so dictated. Each sample was run a number of times at different concentrations but no other ribosomal proteins appeared. No anionic proteins were found

for either subunit but this may be due to the failure to use sufficient amounts of subunit ribosomal proteins.

The gel pattern of the 40S subunit (Fig 55) shows at least 24 distinct spots. The relative intensities of most of these spots were identical with those in the patterns of the intact Hela polysomes (Fig 23).

The 60S subunit pattern is not as distinct as that of the 40S subunit. Twenty-four ribosomal proteins are clearly seen in Fig 56 while 6 more are visible only with larger amounts of sample. As shown in the schematic drawing (Fig 58) of both 40S and 60S subunits, L23 has been regarded as one spot for only with larger amounts of sample does it show two spots (of equal intensity), suggesting a protein concentration effect.

When equal amounts of both subunits are combined and the artificial mixture run on the 2-D gels (Fig 57), a pattern resembling that of the polysomes (Fig 23) was created. This pattern was also a superimposition of both subunit patterns and implies that there are no protein interactions during electrophoresis. It should also be noted that S3, previously regarded as a "tail" of another protein was found to be a ribosomal protein distinct from L5, though running close to it.

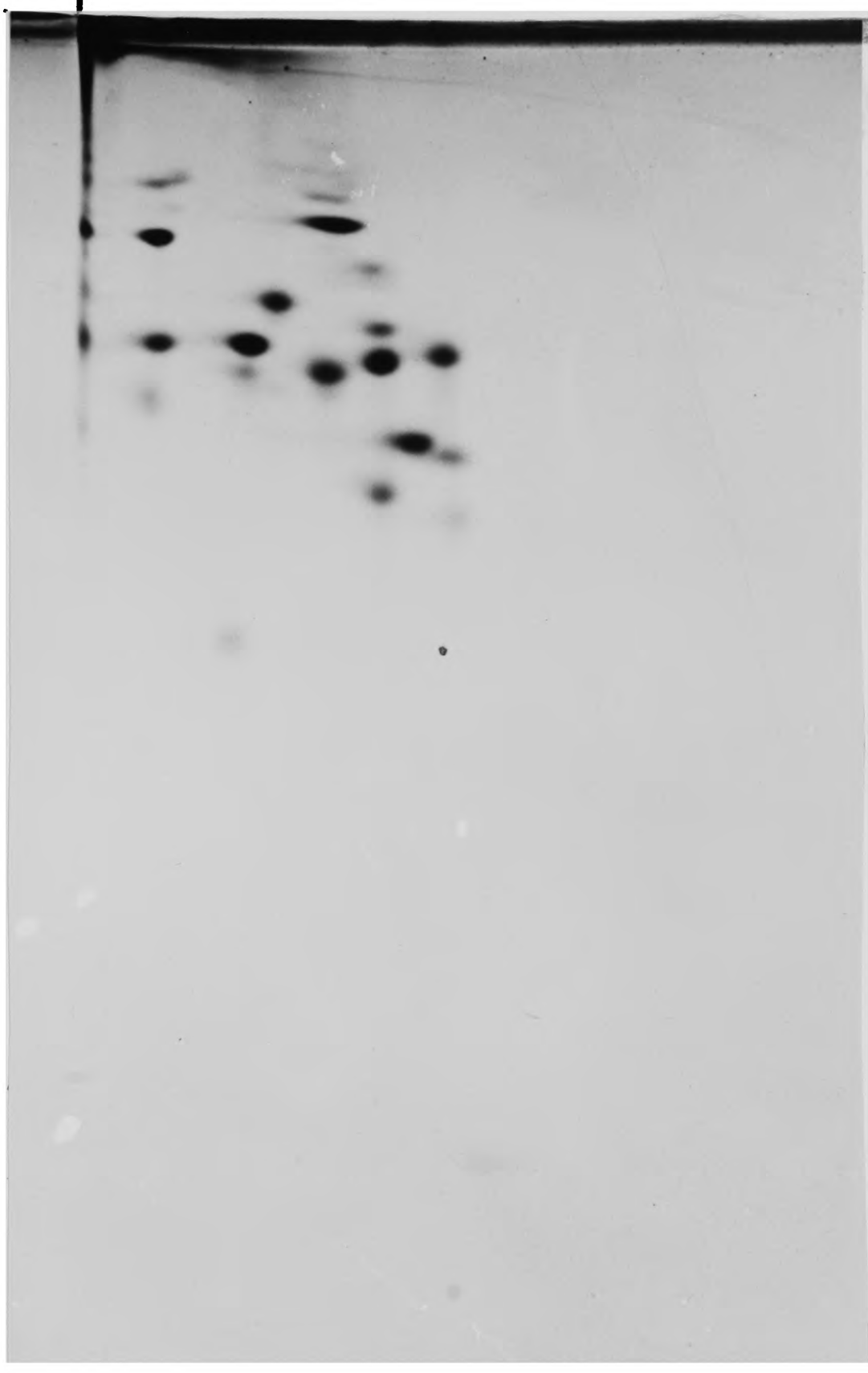
It can be seen that there are at least two areas of overlap between the subunit ribosomal proteins. One such area is that including proteins S13,L13, L14 with L13 and L14 running separately from each other only on the large subunit pattern (Fig 56). The second area is that consisting of proteins L19 and S17 (Fig 58). Others (162,335,336) have found 3 such sets of overlapping proteins, two of which correspond to those here while the third is associated with proteins S7 and L9 (which run separately

+ 2-D

ORIGIN

+ 1-D

- 1-D



- 2-D

Figure 55
Two-dimensional pattern of Hela 40S ribosomal subunit cationic proteins.

Microgel electrophoresis of the protein from 3.2 ODU_{260} of 40S subunits was carried out exactly as in 2.072.

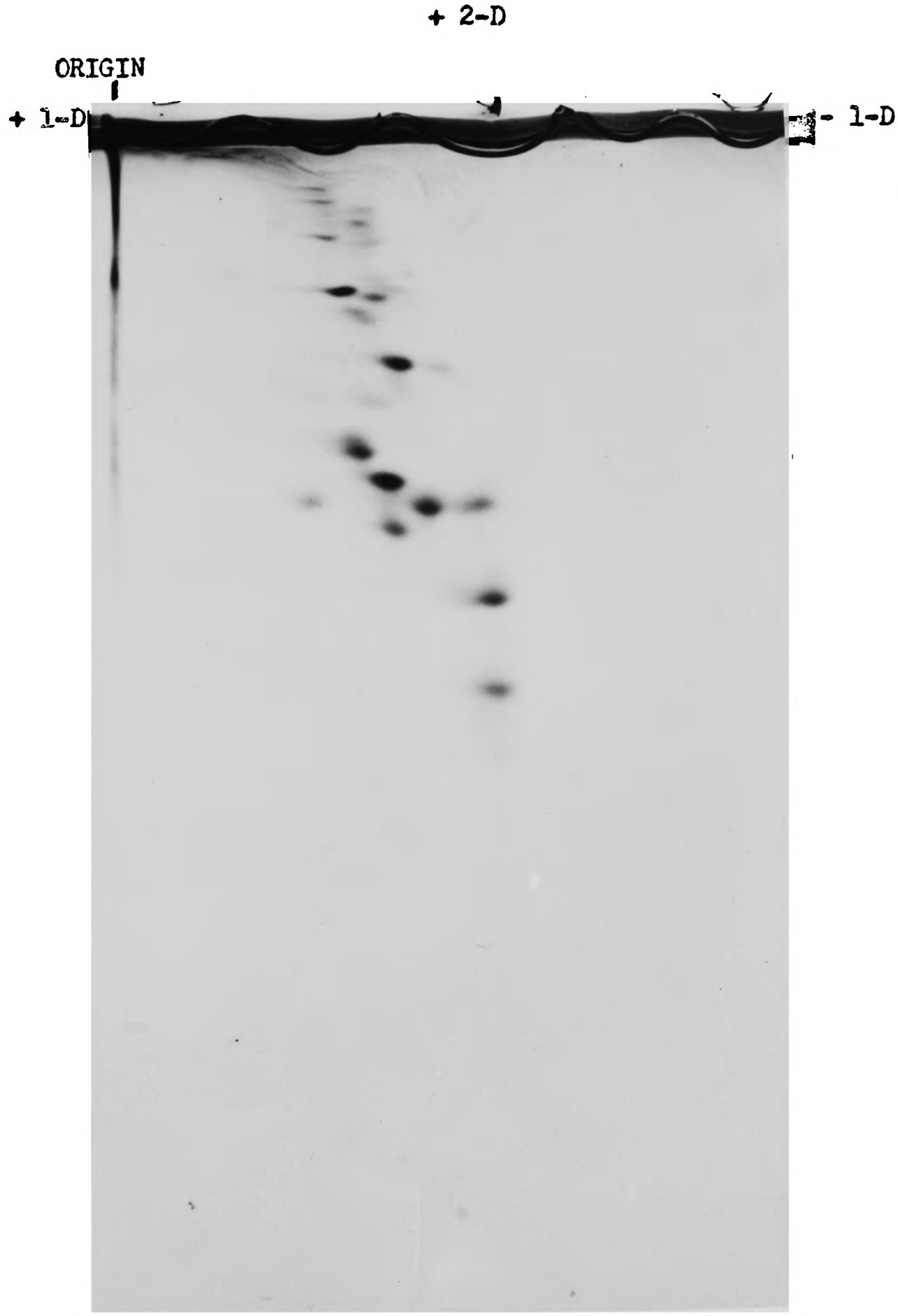


Figure 56
Two-dimensional pattern of Hela 60S ribosomal subunit cationic proteins.

Microgel electrophoresis of the proteins from 7 ODU_{260} of 60S subunits was carried out exactly as in 2.072.



Figure 57
Two-dimensional pattern of the cationic proteins from a mixture of Hela 40S and 60S ribosomal subunits.

Microgel electrophoresis of a mixture of the proteins from 9 ODU_{260} of 60S subunits and 3.2 ODU_{260} of 60S subunits was carried out exactly as in 2.072.

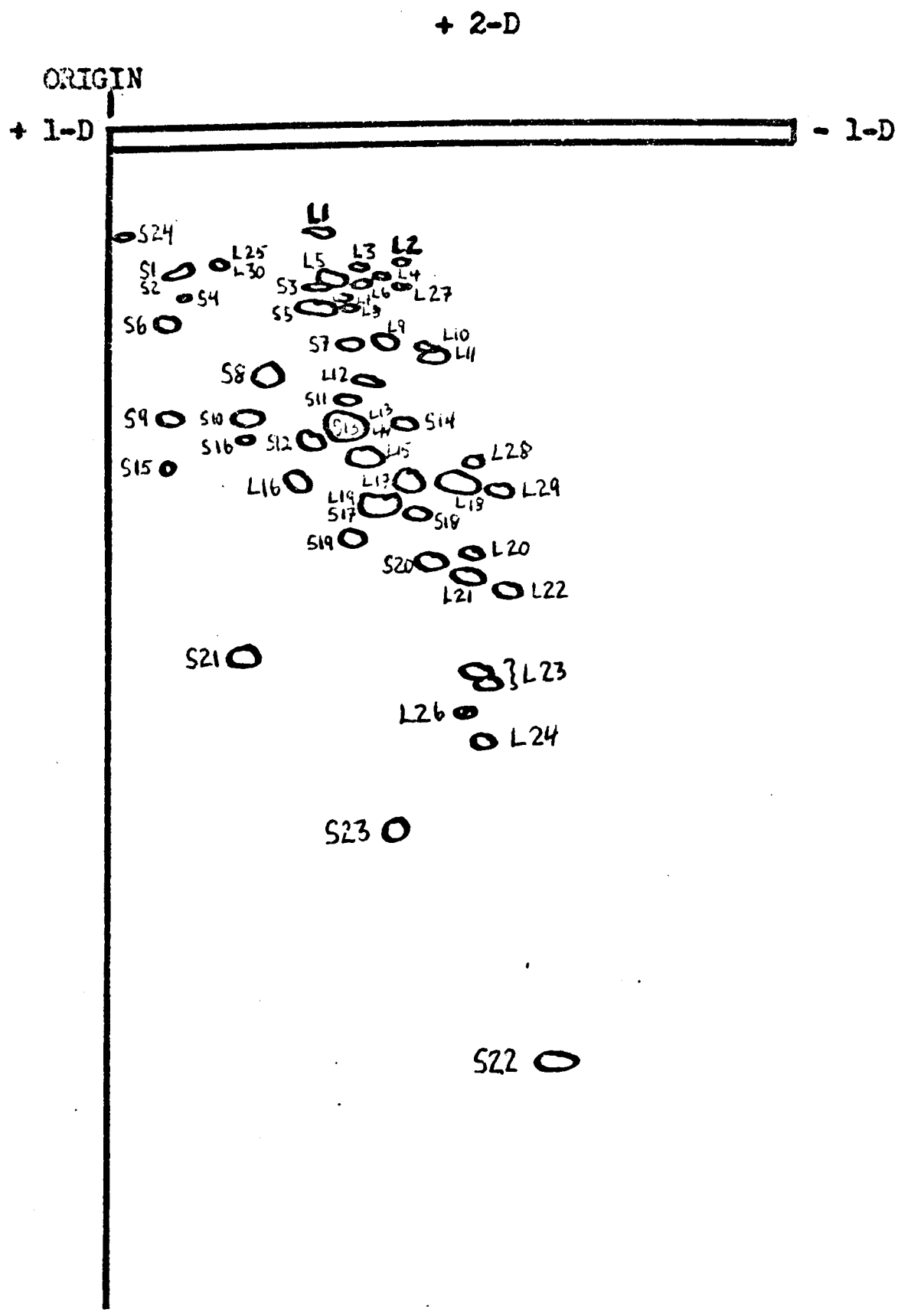


Figure 58
Schematic diagram of cationic ribosomal proteins of Hela subunits.

here). Whether such proteins of identical mobility but of different subunit are truly distinct proteins has not been shown chemically. It is possible, as Wool has shown for another pair of supposedly overlapping ribosomal proteins (336), that such proteins are actually due to a single ribosomal protein species being apportioned to either subunit or both depending solely on the relative amounts of monovalent and divalent cations present during subunit preparation.

Though no new ribosomal proteins have been uncovered in studying these subunits, a number of ribosomal proteins present in the polysome pattern were not found to be present in either subunit. Of the 69 cationic ribosomal proteins of the Hela polysome pattern (Fig 24), only 54 have been accounted for here. This may be due to the fact that these proteins are only associated with the polysome. Many of those ribosomal proteins lost upon subunit preparation were those present less intensely in the polysome pattern and they may be translational factors. Almost all strongly staining ribosomal protein spots on the polysome pattern did not fail to be associated with one or the other subunit in these studies.

Another possibility, of course, is that these "lost" proteins are true ribosomal proteins that were less strongly attached to the particles under these conditions of KCl and Mg and were washed away; those remaining were the more strongly bound ribosomal proteins. Indeed, the 60' particle which is a derivative of the 60S subunit was found to have lost approximately 40% of the ribosomal proteins seen on the 60S pattern (gel not shown). This suggested mechanism of ribosomal protein loss becomes more important in Chapter 7 where it was found that only some of the large subunit

ribosomal proteins were attached to the precursor of the large subunit; those so bound were almost identical to those more strongly stained spots seen in Fig 56.

McConkey (237) has recently "re-evaluated" the composition of mammalian ribosomal subunits and found that subunits prepared with methods identical to those used here were active and yet had considerably less protein than subunits isolated in other ways. The critical factor was thought to be the KCl concentration in the gradients. Whether this was a specific loss of only certain ribosomal proteins or the loss of a fraction of all ribosomal proteins was not investigated. The data here suggests the loss may be specific and may explain why a number of ribosomal proteins were lost from subunits prepared by this method.

6.050 Discussion.

During the course of the present work, two studies appeared that mapped the proteins of human ribosomal subunits. In one, Peeters et al (274) have prepared human placental ribosomal subunits exactly as described here. The 40S picture so obtained was identical to Fig 55 except that an additional protein was found midway between S8 and S5. The 60S gel picture was identical to Fig 56 except for 3 additional ribosomal proteins that appeared the same as spots 20,21 and 67 in Fig 24. From these gels the authors claimed the small subunit to have 28 proteins and the large, 36. The discrepancy between these numbers and the lesser figures claimed in the present work is one of interpretation of the gel patterns. Those subunit patterns presented in this chapter have been conservatively judged when assigning proteins to one or the other subunit while Peeters et al claimed some single spots to

represent two ribosomal proteins.

Ueer et al (381) have analyzed the subunits of ribosomes from human tonsils and found 31 ribosomal proteins associated with the small subunit and 34 with the large. Again, the pictures of the gels were virtually identical to those here with the same exceptions as in the placenta patterns. The additional ribosomal protein spots seen by these authors may be accounted for by some cross-contamination and by the numerous small smudges that are most likely artifacts. A more reasonable counting of these patterns agrees with the results of Peeters et al (16).

The subunit gel pictures obtained here for the Hela ribosomes are also the same as those for rat liver (335) and rabbit reticulocytes (336) except for those few species-specific differences. But though the pictures are the same, the total ribosomal proteins assigned to each subunit are higher than the values mentioned in this chapter [24 and 30 here vs. 31 and 39 (336) for the small and large subunits, respectively] when the more faintly appearing spots were taken into account.

It is curious to note, however, that the subunits prepared in all these studies are supposed to be active in protein synthesis and yet some of the supposedly "ribosomal" proteins are present in such small amounts with respect to the rest. If so, then they must either be able to function without these proteins seen only faintly in the 2-D gel pattern or acquire them anew from the in vitro protein synthesizing system. Alternatively these faint spots may be indicative of heterogeneity of the ribosomes or represent only that minority of ribosomes which are functional in in vitro tests. As discussed before (Chapter 3), how many of these subunit ribosomal proteins are "ribosomal" cannot be determined from 2-D gels alone and must await further studies.

6.060 Summary.

Hela polysomes were dissociated into their 60S and 40S subunits by puromycin in the presence of high concentrations of monovalent cation. The isolated subunits were characterized by their RNA composition and sedimentation profile and found to be at most 2% cross-contaminated with the other subunit. Of the 69 cationic ribosomal proteins appearing in the 2-D gel pattern of the Hela polysomes, 24 were associated with the small subunit in roughly the same intensity as in the polysome pattern. The large subunit contained 30 ribosomal proteins of which 2 overlapped with the ribosomal proteins of the small subunit. Whether the 15 ribosomal proteins not present on either subunit are true ribosomal proteins or are factors of protein synthesis associated only with the polysomes could not be determined here.

CHAPTER 7

THE PROTEIN COMPOSITION OF HELA NUCLEOLAR RIBOSOMAL PRECURSOR PARTICLES

7.000 Introduction.

The nucleolus has long been recognized as the site of ribosome biosynthesis. Early observations of the nucleolus with the electron microscope showed it to be composed of numerous ribonucleoprotein (RNP) particles of 150 Å diameter that were identical to those found in the cytoplasm (14,171,202,303,360). Arranged either loosely (pars amorpha) or tightly compacted with fibrillar material (pars compacta), these granules labelled intensely with radioactive uridine (339). Loss of nucleolar function either through microbeam u.v. irradiation (285), genetic mutation [such as the anucleolate mutant of Xenopus laevis (43)], or low levels of actinomycin D (281) resulted in the absence of new rRNA synthesis.

That the nucleolus contained significant quantities of RNA had been demonstrated by histochemistry (37). Edstrom (92) then showed that the RNA isolated by microdissection from nucleoli and cytoplasm of spider oocytes were of identical nucleotide base composition while that of the nucleoplasm differed. When the amino acid composition of isolated pea nucleolar protein was compared to that of cytoplasmic ribosomes, Birnstiel et al (24,25) found them to be very similar.

But these morphological and chemical studies on the nucleolus only related the nucleolus and ribosome structurally. The advent of actinomycin D permitted more precise kinetic studies to describe the flow of nascent ribosomes from the nucleolus to the cytoplasm and a number of nucleolar rRNA precursors were observed in various

stages of their migration to the cytoplasm (325). With the development of acrylamide gel electrophoresis of RNA (219), a more detailed description of the formation and processing of 45S rRNA was undertaken. [The results of these studies are discussed in numerous reviews (63,278,283,284) and are summarized in Chapter 1.] Other kinetic studies showed that the small subunit entered the cytoplasm more rapidly than the large (118, 282) due to a smaller nucleolar and nuclear pool size. Such new 40S subunits, upon entering the cytoplasm, showed a characteristic density (286) lighter than its cytoplasmic counterpart; presumably they then lost protein to assume the density of mature cytoplasmic subunits (287). Such new 40S subunits were not associated with mRNA (183,213).

Initial attempts to isolate these nuclear ribosome-like particles were of doubtful success due to the inability to eliminate contamination by cytoplasmic ribosomes. Pogo et al (299) reported the isolation of such "nuclear ribosomes" from thymus nuclei and showed with the electron microscope that their extraction correlated with the loss of ribosome-like granules from the tissue. Such preparations showed sedimentation properties, subunits and EDTA sensitivity just like the cytoplasmic ribosomes. Their insensitivity to ribonuclease supposedly proved their non-cytoplasmic origin since cytoplasmic ribosomes were said to be degraded under these conditions. However, such ribonuclease treatment only inactivates, not degrades, polysomes. Given the propensity for cytoplasmic contamination to remain attached to isolated nuclei, it is most likely that these "nuclear ribosomes" were of cytoplasmic origin.

Another major attempt to isolate these nuclear ribosomal

precursors was undertaken by Birnstiel and his coworkers (21, 22,23,24) using plant cells (pea seedlings and cultured tobacco cells). They found three particles with sedimentation coefficients of 81S, 60S and 38S that could be isolated from nuclei and whose amino acid composition and sedimentation characteristics were very similar to those of cytoplasmic ribosomes. Though several attempts were made to show the degree of cytoplasmic contamination, none adequately demonstrated that the plant nuclei so isolated did not contain numerous cytoplasmic "tags" (386).

Tamaoki and Miller (363) were first to provide evidence that identified a RNP isolated from the nucleus as a precursor to the cytoplasmic ribosome. Treatment of whole nuclei with deoxycholate yielded a nuclear precipitate from which 53S particles (containing 45S rRNA) were obtained upon addition of deoxyribonuclease. These particles were said to cosediment with 53S particles from the cytoplasm but actually showed a very broad distribution upon centrifugation. Upon close inspection of their data it may be seen that these particles actually sedimented faster than the 53S reference particle. On Geon column electrophoresis, the nuclear and cytoplasmic particles were identical.

Similar experiments were also conducted by Yoshikawa-Fukada (421) with FL cells. A "presome" fraction was isolated that showed a broad sedimentation profile centered at 80S (between 70 and 90S) and contained ribosomal precursor RNA (32S). These precursors were shown to be distinct from cytoplasmic ribosomes in that they failed to dissociate into subunits in the presence of EDTA and were very susceptible to ribonuclease. Further studies showed the protein part of the precursor to come from a preformed pool, mature in the nucleolus, and then be released to the cytoplasm.

Amphibian oocyte nuclei also yielded RNP particles (313). A 50-55S particle (containing 30-40S RNA) was said to be the precursor to the large cytoplasmic subunit while a 30S particle (containing 20S rRNA) was thought to be the precursor to the small. This is the only instance where such a precursor to the small subunit had been described. But all these results are somewhat suspect due to the fact that these particles were isolated directly from nuclei lysed in deoxycholate with little assurance given that these RNP particles were not due to cytoplasmic contamination or nucleoplasmic precursors (vide infra).

Izawa and Kawashima (169,170) were among the first to show using 1-D urea gels that the protein composition of the RNP isolated from Ehrlich ascites cells was similar to that of cytoplasmic ribosomes. However resemblance was too close in other ways in that only 28S and 18S rRNA were found and, as before, these precursors must have been cytoplasmic contaminants or nucleoplasmic precursors.

Any further characterization of these particles was thus limited by the available cell fractionation techniques. But with the perfection of Penman's cell fractionation techniques (277,279) nuclei could be treated with detergents which successfully removed the nuclear rim of cytoplasm and the outer layer of the nuclear membrane without altering the intranuclear structure (158,277). This successfully eliminated significant contamination by cytoplasmic ribosomes. Upon treatment by deoxyribonuclease in the presence of high salt concentrations, nucleoplasmic and nucleolar fractions could then be isolated (279).

Given these techniques, Warner and Soeiro (393) extracted two ribosomal precursor particles from isolated Hela nucleoli with buffers containing EDTA and a reducing agent. When centrifuged in

the presence of EDTA, both sedimented faster than cytoplasmic ribosomes (which were totally converted to subunits). The 80S precursor particles contained about equal amounts of 45S and 32S rRNA while the 55S precursor particle contained 32S rRNA with only a small amount (5%) of 28S rRNA. When the protein of the 55S particles were coelectrophoresed on 1-D SDS gels with those of the larger cytoplasmic subunit, the similarity was striking. Of the 13 detectable peaks of the large subunit pattern, 12 ran in positions identical with those of the 55S particles. Recent modifications of this technique (196,273) have served to reduce the amount of contamination of the 80S precursor with the 55S precursor and prove that these precursors were not simply an artifact created by the non-specific adherence of basic, nuclear proteins to RNA during the isolation procedure as suggested by the work of Baltimore and Huang (9).

Soeiro (340) has sought to determine those factors which bind the nascent ribosomes into the nucleolar network. Divalent cations were important for maintaining intact protein-protein and/or protein-RNA bridges and were removed only by chelating agents. Electrostatic interactions also seemed to maintain the structure, for only at low ionic strength (and strong repulsive forces) could precursors be extracted. Disulfide bonds did not seem to be as important for increased temperature could replace the requirement for a reducing agent during extraction.

Aside from these precursors which contain the various forms of precursor rRNA and which are located mostly in the nucleolus, two precursors may also be found in the nucleoplasm and appear to be the end-products of nuclear maturation in transit to the cytoplasm. Such precursors exist as subunits (383) and differ from

their cytoplasmic counterparts only in being less dense (having more protein). Whether these subunits become associated with or assist in the transit of mRNA to the cytoplasm is still controversial.

Though these precursors of Warner and Soeiro (393) have been those most carefully defined and most widely used, it is important to mention the experiments of Liau and Perry (212) with similar particles from L cells. These workers have employed entirely different methods to isolate particles under non-chelating conditions, unlike the harsh EDTA treatment of Warner and Soeiro. Three such precursor particles were found: a 62S particle with 32S rRNA and some 28S rRNA; a 78S particle containing 36S rRNA and some 32S rRNA; and a 110S particle with 45S, 36S and 32S rRNA. When centrifuged in the presence of EDTA, the 62S and 110S precursor ran like the 55S and 80S particles previously described. However, the 78S precursor (running at 65S in EDTA) showed no such correlation and was shown to have been degraded by the salt conditions employed in the method of Warner and Soeiro (393). This thus suggests that other nucleolar particles in the maturation step might be isolated and characterized.

In all these experiments concerning the nucleolar ribosomal precursor particles, little consideration has been given to describing their protein composition. Given the present knowledge of the complexity of the eukaryotic ribosome, it is relevant to determine exactly which ribosomal proteins are added to the precursor rRNA at the various stages of maturation. Warner and Soeiro (393) and Pederson and Kumar (273) were able to show correspondence between the 55S precursor and the cytoplasmic large subunit but presented no similar data for the 80S precursor. Since the 80S

precursor contains 45S rRNA which is the precursor to the rRNA of both small and large subunits, it was expected that it would also contain ribosomal proteins of both subunits. But the 1-D SDS gel electrophoresis provides insufficient resolution between proteins of the subunits to make this distinction or even provide a detailed description of all the ribosomal proteins on the 55S particle. Such gels identify 15 bands at most on the large subunit which from 2-D gels is known to have approximately 40 proteins (Chapter 6).

An alternate approach to defining the ribosomal protein composition of both 55S and 80S precursor particles was that of Shepherd and Maden (333) who mapped the peptides of methionine-labelled ribosomal proteins after tryptic digestion. They were able to discern spots on peptide maps characteristic of each cytoplasmic ribosomal subunit and such a map of the 55S precursor was very similar to that of the cytoplasmic large subunit. Of the 11 or so spots characteristic of the 40S subunit, only 3 appeared on the 80S precursor while most of those of the large subunit were present. But those small subunit spots on the 80S precursor patterns were variable in position and intensity and were present in amounts one-third or less than the corresponding molar amounts of large subunit material. Though suggestive that the 80S precursor has some (at most 3) ribosomal proteins of the small subunit, this data is difficult to interpret for in no case does it tell specifically which ribosomal protein(s) of the small subunit (and large subunit) were present.

Aside from these attempts to define the set of ribosomal structural proteins that associate with the precursor, two other classes of protein are of concern with respect to ribosome biosynthesis. The

first consists of a small number of ribosomal proteins that are found in cytoplasmic ribosomes but are not added in the nucleolus. In Hela cells, 5 or 6 such ribosomal proteins of the cytoplasmic ribosome are in rapid equilibrium with protein synthesized during pulse labelling conditions while the other ribosomal proteins only appear after sufficient time for nucleolar processing (195, 390). Similar kinetics were shown by 3 ribosomal proteins of rat liver ribosomes (380) and these proteins were not found in isolated nucleolar precursors. What functional role this cytoplasmic exchange plays is not known.

The second class of proteins consists of those stably associated with the nucleolus which may assist in the processing of nascent ribosomes. They are characterized by their high molecular weight, slow turnover and ability to accumulate in the nucleolus even without RNA synthesis, unlike ribosomal structural proteins (196,340,341,379,380).

Since previous attempts to analyze these three different classes of protein associated with ribosome biosynthesis (ribosomal structural proteins, stable nucleolar proteins, and rapidly equilibrating cytoplasmic ribosomal proteins) have been frustrated by the lack of resolution by 1-D gels, the development of 2-D gel electrophoresis affords a promising alternative. Though the original methods demanded milligram quantities of protein and obviated their convenient use for the study of the Hela precursor particles, various modifications of the 2-D method (2.072) have allowed the analysis of microgram quantities of protein available from isolated precursor particles. The experiments described in this chapter have employed such a method to compare the ribosomal protein patterns of Hela 80S and 55S precursors with those of cytoplasmic 40S and 60S subunits.

7.010 Precursor isolation and purity.

The isolation technique employed here (2.120) is identical to that of Pederson and Kumar (273) and varies only slightly from that of Warner and Soeiro (393) to allow cleaner separation of the two precursors. Routine preparations of precursors were made from an initial quantity of 1.2×10^9 Hela cells. Nuclei showed at most 5% unbroken cells before detergent treatment while detergent-washed nuclei showed at most a 3% (by weight RNA) contamination with cytoplasmic ribosomes when the amount of 18S rRNA was determined from RNA gels. Since some 18S rRNA is normally present within the nucleoplasm of isolated nuclei (383) the true cytoplasmic contamination must be even less.

Upon preparation of nucleoli from washed nuclei, the nucleolar pellet contained no 18S rRNA upon electrophoresis in 2.7% polyacrylamide gels. Since these gels are capable of picking up 18S rRNA contamination as low as 1% of the total RNA, these experiments rule out significant contamination by cytoplasmic ribosomes. Extraction of the nucleoli with buffer containing EDTA and dithiothreitol yielded the pattern shown in Fig 59a after centrifugation. The two peaks, one at 80S and the other at 55S are identical to other preparations previously published (273,333,393). From 1.2×10^9 cells, a typical recovery was 7 ODU_{260} of 55S and 5 ODU_{260} of 80S precursor.

When Hela polysomes were resuspended in the extraction buffer and centrifuged with the nucleolar extract, they ran in the positions indicated in Fig 59a. The small and large subunits run as their EDTA derivatives 40' and 60' with the 60' cytoplasmic subunit running slightly slower than the 55S precursor. Though the RNA composition of these precursors was exactly that previously described (273, 393)

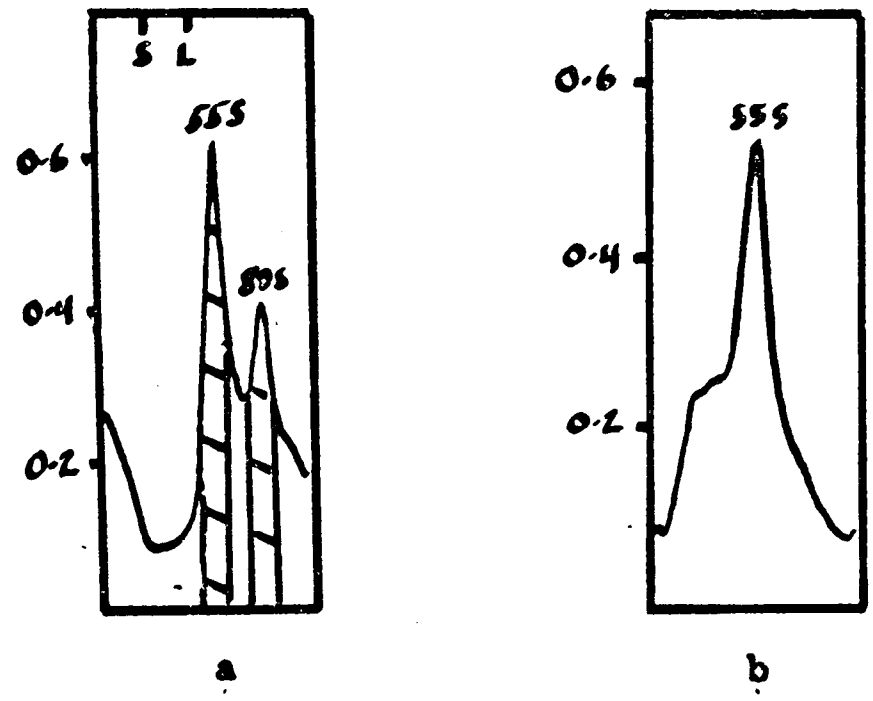


Figure 59
Sedimentation profiles of Hela nucleolar precursor particles.

Abscissa: distance along gradient - top of gradient to left.
Ordinate: A_{254}.
Ribosomal precursor particles extracted from Hela nucleoli by the EDTA method (2.120) were centrifuged on gradient (a) as in 2.120. Hatched fractions denote those collected for analysis on 2-D gels. S and L indicate the locations of peak fractions for Hela small and large subunits, respectively, when centrifuged on these gradients. Nucleolar particles extracted with pyrophosphate (2.120) were run as described in 2.120 on gradient (b).

when preparations were run on 2.7% polyacrylamide gels, it was found that cytoplasmic contamination could be more easily detected by looking for 40' peaks (or 60' shoulders on the 55S peak). Artificial mixing experiments showed that 5% (wt/wt, RNA) contamination by cytoplasmic ribosomes could easily be detected in this way. This became the primary criterion of purity in all these experiments though no such impurity was ever found and all preparations were identical.

Several attempts were made to extract precursors with pyrophosphate instead of EDTA (2.120) and such extracts showed a different profile upon centrifugation (Fig 59b). The 55S subunit was extracted in greater quantities than with the EDTA method but only a faint shoulder on the 55S peak suggested the presence of the 80S precursor. In addition, some material sedimenting slower than 55S formed an ill-defined peak. Whether this is another precursor or not was not determined and these pyrophosphate particles were not further characterized.

7.020 2-D gel electrophoresis of isolated precursors.

Electrophoresis of these ribosomal precursor particles has been frustrated by the failure of extracted proteins to solublize completely in the sample buffer (2.072). Nevertheless, the 2-D gel patterns so obtained were highly reproducible and variations in the times of electrophoresis and amount of sample have not revealed any other spots than those depicted below.

When gels were run to separate the anionic precursor proteins a pattern considerably different from that of the polysomes was found. The 55S precursor pattern (Fig 60) showed 21 spots (some quite faint) none of which ran identically to any of the cyto-



Figure 60
Two-dimensional pattern of anionic proteins of Hela 55S precursor particles.

Microgel electrophoresis of the proteins from 9.2 ODU_{260} of Hela 55S particles run exactly as described in 2.072.

plasmic ribosomal anionic proteins (Figs 30,31). The 80S precursor pattern was almost identical to that of the 55S (Fig 61) except that it lacks three spots (labelled 1,2,3 on the schematic diagram in Fig 64). The small amounts of protein in most of these anionic spots relative to that of the cations makes it difficult to conclude much from these gels except that none of the anionic cytoplasmic ribosomal proteins (including 64, the supposedly important L7/L12 analogue) are present in these precursors.

The cationic ribosomal proteins of the 55S precursor produce a pattern (Fig 62) showing 52 distinct spots. With the exception of the numerous high molecular weight spots on this gel, the overall pattern was remarkably like that of the 60S cytoplasmic subunit (Fig 56) even with respect to the relative intensities of the spots. In fact, 18 spots (usually among the most intensely stained of all the spots on patterns of cytoplasmic ribosomal proteins) on the 55S pattern correspond exactly to the 60S subunit ribosomal proteins as is summarized in Fig 64. Another 5 spots (marked "L" in Figure 64) were also probably large subunit ribosomal proteins. This is based on the fact that though it was not possible to assign these proteins to subunits in Chapter 6, proteins of identical mobility in other species are assigned to the large subunit.

The 80S precursor particle yielded a 2-D cationic ribosomal protein pattern (Fig 63) that was almost identical with that of the 55S precursor except that spots L5, L12, L18 and 4 were missing (see schematic diagram, Fig 64). Spots L10 and L11 were greatly reduced in their intensity. Hence the 80S precursor pattern showed 48 spots of which 15 were distinctly large subunit ribosomal proteins.

Neither the 80S nor the 55S patterns revealed spots that could

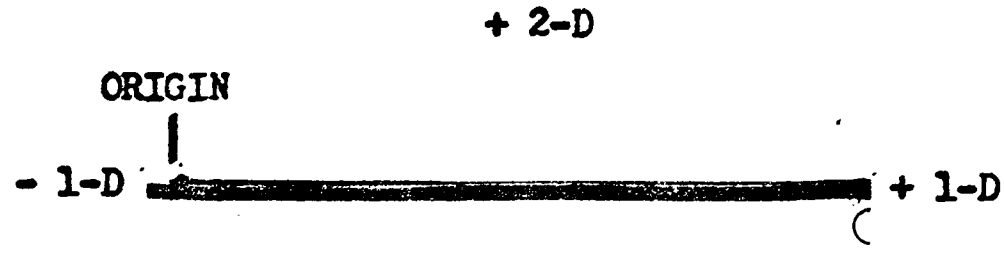


Figure 61
Two-dimensional pattern of anionic proteins of Hela 80S precursor particles.

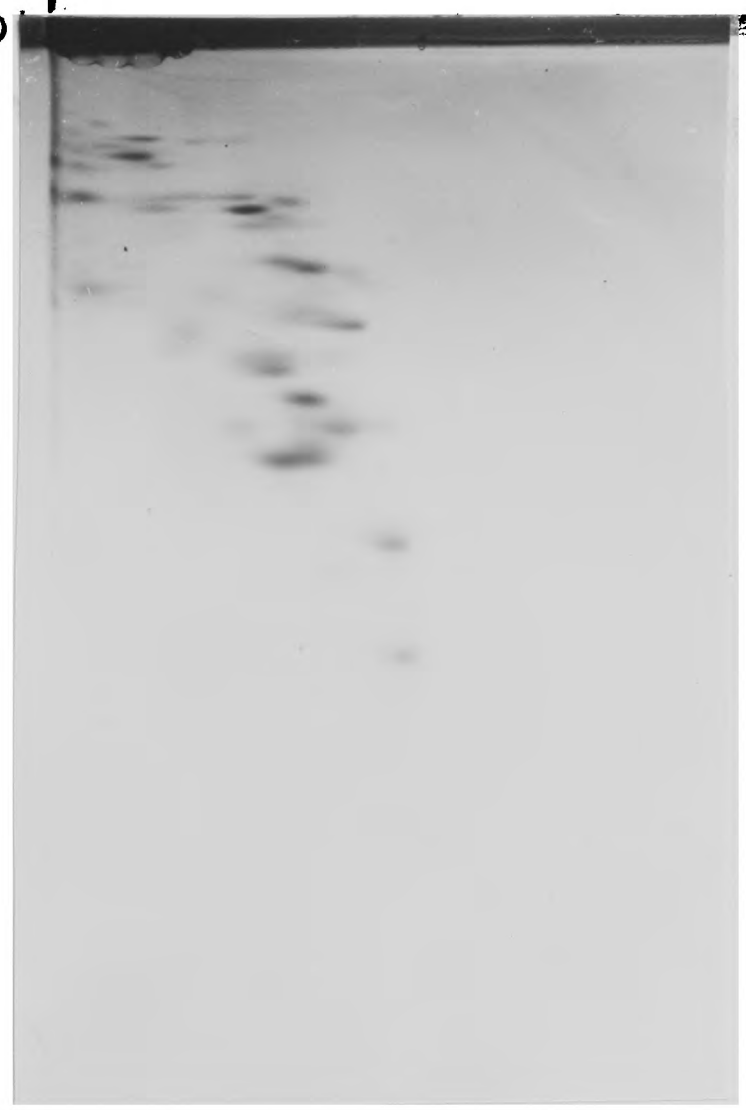
Microgel electrophoresis of the proteins from 7 ODU_{260} of Hela 80S precursor particles run exactly as described in 2.072.

+ 2-D

ORIGIN

+ 1-D

- 1-D



- 2-D

Figure 62
Two-dimensional pattern of cationic proteins of Hela 55S precursor particles.

Microgel electrophoresis of the proteins from 7 ODU_{260} of Hela 55S precursor particles run exactly as in 2.072.

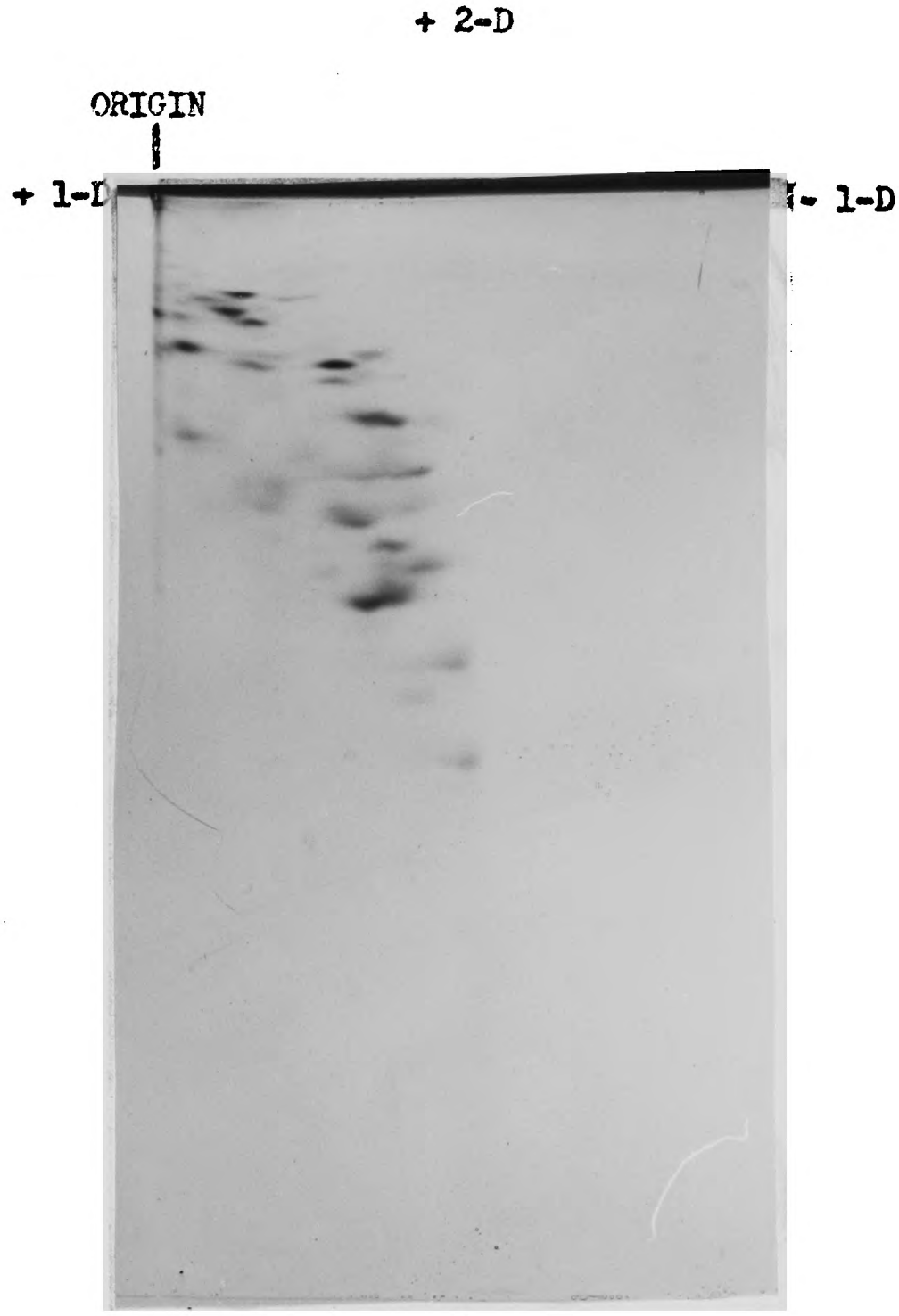


Figure 63
Two-dimensional pattern of cationic proteins of Hela 80S precursor particles.

Microgel electrophoresis of the proteins from 7 ODU_{260} of Hela 80S precursor particles run as in 2.072.

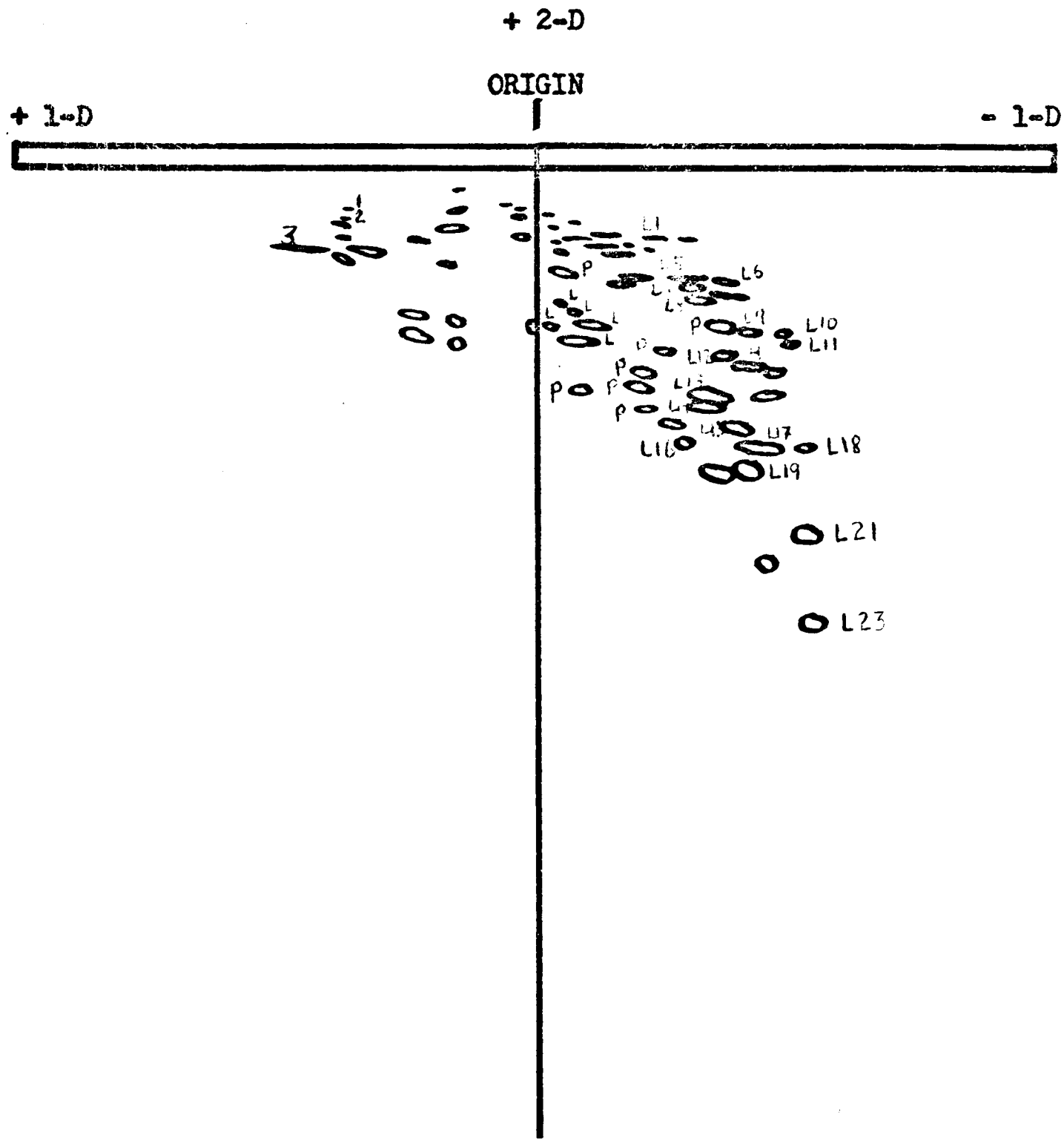


Figure 64
Schematic diagram of the proteins of Hela precursor particles.

Diagram shows all the proteins of the 55S precursor. The 80S precursor is identical except for the loss of several proteins mentioned in the text.

be identified as small subunit ribosomal proteins. However, there was some ambiguity about several spots found on both precursor patterns. Those spots designated "P" in Figure 64 ran very close to where the small subunit ribosomal proteins S1,S7,S8,S9,S10,S15 and S16 ran (Fig 58) but due to the presence of only small amounts of these proteins, they could not be resolved from 40S subunit ribosomal proteins upon coelectrophoresis. Upon close comparison, all but one of these spots seemed to migrate slightly differently from the small subunit ribosomal proteins and were felt not to be identical. One spot seemed to run with the same mobility as small subunit ribosomal protein S1/S2; whether they are identical remains to be determined.

Both precursor patterns show a number of high molecular weight spots as well as one or two lower molecular weight spots not seen on gel patterns of polysomes. These high molecular weight spots were not the result of aggregation by disulfide links since they were not removed or altered upon oxidation with performic acid. These proteins (and perhaps also the high molecular weight anionic ribosomal proteins) may be members of that set of proteins referred to as "stable nucleolar proteins" and may assist in the processing of the precursors. It has been shown (195,196,340,341, 379,380) that such stable nucleolar proteins show such characteristic high molecular weight.

7.030 Discussion.

These experiments have sought to define the protein composition of the ribosome precursor particles isolated from Hela nucleoli and especially to determine whether the 80S precursor contains proteins of the small as well as the large cytoplasmic subunit. Since 45S rRNA is the precursor to both 18S and 28S rRNA, it had been

expected that the 80S precursor particle would contain some ribosomal proteins of both subunits and that the 55S precursor would have only large subunit ribosomal proteins. The results of this chapter have been unable to prove this entirely. Both the precursors contain a number of distinct large subunit ribosomal proteins but the 80S precursor shows no ribosomal proteins of the small subunit. This latter conclusion remains somewhat in doubt since several very faint protein spots of the 80S pattern have not yet been adequately shown to be of different mobility from some small subunit ribosomal proteins. But if identity were shown, this would complicate the interpretation even more since these spots are common to both 55S and 80S precursors.

This failure to demonstrate any small subunit ribosomal proteins (as well as other proteins of the large subunit) may indeed reflect their absence from the 80S precursor and suggest that small subunit proteins are added after the small subunit rRNA is cleaved from the 45S rRNA precursor. These proteins might then be added in the nucleolus, nucleoplasm and/or in the cytoplasm. The large subunit ribosomal proteins not found in the 55S precursor might likewise be acquired. The numerous proteins present on both precursors might bind specific RNA sites on the non-conserved RNA and on the 18S and 28S rRNA sequences as well as bind to certain other proteins.

However the conditions of purification of these precursor particles are relatively harsh and considerable protein may be lost. That this loss would affect the protein of the small subunit more than those of the large is suggested by the observation that isolated small cytoplasmic subunits (but not large subunits) are rapidly degraded under the conditions used for extraction of

these precursor particles. Likewise Daskal et al (64) have shown that precursors extracted by the EDTA method appear altered from their in vivo form as seen in the electron microscope. They were less compact, more porous, and had lost specific ribosomal proteins (305).

It should be recalled that a somewhat similar situation may have occured during the repeated dissociation steps in the preparation of 60S subunits; a number of ribosomal proteins were felt to have been washed off by the conditions of KCl and Mg++. Most of the 60S subunit ribosomal proteins remaining after subunit preparation appear to be the same proteins that stain most intensely on the precursor patterns and in the polysome patterns. This may thus reflect the increased stability of the association of these ribosomal proteins with the rRNA and explain why only these survived extraction from nucleoli.

Furthermore, the extraction of these precursors is the result of a partitioning effect of the salt, EDTA and dithiothreitol and in any interpretation of these results it must be borne in mind that particles are in effect being ripped from the nucleoli. Vesco and Penman (280,384) have even found such a partitioning effect with respect to the nucleolar isolation itself. With increasing ionic strength the more loosely bound elements were released and the nucleoli isolated at high ionic strength (as here) showed altered nucleolar integrity. Though the precursor RNA is still almost totally removed, whether some proteins (ribosomal structural proteins as well as the stable nucleolar proteins) remain in the nucleoli after extraction is not known. The absence of all the small subunit ribosomal proteins and some of the large subunit proteins may simply be due to this. Future studies might profit by analysis of

the protein patterns of nucleoli before and after extraction by EDTA.

Still another possibile explanation for the failure to see more structural ribosomal proteins in these precursors is that in the nucleolus the small subunit ribosomal proteins (and some large subunit ones) are modified so as to alter their mobility (see 3.090). Some rat hepatoma ribosomal proteins have been found to vary in their degree of phosphorylation (and hence mobility) depending on whether they are in the nucleolus or in the cytoplasm (268). This may suggest a relationship between that group of ribosomal proteins marked "P" in Fig 64 and those several small subunit proteins of closely related mobility.

The interpretation of these two-dimensional gel patterns must be tempered by the fact that proteins of identical mobility need not necessarily possess the same amino acid sequence (3.090). In determining the degree of homology between two sets of proteins it is the number of proteins with identical mobility that reduces the potential for error. Hence it can be said with confidence that both precursor particles do indeed contain ribosomal proteins of the large subunit because 18 such coelectrophoresing proteins were found. Had only 1 or 2 proteins possessed identical mobility, this conclusion might not be justified.

These gel patterns of the Hela precursor particles are the first of their kind and represent the initial attempts to analyze the biosynthesis of the ribosomal proteins of the Hela ribosome. Using precursors extracted from rat liver nucleoli by different methods, others have reported similar 2-D gel analyses. Tsurugi et al (380) have published a 2-D gel of 60S nucleolar particles that is virtually identical to that in Fig 62. These workers also

found a number of faintly staining spots of unknown origin and a number of nucleolar proteins stably associated with the precursors that were identical to those seen in this chapter. Of 36 major protein spots in the precursor pattern, 33 were of mobility identical to large subunit ribosomal proteins.

Prestayko et al (305) have used the EDTA extraction technique to isolate precursors from NHAC cells. Upon 2-D electrophoresis the precursor preparation was found to contain 60 proteins of which 21 were of the large and 10 of the small subunit. However the nature of these precursors was, at best, ambiguous. The EDTA nucleolar extract was centrifuged through a gradient to form a pellet which was subsequently the source of sample for these gels. No evidence has been published to show the RNA composition of these precursors nor has there been an attempt to isolate the separate sedimentation classes of precursors and determine their protein composition. Indeed, the published RNA composition of identical particles isolated by an "improved" technique (306) stated that they contained 28S rRNA only; in fact they contained 28S rRNA and 32S rRNA (see Fig 4 in ref 306) and perhaps some 18S rRNA. These particles also showed a sedimentation coefficient of 78S (in non-EDTA buffers) with aggregates also at 110S and about 150S [as did Liau and Perry (212)]. It is clear that none of these precursors is equivalent to the 80S precursor of Warner and Soeiro (393) for there is no particle containing 45S rRNA.

What Busch has isolated appears to be a mixture of a 55S equivalent (32S rRNA) and the large cytoplasmic (or nucleoplasmic) subunit (28S rRNA). Close analysis of the published 2-D gels showing the presence of small subunit ribosomal proteins shows 5 of 10 to be very faint and only 3 to be of average intensity.

Why such small subunit ribosomal proteins should be present on these 55S equivalent particles is not understood and possible cytoplasmic contamination must be borne in mind.

In general, the results of this chapter and those of others are consistent with a theory of ribosome biosynthesis whereby certain ribosomal proteins migrate to the nucleolus and bind to precursor rRNA along with more stable "nucleolar" proteins. During RNA maturation, the protein composition is also altered. When and where both subunits acquire the rest of their structural proteins is not known. Certainly a small number of what are defined as ribosomal proteins are added in the cytoplasm. Further studies of these particles will demand different isolation techniques to study their changing protein composition as well as their possible participation in messenger RNA selection and transport.

7.040 Summary.

Nucleolar ribosomal precursor particles (80S and 55S) have been isolated from Hela cells with standard methods and are identical to those preparations of other workers. The two-dimensional gel electrophoresis pattern of the 55S precursor showed 21 anionic and 52 cationic proteins. Of the cations, 18 corresponded to the ribosomal proteins of the large subunit. The 80S precursor pattern was identical to the 55S pattern except 3 anions and 4 cations were absent. Of those missing cations, 3 were from the large subunit. No small subunit ribosomal proteins were found on either precursor. Their absence may be due to the extraction conditions or reflect the true state of these precursors. No anionic proteins of the cytoplasmic ribosomes were found in either precursor.

CHAPTER 8

THE SYNTHESIS OF RIBOSOMAL PROTEINS AND rRNA IN HYBRID CELLS

8.000 Introduction.

Ribosome structure and function have been the subject of considerable attention during recent years and hence much is known about these topics for both eukaryotic and prokaryotic systems. Considerably less, however, is known about the genetics of the ribosome and the regulation of its synthesis. Such studies have as their goals the identification of those genes coding for the structural components of the ribosome itself, those genes responsible for modification and assembly, as well as those genetic systems regulating ribosome biosynthesis. Already clever biochemical genetics has succeeded in learning the structure of the rRNA cistrons in certain eukaryotic organisms (Chapter 1) but progress has been slower in most other areas.

As in so many other instances, the bacterium offers a convenient starting point and describes such a genetic approach to the ribosome (for review, see 67). Genes for rRNA (5S, 16S, 23S) have been shown by RNA-DNA hybridization to be present in amounts of 5-10 copies per genome (420). Though the copies of 5S rRNA genes show microheterogeneity (47,172) this does not seem to alter their function. Whether such is also true of 16S and 23S rRNA is uncertain. About half of the rRNA genes lie near minute 74 of the *E. coli* map (20) and the other half appear to be scattered over the genome, some in the 54-59 minute span (269) and some perhaps in association with the ribosomal protein genes. But unlike in higher organisms, the rRNA genes do not appear in clusters; instead single units consisting of genes for 16S, 23S and 5S (in

this order) are separated from each other by other functioning genes (67) and each rRNA unit is transcribed as one piece.

Though mapping of ribosomal protein genes has been hindered by the lack of adequate mutants, the additional use of interspecific merodiploids has increased the number of ribosomal protein markers. Twenty-one (11 50S and 10 30S) ribosomal proteins were thus found to map distinct from the rRNA genes at minute 64 of the E. coli map (34,70,71,207,260,361,362) and were partly ordered as follows: crp - S7 - S12 - S4 - L4 - L22 - S5 - S6 - aro E (46). The ribosomal proteins of both subunits are thus intermingled. Nomura (173) has recently developed several specialized λ transducing phages that carry about half the ribosomal protein genes of E. coli and show that about 33 ribosomal proteins map near the str A locus. However S18 is known to map outside this area at minute 84 (35,83) and 5 other ribosomal protein genes map near rif (174). Whether more ribosomal proteins will map outside of minute 64 will require further analysis -- the total map at minute 64 (crp - aroE) has room for 1% of the E. coli genome (~100 genes).

This clustering of ribosomal protein genes suggested their possible functioning as a single transcriptional unit and the original experiments of Nomura and Engback (260) had supported this since bacteriophage Mu insertions into this cluster showed polar effects on the "downstream" ribosomal protein genes. Cabezon et al (50) have reevaluated these experiments and by controlling adventitious deletions found no such polar effects with respect to these ribosomal proteins. Similar conclusions were reached by other experimental approaches as well (245). Nomura (174) has recently found that insertion mutations inactivate some but not all ribosomal proteins in the str - spc region contained in λ transducing phages.

At least 4 different transcriptional units are thus found for E. coli ribosomal proteins.

The synthesis of bacterial ribosomes appears closely regulated (78,377). Under varying growth conditions (such as altering the carbon source) neither ribosomal proteins* nor rRNA accumulate (114, 326). Controls of two aspects of ribosome biosynthesis might be suggested:

a) That which coordinates the relative amounts of rRNA and ribosomal protein synthesized to maintain balanced quantities;
b) That which coordinates these balanced amounts of protein and RNA with the growth rate to keep their balanced synthesis proportional to the growth rate.

Since all three rRNA species compose a single precursor RNA (89, 117,255), this assures a balanced production of the RNA species with respect to each other and is the likely site for coordination of rRNA synthesis with the growth rate. For ribosomal proteins, the several transcriptional units may be the sites of the controlling factors though translational control has been found (79). In any case, what is of importance is that the synthesis of ribosomal proteins [and other factors like EF-G (79)] yields the appropriate ratio of these different proteins and is a sensitive function of the bacterial growth rate (79,245,326).

These two aspects of the control of ribosome biosynthesis might be explained simply as due to "metabolic control," ie. the general increase or decrease in RNA and protein synthesis associated with altered growth rate is coupled with a rapid turnover of ribosomal protein and rRNA that do not bind to each other. Formation of a RNP complex would thus be favored by increasing amounts of rRNA and ribosomal protein, with the stabilized products being

*In these experiments, ribosomal protein levels were not accurately examined; only those proteins on isolated ribosomes were measured. Thus it was possible to miss completely ribosomal proteins synthe-

less susceptible to degradation. The association constant for this complex would determine the relative stability of the ribosomal proteins with respect to the other proteins and may be of such an order as to explain why in conditions favoring increased growth, ribosomal proteins increase more than other proteins.

However the existence of "stringency" in terms of the "rel" gene in E. coli suggests that there is a specific controlling system involved. Mutation in this gene effectively uncouples the balanced synthesis of ribosomal proteins and rRNA from the growth conditions (eliminating control "b" above). Wild-type (rel+) strains restrict rRNA accumulation during periods of deprivation for an essential amino acid while rel- strains do not and are said to be "relaxed."

This stringency response has recently become better understood and helps explain one level of control of ribosome biosynthesis. During amino acid starvation, rel+ cells accumulate ppGpp and the rates of rRNA production (105) and ribosomal protein synthesis decrease. Likewise the rate of production of mRNA for ribosomal proteins is inversely correlated with the intracellular level of ppGpp (11,80,81). But this proves only one aspect of the control of ribosome biosynthesis: that the synthesis of rRNA and ribosomal protein are each a function of ppGpp concentration (itself a function of the growth conditions) acting at the transcriptional level. These ppGpp levels may not explain what keeps the balance between rRNA and ribosomal proteins -- there may be other control mechanisms for this and also for controlling the relative amounts of the different ribosomal proteins (a likely translational control).

Analysis similar to these have been attempted for eukaryotic organisms. The fine structure of the ribosomal DNA has been deter-

mined (Chapter 1) and differs from the bacterial rDNA in that rDNA genes are clustered and do not include the genes for 5S rRNA. In the human, the 18S and 28S rRNA genes are aggregated at the 2° constriction on chromosomes 13,14,15,21,22 (154) as revealed by in situ hybridization. Though cytologists have long correlated 2° constrictions with nucleolar organizers, very recent work with the Indian muntjac (271) has shown this to be not always true; hybridization may reveal rDNA loci too small to give a 2° constriction during metaphase.

The multiple clusters of 5S rRNA genes are at chromosome sites distant from those for 18S and 28S rRNA (211,407,417) and in the human karyotype, in situ hybridization has localized these cistrons mostly on chromosome number 1 but also on 9 and 16 (353). Though on chromosomes separate from those for the other rRNA species, in interphase cells the 5S genes are found clustered about the nucleoli (270,353), an association of obvious advantage for effective ribosome assembly. This may suggest that there is considerable order in the interphase nucleus.

The location of genes coding for ribosomal proteins has not been determined. Steffensen (352,417) has suggested that 8 ribosomal proteins from both ribosomal subunits of D. melanogaster map within segment 19E1-4 on the X chromosome but the data to support this conclusion have not yet been published. Like so many other areas of eukaryotic genetics, the analysis of ribosomal structure is hampered by a lack of mutants. Antibiotic resistant mutants of eukaryotic cells have only just been developed for Saccharomyces cerevisiae (56), Physarum polycephalum (149), Neurospora (300) and Chinese hamster cells (297,298). As in bacteria, they exert their effect at the level of the ribosome.

With respect to the control of ribosome synthesis or precursor processing, even fewer mutations have been described. Some temperature-sensitive BHK 21 cells seem to be blocked in the conversion of 32S rRNA precursor to 28S rRNA at restrictive temperature (239, 375). Warner has described a second sort of temperature-sensitive mutation in yeast (Saccharomyces cerevisiae) that seems analogous to the stringent control system in bacteria (394). At the non-permissive temperature, precursor rRNA and all ribosomal proteins are not synthesized, processing is stopped and the rRNA degraded. The nature of these pleiotropic effects is unknown.

This dearth of eukaryotic mutations has led to the use of alternative methods to analyze the regulation of ribosome biosynthesis. One approach has been to observe the effect of inhibitors of rRNA or protein synthesis upon ribosome formation. While inhibitors of RNA synthesis (actinomycin D) stopped transcription of rRNA precursors, the synthesis of ribosomal protein was unaffected (59). But ribosomal proteins synthesized during the block on RNA production were not incorporated into ribosomes upon removal of the block and were presumed to be degraded. This explains why Craig (58) failed to find newly synthesized ribosomal proteins in ribosomes isolated from actinomycin D treated L cells. Experiments with other inhibitors of RNA synthesis have shown that concurrent transcription of 45S rRNA is necessary for maintaining later stages of processing (197).

As it is separate from the genes for 18S and 28S rRNA, 5S rRNA also seems separately controlled, for in the presence of actinomycin D its synthesis is maintained. But like the ribosomal proteins synthesized during the actinomycin D pulse, the 5S rRNA produced seems more labile and was not incorporated into new ribosomes

assembled after removal of actinomycin D (288).

Blocking protein synthesis initially affects processing of the precursors (273) but doesn't alter their transcription. There is an immediate drop in the protein content of precursors and it seems that such deficient particles take more time to process, hence an overall reduction in ribosome biosynthesis. This occurs in spite of the supposedly large pool of ribosomal proteins*(390,392) and suggests that perhaps some proteins are present in limiting quantities. Prolonged inhibition of protein synthesis (224,416) results in a decrease in 45S rRNA formation as well as increased precursor processing time. Thus the common message of all these experiments is that rRNA and ribosomal protein are not coordinately synthesized: rRNA synthesis continues (for a while) without ribosomal protein synthesis and ribosomal protein synthesis continues in the presence of actinomycin D.

But these experiments do not exclude the possibility of some genetic control. In fact, the situations created by these inhibitors are highly artificial and controls like the stringent mechanism might have been completely overlooked by such experiments in bacteria. Instead, what these inhibition studies illustrate is the metabolism of rRNA and ribosomal proteins when not stabilized in the nucleolus and the necessity for concurrent synthesis of the two.

A more physiological approach to the regulation of ribosome biosynthesis does suggest there is some coordinate regulation. Developing Xenopus laevis embryos synthesize no detectable 18S and 28S rRNA until gastrulation. The rate of rRNA and ribosome syntheses gradually increase as development proceeds. Hallberg and Brown (131) have also shown that such cleaving embryos do not synthesize or

*Wool and Stoffler (419) have not been able to confirm the existence of such a pool of ribosomal proteins when assayed by antibodies to specific ribosomal proteins.

or accumulate 10 different ribosomal proteins and anucleolate Xenopus laevis embryos (deleted of genes for 28S and 18S rRNA) make only 4% as much ribosomal protein as do the normal embryos. These results suggest that during cleavage 18S rRNA, 28S rRNA and these ribosomal proteins are coordinately synthesized. But 5S rRNA is not so regulated. 5S rRNA is made by normal blastula and anucleolate mutants at the same rate as in later developmental stages (241) but in both instances has a reduced half-life (242). It thus appears that 5S rRNA synthesis is not regulated by the same mechanisms as ribosomal proteins and the other rRNAs but may have some other roles.

In another developing system, the embryonic cleavage of Strongylocentrotus purpuratus, though 18S and 28S rRNA are not made, ribosomal proteins are synthesized (184). In general, the ribosomal proteins themselves showed no coordinate synthesis either with respect to rRNA or to each other. A low level of ribosomal protein synthesis yielded ribosomal proteins that appeared to be in a dynamic equilibrium with the oocyte ribosomes already present. This conflicts with the Xenopus data above but is consistent with the experiments of Dice and Schimke (84) who also found many ribosomal proteins on ribosomes to be rapidly exchanged with those in the cytoplasm. However such exchange experiments have not been confirmed (312,358).

An entirely different approach to the analysis of the genetics of the eukaryotic ribosome is the use of interspecific hybrid cells, a parallel to the use of interspecific merodiploids with bacteria (E. coli x S. marcescens). A number of these hybrid cells were the result of breeding experiments between different plant or animal species. It was long noted that interspecies hybrids within

the plant genera Crepis, Ribes and Solix showed "nucleolar dominance" (186,248,389,415), ie. though the parental species contained two nuclecli, the F1 hybrids had only one and suggested that one or other nucleolar organizer was inactivated. Similar observations were reported by Blackler and Gecking (27) with F1 hybrids of X. laevis and X. mulleri. Analysis of these hybrids has shown that though both sets of parental rDNA are present, only X. laevis rRNA is made. But when the X. laevis parent contributed a genome with the rDNA genes deleted (the anucleolate mutant), the X. mulleri rDNA was not repressed (159).

Though the evidence for such a repressing substance is not strongly convincing, it seems that the repressing effect may be reversed if the two genomes are separated by a nuclear membrane. Hence in certain Crepis hybrids (389), the repressed genome could be reactivated to form nucleoli during pollen formation when a nuclear membrane separated it from the dominant genome. Likewise a hybrid wheat nucleolar organizer was reactivated when segregated to a micronucleus. In heterokaryons formed between Hela cells and mouse cells both rDNA genes continue to be transcribed (230) until the separate nuclei fuse to form a synkaryon, at which time only the mouse rRNA is synthesized. Attempts to reexpress the human genome by micronucleation of such synkaryons have failed (228,230). What function the nuclear membrane serves in maintaining the integrity of expression of the separate genomes is not known.

Hybrid cells created by cell fusion (for reviews see 65,66, 135,143,318) have been more convenient for study. Early experiments showed that human-mouse hybrid cells containing up to 35 human chromosomes (including some with 2^{o} constrictions) produced no detectable human rRNA (38,97) and suggested "nucleolar dominance"

as in Xenopus or plant hybrids. Such hybrids did produce human enzymes and human HnRNA (85). However in hamster-mouse (95,96, 350) somatic cell hybrids both parental types of 28S rRNA were made. Since the 18S rRNA of the two species cannot be separated on acrylamide gels, the source(s) of the 18S rRNA species could not be distinguished.

Recently a response like that mediated by ppGpp in stringent controlled bacteria has been identified for eukaryotes. During the differentiation phase of the slime mold Dictyostelium discoideum, rRNA synthesis is prevented (188) and the inhibition correlated with the accumulation of ppGpp (191). Other work (167) has suggested that ppGpp has still other functions during development.

Thus at the start of the studies described in this chapter the genetics of the eukaryotic ribosome could be summarized as follows:

a) The major genetic loci for the rRNA (5S,18S,28S) were known.
b) The effects of inhibiting RNA or protein synthesis showed the importance of stabilizing the newly synthesized components as nascent ribosomes.
c) Nucleolar dominance in interspecific hybrids was a common phenomenon.

Given these aspects of ribosome biosynthesis, experiments were begun whose ultimate aim was to assign chromosomal locations for the genes for some ribosomal proteins. Since a number of interspecific ribosomal protein differences had been previously determined (Chapter 3), interspecific hybrids of these were constructed and analyzed for the presence of parental rRNA and ribosomal protein. Together with mapping ribosomal proteins, a second aspect of this work was to determine if nucleolar dominance also affected ribosomal protein synthesis and, if not, whether nucleolar dominance acted differently on ribosomal protein genes than rRNA genes. Finally,

it was hoped that by introducing into the genome a second functional ribosome synthesizing system some principles of regulation of ribosome synthesis might be learned.

Only Houssais (161) has made similar attempts at analyzing the ribosomal proteins in hybrid cells and found, in only one case, that a hamster-mouse hybrid line contained one ribosomal protein specific for each parent. However these results are difficult to analyze since the marker ribosomal proteins were poorly defined using one-dimensional SDS gels. With the 2-D gel system it was felt that a more conclusive demonstration of the ribosomal proteins of hybrid cells might be made.

8.010 Selection and karyotype analysis of hybrids.

When A9HT and rat leukemia cells are fused together by the Sendai virus technique and grown in HAT (2.020) the unfused parental A9HT cells (which are deficient in inosinic acid pyrophosphorylase) are unable to grow. Since rat leukemia cells are unable to grow in vitro, any cells growing under these conditions are hybrid cells. Of the seven clones isolated from this population only one clone grew well in roller culture (clone 2) and it is this clone which is described in detail here.

To determine whether this clone was indeed a rat-mouse hybrid, its karyotype and that of the parental lines were compared using the C-banding technique (2.033) to help distinguish between rat and mouse chromosomes. C-banding is thought to be due to the staining of residual protein at centromeres (and 2° constrictions) which is left after the extraction of most of the other chromosomal proteins by the conditions of heat, salt and formamide (55). The retention of protein at these specific locations probably reflects unique properties of the underlying base sequences. Under the conditions

of extraction here, there appears to be a different effect on mouse chromosomes than on human, rat, chicken, Syrian hamster, Chinese hamster, vole and toad chromosomes (229), only the mouse chromosomes showing C-banding. C-banded chromosome preparations show different intensities of centromeric banding and these degrees of staining have been described here as being either strong, faint, or unbanded.

If not treated for C-bands, A9HT metaphase spreads (Fig 65) show a modal chromosome number of 52 consisting of 26 biarmed, 24 acrocentric/telocentric and 2 "dot" chromosomes. When C-banded, this line shows strong and highly reproducible banding at the centromeres (and at some secondary constrictions) of most chromosomes (Fig 66). All biarmed and "dot" chromosomes show a strong centromeric band; the remaining chromosomes are all strongly banded except for 2-6 acrocentric chromosomes which show only faint C-bands. Under these conditions of staining no mouse chromosome is ever totally devoid of a C-band. In addition, the banding at the 2^{o} constrictions of two chromosomes (arrows in Fig 66) reveals two distinct A9HT marker chromosomes.

The modal number of 52 for the A9HT line is somewhat lower than the number of 55 that Allderdice et al (3) have shown in their detailed studies of the A9HT karyotype; but the A9HT line used in the fusions with the rat leukemia line has been grown extensively in culture since the experiments of Allderdice et al and this altered karyotype is not unexpected.

As in other experimentally-induced leukemias (263), this rat leukemia has a normal diploid karyotype (Fig 67) of 42. Under C-banding conditions identical to those for A9HT, the rat chromosomes show no banding at all. However as with other leukemias (263, 264), chromosome preparations suitable for banding were difficult to obtain from this line. To make certain that the lack of banding

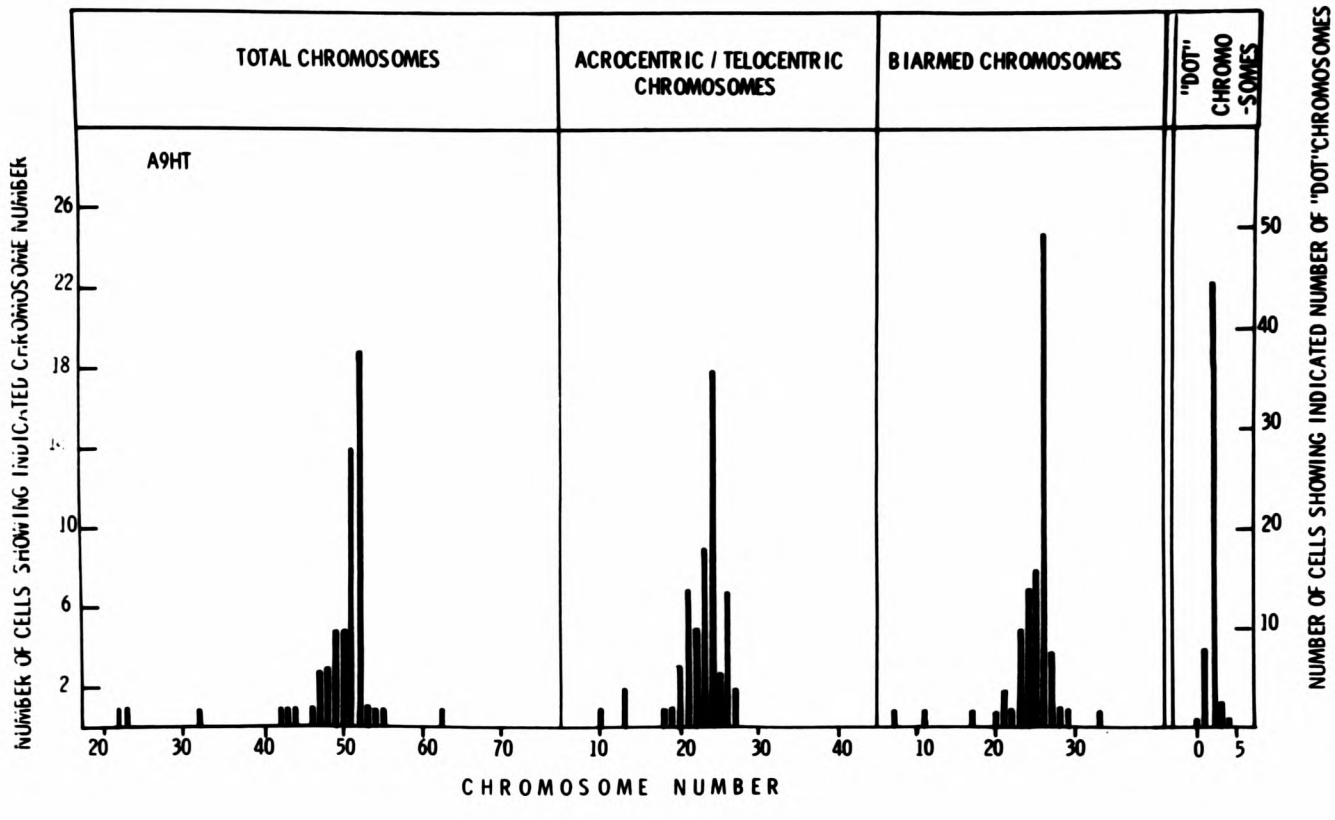




Figure 65
Karyotyre analysis of A9HT (unbanded).

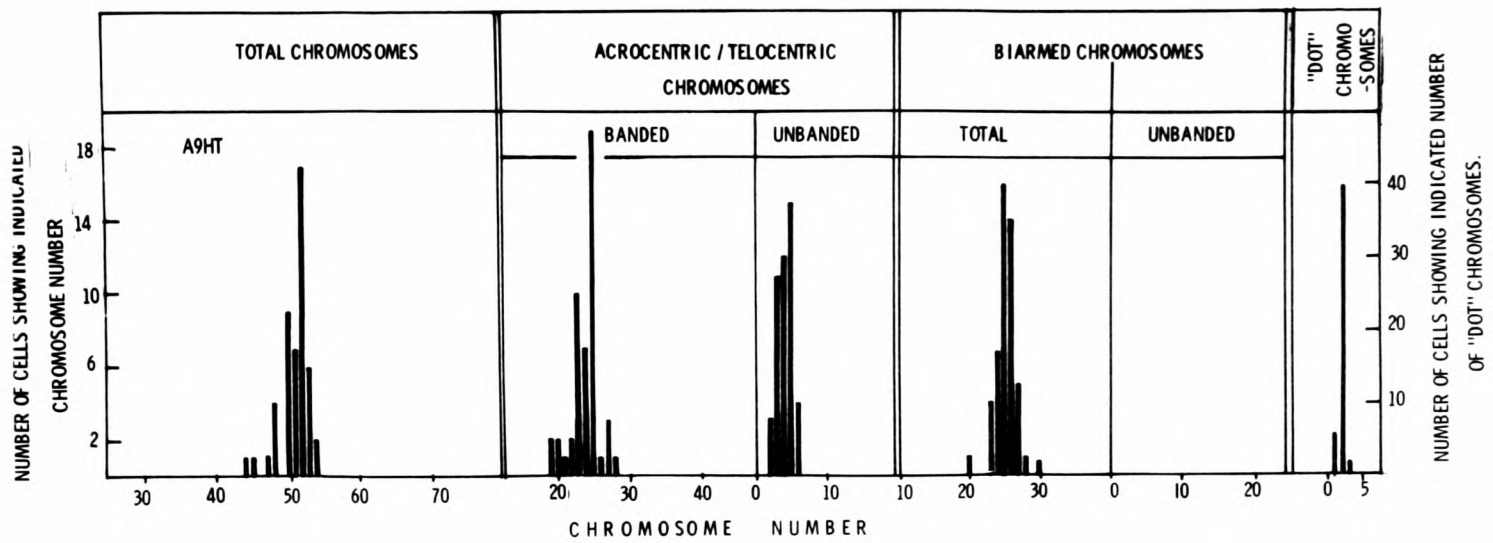
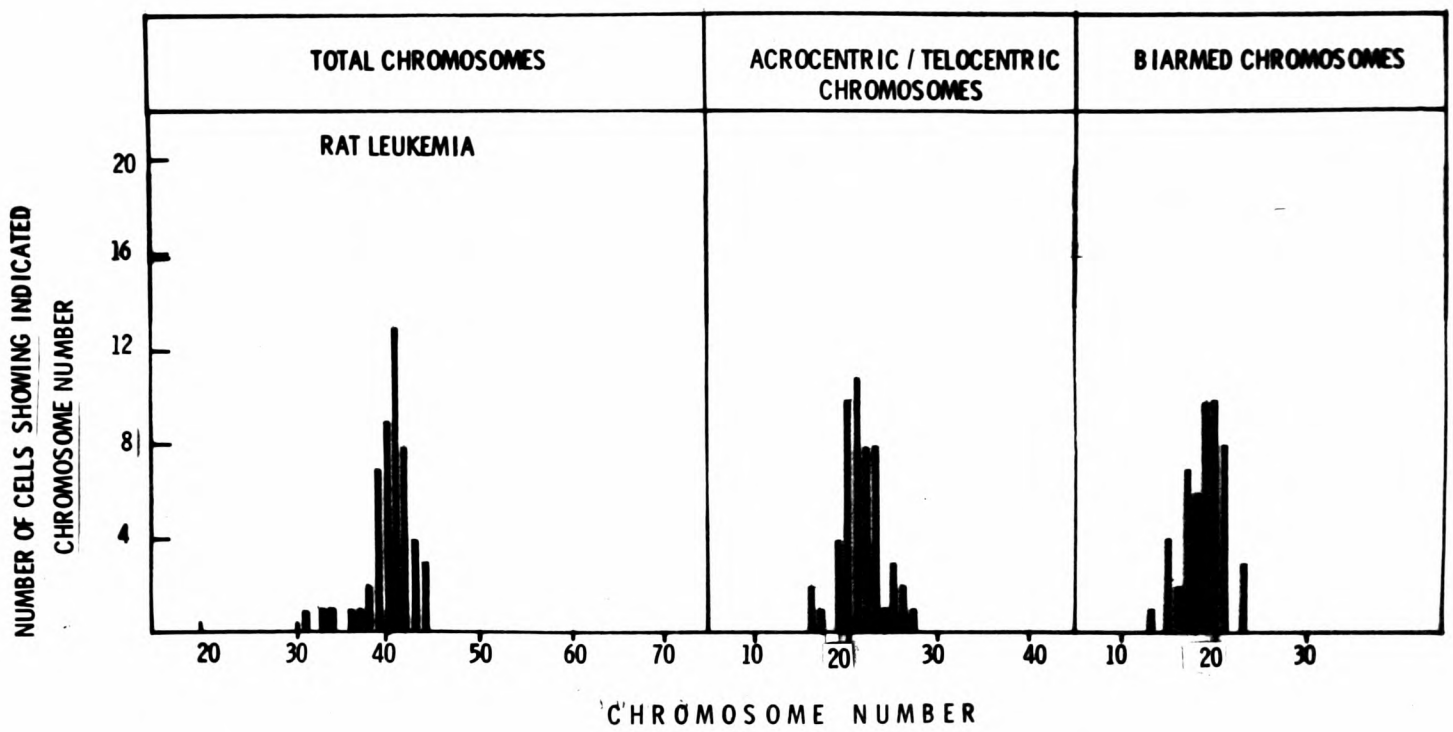




Figure 66
Karyotype analysis of A9HT (C-banded).

Arrows indicate mouse marker chromosomes.



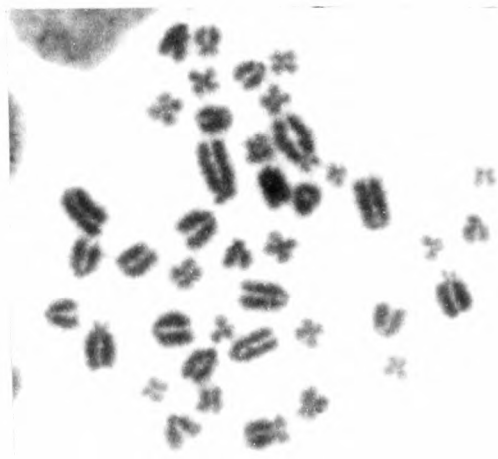


Figure 67
Karyotype analysis of rat leukemia (C-banded).

by the rat leukemia was a species phenomenon and not due to the poorer spreads, rat embryo fibroblasts were analyzed.

Rat embryo fibroblasts have a normal diploid rat chromosome number of 42 (20 acrocentrics and 22 biarmed chromosomes)(Fig 68). When treated for C-bands (Fig 69), about half the chromosomes remain unbanded; the rest of the chromosomes showed banding of a much weaker intensity than the strong banding in A9HT and comparable to that in the 2-6 faintly banded A9HT chromosomes. Unakul and Hsu (382) have found such faint banding for NHAC and Rattus norvegicus as well. Thus with the exception of the faintly banded rat acrocentrics which cannot be distinguished from the faintly banded mouse acrocentrics by this technique, it should be possible to identify in rat-mouse hybrids some of the chromosomes from each parent. Any strongly banded chromosomes are of mouse origin, any totally unbanded chromosomes of rat origin, and the faintly banded chromosomes are of uncertain origin unless morphologically distinct.

Unbanded A9HT/rat leukemia cells (Fig 70) show a unimodal chromosomal distribution of 69 which contains 36 acrocentric/telocentrics, 29 biarmed and 3 "dot" chromosomes. (The sum of the different chromosome types does not equal the total since the distributions were broad and the modal numbers vary slightly.) When C-banded preparations were analyzed, specific rat and mouse chromosomes may be clearly seen (Fig 71). The two mouse marker chromosomes (⟶) are present as are most of the other strongly banded mouse chromosomes. Three rat small metacentric chromosomes (⟶), two rat subacrocentric chromosomes (⟶) and 4 other unbanded acrocentrics (⟶) may be discerned. Several other weakly banded acrocentric chromosomes are visible but their origin cannot be

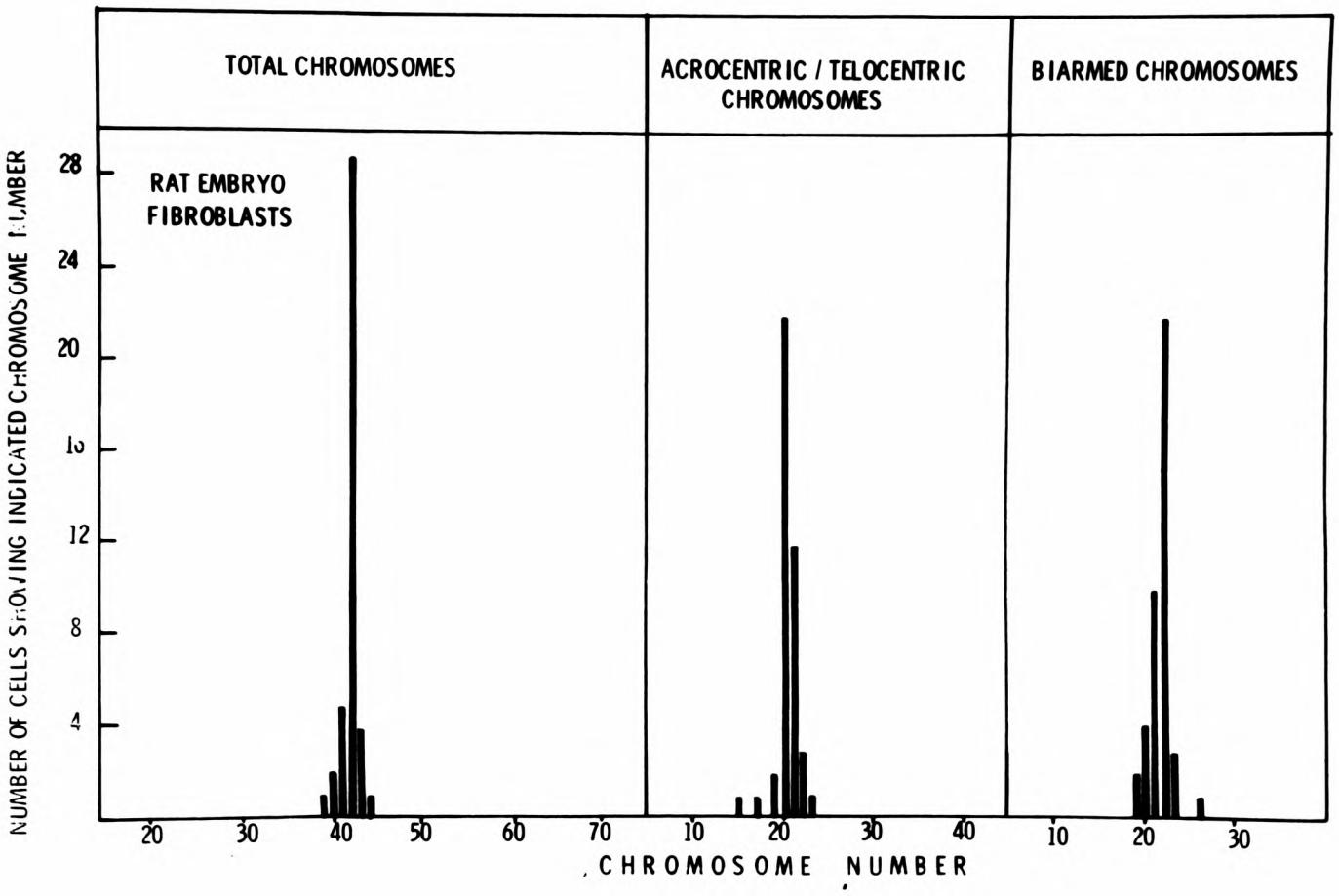




Figure 68
Karyotype analysis of rat embryo fibroblasts (unbanded).

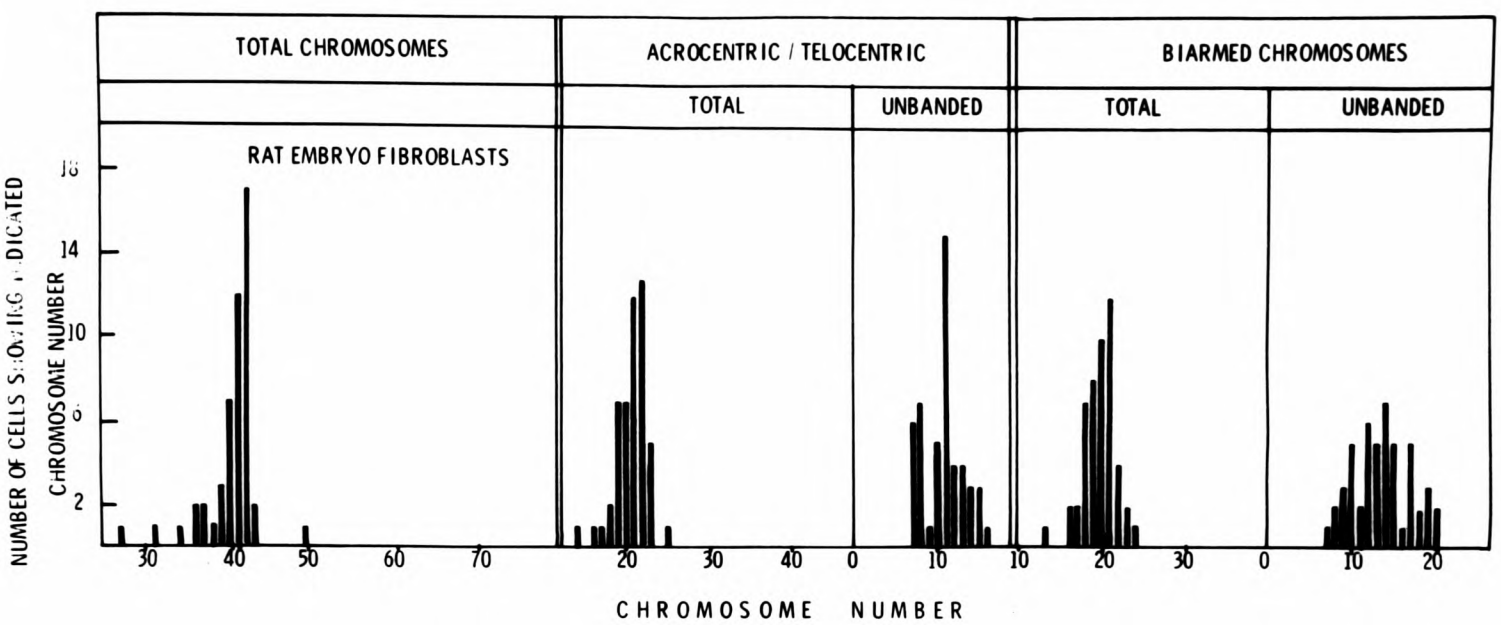




Figure 69
Karyotype analysis of rat embryo fibroblasts (C-banded).

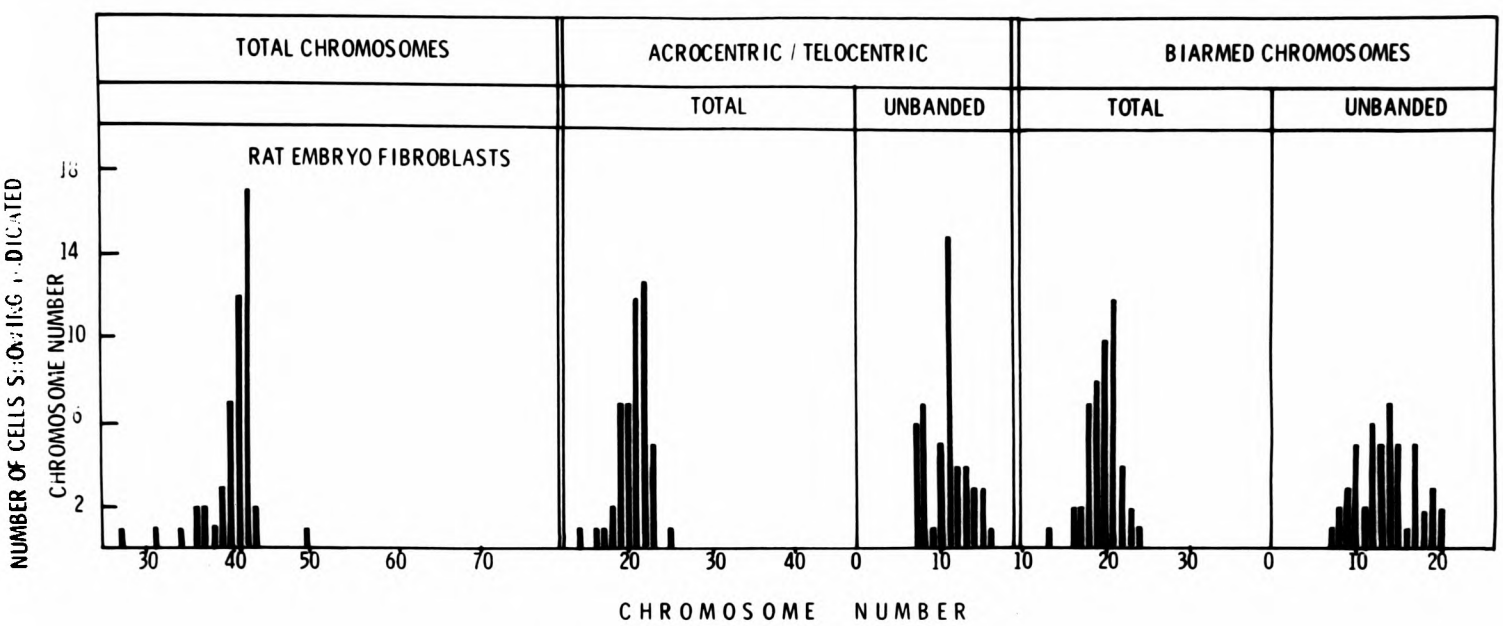
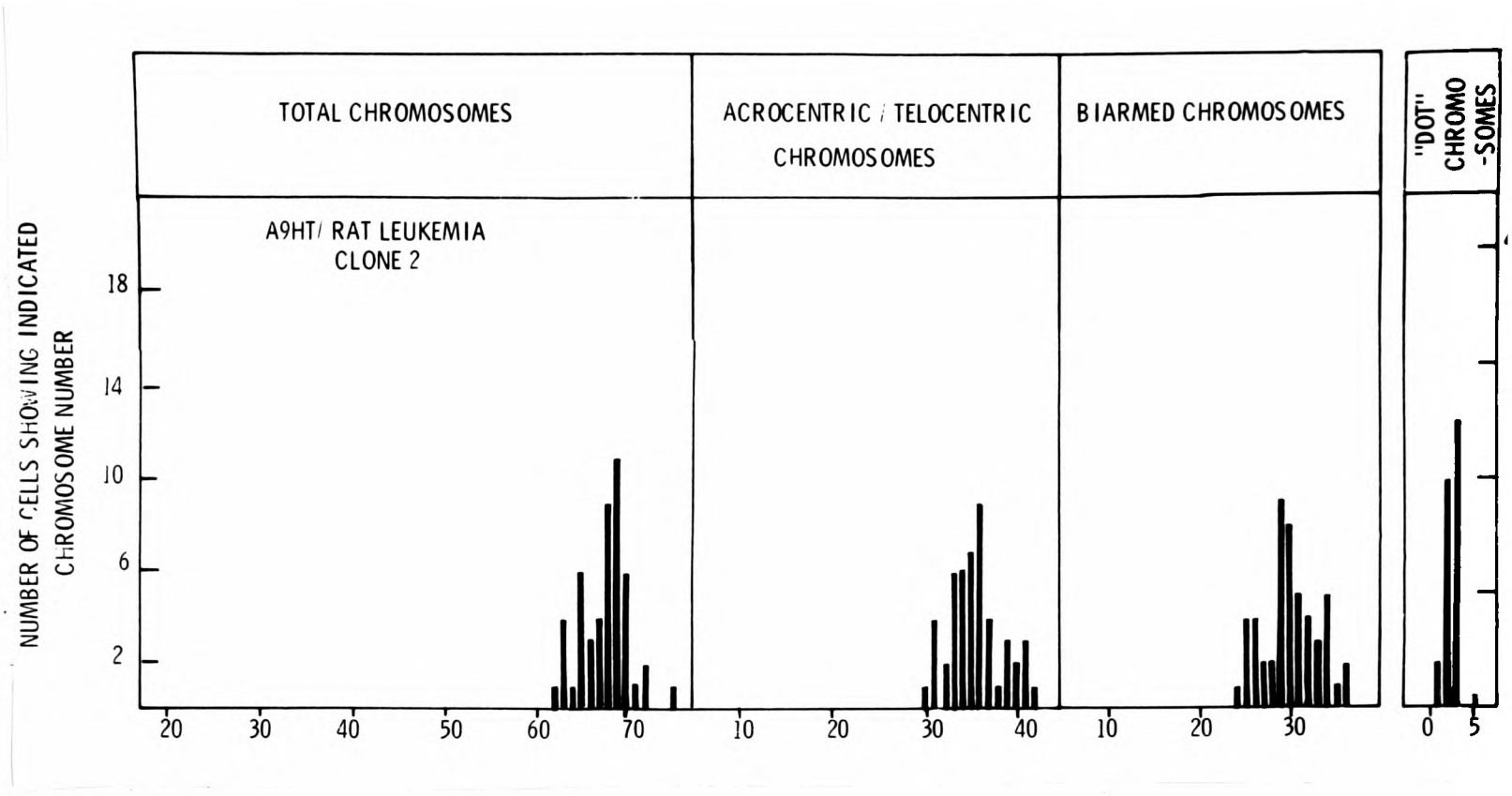




Figure 69
Karyotype analysis of rat embryo fibroblasts (C-banded).



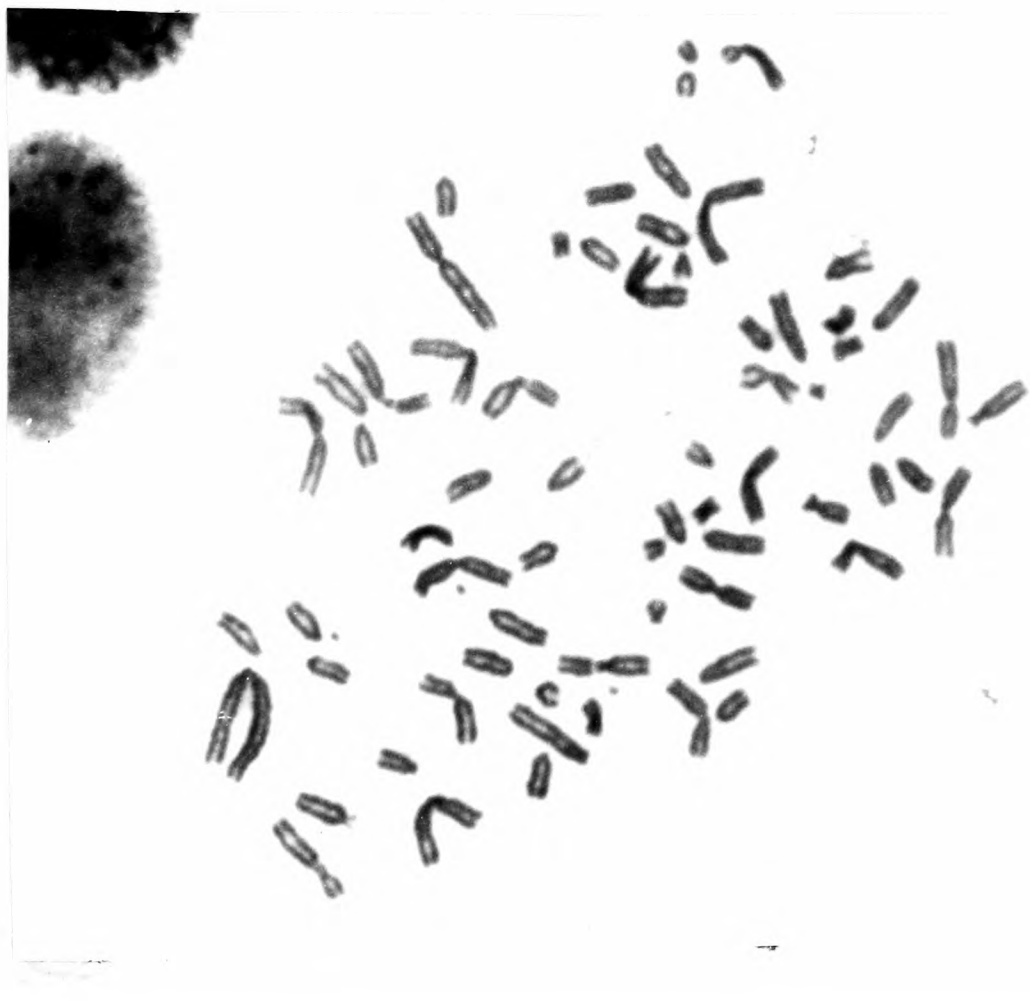


Figure 70
Karyotype analysis of A9HT/rat leukemia (unbanded).

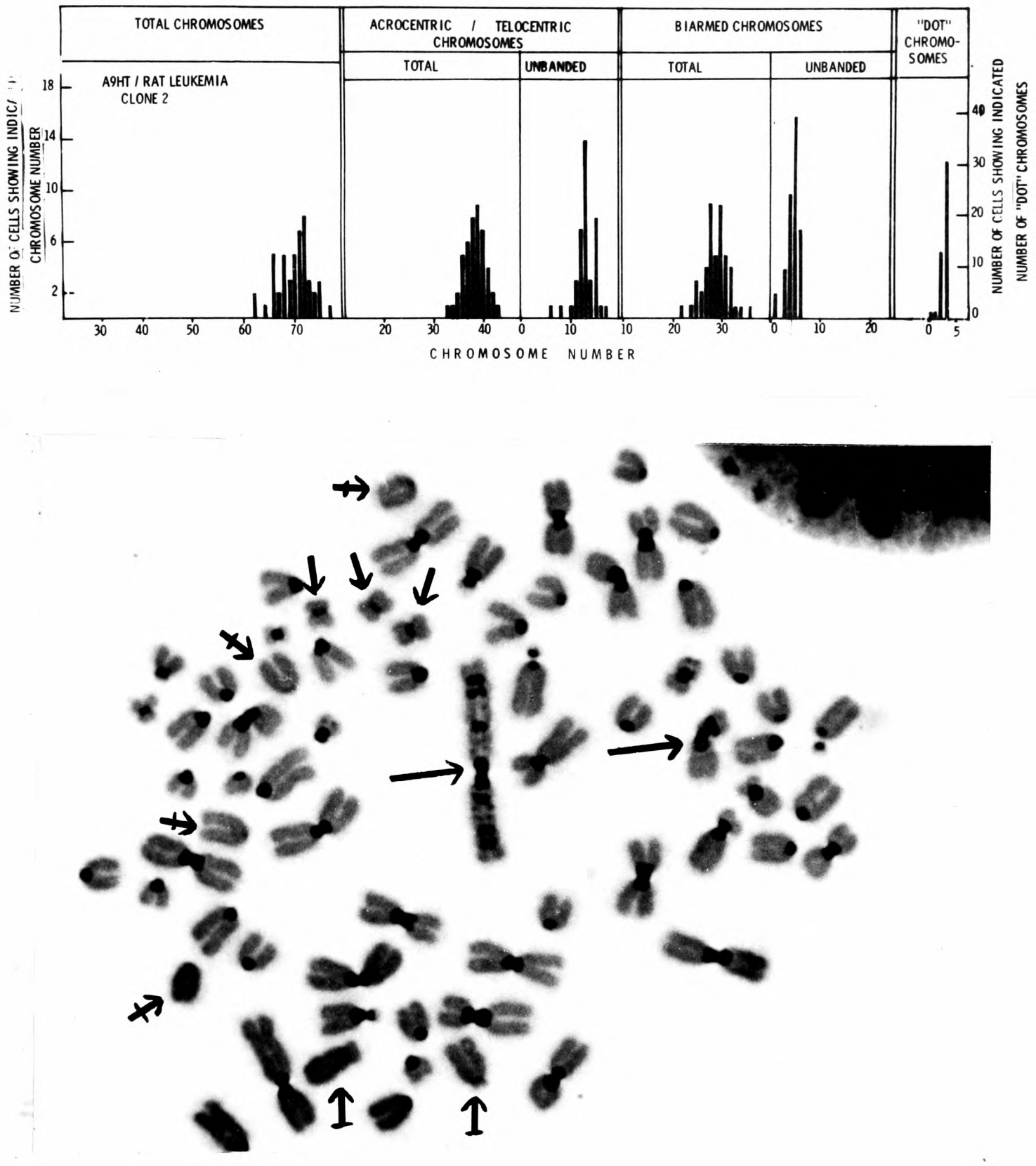


Figure 71
Karyotype analysis of A9HT/rat leukemia (C-banded).

All arrows are described in the text.

discerned. Several other weakly banded acrocentric chromosomes are visible but their origin cannot be determined because weakly banded rat and mouse acrocentrics look alike.

Table II summarizes the distribution of chromosomes in the hybrid. In the rat embryo fibroblasts (and presumably in the rat leukemia) the number of chromosomes classified as "unbanded" indicates chromosomes totally devoid of banding; for the acrocentric and telocentric chromosomes of A9HT and the A9HT/rat leukemia hybrid, "unbanded" chromosomes include both the faintly banded and completely unbanded chromosomes . In the hybrid a modal number of 13 of the 39 acrocentric/telocentric chromosomes are unbanded, as are 5 of the 30 biarmed chromosomes (Table II and Fig 71). Because all the A9HT biarmed chromosomes are strongly banded, these 5 unbanded chromosomes can be attributed to the rat parent.

Since 5 of the A9HT acrocentric/telocentric chromosomes are too faintly banded to be distinguished from the 11 faintly banded rat chromosomes, not all of the 13 unbanded acrocentrics in the hybrid may be of rat origin. The difference between these totals may represent the number of rat chromosomes in the hybrid. When morphological comparisons are taken into account, there are 8 distinguishable rat acrocentric/telocentric chromosomes present in this hybrid clone. These studies thus confirm that the cell line is indeed a rat-mouse hybrid and that the hybrid contains at least 13 rat chromosomes (5 biarmed and 8 acrocentric/telocentric) as well as the mouse genome. Any 2^{o} constrictions on these rat chromosomes could not be detected.

As found in other experiments with rat-mouse hybrids (57, 399), A9HT/rat leukemia hybrids preferentially lose the rat chromo-

CELL TYPE	TOTAL CHROMOSOME NO.		NO. OF ACROCENTRIC / TELOCENTRIC CHROMOSOMES				NO. OF BIARMED CHROMOSOMES				NO. OF "DOT" CHROMOSOMES	
			TOTAL NUMBER		NO. UNBANDED		TOTAL NUMBER		NO. UNBANDED			
	MODE	RANGE	MODE	RANGE	MODE	RANGE	MODE	RANGE	MODE	RANGE	MODE	RANGE
A9HT	52	44 - 54	25	19 - 28	5	2 - 6	25	20 - 28	N O N E		2	1 - 3
RAT EMBRYO FIBROBLASTS	42	36 - 43	22	17 - 23	11	7 - 16	21	16 - 24	14	7 - 20	N O N E	
A9HT / RAT LEUKEMIA CLONE 2	72	66 - 75	39	35 - 42	13	10 - 16	30	24 - 34	5	1 - 6	3	1 - 3

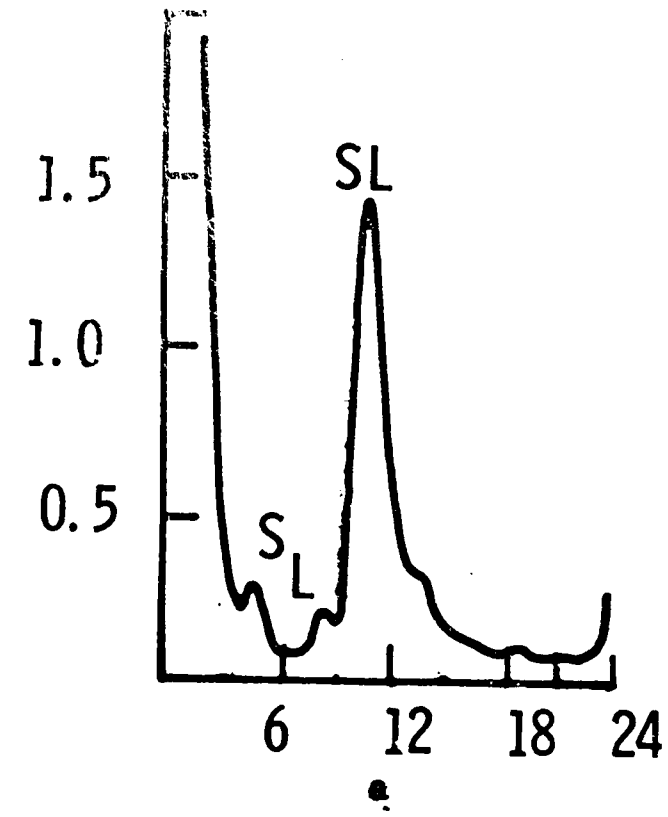
TABLE II
Summary of C-banded chromosomes of A9HT-rat leukemia and its parents.

somes. But during the course of the present experiments, the hybrid clone described here showed little change in karyotype.

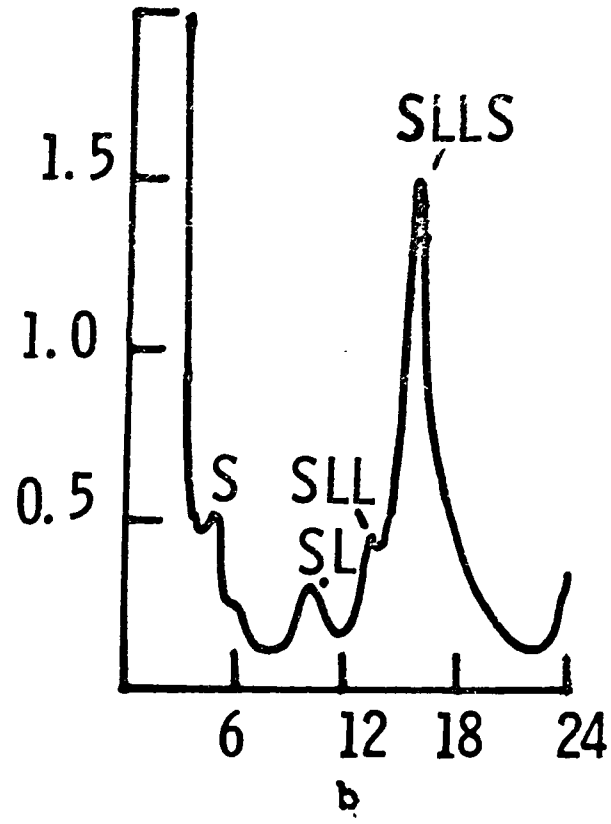
A second hybrid line used in these experiments, A9/RBC (2.012) is a mouse-chicken hybrid with only the normal A9 chromosome complement present. The fact that it expresses chick IMPase suggests that a small amount of chick genetic material is stably associated with the A9 genome (331). But since the chick karyotype consists mostly of tiny, dot chromosomes, it is possible to underestimate the amount of morphologically distinct chick genetic material present.

8.020 Ribosomal RNA in A9HT-rat leukemia hybrid cells.

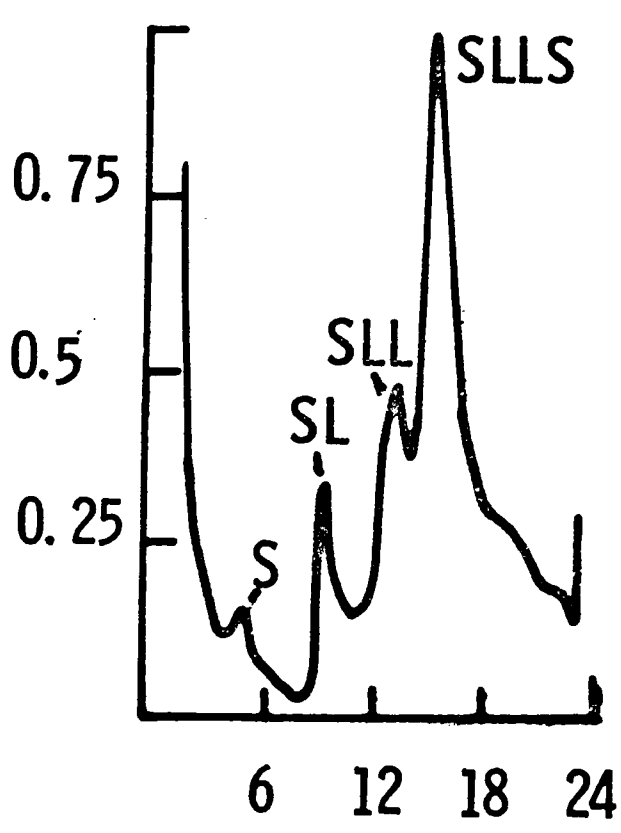
Rat and mouse 28S rRNA can be separated only slightly in low percentage polyacrylamide gels (228). However Reeder and Stanners (309) have shown that ribosomes from different species vary in the extent to which they form dimers at certain concentrations of salt and divalent cations. This is apparently entirely the result of interactions between the 28S rRNA component of free, intact ribosomes or 60S subunits (35). Under these conditions, A9HT ribosomes (Fig 72a) sediment essentially as the monomer with only about 3-5% of the ribosomal optical density greater than the monomer. On the other hand, rat leukemia ribosomes (Fig 72b) sediment as a dimer with no more than 10% of the ribosomal optical density remaining in the monomer peak. Other rat cell lines [NHAC (Fig 72c), rat embryo fibroblasts (Fig 72d) and rat liver] show similar patterns. The several rat lines vary somewhat in the amount in the monomer peak (from 5-20% of the total ribosomal optical density) and this is probably due to the relative exposures to ribonuclease. The dimerization of rat ribosomes is stopped by the addition of ribonuclease before, but not after, dimerization



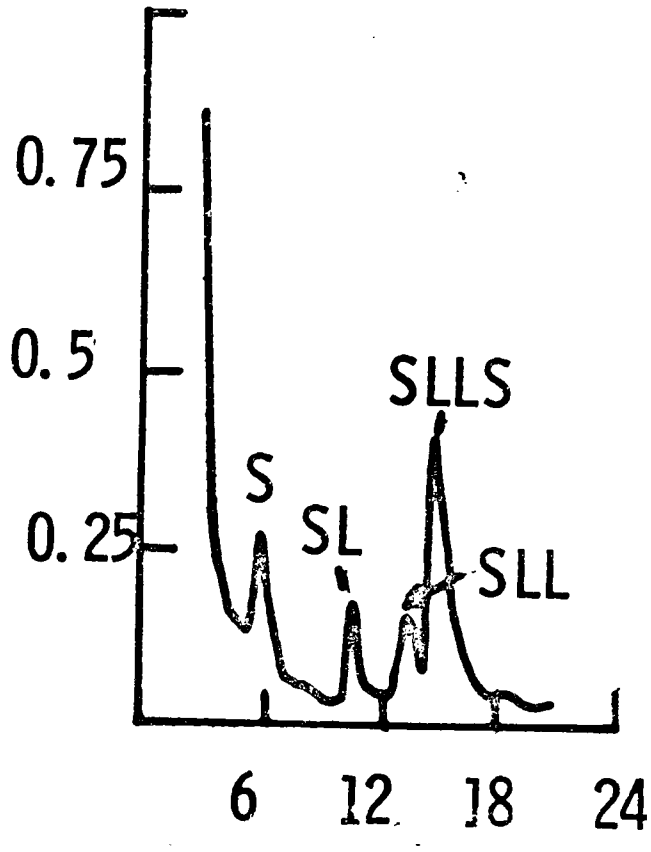
a



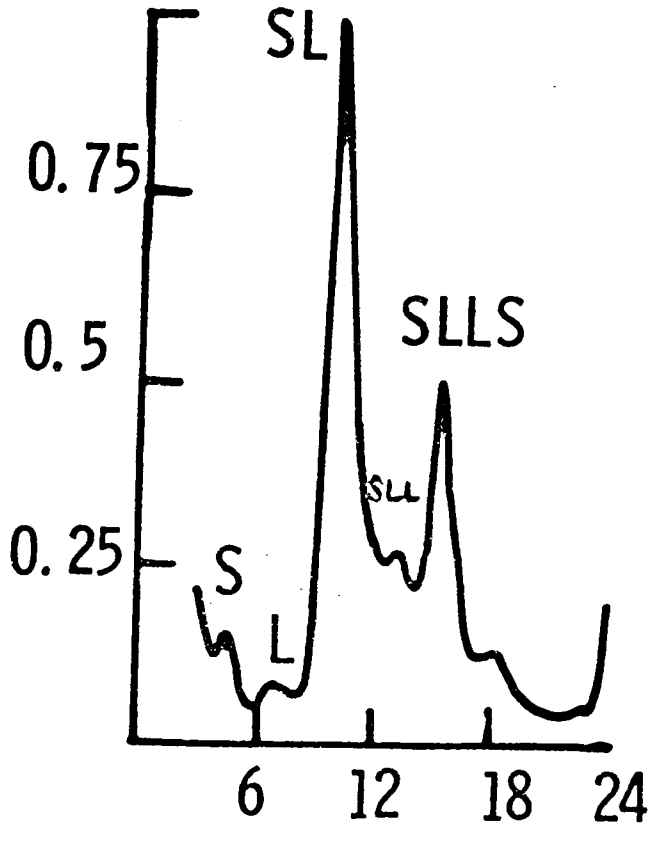
b



c



d



e

Figure 72
Sedimentation profiles of ribosome dimers.
Abscissa: fraction number.
Ordinate: A_{254}.
Ribosome dimers were prepared and centrifuged as in 2.160. (a) A9HT, (b) rat leukemia, (c) HeLa, (d) rat embryo fibroblasts, (e) A9HT-rat leukemia. L designates the larger subunit and S, the smaller.

and by elevation of the temperature above 10°. These limits to dimer formation suggest that it is the interactions between intact RNA molecules that are important.

Using the presence of a dimer peak to detect rat 28S rRNA, the A9HT/rat leukemia hybrid was examined. As indicated in the optical density profile in Fig 72e, a substantial dimer peak was seen. Marshall (228) has correlated the presence of this peak with the presence of rat 28S rRNA on low percentage polyacrylamide gels.

In the hybrid and rat leukemia profiles other peaks are seen. Those sedimenting faster than the dimer were not reproducible. But by analogy with the results of Eliceiri (95), the particles (SLL) sedimenting between the monomer and dimer peaks (Fig 72) may consist of 2 rat large subunits and one small subunit of either rat or mouse origin. On this basis the relative amounts of rat and mouse 28S rRNA may be calculated to be 45% rat (peaks SLLS + SLL) and 55% mouse (peaks S + L + SL).

In the chick-mouse hybrid no 28S rRNA of the chick parent was detected though both species of 28S rRNA can be separated on polyacrylamide gels.

8.030 Ribosomal proteins in hybrid cells.

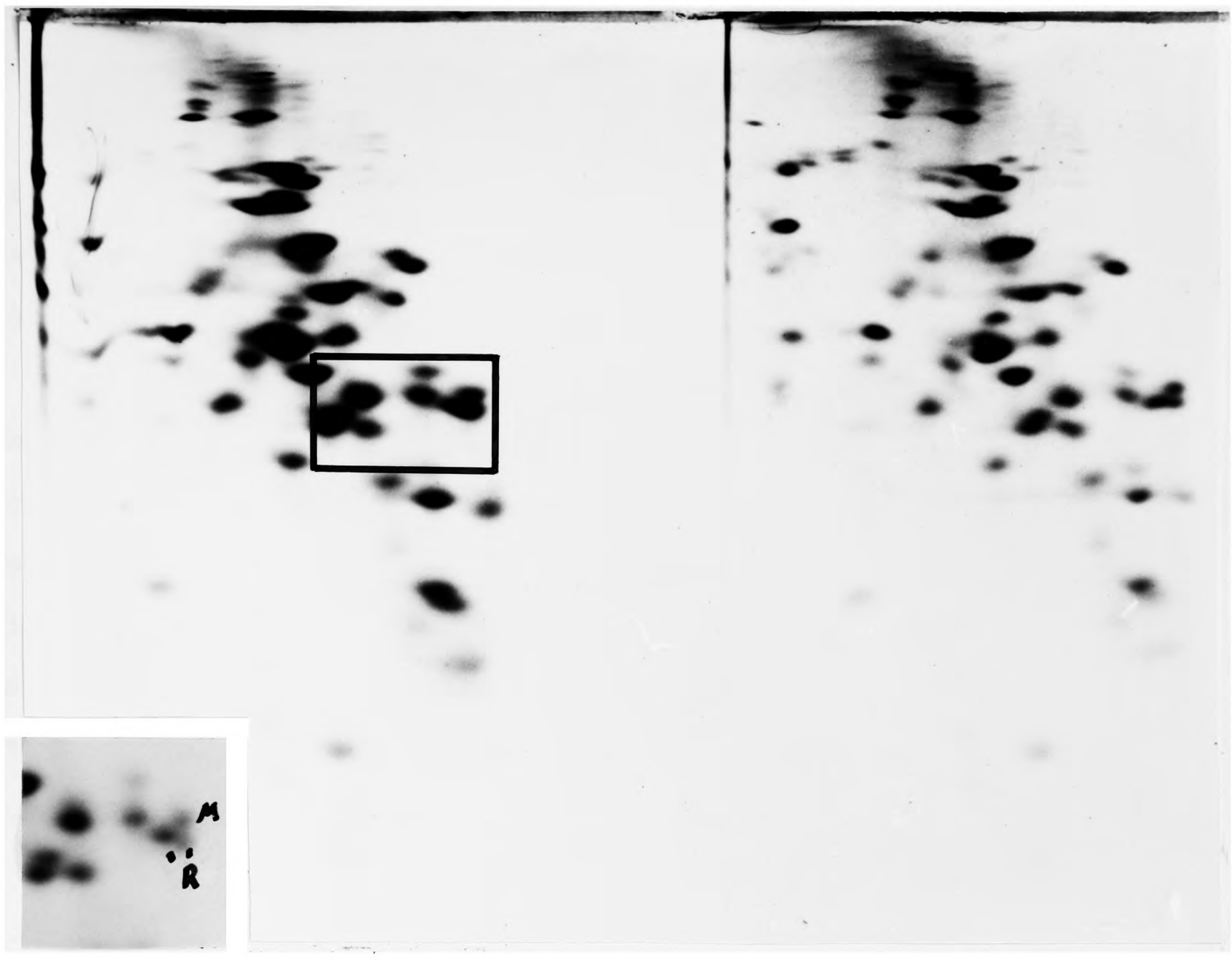
8.031 A9HT/rat leukemia hybrid.

It has previously been shown that there are several significant differences between the ribosomal protein patterns of rat (3.060) and mouse (3.050) lines. Rat patterns show two distinct marker proteins, 77 and 48c (Fig 22) while mouse patterns show a different variant of 48, 48b (Fig 9). Other differences between these species have not been easy to distinguish upon coelectrophoresis and reference will not be made to them.

These species-specific markers are more clearly distinguished upon electrophoresis of a mixture of equal amounts of rat leukemia and A9HT ribosomal protein (Fig 73b). Protein 77 has unfortunately been obscured by a discontinuity in this gel but is clearly evident in Figs 18 and 21. More importantly, this gel affords a good comparison between ribosomal proteins 48b and 48c. The mouse protein migrates less rapidly in the 2nd dimension than does the rat protein.

It was noted that the rat-specific protein has an irregular shape on this and other (Figs 18,21) rat gel patterns. When smaller amounts of protein (50-200 ug) are electrophoresed, the rat-specific spot appears to be composed of 2 partly overlapping spots. Longer times of electrophoresis in the 2nd dimension (Fig 73a, inset) resolve these rat spots more completely and show them to be distinct from the mouse-specific spot. Though Sherton and Wool (335,336) have not observed this, the 2-D patterns of rat liver ribosomes by Welfle et al (404) are identical to those here and indicate that this rat-specific spot is composed of two spots (L27 and L29 in their nomenclature). In addition, Stahl et al (349) have found that both these proteins may be phosphorylated in vitro. Though such has not been shown in vivo, this data does suggest that these two spots may be phosphorylated derivatives of each other. However, these two spots may simply be oxidized and reduced forms of each other. Such artifacts of preparation of these proteins have been known to cause such closely paired spots (151,265).

The ribosomal proteins extracted from ribosomes of the A9HT/ rat leukemia cell line show both mouse-specific and rat-specific protein 48 (Fig 73a). However rat-specific ribosomal protein 77



a b

Figure 73
Two-dimensional patterns of cationic proteins of hybrid ribosomes.

Microgel electrophoresis was as described in 2.072. Samples were (a) A9HT/rat leukemia ribosomal protein (200 ug) and (b) an artificial mixture of A9HT (220 ug) and rat leukemia (210 ug) ribosomal protein. (a, inset) A9HT/rat leukemia ribosomal protein (600 ug) electrophoresed as in (a) except run for 37 hours in the second dimension. The area shown is equivalent to that enclosed by lines in (a). M and R denote the mouse-specific and rat-specific ribosomal proteins, respectively.

was not detected even when much greater amounts of sample were electrophoresed. The ribosomal protein pattern of the hybrid may be directly compared to the artificial mixture of rat and mouse ribosomal proteins since both samples were run on the same gel slab for the 2nd dimension electrophoresis.

8.032 Mouse-chick hybrid cells (A9/RBC).

In Chapter 3 a number of differences between chick and mouse ribosomal protein patterns were established. Of these, a pair of such ribosomal protein variants was conveniently distinguished upon coelectrophoresis: 45b and 45c for mouse (Fig 9) and 45 for chick (Fig 8). Upon electrophoresis of ribosomal protein from the hybrid, however, only ribosomal proteins 45b and 45c were detected (Fig 74). None of the other chick ribosomal proteins could be detected.

8.040 Do hybrid cells have hybrid ribosomes?

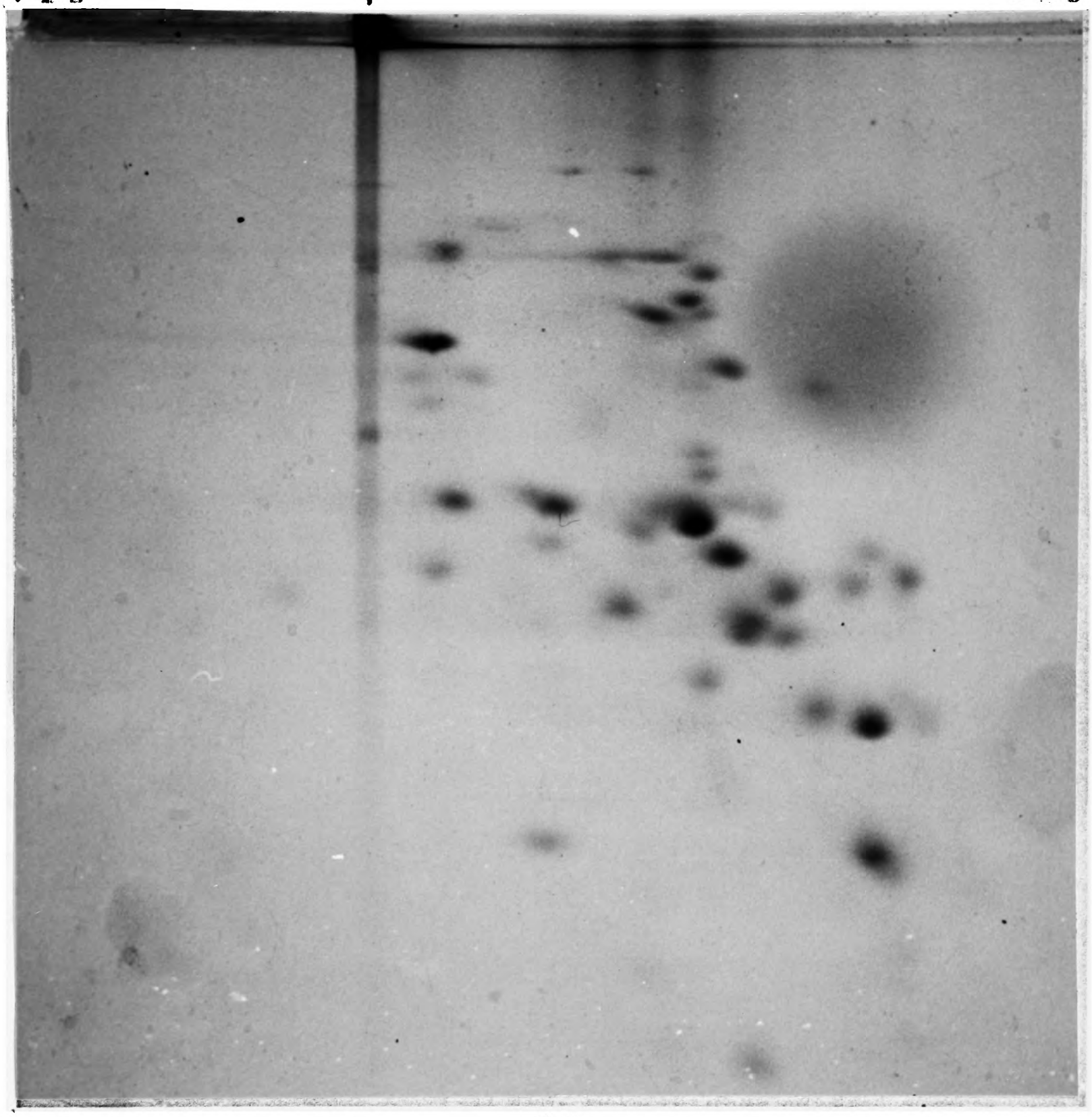
From studies of bacterial ribosomes it was found that the ribosomal proteins from E. coli ribosomes could reconstitute intact, functional ribosomes with 16S rRNA of A. vinelandii or B. stearothermophilus (262) even though such rRNA varied in base composition from E. coli 16S rRNA. Also the 5S rRNA of one bacterial species could replace that of another to give functional ribosomes even though it was somewhat different in sequence. These studies thus suggested that perhaps in the rat-mouse hybrid lines the ribosomal proteins of one species might be associated with the rRNA of another. To test this, use was made of the dimerization of rat ribosomes to isolate particles containing only rat 28S rRNA. Since variants of protein 48 were associated with the large subunit, the ribosomal proteins from such dimers could then be analyzed to see if rat- and/or mouse-specific ribosomal proteins were present.

+ 2-D

ORIGIN

+ 1-D

- 1-D



- 2-D

Figure 74

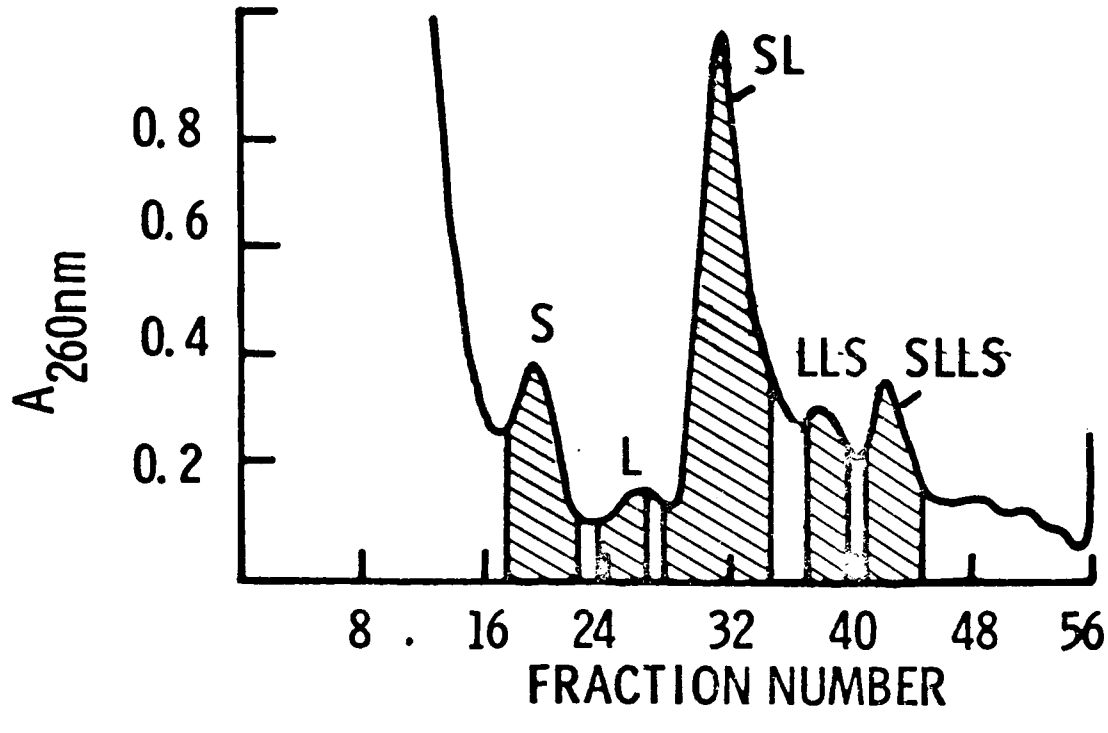
Two-dimensional pattern of the proteins of A9/chick ribosomes.

Standard method electrophoresis (2.071) of 3 mg protein run for 24 hours at 100 V in the first dimension and 36 hours at 100 V in the second dimension.

A9HT/rat leukemia hybrid cells that had been grown in ^{14}C amino acids were used for bulk preparation of ribosome dimers as in 2.160. Monosome and dimer fractions were collected as indicated in Fig 75a and pelleted by centrifugation. The acetic acid extracted ribosomal proteins for the monomer and dimer fractions were run on 2-D gels along with 150 ug of unlabelled hybrid ribosomal proteins in order to reveal ribosomal protein spots upon staining. Spots corresponding to the rat- or mouse-specific ribosomal protein 48 along with an equal sized piece of unstained gel were cut out and prepared for counting. The results (Fig 75b) show that the dimer peak contains almost entirely the rat-specific protein 48c. The monomer data suggests there is more rat-specific marker ribosomal protein on the mouse 28S rRNA than mouse-specific marker. But this latter conclusion is valid only if dimer formation in these bulk preparations is as efficient as in the smaller ones. When the relative staining of the rat- and mouse-specific spots from the hybrid were compared, it also seemed that there was overall significantly more rat- than mouse-specific ribosomal protein.

8.050 Discussion.

With either rat-mouse or mouse-chick hybrids it has not been possible to assign a chromosomal location to those genes coding for ribosomal proteins or rRNA. Other rat-mouse hybrids with fewer rat chromosomes have not yet been isolated. Though additional hybrids need to be analyzed, these first attempts suggest several drawbacks to this approach. For example, those species between which there are an adequate number of species-specific ribosomal proteins (chick-mouse) as revealed by 2-D gel electrophoresis fail to form suitable hybrids. Such hybrid lines lose chick chromosomes too rapidly in culture to permit genetic



a

Gradient fraction	Rat marker 48c (cpm*)	Mouse marker 48b (cpm*)	Control (cpm*)
Monomer (SL)	383.5	134.3	5.4
Dimer (SLLS)	61.1	5.5	5.0

*after correction for background of 20.3 cpm.

b

Figure 75
The presence of hybrid ribosomes in hybrid cells.

(a) Absorbance profile of preparative dimer gradient as described in 2.160. Hatched fractions were those collected and concentrated.
(b) The relative amounts of ^{14}C-labelled protein in rat or mouse specific protein 48 separated by two-dimensional gels from the dimers and monomers of rat-mouse hybrid cells separated by the gradient in (a).

analysis. But more stable hybrid lines obtainable between more closely related species (rat-mouse, mouse-hamster) are limited by the very few electrophoretic differences between their ribosomal protein patterns. Even the stable human-mouse hybrids, though possessing sufficient ribosomal protein differences, fail to synthesize human rRNA, probably due to nucleolar dominance.

Thus the mapping of these ribosomal proteins with interspecific hybrids will demand the elucidation of species which show adequate ribosomal protein differences and form reasonably stable hybrids. Alternatively, the newly-developed antibiotic-resistant lines offer the ability to select for the retention of specific chromosomes in hybrids unlike the hybrids used here which were selected only for the presence of the rat X chromosome (IMPase).

However it is important that while one rat-specific ribosomal protein (48c) was synthesized, the other (protein 77) wasn't. This may indeed reflect the loss of genetic material coding for that ribosomal protein and show that the ribosomal protein genes are on different chromosomes. Since these are the only ribosomal protein markers for the rat ribosome, these studies throw no light on the origin of the other 70 or so ribosomal proteins in the ribosome. But even this interpretation of the absence of linkage between two ribosomal proteins must be approached with caution. The failure to synthesize the protein of one of the parents may just as well be due to the loss of a regulatory gene or to the ill-understood concept of "nucleolar dominance." Since these experiments look only at what stably associates with the ribosomes and not what is synthesized, the missing rat marker might also be synthesized but not stabilized in a ribosome. Thus the fact that protein 77 was

not found in this hybrid could be due to any of these four possibilities.

Though these experiments say little about where ribosomal proteins map, they do yield some information regarding several aspects of the regulation of ribosome synthesis. They show that the nucleolar dominance observed in other somatic cell hybrids (38,97) and in Xenopus (159) is not found in rat-mouse hybrids. The expression of both rat and mouse rRNA in hybrid cells confirms the results of others that selective transcription of only one set of parental rDNA cistrons is not the rule in hybrid cells. Moreover, at least one ribosomal protein gene from each parent is functional in the hybrid.

Nucleolar dominance is not unique to nucleolar function. "Allelic repression" and "allelic exclusion" are synonymous terms that refer to phenomena whereby in F1 hybrids one of the parental gene products is not expressed (53), such as the synthesis of only one immunoglobulin type in a cell heterozygous for two types. Nucleolar dominance thus seems to be allelic repression of rDNA. Since in some hybrids allelic repression seems to be attributable to a chromosome of the dominant parent whose loss results in the reexpression of the other parent's genome (190), it is reasonable to suggest that the lack of nucleolar dominance in the rat-mouse hybrids here may be due to the loss of mouse genetic material suppressing the rDNA of the rat genome or the loss of rat genetic material responsive to this control. Whether the initial fusion product containing the complete mouse and rat genomes shows such repression is not known.

Aside from the hybrids described here, six other rat-mouse hybrid clones were tested for the loss of rRNA or ribosomal protein

of either parent. In no case was such found. Whether ribosomal proteins and rRNA are thus coordinately synthesized is uncertain. However if the relative amounts of the rRNA and specific ribosomal protein are compared, it is found that while the hybrid has about equal amounts of 28S rRNA from each parent (8.020), there is considerably more rat-specific ribosomal protein 48c than mouse-specific protein 48b (8.040). One interpretation of this is that the syntheses of ribosomal protein and rRNA are not balanced. (mechanism "a" in 8.000 is not operative). Hence more rat-specific protein was made than rat rRNA and less mouse-specific protein than mouse rRNA.

However these experiments have not looked at synthesis rates but at the amounts on the final ribosomes themselves. It may thus be that for each parental genome in the hybrid the rRNA and ribosomal proteins are synthesized in balanced amounts (mechanism "a" works) but that the rat protein 48c has a higher affinity for both rRNA species and is more stably bound by both 28S rRNAs while the mouse 48b is turned over more.

Ribosomal proteins 48b and 48c may be unusual in that they show more sequence variation than other ribosomal proteins (Chapter 3). This may reflect their binding directly to the 28S rRNA (since they are large subunit ribosomal proteins) and to sequences that are unique for the species. Other ribosomal proteins would bind those more conserved rRNA sequences. If so, this might explain why the rat 28S rRNA bound only to its own ribosomal protein, 48c, in the hybrid ribosomes (8.040). This behavior is interesting in light of the fact that ribosomal protein 48 is not found in nucleolar particles (7.020, ref 380) and appears to be added on in the cytoplasm where it rapidly equilibrates with newly synthesized ribosomal

protein (380).

8.060 Summary.

A rat-mouse hybrid cell line was examined for the presence of rRNA and ribosomal proteins from both parents. As demonstrated by banding of centromeric heterochromatin, the hybrid cell line contained most of the mouse genome and at least 13 rat chromosomes. The ability of rat, but not mouse, ribosomes to dimerize was used to show that the hybrid contained both rat and mouse 28S rRNA. Two-dimensional polyacrylamide gel electrophoresis of ribosomal proteins indicated the presence of one rat and one mouse marker ribosomal protein. The synthesis of one but not a second rat marker protein suggested that at least two ribosomal proteins were not linked. In the hybrid the rat 28S rRNA bound only the rat-specific marker ribosomal protein while the relative amounts of ribosomal protein and rRNA of the two species implied that their syntheses were not balanced with respect to each other.

CHAPTER 9

FURTHER DIRECTIONS

The experiments discussed in the previous chapters have described a methodology which has enabled a number of initial studies to be conducted on the eukaryotic ribosome. Many of these studies were a direct adaptation of those techniques and experiments conducted upon bacterial ribosomes. However the analogy with the bacterial systems cannot be complete. Functions and responses (such as to hormones) foreign to bacteria have been attributed to the eukaryotic ribosome. The increased number of ribosomal proteins and size of the eukaryotic ribosome have also been attributed to evolutionary advance (220). Whether functions may be assigned to those elements of increased complexity is yet uncertain. It is thus the purpose of this chapter to suggest those (often obvious) extensions of the bacterial ribosome work but also to show new areas where research may pursue those problems which are the sole concern of the eukaryotic ribosome.

By now sufficient studies have been completed to enable some consensus about the 2-D protein patterns of ribosomes of different species as well as their subunit distribution. Further work will undoubtedly be concerned with establishing the structural relations of these ribosomal proteins within the ribosome and their exposure to the external environment. Already the use of protein kinases (3.090) has allowed the specific phosphorylation of those ribosomal proteins on the "outside" of the ribosome and their identification after autoradiography on 2-D gels. Additional proteins became phosphorylated upon dissociation into subunits (348). Enzyme iodination with lactoperoxidase and hydrogen peroxide has also been

so used, however it labels all the ribosomal proteins (205,401) and suggests they are all exposed to the surface in some way.

More functional relations may be established using chemicals that specifically modify certain chemical groups. Iodoacetamide may be used to react with and label sulfhydryl and carboxylate groups while methyl-acetimidate could be used for amino groups (409). The resulting radioactively labelled ribosomal proteins might then be detected with autoradiography. Similar use of specifically reactive antibiotics (301) or other affinity labels (119,193,194,223) might also be employed to identify specific ribosomal protein sites.

More precise relations between eukaryotic ribosomal proteins may be established with the use of covalent cross-linking substances like dimethyl suberimidate which have proved so useful in elucidating the ribosomal protein "neighborhoods" in prokaryotic ribosomes (33). Newer cross-linking reagents which contain a reducible disulfide bond may be of even more use.

The development of antibodies to single isolated ribosomal proteins gives the most precise tool for probing the surface of the ribosome and they have been used extensively to determine the surface topography of bacterial ribosomes (354). When coupled with electron microscopy, this technique allows a three-dimensional location to be assigned to certain ribosomal proteins (372,373,385). Though the development of such specific antisera for eukaryotic ribosomal proteins has been slow (3.090), a similar 3-D structure might be developed by immune electron microscopy.

The ability to isolate various precursors from the nucleoli of eukaryotic cells allows an extension of the chemical techniques above (phosphorylation, immune electron microscopy, affinity labels)

to the analysis of these precursors. Such studies might show alterations in protein neighbors or modification of various ribosomal proteins during precursor processing. The development of appropriate antisera might also allow a more convenient determination of which ribosomal proteins of both subunits accumulate in the nucleolus (as detected by immunofluorescence of unfractionated cells), a method that would avoid any artifacts incurred previously in precursor preparation (7.030). Whether small subunit ribosomal proteins do enter the nucleolus might then be detected.

Another potential use of such sera would be in analysis of the reactivation of the chick erythrocyte, a phenomenon which occurs when nucleated chick erythrocytes are fused into dividing cells to form heterokaryons. The chick nucleus soon begins to synthesize RNA and DNA (140,141) and when a nucleolus forms, mRNA exits to the cytoplasm (146,338). These events have been correlated with the passage of specific antigens to the chick nucleolus from the host cytoplasm (51,52,310). Whether any of these antigens are ribosomal proteins might be determined with antisera to ribosomal proteins.

This correlation of nucleolar function with mRNA transport is a puzzling phenomenon the existence of which is strengthened by the fact that microbeam u.v. irradiation of nucleoli (68,69,338) stops transport of all classes of RNA to the cytoplasm. Since HnRNA is synthesized outside of the nucleolus using different polymerase(s) (384), the inactivated nucleolar mechanism seems to be responsible for the transport of mRNA to the cytoplasm. Some early workers suggested that the small ribosomal subunit was responsible for this transport (155,175,238). Hence irradiation of the nucleolus would stop formation of new ribosomal precursors and

also messenger transpcrt. But the original experiments were hampered by the fact that the polydisperse, rapidly labelled RNA might only be fortuitously sedimenting with the small subunit. Though more recent workers do not favor this concept, it is important to reanalyze this proposition with more modern methods. Detergent-cleaned nuclei might be fractionated as done by Vaughan et al (383) and the nucleoplasmic precursors to the small and large subunit isolated. After purification on metrizamide gradients, the presence of poly A-containing material might be assayed to see if these nucleoplasmic ribosomal subunits were associated with significant amounts of messenger. If so, then further work using 2-D gels might give further insight into the protein composition of these particles.

Other workers have isolated from cells three different types of RNP particles associated with RNA metabolism. The first class consists of 30S particles isolated from nuclei that consist of HnRNA and associated protein and have been called "informofers" (126,320,321). However the protein composition of these particles varies with the gel technique -- only a few protein bands are detected on urea gels (19,253,254) but many (30-50) with SDS gels (2,106,253,254,272). Given the complexity of these proteins and the fact that some are felt to be ribosomal proteins (19), analysis of these proteins using the 2-D methods may determine if ribosomal proteins play a role in these particles.

A second class of nuclear RNP particles are distinct from the informofers in that they contain poly A and have been isolated on this basis (106,308). Like the informofers these also show a complex protein composition whose resolution would be aided by 2-D gel electrophoresis. Such a 2-D analysis here and for the informofers

would help eliminate any uncertainty that these particles are derivatives of nucleoplasmic ribosomal subunits (or their precursors) as well as to establish their precise protein composition.

Treatment of polysomes with either EDTA (164,206), puromycin-salt (28,30), or heat (328) releases a third class of particles which are composed of mRNA and proteins. Four or more proteins were associated with those prepared using EDTA (10,30,153,168) but included the only two proteins found after puromycin-salt (6, 30,153). One of these two latter proteins (MW 73,000) is a poly A binding protein while the other (MW 50,000) is phosphorylated. Since both are from the polysomes they should correspond with some ribosomal protein spots in a typical 2-D gel pattern and future experiments with these isolated particles may indicate which ribosomal proteins. Similar 2-D gel comparisons could also be made to establish if there were any similarities between the various nuclear proteins associated with mRNA and those associated with it on polysomes. Studies of this nature would thus give a clearer understanding of those structures associated with the transportation of messenger from the nucleus to the cytoplasm.

Chapter 3 has described the great conservation of ribosomal protein patterns between the many different tissues and species but in so doing, the great specificity of each translational system has been overlooked. In using such well washed ribosome preparations, many specific protein synthesis factors have been removed. The analysis of these may be particularly important in understanding developing organisms. Due to its ability to resolve a great number of different proteins, a 2-D analysis of these proteins may help describe developmental changes at the translational level.

Such an analysis of the factors associated with ribosomes may

also be of interest with respect to the action of hormones or interferon upon translation. For example, interferon makes the treated cells' protein synthesizing machinery many times more sensitive to inhibition by double-stranded RNA, perhaps accounting for some of the antiviral properties (187). That this effect is not due to modification or substitution of a ribosomal protein may be determined by analyzing the gel pattern of the washed ribosomes. But it is perhaps more likely that this effect is mediated via a factor binding to the ribosomes, in which case the pattern of the salt wash itself may demonstrate some altered protein composition.

The emphasis in all these suggestions for future work has been on the use of two-dimensional gel methods to analyze and compare multi-protein complexes, especially those associated with ribosomal proteins. The goal has been to determine the protein composition of those nuclear and cytoplasmic RNP particles which are important for messenger RNA transport and translation. More recent 2-D gel systems such as that of Mets and Bogorad (240) or O'Farrell (265) may be even more convenient than those methods used here. But these 2-D gel methods cannot be the sole approach to these problems and those discussed in earlier chapters. Future studies on eukaryotic ribosomal proteins must also consider genetic (perhaps using the many potential drug sensitive lines) as well as synthetic (with reconstitution of ribosomal precursors from purified rRNA and protein) approaches to determine structure and function.

REFERENCES

1 Alberghina, F A M & Suskind, S R, J bact 94 (1967) 630.
2 Albrecht, C & van Zyl I M, Exptl cell res 76 (1973) 8.
3 Allderdice, P W, Miller, O J, Miller, D A, Warburton, D, Pearson, P L, Klein, G & Harris, H, J cell sci 12 (1973) 263.
4 Allderdice, P W, Miller, O J, Pearson, P L, Klein, G & Harris, H, J cell sci 12 (1973) 809.
5 Ashby, C D & Roberts, S, J biol chem 250 (1975) 2546.
6 Auerbach, S & Pederson, T, Biochem biophys res comm 63 (1975) 149.
7 Auger, M-A & Tiollais, P, Eur j biochem 48 (1974) 157.

8 Bailey, J L, Techniques in Protein Chemistry (2nd Revised and Expanded Edition), Elsevier (New York, 1967), p 340.
9 Baltimore, D & Huang, A, J mol biol 47 (1970) 293.
10 Barrieux, A Biochemistry 14 (1975) 1815.
11 Bennett, P M & Maaløe, O, J mol biol 90 (1974) 541.
12 Benzer, S, Proc natl acad sci US 47 (1961) 403.
13 Berger, E, Mol gen genet 128 (1974) 1.
14 Bernhard, V & Granboulan, N, Exptl cell res supp 9 (1963) 19.
15 Berns, A, Salden, M, Bogdanovsky, O, Raymondjean, M, Schapira, G & Bloemendal, H, Proc natl acad sci US 72 (1975) 714.
16 Bickle, T A & Traut, R R, J biol chem 246 (1971) 6828.
17 Bielka, H, Stahl, J & Welfle, H, Arch geschwulstforch 38 (1971) 109.
18 Bielka, H & Welfle, H, Mol gen genet 102 (1968) 128.
19 Billings, P B & Martin, T E, J cell biol 59 (1973) 249.
20 Birnbaum, L S & Kaplan, S, Proc natl acad sci US 68 (1971) 925.
21 Birnstiel, M L, Ann rev plant physiol 18 (1967) 25.
22 Birnstiel, M L, Chipchase, M I H & Flamm, W G, Biochim biophys acta 87 (1964) 111.
23 Birnstiel, M L, Chipchase, M I H & Hyde, B B, Biochim biophys acta 76 (1963) 454.
24 Birnstiel, M L & Flamm, W G, Biochim biophys acta 87 (1964) 101.
25 Birnstiel, M L & Hyde, B B, J cell biol 18 (1963) 41.
26 Bjork, I & Fish, W F, Biochemistry 10 (1971) 2844.
27 Blackler, A W & Gecking, C A, Dev biol 27 (1972) 385.
28 Blobel, G, Proc natl acad sci US 68 (1971) 832.
29 Blobel, G, Proc natl acad sci US 68 (1971) 1881.
30 Blobel, G, Biochem biophys res comm 47 (1972) 88.
31 Blobel, G & Sabatini, D D, J cell biol 45 (1970) 130.
32 Blobel, G & Sabatini, D D, Proc natl acad sci US 68 (1971) 390.
33 Bode, U, Lutter, L C, Kurland, C G, Zeichhardt, H & Stoffler, G, Acta biol med germ 33 (1974) 625.

34 Bollen, A, Davies, J, Ozaki, M & Mizushima, S, Science 165 (1969) 85.
35 Bollen, A, Faelen, M, Lecoaq, J P, Herzog, A, Zengel, J, Kahan, L & Nomura, M, J mol biol 76 (1973) 463.
36 Bourque, D P & Wildman, S G, Biochem biophys res comm 50 (1973) 532.
37 Brachet, J, Compt rend soc biol 133 (1940) 88.
38 Branwell, M E & Handmaker, S D, Biochim biophys acta 232 (1971) 580.
39 Bray, G A, Anal biochem 1 (1960) 279.
40 Brot, N & Weissbach, H, Biochem biophys res comm 49 (1973) 673.
41 Brouwer, J & Planta, R J, FEBS letters 53 (1975) 73.
42 Brown, D D & Blackler, A W, J mol biol 63 (1972) 75.
43 Brown, D D & Gurdon, J B, Proc natl acad sci US 51 (1964) 139.
44 Brown, D D, Wensink, P C & Jordan, E, J mol biol 63 (1972) 57.
45 Brown, D G & Abrams, A, Biochim biophys acta 200 (1970) 522.
46 Brown, M E & Apirion, D, Mol gen genet 133 (1974) 317.
47 Brownlee, G G, Sanger, F & Barrell, B G, Nature 215 (1967) 735.
48 Busch, H, Busch, R K, Spohn, W, Wikman, J & Daskal, Y, Proc soc exp med biol 137 (1971) 1470.
49 Busch, R K, Spohn, W H, Daskal, Y & Busch, H, Proc soc exp med biol 140 (1972) 1030.

50 Cabezon, T, Faelen, M, deWilde, M, Bollen, A & Thomas, R, Mol gen genet 137 (1975) 125.
51 Carlsson, S-A, Luger, O, Ringertz, N R & Savage, R E, Exptl cell res 84 (1974) 47.
52 Carlsson, S-A, Ringertz, N R & Savage, R E, Exptl cell res 84 (1974) 255.
53 Castro-Sierra, E & Ohno, S, Biochem genet 1 (1968) 323.
54 Chantrenne, H, The Biosynthesis of Proteins, Pergamon Press (New York, 1961), p 48.
55 Comings, D E, Avelino, E, Okada, T A & Wyandt, H E, Exptl cell res 77 (1973) 469.
56 Cooper, D, Banthorpe, D V & Wilkie, D, J mol biol 26 (1967) 347.
57 Cotton, R G H & Milstein, C, Nature 244 (1973) 42.
58 Craig, N C, J mol biol 55 (1971) 129.
59 Craig, N C & Perry, R P, Nature new biol 229 (1971) 75.
60 Creusot, F, Delaunay, J & Schapira, G, Biochemie 57 (1975) 167.

61 Dahlberg, A E, J biol chem 249 (1974) 7673.
62 Darnbrough, C, Legon, S, Hunt, T & Jackson, R T, J mol biol 76 (1973) 379.
63 Darnell, J R, Bact rev 32 (1968) 262.
64 Daskal, Y, Prestayko, A W & Busch, H, Exptl cell res 88 (1974) 1.
65 Davidson, R L, Ann rev genet 8 (1974) 195.

66 Davidson, R L & de la Cruz, F F, Somatic Cell Hybridization, Raven Press (New York, 1974).
67 Davies, J & Nomura, M, Ann rev genet 6 (1972) 203.
68 Deák, I, J cell sci 13 (1973) 395.
69 Deák, I, Sidebottom, E & Harris, H, J cell sci 11 (1972) 379.
70 Dekio, S, Mol gen genet 113 (1971) 20.
71 Dekio, S, Takata, R & Osawa, S, Mol gen genet 109 (1970) 131.
72 Delaunay, J, Creusot, F & Schapira, G, Eur j biochem 39 (1973) 305.
73 Delaunay, J, Creusot, F & Schapira, G, Biochemie 56 (1974) 1459.
74 Delaunay, J, Loeb, J E, Pierre, M & Schapira, G, Biochim biophys acta 312 (1973) 147.
75 Delaunay, J, Mathieu, C & Schapira, G, Eur j biochem 31 (1972) 531.
76 Delaunay, J & Schapira, G, Biochim biophys acta 259 (1972) 243.
77 Delovitch, T L, Davis, B K, Holme, G & Sehon, A H, J mol biol 69 (1972) 373.
78 Dennis, PP, Nature new biol 232 (1971) 43.
79 Dennis, PP, J mol biol 89 (1974) 223.
80 Dennis, PP & Nomura, M, Proc natl acad sci US 71 (1974) 3819.
81 Dennis, PP & Nomura, M, Nature 255 (1975) 460.
82 Dev, V G, Miller, D A, Allderdice, P W & Miller, Exptl cell res 73 (1972) 259.
83 deWilde, M, Michel, F & Broman, K, Mol gen genet 133 (1974) 329.
84 Dice, F J & Schimke, R, J biol chem 247 (1972) 98.
85 DiCioccio, R A, Voss, R, Krim, M, Grzeschik, K H & Siniscalco, M, Proc natl acad sci US 72 (1975) 1868.
86 Dulbecco, R & Freeman, G, Virology 8 (1959) 396.
87 Dulbecco, R & Vogt, M, J exptl med 99 (1957) 167.
88 Duncan, M J & Gorini, L, Proc natl acad sci US 72 (1975) 1533.
89 Dunn, J J & Studier, F W, Proc natl acad sci US 70 (1973) 3296.

90 Eagle, H, Science 130 (1959) 432.
91 Earle, W, J natl cancer inst 4 (1943) 165.
92 Edstrom, J E, J biophys biochem cytol 8 (1960) 47.
93 Eil, C & Wool, I, J biol chem 248 (1973) 5122.
94 Eil, C & Wool, I G, J biol chem 248 (1973) 5130.
95 Eliceiri, G L, J cell biol 53 (1972) 177.
96 Eliceiri, G L, Biochim biophys acta 269 (1972) 450.
97 Eliceiri, G L & Green, H, J mol biol 41 (1969) 253.
98 Erdmann, V A, Fahnestock, S, Higo, K & Nomura, M, Proc natl acad sci US 68 (1971) 2932.
99 Erdmann, V A & Horne, J R, Mol gen genet 119 (1972) 337.
100 Erdmann, V A & Nomura, M, Nature 228 (1970) 744.

101 Falvey, A K & Staehelin, T, J mol biol 53 (1970) 1.
102 Fazekas de St Groth, S, Webster, R G & Datyner, A, Biochim biophys acta 71 (1963) 377.
103 Fehlmann, M, Bellemare, G & Godin, C, Biochim biophys acta 378 (1975) 119.

104 Feit, H, Slusarek, L & Shelanski, M L, Proc natl acad sci US 68 (1971) 2028.
105 Fiil, N P, von Meyenburg, K & Friesen, J D, J mol biol 71 (1972) 769.
106 Firtel, R A & Pederson, T, Proc natl acad sci US 72 (1975) 301.
107 Flamm, W G & Birnstiel, M L, Biochim biophys acta 87 (1964) 101.
108 Freienstein, C & Blobel, G, Proc natl acad sci US 71 (1974) 3435.
109 Fridlender, B R & Wettstein, F A, Biochem biophys res comm 39 (1970) 247.
110 Funatsu, G, Nierhaus, K & Wittmann, H G, Biochim biophys acta 287 (1972) 282.

111 Gallinaro-Matringe, H, Stevenin, J & Jacob, M, Biochemistry 14 (1975) 2547.
112 Garrett, R A, Schulte, C, Stoffler, G, Gray, P & Monier, R, FEBS letters 49 (1974) 1.
113 Garrett, R A & Wittmann, H G, Advances in prot chem 27 (1973) 277.
114 Gausing, K, Mol gen genet 129 (1974) 61.
115 Geisser, M, Tischendorf, G W & Stoffler, G, Mol gen genet 127 (1974) 129.
116 Geisser, M, Tischendorf, G W, Stoffler, G & Wittmann, H G, Mol gen genet 127 (1974) 111.
117 Ginsburg, D & Steitz, J A, J biol chem 250 (1975) 5647.
118 Girard, M, Latham, H, Penman, S & Darnell, J E, J mol biol 11 (1965) 187.
119 Girshovich, A S, Bochkareva, E S & Pozdnyakov, V A, Acta biol med germ 33 (1974) 639.
120 Glass, R D & Doyle, D, J biol chem 247 (1972) 5234.
121 Godwin, E, Cox, R A & Huvos, P, Acta biol med germ 33 (1974) 733.
122 Goldstein, E S & Penman, S, J mol biol 80 (1973) 243.
123 Goodman, R M & Benjamin, W B, Exptl cell res 77 (1973) 63.
124 Gould, H, Nature 227 (1970) 1145.
125 Gray, P N, Bellemare, G, Monier, R, Garrett, R A & Stoffler, G, J mol biol 77 (1973) 133.
126 Greenberg, J R, J biol chem 64 (1975) 269.
127 Gressner, A M & Wool, I G, J biol chem 249 (1974) 6917.
128 Groner, Y, Pollack, Y, Borissi, H & Revel, M, Nature new biol 239 (1972) 16.
129 Groner, Y, Scheps, R, Kamen, R, Kolakofsky, D & Revel, M, Nature new biol 239 (1972) 19.
130 Grumnt, F & Grumnt, I, FEBS letters 42 (1974) 343.

131 Hallberg, R L & Brown, D D, J mol biol 46 (1969) 393.
132 Hamilton, M G, Pavlovec, A & Petermann, M L, Biochemistry 10 (1971) 3424.
133 Hamilton, M G & Ruth, M E, Biochemistry 6 (1967) 2585.
134 Hamilton, M G & Ruth, M E, Biochemistry 8 (1969) 851.
135 Handmaker, S D, Ann rev micro 27 (1973) 139.
136 Hanna, N, Bellemare, G & Godin, C, Biochim biophys acta 331 (1973) 141.

137 Hanna, N & Godin, C, Biochim biophys acta 374 (1974) 342.
138 Hanson, M R, Davidson, J N, Mets, L J & Bogorad, L, Mol gen genet 132 (1974) 105.
139 Hardy, SJS, Kurland, C G, Voynow, P & Mora, G, Biochemistry 8 (1969) 2897.
140 Harris, H, Nature 206 (1965) 583.
141 Harris, H, J cell sci 2 (1967) 23.
142 Harris, H, Harvey lect 65 (1969) 1.
143 Harris, H, Cell Fusion, Clarendon Press (Oxford, 1970).
144 Harris, H, Proc royal soc Lond (B) 179 (1971) 1.
145 Harris, H, Miller, O J, Klein, G, Worst, P & Tachibana, T, Nature 223 (1969) 363.
146 Harris, H, Sidebottom, E, Grace, D M & Bramwell, M E, J cell sci 4 (1969) 499.
147 Harris, H & Watkins, J F, Nature 205 (1965) 640.
148 Haselkorn, R & Rothman-Denes, L B, Ann rev biochem 42 (1973) 397.
149 Haugli, F B & Dove, W F, Mol gen genet 118 (1972) 97.
150 Heady, J E & McConkey, E H, Biochem biophys res comm 40 (1970) 30.
151 Held, W A, Mizushima, S & Nomura, M, J biol chem 248 (1973) 5720.
152 Held, W A & Nomura, M, J biol chem 250 (1975) 3179.
153 Hellerman, J G & Shafritz, D A, Proc natl acad sci US 72 (1975) 1021.
154 Henderson, A S, Warburton, D & Atwood, K C, Proc natl acad sci US 69 (1972) 1464.
155 Henshaw, E, Revel, M & Hiatt, H, J mol biol 14 (1965) 241.
156 Herr, W & Noller, H F, FEBS letters 53 (1975) 248.
157 Holme, G, Delovitch, T L, Boyd, S L & Sehon, A H, Biochim biophys acta 247 (1971) 104.
158 Holtzman, E, Smith, I & Penman, S, J mol biol 17 (1966) 131.
159 Honjo, T & Reeder, R H, J mol biol 80 (1973) 217.
160 Horne, J R & Erdmann, V A, Proc natl acad sci US 70 (1973) 2870.
161 Houssais, J F, Eur j biochem 24 (1971) 232.
162 Howard, G A, Traugh, J A, Croser, E A & Traut, R R, J mol biol 93 (1975) 391.
163 Howard, G A & Traut, R A, FEBS letters 29 (1973) 177.
164 Huez, G, Burney, A, Marbaix, G & Lebleu, B, Biochim biophys acta 145 (1967) 629.
165 Huynh-van-tan, Delaunay, J & Schapira, G, FEBS letters 17 (1971) 163.

166 Inouye, H, Pollack, Y & Petre, J, Eur j biochem 45 (1974) 109.
167 Irr, J D, Kaulenas, M S & Unsworth, B R, Cell 3 (1974) 249.
168 Irwin, D, Kumar, A & Malt, R A, Cell 4 (1975) 157.
169 Izawa, M & Kawashima, K, Biochim biophys acta 155 (1967) 51.
170 Izawa, M & Kawashima, K, Biochim biophys acta 174 (1969) 124.

171 Jacob, J & Sirlin, J L, J cell biol 17 (1963) 153.
172 Jarry, B & Rosset, R, Mol gen genet 113 (1971) 43.
173 Jaskunas, S R, Lindahl, L & Nomura, M, Proc natl acad sci US 72 (1975) 1.

174 Jaskunas, S R, Lindahl, L & Nomura, M, Nature 256 (1975) 183.
175 Joklik, W K & Becker, Y, J mol biol 13 (1964) 511.

176 Kabat, D, Biochemistry 9 (1970) 4160.
177 Kabat, D, Biochemistry 10 (1971) 197.
178 Kaltschmidt, E, Anal biochem 43 (1971) 25.
179 Kaltschmidt, E & Wittmann, H G, Anal biochem 36 (1970) 401.
180 Kaltschmidt, E & Wittmann, H G, Proc natl acad sci US 67 (1970) 1276.
181 Kanai, H, Castles, J J, Wool, I G, Stirewalt, W S & Kanai, A, FEBS letters 5 (1969) 68.
182 Kanda, F, Comiai, H & Iwabuchi, M, Eur j biochem 44 (1974) 469.
183 Katchalski, E, Schultz, G & Chen, D, Nature new biol 231 (1971) 69.
184 Kaulenas, M S & Unsworth, B R, Mol gen genet 135 (1974) 231.
185 Kawashima, K, Izawa, M & Sato, S, Biochim biophys acta 232 (1971) 192.
186 Keep, E, Can j genet cytol 4 (1962) 206.
187 Kerr, I M, Brown, R E & Ball, L A, Nature 250 (1974) 57.
188 Kessin, R H, Dev biol 31 (1973) 242.
189 King, H W S, Gould, H J & Shearman, J J, J mol biol 61 (1971) 143.
190 Klebe, R J, Chen, T & Ruddle, F H, Proc natl acad sci US 66 (1970) 1220.
191 Klein, C, FEBS letters 38 (1974) 149.
192 Klucis, E S & Gould, H J, Science 152 (1966) 378.
193 Knorre, D G, Acta biol med germ 33 (1974) 649.
194 Kuechler, E, Hauptmann, R, Czernilofsky, A P, Fiser, I, Barta, A, Voorma, H O, Stoffler, G & Scheit, K H, Acta biol med germ 33 (1974) 633.
195 Kumar, A & Subramanian, A R, J mol biol 94 (1975) 409.
196 Kumar, A & Warner, J R, J mol biol 63 (1972) 233.
197 Kumar, A & Wu, R S, J mol biol 80 (1973) 265.
198 Kung, H-F, Fox, J E, Spears, C, Brot, N & Weissbach, H, J biol chem 248 (1973) 5012.
199 Kuter, D J & Rodgers, A, Exptl cell res 87 (1974) 186.
200 Kuter, D J & Rodgers, A, Exptl cell res 91 (1975) 317.
201 Kuter, D J & Rodgers, A, In preparation.

202 Lafontaine, J G & Chouinard, L A, J cell biol 17 (1963) 167.
203 Lane, C D, Marbaix, G & Gurdon, J B, J mol biol 61 (1971) 73.
204 Lazardi, P M & Luck, D J, J cell biol 54 (1972) 56.
205 Leader, D P, Acta biol med germ 33 (1974) 685.
206 Lebleu, B, Marbaix, G, Huez, G, Temmerman, J, Burny, A & Chantrenne, H, Eur j biochem 19 (1971) 264.
207 Leboy, P S, Cox, E C & Flaks, J G, Proc natl acad sci US 52 (1964) 1367.
208 Leibowitz, M J & Soffer, R L, Proc natl acad sci US 68 (1971) 1866.
209 Leister, D E & Dawid, I B, J biol chem 249 (1974) 5108.

210 Levisohn, S R & Thompson, E B, Nature new biol 235 (1972) 102.
211 Lewin, B, Nature 250 (1974) 619.
212 Liau, M C & Perry, R P, J cell biol 42 (1969) 272.
213 Liautard, J-P & Kohler, K, Anal biochem 60 (1974) 581.
214 Liew, C-C & Gornall, A G, J biol chem 248 (1973) 977.
215 Lin, A & Wool, I G, Mol gen genet 134 (1974) 1.
216 Littlefield, J W, Proc natl acad sci US 50 (1963) 568.
217 Littlefield, J W, Science 145 (1964) 709.
218 Loening, U E, J mol biol 38 (1965) 355.
219 Loening, U E, Biochem j 102 (1967) 251.
220 Loening, U E, J mol biol 38 (1968) 355.
221 Loening, U E, Biochem j 113 (1969) 131.
222 Lowry, O H, Rosenbrough, N J, Farr, A L & Randall, R J, J biol chem 193 (1951) 265.

223 Maassen, J A & Moller, W, Acta biol med germ 33 (1974) 621.
224 Maden, B E H, Vaughan, M H, Warner, J R & Darnell, J E, J mol biol 45 (1969) 265.
225 Maisel, J C & McConkey, E H, J mol biol 61 (1971) 251.
226 Majumder, G C & Turkington, R W, J biol chem 247 (1972) 7207.
227 Markey, F, (1974) personal communication.
228 Marshall, C J, D Phil thesis, Oxford, 1973.
229 Marshall, C J, Exptl cell res 91 (1975) 464.
230 Marshall, C J, Handmaker, S D & Bramwell, M E, J cell sci 17 (1975) 307.
231 Martin, T E, Rolleston, F S, Low, R B & Wool, I G, J mol biol 43 (1969) 135.
232 Martin, T E, Wool, I G & Castles, J J, In K Moldave and L Grossman (eds), Methods in Enzymology, Vol XX, Academic Press (New York, 1971), p 417.
233 Martini, O H W & Gould, H J, J mol biol 62 (1971) 403.
234 Martini, O H W & Gould, H J, Biochim biophys acta 295 (1973) 621.
235 Mathews, M B, Osborn, M, Berns, A J M & Bloemendal, H, Nature new biol 236 (1972) 5.
236 Mathews, M B, Osborn, M & Lingrel, J B, Nature new biol 233 (1971) 206.
237 McConkey, E, Proc natl acad sci US 71 (1974) 1379.
238 McConkey, E H & Hopkins, J W, J mol biol 14 (1965) 257.
239 Meiss, H K & Basilico, C, Nature new biol 239 (1972) 66.
240 Mets, L & Bogorad, L, Anal biochem 57 (1974) 200.
241 Miller, L, J cell biol 59 (1973) 624.
242 Miller, L, Cell 3 (1974) 275.
243 Miller, O L, Hamkalo, B A & Thomas, C A, Science 169 (1970) 392.
244 Moav, B & Harris, T N, J immun 104 (1970) 957.
245 Molin, S, von Meyenburg, K, Gulløv, K & Maaløe, O, Mol gen genet 129 (1974) 11.
246 Morell, P & Marmur, M, Biochemistry 7 (1968) 1141.

247 Nakaya, K, Ranu, R S & Wool, I G, Biochem biophys res comm 59 (1974) 237.

248 Navashin, M, Cytolcgia 5 (1934) 169.
249 Nazar, R N, Owens, T W, Sitz, T O & Busch, H, J biol chem 250 (1975) 2475.
250 Nierhaus, D & Nierhaus, K H, Proc natl acad sci US 70 (1973) 2224.
251 Nierhaus, K H & Dohme, F, Proc natl acad sci US 71 (1974) 4713.
252 Nierhaus, K H, Montejo, V, Nierhaus, D, Diertich, S & Schrandt, I, Acta biol med germ 33 (1974) 613.
253 Niessing, J & Sekeris, C E, FEBS letters 18 (1971) 39.
254 Niessing, J & Sekeris, C E, Biochim biophys acta 247 (1971) 391.
255 Nikolaev, N, Silengo, L & Schlessinger, D, Proc natl acad sci US 70 (1973) 3361.
256 Noll, F & Bielka, H, Mol gen genet 106 (1970) 106.
257 Noll, F, Heumann, W & Bielka, H, Immunochem 10 (1973) 9.
258 Noll, F, Theise, H & Bielka, H, Acta biol med germ 33 (1974) 547.
259 Nomura, M, Bact rev 34 (1970) 228.
260 Nomura, M & Engback, F, Proc natl acad sci US 69 (1972) 1520.
261 Nomura, M, Tissieres, A & Lengyel, P (eds), Ribosomes, Cold Spring Harbor Monograph Series, Cold Spring Harbor Lab, (Cold Spring Harbor, N Y, 1974).
262 Nomura, M, Traub, P & Bechmann, H, Nature 219 (1968) 793.
263 Nowell, P C, Ferry, S & Hungerford, D A, J natl cancer inst 30 (1963) 687.
264 Nyormoi, O, Sinclair, J H & Klein, G, Exptl cell res 82 (1972) 241.

265 O'Farrell, P H, J biol chem 250 (1975) 4007.
266 Ogata, K, Tsurugi, K & Nabeshima, Y, Acta biol med germ 33 (1974) 963.
267 Oger, J, Arnason, B G W, Pantazis, N, Lehrich, J & Young, M, Proc natl acad sci US 71 (1974) 1554.
268 Olson, M O J, Prestayko, A W, Jones, C E & Busch, H, J mol biol 90 (1974) 161.

269 Pace, N R, Bact rev 37 (1973) 563.
270 Pardue, M L, Brown, D D & Birnstiel, M L, Chromosoma 42 (1973) 191.
271 Pardue, M L & Hsu, T C, J cell biol 64 (1975) 251.
272 Pederson, T, J mol biol 83 (1974) 163.
273 Pederson, T & Kumar, A, J mol biol 61 (1971) 655.
274 Peeters, B, Vanduffel, L, Depuydt, A & Rombants, W, FEBS letters 36 (1973) 217.
275 Pemberton, R E, Housman, D, Lodish, H F & Baglioni, C, Nature new biol 235 (1972) 99.
276 Pene, J J, Knight, E & Darnell, J E, J mol biol 33 (1968) 609.
277 Penman, S, J mol biol 67 (1966) 117.
278 Penman, S, N E j med 276 (1967) 502.
279 Penman, S, Smith, I & Holtzman, E, Science 154 (1966) 786.
280 Penman, S, Vesco, C & Penman, M, J mol biol 34 (1968) 49.

281 Perry, R P, Proc natl acad sci US 48 (1962) 2179.
282 Perry, R P, Natl cancer inst monog 23 (1966) 527.
283 Perry, R P, Progr nucleic acid res 6 (1967) 228.
284 Perry, R P, In A Lima-de-Faria (Ed) Handbook of Molecular Cytology, North Holland Publ. Co (Amsterdam, 1969) p 620.
285 Perry, R P, Hell, A & Errera, M, Biochim biophys acta 49 (1961) 47.
286 Perry, R P & Kelly, D E, J mol biol 16 (1966) 255.
287 Perry, R P & Kelly, D E, Biochem biophys res comm 24 (1966) 459.
288 Perry, R P & Kelly, D E, J cell physiol 72 (1968) 235.
289 Pestana, A & Pitot, H C, Biochemistry 14 (1975) 1397.
290 Petermann, M L, In K Moldave and L Grossman (eds), Methods in Enzymology, Vol XX, Academic Press (New York, 1971), p 433.
291 Petermann, M L, Hamilton, M G & Pavlovec, A, Biochemistry 11 (1972) 2323.
292 Petermann, M L & Pavlovec, A, Biochim biophys acta 114 (1966) 264.
293 Petermann, M L & Pavlovec, A, Fed proc 28 (1969) 725.
294 Petermann, M L & Pavlovec, A, Biopolymers 7 (1969) 73.
295 Pfitsyn, O B, Denesynk, A I, Finkelstein, A V & Lim, V T, FEBS letters 34 (1973) 55.
296 Pierre, M, Creuzet, C & Loeb, J E, FEBS letters 45 (1974) 88.
297 Poche, H, Junghahn, I, Geissler, E & Bielka, H, Mol gen genet 138 (1975) 173.
298 Poche, H, Varshaver, N B & Geissler, E, Mut res 27 (1975) 399.
299 Pogo, A O, Pogo, B G T, Littau, V C, Allfrey, V G & Mirsky, A E, Biochim biophys acta 55 (1962) 849.
300 Pongratz, M & Klingmuller, W, Mol gen genet 124 (1973) 359.
301 Pongs, O, Acta biol med germ 33 (1974) 629.
302 Pongs, O, Bald, R & Erdmann, V A, Proc natl acad sci US 70 (1973) 2229.
303 Porter, K R, In Proceedings of the 4th International Conference of Electron Microscopy, Berlin 1958, Vol 2, Springer (Berlin, 1960), p 189.
304 Pratt, H & Cox, R A, Biochim biophys acta 310 (1973) 188.
305 Prestayko, A W, Klomp, G R, Schmoll, D J & Busch, H, Biochemistry 13 (1974) 1945.
306 Prestayko, A W, Lewis, B C & Busch, H, Biochim biophys acta 269 (1972) 90.
307 Prestayko, A W, Olson, M D J & Busch, H, FEBS letters 44 (1974) 131.

308 Quinlan, T J, Billings, P B & Martin, T E, Proc natl acad sci US 71 (1974) 2632.

309 Reader, R W & Stanners, C P, J mol biol 28 (1967) 211.
310 Ringertz, N R, Carlsson, S-A, Ege, T & Bolund, L, Proc natl acad sci US 68 (1971) 3228.
311 Rodgers, A, Biochim biophys acta 294 (1973) 292.

312 Rodgers, A, (1973) unpublished results.
313 Rogers, M E, J cell biol 36 (1968) 421.
314 Rolleston, F S, Sub-cell biochem 3 (1974) 91.
315 Roser, B & Ford, W L, Aust j exptl biol med sci 50 (1972) 165.
316 Rothfels, K H & Siminovitch, L, Stain tech 33 (1958) 73.
317 Rothschild, H, Itikawa, H & Suskind, S R, J bact 94 (1967) 1800.
318 Ruddle, F H, Nature new biol 242 (1973) 165.

319 Sabatini, DD & Blobel, G, J cell biol 45 (1970) 146.
320 Samarina, O P, Krichevskaya, A A & Georgiev, G P, Nature 210 (1966) 1319.
321 Samarina, O P, Lukanidin, E M, Molnar, J & Georgiev, G P, J mol biol 33 (1968) 251.
322 Sanford, K K, Earle, W R & Likely, G D, J natl cancer inst 9 (1948) 229.
323 Sarkar, P K & Som, S, Biochem biophys res comm 37 (1969) 767.
324 Scheinbuks, J, Kaltschmidt, E & Marcus, L, Biochim biophys res comm 281 (1972) 141.
325 Scherrer, K, Latham, H & Darnell, J E, Proc natl acad sci US 49 (1963) 240.
326 Schleif, R, J mol biol 27 (1967) 41.
327 Schmitt, H, FEBS letters 26 (1972) 215.
328 Schochetman, G & Perry, R P, J mol biol 63 (1972) 577.
329 Schreier, M H & Staehelin, T, Nature new biol 242 (1973) 35.
330 Schur, P H, Moroz, L A & Kunkel, H G, Immunochem 4 (1967) 447.
331 Schwartz, A G, Cook, P R & Harris, H, Nature new biol 230 (1971) 5.
332 Shapiro, A L, Vinuela, E & Maizel, J V, Biochem biophys res comm 28 (1967) 815.
333 Shepherd, J & Maden, B E H, Nature 236 (1972) 211.
334 Sherman, J H & Petermann, M L, Biochim biophys acta 47 (1961) 188.
335 Sherton, C C & Wool, I G, J biol chem 247 (1972) 4460.
336 Sherton, C C & Wool, I G, J biol chem 249 (1974) 2258.
337 Sherton, C C & Wool, I G, Mol gen genet 135 (1974) 97.
338 Sidebottom, E & Harris, H, J cell sci 5 (1969) 351.
339 Sirlin, J L & Schor, N D, Exptl cell res 27 (1962) 363.
340 Soeiro, R, In F T Kenney, B A Hamkalo, G Favelukes and J A August (eds), Gene Expression and Its Regulation Plenum Press (New York, 1973), p 277.
341 Soeiro, R & Basilo, C, J mol biol 79 (1973) 507.
342 Soffer, R L, J biol chem 245 (1970) 731.
343 Soffer, R L, J biol chem 246 (1971) 1602.
344 Soffer, R L, J biol chem 248 (1973) 2918.
345 Soffer, R L, Horiniski, H & Leibowitz, M T, Cold spr harb symp quant biol 34 (1969) 529.
346 Spirin, A S, In G D Fasman and S N Timasheff (eds), Subunits in Biological Systems, Part B, Marcel Dekker Inc (New York, 1973), p 72.
347 Spirin, A S, Belitsina, N V & Lerman, M I, J mol biol 14 (1965) 611.

348 Stahl, J, Bohm, H & Bielka, H, Acta biol med germ 33 (1974) 667.
349 Stahl, J, Welfle, H & Bielka, H, FEBS letters 26 (1972) 233.
350 Stanners, C P, Eliceiri, G L & Green, H, Nature new biol 230 (1971) 52.
351 Stavnezer, J & Huang, R C C, Nature new biol 230 (1971) 172.
352 Steffensen, D M, (1974), unpublished results.
353 Steffensen, D M, Duffey, P & Prensky, W, Nature 252 (1974) 741.
354 Stoffler, G, Hasenbank, R, Lutgehaus, M, Maschler, R, Morrison, C A, Zeichhardt, H & Garrett, R A, Mol gen genet 127 (1973) 89.
355 Stoffler, G & Wittmann, H G, Proc natl acad sci US 68 (1971) 2283.
356 Stoffler, G, Wool, I G, Lin, A & Rak, K-H, Proc natl acad sci US 71 (1974) 4923.
357 Subramanian, A R, Eur j biochem 45 (1974) 541.
358 Subramanian, A R, Biochim biophys acta 374 (1974) 400.
359 Subramanian, A R, Gilbert, J M & Kumar, A, Biochim biophys acta 383 (1975) 93.
360 Swift, H, Brookhaven symp biol 12 (1959) 134.

361 Takata, R, Mol gen genet 118 (1972) 363.
362 Takata, R, Dekio, S, Otaka, E & Osawa, S, Mol gen genet 105 (1969) 113.
363 Tamaoki, T & Mueller, G C, Biochim biophys acta 108 (1965) 81.
364 Tashiro, Y & Siekevitz, P, J mol biol 11 (1965) 149.
365 Tashiro, Y & Siekevitz, P, J mol biol 11 (1965) 166.
366 Tashiro, Y & Yphantis, D A, J mol biol 11 (1965) 174.
367 Terao, K & Ogata, K, Biochim biophys acta 285 (1972) 473.
368 Terhorst, C, Moller, W. Laursen, R & Wittmann-Liebold, B, FEBS letters 28 (1972) 325.
369 Thammana. P, Kurland, C G, Deusser, E, Weber, J, Maschler, R, Stoffler, G & Wittmann, H G, Nature new biol 242 (1973) 47.
370 Theise, H, Noll, F & Bielka, H, Acta biol med germ 33 (1974) 565.
371 Thomas, H, Exptl cell res 77 (1973) 298.
372 Tischendorf, G W, Zeichhardt, H & Stoffler, G, Mol gen genet 134 (1974) 187.
373 Tischendorf, G W, Zeichhardt, H & Stoffler, G, Mol gen genet 134 (1974) 209.
374 Tissieres, A, Schlessinger, D & Gros, F, Proc natl acad sci US 46 (1960) 1450.
375 Toniolo, D, Meiss, H K & Basilico, C, Proc natl acad sci US 70 (1973) 1273.
376 Traugh, J A & Traut, R R, In D M Prescott (ed), Methods in Cell Physiology, Vol 7, Academic Press (New York, 1973), p 67.
377 Travers, A A, Nature 255 (1975) 523.
378 Ts'o, P O P & Sato, C S, J biophys biochem cytol 5 (1959) 59.
379 Tsurugi, K, Morita, T & Ogata, K, Eur j biochem 25 (1972) 117.
380 Tsurugi, K, Morita, T & Ogata, K, Eur j biochem 32 (1973) 555.

381 Ucer, U & Bermek, E, Eur j biochem 50 (1974) 183.
382 Unakul, W & Hsu, T C, J natl cancer inst 49 (1972) 1425.

383 Vaughan, M H, Warner, J R & Darnell, J E, J mol biol 25 (1967) 235.
384 Vesco, C & Penman, S, Biochim biophys acta 169 (1968) 188.

385 Wabl, M R, J mol biol 84 (1974) 241.
386 Wagner, E K, Katz, L & Penman, S, Biochem biophys res comm 28 (1967) 152.
387 Wahba, A J, Miller, M J, Niveleau, A, Landers, T A, Carmichael, G G, Weber, K, Hawley, D A & Slobin, L I, J biol chem 249 (1974) 3314.
388 Walker, T A, Pace, N R, Erikson, R L, Erikson, E & Behr, F, Proc natl acad sci US 71 (1974) 3390.
389 Wallace, H & Langridge, W H R, Heredity 27 (1971) 1.
390 Warner, J R, J mol biol 19 (1966) 383.
391 Warner, J R, J biol chem 246 (1971) 447.
392 Warner, J R, Girard, M, Latham, H & Darnell, J E, J mol biol 19 (1966) 373.
393 Warner, J R & Soeiro, R, Proc natl acad sci US 58 (1967) 1984.
394 Warner, J R & Udem, S A, J mol biol 65 (1972) 243.
395 Weber, K & Kuter, D J, J biol chem 246 (1971) 4504.
396 Weber, K & Osborn, M, J biol chem 244 (1969) 4406.
397 Weinberg, R & Penman, S, J mol biol 38 (1968) 289.
398 Weinberg, R A & Penman, S, J mol biol 47 (1970) 169.
399 Weiss, M C & Ephrussi, B, Genetics 54 (1966) 1095.
400 Welfle, H, Acta biol med germ 27 (1971) 211.
401 Welfle, H, Acta biol med germ 33 (1974) 677.
402 Welfle, H, Bielka, H & Bottger, M, Mol gen genet 104 (1969) 165.
403 Welfle, H, Stahl, J & Bielka, H, Biochim biophys acta 243 (1971) 416.
404 Welfle, H, Stahl, J & Bielka, H, FEBS letters 26 (1972) 228.
405 Wellauer, P K & Dawid, I B, J mol biol 89 (1974) 379.
406 Wellauer, P K, Dawid, I B, Kelley, D E & Perry, R P, J mol biol 89 (1974) 397.
407 Wen, W-N, Leon, P E & Hague, D R, J cell biol 62 (1974) 132.
408 Westermann, P, Bielka, H & Bottger, M, Mol gen genet 104 (1969) 157.
409 Westermann, P, Gross, B & Heumann, W, Acta biol med germ 33 (1974) 699.
410 Wettenhall, R E H, Nakaya, K & Wool, I G, Biochem biophys res comm 59 (1974) 230.
411 Wettstein, F O, Staehelin, T & Noll, H, Nature 197 (1963) 430.
412 Wiener, F, Klein, G & Harris, H, J cell sci 12 (1973) 253.
413 Wikman-Coffelt, J, Anal biochem 48 (1972) 339.
414 Wikman-Coffelt, J, Howard, G A & Traut, R R, Biochim biophys acta 277 (1972) 671.
415 Wilkinson, J, Ann bot 8 (1944) 269.
416 Willems, M, Penman, M & Penman,S, J cell biol 41 (1969) 177.

417 Wimber, D E & Steffensen, D M, Ann rev genet 7 (1973) 205.
418 Wittmann, H G & Wittmann-Liebold, B, Acta biol med germ 33 (1974) 571.
419 Wool, I G & Stoffler, G, In M Nomura, A Tissieres and P Lengyel (eds), The Ribosome, Cold Spring Harbor Lab (Cold Spring Harbor, New York, 1974), p 417.

420 Yankofsky, S A & Spiegelman, S, Proc natl acad sci US 48 (1962) 1466.
421 Yoshikawa-Fukada, M, Biochim biophys acta 145 (1967) 651.

ABSTRACT

The experiments described in this thesis attempt to analyze the proteins of eukaryotic ribosomes using both biochemical and genetic approaches. The primary technique employed was a two-dimensional gel electrophoresis method that was a micro-modification of the standard method of Kaltschmidt and Wittmann [Anal biochem 36 (1970) 401] and allowed microgram quantities of protein to be separated accurately in much shorter time. When the ribosomal proteins of different cell lines or tissues from chick embryos, rats, mice and humans were electrophoresed on these 2-D gels, a total of 97 different protein spots were localized. Of these, each individual pattern contained 71-76 protein spots which were mostly cations.

Though two-thirds of the proteins were common to all the different species, most species examined possessed only one or two species-specific marker ribosomal proteins (all cations). In most cases, the species-specific proteins were associated with only certain areas of the gel pattern. For example, a protein, 48, showed noticeably different mobility in each of several different species (mouse, rat, chick, Syrian hamster) and was absent in Chinese hamster. It was suggested that such areas might represent those proteins less rigorously conserved; hence these species variants might be sequence variations of the same primitive ribosomal protein.

In general, ribosomal proteins were seen to be highly conserved, with the more evolutionary distant species showing fewer proteins in common. Within each species, there seemed to be a few (1-3 proteins) differences in patterns between different cell lines or tissues. These variations of cationic ribosomal proteins could not

be found to correlate with differences in growth rate, state of differentiation or malignancy.

When closely analyzed on 2-D gels, all eukaryotic ribosomes examined carried the same complement of 8 anionic ribosomal proteins. No species-specific differences were found but several anions varied in their staining intensities depending on whether they were isolated from rapidly growing or non-growing cells. A group of 3 high molecular weight ribosomal proteins were present only in small amounts in non-growing cells while actively growing cells showed much greater amounts of them. One of the anions, 64, appears to be identical to a ribosomal protein described by others as a eukaryotic version of E. coli ribosomal proteins L7/L12.

Since none of the eukaryotic ribosomal proteins identified by 2-D gels have been specifically assigned a function, experiments were conducted to identify which protein on the pattern bound to 5S rRNA. This particular function was selected for study because it was known that upon exposure of intact polysomes to EDTA, subunit particles were formed accompanied by the release of a complex consisting of 5S rRNA and a single protein which possessed GTPase activity. To confirm the kinetics of this release, the removal of complex at several concentrations of EDTA was analyzed and it was shown that the release of 5S rRNA does not solely account for the formation of the EDTA subunits nor does the release of 5S rRNA occur at the same EDTA concentration as the release of the 5S rRNA binding protein. The binding protein was observed to possess a molecular weight of 40,000 $\pm$ 500 and to be associated with most of the 5S rRNA released under the conditions employed.

One-dimensional gel electrophoresis of EDTA-treated ribosomes showed the loss of only one protein, but 2-D gel electrophoresis

of treated ribosomes showed them to be deficient in approximately 8 different ribosomal proteins of which only one was of the appropriate molecular weight and charge to be identified, tentatively, as the binding protein. Attempts to run the isolated 5S rRNA complex on 2-D gels failed, however, to show any protein spot.

Though the processing and maturation of rRNA during ribosome biosynthesis have been adequately documented, few studies have determined the protein composition of the nucleolar ribosomal precursor particles at the several stages of their nucleolar maturation. Since such ribosomal precursor particles could readily be isolated from Hela cells, experiments were conducted to determine their protein composition in comparison to the cytoplasmic ribosomal subunits. Since one of the precursors (80S) contained 45S rRNA, the precursor for both 18S and 28S rRNA, it was thought that this precursor might have ribosomal proteins of both small and large cytoplasmic subunits while the other one (55S), containing only precursor rRNA of the large subunit, was thought to be associated only with large subunit ribosomal proteins.

To enable such studies, the 40S and 60S Hela cytoplasmic subunits were first prepared by puromycin dissociation in the presence of high concentrations of monovalent cations. Isolated subunits were characterized by their RNA composition and sedimentation profile and found to be at most 2% cross-contaminated with the other subunit. Of the 69 cationic ribosomal proteins appearing in the 2-D gel pattern of the Hela polysomes, 24 were associated with the small subunit and were present in roughly the same relative intensities as in the polysome patterns. The large subunit contained 30 ribosomal proteins of which 2 overlapped with ribosomal proteins of the small subunit. Whether the 15 ribosomal proteins not associated

with either subunit pattern are true ribosomal proteins or are associated factors could not be determined. The high KCl concentration used in their isolation might have removed some "true" ribosomal proteins.

When nucleolar ribosomal precursor particles (80S and 55S) were isolated from Hela cells and their proteins electrophoresed, the 2-D pattern of the 55S precursor showed 21 anionic and 52 cationic proteins. Of the cations, 18 corresponded to the ribosomal proteins of the large subunit. The 80S precursor was identical to the 55S patterns except 3 anions and 4 cations were absent. Of those missing cations, 3 were from the large subunit. No small subunit ribosomal proteins were found on either precursor though their absence may be due to the extraction conditions. No anionic proteins of the cytoplasmic ribosomes were found in either precursor. A number of high molecular weight proteins found in both precursors were most likely those proteins stably associated with the nucleolus and may be concerned with the processing of the ribosomal precursors.

Given the several species-specific marker ribosomal proteins described above, experiments were designed to determine whether the genes coding for ribosomal proteins might be mapped using interspecific cell hybrids. In addition, the formation of such hybrid lines was used to gain some insight into the regulation of ribosome synthesis. When a rat-mouse hybrid line was examined for the presence of rRNA and ribosomal proteins from both parents, both parental 28S rRNAs were present. Two-dimensional gel electrophoresis of ribosomal proteins indicated the presence of one rat and one mouse marker ribosomal protein. The synthesis of one but not a second rat marker protein suggested that at least 2

ribosomal proteins are not linked. Further genetic analysis using interspecific hybrids is hindered by the inability to determine sufficient ribosomal protein differences between species that yield workable hybrids.

In the hybrid, rat 28S rRNA bound only the rat-specific marker ribosomal protein. When the relative amounts of rat and mouse marker proteins and 28S rRNA were compared, it was found that though both parental 28S rRNAs were present in equal amounts there was considerably more rat-specific than mouse-specific marker ribosomal protein. This suggests a model for the regulation of ribosome synthesis whereby the amounts of rRNA and ribosomal protein are not coordinated with respect to each other but simply are rapidly turned over if they fail to form a complex. In addition, the synthesis of both parental 28S rRNAs and specific ribosomal proteins suggests there is no "nucleolar dominance" in these rat-mouse hybrids.

Printed in Sweden
Copyright © 1974 by Academic Press, Inc.
All rights of reproduction in any form reserved

Experimental Cell Research 87 (1974) 186–194

RIBOSOMAL PROTEIN DIFFERENCES BETWEEN ANIMAL CELLS

D. J. KUTER and A. RODGERS

Sir William Dunn School of Pathology, University of Oxford, Oxford OX1 3RE, UK

SUMMARY

Ribosomal proteins of human (HeLa), Syrian hamster, Chinese hamster, chick (embryo) and rat (Novikoff hepatoma) cells have been examined by two-dimensional polyacrylamide gel electrophoresis. The results show that although there are many similarities between the electrophoretic patterns, species-specific marker proteins can be identified for Syrian hamster, chick, rat and possibly HeLa cells, which could be used in genetic analysis. No specific protein marker has been identified for Chinese hamster. The similarity in electrophoretic mobility of the hamster, chick and rat marker proteins suggests an overall structural relationship between them.

Genetic analysis of ribosomal proteins in bacteria has shown that in *E. coli*, about 20 of the 55 proteins are located around minute 64 of the genetic map [1] and one (S18) maps at minute 84 [2]. The location of the others is unknown at the present time. Such analyses have involved crosses between species showing differences in the parental ribosomal protein complement. For example, in the *E. coli* – *S. marcescens* system, nine 30S and six 50S ribosomal protein differences were discovered by Takata [1], and Sypherd et al. [3] described three differences in the 30S and three in the 50S subunits between *E. coli* and *S. typhimurium*. Both systems were used in mating experiments.

An analogous analysis might be performed with mammalian cells by the use of the Sendai virus technique for cell fusion and the production of interspecific hybrids [4]. The frequent chromosome losses which such hybrids undergo might permit the assignation of the genes for some ribosomal proteins to particular chromosomes. A prerequisite of this analysis is clearly the identification of suitable species for cell fusion. Others [5, 6] have examined Syrian hamster, mouse, rat, human and rabbit ribosomal proteins by disc electrophoresis but the resolution of this method is unsatisfactory. Only a very limited number of species has so far been examined by the two-dimensional gel electrophoresis method [7–11]. We have used 2D polyacrylamide gel electrophoresis to survey the ribosomal protein complements of a number of animal species whose cells have been used in fusion experiments, to discover those with maximal differences in their ribosomal proteins and to identify species-specific marker proteins.

The experiments in this paper show that ribosomal proteins are very strongly conserved: the species-specific differences are localised in only a few regions of the 2D gel pattern. Specific protein markers have been identified for Syrian hamster, rat, chick and mouse [11]. A protein has been found in HeLa cells which may be suitable as a marker although it differs only in its mobility in the

first dimension from similar proteins in other species. No marker protein has been identified in Chinese hamster cells.

METHODS

HeLa cells were grown in spinner culture at 37°C in Earle's salts [12] solution with added yeast extract, nutrient broth and rabbit serum. Cells were grown in 2-litre batches and harvested at 1.2×10^6 cells/ml. They were washed in 0.9% sodium chloride solution and the pelleted cells prepared for homogenisation by freezing and thawing twice, to disrupt them.

Syrian hamster cells (BHK 21 line) were grown in roller culture in minimum essential medium (MEM) (Flow Laboratories) with 10% foetal calf serum. Cells were harvested at near confluence by trypsinisation, washed with 0.9% saline, pelleted and prepared for homogenisation by freezing and thawing twice.

Twelve day old chick embryos were carefully removed from the egg without breaking the yolk sac, decapitated and rinsed in Dulbecco's phosphate-buffered saline (PBS) [13].

Rat livers were obtained from 150 g Wistar rats.

Chinese hamster livers were obtained from 40 g animals.

A line of Novikoff hepatoma B/P6 cells was obtained from the Chester Beatty Institute, London, and grown as an ascites tumour in 150 g Wistar rats. Cells were washed in PBS, separated from any contaminating red blood cells by differential centrifugation, and the pelleted cells frozen and thawed twice before homogenisation.

All cells and tissues were homogenised with 2 vol of 0.2 M sucrose in Buffer A [14], and nuclei and mitochondria removed by centrifugation at 17 000 g for 15 min. For increased yields of ribosomes from small amounts of cells, the post-mitochondrial pellet was homogenised a second time with 2 vol of 0.2 M sucrose in Buffer A, and the post-mitochondrial supernatants combined. The post-mitochondrial supernatant was made 0.5–1.3% (w/v final concentration) in sodium deoxycholate by the addition of a 10% aqueous solution. Ribosomes were then pelleted by centrifugation at 100 000 g for 4½ h, through a discontinuous sucrose gradient, according to the method of Falvey & Staehalin [14]. The ribosomal pellet was resuspended in 3 mM Mg^{2+}, 5 mM tris buffer, pH 7.2 containing either 0.3 M KCl or 0.6–0.7 M KCl, and again centrifuged at 100 000 g for 2½ h. The pellet of washed ribosomes was suspended in 1 mM Mg^{2+}, 1 mM Tris buffer, pH 7.2, clarified by centrifugation at 8 000 g for 10 min and 2 vol of glacial acetic acid added to precipitate the RNA. After standing at 0°C for 1 h, the RNA precipitate was removed by centrifugation and the acetic acid extract freeze dried.

Ribosomal protein patterns were made by the 2D acrylamide gel electrophoresis method of Kaltschmidt & Wittmann [15] using pH 8.6 in the first dimension and pH 4.3 in the second. Gels were stained with amido black and destained in 1% acetic acid solution.

RESULTS

The ribosomes examined in these experiments were obtained from cells grown in culture (HeLa, Syrian hamster), from homogenised tissues (Chinese hamster and rat livers, chick embryos), or from cells grown in vivo (Novikoff hepatoma). All of the ribosomes were subjected to the same isolation and washing procedure and consisted mostly of monosomes. All of the early preparations were examined ultracentrifugally, until it became clear that the standard preparative method produced an identical ribosome profile in each case.

A comparison of the gel patterns reproduced here with those previously published from rat hepatoma [8], rat liver [10] and mouse [11] shows that they have many features in common. For example, most of the proteins illustrated are easily recognisable in the composite diagram of Sherton & Wool [10], although protein S27 and its 3 satellites were not visible in our gels: proteins S29, S30 and L39 [10] were also not visible in our gels. Although under the electrophoresis conditions used here they would have migrated off the lower right-hand corner of the gel, shorter times of electrophoresis also failed to disclose them. Monosomes were not separated into subunits in our experiments and since it is possible that some spots contain more than one protein, both the number of differences and the number of proteins represent minimum values. Our ribosomal protein patterns, from ribosome preparations containing mostly monomer, contain 61–63 spots, which is close to what others have reported for the 80S ribosome in this electrophoresis system. The number of visible faint spots depends to some extent on the amount of protein on the gel and therefore not all of the possible spots appear on any one photograph. Complete analysis of the patterns

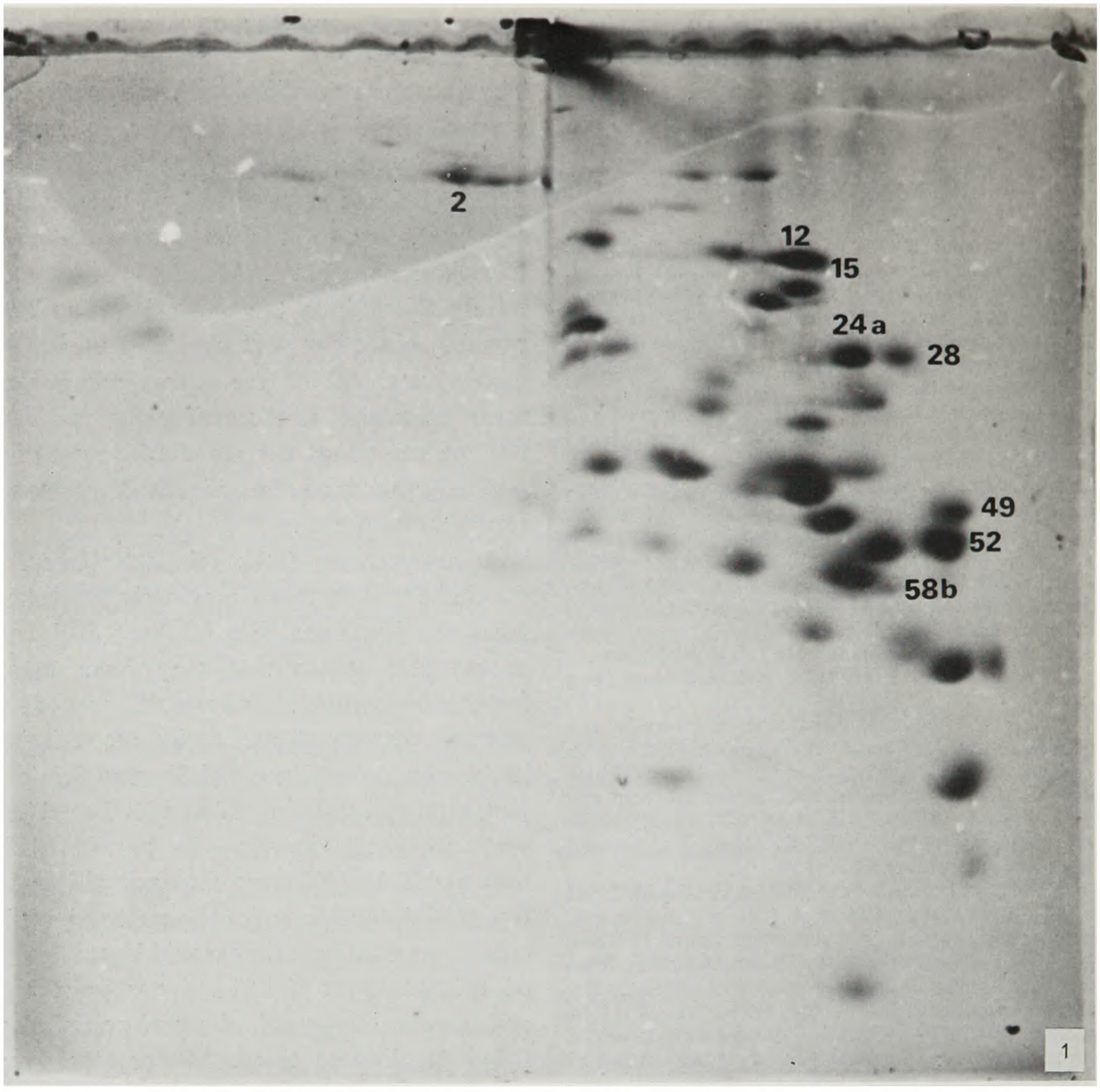


Fig. 1. Two-dimensional polyacrylamide gel electrophoresis of HeLa ribosomal proteins (3 mg). First dimension pH 8.6. Running time 24 h at 100 V. Second dimension pH 4.3. Running time 35 h at 110 V. Acidic proteins move from centre to left, basic proteins move from centre to right. Numbered proteins are discussed in the text.

of ribosomal proteins to separate overlapping spots requires electrophoretic runs for different lengths of time in each of the two dimensions. The system for numbering the proteins follows that used previously [11].

A typical 2 D gel pattern of HeLa ribosomal proteins is shown in fig. 1. The group of three acidic proteins at the positive (left-hand) side of the gel is not always seen, and is not included in the ribosomal protein catalogue. Protein 2 is present in all of the HeLa preparations and is classified as a ribosomal protein: experiments with mouse cells suggest that it may be related to growth conditions [11]. It is not species-specific. Protein 24 shows an altered (increased) mobility at pH 8.6 compared with the same protein in other cells, and it may be useful as a marker

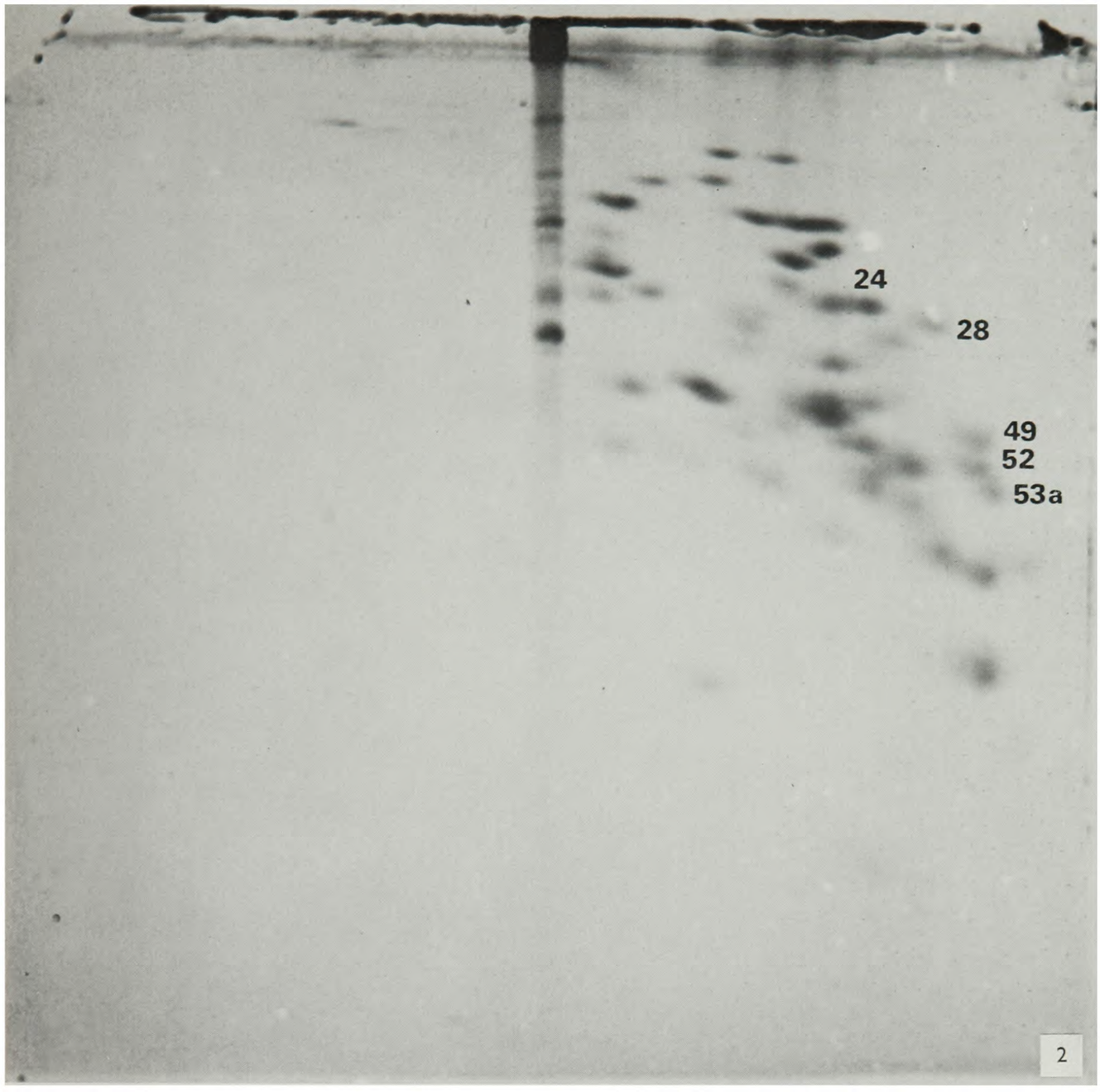


Fig. 2. Two-dimensional polyacrylamide gel electrophoresis of Syrian hamster ribosomal proteins (3 mg). Conditions of electrophoresis as in fig. 1. Numbered proteins are discussed in the text: 53a is Syrian hamster-specific.

protein (24a). Though lacking protein 53, HeLa ribosomes possess protein 58b as found in A9 cells [11].

Syrian hamster ribosomal proteins (fig. 2) are again very similar to the other species. Protein 24 migrates at a slower rate at pH 8.6 compared with HeLa, but very similar to that in other species. A new protein, 53a occurs below 52, which has not been observed in the other species tested, and may therefore be a species-specific marker. No other consistent differences have been observed.

Chinese hamster ribosomal proteins (fig. 3) show the characteristic pattern of the other species but the following differences may be noted. Protein 24 migrates more slowly at pH 8.6 than the comparable protein in HeLa, and is identical with the mouse and

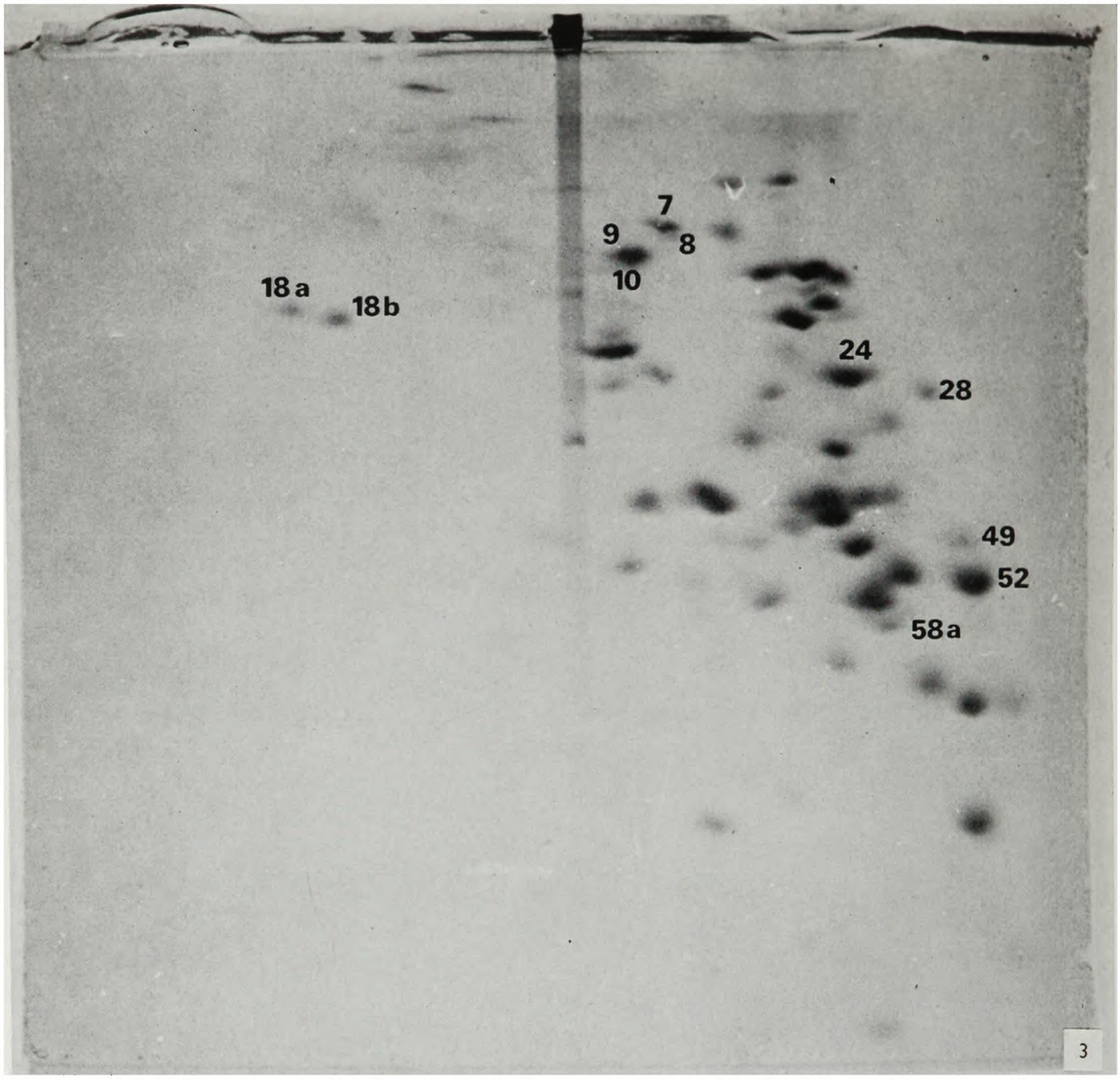


Fig. 3. Two-dimensional polyacrylamide gel electrophoresis of Chinese hamster ribosomal proteins (3 mg). First dimension pH 8.6. Running time 24 h at 100 V. Second dimension pH 4.3. Running time 30 h at 120 V. Numbered proteins are discussed in the text.

rat in this respect. No proteins suitable as markers have been found in Chinese hamster cells, except perhaps the slight variation in protein 58a which (as in mouse liver [11]) migrates slightly faster at pH 8.6. The two acidic proteins 18a and 18b have been found in several Chinese hamster liver preparations although not in cultured cells, and their significance is unclear.

Chicken embryo ribosomes (fig. 4) present a different pattern of ribosomal proteins. Thus, protein 28 is missing and 28a, 30a, 32a, 34a move differently from the other species examined. Also the relative positions of 57a and 58b are altered, with 57a running more slowly at pH 8.6 and 58b more quickly. Protein 58b runs as in A9 cells [11], while 57a has not been previously encountered.

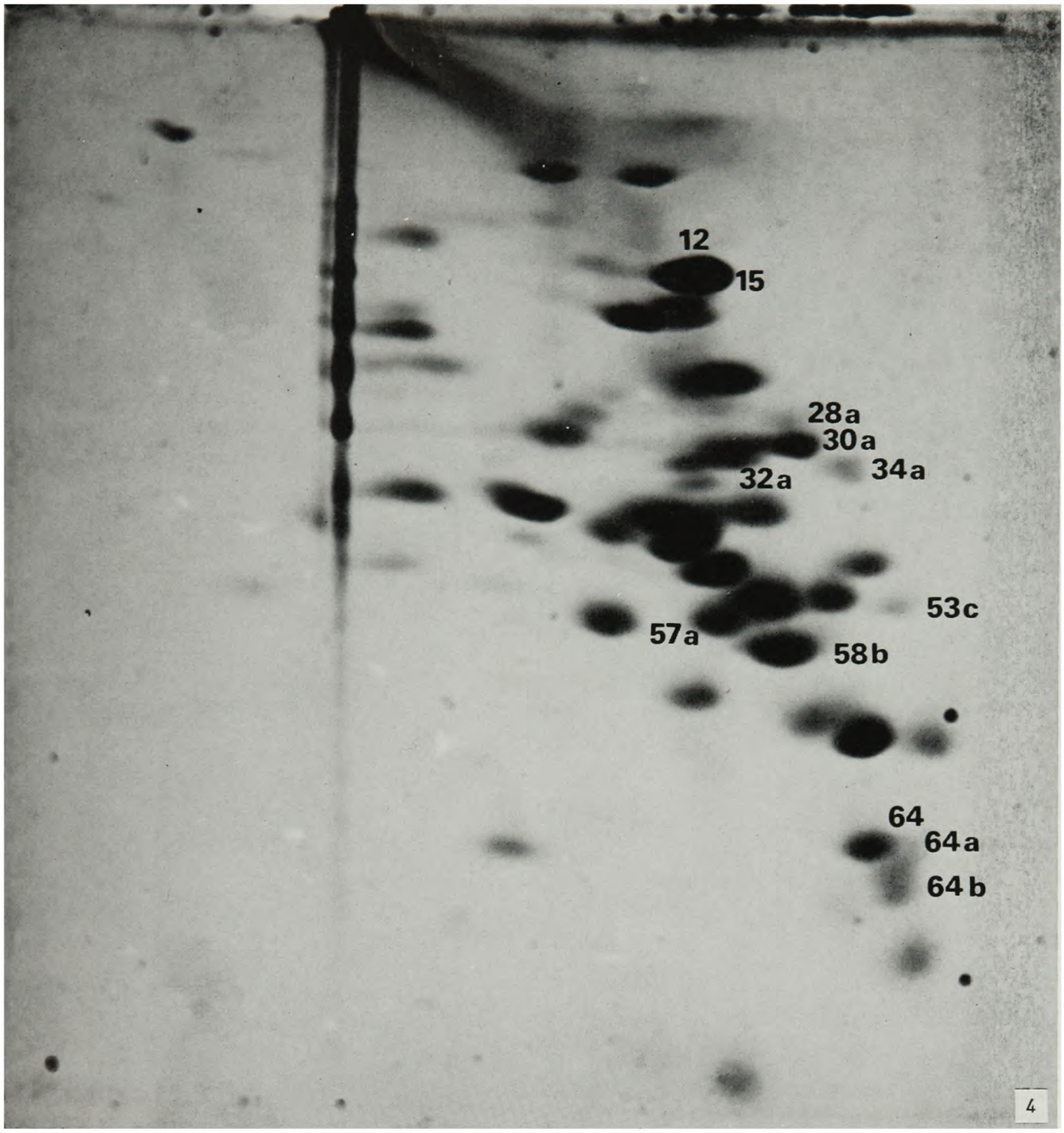


Fig. 4. Two-dimensional polyacrylamide gel electrophoresis of chick embryo ribosomal proteins (4 mg). Conditions of electrophoresis as in fig. 1 except that second dimension running time was 40 h. Chick-specific proteins are 34a, 57a, 64a, 64b. Numbered proteins are discussed in the text.

In the other species examined, proteins 64 and 64a are largely overlapping, but in chick, 64, 64a and 64b are well separated in the second dimension. Protein 53, variants of which are specific markers for several species (see Discussion), is present here in small amounts as a new form, 53c which differs slightly from 53b in the rat on co-electrophoresis. Thus four specific marker proteins, 34a, 57a, 64a and 64b may be distinguished in the chick.

The Novikoff hepatoma ascites ribosomal protein pattern (fig. 5) resembles that of rat liver obtained by ourselves and by Sherton &

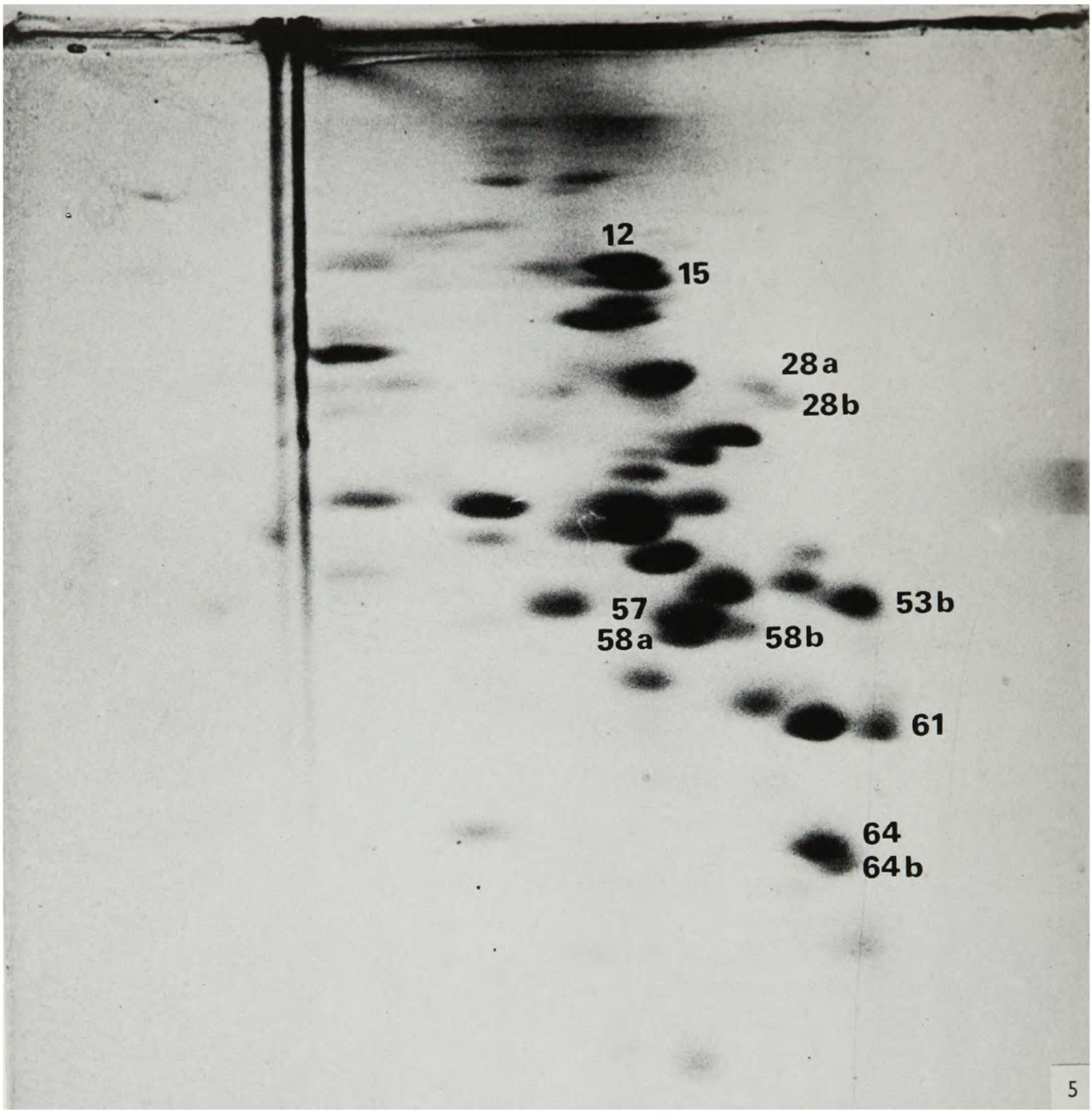


Fig. 5. Two-dimensional polyacrylamide gel electrophoresis of Novikoff hepatoma ascites ribosomal proteins (3 mg). Conditions of electrophoresis as in fig. 4. Rat-specific protein is 53b; other numbered proteins are discussed in the text.

Wool [10], except that their L14 resolves into 2 proteins, 28a and 28b in our experiments. 28a and 28b might be oxidised and reduced forms of the same protein, however. Though present in only trace amounts in rat liver, protein 61 in Novikoff hepatoma ribosomes has the usual intensity found in other species: similarly, hepatoma-liver differences in proteins 57, 58a and 58b are only quantitative. A qualitative difference occurs with protein 64 however, which is a single spot in liver but resolves into an additional spot, 64b, in hepatoma. Both liver and hepatoma show the characteristic rat-specific ribosomal protein 53b which runs further in the second dimension than the mouse-specific protein 53.

DISCUSSION

These experiments show that although there are differences between the ribosomal protein patterns of various species, these differences are quite small. Non-ribosomal protein variants between species are common, but similarities in the T_1 digest fingerprints of rRNA from various species [16], and the ribosomal protein results reported here imply strong conservation of the ribosome structure. All of the cell types examined so far, however, except for Chinese hamster, have at least one ribosomal protein which characterises the species and is therefore useful as a marker. These variations in the protein composition are not distributed throughout the 2D gel pattern, but are localised in four major areas.

(1) The most easily identifiable differences occur in the region of the protein triplet 49, 52, 53a in the Syrian hamster. Thus, in the mouse [11] this triplet is 49, 52, 53; in the chicken (fig. 4) 49, 52, 53c; in the rat 49, 52, 53b; and in Chinese hamster and HeLa, 49 and 52 only. These proteins are probably large subunit proteins as characterised by Sherton & Wool [10] in the rat (L24, L28, L29 in their system).

(2) The region of proteins 57 and 58 is associated with chick- and mouse-specific proteins. Although previous results [11] have shown tissue specific variations of mouse ribosomes in this area, none of the mouse variants is identical to the chick-specific 57a.

(3) Variations also occur in the region of proteins 24 and 28, where the HeLa-specific protein 24a (fig. 1) and the chick variation (absence of protein 28, fig. 5) may be found. In the case of HeLa, the change involves a difference in mobility in the first dimension only.

(4) Proteins 12 and 15 may also vary. In HeLa and chick these proteins are almost totally overlapping (figs 1, 4) while in the mouse and rat ([11] and fig. 5), they are separated in the second dimension.

The variations described above imply an overall structural similarity of the altered proteins in terms of their mobilities in this 2D gel electrophoresis system although not necessarily amino-acid sequence. If the structure of the ribosome is not to be altered, then one protein is likely to be replaced by another of similar charge and molecular size, particularly if the protein binds to rRNA. It would be informative to know if sequence homologies exist between proteins 53 (mouse), 53a (Syrian hamster), 53b (rat) and 53c (chick), and, if these proteins bind directly to rRNA, whether a corresponding change in the base sequence of the rRNA binding site has occurred.

In interpreting these species variations, it is also important to remember that altered mobilities on the gels may correspond to modifications of existing ribosomal proteins and not necessarily a new polypeptide species. For example, *E. coli* ribosomal proteins L7 and L12 are separable in this 2D system [17], but differ only by an acetyl group on the N-terminal serine of L7 [18]. Similar modifications are thought to occur on mammalian ribosomes [19, 20, 21]. Whether this type of modification is responsible for the species differences reported here must await more detailed analysis of the proteins themselves. A further possible modification found in *E. coli*, involves oxidation of the protein, causing a second, neighbouring spot to appear. Although this might account for the double spots we find near proteins 7 and 9, it is unlikely to account for the species-specific differences which never show such close pairing.

Though used successfully with bacteria, genetic analysis of ribosomal proteins presents additional problems in mammalian systems. Firstly, as these experiments show, the small

number of ribosomal protein variants between species limits the genetic analysis. Secondly, present evidence suggests that given the appropriate factors (when required) the ribosomes of one species can translate the mRNA's of another [22, 23, 24], and there is therefore little selective advantage in preserving the ribosomes of both parents if the cell is to survive as a hybrid. Thirdly, even in favourable cases where several ribosomal protein differences may exist (e.g. chick and rodent), formation of the hybrid cell may result in extensive loss of chromosomes of one parent, the remaining fragment of genetic material being indistinguishable and analysis not possible. However, ribosomal proteins in two somatic cell hybrid systems have been investigated and will be reported later (ms in preparation).

REFERENCES

1. Takata, R, Mol gen genet 118 (1972) 363.
2. Bollen, A, Faelen, M, Lecocq, J P, Herzog, A, Zengel, J, Kahan, L & Nomura, M, J mol biol 76 (1973) 463.
3. Sypherd, P S, O'Neill, D M & Taylor, M M, Cold Spring Harbor symp quant biol 34 (1969) 77.
4. Harris, H & Watkins, J F, Nature 205 (1965) 640.
5. Houssais, J F, Eur j biochem 24 (1971) 232.
6. Gould, H J, Nature 227 (1970) 1145.
7. Welfle, H, Stahl, J & Bielka, H, Biochim biophys acta 243 (1971) 416.
8. Delaunay, J & Schapira, G, Biochim biophys acta 259 (1972) 243.
9. Huynh-Van-Tan, Delaunay, J & Schapira, G, FEBS letters 17 (1971) 163.
10. Sherton, C C & Wool, I G, J biol chem 247 (1972) 4460.
11. Rodgers, A, Biochim biophys acta 294 (1973) 292.
12. Earle, W, J natl cancer inst 4 (1943) 165.
13. Dulbecco, R & Vogt, M, J exptl med 99 (1957) 167.
14. Falvey, A K & Staehelin, T, J mol biol 53 (1970) 1.
15. Kaltschmidt, E & Wittmann, H G, Anal biochem 36 (1970) 401.
16. Pinder, J C, Gould, H J & Smith, I, J mol biol 40 (1969) 289.
17. Kaltschmidt, E & Wittmann, H K, Proc natl acad sci US 67 (1970) 1276.
18. Terhorst, C, Möller, W, Laursen, R & Wittmann-Liebold, B, FEBS letters 28 (1972) 325.
19. Liew, C-C & Gornall, A G, J biol chem 248 (1973) 977.
20. Martini, O H W & Gould, H J, Biochim biophys acta 295 (1973) 621.
21. Eil, C & Wool, I C, J biol chem 248 (1973) 5122.
22. Sampson, J, Mathews, M B, Osborn, M & Borghetti, A F, Biochemistry 11 (1972) 3636.
23. Stavnezer, J & Huang, R R C, Nature new biol 230 (1971) 172.
24. Mathews, M B, Osborn, M, Berns, A J M & Bloemendal, H, Nature new biol 236 (1972) 5.

Received December 19, 1973
Revised version received March 18, 1974

Printed in Sweden
Copyright © 1975 by Academic Press, Inc.
All rights of reproduction in any form reserved

Experimental Cell Research 91 (1975) 317–325

THE SYNTHESIS OF RIBOSOMAL PROTEIN AND RIBOSOMAL RNA IN A RAT–MOUSE HYBRID CELL LINE

D. J. KUTER and A. RODGERS

Sir William Dunn School of Pathology, Oxford, UK

SUMMARY

A rat–mouse hybrid cell line was examined for the presence of ribosomal RNA and ribosomal proteins from both parents. As demonstrated by banding of centromeric heterochromatin, the hybrid cell line contained most of the mouse genome and at least 13 rat chromosomes. The ability of rat, but not mouse, ribosomes to dimerize was used to show that the hybrid contained both rat and mouse 28S ribosomal RNA. Two-dimensional polyacrylamide gel electrophoresis of ribosomal proteins indicated the presence of both rat and mouse ribosomal proteins.

The regulation of ribosome biosynthesis in eukaryotic cells may be analysed by the use of hybrid cell lines. Earlier experiments showed that human–mouse hybrid cells containing up to 35 human chromosomes produced no detectable human ribosomal RNA (rRNA) and suggested that the human 28S rRNA genes were repressed [1, 2]. Similarly, *Xenopus* embryos produced in crosses between *Xenopus laevis* and *Xenopus mulleri* made only *X. laevis* rRNA [3]. However, in hamster–mouse [4, 5] and rat–mouse [6] somatic cell hybrids both parental types of 28S rRNA were made.

Since ribosome biosynthesis in eukaryotic cells demands the concurrent synthesis of ribosomal protein and ribosomal RNA [7, 8], we have sought to determine what ribosomal proteins are synthesized in hybrid cells. Houssais [9] has previously analysed the ribosomal proteins of several interspecific hybrids with one-dimensional gel electrophoresis and, in one case, reported that a hamster–mouse hybrid line contained one ribosomal protein specific for each parent. However, the two-dimensional gel electrophoresis system of Kaltschmidt & Wittmann [10] allows a more detailed analysis of ribosomal proteins. This technique has shown that there are only a few species differences between cell lines commonly used in cell fusion experiments, though rat and mouse lines do each possess one specific ribosomal protein [11].

We describe here a rat–mouse hybrid cell line, having 13 identifiable rat chromosomes and most of the mouse genome, which synthesizes both rat and mouse 28S rRNA and both rat and mouse marker ribosomal proteins.

MATERIALS AND METHODS

Cell lines and cell culture

A9HT, a malignant derivative of A9 [12], deficient in inosinic acid pyrophosphorylase, was grown in Falcon flasks (Becton, Dickinson Co., USA) with a

surface area of 75 cm^2 containing Eagle's Minimal Essential Medium (MEM, Flow Laboratories) supplemented with 10% foetal calf serum (Flow Labs). Rat leukemia cells [13] were propagated in 150 g PGV/c hooded rats and were kindly supplied by Dr M. Muirhead, Department of Biochemistry, Oxford. Large quantities of rat leukemia cells for the preparation of ribosomes were isolated from peripheral blood and dissected lymph nodes of leukemic rats. Leukemia cells were freed from the surrounding nodal tissues by gentle pipetting in phosphate buffered saline (PBS, [14]) and pelleted by differential centrifugation.

An uncloned population of A9HT/rat leukemia hybrid cells was obtained from Professor H. Harris. These hybrids had been produced by the Sendai virus technique [15] and selected in HAT [16]. The initial hybrid population was cloned one month after fusion by inoculating 10^2 cells into a small Falcon flask containing MEM–HAT plus 20% foetal calf serum. After 2 weeks, individual clones were removed by trypsinization in a small metal cylinder attached to the floor of the flask, and the resulting cell suspension was introduced into a small plastic flask. Seven hybrid clones were thus isolated and were maintained in culture in MEM–HAT plus 10% foetal calf serum.

Rat embryo fibroblasts were prepared from minced 10-day-old embryos, grown in MEM plus 10% foetal calf serum, and subcultured three times a week.

Large quantities of A9HT and hybrid cells were grown in roller bottles.

Rat livers were obtained from 150 g PGV/c rats and frozen at −70°C until use.

Chromosome analysis

Colcemid was added to 2-day-old cell cultures in Falcon flasks to a final concentration of 0.01 μg/ml and the cultures were incubated for 1 h at 37°C. Cells were removed with trypsin, rinsed three times in PBS, resuspended in hypotonic (0.075 M KCl) solution at 20°C for 8 min, and fixed in several changes of glacial acetic acid – methanol (1 : 3). Small drops of cell suspension were placed on glass slides and air-dried. Rat leukemia cells were treated as above except that 5 ml of peripheral blood from a leukemic rat were incubated for 2 h in 20 ml of MEM containing the above concentration of Colcemid before the PBS wash.

After 'ageing' for 5–7 days at 20°C in the dark, slides were banded for centromeric heterochromatin (C-banding) as described by Allderdice et al. [17] with the slight modifications introduced by Marshall [6]. This method involves treating the chromosome population with 95% formamide in SSC (0.15 M NaCl, 0.015 M trisodium citrate, pH 7.0) at 65°C for 7–8 min and then staining in Giemsa [10 ml of Giemsa (G. T. Gurr) diluted with 90 ml of pH 6.8 buffer (Gurr)] for 40–50 min.

Slides were examined with a Leitz Orthoplan microscope incorporating the Leitz side-arm tracing device. Chromosomes were divided into three categories: biarmed, acrocentric/telocentric, and 'dot'. All calculations of the modal chromosome number are based on 50–60 metaphase spreads.

Ribosome dimers

About 10^7 cells from either leukemic rats or tissue culture was washed three times at room temperature with PBS and then prepared as previously described [4]. Briefly, the pelleted, washed cells (0.1 ml packed volume) were resuspended to a volume of 0.9 ml with ice-cold NaH buffer (0.1 M NaCl, 0.02 M $MgCl_2$, 0.01 M Tris pH 7.4 at 20°C), incubated for 30 min at 4°C, made 0.5% (w/v) in Lubrol W and homogenized in a small, loose-fitting Dounce homogenizer (15 strokes). The homogenate was centrifuged at 15 000 g_{ave} for 15 min and the supernatant then placed on top of 15% to 30% (w/v) sucrose gradients prepared in NaH buffer. Gradients were centrifuged for 190 min at 35 000 rpm at 4°C in the SW41 rotor on a Beckman L2-65B centrifuge, and fractionated with an ISCO (Instrumentation Specialty Co., Lincoln, Nebr.) Density Gradient Fractionator.

Two-dimensional electrophoresis of ribosomal proteins

Ribosomes were prepared according to Falvey & Staehelin [18] and the ribosomal protein extracted as previously described [11]. Electrophoresis was done according to Kaltschmidt & Wittmann [10] with the following modifications. Lyophilized proteins were dissolved directly in sample gel [10] without polymerization catalysts and 30 μl samples (100–400 μg) placed on top of 0.45 · 9.0 cm first-dimension separation gels. Electrophoresis in the first dimension was for 1 h at 3 mA/tube followed by 5 h at 6 mA/tube (constant current). Gels were then dialyzed in 500 ml of dialysis buffer [10] for 1½ h with two changes of dialysis buffer and then polymerized on top of gel slabs in the device of Kaltschmidt & Wittmann, but with slabs only 2 mm wide. Except where otherwise stated, gels were run in the second dimension for ½–1 h at 40 V and 20 h at 100 V (constant voltage) and then stained and destained according to Howard & Traut [19].

RESULTS

Selection and karytopic analysis of an A9HT/rat leukemia hybrid clone

When A9HT and rat leukemia cells are fused together by the Sendai virus technique and grown in HAT, the unfused parental A9HT cells (which are deficient in inosinic acid pyrophosphorylase) are unable to grow. Since rat leukemia cells are unable to grow in vitro, any cells growing under these conditions are hybrid cells. Of the seven clones isolated from this population only one grew well in roller culture and it is this clone which is described in detail here.

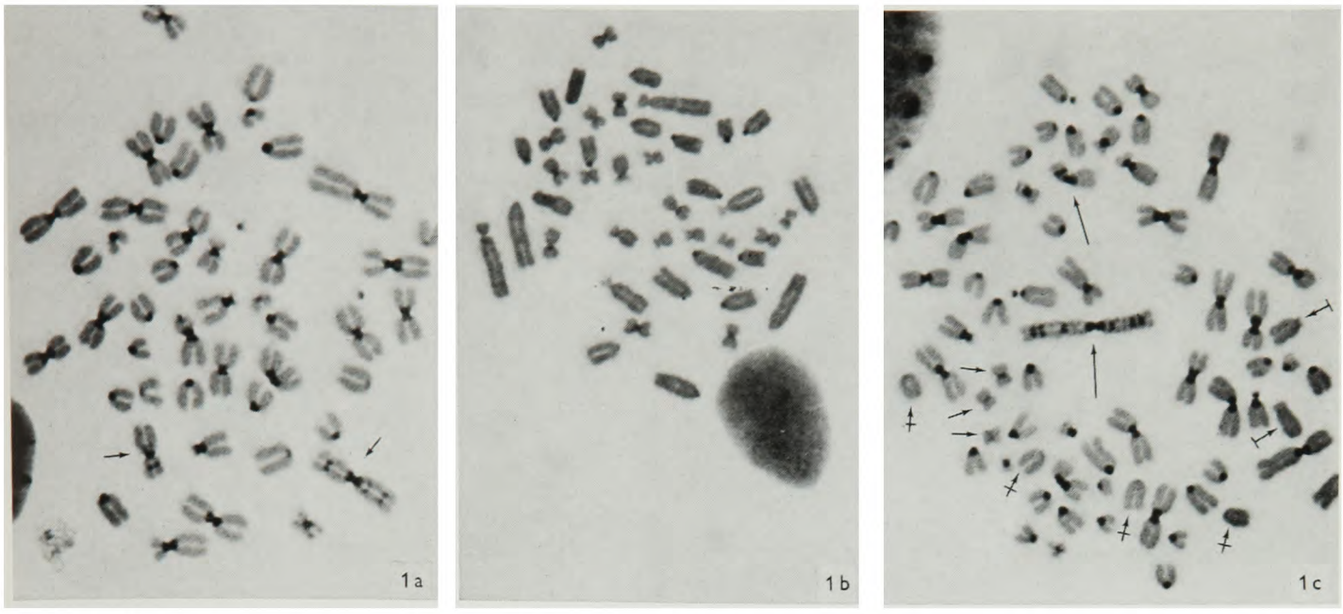


Fig. 1. C-banded preparations of metaphase chromosomes from (*a*) A9HT; (*b*) rat embryo fibroblasts; (*c*) A9HT/rat leukemia hybrid. All arrows are explained in the text.

To determine whether the clone was indeed a rat–mouse hybrid, its karyotype and that of the parental lines were compared using the C-banding technique to help distinguish between rat and mouse chromosomes. Banded chromosome preparations show different intensities of centromeric banding and these degrees of staining have been described as being either strong, faint, or unbanded. A9HT (fig. 1*a*) shows strong and highly reproducible banding at the centromeres (and at some secondary constrictions) of most chromosomes. All biarmed and 'dot' chromosomes show a strong centromeric band; the remaining chromosomes are all strongly banded except for 2–6 acrocentric chromosomes which show only faint C-bands. Under these conditions of staining, no mouse chromosome is ever totally devoid of a C-band. In addition, the banding at the secondary constrictions of two chromosomes (arrows in fig. 1*a*) reveals two distinct A9HT marker chromosomes.

As in other experimentally-induced leukemias [25], this rat leukemia has a normal diploid karyotype. Under C-banding conditions identical with those for A9HT, the rat leukemia chromosomes show no banding at all. However, as with other leukemias [25], chromosome preparations suitable for banding were difficult to obtain from this leukemia line. When rat embryo fibroblasts were banded (fig. 1*b*), about half the chromosomes were unbanded; the rest of the chromosomes showed banding of a much weaker intensity than the strong banding in A9HT and comparable to that in the 2–6 faintly banded A9HT chromosomes. Thus with the exception of the faintly banded rat acrocentrics, which cannot be distinguished from the faintly banded mouse acrocentrics by this technique, it should be possible to identify in rat–mouse hybrids some of the chromosomes from each parent. Any strongly banded chromosomes are of mouse origin, any totally unbanded chromosomes of rat origin, and the faintly banded chromosomes are of uncertain origin unless morphologically distinct.

When these criteria are applied to C-banded preparations of A9HT/rat leukemia hybrids, specific rat and mouse chromosomes may

Table 1. *Chromosomal analysis of hybrid and parental cell lines*

	Total chromosome no.		No. of acrocentric/telocentric chromosomes				No. of biarmed chromosomes				No. of 'dot' chromosomes	
			Total number		No. unbanded		Total number		No. unbanded			
Cell type	Mode	Range	Mode	Range	Mode	Range	Mode	Range	Mode	Range	Mode	Range
A9HT	52	44–54	25	19–28	5	2– 6	25	20–28	None		2	1–3
Rat embryo fibroblasts	42	36–43	22	17–23	11	7–16	21	16–24	14	7–20	None	
A9HT/rat leukemia clone 2	72	66–75	39	35–42	13	10–16	30	24–34	5	1–6	3	1–3

be clearly seen (fig. 1*c*). The two mouse marker chromosomes are present as are most of the other strongly banded mouse chromosomes. Three rat small metacentric chromosomes (⟶), two rat subacrocentric chromosomes (↦) and four other unbanded acrocentrics (↔) may be discerned. Several other weakly banded acrocentric chromosomes are visible but their origin cannot be determined because weakly banded rat and mouse acrocentrics look alike.

Table 1 summarizes the distribution of chromosomes in the hybrid. In the rat embryo fibroblasts and the rat leukemia the number of chromosomes classified as 'unbanded' indicates chromosomes totally devoid of banding; for the acrocentric and telocentric chromosomes in A9HT and the A9HT/rat leukemia hybrid, 'unbanded' chromosomes include both the faintly banded and completely unbanded chromosomes. In the hybrid a modal number of 13 of the 39 acrocentric or telocentric chromosomes are unbanded, as are 5 of the 30 biarmed chromosomes. Because all the A9HT biarmed chromosomes are strongly banded, these 5 unbanded biarmed chromosomes can be attributed to the rat parent.

Since five of the A9HT acrocentric or telocentric chromosomes are too faintly banded to be distinguished from the 11 faintly banded rat chromosomes, not all of the 13 unbanded acrocentrics in the hybrid may be of rat origin. The difference between these totals may represent the number of rat chromosomes in the hybrid. When morphological comparisons are taken into account, there are 8 distinguishable acrocentric or telocentric chromosomes present in this hybrid clone. These studies thus confirm that the cell line is indeed a rat–mouse hybrid and that the hybrid contains at least 13 rat

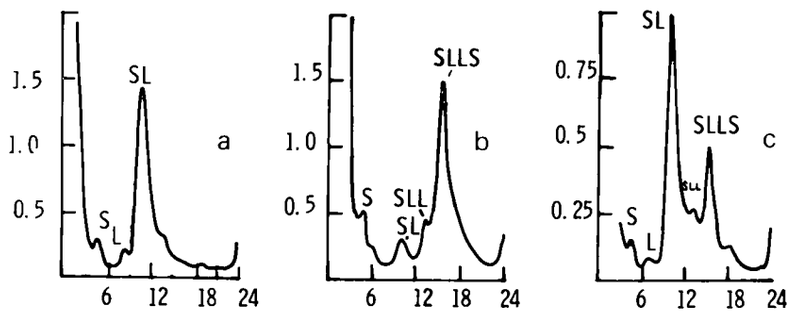
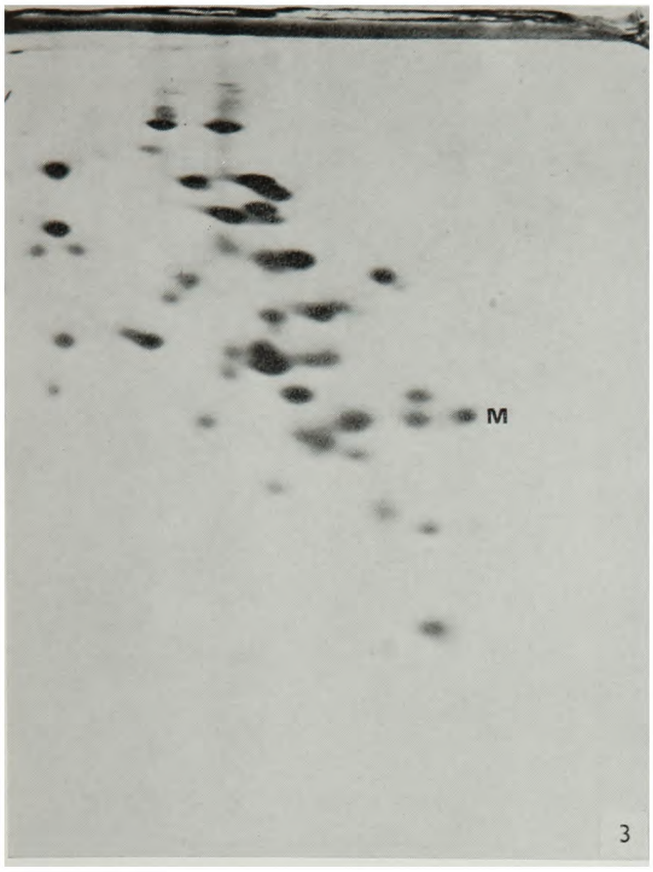


Fig. 2. Abscissa: fraction no.; *ordinate:* absorbance (A_{254}) profile.

Sedimentation analysis of ribosomes of (*a*) A9HT; (*b*) rat leukemia cells; (*c*) A9HT/rat leukemia prepared as described in the text. *L*, larger; *S*, smaller ribosome subunit.



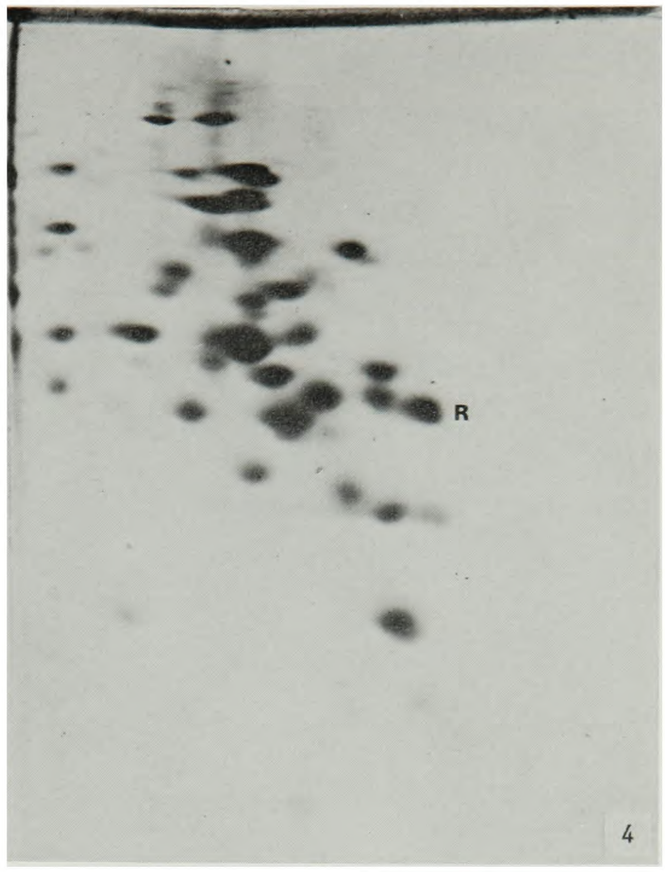


Fig. 3. Two-dimensional polyacrylamide gel electrophoresis of A9HT ribosomal proteins (220 μg). Conditions of electrophoresis are as described in Materials and Methods. *M*, mouse-specific ribosomal protein.
Fig. 4. Two-dimensional polyacrylamide gel electrophoresis of rat liver ribosomal proteins (360 μg). Conditions of electrophoresis as in fig. 3. *R*, rat-specific ribosomal protein.

chromosomes (5 biarmed and 8 acrocentric or telocentric) as well as the mouse genome.

As found in other experiments with rat–mouse hybrids [26], A9HT/rat leukemia hybrids preferentially lose the rat chromosomes. But during the course of the present experiments, the hybrid clone described here showed little change in karyotype.

Ribosomal RNA in hybrid cells

Rat and mouse 28S ribosomal RNA can be separated only slightly on low percentage polyacrylamide gels [6]. However, Reader & Stanners [21] have shown that ribosomes from different species vary in the extent to which they form dimers at certain concentrations of salt and divalent cation. This has been shown to be a property only of the 28S rRNA [4]. Under these conditions, mouse ribosomes (fig. 2*a*) sediment essentially as the monomer with only about 1–3% of the ribosomal optical density greater than the monomer. On the other hand, rat leukemia ribosomes (fig. 2*b*) sediment as a dimer with no more than 10% of the ribosomal optical density remaining in the monomer peak. Other rat cell lines (Novikoff hepatoma, rat embryo fibroblasts, rat liver) show an identical pattern.

Using the presence of a dimer peak to detect rat 28S ribosomal RNA, the A9HT/rat leukemia hybrid was examined. As indicated in the optical density profile in fig. 2*c* a substantial dimer peak is seen.

In the hybrid and rat leukemia profiles other peaks are seen. Those sedimenting faster than the dimer were not reproducible. By analogy with the results of Eliceiri [34],

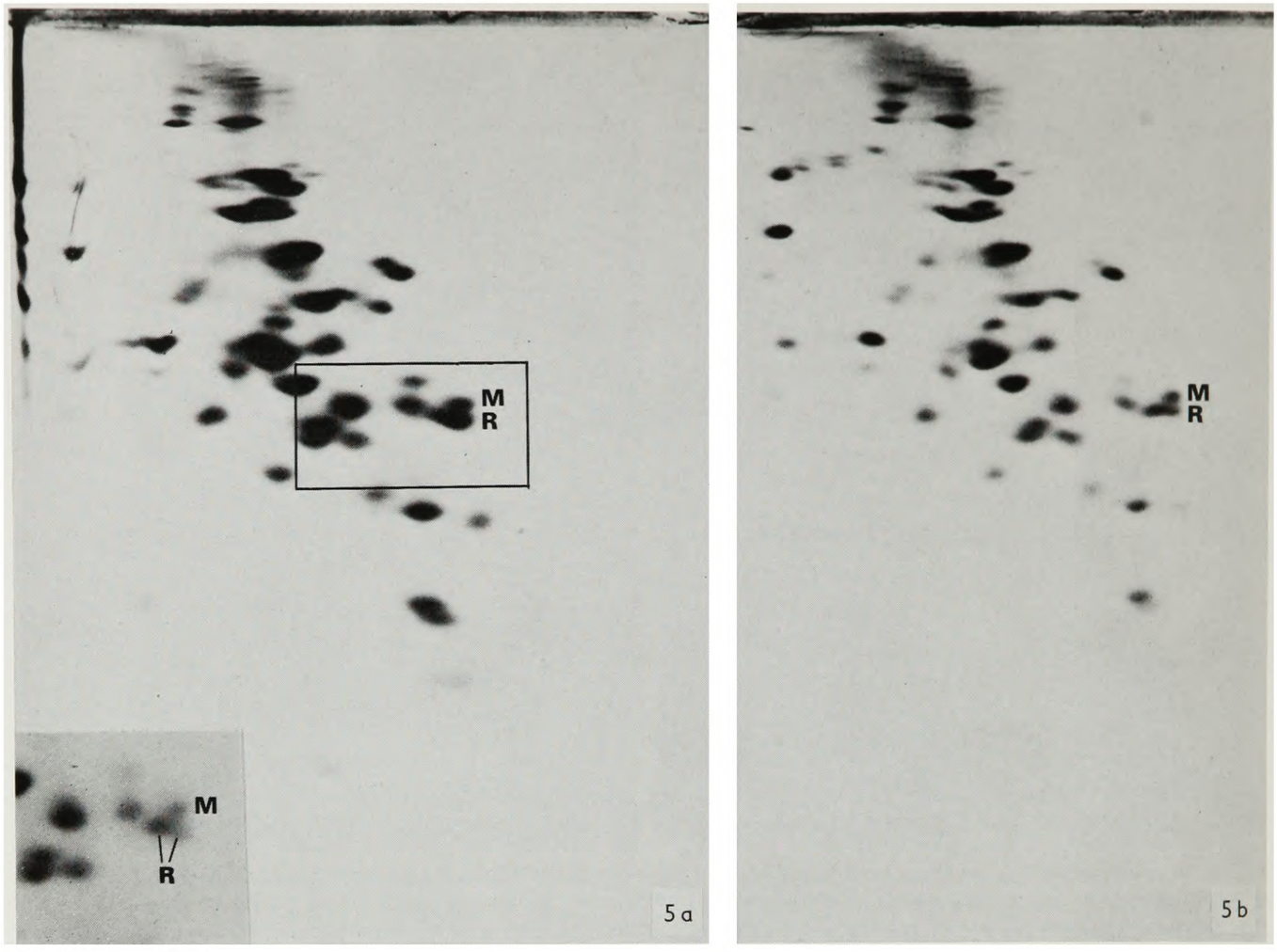


Fig. 5. Two-dimensional polyacrylamide gel electrophoresis of (*a*) A9HT/rat leukemia ribosomal protein (200 μg); (*b*) an artificial mixture of A9HT (220 μg) and rat leukemia (210 μg) ribosomal proteins. Conditions of electrophoresis are as in fig. 3. (*a, inset*) A9HT/rat leukemia ribosomal proteins (600 μg) electrophoresed as above except run 37 h in the second dimension. The area shown is equivalent to that enclosed by lines in (*a*). *M*, mouse-specific; *R*, rat-specific ribosomal proteins.

the particles (SLL) sedimenting between the monomer and dimer peaks may perhaps consist of two rat large subunits and one small subunit of either rat or mouse origin.

Ribosomal proteins in hybrid cells

Two-dimensional polyacrylamide gel electrophoresis of the ribosomal proteins is highly reproducible and separates the ribosomal proteins according to charge and size. The modified Kaltschmidt & Wittmann procedure used here avoids the polymerization of the sample in sample gel [10] and allows smaller amounts of protein to be run without any alteration in the two-dimensional pattern. The patterns presented here consist only of the ribosomal proteins which run towards the cathode in the first dimension. In other experiments [11, 22] several proteins running toward the anode have been described but none of these is a species-specific marker. The dark, streaky shadows often found near the top of the gel patterns have been discussed by others [23, 24] and are most likely caused by aggregates of ribosomal protein linked by disulfide bonds.

Fig. 3 shows the two-dimensional gel pattern for A9HT ribosomal proteins. It is very similar to those previously published [11, 22] for other mouse cell lines or tissues

and shows the mouse-specific marker ribosomal protein (designated M). Rat leukemia ribosomal proteins yield a pattern identical with that presented in fig. 4 for rat liver ribosomes. This pattern is comparable to others previously published for rat tissues [11, 29] and is identical with the A9HT patterns except for the rat-specific marker protein (designated R). This rat protein migrates only slightly farther in the second dimension than does the mouse marker. Previous experiments with these lines and other mouse (A9, L, mouse liver) and rat (Novikoff hepatoma, rat liver) cell lines and tissues have confirmed that these are the only reproducible differences between the patterns of rat and mouse ribosomal proteins.

This species difference is seen more clearly by mixing equal amounts of rat leukemia and A9HT ribosomal proteins and running the 2-D gels (fig. 5*b*). The rat and mouse spots are clearly separated from each other in the second dimension.

Ribosomal proteins extracted from ribosomes of the hybrid cell line show both mouse-specific and rat-specific proteins (fig. 5*a*). The ribosomal protein pattern of the hybrid may be directly compared to the artificial mixture of rat and mouse ribosomal protein (fig. 5*b*) since both samples were run on the same gel slab for the second dimension electrophoresis. Though differing in the amount of protein run, the gel patterns are identical.

As shown in fig. 4, the rat-specific protein has an irregular shape on the gel patterns. When smaller amounts of protein (50–200 μg) are electrophoresed, the rat-specific spot appears to be composed of two partly overlapping spots as shown in fig. 5*b*. Longer times of electrophoresis in the second dimension (fig. 5*a*, inset) resolve these rat spots more completely and show them to be distinct from the mouse-specific spot. All other rat cell lines and tissues examined show an identical pattern: short times of electrophoresis (20 h) in the second dimension show only an irregular, large spot while longer times of electrophoresis (37 h) show two spots.

DISCUSSION

The mouse parent, A9HT, in these experiments has a modal chromosome number of 52. This is somewhat lower than the number of 55 that Allderdice et al. [20] have shown in their detailed studies of the A9HT karyotype; but the A9HT line used in these fusions has been grown extensively in culture since the experiments of Allderdice et al. and this altered karyotype is not unexpected.

The dimerization of ribosomes is a species-specific phenomenon [21] and is apparently entirely the result of interactions between the 28S rRNA component of free, intact ribosomes or 60S subunits [4]. Since rat ribosomes dimerize almost completely (greater than 90%), and mouse ribosomes do not, we have used the presence of a dimer peak in the hybrid to identify rat 28S rRNA. Marshall [6] has correlated the appearance of this dimer peak with the presence of rat 28S rRNA on low percentage polyacrylamide gels.

Attempts to analyse the ribosomal proteins of hybrid cells on one-dimensional gels [9] have not been adequate to detect small differences between the parental ribosome protein patterns. As previously reported [11], the differences between ribosomal proteins are minimal and few species-specific proteins exist. Such species-specific ribosomal proteins (at least in chick, Syrian hamster, rat and mouse) seem to be electrophoretic variants of one particular ribosomal protein. Rat and mouse ribosomes each have a specific ribosomal protein which clearly differs from the other only in its mobility in the second dimension (fig. 5). Whether these variant

proteins are amino acid sequence variants of each other remains to be shown. In any case, both rat and mouse specific ribosomal proteins are found in the ribosomes isolated from hybrid cells.

The irregular shaped rat-specific spot in fig. 4 seems to be composed of two overlapping spots as shown in fig. 5*a*; this is also observed in rat liver, Novikoff hepatoma, and rat leukemia ribosomes. Though Sherton and Wool [23, 29] have not observed this, the two-dimensional patterns of rat liver ribosomes by Welfle et al. [31] are identical to that in fig. 4. They also indicate that the rat-specific spot is composed of two spots (L27 and L29 in their nomenclature). In addition, Stahl et al. [32] have found that both these proteins may be phosphorylated in vitro. This suggests that the two spots may be phosphorylated derivatives of each other. However, these two spots may simply be oxidized and reduced forms of each other. Oxidation of ribosomal proteins has been known to cause such an appearance [28].

Since there is only one consistent ribosomal protein difference between rat and mouse ribosomes, these studies throw no light on the origin of the other 70 or so ribosomal proteins in the ribosome. Hybrids formed with other species (chick–mouse) between which there are several species-specific differences in ribosomal proteins as revealed by two-dimensional gel electrophoresis [11], have also been analyzed (Kuter, unpublished). However, these hybrid lines lose the chick chromosomes too rapidly in culture to permit genetic analysis. Stable hybrid lines are obtainable between more closely related species (rat–mouse, mouse–hamster), but there appears to be only one electrophoretic difference between the ribosome patterns of these cell lines. Other hybrids (human–mouse), though possessing chromosomes of both species, nevertheless fail to synthesize human ribosomal RNA. Although the location of many genes coding for *E. coli* ribosomal proteins has been accomplished by the use of partial diploid strains formed between *E. coli* and *S. marcescens*, each of which has many distinct ribosomal proteins [33], any attempt to map the genes for the ribosomal proteins of eukaryotic cells by means of interspecific hybrid cell lines must acknowledge the problems mentioned above.

The results of the present experiments bear on two other aspects of ribosome biosynthesis. The first is the phenomenon of 'allelic repression' [3] observed in other somatic cell hybrids [1, 2] and in *Xenopus* hybrid embryos [3]. The expression of both rat and mouse rRNA in the A9HT/rat leukemia hybrids confirm the findings of others [4, 5, 6] that selective transcription of only one set of parental rDNA cistrons is not the rule in hybrid cells. Moreover, at least one ribosomal protein gene from both parents is functional in the hybrid. Whether this is true for other ribosomal protein genes remains undetermined.

Secondly, since the synthesis of ribosomes demands the co-ordinated synthesis of both ribosomal proteins and rRNA, the mechanism of the regulation is of interest. Attempts to study this have usually involved the use of inhibitors of RNA and protein synthesis in order to dissociate the synthesis of ribosomal protein from rRNA. Inhibitors of protein synthesis stop synthesis of rRNA but inhibitors of rRNA synthesis do not stop ribosomal protein synthesis [7]. However, in *Xenopus* embryos ribosomal protein and rRNA synthesis appear to be coupled; when rRNA synthesis declines, ribosomal protein also declines [30]. The use of hybrid cell lines may overcome the disadvantages of using inhibitors of this kind. For example, segregants might be found which synthesize only rat rRNA but not rat ribosomal protein.

Such rRNA segregants have not yet been found in six other rat–mouse hybrid clones so far examined.

REFERENCES

1. Eliceiri, G L & Green, H, J mol biol 41 (1969) 253.
2. Bramwell, M E & Handmaker, S D, Biochim biophys acta 232 (1971) 580.
3. Honjo, T & Reeder, R H, J mol biol 80 (1973) 217.
4. Stanners, C P, Eliceiri, G L & Green, H, Nature new biol 230 (1971) 52.
5. Eliceiri, G L, J cell biol 53 (1972) 177.
6. Marshall, C J, Dr Phil. thesis, Oxford University (1973).
7. Craig, N & Perry, R P, Nature new biol 229 (1971) 75.
8. Wu, R S, Kumar, A & Warner, J R, Proc natl acad sci US 68 (1971) 3009.
9. Houssais, J F, Eur j biochem 24 (1971) 232.
10. Kaltschmidt, E & Wittmann, H G, Anal biochem 36 (1970) 401.
11. Kuter, D J & Rodgers, A, Exptl cell res 87 (1974) 186.
12. Weiner, F, Klein, G & Harris, H, J cell sci 12 (1973) 253.
13. Roser, B & Ford, W L, Aust j exptl biol med sci 50 (1972) 165.
14. Dulbecco, R & Vogt, M, J exptl med 99 (1957) 167.
15. Harris, H & Watkins, J F, Nature 205 (1965) 640.
16. Engel, E, McGee, B J & Harris, H, J cell sci 5 (1969) 93.
17. Allderdice, P W, Miller, O J, Pearson, P L, Klein, G & Harris, H, J cell sci 12 (1973) 809.
18. Falvey, A K & Staehelin, T, J mol biol 53 (1970) 1.
19. Howard, G A & Traut, R R, FEBS lett 29 (1973) 177.
20. Allderdice, P W, Miller, O J, Miller, D A, Warburton, D, Pearson, P L, Klein, G & Harris, H, J cell sci 12 (1973) 263.
21. Reader, R W & Stanners, C P, J mol biol 28 (1967) 211.
22. Rodgers, A, Biochim biophys acta 294 (1973) 292.
23. Sherton, C C & Wool, I G, J biol chem 247 (1972) 4460.
24. Kaltschmidt, E & Wittmann, H G, Proc natl acad sci US 67 (1970) 1276.
25. Nowell, P C, Ferry, S & Hungerford, D A, J natl cancer inst 30 (1963) 687.
26. Weiss, M C & Ephrussi, B, Genetics 54 (1966) 1095.
27. Unakul, W & Hsu, T C, J natl cancer inst 49 (1972) 1425.
28. Held, W A, Mizushima, S & Nomura, M, J biol chem 248 (1973) 5720.
29. Sherton, C C & Wool, I G, J biol chem 249 (1974) 2258.
30. Hallberg, R L & Brown, D D, J mol biol 46 (1969) 393.
31. Welfle, H, Stahl, J & Bielka, H, FEBS lett 26 (1972) 228.
32. Stahl, J, Welfle, H & Bielka, H, FEBS lett 26 (1972) 233.
33. Takata, R, Mol gen genet 118 (1972) 363.
34. Eliceiri, G L, Biochim biophys acta 269 (1972) 450.

Received June 26, 1974