Studies of Rat Liver Polyribosomes and RNA Metabolism

by

Barry M.R.N.J. Woloski

A Thesis Submitted to
the Faculty of Graduate Studies and Research
of the University of Manitoba
in partial fulfillment
of the requirements of the degree
Master of Science

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Abstract

The significance of segregation of mammalian polyribosomes into bound and free classes is a topic of research capable of lending insight into many biochemical processes. This segregation seems to be important in consideration of evidence for preferred synthesis of secretory proteins on bound polyribosomes and intracellular proteins on free polyribosomes. The effect of induction of acute phase reactant synthesis was examined on rat hepatic RNA metabolism and the hepatic distribution of polyribosomes. Emphasis was placed on α_1-acid glycoprotein as a typical example of an acute phase reactant. The effects on polyribosome distribution of induction of ferritin synthesis by iron administration and metallothionein synthesis by zinc administration were also examined. Ferritin and metallothionein were considered to be examples of intracellular proteins.

Quantitation of bound and free polyribosome levels revealed that acute inflammation resulted in a shift of polyribosomes into the bound class as a result of increased levels of heavier aggregates of polyribosomes 18 h after inflammation. Induced synthesis of intracellular proteins did not induce a similar shift of polyribosomes into the free class.

Synthesis of α_1-acid glycoprotein and albumin were shown to occur preferentially on bound polyribosomes by immunochemical reactivity of nascent polypeptide chains on polyribosomes. These studies showed that inflammation elevated α_1-acid glycoprotein polyribosomal mRNA levels. Studies using an *in vitro* translation system suggested

preferential synthesis of α_1-acid glycoprotein on bound polyribosomes. Studies of orotate incorporation showed that rRNA levels increased as early as 4 h, while mRNA levels increased about 8 h after inflammation.

Changes in the levels of mRNA transcripts alone could not account for all the observed changes in polyribosome distribution. Assays of total alkaline ribonuclease levels showed that while this enzyme may be important to hepatic polyribosome distribution during acute inflammation, it does not account for the changes following iron and zinc administration.

Acknowledgements

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Finally, I am indebted to the National Research Council of Canada, the University of Manitoba and the Department of Chemistry, University of Manitoba for financial assistance.

List of abbreviations used

A	adenine
AA-tRNA	aminoacylated transfer RNA
ATP	adenosine triphosphate
DNA	deoxyribonucleic acid
dT	2'-deoxythymine
GTP	guanosine triphosphate
hnRNA	heteronuclear RNA
mRNA	messenger RNA
mRNP	messenger ribonucleoprotein particle
RNA	ribonucleic acid
rRNA	ribosomal RNA
tRNA	transfer RNA

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Introduction

Site of protein synthesis

The mammalian liver is an organ capable of modulating many biochemical and physiological changes that mammals are required to contend with. Biochemical mechanisms which accomplish these changes often involve biosynthesis of intracellular proteins and proteins which are secreted into the plasma. Control mechanisms for the biosynthesis of these proteins are not fully understood at this time.

Proteins are synthesized on polyribosomes formed by the aggregation of ribosomal subunits on strands of mRNA. There are two classes of mammalian polyribosomes, bound polyribosomes which are attached to the endoplasmic reticulum and free polyribosomes which are found in the cytosol. Examination of the site of synthesis of proteins (1-3), as is shown in Table 1, leads to the conclusion that secretory proteins are synthesized primarily on bound polyribosomes. Evidence for stating that intracellular proteins are synthesized on free polyribosomes is not as convincing.

The significance and consequences of the segregation of polyribosomes into two classes is a topic of research that is capable of lending insight into many other biochemical processes. Secretory proteins must pass through a membrane before they are secreted into the sinusoids of the liver. Modifications such as partial proteolysis, glycosylation and hydroxylation often occur before secretion. Both secretion and post-translational modification are processes associated with membrane structures and as such localization of secretory

Table 1

Synthesis of Proteins on Free and Bound Polyribosomes

Protein*	Synthesis of protein per mg RNA in bound:free polyribosomes
Secretory Proteins	
Serum proteins	7:1
Albumin	2.5:1
Albumin	1:5
Albumin**	24:1
α-globulins	4:1
α-globulins	77:1
β-lactoglobulin	20:1
α_1-acid glycoprotein***	6.6:1
Intracellular Proteins	
Ferritin	1:1.5
Ferritin	1:5
Arginase	1:7.7
Myosin	1:5
Serine dehydrogenase	1.2:1
Serine dehydrogenase	15:1
Catalase	1:1
Catalase	1:1
Catalase	1:1
Membrane Proteins	
NADP-cytochrome c-reductase	1:1
NADP-cytochrome c-reductase	0.14-1.5:1

* Unless otherwise noted, examples were taken from (1) which cites the work of several authors. Different values cited represent differences in tissues, assay techniques or physiological states.
** Taken from (2) where albumin mRNA was directly assayed.
*** Taken from (3).

protein synthesis on membrane-bound polyribosomes has a special significance. Similarly, localization of the synthesis of intracellular proteins, which are neither secreted nor modified as secretory proteins are, would seem to have a significance. There are many factors which determine the distribution of ribosomal RNA between bound and free polyribosomes as well as between ribosomal subunits and polyribosome aggregates of various sizes. If, under a set of conditions, the distribution of ribosomal RNA is controlled primarily by the mRNA transcripts initiating protein synthesis, then analyses of polyribosomes could provide some insight into mRNA metabolism. Experiments with animals in other physiological states could provide insight into the capacity of the liver to meet requirements for protein synthesis and the mechanisms by which the translation process is controlled. The work presented in this thesis has attempted to more fully elucidate these aspects of the biochemistry of the mammalian liver.

Mammalian RNA metabolism

RNA metabolism in mammalian systems involves a complex interrelationship of all forms of nucleic acids as is shown in Figure 1. Species of RNA are transcribed as precursors which are later processed in the cell nucleus into forms that are active in the cytoplasm. Both the transcriptional and processing events offer opportunity for regulation of later cytoplasmic events. Transcription and processing of mRNA are of special relevance to the topic of differential expression of genes. Transcription of mRNA has been reviewed by Goldberger

Fig. 1. General Scheme of Mammalian RNA Metabolism

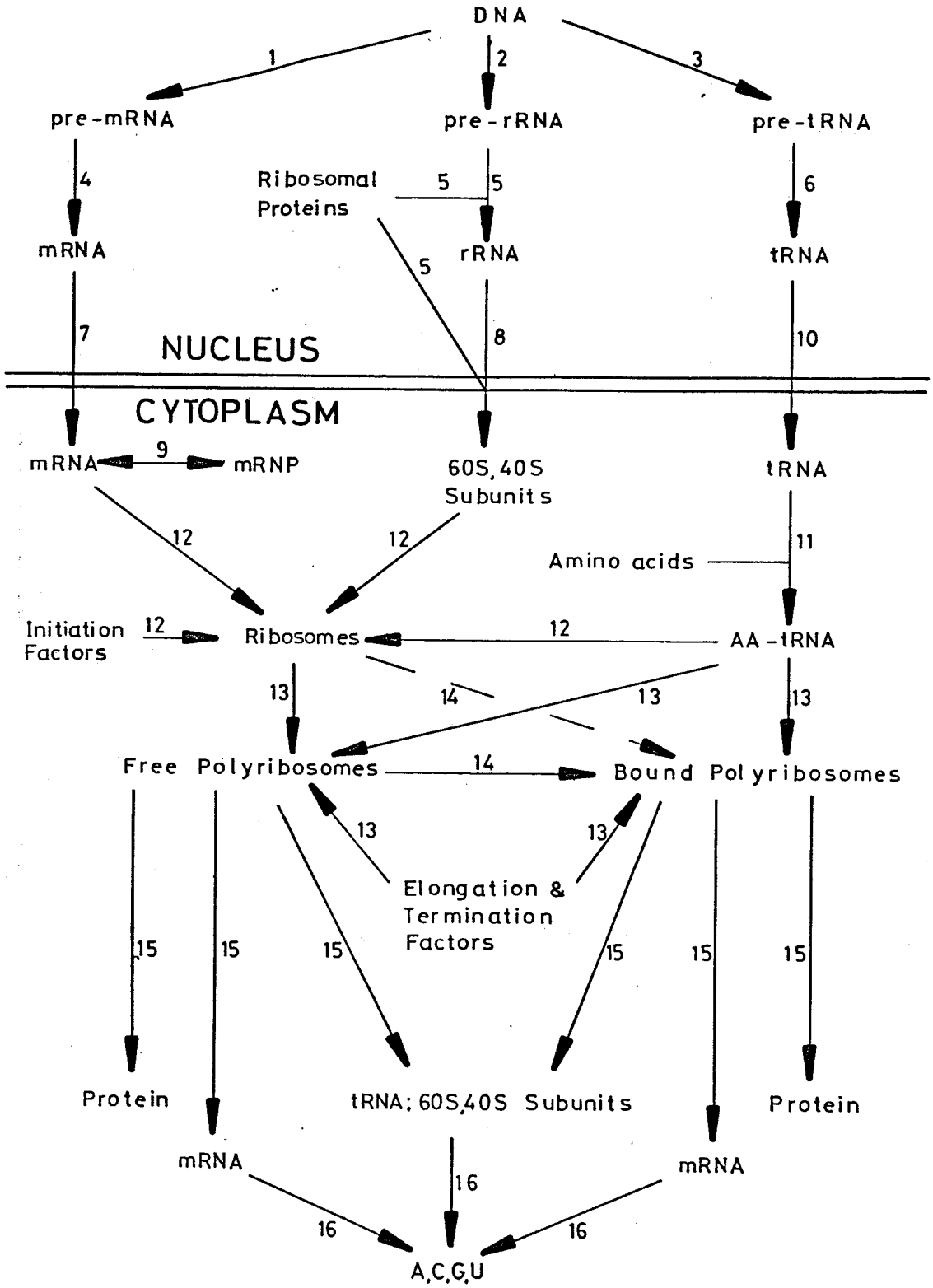
Adapted primarily from the text of (7) and (8)

Abbreviations used are:

pre	precursor form of RNA
mRNA	messenger RNA
rRNA	ribosomal RNA
tRNA	transfer RNA
A,C,G,U	nucleotides
mRNP	messenger ribonucleoprotein particle
60S & 40S	large and small ribosomal subunits
AA	amino acyl unit

Processes involved in this scheme are:

1,2,3	transcription
4,5,6	RNA processing
7,8,10	RNA transport across nuclear membrane
9	mRNA-protein association/dissociation
11	amino acylation of tRNA
12	initiation of polypeptide synthesis
13	elongation of polypeptides
14	segregation of bound polyribosomes
15	termination of polypeptide synthesis
16	cytoplasmic RNA degradation


DNA
1
2
3
pre-mRNA
pre-rRNA
pre-tRNA
4
Ribosomal Proteins
5
5
6
mRNA
rRNA
tRNA
5
7
NUCLEUS
8
10
CYTOPLASM
mRNA
9
mRNP
60S, 40S Subunits
tRNA
12
12
Amino acids
11
Initiation Factors
12
Ribosomes
12
AA-tRNA
13
14
13
13
Free Polyribosomes
14
Bound Polyribosomes
13
13
Elongation & Termination Factors
15
15
15
15
15
15
Protein
tRNA; 60S,40S Subunits
Protein
mRNA
16
mRNA
16
16
A,C,G,U

(4), Stein et al. (5) and Potter (6). The topic of processing of mRNA has been reviewed by Greenberg (7) and Revel and Groner (8).

Primary transcripts of mRNA or pre-mRNA are generally accepted (7) to be heteronuclear RNA (hnRNA). Molecules of hnRNA may be larger than 10^7 daltons while mRNA molecules are generally smaller than 2×10^6 daltons. The majority of hnRNA does not enter the cytoplasm but rather is degraded in the nucleus. Those molecules of mRNA that are transported into the cytoplasm are not necessarily translated as a matter of consequence. Control can be exerted over the processes of initiation, elongation and termination of polypeptide synthesis, as well as degradation of mRNA which will prevent entry or re-entry into the pool of translatable mRNA.

It is known that mRNA may bind specific classes of protein. There have been a number of functions suggested for these messenger ribonucleoprotein particles (mRNP) which include involvement in messenger storage, transport and initiation (9). These mRNP have sedimentation properties similar to ribosomal subunits (7) and thus mRNP and ribosome subunits are indistinguishable under some conditions for sedimentation analyses of polyribosome populations. It has been suggested that binding of specific proteins may result in an untranslatable mRNP, whereas dissociation of binding proteins results in translation of the free mRNA transcript (7). Translation of rat liver ferritin may be controlled post-transcriptionally in this manner (10). There also is evidence to suggest that proteins associated with mRNA transcripts may be involved in other ways in the translation process (9).

One of the differences between eukaryotes and prokaryotes is that eukaryotes have polyribosomes which are bound to the endoplasmic reticulum while prokaryotes do not have this membrane structure. However, it should not be forgotten that there is evidence that the synthesis of some bacterial proteins occurs with the nascent chain attached to the cell membrane (11). The experimental data presented in Table 1 suggests that segregation of polyribosomes into free and bound classes is a process which may be capable of determining the fate of proteins.

Many of the features of cytoplasmic mRNA are acquired during the maturation process (8). The enzymic activities involved in the processing events are presented in Figure 2. The primary transcripts of mRNA contain sequences which will appear in the processed mRNA separated by intervening sequences, which will be cleaved from the molecule. A stretch of fifty to two hundred adenine residues is added to the 3'-terminus of the primary transcript as a post-transcriptional event. This may occur as a nuclear event prior to the processing of hnRNA, or as a cytoplasmic event (8). It is currently thought that at an early step of the maturation process, some internal adenine residues are methylated at the 6 position of the nitrogenous base. A guanine residue is also added at the 5'-terminus and then methylated at the 7 position of the nitrogenous base. Two nucleotides adjacent to the 5'-terminal guanine are methylated at the 2' position of ribose to form a "cap" structure with the 5'-terminus methylated guanine residue. The 5' cap structure has at least two functions in

Fig. 2. Maturation of messenger RNA

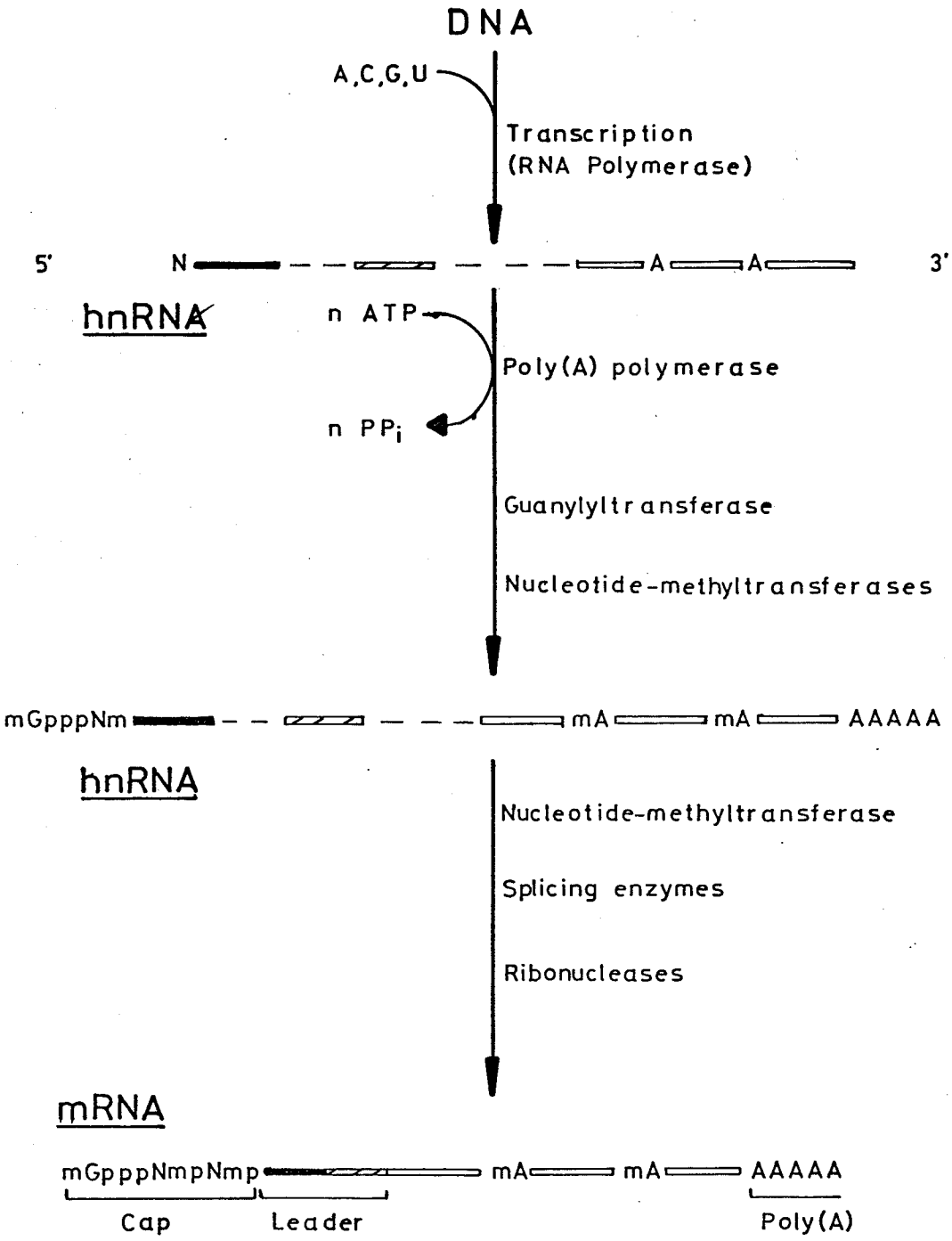
Adapted from (8)

Abbreviations used are:

DNA	deoxyribonucleic acid
hnRNA	heteronuclear ribonucleic acid
mRNA	messenger RNA
A	adenine
C	cytosine
G	guanine
U	uracil
N	ribonucleotide
ATP	adenosine triphosphate
PP_i	pyrophosphate
AAAAA	poly(A) tract
P	phosphate
m	methyl group, nitrogenous base is methylated if written on left side of the base, sugar is methylated if written on right

Symbols used are:

▭	translatable transcript
▬▨	leader sequence
— — —	sequences which are cleaved from hnRNA during processing


DNA
A,C,G,U
Transcription
(RNA Polymerase)
5'
N
A
A
3'
hnRNA
n ATP
Poly(A) polymerase
n PPi
Guanylyltransferase
Nucleotide-methyltransferases
mGpppNm
mA
mA
AAAAA
hnRNA
Nucleotide-methyltransferase
Splicing enzymes
Ribonucleases
mRNA
mGpppNmpNmp
mA
mA
AAAAA
Cap
Leader
Poly(A)

mRNA translation. The 5'-terminal methylated guanine is recognized during initiation and the cap itself protects the mRNA from 5'-exonucleolytic degradation (8). At a later stage of the maturation process, intervening sequences are cleaved out of the transcript to leave a "leader sequence" on the 5' side of the initiation codon. The leader sequence has been implicated in such processes as initiation and the binding of protein to form mRNP. The processed mRNA that results from the maturation process is the form of mRNA which will be transported into the cytoplasm to be translated.

Translation of messenger RNA

Initiation of protein synthesis is essentially a two step process as is shown in Figure 3. First, the initiation region of a mRNA transcript binds to a complex of 40S ribosomal subunit, initiation factor 2 and GTP. This process requires the initiation factors usually referred to as 1, 2, 3, 4A, 4B and 4C as well as the expenditure of energy as ATP. Binding of the 60S ribosomal subunit is the second step of initiation and requires initiation factor 5 which also has a GTPase activity. The 80S monoribosome formed by the aggregation of ribosomal subunits is incapable of protein synthesis without an adequate supply of AA-tRNAs and transfer factors.

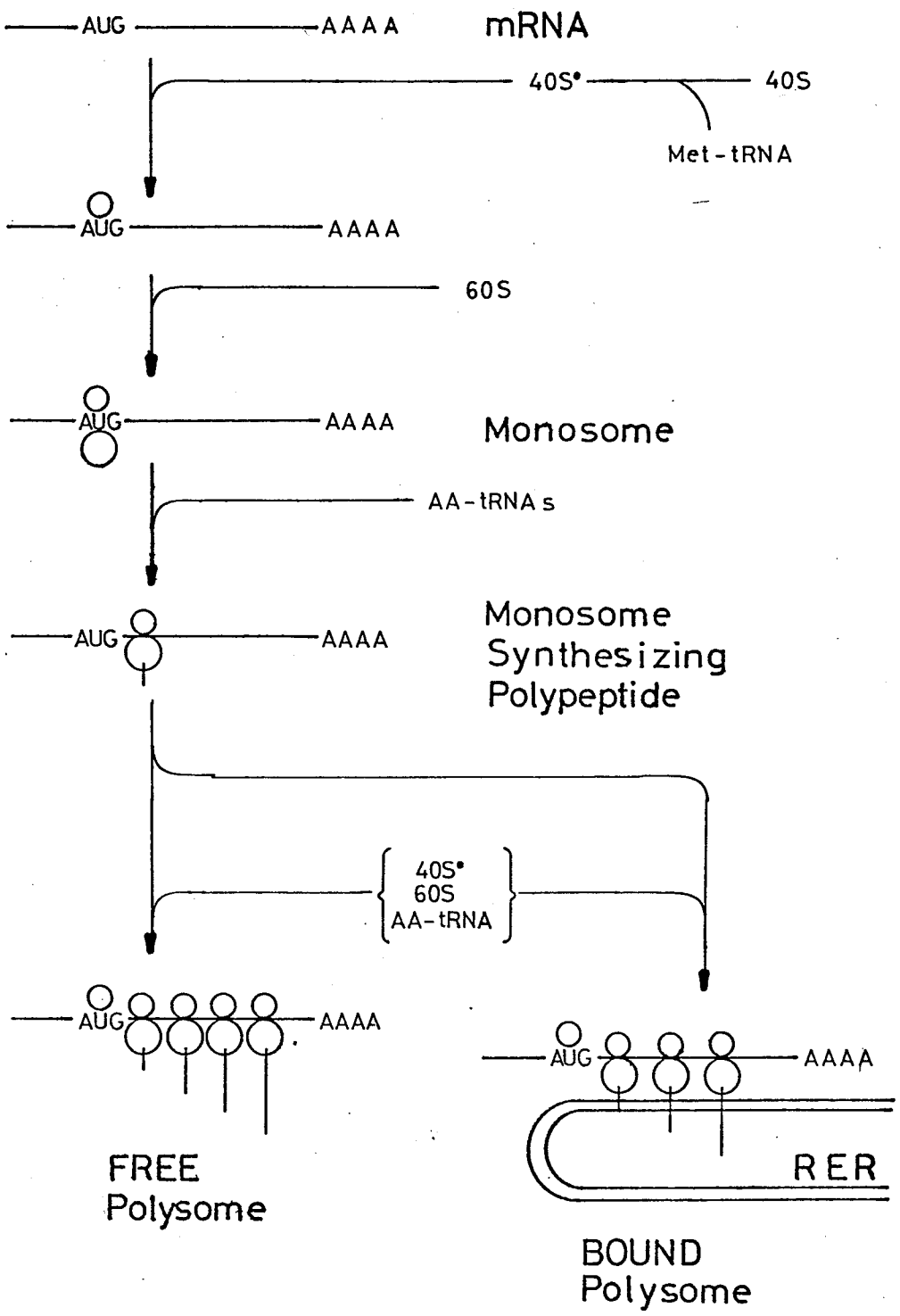
There is a growing amount of evidence that the initiation step of protein synthesis allows for a selection and discrimination of mRNA transcripts as is discussed by Revel and Groner (8). In a reticulocyte lysate translation system, increasing levels of hemoglobin mRNA decrease the ratio of synthesis of α- to β-hemoglobin. An

Fig. 3. Initiation and Translation of messenger RNA

Adapted from (8,12 & 15)

Abbreviations used are:

AUG	initiation codon
AAAA	poly(A) tract
40S	small ribosomal subunit
Met-tRNA	methionyl-tRNA
40S'	complex of 40S, Met-tRNA, GTP and initiation factor 2
60S	large ribosomal subunit
AA-tRNA	amino acyl-tRNA
RER	rough endoplasmic reticulum


AUG
AAAA
mRNA
40S*
40S
Met-tRNA
AUG
AAAA
60S
AUG
AAAA
Monosome
AA-tRNAs
AUG
AAAA
Monosome
Synthesizing
Polypeptide
40S*
60S
AA-tRNA
AUG
AAAA
FREE
Polysome
AUG
AAAA
RER
BOUND
Polysome

explanation invoked to explain this observation relies on the argument that α-globin mRNA depends on cap recognition while β-globin mRNA depends on initiation factor 4B binding during the initiation event. Other examples of apparent selection and discrimination support models of initiation control.

Elongation of polypeptides requires amino acyl tRNAs, elongation or transfer factors and the expenditure of energy as GTP. Elongation factor 1, which previously had been referred to as transferase 1, is responsible for the transfer of AA-tRNA into the acceptor site on the ribosome. A component of the large ribosomal subunit catalyzes the formation of the peptide bond and elongation factor 2 translocates the peptidyl-tRNA into the donor site of the ribosomes. A more detailed discussion is presented by Wainwright (12).

As previously mentioned, polyribosomes synthesizing polypeptides can exist in one of two states, free in the cytosol or bound to a membrane such as the endoplasmic reticulum. Discussions on the nature of the interaction between ribosomes and membrane are presented by Rolleston (1), Shore and Tata (13) and McIntosh and O'Toole (14). These authors discuss evidence to support the existence of interactions between components in the membrane and the leader sequence or poly(A) tract of mRNA, specific sites on ribosomal subunits or the nascent chain synthesized by the polyribosomes.

A model that offers an explanation for the synthesis of secretory proteins on bound polyribosomes (Table 1) has been proposed by Blobel and Dobberstein (15) and elaborated upon by Blobel (16). These workers

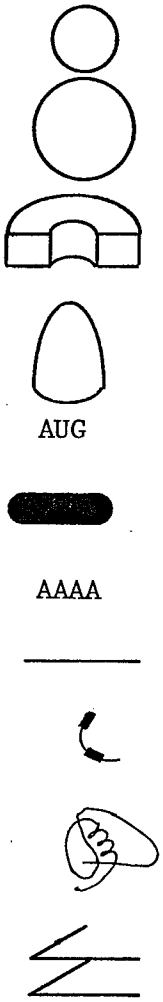
and others (15,17) have found that primary translation products of mRNA coding for secretory proteins are larger than the products secreted *in vivo*. The "signal" hypothesis proposed by Blobel and Dobberstein, and shown in Figure 4, proposes that there exist "signal" codons 3' to the initiation codon for a "signal" sequence which will interact with membrane proteins prior to completion of the nascent polypeptide chain. In this model, the "signal" peptide-membrane protein interaction brings about the aggregation of some membrane proteins to form a tunnel through which the nascent chain can pass into the lumen of the endoplasmic reticulum. It is also proposed that the interaction between the nascent chain and membrane proteins is further stabilized by a ribosome-membrane protein interaction. Blobel and Dobberstein found that during immunoglobulin synthesis, proteolysis of the "signal" peptide occurred prior to completion and release of the polypeptide from the ribosome and suggest that this may be an universal occurrence. This model has been extended by Blobel beyond the insertion of secretory proteins through the endoplasmic reticulum to include the insertion of membrane proteins into the endoplasmic reticulum and mitochondrial membrane, and the process by which the conformation of these proteins in these membranes is assumed (16).

A number of amino terminals of secretory proteins have been identified from the products of *in vitro* translation systems utilizing purified exogenous mRNA (15,17). These sequences are generally hydrophobic in nature, and are apparently cleaved before completion and release of the nascent chain from the ribosomes. In addition to these

Fig. 4. The "Signal" Hypothesis of Blobel and Dobberstein

Taken from (15)

Symbols used are:



small ribosomal subunit

large ribosomal subunit

membrane receptor protein for signal sequence

general membrane protein

initiation codon

signal codons

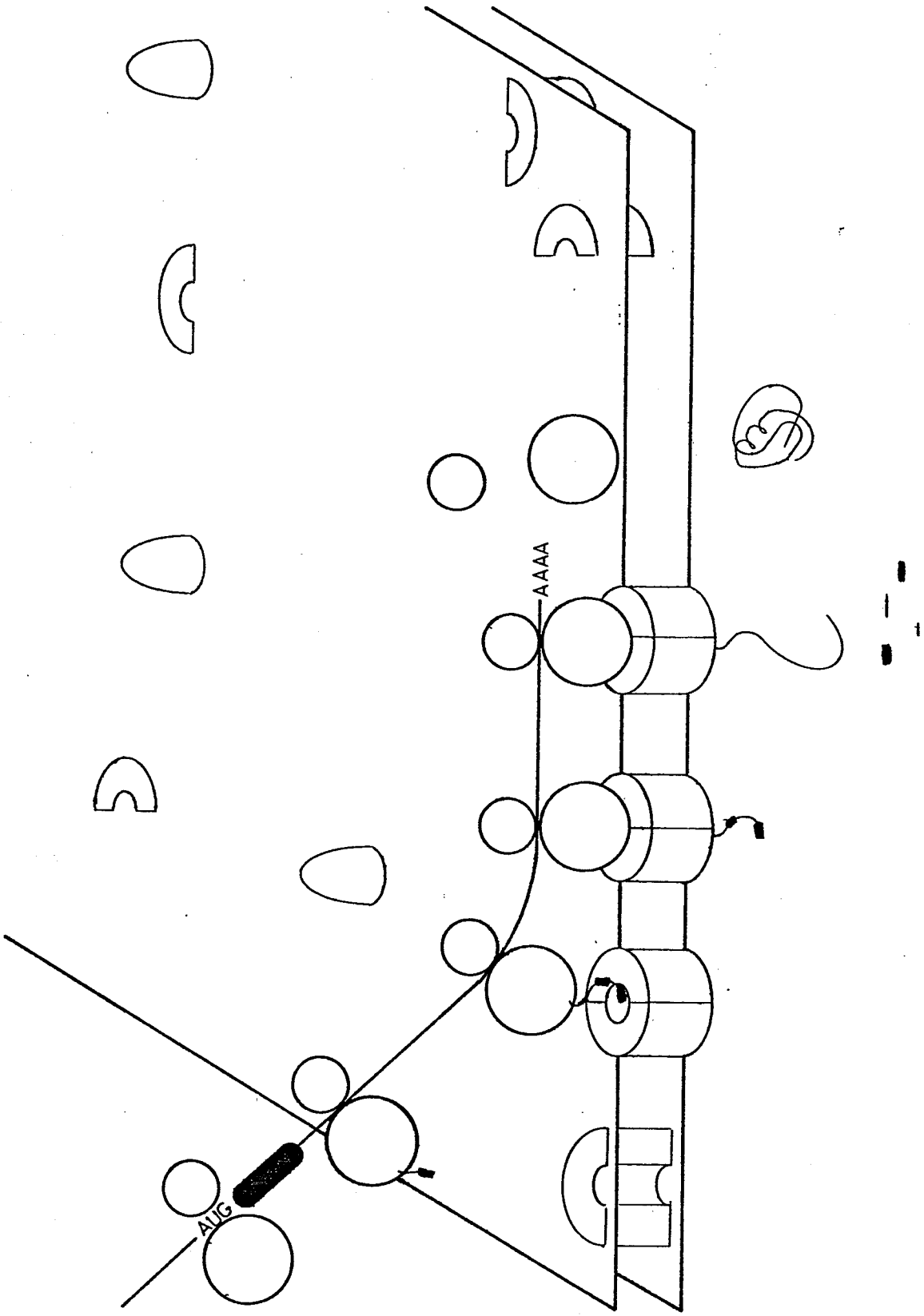
Poly(A) tract

mRNA

signal peptide

polypeptide

rough endoplasmic reticulum


AAAA
AUG

sequences, proteins such as albumin, parathyroid hormone, trypsin, insulin and glucagon are known to contain extra sequences which are cleaved from the translation products (17,19). Although no generally accepted nomenclature exists for precursor proteins, it has been suggested that the designation "pre" should be adapted for precursor proteins with a "signal region" cleaved by microsomal peptidases, whereas "pro" should refer to protein segments cleaved by either intracellular (localized in Golgi and condensing vacuole membranes) or extracellular peptidases (17). It has also been proposed that precursor proteins be grouped into four structural classes (17). Type 1 contains only a presegment, type 2 contains a presegment and an amino terminal prosegment, type 3 contains a presegment and an internal prosegment and type 4 contains a presegment and a carboxyl terminal prosegment. Examples of these sequences are shown in Table 2. Although the existence of presegments at amino terminals of translation products is explained by the signal hypothesis, not all prosegments share a common function. Prosegments seem to be intricately involved in controlling the biological activity of proteins such as insulin, collagen, trypsin and chymotrypsin. Functions also suggested for the prosegments of albumin include mediation of membrane binding, masking of binding sites, regulation of degradation or synthesis, and facilitation of tertiary structure formation.

The secretion of secretory proteins involves passage of the proteins from the rough endoplasmic reticulum to the smooth endoplasmic reticulum to the Golgi apparatus to secretory vesicles to the plasma

Table 2

Presegments

Serum Albumin (rat)	Met Lys Trp Val Thr Phe Leu Leu Leu Leu Phe Ile Ser Gly Ser Ala Phe Ser
Trypsin (dog)	Ala Lys Leu Phe Leu Phe Leu Ala Leu Leu Leu Ala Tyr Val Ala Phe
Insulin (rat)	X Leu Lys Met X Phe Leu Phe Leu Leu Lys Leu Leu X Leu X X X X X X X X
Ovomucoid (chicken)	Ala Met Ala Gly Val Phe Val Leu Phe Ser Phe Val Leu X Gly Phe Leu Pro Asp Ala Ala Phe Gly

Prosegments (type 2)

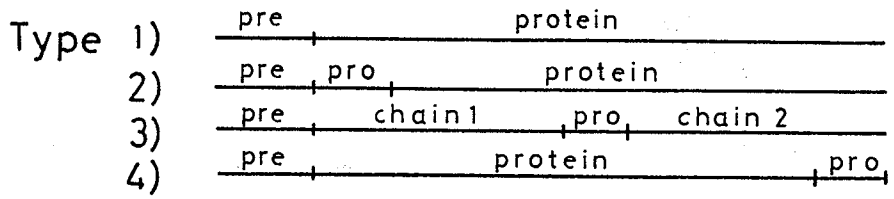
Albumin	Arg Gly Val Phe Arg Arg
Parathyroid Hormone	Lys Ser Val Lys Lys Arg

Prosegment (type 3)

Glycophorin	Arg Arg Leu Ile Lys Lys

Prosegment (type 4)

Glucagon	Thr Lys Arg Asn Lys Asn Asn Ile Ala



The amino end of these segments is on the left side of the Table while the carboxyl end of the segments is on the right side. The precursors are classified by the scheme shown immediately above. The code used to indicate the amino acids is: Ala, alanine; Arg, arginine; Asn, asparagine; Asp, aspartate; Gly, glycine; Ile, isoleucine; Leu, leucine; Lys, lysine; Met, methionine; Phe, phenylalanine; Pro, proline; Ser, serine; Thr, threonine; Trp, tryptophan; Tyr, tyrosine; Val, valine; X, unidentified amino acid.

These sequences were taken from (17) and (19). See text for further explanation.

(13,17,18). If the protein is destined to be a glycoprotein, glycosylation also occurs during this passage.

Glycosylation of proteins

Concepts of the processes involved in the synthesis of glycoproteins have been subject of several alterations in the past few years. Figure 5 shows a scheme of what is believed to be involved in the synthesis and processing of glycoproteins which are destined to be secreted into the plasma. Vesicular stomatitis virus G protein is shown in this scheme as a typical example of a secretory protein which also has been well characterized (20).

The weight of current evidence suggests that the biosynthesis of complex oligosaccharide units is initiated by the en bloc transfer of a high molecular weight oligosaccharide from a lipid carrier to the nascent polypeptide chain (20). For the serum type of glycoprotein, this transfer is to an asparagine residue in the polypeptide (21). The attachment of carbohydrates to the polypeptide seems to be fixed by the amino acid sequence adjacent to the asparagine involved in the linkage with carbohydrate. The amino acid sequence which codes for the attachment of carbohydrate has been referred to as the "sequon" (21). The sequon for the serum type of glycoprotein is believed to be Asn-X-Ser(Thr) where any amino acid can occupy the intermediate position between asparagine and serine or threonine. The lipid involved in the transfer is dolichol diphosphate and the high molecular weight oligosaccharide has a high mannose content and contains glucose. Glucose and most of the mannose are cleaved from the complex carbohydrate in an

Fig. 5. Incorportation of carbohydrate into Glycoproteins

Adapted from (20)

Abbreviations used are*:

Dol-P-P	dolichol diphosphate
GlcNAc	N-acetyl glucosamine
M	mannose
G	glucose
AAA	poly(A) tract
Fuc	fucose
Gal	galactose
NANA	N-acetyl neuraminic acid

Symbols used are:

signal peptide

nascent polypeptide chain

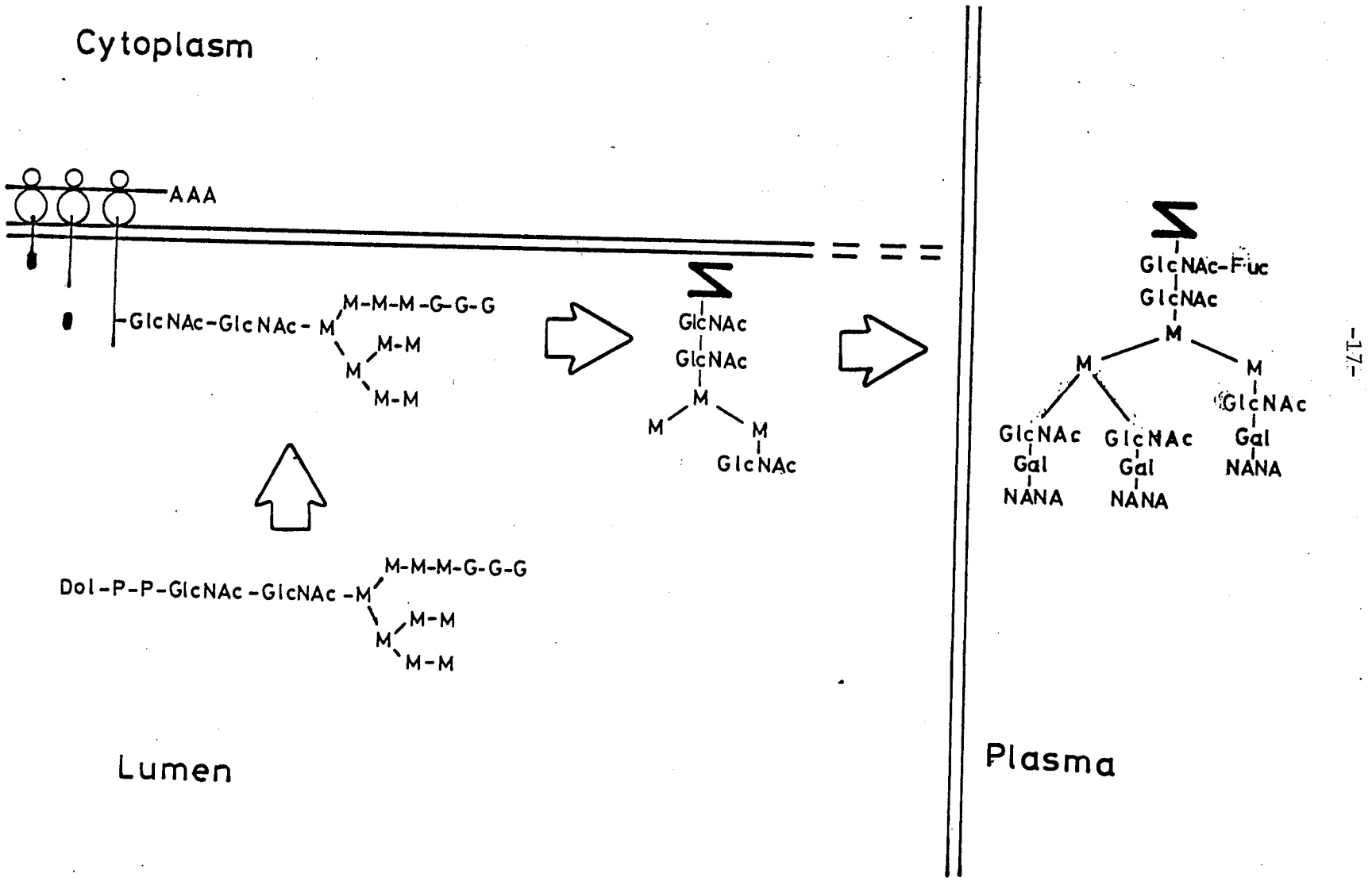
mRNA

membrane of cytoplasmic reticulum

plasma membrane

polypeptide in folded conformation

* Although man and glc are the accepted abbreviations of mannose and glucose, M and G were used for the sake of clarity in graphical presentation.


Cytoplasm
AAA
-GlcNAc-GlcNAc-
M-M-M-G-G-G
M-M
M-M
GlcNAc
GlcNAc
GlcNAc
Dol-P-P-GlcNAc-GlcNAc-
M-M-M-G-G-G
M-M
M-M
Lumen
GlcNAc-Fuc
GlcNAc
GlcNAc
GlcNAc
Gal
NANA
GlcNAc
Gal
NANA
GlcNAc
Gal
NANA
Plasma

event that has been referred to as processing (20). After the glycoprotein is processed, sugars are sequentially added to give the secreted form of the glycoprotein.

Although the incorporation of carbohydrate into vesicular stomatitis virus G protein represents a well characterized system, the scheme proposed by Kornfeld et al. (20) may not be generally true for the incorporation of carbohydrate into all glycoproteins. Jamieson (3) has shown that initial glycosylation of rat α_1-acid glycoprotein occurs mainly as a post ribosomal event. As this author suggests, there may exist more than one mechanism for the initial glycoslyation of polypeptides destined to become glycoproteins. Similarly, it should not be assumed that later events during glycosylation are the same for all glycoproteins.

The acute inflammatory response

In mammals, inflammation may be caused by a wide variety of conditions including chemical inflammatory agents, neoplastic diseases, bacterial infections and rheumatoid arthritis (22,23). Glenn et al. (24) have suggested that the acute inflammatory response should be divided into two reactions, the local reaction at the immediate site of tissue damage and the systemic reaction induced by the local reaction.

Granulomatous inflammation may be caused by tuberculosis, syphylis, parasitic worm infestation or foreign bodies such as talc, asbestos, plastic beads or turpentine (25). Granulomas are tumor-like masses of leukocytes and collagen. Granuloma formation is a rapid process beginning as early as 6 hours after inflammation and reaching a peak at

about 48 hours after inflammation. Acute inflammation in response to the deposition of a foreign body is quite similar to that in response to an infectious agent. A more complete discussion of the roles of cellular and humoral components involved in the formation of granulation tissue is given by Ross (26).

According to the scheme of Glenn et al. (24), the formation of granulation tissue at the site of injury is accompanied by an induction of a systemic reaction by factors released from the site of injury. It is believed that hormones mediate this reaction and that prostaglandins represent at least one class of the chemical mediators that are involved (27-30). One aspect of the systemic reaction of acute inflammation is represented by characteristic changes of some of the plasma proteins (23,31) as is shown in Table 3. Those proteins which increase in concentration during acute inflammation are referred to as acute phase reactants and are almost all glycoproteins. The most important acute phase reactants in humans and rats are α_1-acid glycoprotein, α_2-macroglobulin, α_2-(acute phase) globulin, haptoglobulin, ceruloplasmin, transferrin and fibrinogen (23,31). It has been established that the liver is the site of synthesis of these acute phase reactants (32-35).

Many of the inflammation-related changes in serum proteins have been attributed to increased hepatic translation of acute phase reactants (23,36). This is accompanied by a significant alteration in the RNA metabolism in rat liver. Thompson and Wannemacher (37) showed that infection with Diplococcus pneumoniae resulted in an increase of incorporation of orotate into hepatic RNA. Most of the newly incorporated

Table 3

Proteins of Human Plasma Showing Altered Concentration after Trauma

Taken from (23)

Protein	Concentration in Plasma (% of preoperative values)
Fibrinogen	>200
Haptoglobulin	206
Orosomucoid	>200
C-reactive protein	>200
α_1-antitrypsin	>200
Slow β-globulin	173
Inter α-globulin	189
Complement C'3	122
Caeruloplasmin	124
Easily precipitable glycoprotein	140
Thyroxine binding prealbumin	69
β-lipoprotein	77
Transferrin	78
Albumin	80

orotate was associated with the bound ribosomal RNA fraction. Chandler and Neuhaus (38) found that laparotomy resulted in increased synthesis of RNA which could be blocked by administration of actinomycin D. These authors also found that injury had no detectable influence on DNA synthesis nor on the activity of RNA polymerase. Liu and Neuhaus (39) have shown that laparotomy results in a decrease in hepatic alkaline ribonuclease activity as well as a shift in hepatic polyribosomes to higher states of aggregation. Although similarities such as increased synthesis of acute phase reactants have been observed following laparotomy, infection with D. pneumoniae and acute inflammation, it has not been established that these stress situations result in alterations in RNA metabolism which reflect common biochemical mechanisms.

In view of the alterations in concepts of RNA and protein metabolism, as well as the appearance of more sophisticated techniques for examining these phenomena, re-examination of the acute phase response may be fruitful and forms the subject of a part of this thesis.

Introduction to the present work

Part of the work presented in this thesis is concerned with the control of biosynthesis of a specific acute phase reactant, α_1-acid glycoprotein, which has been isolated from rat serum (40). It has been shown that the content of α_1-acid glycoprotein associated with the microsome fraction is approximently four-fold greater in animals suffering from induced inflammation than is found in control animals (33). This is believed to result from a greater capacity for synthesis of

α_1-acid glycoprotein by the liver in response to inflammation (33).

Ferritin is an iron storage protein localized in many tissues including the liver (41). Administration of a single intraperitoneal injection of iron to rats results in an increased synthesis of hepatic ferritin accompanied by an increase in translatable mRNA coding for ferritin (10,41). This response was not blocked by administration of actinomycin D and cordycepin* which are known to block the synthesis of mRNA and processing of hnRNA, respectively (10). Zahringer et al. (10) have proposed that the induction of ferritin synthesis is accomplished by an iron-induced dissociation of ferritin sybunits from mRNP coding for ferritin.

Metallothioneins are inducible cytoplasmic proteins synthesized in response to transition metal ions in certain mammalian tissues (42). Administration of a single intraperitonal injection of zinc to rats results in an increase in hepatic synthesis of metallothionein and mRNA coding for metallothionein which is inhibited by actinomycin D (42).

The responses to turpentine, iron and zinc administration represent three different physiological states where qualitative changes in protein synthesis are known to occur. The synthesis of α_1-acid glycoprotein is known to occur preferentially on bound polyribosomes (3). Ferritin has been cited as an example of an intracellular protein synthesized primarily on free polyribosomes (1,10,13). The site

* Cordycepin or 3'-deoxyadenosine allegedly inhibits addition of poly(A) to hnRNA and prevents transport of newly synthesized mRNA into the cytoplasm (10).

of synthesis of the intracellular protein metallothionein has not been reported as yet. In view of the different sites of synthesis of α_1-acid glycoprotein and ferritin, it was hoped that induction of synthesis of these proteins would result in changes in distribution of polyribosomes between the bound and free classes. Zinc induction of metallothionein synthesis was also examined under the assumption that as an intracellular protein, metallothionein may have a bias for synthesis on free polyribosomes.

As previously mentioned, acute inflammation results in a shift of the hepatic polyribosomes to heavier aggregates. The results reported by Liu and Neuhaus (39) provide a limited amount of information about processes during acute inflammation as the sedimentation analyses were performed on total polyribosomal populations. In this thesis, the effect of inflammation on both bound and free polyribosome profiles is reported. Those polyribosomes within the polyribosome profile which synthesize α_1-acid glycoprotein and albumin were also identified utilizing antibodies prepared against these proteins. Albumin was considered to be an example of a protein which decreases in concentration during the acute phase response and α_1-glycoprotein was considered to be an example of an acute phase reactant in these experiments.

Liu and Neuhaus have suggested that the decrease in alkaline ribonuclease activity following laparotomy had a stabilizing effect on polyribosomes which accounted for the observed increase in bound ribosomal RNA (39). This also represents a possible mechanism by which alterations in the distribution of RNA between bound and free poly-

ribosomes might occur following iron and zinc administration and as such, the effects of acute inflammation, iron administration and zinc administration on this enzymic activity were also examined.

As previously mentioned, stress has been shown to increase the hepatic incorporation of orotate into RNA. Although orotate incorporation does not normally reflect isolated events of RNA metabolism, experiments of this nature are useful in providing insight into synthetic and degradative aspects of RNA metabolism in conjunction with information from related experiments. In this thesis, incorporation of orotate is reported into polyribosomal fractions separated by oligo(dT)-cellulose chromatography. This method of RNA separation avoids the problems associated with the phenol methods of RNA isolation (43).

The observation that administration of actinomycin D blocked laparotomy-induced seromucoid synthesis suggests that induced synthesis of the acute phase reactants involves synthesis of new mRNA transcripts coding for these proteins (38). However, this observation does not provide direct evidence of mRNA involvement in the acute phase response. In this thesis, the products synthesized by an <u>in vitro</u> translation system in response to the addition of purified mRNA fractions were examined immunochemically. The amount of material precipitated by an antiserum in these assays should be proportional to the levels of mRNA added to the translation system and translated into the antigen. This type of experiment provides a more direct measure of mRNA levels than do experiments utilizing actinomycin D. Antisera prepared against

albumin and α_1-acid glycoprotein were utilized in these experiments with the rationale that albumin was representative of those proteins which decreased in concentration during the acute phase response and α_1-acid glycoprotein was representative of the acute phase reactants. Similar experiments were performed utilizing mRNA fractions prepared from bound and free polyribosomes in an attempt to determine the distribution of mRNA transcripts for these proteins between bound and free polyribosomes.

The experiments reported in this thesis were performed with the intent of providing insight into the processes modulating acute inflammation as well as into the validity of present concepts of the significance of the division of mammalian polyribosomes into two classes.

Experimental

Materials

Radioactive compounds

Sodium [^{3}H]-orotate (19 Ci/mmole), sodium [^{125}I] (1 mCi/10 μℓ), Amersham Corp., Oakville, Ontario.

Chemicals for liquid scintillation counting

Aqueous counting scintillant (ACS), Amersham Corp., Oakville, Ontario.

Proteins

Bovine serum albumin (crystalline), Sigma Chemical Co., St. Louis, Mo.

Chromatography media

Sephadex G-50, DEAE-Sephadex A-50, Pharmacia, Uppsula, Sweden; CM-cellulose, Sigma Chemical Co., St. Louis, Mo.; Oligo(dT)-cellulose, Collaborative Research, Waltham, Mass.

Electrophoresis media

Acrylamide, N,N'-methylenebisacrylamide (bis), N,N,N',N'-tetramethylethylenediamine (TEMED), ammonium persulfate, BioRad Laboratories, Richmond, Calif.

Other chemicals were obtained as follows: Trizma Base, sodium dodecyl sulfate (SDS), glycine (crystalline), Triton X-100, sodium deoxycholate, glutathione (crystalline), diethyl pyrocarbonate, agarose, thimerosal, sucrose, Sigma Chemical Co., St. Louis, Mo.; ampholine carrier ampholytes, LKB Produkter AB, Stockholm-Bromma 1, Sweden; grade A high molecular weight RNA (sodium salt), Calbiochem-Behring

Corp., La Jolla, Calif.; Nobel agar, Difco Laboratories, Detroit, Mich.; sodium parachloromercuribenzoate (PCMB), United States Biochemical Corp., Cleveland, Ohio; N-2-hydroxyethyl piperazine-N-2-ethane sulfonic acid (HEPES), heparin, Nutritional Biochemical Corp., Cleveland, Ohio; turpentine oil (double rectified), ethylenediaminotetraacetate (EDTA), Fisher Scientific Co., Toronto, Ontario. Reagent grade 1 sucrose, which was obtained from Sigma Chemical Co., St. Louis, Mo. was used in all experiments where contamination with ribonuclease would represent a problem. Other chemicals were of analytical grade obtained from local suppliers.

Physical and chemical methods

Absorbances in the ultraviolet region of the spectrum were measured with a Gilford 2400-2 spectrophotometer. Measurements of pH were made with a Radiometer 28b pH meter. Radioactivity was determined with a Packard model 3003 Tri-carb liquid scintillation spectrometer. Aqueous samples were counted in 10 ml ACS scintillation cocktail. For measurement of total [^{3}H]-leucine incorporation during *in vitro* translation, 2 cm x 2 cm strips of Whatman 3MM paper (containing TCA precipitates) were added to 10 ml ACS scintillation cocktail and counted. The red channel was set at a pulse height of 50-1000 divisions (60% gain) to count [^{3}H] and the green channel was set at a pulse height of 50-1000 divisions (4.5% gain) to count [^{125}I].

Immunoelectrophoresis was carried out on equipment supplied by Shandon Scientific Co. Ltd., London.

For isoelectric focusing experiments a 110 ml isoelectric focusing column (LKB 8100-10) and a gradient mixing device obtained from LKB Produkter AB, Stockholm-Bromma 1, Sweden were used. Temperature was maintained at 2°C by circulating 10% ethanol from a Haake FKN Circulating Bath with a Haake F4391 Line Heater through the cooling jacket. Power was supplied from a Vokam Mk IV power supply.

Gel electrophoresis was performed in a BioRad model 155 Electrophoresis Cell connected to a Heathkit IP-17 Regulated HV Power Supply. Temperature was maintained at 4°C as described above. Gel staining and destaining was performed in a BioRad model 172A Diffusion Destainer. Scans of gels were performed on a Gelman DCD-16 Densitometer. Gel slicing was performed with a BioRad model 190 Gel Slicer.

Sucrose solutions for sedimentation analysis were formed with the LKB gradient mixing device. Gradients were removed from tubes by upward displacement at a rate of 0.6 ml/min using an ISCO model 182 Density Gradient Fractionator.

Protein was assayed as described by Lowry et al. (44), but with modified reagents and volumes described by Miller (45). Crystalline bovine serum albumin was used as the standard. RNA was assayed as described by Munro and Fleck (46).

Isolation of α_1-acid glycoprotein

A method based on a procedure previously described by Simkin et al. (47) was used to isolate α_1-acid glycoprotein. Serum was obtained from rats which received a subcutaneous injection of 0.5 ml turpentine oil per 100 g body weight into the dorsolumbar region. Turpentine

administration resulted in an acute phase response which elevated serum levels of α_1-acid glycoprotein about four-fold. Nine volumes 0.15M sodium chloride were added to the serum and stirred rapidly with a magnetic stirrer at room temperature. This solution was made to 0.6M perchloric acid by slow addition of 1.8M perchloric acid, allowed to stand 10 min and centrifuged in a Sorvall RC2-B fitted with a GSA rotor for 20 min at 2,000 r.p.m. (650 g_{av}). The supernatant was decanted and neutralized in an ice bath to pH 6.5 with 2M sodium hydroxide, dialyzed exhaustively against distilled water for 3 days and concentrated to 50 ml using an Amicon model 202 Ultrafiltration Cell fitted with an UM10 62 mm Diaflo filter. Material retained by the filter was designated PCA-soluble fraction and freeze-dried.

The PCA-soluble fraction was purified by chromatography on CM-cellulose as described by Jamieson et al. (40). The fine particles were first removed from the CM-cellulose by resuspension in water followed by decanting. CM-cellulose was regenerated by washing twice with 0.25M sodium hydroxide containing 0.25M sodium chloride and then with distilled water until neutral. CM-cellulose was suspended in 50mM sodium acetate buffer, pH 4.9 and a 45 cm x 2.5 cm column was prepared and equilibrated with the same buffer. A 250 mg sample of the PCA-soluble fraction was dissolved in 5 ml 50mM acetate buffer, pH 4.9 and applied to the CM-cellulose column. The column was eluted with the same buffer and 60 drop fractions were collected. Protein was detected by monitoring the column effluent automatically at 254 nm with a LKB 4701A Uvicord. Protein that was eluted with acetate buffer

was pooled, dialyzed overnight against 4 ℓ water and freeze-dried. This fraction was designated CMC-1.

CMC-1 was subjected to isoelectric focusing on a pH 1-3 gradient as described by Jamieson et al. (40). A dense electrode solution containing 0.15 ml concentrated sulfuric acid, 16 ml glycerol and 4 ml distilled water was added to the anode at the bottom of the column. A glycerol gradient containing ampholytes was introduced into the column using the LKB gradient device to produce a linear glycerol gradient. The dense electrode solution contained 0.1 g monochloroacetic acid, 0.1 g orthophosphoric acid, 0.1 g dichloroacetic acid, 35 ml glycerol and 20 ml distilled water. The light gradient solution contained 0.1 g acetic acid, 0.1 g formic acid, 0.1 g citric acid, 0.05 g glutamic acid, 0.05 g aspartic acid, 0.30 ml ampholine carrier ampholytes, pH 5-8 and 60 ml distilled water. After about 50 ml of the gradient had entered the column, flow from the gradient device was stopped and 20-30 mg CMC-1 was dissolved in about 1 ml gradient solution from the gradient device. This material was carefully layered on the gradient solution already in the column using a Pasteur pipette and flow of the gradient solution to the column was resumed. When the column had filled, 10 ml light electrode solution containing 0.1M sodium hydroxide was added to the cathode at the top of the column. During isoelectric focusing, potential applied to the column was slowly increased to 400 V while keeping the current below 15 mA. The column was run until the current dropped below 1 mA. When isoelectric focusing was complete, the valve at the bottom of the column was closed and 30

drop fractions were collected from the lower exit of the column. The pH and absorbance at 280 nm of each sample was determined. Protein which focused at pH 2.95 was pooled, dialyzed against several changes of distilled water for 3 days and freeze-dried.

The above preparation gave single precipitin lines using antisera to α_1-acid glycoprotein and whole rat serum in double diffusion analysis in agar gels as described by Ouchterlony (48). By this criterion, the preparation was considered to be essentially pure α_1-acid glycoprotein.

Polyacrylamide gel electrophoresis

Protein samples were examined by gel electrophoresis on 7.5% polyacrylamide gels. Prior to casting the gels, gel tubes were soaked overnight in chromic acid, repeatedly rinsed with distilled water and oven-dried. Acrylamide solution A was prepared by dissolving 22.2 g acrylamide and 0.6 g N,N'-methylenebisacrylamide in distilled water to give 100 ml of solution, filtered through Whatman No. 1 filter paper and stored in a dark bottle at 4°C. An ammonium persulfate solution of 15 mg/ml and a 0.376 Tris/glycine, pH 8.9 gel buffer was prepared shortly before casting of the gels. A mixture of 10.1 parts acrylamide solution A, 3.4 parts water and 15 parts gel buffer was deaerated under vacuum in a side arm flask for about 15 min. Ammonium persulfate solution and TEMED were added at volumes of 1.5 parts and 0.045 parts, respectively, and about 3 ml of the mixture was added with a Pasteur pipette to gel tubes 12.5 cm long with an inner diameter of 5 mm. A drop of distilled water was added to the tops of the gels to ensure complete polymerization and the gels were allowed to polymerize for at

least 3 h.

Gels were equilibrated by electrophoresis in a tank buffer of 0.188M Tris/glycine, pH 8.9 for 2 h using a current of 1 mA/gel. Protein samples of 5-40 µg were dissolved in 10-50 µl tank buffer containing 0.05% bromophenol blue and a few crystals of sucrose. These solutions were carefully layered on the tops of gels in the electrophoresis cell so that they displaced the tank buffer. A potential of 50 V was applied to the gels for 15 min to allow the samples to enter the gels. The potential was then increased to give a current of 2-3 mA/gel. Electrophoresis was continued until the bromophenol blue had migrated to the bottom of the gels. Gels were removed from the tubes by injecting hot water through a 18 gauge needle along the inner walls of tubes.

The staining procedure followed was based on a method previously reported by Fairbanks et al. (49). Gels were first stained overnight in a solution containing 0.03% Coomassie Brilliant Blue R250, 10% acetic acid and 25% isopropanol. Gels were then transferred to a second stain containing 0.0025% Coomassie Brilliant Blue R250, 10% acetic acid and 10% isopropanol and left in this stain for 4-6 h. Gels were destained in a destaining solution containing 10% acetic acid until the background appeared clear.

In those experiments where slices of polyacrylamide gels were counted for radioactivity, slices of the gels were transferred to scintillation vials and 0.5 ml 30% hydrogen peroxide added. Vials were

tightly capped and heated at 70°C in an oven until the slices had dissolved. A volume of 10 ml ACS scintillation cocktail was added to the vials and radioactivity determined by scintillation counting as previously described.

Immunological techniques

Antisera were prepared essentially as described by Simkin et al. (50). Male Full Lop Albino rabbits were used for the preparation of antisera. An emulsion of 0.75 mg antigen, 0.25 ml sterile 0.15M sodium chloride and 0.4 ml Freund's adjuvant was injected intramuscularly, one-half of the dose being injected into each thigh of the rabbit. After 6 days, a second preparation containing 1.25 mg antigen with Freund's adjuvant was injected as before. After a further 22 days, a series of intravenous injections was given of a suspension of a coprecipitate of antigen with aluminium hydroxide. Samples of 0.05 ml, 0.10 ml and 0.25 ml were injected on alternate days. The rabbits were bled 5 days after the final injection. To prepare the coprecipitate, 0.4 ml 10% potassium alum was added to a mixture of 6 mg antigen in 0.2 ml water to which 0.08 ml 1M sodium bicarbonate had been added. Sodium bicarbonate, at a concentration of 1M, was added to neutrality and the mixture was allowed to stand 16 h at 2°C. The precipitate was collected by centrifugation and the pellet suspended in 0.4 ml sterile 122mM disodium phosphate-28mM monosodium phosphate-0.01% thimerosal, pH 7.4.

Double diffusion analysis of test solutions and antisera was performed as described by Ouchterlony (48). The medium used consisted

of 1.25% Nobel agar and 0.9% sodium chloride. The plates were allowed to develop at room temperature in a humidity cabinet. Photographs were taken with the aid of an illumination device as described by Hunter (51).

Immunoelectrophoresis was performed as described by Ashton et al. (36). Immunoelectrophoresis buffer contained 55mM sodium acetate, 48.5mM sodium diethylbarbiturate, and 65mM hydrochloric acid, pH 8.6. The gels consisted of 1% agarose in a 2:1 mixture of distilled water to immunoelectrophoresis buffer. A 1 mm layer of gel was prepared on microscope slides using equipment supplied by Shandon Scientific Co. Ltd. and allowed to set first at room temperature for 45 min and then at 4°C for a further 2 h. Wells were cut with the aid of a template supplied by Shandon Scientific Co. Ltd. A potential of 80 V was applied for 90 min and then the center well was filled with antiserum and allowed to develop in a humidity cabinent until precipitin lines appeared. Photographs were taken with the aid of an illumination system as described by Hunter (51).

The procedure used for collecting immune precipitates was based on a method previously reported by Jamieson et al. (31). Samples containing antigen were made up to a volume of 0.45 ml in 0.15M sodium chloride, 1mM sodium azide and 4.7% Dextran T70. Volumes of 0.05 ml antiserum to rat serum albumin or 0.15 ml antiserum to rat α_1-acid glycoprotein were added to the samples. Mixtures were incubated at 37°C for 45 min and allowed to stand at 4°C for 48 h. Precipitates were collected by centrifuging at 12,000 r.p.m. (8,000 g_{av}) in an

Eppendorf model 3200 microcentrifuge. Pellets were washed with 0.3 ml ice cold 0.15M sodium chloride, 4% Dextran T70 and then three times with 0.3 ml 0.15M sodium chloride. Precipitates were dissolved in 1 ml 0.1M sodium hydroxide for determination of protein and radioactivity by scintillation counting.

Immunoglobulin G fractions of antisera were prepared by chromatography on DEAE-Sephadex. Samples of 20-30 ml antisera were dialyzed overnight against 0.02M sodium phosphate buffer, pH 7.4. The antiserum was then applied to a 2.5 cm x 40 cm column of DEAE-Sephadex previously equilibrated with the same buffer. The immunoglobulin G fraction was eluted from the column with 0.02M sodium phosphate buffer, pH 7.4. Protein was detected by monitoring the column effluent automatically at 254 nm with a LKB 4701A Uvicord. Those fractions which absorbed light at 254 nm were pooled, dialyzed overnight against 4 ℓ distilled water and freeze-dried.

Immunoglobulin G samples were iodinated with [^{125}I] as described by Greenwood et al. (52). Immediately before the iodination reaction, solutions of 8 mg/ml chloramine T, 2.4 mg/ml sodium bisulfite and 10 mg/ml potassium iodide, all in 0.05M sodium phosphate buffer, pH 7.4, were prepared. Protein samples of 0.5-1.5 mg in 0.3M sodium phosphate buffer, pH 7.4 and 0.025 ml chloramine T solution were added to vials containing sodium [^{125}I]. This preparation was mixed and allowed to stand for 3 min at room temperature and then 0.2 ml sodium bisulfite solution and 0.2 ml potassium iodide solution were added. This sample was transferred to a 20 cm x 1 cm Sephadex G-50 column and eluted

first with 0.4 ml potassium iodide solution and then with 0.05M sodium acetate buffer, pH 7.4. Fractions of 1 ml were collected and the radioactive material excluded from the gel was pooled, dialyzed against distilled water and freeze-dried.

Treatment of experimental animals

Acute inflammation and the physiological states induced by iron and zinc administration were examined in the studies reported in this thesis. Acute inflammation in experimental rats was induced by a single subcutaneous injection of 0.5 ml turpentine oil per 100 g body weight into the dorsolumbar region. Synthesis of ferritin was induced by a single intraperitoneal injection of iron as 2.5 mg ferric ammonium citrate in 0.9% sodium chloride per 100 g body weight. Synthesis of metallothionein was induced by a single intraperitoneal injection of zinc as 0.25 mg zinc sulfate in 0.9% sodium chloride per 100 g body weight. Control animals received either subcutaneous or intraperitoneal injections of 0.5 ml 0.9% sodium chloride. In the studies on *in vivo* incorporation of orotate, sodium [^{3}H]-orotate was administered as an intraperitoneal injection. Iron and zinc solutions were prepared using sterile distilled water and oven-dried glassware. These solutions were adjusted to pH 7.4 with 1M sodium hydroxide. Rats were lightly etherized prior to all injections.

It is known that hormonal levels, nutritional state, age and time of sacrifice affect the distribution of polyribosomes (14). In the studies reported here, male Long-Evans Hooded rats of approximately 250 g body weight were used and maintained in an environment of constant

light. These rats were maintained on a diet of Purina Rat Chow and starved 16 h prior to sacrifice to deplete hepatic glycogen stores which could interfere with polyribosome isolation. Rats were sacrificed between 9.00 a.m. and 11.00 a.m.

Quantitative isolation of free and bound polyribosomes

A modification of the procedure described by Ramsey and Steele (53) was used to quantitatively isolate free and bound polyribosomes. Rats were sacrificed by cervical dislocation and livers perfused via the portal vein with ice-cold 0.25M sucrose containing 1mM magnesium chloride. Livers were excised into ice-cold polyribosome buffer containing 0.25M sucrose and 3mM glutathione. Polyribosome buffer contained 50mM HEPES, 0.25M potassium chloride, 5mM magnesium chloride, pH 7.4. All subsequent operations were performed at 0-4°C. Samples of 4 g wet weight liver were minced, blotted and homogenized with 3 volumes of the same solution using eight strokes of a Potter-Elvehjem homogenizer rotating at 2,000 r.p.m. This homogenate was first centrifuged for 2 min at 700 r.p.m. (700 g_{av}) and then 12 min at 26,000 r.p.m. (135,000 g_{av}) in a Beckman L5-50 Ultracentrifuge fitted with a SW 27.1 rotor. The supernatant containing free polyribosomes was decanted and stored at 0°C. The pellet containing bound polyribosomes was suspended in 12 ml polyribosome buffer containing 0.25M sucrose and 3mM glutathione by manually rotating a loosely fitting pestle in a Potter homogenizing vessel. These homogenates were designated crude free polyribosomes and crude bound polyribosomes.

The crude bound polyribosome preparation was made to 1% in Triton X-100 and the sample manually homogenized as before. Nuclei were removed by centrifuging for 5 min at 3,500 r.p.m. (1,470 g_{av}) in a Sorvall RC2-B fitted with a SS-34 rotor. The supernatant was decanted and treated with 1.3 ml 13% sodium deoxycholate and homogenized manually as before. This solution was centrifuged for 10 min at 15,000 r.p.m. (27,000 g_{av}) in a SS-34 fotor to remove deoxycholate-insoluble material. The supernatant containing bound polyribosomes together with the crude free polyribosomes were layered over discontinuous sucrose gradients composed of 5 ml 1.38M sucrose layered over 5 ml 2M sucrose all in polyribosome buffer and centrifuged for 20 h at 49,000 r.p.m. (174,000 g_{av}) in a Beckman L5-50 Ultracentrifuge fitted with a 60Ti rotor. All material above the 2M sucrose layer was aspirated and discarded. The pellet and 2M sucrose layer were pooled and assayed for RNA content.

Polyribosome profiles

A modification of the procedures described by Bouma et al. (54) and Ramsey and Steele (53) was used to isolate free and bound polyribosomes for sedimentation analyses. Crude free polyribosomes and crude bound polyribosomes were prepared as previously described. These preparations were centrifuged for 10 min at 10,000 r.p.m. (12,000 g_{av}) in a Sorvall RC2-B fitted with a SS-34 rotor to remove nuclei and mitochondria. The supernatants were adjusted to 1% with respect to Triton X-100 and sodium deoxycholate and manually homogenized as before. These preparations were layered over discontinuous sucrose density gradients composed of 2 ml 0.5M sucrose layered over 14 ml 1M sucrose

layered over 6 ml 2.5M sucrose all in polyribosome buffer and centrifuged for 3.5 h at 26,000 r.p.m. (120,000 g_{av}) in a Beckman L5-50 Ultracentrifuge fitted with a SW 27 rotor. The polyribosomes which appeared as an opaque band in the 2.5M sucrose layer were carefully aspirated with a Pasteur pipette and concentrated overnight with concurrent dialysis against 5mM HEPES, 0.25M potassium chloride, 5mM magnesium chloride, 0.5mM EDTA, pH 7.4.

Sedimentation analyses of the polyribosomes were performed in 36 ml 0.5M to 1.5M sucrose continuous linear gradients containing polyribosome buffer and 0.5mM EDTA. Free and bound polyribosome samples of 30 A_{260} units in 1 ml volumes were prepared as described above and carefully layered on the tops of these gradients and centrifuged for 3 h at 26,000 r.p.m. (120,000 g_{av}) in a SW 27 rotor. Gradients were displaced by pumping 85% sucrose into the bottom of the tubes at a rate of 0.6 ml/min and 7 drop fractions were collected. A volume of 1 ml distilled water was added to each tube and the absorbance at 260 nm determined.

All solutions used in the isolation and sedimentation analyses of polyribosomes were sterilized by treatment with 0.05% diethyl pyrocarbonate which destroyed residual ribonuclease activity. Three cycles of heating in a boiling water bath and vigorous shaking were used to decompose unreacted diethyl pyrocarbonate. EDTA was included in the sedimentation analysis media as preliminary experiments indicated that a loss of the heavier aggregates of polyribosomes occured in its abence. It has been shown that low concentrations of EDTA prevented the

inactivation of an inhibitor of ribonuclease activity even in the presence of excess magnesium (55). It may be important with regard to this observation that EDTA is a better chelating agent for metal ions such as aluminium, calcium, cadmium, cobalt, copper, iron, manganese, mercury, nickel, lead and zinc than for magnesium. At pH 7, the stability constants of complexes of these metals with EDTA are greater than the stability constant of the EDTA-Mg^{2+} complex by factors of 10^2 to 10^{10} (56). Complexes of magnesium and EDTA are also capable of preventing lipid peroxidation which occurs during the isolation of bound polyribosomes (14). Perhaps one or both of these observations accounts for the stabilizing effect of low concentrations of EDTA on polyribosomes observed in the results reported here as well as by other authors (53,57,58).

The procedure used to identify those fractions within a polyribosome profile which were synthesizing α_1-acid glycoprotein and albumin involved the use of specific antisera prepared against these proteins. Immunoglobulin G fractions of anti-rat serum albumin and anti-rat α_1-acid glycoprotein were prepared and labeled with [^{125}I] as previously described. Free and bound polyribosomes were prepared as described above and after concentration with concurrent dialysis, 10 A_{260} units of the polyribosomes were mixed with the labeled immunoglobulin G fraction of either anti-rat serum albumin or anti-α_1-acid glycoprotein and allowed to react at 0°C for 30 min. These preparations were layered on the continuous density gradients and sedimentation analysis performed as described previously.

Alkaline ribonuclease assays

For initial studies of alkaline ribonuclease activity, high molecular weight RNA, which was used as the substrate for enzyme assays, was prepared by a method based on that previously reported by Kirby (55). Rats were sacrificed by cervical dislocation and the livers excised and immediately dropped onto solid carbon dioxide. Pooled livers were thawed and homogenized in 3 volumes distilled water in a Waring Blender for 1 min. The homogenate was stirred at room temperature while an equal volume of 90% phenol (W/V with water) was added and allowed to mix for 1 h. The mixture was centrifuged for 1 h at 2,000 r.p.m. (480 g_{av}) in a Sorvall RC2-B fitted with a SS-34 rotor. The cloudy aqueous layer was aspirated and saved. The phenol layer was washed three times with distilled water, the aqueous layer being separated by centrifuging for 45 min at 2,000 r.p.m. in the SS-34 rotor. Combined aqueous extracts were made up to 2% in potassium acetate and the RNA was precipitated by addition of 2 volumes ethanol. The precipitate was collected by centrifuging for 20 min at 2,000 r.p.m. in the SS-34 rotor and washed once with a 3:1 ethanol:water mixture. The precipitate was dissolved in 25-100 ml water and ethanol removed by extracting three times with equal volumes of ether. Ether was removed by blowing nitrogen through the aqueous solution. This preparation represented a protein-free RNA sample.

To remove polysaccharides, the above aqueous solution was mixed with an equal volume of 2.5M dipotassium phosphate, 0.05 volume of 33% phosphoric acid and an equal volume of 2-methoxyethanol. The top

layer which separated upon standing was collected using a separatory funnel and then centrifuged for 1 h at 10,000 r.p.m. (12,000 g_{av}) in the SS-34 rotor. A few drops of toluene were added to the supernatant and the preparation was exhaustively dialyzed against distilled water for 3 days. Contents of the dialysis bag were made up to 2% potassium acetate and precipitated by the addition of 2 volumes ethanol. The precipitate was collected by centrifuging for 20 min at 2,000 r.p.m. in the SS-34 rotor and washed three times with a 3:1 ethanol:water mixture. The precipitate was dried in a vacuum desiccator over sodium hydroxide.

The procedure used for the determination of alkaline ribonuclease activity was a modification of the procedure previously reported by Liu and Neuhaus (39). Male Long-Evans Hooded rats were sacrificed by cervical dislocation and livers perfused via the portal vein with ice-cold 0.15M sodium chloride and excised into ice-cold 0.15M sodium chloride. All subsequent operations other than freeze-thawing were performed at 0-4°C. Samples of 2 g wet weight liver were trimmed, minced, blotted and homogenized with 2 volumes 0.44M sucrose and stored at -20°C. Homogenates were subjected to several freeze-thaw cycles at -20°C and 37°C to inactivate hepatic ribonuclease inhibitor. Homogenates were diluted with 9 volumes distilled water and added to tubes containing 0.2 ml 0.03M barbiturate buffer, pH 7.8 and 0.1 ml 1.7mM PCMB; PCMB was added to inactivate hepatic ribonuclease inhibitor. Either 0.2 ml 1% high molecular weight RNA or 0.2 ml water was added to the tubes which were immediately mixed and placed in a 37°C water bath.

After 30 min of incubation, tubes were removed from the 37°C bath, placed on ice and 0.5 ml 1M hydrochloric acid, 76% ethanol added to stop the reaction and precipitate undigested RNA. The mixture was allowed to sit for 30 min at 4°C and insoluble material removed by centrifuging for 5 min at 12,000 r.p.m. (8,000 g_{av}) in an Eppendorf model 3200 centrifuge. The supernatant was diluted with distilled water and the absorbance at 260 nm determined. Tissue blanks, to which no exogenous substrate was added, were subtracted from samples.

Isolation of mRNA

Polyribosomes were isolated by a magnesium precipitation technique reported previously by Palmiter (60). Buffers were prepared from a stock buffer containing 0.25M Tris, 0.25M sodium chloride and 0.05M magnesium chloride, pH 7.5. Medium A contained 1 mg/ml heparin, 2% Triton X-100 and 10% stock buffer. Medium B was prepared using 4 volumes medium A and 1 volume 1M magnesium chloride. Medium C contained 0.2M sucrose and 10% stock buffer. All solutions were sterilized with diethyl pyrocarbonate as previously described. Male Long-Evans Hooded rats were sacrificed by cervical dislocation, livers were perfused *via* the portal vein with ice-cold 0.15M sodium chloride and excised into ice-cold medium A. Finely minced liver samples were mixed with 9 volumes medium A and homogenized in a Potter-Elvehjem homogenizer with several strokes of a loosely fitting pestle followed by 10-20 strokes of a tightly fitting pestle rotating at 2,000 r.p.m. This homogenate was centrifuged for 5 min at 15,000 r.p.m. (27,000 g_{av}) in a Sorvall RC2-B fitted with a SS-34 rotor. The supernatant was decanted and an equal

volume of medium B added. The preparation was incubated for 90 min at 0°C, layered over 0.25 volumes of medium C and centrifuged for 10 min at 27,000 g_{av} as before. The supernatant and the upper portion of the medium C layer were aspirated and discarded. The walls of the tubes were washed with sterilized distilled water and the water aspirated. The tubes were inverted for a few minutes and the walls wiped dry with cottom swabs. The pellet was dissolved in 10mM Tris, 0.5M potassium chloride, 0.1% SDS, pH 7.5.

Poly(A)-containing RNA was isolated by oligo(dT)-cellulose chromatography as described by Aviv and Leder (61). Application buffer contained 10mM Tris, 0.5M potassium chloride, pH 7.5; elution buffer A contained 10mM Tris, 0.1M potassium chloride, pH 7.5 and elution buffer B contained 10mM Tris, pH 7.5. All solutions were sterilized with diethyl pyrocarbonate as previously described. Oligo(dT)-cellulose was washed extensively with application buffer and a column prepared in a 10 ml sterile syringe. Polyribosomal RNA prepared by the magnesium precipitation technique or free and bound polyribosomes prepared by the procedure used for the isolation of polyribosomes for sedimentation analyses, was fractionated into poly(A)-containing and poly(A)-lacking RNA. RNA samples of 50 A_{260} units/gm dry weight oligo (dT)-cellulose in application buffer were applied to the column and washed with 10 bed volumes application buffer at a flow rate of 1 ml/min and then with 10 bed volumes elution buffer A. Poly(A)-containing RNA was eluted from the column with elution buffer B. Poly(A)-containing RNA samples were made to 2% in sterile potassium acetate and precipi-

tated overnight at 4°C by the addition of 2 volumes ethanol. The precipitates were collected by centrifuging for 5 min at 12,000 r.p.m. (8,000 g_{av}) in an Eppendorf model 3200 centrifuge, washed three times with a 3:1 ethanol:water mixture, dissolved in sterile distilled water, freeze-dried and stored at -20°C. The oligo(dT)-cellulose was regenerated for repeated use by elution with 5 volumes 0.1M potassium hydroxide, then 20 volumes distilled water and 10 volumes ethanol. Oligo(dT)-cellulose was dried in a vacuum desiccator over sodium hydroxide and stored at -20°C.

In vitro translations

In vitro translation experiments were performed using materials supplied by New England Nuclear Corp. in a Reticulocyte Lysate Translation Kit. A premix was prepared of [^{3}H]-leucine, translation cocktail, 1M potassium acetate and stock magnesium acetate in volume ratios of 34:8:8:1, respectively. Preparations of poly(A)-containing RNA dissolved in 5 µℓ water provided by New England Nuclear Corp. were added to 25 µℓ of the premix, vortexed and centrifuged for 1 min at 12,000 r.p.m. (8,000 g_{av}) in an Eppendorf model 3200 centrifuge. A volume of 20 µℓ of reticulocyte lysate was added to the tubes which were vortexed and centrifuged as above and then placed immediately in a 37°C water bath. At appropriate times of incubation, aliquots were withdrawn for TCA precipitation. At the end of the incubation period, the tubes were placed on ice,diluted with distilled water and subjected to electrophoretic analyses or immunoprecipitation as previously described.

TCA precipitation was performed by spotting 5 µℓ aliquots on 2 cm x 2 cm squares of Whatman 3MM paper which were immersed in a beaker of 10% TCA. The TCA was boiled for 10 min and ice added to the TCA. The TCA was decanted and the papers were washed twice with distilled water, ethanol and acetone; air-dried and the radioactivity determined as previously described.

Results

Immunochemical analyses of purity

Several experiments reported in this thesis utilized antisera prepared against rat serum albumin and rat α_1-acid glycoprotein. The purity of the antisera used in these experiments is important in the interpretation of the results obtained from these experiments and as such the preparations were analyzed to determine if they were monospecific. The results of double diffusion analysis of the preparation of α_1-acid glycoprotein used to prepare antiserum and the anti-α_1-acid glycoprotein are shown in Figures 6a and 6b, respectively; Figure 6c shows the results of immunoelectrophoresis using this antiserum. The results of double diffusion analysis and immunoelectrophoresis of anti-rat serum albumin previously prepared in this laboratory are shown in Figures 7a and 7b, respectively.

As previously mentioned, α_1-acid glycoprotein prepared for immunization of rabbits was considered to be essentially pure by virtue of the presence of single precipitin lines formed against anti-α_1-acid glycoprotein and anti-whole rat serum. Anti-α_1-acid glycoprotein and anti-rat serum albumin formed single precipitin lines against their respective antigens and rat serum in double diffusion analysis and immunoelectrophoresis experiments and as such were considered to be monospecific.

Quantitative analyses of bound and free polyribosomes

It has been reported that acute inflammation (33), iron administration (10,41) and zinc administration (42) result in changes

Fig. 6. Immunochemical Analyses of α_1-acid glycoprotein and Anti-α_1-acid glycoprotein

(a) Double diffusion analysis of α_1-acid glycoprotein

top well	α_1-acid glycoprotein
bottom right well	anti-α_1-acid glycoprotein
bottom left well	anti-whole rat serum

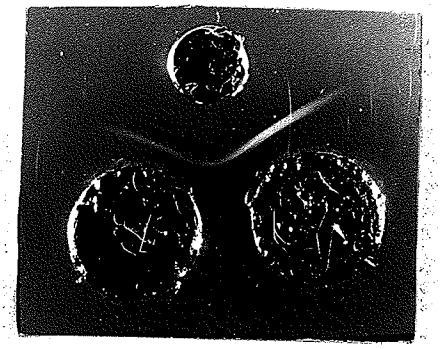
(b) Double diffusion analysis of anti-α_1-acid glycoprotein

top well	anti-α_1-acid glycoprotein
bottom right well	whole rat serum
bottom left well	α_1-acid glycoprotein

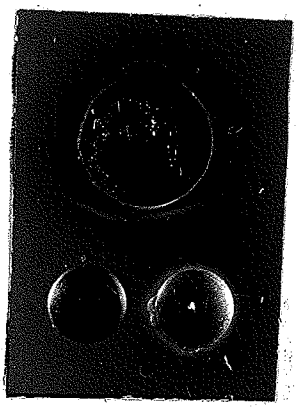
(c) Immunoelectrophoretic analysis of anti-α_1-acid glycoprotein

well	whole rat serum
trough	anti-α_1-acid glycoprotein

(a)



(b)



(c)

origin

Fig. 7. Immunochemical Analyses of Anti-Rat Serum Albumin

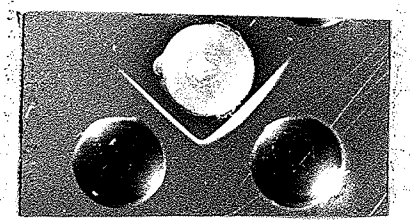
(a) Double diffusion analysis of anti-rat serum albumin

top well	anti-rat serum albumin
bottom right well	rat serum albumin
bottom left well	whole rat serum

(b) Immunoelectrophoretic analysis of anti-rat serum albumin

well	whole rat serum
trough	anti-rat serum albumin

(a)



(b)

origin

in protein synthesis in rat liver. As previously mentioned, acute inflammation induces increased synthesis of secretory proteins, such as α_1-acid glycoprotein, which are synthesized primarily on bound polyribosomes. Iron administration induces the synthesis of ferritin which is an intracellular protein believed to be synthesized primarily on free polyribosomes. Zinc administration induces the synthesis of metallothionein, which is an intracellular protein.

An explanation of the segregation of the synthesis of secretory and intracellular proteins into bound and free polyribosomes has been proposed by Blobel and Dobberstein in their "signal" hypothesis (15). A simplistic interpretation of this model could lead to the prediction that qualitative changes in the levels of synthesis of secretory proteins or intracellular proteins would lead to a redistribution of polyribosomes between the bound and free states. This prediction relies primarily on the assumptions that bound and free polyribosomes share common precursor pools of ribosomal subunits, tRNAs, elongation factors and termination factors and that the only significant difference in the precursors to the polyribosome complexes is in the mRNA transcripts which will be associated with the polyribosomes. Increases in the levels of synthesis of secretory proteins made primarily on bound polyribosomes should bring about a shift of polyribosomes into the bound class. Increases in the levels of synthesis of proteins made primarily on free polyribosomes should bring about a shift of polyribosomes into the free class. Inherent in this interpretation is the assumption that the level of synthesis of proteins is dependent upon the levels of mRNA

transcripts coding for the protein being synthesized.

In order to determine if this interpretation represents a realistic view of the segregation of polyribosomes and protein synthesis, levels of bound and free polyribosomes were assayed at various times after induction of experimental inflammation, iron administration and zinc administration. The results of the experiments with inflammation, iron administration and zinc administration are shown in Tables 4,5 and 6, respectively, and presented graphically in Figures 8,9 and 10,respectively. The results of the assays on animals which were grouped together within an individual experiment are indicated as such by letter groupings in these tables.

The results shown in Table 4 and Figure 8 indicate that inflammation results in a significant shift of the polyribosomes into the bound class. This shift is reproducible and maximal about 16 h after inflammation. The change in the ratio of bound to free polyribosomes seems to be preceded by an overall increase in the amount of RNA recovered as polyribosomes as is shown by the increase in both bound and free polyribosomes prior to 16 h after inflammation.

It is difficult to assess the consequences of iron administration on the distribution of polyribosomes between the bound and free classes from the data presented in Table 5 and Figure 9. An increase was not observed in the amount of RNA recovered as free polyribosomes relative to the amount of bound polyribosomes. An apparent loss of free polyribosomes at early times after treatment occurs, but there seems to be a large variation in the distribution of polyribosomes at later times

Table 4

Effect of Inflammation on Polyribosome Populations in Rat Liver

Experiment	Time*	Total liver RNA content**	RNA recovered as polyribosomes**		Bound/Free polyribosome ratio
			Bound	Free	
A	0	NA	1.97	1.80	1.09
	0	NA	2.15	2.13	1.01
	16	NA	2.89	1.62	1.78
B	0	NA	2.73	2.09	1.31
	16	NA	3.84	1.49	2.58
C	0	5.79	2.25	1.91	1.18
	16	4.44	2.91	1.44	2.02
D	0	6.52	2.42	2.18	1.11
	4	6.34	2.50	2.07	1.21
E	0	6.53	3.15	2.64	1.19
	8	6.27	3.42	2.78	1.23
	24	7.05	4.02	2.26	1.78
	40	8.01	3.34	2.16	1.55
F	0	6.36	2.54	2.13	1.19
	12	6.78	3.34	2.66	1.27

* Time in h after subcutaneous injection of turpentine oil.
** Expressed as mg RNA/g liver.
NA - Not assayed.

Fig. 8. Effect of Inflammation on Polyribosome Pool Sizes

Pool sizes are expressed as percentages of the control values for the individual experiment.

(a) Free polyribosomes

(b) Bound polyribosomes

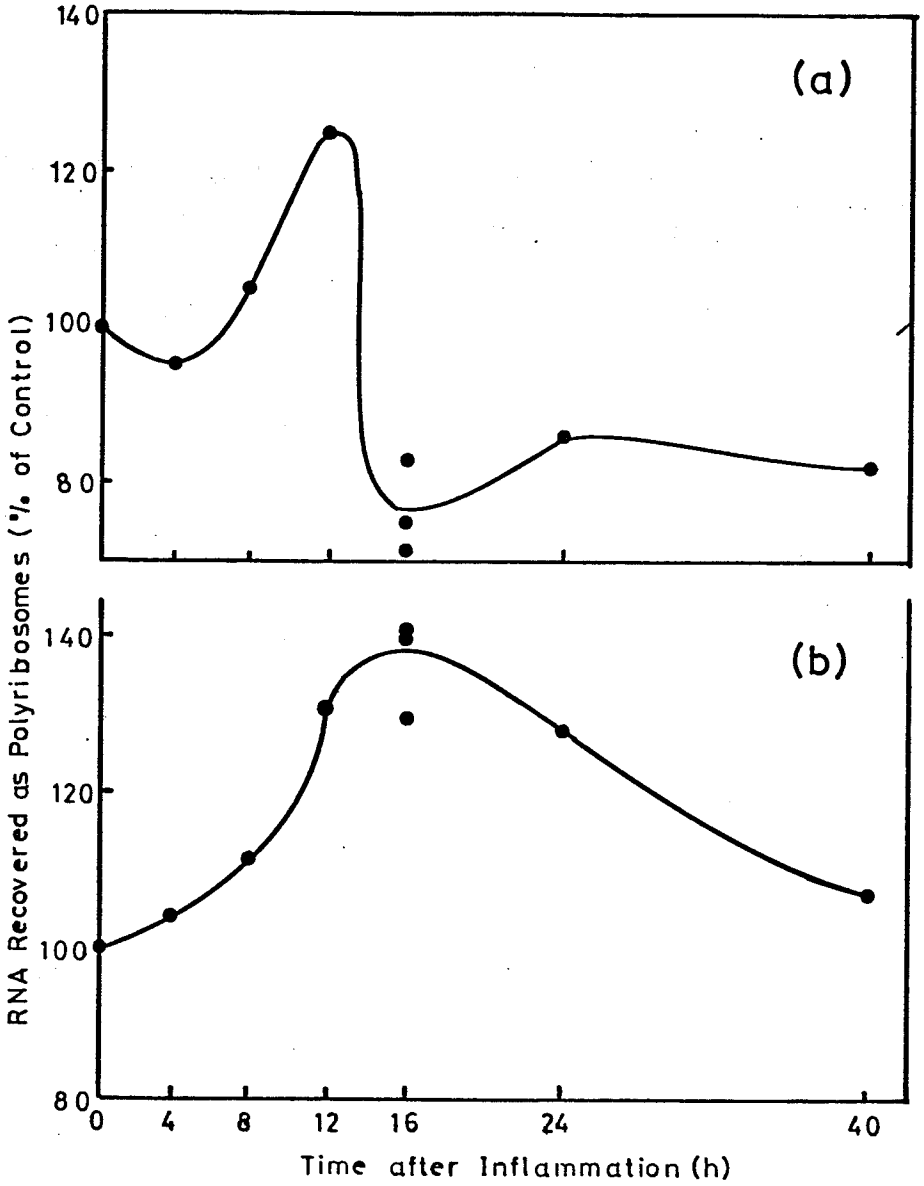

(a)
(b)
140
120
100
80
RNA Recovered as Polyribosomes (% of Control)
0
4
8
12
16
24
40
Time after Inflammation (h)

Table 5

Effect of Iron Administration on Polyribosome Populations in Rat Liver

Experiment	Time*	Total liver RNA content**	RNA recovered as polyribosomes**		Bound/Free polyribosome ratio
			Bound	Free	
A	0	5.79	2.25	1.91	1.18
	14	5.05	2.24	2.12	1.05
B	0	7.87	2.74	2.58	1.06
	10	7.71	2.84	2.71	1.05
	20	8.05	2.58	2.40	1.08
C	0	10.78	3.56	3.88	0.92
	2	9.28	3.07	3.54	0.84
	4	9.82	3.64	3.26	1.12
	4	10.06	3.99	3.22	1.24
D	0	8.41	2.98	2.63	1.13
	5	8.15	2.98	2.59	1.15
	16	8.92	3.42	2.58	1.33
	24	7.85	3.17	2.06	1.54
E	0	NA	1.75	2.12	0.83
	24	NA	1.94	2.22	0.87
	48	NA	1.91	2.13	0.90
	72	NA	2.15	1.96	1.10

* Time in h after intraperitoneal injection of iron.
** Expressed as mg RNA/g liver.
NA - Not assayed.

Fig. 9. Effect of Iron Administration on Polyribosome Pool Sizes

Pool sizes are expressed as percentages of the control values for the individual experiments.

(a) Free polyribosomes

(b) Bound polyribosomes

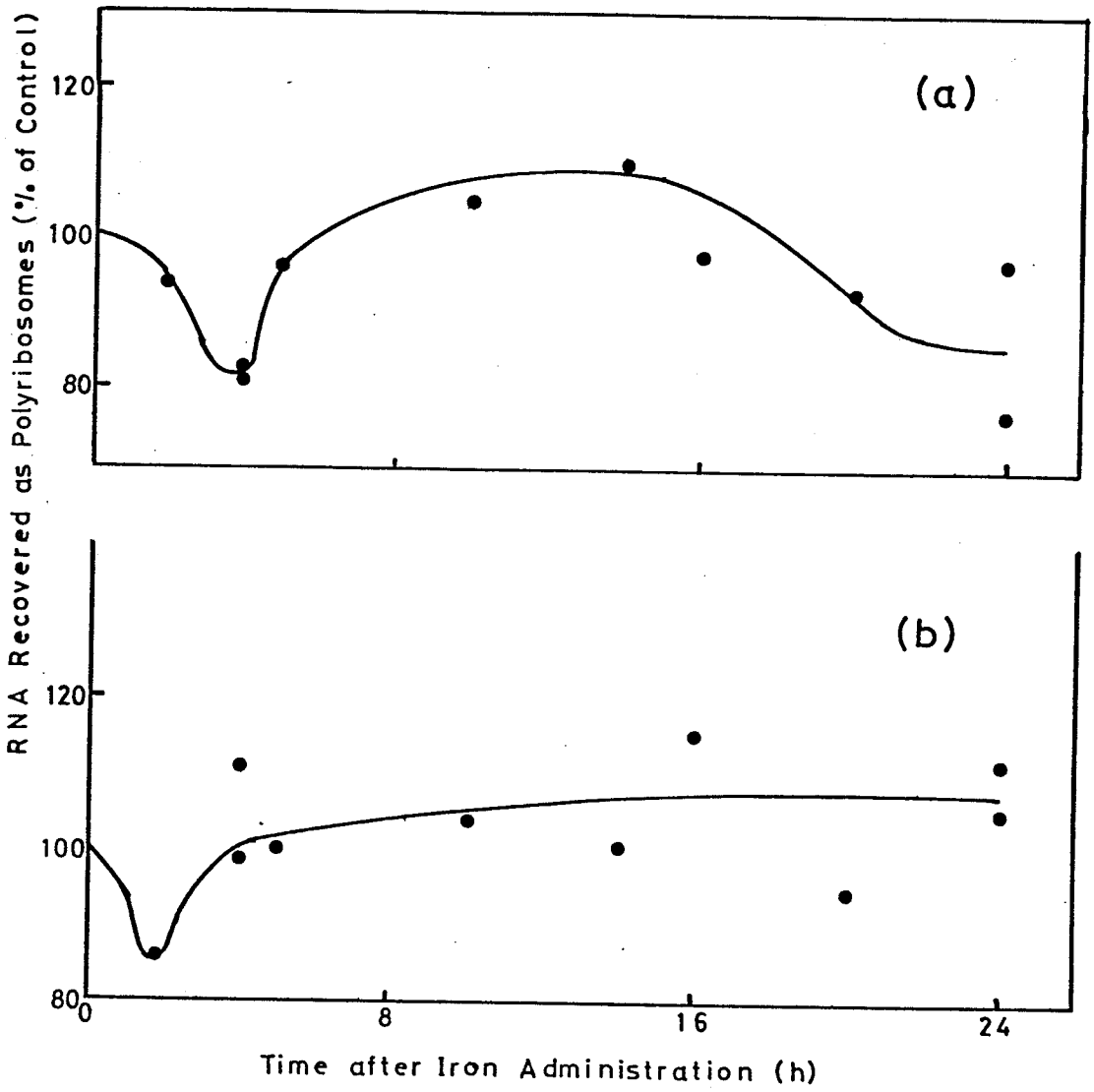

(a)
(b)
120
100
80
120
100
80
0
8
16
24
RNA Recovered as Polyribosomes (% of Control)
Time after Iron Administration (h)

Table 6

Effect of Zinc Administration on Polyribosome Populations in Rat Liver

Experiment	Time*	Total liver RNA content**	RNA recovered as polyribosomes**		Bound/free polyribosome ratio
			Bound	Free	
A	0	7.10	2.53	2.35	1.08
	0	6.98	2.56	2.43	1.03
	14	6.92	3.48	2.80	1.24
	18	6.63	3.07	2.64	1.16
B	0	7.41	2.40	2.84	0.97
	18	7.14	2.65	2.41	1.10
	24	7.07	2.68	2.46	1.09
C	0	NA	3.58	3.30	1.08
	4	NA	3.59	3.10	1.09

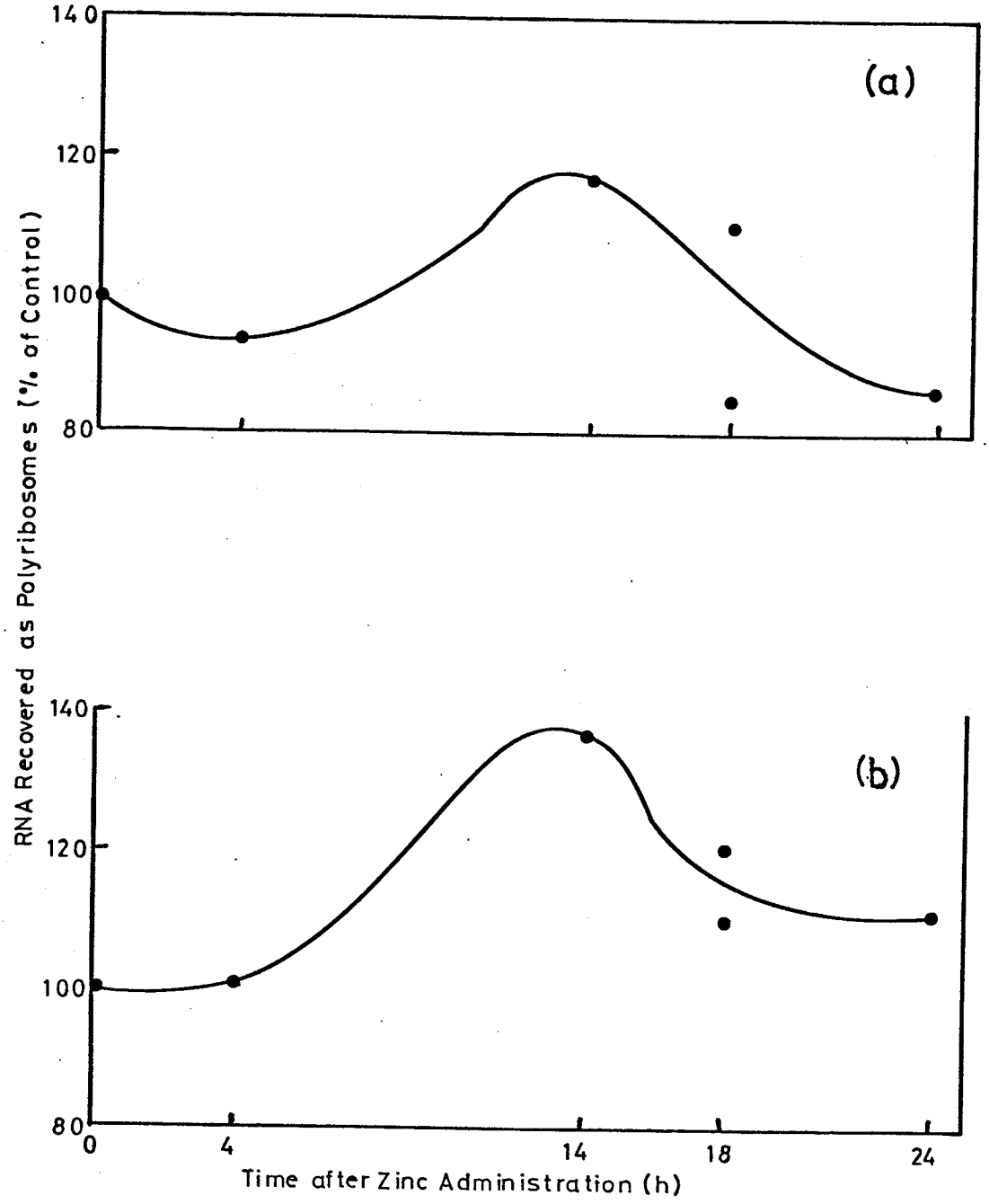
* Time in h after intraperitoneal injection of zinc.
** Expressed as mg RNA/g liver.
NA - Not assayed.

Fig. 10. Effect of Zinc Administration on Polyribosome Pool Sizes

Pool sizes are expressed as percentages of the control values for the individual experiments.

(a) Free polyribosomes

(b) Bound polyribosomes


140
120
100
80
(a)
RNA Recovered as Polyribosomes (% of Control)
140
120
100
80
(b)
0
4
14
18
24
Time after Zinc Administration (h)

after iron administration.

Zinc administration results in an increase in the amount of RNA recovered as polyribosomes as is shown in Table 6 and Figure 10. It would appear that the bound polyribosome pool enlarges to a greater extent after treatment than does the free polyribosome pool.

It may also be significant that inflammation, iron administration and zinc administration all result in a decrease in the hepatic free polyribosome content 4 h after treatment.

The three physiological states studied in this thesis all resulted in detectable changes in hepatic bound and free polyribosome levels. The mechanisms by which these changes occur are not evident from the results presented here. Interpretation of these results should not neglect the fact that, unlike acute inflammation, induction of qualitative changes in hepatic protein synthesis by iron and zinc administration involves a direct challenge of the liver with the stimulating factor rather than mediation of the response by endogenous factors such as hormones. This may account for the apparent lack of reproducibility in the alterations in the polyribosome levels after iron administration.

Sedimentation analyses of bound and free polyribosomes

As previously mentioned, inflammation has been reported to induce a shift of polyribosomes into heavier aggregates (39). In view of the significant difference between the bound to free polyribosome ratio in a 16 h inflamed animal as compared to a control animal, bound and free polyribosomes were isolated as previously described and subjected to

sedimentation analyses. The results of sedimentation analyses of bound polyribosomes from control and 16 h inflamed animals are shown in Figures 11a and 11b, respectively. The free polyribosome profiles from control and 16 h inflamed animals are shown in Figures 12a and 12b, respectively.

The location of monoribosomes within these polyribosome profiles were estimated as described by Griffith (62). A value of Z_0 for the rotor and gradient was calculated from the formula

$$Z_0 = \frac{Z_1 r_2 - Z_2 r_1}{r_2 - r_1} \qquad [1]$$

Where Z_1 = minimum % W/V of sucrose solution (16.1%)

Z_2 = maximum % W/V of sucrose solution (43.2%)

r_1 = meniscus of gradient, i.e. minimum radial distance from centrifugal axis (8.0 cm)

r_2 = bottom of tube, i.e. maximum radial distance from centrifugal axis (16.1 cm)

Z_0 = solute concentration corresponding to extrapolation of a linear gradient to zero radius

A value for the Time Integral change (ΔI) for the sedimentation conditions used in these experiments is calculated from the formula

$$\Delta I = s_{20,w}\omega^2 t \qquad [2]$$

Where $\omega^2 = (0.10472 \times \text{r.p.m.})^2 = 7.413 \times 10^6 \text{ sec}^{-2}$

$t = 3.5 \text{ h} = 12{,}600 \text{ sec}$

$s_{20,w} = 80 \times 10^{-13}$ sec (or 80S)

$\Delta I = 0.74723$

Fig. 11. Effect of Inflammation on Bound Polyribosome Profiles

The position of monoribosomes in these profiles is indicated by an arrow.

(a) Bound polyribosome profile from a control animal

(b) Bound polyribosome profile from a 16 h inflamed animal

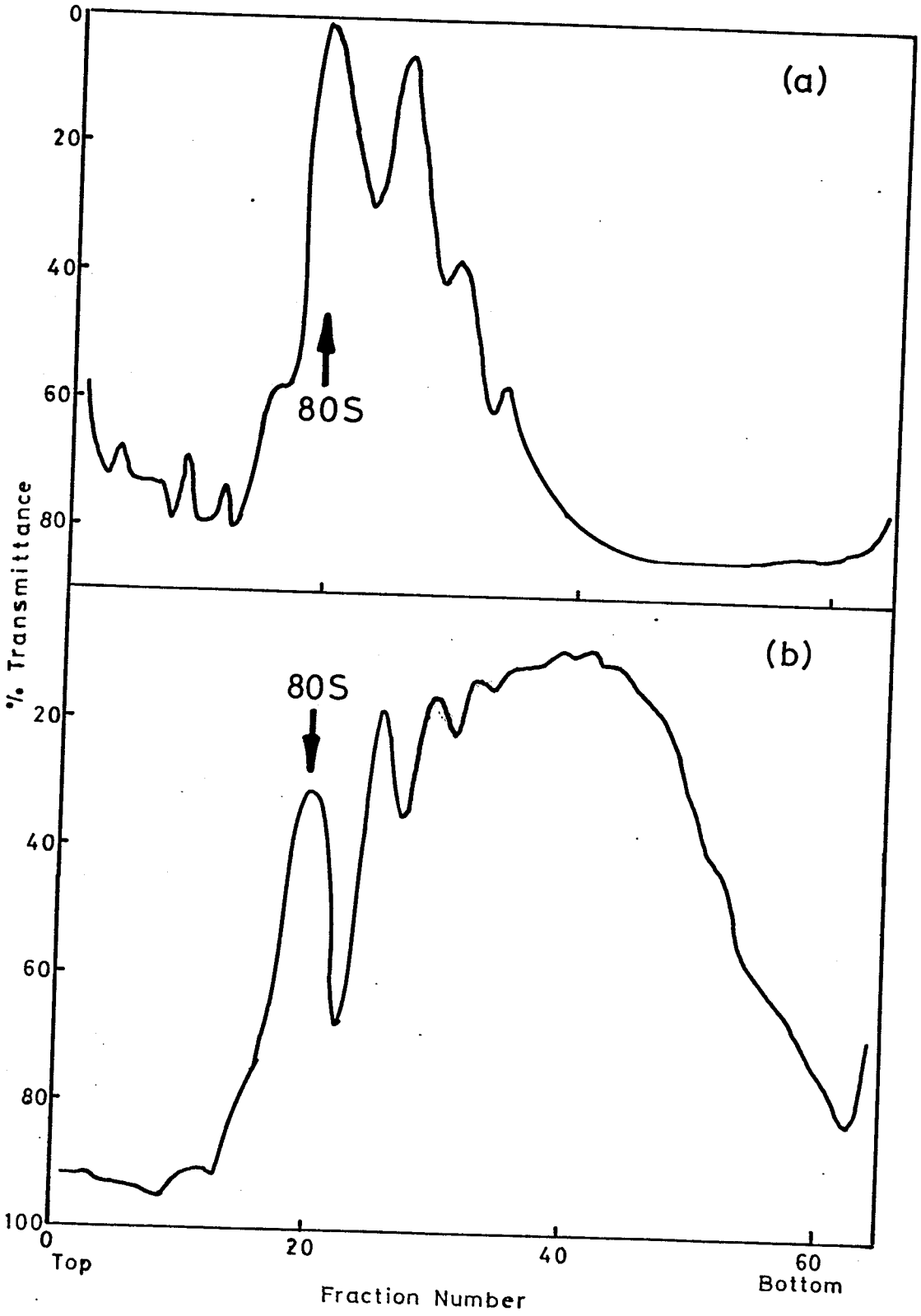
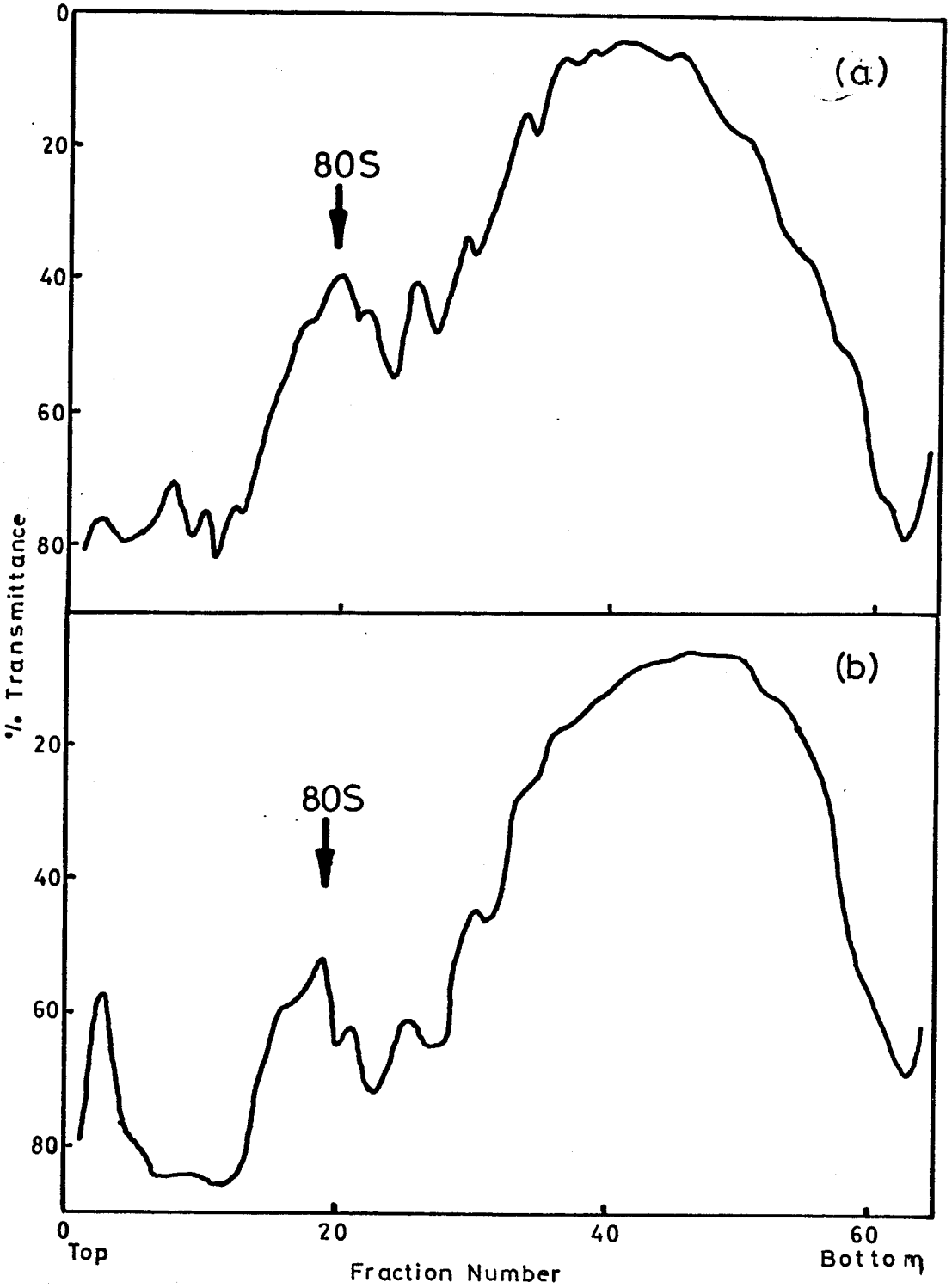

(a)
80S
(b)
80S
% Transmittance
0
20
40
60
80
100
Top
Bottom
Fraction Number

Fig. 12. Effect of Inflammation on Free Polyribosome Profiles

The position of monoribosomes in these profiles is indicated by an arrow.

(a) Free polyribosome profile from a control animal

(b) Free polyribosome profile from a 16 h inflamed animal


(a)
80S
(b)
80S
% Transmittance
0
20
40
60
80
Top
Fraction Number
Bottom

Assuming a particle density of 1.50 g/cc for polyribosomes and using the Z_0 value calculated with formula [1], the Time Integral for a 16% W/V sucrose solution (I(16%)) is tabulated (62) as 1.2037. The Time Integral for a 80S monoribosome under these conditions should be 1.9509, which is the sum of the Time Integral change (ΔI) and I(16%). This corresponds to a sucrose concentration of 21% W/V (62). The position of the 80S monoribosome in the polyribosome profiles is indicated in Figures 11-16 assuming that it does band at 21% W/V sucrose within the linear gradient.

It was also of interest to know which polyribosome aggregates within the polyribosome profiles were involved in the synthesis of the acute phase reactant α_1-acid glycoprotein. With this in mind, polyribosome preparations were reacted with [^{125}I]-immunoglobulin G fraction prepared from antiserum to α_1-acid glycoprotein as previously described. The same procedure was used with [^{125}I]-immunoglobulin G fraction of anti-rat serum albumin to identify those polyribosome aggregates with nascent chains of albumin and provide a basis of comparison for the experiments with anti-α_1-acid glycoprotein.

The results of sedimentation analyses of polyribosome preparations reacted with [^{125}I]-immunoglobulin G fractions of either anti-α_1-acid glycoprotein or anti-rat serum albumin are shown in Figures 13-16.

The polyribosome profiles presented in Figures 11a and 11b indicate that a marked increase in the heavier aggregates of bound polyribosomes is induced by inflammation. The polyribosome profile of bound polyribosomes prepared from control animals is quite striking in that the

Fig. 13. Immunological Localization of Bound Polyribosome Aggregates Synthesizing α_1-acid glycoprotein

(a) Bound polyribosomes prepared from control animals and reacted with [^{125}I]-immunoglobulin G fraction of anti-α_1-acid glycoprotein prior to sedimentation.

(b) Bound polyribosomes prepared from 16 h inflamed animals and reacted with [^{125}I]-immunoglobulin G fraction of anti-α_1-acid glycoprotein prior to sedimentation.

The position of monoribosomes in these polyribosome profiles is indicated by an arrow.

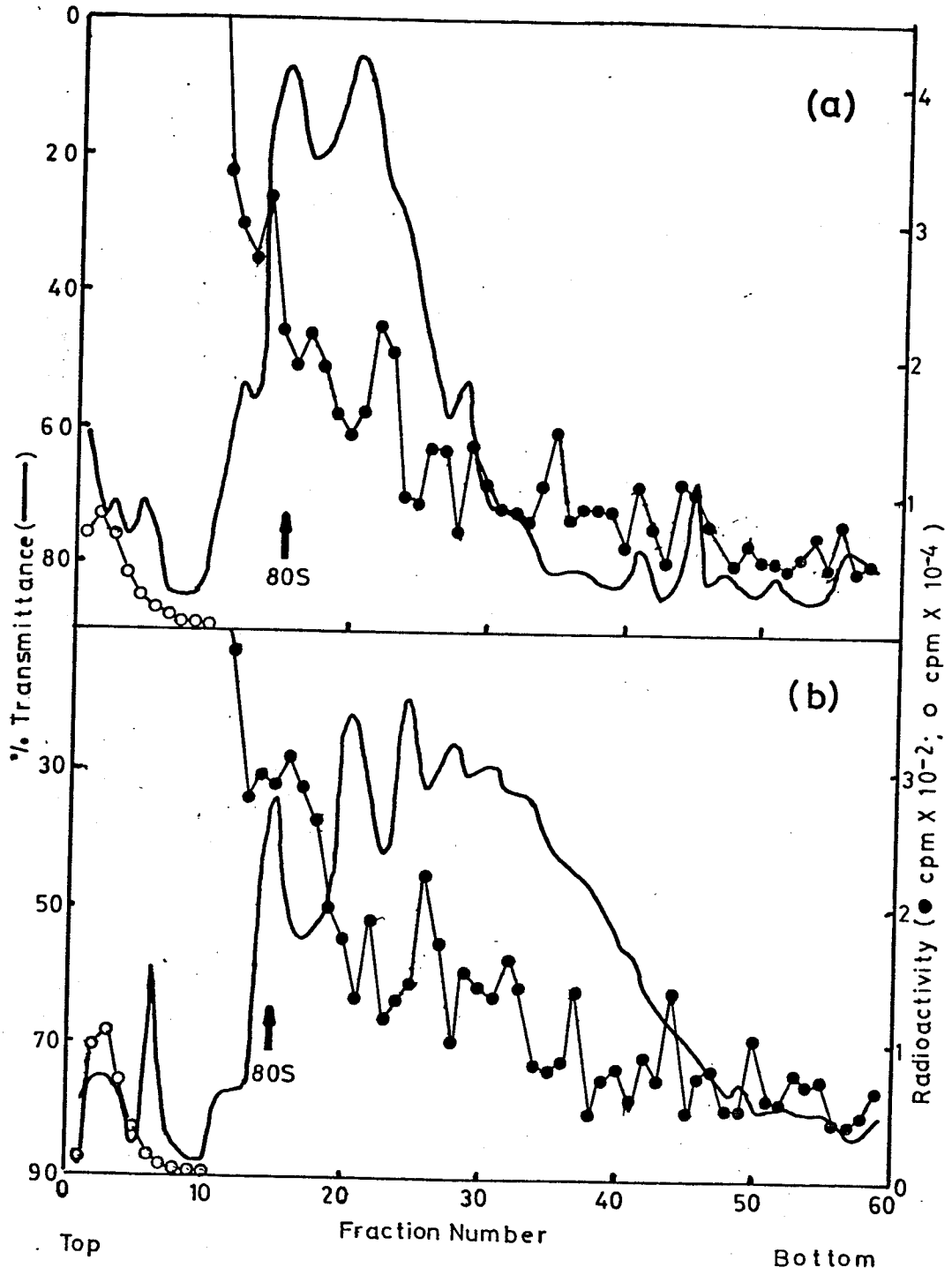

(a)
(b)
80S
80S
% Transmittance (——)
Radioactivity (● cpm X 10⁻²; o cpm X 10⁻⁴)
Fraction Number
Top
Bottom
0
20
40
60
80
30
50
70
90
10
30
40
50
60
1
2
3
4

Fig. 14. Immunological Localization of Free Polyribosome Aggregates Synthesizing α_1-acid glycoprotein

Free polyribosomes were prepared from control animals and reacted with [^{125}I]-immunoglobulin G fraction of anti-α_1-acid glycoprotein prior to sedimentation.

The position of monoribosomes in these polyribosome profiles is indicated by an arrow.

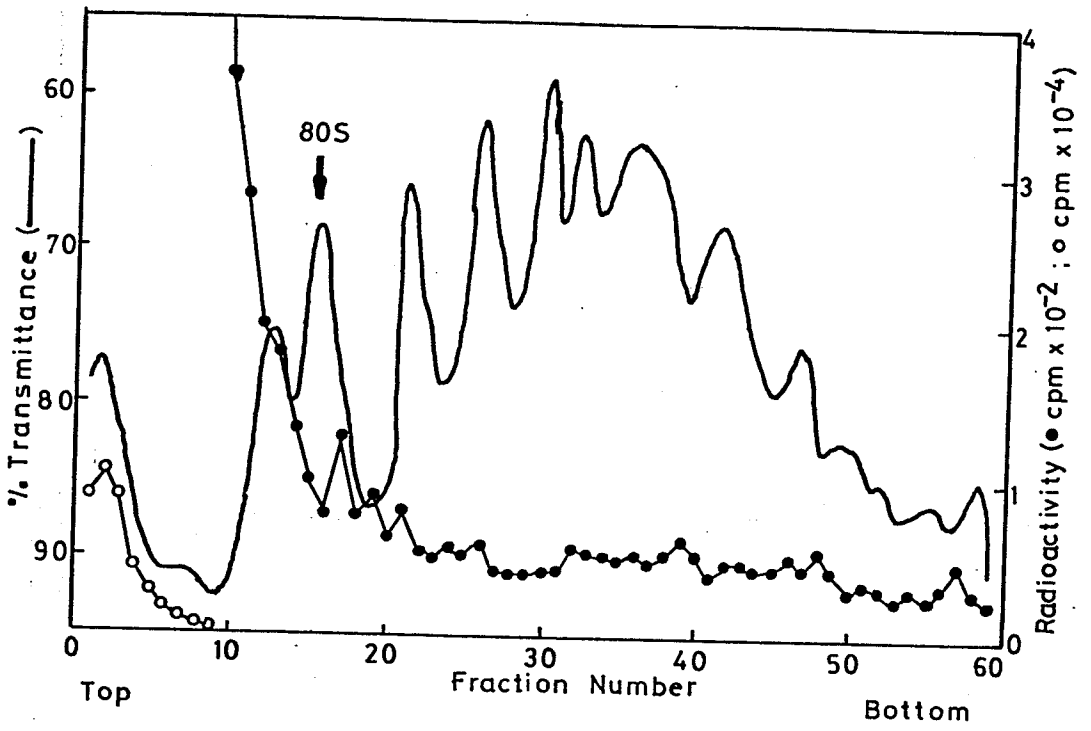

80S
% Transmittance (——)
Radioactivity (• cpm x 10^{-2} ; o cpm x 10^{-4})
Fraction Number
Top
Bottom
60
70
80
90
0
10
20
30
40
50
60
1
2
3
4

Fig. 15. Immunochemical Localization of Bound Polyribosome Aggregates Synthesizing Albumin

Bound polyribosomes were prepared from control animals and reacted with [^{125}I]-immunoglobulin G fraction of anti-rat serum albumin prior to sedimentation.

The position of monoribosomes in these polyribosome profiles is indicated by an arrow.

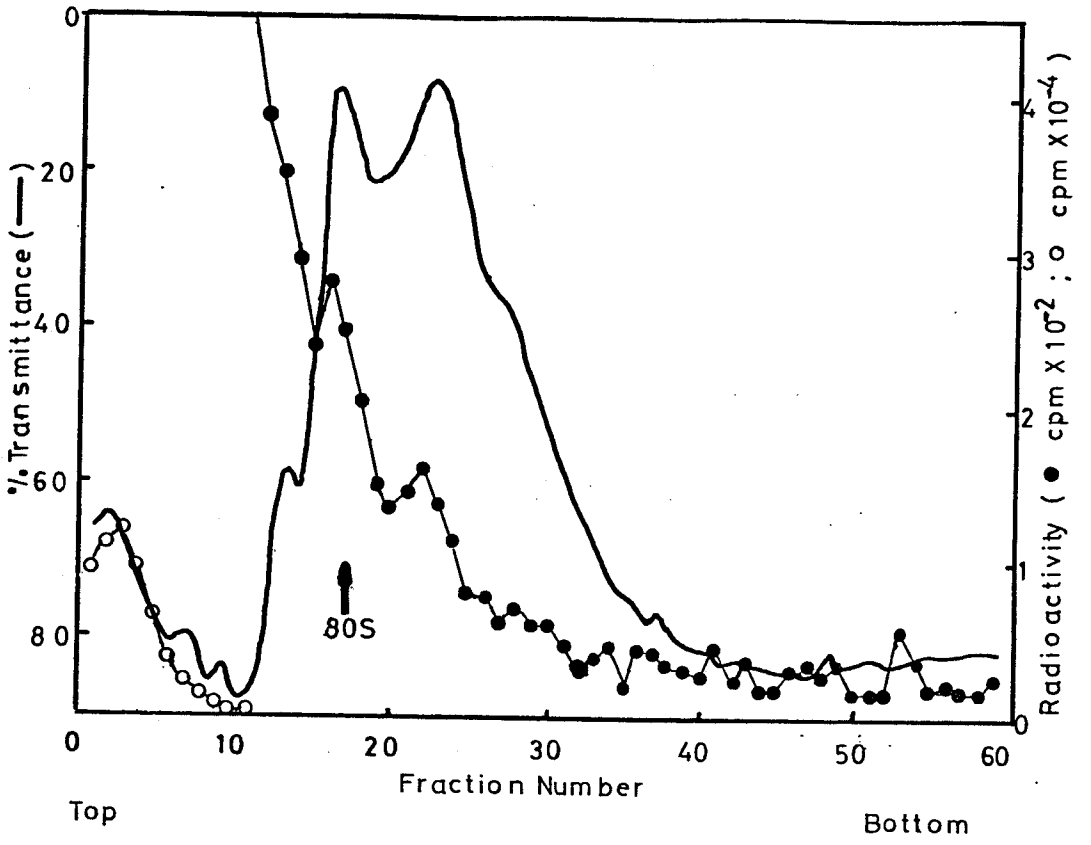
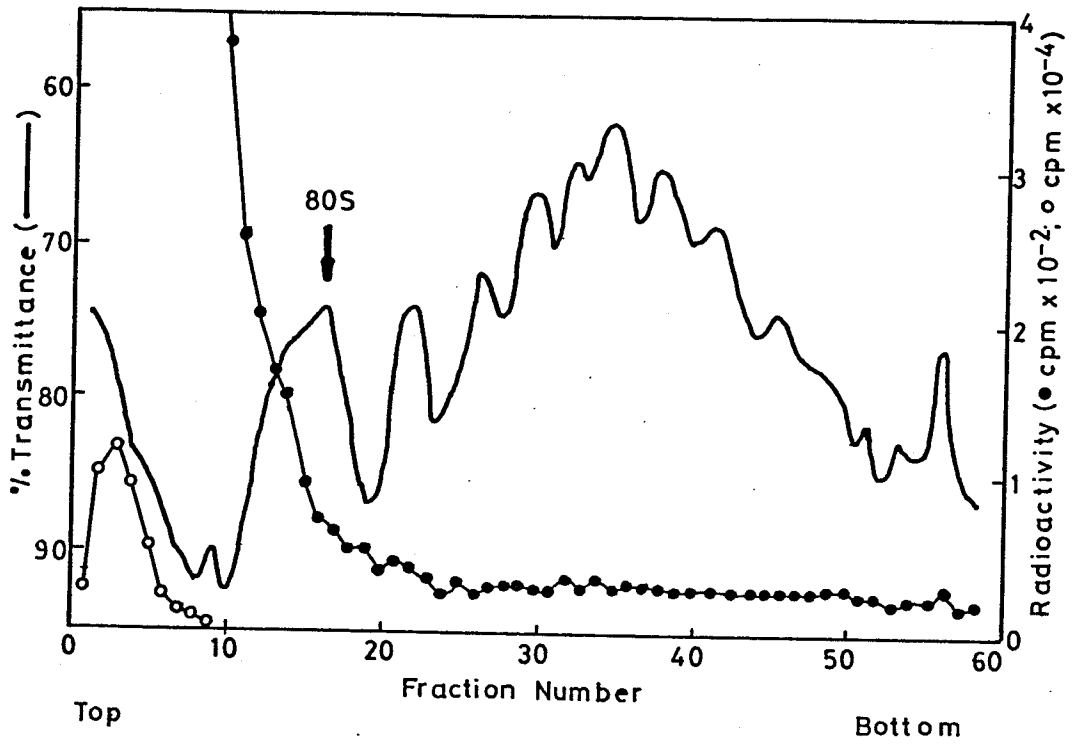

0
20
40
60
80
% Transmittance (—)
80S
0
10
20
30
40
50
60
Fraction Number
Top
Bottom
Radioactivity (● cpm X10⁻² ; o cpm X10⁻⁴)
0
1
2
3
4

Fig. 16. Immunochemical Localization of Free Polyribosome Aggregates Synthesizing Albumin

Free polyribosomes were prepared from 16 h inflamed animals and reacted with [^{125}I]-immunoglobulin G fraction of anti-rat serum albumin prior to sedimentation.

The position of monoribosomes in these polyribosome profiles is indicated by an arrow.


% Transmittance (——)
60
70
80
90
80S
0
10
20
30
40
50
60
Fraction Number
Top
Bottom
Radioactivity (• cpm x 10⁻²; o cpm x10⁻⁴)
4
3
2
1
0

preparation seems to be composed almost entirely of material sedimenting either as mono- or di-ribosomes.

There does not seem to be a sizable difference betweeen the free polyribosome profiles prepared from control and 16 h inflamed animals as is shown in Figures 12a and 12b. However, there does seem to be a slight shift of the profile to heavier aggregates of polyribosomes in the 16 h inflamed rat as compared to the control rat.

It is noteworthy that peaks of radioactivity in the polyribosome profiles shown in Figures 13-16 do not align with peaks of material which absorb light at 260 nm. The profiles reported here show a significant degree of binding of [^{125}I]-immunoglobulin G to material within the polyribosome profile, however, the nature of the complexes formed by this binding has not been identified. The observation that very little radioactivity appears at the bottom of the profiles would seem to rule out the possibility of formation of large amounts of insoluble immune complexes under the conditions utilized in this study. Examination of Figures 14 and 16 seems to indicate that a significant amount of nonspecific binding of [^{125}I]-immunoglobulin to material in the polyribosome preparation does not occur. In consideration of these observations, the peaks of radioactivity in fractions 15-50 of the profiles would seem to be attributable primarily to the formation of soluble immune complexes between immunoglobulin G and the nascent chains of α_1-acid glycoprotein and albumin. The nonalignment of radioactivity and A_{260} peaks is probably accounted for by an increase in sedimentation coefficients of polyribosome aggregates upon binding of

immunoglobulin G.

Examination of the results shown in Figures 13a and 13b indicates that anti-α_1-acid glycoprotein binds to most of the bound polyribosome aggregates. However, there does seem to be a greater degree of binding to material which sediments as smaller aggregates of polyribosomes. A close examination of the two profiles also suggests that inflammation results in an increased binding of immunoglobulin, especially to material which sediments as smaller aggregates. This binding is difficult to assess quantitatively due to the large amount of unreacted immunoglobulin which remains at the top of the profile.

Comparison of Figures 13a, 13b and 14 indicates that a greater degree of binding of the immunoglobulin G fraction of anti-α_1-acid glycoprotein occurs to bound polyribosomes as compared to free polyribosomes. This observation suggests that α_1-acid glycoprotein is synthesized primarily on bound polyribosomes, which is a conclusion drawn from an independent study from this laboratory (3).

The results shown in Figure 15 suggest that the lighter aggregates of bound polyribosomes also contain nascent chains of albumin. Comparison of the profiles shown in Figures 15 and 16 leads to the conclusion that the synthesis of albumin occurs primarily on bound polyribosomes, which agrees with the results of reports summarized in Table 1.

Acute inflammation induced by subcutaneous turpentine injection results in an increase in heavier aggregates of polyribosomes. This effect is seen primarily in the pool of bound polyribosomes. Acute

inflammation also seems to result in an increased content of the acute phase reactant α_1-acid glycoprotein associated with the bound polyribosomes although this increase is not seen in the heavier aggregates of polyribosomes resulting from this stress situation. From the results presented here, it is difficult to assess whether the stress-related shift of polyribosomes to heavier states of aggregation is related specifically to the metabolism of acute phase reactants such as α_1-acid glycoprotein, or, generally to other biochemical and ultrastructural changes which occur during inflammation.

Alkaline ribonuclease activity

Liu and Neuhaus (39) have reported similar effects of laparotomy to the effects of inflammation reported here on the polyribosome pools. The study of Liu and Neuhaus also reported a decrease in rat liver alkaline ribonuclease activity which paralleled the stress-related increase in heavier aggregates of hepatic polyribosomes. The proportion of polyribosomes recovered as heavier aggregates was maximal at 18 h after injury. This time also exhibited the minimum alkaline ribonuclease activity. Liu and Neuhaus argued that as stress-related RNA synthesis occurs prior to and not during the formation of heavier aggregates of polyribosomes, increased aggregation 18 h after injury is attributable to increased stability of polyribosomes rather than elevated RNA levels alone. These authors suggested that the increased stability of polyribosomes following laparotomy resulted from decreased alkaline ribonuclease activity.

The increase in the bound to free polyribosome ratio reported in

this thesis seems to result from an increase in bound polyribosomes recovered as heavier aggregates. In order to determine if changes in alkaline ribonuclease activity could account for the changes in bound to free polyribosome ratios reported here, alkaline ribonuclease activity was assayed in rats suffering from acute inflammation, iron administration and zinc administration as is shown in Table 7.

Rats suffering from inflammation for 18 h had a lower activity of alkaline ribonuclease than control rats. Iron administration did not markedly change alkaline ribonuclease activity although the ratio of bound to free polyribosomes was relatively constant in those animals tested. There was a significant increase in rat liver alkaline ribonuclease activity at early times after administration of zinc.

A decrease in the level of alkaline ribonuclease activity was observed at a time after inflammation which showed a reproducible increase in the amount of bound polyribosomes. It may also be noteworthy that basal levels of alkaline ribonuclease activity varied as did basal levels of bound and free polyribosome pool sizes in rats sacrificed on different days. Both of these observations give credence to the suggestion that the size of the bound polyribosome pool is related to decreases in alkaline ribonuclease activity.

At a time 72 h after administration of iron to rats, there was a slight increase in the level of alkaline ribonuclease activity, increases in both the bound and free polyribosome pool sizes and an increase in the ratio of bound to free polyribosomes. The activity of alkaline ribonuclease increased roughly 50% by 4 h after administration

Table 7

Effects of Inflammation, Iron Administration and Zinc Administration on Rat Liver Alkaline Ribonuclease

Time after treatment (h)	Total alkaline ribonuclease activity*
Inflammation	
0	0.056
18	0.036
Iron Administration	
0	0.048
24	0.043
48	0.046
72	0.058
Zinc Administration	
0	0.062
2	0.073
4	0.097
18	0.058

* Samples for the iron administration experiment were taken from rats used in experiment E described in Table 5. Other values represent the mean of three liver samples. All assays were performed in triplicate and the reproducibility was within ±10%. Units of enzyme activity are ΔA_{260}/min/mg protein.

of zinc while very little change was observed in either the size of the polyribosome pools or the bound to free polyribosome ratio. These observations argue against the suggestion that alkaline ribonuclease activity is involved in determining polyribosome pool sizes.

It would appear that, if the physiological states resulting from acute inflammation, iron administration and zinc administration share common factors important in determining the distribution of polyribosomes between the two classes, alkaline ribonuclease is not one of these factors.

Effect of inflammation on mRNA metabolism

Chandler and Neuhaus (38) reported experiments using phenol methods of extraction of RNA which suggested that laparotomy resulted in stimulated synthesis of both rRNA and mRNA which is maximal about 8 h after injury. These authors also reported that administration of actinomycin D, prior to but not after 8 h following laparotomy, blocked stimulation of RNA synthesis and seromucoid synthesis. It may be inferred, therefore, that injury stimulates the synthesis of hepatic RNA specific for the increase in synthesis of serum glycoproteins. However, these studies should be interpreted with caution as the effects of actinomycin D are complex and subject to other explanations.

It was of interest to know the effect of acute inflammation induced by subcutaneous injection of turpentine on the transcription process and hepatic RNA levels. Although hepatic RNA metabolism during acute inflammation may closely resemble that following laparotomy, this similarity has not been previously established. In consideration of

the effects of acute inflammation on hepatic polyribosome pools reported in this thesis, as well as the major role mRNA plays in the formation of polyribosome complexes, studies were also performed which were aimed at correlating qualitative differences in mRNA levels with the segregation of polyribosomes into bound and free classes.

The rates of synthesis of hepatic RNA fractions were examined during acute inflammation induced by subcutaneous turpentine administration. Rats received an intraperitoneal injection of 40 μCi [^{3}H]-orotate 3 h prior to sacrifice as preliminary studies indicated that this represented sufficient time for orotate to become evenly distributed in hepatic RNA. Polyribosomes were isolated by magnesium precipitation and chromatographed on oligo(dT)-cellulose. Recovery of the tritium label in various RNA fractions was determined by liquid scintillation counting. The results from this experiment are presented in Table 8. During affinity chromatography, rRNA and tRNA was eluted from the oligo(dT)-cellulose column in the application buffer. Polyribosomal RNA, which was not eluted in the application buffer and did not contain a lengthy poly(A) tract, was eluted in elution buffer A. Poly(A)-containing RNA, which represented a mRNA enriched fraction, was eluted in elution buffer B.

Magnesium precipitated RNA fractions, prepared from 4 h and 8 h inflamed rat livers, showed higher specific radioactivity than did the magnesium precipitated RNA fraction of control rat liver. This observation suggests that acute inflammation results in an elevated rate of RNA synthesis at early times after turpentine injection. It

Table 8

Effect of Inflammation on [^{3}H]-orotate Incorporation into Rat Liver RNA

RNA fraction*	Control	4 h inflamed	8 h inflamed
Total liver homogenate			
Radioactivity	522	524	684
Magnesium precipitated			
Specific radioactivity	0.451	0.646	0.557
Radioactivity	45.3	50.9	45.2
A_{260} units	100	79	81
Eluted in application buffer			
Specific radioactivity	0.438	0.541	0.573
Radioactivity	36.3 (80)	37.9 (74)	39.4 (87)
A_{260} units	82 (82)	70 (89)	69 (85)
Eluted in elution buffer A			
Specific radioactivity	0	0	0.25
Radioactivity	0 (0)	0 (0)	1.1 (2.4)
A_{260} units	1.8 (1.8)	1.1 (1.4)	4.1 (5.1)
Eluted in elution buffer B			
Specific radioactivity	2.14	2.08	1.31
Radioactivity	4.8 (11)	3.2 (6)	4.6 (10)
A_{260} units	2.2 (2.2)	1.5 (1.9)	3.5 (4.3)

* The RNA fractions were prepared as described in text. Total liver homogenate represents perfused liver samples homogenized in buffer A. Other fractions represent partially purified RNA samples. The amount and specific radioactivity of tritium in the fractions are in units of nCi and nCi/A_{260} unit, respectively. Recovery of material eluted from oligo(dT)-cellulose is also shown in parentheses as percentages of the applied material.

also may be significant that total liver homogenate prepared from 8 h inflamed rat liver contained a higher level of radioactivity than the other samples, as this may reflect an alteration in the uptake of orotate into hepatocytes.

Of the 4 h inflamed rat liver RNA fractions prepared by affinity chromatography, only that fraction eluted in application buffer had a higher specific activity than the corresponding fraction from control rat liver. The other fractions contained less material than control fractions. The major RNA component of the material eluted in application buffer is rRNA. If the 4 h inflamed rat liver showed an elevated rate of transcription, then the above observations suggest that newly synthesized RNA appeared in the rRNA fraction and not in the mRNA fraction.

Of the 8 h inflamed rat liver RNA fractions prepared by affinity chromatography, the fractions eluted with application buffer and elution buffer A had higher specific activity and contained a higher proportion of the polyribosomal RNA than did the corresponding control fractions. The poly(A)-containing RNA fraction of 8 h inflamed rat liver contained more material absorbing light at 260 nm than the control poly(A)-containing RNA fraction, however, both fractions exhibited similar levels of incorporation of [^{3}H]-orotate. One explanation that could be offered for these observations is that while inflammation-related increases in RNA synthesis in the 8 h inflamed animal resulted primarily in increased rRNA content, elevation of mRNA levels in this animal resulted from decreased degradation of mRNA.

This interpretation is consistent with the relatively low specific radioactivity and elevated levels of material absorbing light at 260 nm in the poly(A)-containing RNA fraction. It is also conceivable that a decrease in endonucleolytic degradation without an alteration in the exonucleolytic degradation of mRNA would result in an increase in poly(A)-lacking mRNA. This material would appear in the fraction eluted from oligo(dT)-cellulose with elution buffer A.

While the above explanations represent plausible interpretations of the results presented here, it should not be neglected that other factors, such as RNA degradation prior to and during affinity chromatography, may account in part for the observations made despite efforts made to control these factors. It should also be noted that the incorporation of [^{3}H]-orotate into RNA may also be affected by aspects of RNA metabolism, such as alterations in pool sizes, not discussed here.

Purified fractions of rat liver mRNA were used in in vitro translation assays in an attempt to determine what relationship, if any, mRNA transcripts had to the increase in the bound to free polyribosome ratio induced by acute inflammation. Two controls and two experimental animals suffering from inflammation for 16 h were sacrificed and the hepatic poly(A)-containing RNA isolated as before. Experimental animals inflamed for 16 h were used in this experiment with the rationale that, if levels of mRNA transcripts are related to bound and free polyribosome pool sizes, then specific changes in mRNA levels should be observed when the alteration in bound to free

polyribosome ratio is maximum. For acute inflammation, this has been shown in the results presented in this thesis to occur 16 h after subcutaneous injection of turpentine.

Material recovered from 5 g liver samples as poly(A)-containing RNA was divided into two equal parts and assayed in an in vitro translation system as previously described. Water and mRNA supplied with the translation kit served as controls for the assays. A stock solution of 32.5mM magnesium acetate was used in the preparation of the premix for the translation assay. TCA precipitation was performed with 5 µℓ samples removed from the incubation mixture after 15 min, 30 min and 60 min of incubation. The results of this experiment are shown in Table 9.

The largest amount of incorporation of [^{3}H]-leucine into TCA precipitates occurs at about 30 min of incubation in the in vitro translation system. It was also observed that a substantial amount of [^{3}H]-leucine was incorporated in this assay system in the absence of exogenous mRNA as is shown by the results of the sample labeled "water". This probably reflects translation of mRNA for hemoglobin which is endogenous to reticulocyte lysate systems (8). The addition of mRNA supplied with the translation kit stimulated the incorporation of [^{3}H]-leucine into TCA precipitates. The addition of poly(A)-containing mRNA isolated from rat liver slightly inhibited the incorporation of [^{3}H]-leucine into TCA precipitates. This may be due to addition of material with the poly(A)-containing RNA which decreases the efficiency of translation, or, may reflect the efficiency that

Table 9

Time Course of Incorporation of [^{3}H]-leucine into TCA Precipitates of *in vitro* Translation Products

Sample*	[^{3}H]-leucine incorporated into TCA precipitates at 15-60 min of incubation**		
	15 min	30 min	60 min
Control A	1,674	1,865	1,810
Control B	1,490	2,335	1,939
Experimental A	1,285	1,803	1,936
Experimental B	1,852	2,306	1,996
Water	NA	NA	2,528
mRNA	NA	NA	18,774

* Poly(A)-containing samples from control and experimental rat liver RNA were divided into two equal parts, A and B, and each part was assayed in duplicate. Results shown are the average of the duplicate samples. Samples labeled "water" and "mRNA" indicate the material from the translation kit which was added to some assay mixtures as controls.
** Units are counts per min. Reproducibility was within ±10%.
NA - Not assayed.

these mRNA preparations were translated under these conditions.

It is difficult to comment on the levels of rat liver mRNA using the data presented in Table 9. However, these results indicate that efficient translation of mRNA can occur in this system as is shown by the sample labeled "mRNA" which was supplied with the translation system. The addition of exogenous mRNA prepared from rat liver, though, does not stimulate translation above that seen without the addition of exogenous mRNA. This may be due to the preparation of the mRNA transcripts which were added, or may reflect the ability of the incubation mixture to translate these mRNA transcripts from rat liver.

An experiment was performed with the intent of identifying some of the factors responsible for the low translational activity resulting in the data presented in Table 9. This experiment was also aimed at determining the distribution between bound and free polyribosomes of mRNA transcripts coding for α_1-acid glycoprotein and albumin at a time after acute inflammation which best reflects changes in protein synthesis which are induced by stress. Animals inflamed for 16 h prior to sacrifice were used in this experiment as results reported in this thesis have shown that the largest change in polyribosome pool sizes occurs at this time after turpentine injection.

Bound and free polyribosomes were isolated using the method described for the preparation of polyribosomes for sedimentation analyses. Polyribosomes were dialyzed against application buffer and poly(A)-containing RNA was prepared by oligo(dT)-cellulose chromatography. Aliquots of the purified poly(A)-containing RNA were assayed in the

in vitro translation system with water serving as the control. Sample sizes were estimated using $E^{1\%}_{260} = 200$ for high molecular weight RNA. A stock solution of 8mM magnesium acetate was used in the preparation of the premix and the translation mixture was incubated for 30 min. TCA precipitation was performed with 5 µℓ samples and immunoprecipitation with 7.5 µℓ samples. Samples of 20 µg CMC-1 were added to the tubes to act as a carrier for the immunoprecipitation of α_1-acid glycoprotein. The results of TCA- and immuno-precipitation are shown in Table 10.

Polyacrylamide gel electrophoresis was performed on the products of in vitro translation in order to determine if there were significant qualitative differences in the products synthesized in response to the addition of different poly(A)-containing RNA samples. The results of electrophoresis of the translation products of samples B and C from bound polyribosomes, described in Table 10, are compared to the "water" control in Figure 17. Similarly, translation products of samples B and C from free polyribosomes are compared to the "water" control in Figure 18.

Examination of the results presented in Table 10 indicates that larger amounts of [^{3}H]-leucine were incorporated into TCA precipitates than in the experiment presented in Table 9. This may be explained by the lower magnesium concentration used in this experiment, or, may reflect differences in the poly(A)-containing RNA samples. The increase in translational activity is accounted for primarily by an increase in the translation of endogenous mRNA as is shown by the results of the

Table 10

Differential Incorporation of [^{3}H]-leucine into TCA and Immuno-Precipitated Products of *in vitro* Translation

Sample*	Radioactivity incorporated into material precipitated by**		
	TCA	Anti-α_1-acid glycoprotein	Anti-rat serum albumin
Bound			
A (1)	5,520	1,146	433
B (2)	4,576	1,278	426
C (5)	4,991	1,178	806
D (5)	5,569	1,075	446
Free			
A (0.8)	7,445	1,509	3,705
B (1.6)	7,131	976	2,409
C (4)	7,979	948	593
D (4)	5,088	622	447
Water	6,160	912	363

* Numbers enclosed in parentheses represent an estimate of sample size in units of µg RNA. Individual samples are distinguished by a letter code.

** Units were counts per minute. TCA precipitation was performed in triplicate and immunoprecipitation in duplicate. Reproducibility was within ±10%.

Fig. 17. Polyacrylamide Gel Electrophoresis of *in vitro* Translation Products of Bound Polyribosomal mRNA

The samples used here are described in Table 10.

(a) Products of bound polyribosomal mRNA sample B.

(b) Products of bound polyribosomal mRNA sample C.

(c) Products of *in vitro* translation system not supplemented with exogenous mRNA.

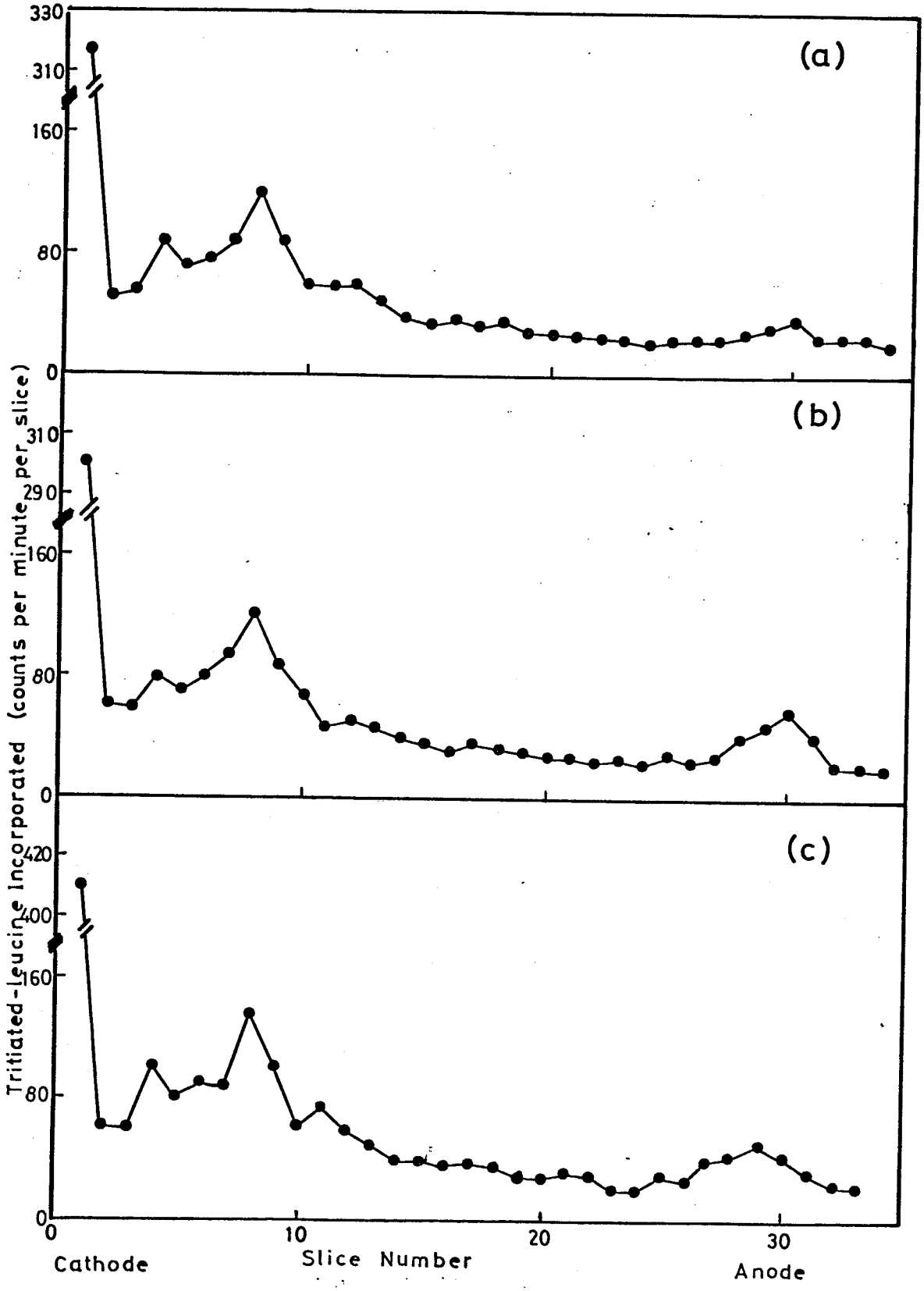

(a)
(b)
(c)
330
310
160
80
0
310
290
160
80
0
420
400
160
80
0
Tritiated-Leucine Incorporated (counts per minute per slice)
0
10
20
30
Cathode
Slice Number
Anode

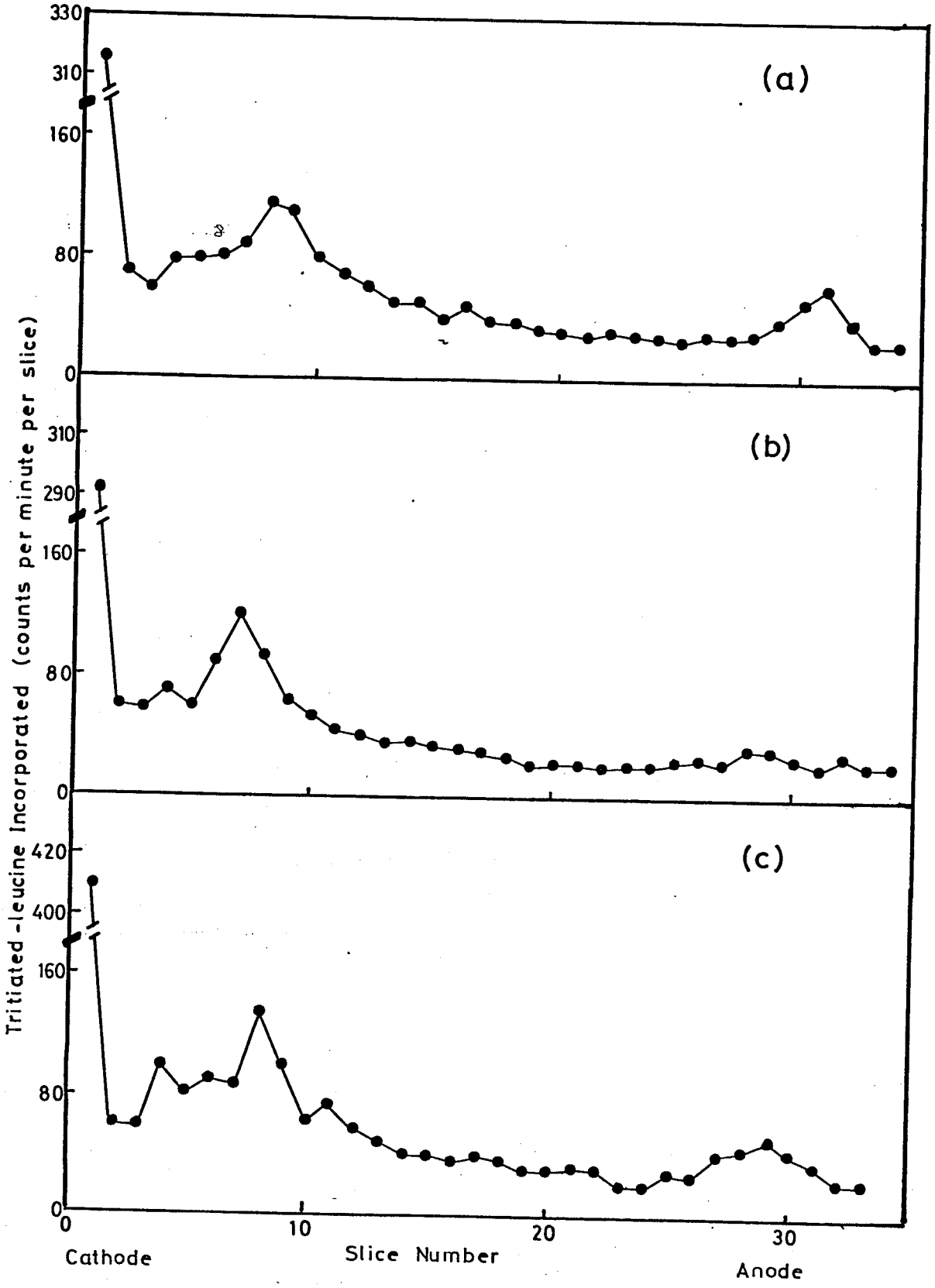
Fig. 18. Polyacrylamide Gel Electrophoresis of *in vitro* Translation Products of Free Polyribosomal mRNA

The samples used here are described in Table 10.

(a) Products of free polyribosomal mRNA sample B.

(b) Products of free polyribosomal mRNA sample C.

(c) Products of *in vitro* translation system not supplemented with exogenous mRNA.


330
310
160
80
0
(a)
310
290
160
80
0
(b)
420
400
160
80
0
(c)
Tritiated-leucine Incorporated (counts per minute per slice)
0
10
20
30
Cathode
Slice Number
Anode

the "water" sample. This observation would seem to rule out the possibility that differences in poly(A)-containing RNA samples alone account for this increased translational activity.

Immunoprecipitates, formed by the products of in vitro translation reacting with anti-α_1-acid glycoprotein and anti-rat serum albumin, contained high levels of background radioactivity which was not associated with polypeptide immunologically similar to either α_1-acid glycoprotein or rat serum albumin. The reticulocyte lysate was prepared from rabbit tissue and should not contain material which would react with antisera prepared in rabbits. It would seem that some of the radioactivity associated with the immunoprecipitates, particularly in that sample which did not contain exogenous mRNA, must be accounted for by non-specific adsorption of material to immune complexes. However, if it can be assumed that a relatively constant amount of non-specific precipitation of radioactive material occurs in these samples, then perhaps some conclusions may be drawn from these results.

It was observed that a greater amount of radioactive material was incorporated into material precipitated by anti-α_1-acid glycoprotein from those samples translated from bound polyribosomal mRNA than from samples translated from free polyribosomal mRNA despite the fact that bound polyribosome poly(A)-containing RNA generally stimulated lower incorporation of [^{3}H]-leucine into TCA precipitates. There also was a greater amount of radioactive material incorporated into anti-rat serum albumin immunoprecipitates in those samples translated

from free polyribosomal mRNA than from bound polyribosomal mRNA. Larger immune precipitates formed by anti-α_1-acid glycoprotein due to the presence of carrier CMC-1 may account for generally higher levels of radioactivity in anti-α_1-acid glycoprotein immunoprecipitates than anti-rat serum albumin immunoprecipitates.

The electrophoretic patterns shown in Figures 17 and 18 do not reveal any striking differences between the translation products synthesized in response to different poly(A)-containing RNA preparations. All gels show a large peak of radioactivity which remained at the cathode end of the gels during electrophoresis. This material would represent high molecular weight aggregates, material insoluble in tank buffer and material which has an isoelectric point near 8.9. All gels show sizable peaks of radioactivity around slice number 8. This material probably represents hemoglobin synthesized from mRNA transcripts endogenous to the reticulocyte lysate. The gels also contain some radioactivity at the anode end of the gels. This could be polypeptide with a very low isoelectric point. It could also be material with a low molecular weight and as such not appreciably hindered in migration by the sieving properties of polyacrylamide. This material may represent polypeptide released from ribosomal complexes prior to completion of translation of mRNA transcripts. Although the size of the peak at the anode end of the gels may be artefactual, it may also reflect translational activity of the reticulocyte lysate system during incubation.

Results presented here suggest that acute inflammation results in increased rRNA synthesis prior to 4 h after turpentine injection. Elevation in mRNA levels apparently occurs between 4 h and 8 h after inflammation and seems to result from decreased RNA degradation. If the results of *in vitro* translation assays presented here are accepted as being representative of *in vivo* mRNA levels, then it would appear that mRNA transcripts coding for α_1-acid glycoprotein are localized primarily on bound polyribosomes while mRNA transcripts coding for albumin are localized on free polyribosomes in livers of 16 h inflamed rats.

Results of *in vitro* translation assays presented here have identified endogenous translational activity of reticulocyte lysate as a problem associated with meaningful assessment of *in vivo* levels of rat liver mRNA. Further studies of incubation conditions and preparation of mRNA fractions would be necessary to obtain suitable translational activity in this *in vitro* translation system. It is possible that other *in vitro* translation systems may be more suitable for assays of this type. It is also evident that immunological identification of translation products of these systems present problems which would have to be overcome.

Discussion

The distribution of RNA between the two classes of mammalian polyribosomes is not static. The distribution of RNA in polyribosome aggregates of different sizes is also subject to variation. Studies of these variations in RNA metabolism provide one avenue through which a better understanding of the homeostatic mechanisms of mammalian systems can be obtained.

There have been several reports on alterations of hepatic RNA levels in polyribosomes under different physiological states. Increases have been observed in the hepatic bound and free polyribosome pools following laparotomy and during liver regeneration following partial hepatectomy (63). Infection with D. pneumoniae has been reported to stimulate an increased uptake of orotate into bound polyribosomal rRNA above that observed into free polyribosomal rRNA (37). Conditions inducing adaptive synthesis of rat liver fatty acid synthetase may result in a shift of polyribosomes into the free pool, which may be blocked by acute inflammation, although quantitation of polyribosomes under conditions inducing the synthesis of fatty acid synthetase may yield ambiguous results (64). Conditions resulting in hepatocyte cell injury, such as administration of carbon tetrachloride or ethionine, have been shown to result in degranulation of the rough endoplasmic reticulum (65,66,67). All of these physiological states are also accompanied by changes in hepatic translational activity.

The mechanisms, which are believed to accomplish these changes in hepatic polyribosome content, vary with the physiological state.

Although ultrastructural changes during cell injury are not fully characterized, it is evident that the process of polyribosome disaggregation in ethionine-treated rats does not disrupt the mRNA-endoplasmic reticulum interaction (65). It seems that the main effects of ethionine administration are probably attributable to replacement of methionine by ethionine and a subsequent disruption of nucleotide biosynthesis (67). In contrast to hepatocyte cell injury, conditions which stimulate increases in hepatic capacity for protein synthesis are believed to be mediated by different mechanisms. It has been suggested that elevation in the translation of proteins synthesized preferentially on bound or free polyribosomes results in increased levels of the class of polyribosomes synthesizing these proteins (37, 63,64). Other authors have suggested that the stress-induced redistribution of polyribosomes between bound and free classes is accounted for by a decrease in alkaline ribonuclease activity (39). It was of interest to study the induction of increased synthesis of secretory and intracellular proteins to determine if common mechanisms responsible for redistribution of polyribosome were shared by these different physiological states.

It is important to bear in mind during interpretation of biochemical data on hepatocyte protein synthesis that, despite morphological similarities, all hepatocytes in the liver may not perform identical biochemical roles. It has been reported that plasma albumin is found in 15% (68), fibrinogen in 1% (68), prothrombrin in 30% (69) and haptoglobin in 2.4% (70) of the hepatocyte population. Stimulation

by injury increases the number of cells producing haptoglobin to 11.2% (70) of the hepatocyte population while stimulation of prothrombrin and fibribogen synthesis reportedly shows that all parenchymal cells are capable of producing these proteins (69,71). These observations were obtained from fluorescent antibody staining techniques and may be subject to alternative interpretations. In this context, the apparent reproducibility of experiments on hepatic translation which seem to be independent of the size of liver sample used, is also important to interpretation of data.

Work presented in this thesis has shown that acute inflammation resulted in an elevation of the bound to free polyribosome ratio which was maximal at about 16 h after turpentine injection (Table 4, Figure 8). Iron may have elevated the bound to free polyribosome ratio (Table 5, Figure 9) while zinc adminstration resulted in an overall increase of both bound and free polyribosomes (Table 6, Figure 10). Although the results obtained for iron and zinc administration may not have been as reproducible as those obtained for acute inflammation, it is evident that conditions which stimulate the synthesis of intracellular proteins do not necessarily induce a shift of polyribosomes into the free class. It may be noteworthy that all three physiological states resulted in decreases in free polyribosomes 4 h after treatment. This is an observation which is more dramatic, and perhaps more significant, after iron administration. This observation may also reflect a common biochemical mechanism involved in the distribution of polyribosomes between the bound and

free classes during the three physiological states tested.

The changes that are reported here are important in relation to other biochemical changes that are known to occur during the physiological states tested.Stress has been reported to result in elevation of seromucoid mRNA transcripts between 4 h and 8 h after laparotomy (38), whereas 5 h after zinc administration, metallothionein mRNA transcripts are increased (72). Synthesis of the acute phase reactant α_1-acid glycoprotein is maximal 24 h after acute inflammation (73), while ferritin synthesis is maximal 5 h after iron administration (74) and metallothionein synthesis is maximal 6 h after zinc administration (75). Hepatic tissue bound α_1-acid glycoprotein levels are maximal 12 h after acute inflammation (73), ferritin levels 12 h after iron administration (76) and metallothionein levels 18-25 h after zinc administration (77,78,79). The alterations in hepatic polyribosome pools reported here must be synchronized with these events in order to efficiently respond to acute inflammation, iron administration and zinc administration.

It would seem that the shift of polyribosomes into the bound class during acute inflammation does not depend just on qualitative changes in the levels of hepatic mRNA transcripts. Sequestration of polyribosomes into the bound class apparently occurs about 8 h after the synthesis of mRNA transcripts for acute phase reactants. This shift of polyribosomes also seems to occur prior to the increased hepatic synthesis of acute phase reactants such as α_1-acid glycoprotein. It would seem that this shift in polyribosomes during acute

inflammation reflects the events that segregate transcription of acute phase reactant mRNA transcripts and translation of these transcripts. Maximum hepatic tissue bound levels of the acute phase reactant α_1-acid glycoprotein are observed prior to the maximum synthesis of this glycoprotein during acute inflammation. However, hepatic tissue bound content and synthesis of albumin, a protein which does not contain covalently-linked carbohydrate, decrease concurrently in similar experiments (73). It may be that increased levels of bound polyribosomes reflect increases in post-translational events, such as partial proteolysis of precursors and glycosylation of glycoproteins, which occur prior to secretion of glycoproteins. If this suggestion is true, then the apparent paradox presented by elevated tissue bound levels of α_1-acid glycoprotein prior to the maximum synthesis of this glycoprotein may be accounted for by an increase in glycosylation and processing of glycoproteins about 16 h after acute inflammation.

It seemed that alteration in polyribosome pool sizes following iron and zinc administration were not dependent solely on levels or activity of mRNA transcripts responsible for induced synthesis of ferritin or metallothionein. There appeared to be a loss of polyribosomes during those times after iron administration that untranslatable mRNP coding for ferritin is being integrated into the pool of translatable mRNA. While this loss, particularly of free polyribosomes, may have reflected other biochemical processes, it is also possible that elevated iron levels were involved. It is difficult to explain how increased translation of an intracellular protein would have resulted

in decreased polyribosome content. It is possible that iron plays a role in disrupting polyribosome integrity. This is conistent with the observation that 12 h after iron administration, when hepatic ferritin levels would have been maximal, polyribosome pool sizes were near control values or slightly elevated. Increases in metallothionein mRNA translation or translation of these transcripts around 5 h after zinc administration seemed to have had little effect on polyribosome pool sizes. However, there did seem to be an increase in polyribosome pools, particularly the bound class, around that time after zinc administration when metallothionein, and thus hepatic zinc binding capacity, are known to be elevated. While the results of quantitation of bound and free polyribosome levels following iron and zinc administration have not identified roles for mRNA coding for ferritin and metallothionein in the distribution of polyribosomes, these results have raised the possibility that transition metals can play a role in disruption of polyribosome integrity. While the results presented here do not lead to this conclusion alone, the observation that low levels of EDTA, which has a high stability constant for complexes formed with both iron and zinc, results in greater polyribosome stability, gives credence to the suggestion.

While the significance of the alterations in polyribosome pool sizes reported here is not clear, this is due, in part, to a relative ignorance of alterations in other aspects of RNA metabolism during the physiological states tested. However, there is reason to believe that during acute inflammation changes occur in the sedimentation propetties

of polyribosomes. Liu and Neuhaus (39) have reported that laparotomy results in a shift of polyribosomes to heavier aggregates which is maximal 18 h after operation. However, Zweig and Grisham (63) reported that no change in the shapes of polyribosome profiles occurs following laparotomy. In view of this discrepancy, the effect of stress induced by subcutaneous injection of turpentine was examined on the sedimentation properties of bound and free polyribosomes. Results from these polyribosome profiles should more clearly identify changes in polyribosome pools during acute inflammation.

The results presented in this thesis indicate that acute inflammation induced by subcutaneous injection of turpentine results in shifts of bound and free polyribosomes to heavier states of aggregation (Figures 11 & 12). Although this effect is slight in the free polyribosome preparations, it is quite marked in the bound polyribosome preparations. An increase in the proportion of heavier aggregates of polyribosomes would seem to account for the increased bound polyribosome pool size during acute inflammation. As previously mentioned, the bound polyribosomes from control animals are unique in that they are composed almost entirely of lighter aggregates of polyribosomes. it is also difficult to argue the artefactual nature of this observation in view of the consistency with which the pattern was observed and the fact that bound polyribosomes from inflamed animals, which were prepared under identical circumstances, contained a high proportion of heavier aggregates of polyribosomes.

There are several explanations that could be offered to explain the results of sedimentation analysis of bound polyribosomes prepared from control animals. Although it has been reported that short-term fasts do not alter the bound to free polyribosome ratio (57), there is evidence to suggest that some disaggregation of polyribosomes into lighter aggregates does occur during starvation (58,80). This disaggregation has a greater effect on bound than free polyribosomes (58). Starvation also results in a loss of some of the albumin mRNA transcripts from the bound polyribosome fraction, most of which appears as mRNP free in the cytoplasm (58). Although starvation of animals prior to preparation of polyribosomes reduces heavier aggregates of bound polyribosomes, the reports on this effect do not account entirely for the almost total absence of heavier aggregates of bound polyribosomes from control animals. It is also difficult to find a simple explanation for the inflammation-induced increase of heavier aggregates of bound polyribosomes while working under the assumption that the translational efficiency of hepatocytes is incapacitated by starvation. The media used in the preparation and sedimentation analysis of bound and free polyribosomes is that shown by Ramsey and Steele (53) to quantitatively separate polyribosomes into bound and free classes with the "loosely bound*" polyribosomes being recovered in the free state of polyribosomes. These authors have provided evidence that loosely bound polyribosomes are identical to free polyribosomes and

* Loosely bound polyribosomes are defined as those polyribosomes released from membranes by treatment with high concentrations of potassium chloride alone (13).

not bound polyribosomes on the basis of protein content of ribosomal subunits, sedimentation properties and *in vitro* translation activity. Media used by other authors result in variable recoveries of loosely bound polyribosomes in the bound polyribosome preparations (1). It is possible that the omission of the loosely bound polyribosomes accounts, in part, for the loss of the heavier aggregates of polyribosomes previously observed in bound polyribosome profiles from this strain of rat. It is possible that elevated levels of alkaline ribonuclease activity accounts for the low proportion of larger-sized polyribosomes, as has been suggested for rat kidney polyribosome preparations (81).

Closely related to the study mentioned above was a study undertaken to immunologically identify those polyribosome aggregates synthesizing α_1-acid glycoprotein and albumin. Bound polyribosomes prepared from inflamed animals showed a greater tendency to bind anti-α_1-acid glycoprotein than did bound polyribosomes from control animals (Figures 13a & 13b). It was also observed that bound polyribosomes bound more anti-α_1-acid glycoprotein than did free polyribosomes (Figures 13a,13b & 14). These observations imply preferential synthesis of α_1-acid glycoprotein on bound polyribosomes and elevated levels of mRNA transcripts coding for α_1-acid glycoprotein during inflammation. This is consistent with results previously reported in this laboratory (3,33). It was also observed that bound polyribosomes bound more anti-rat serum albumin than free polyribosomes (Figures 15 & 16) which is indicative of preferential synthesis of this protein by bound polyribosomes. This is an observation made by numerous other authors (Table 1).

Acute inflammation, iron administration and zinc administration all resulted in alterations in bound and free polyribosome pool sizes. The increase of bound polyribosomes during acute inflammation seemed to result from a marked increase in heavier aggregates of bound polyribosomes which did not seem to be related to changes in levels of mRNA transcripts alone. Liu and Neuhaus have suggested that increases in heavier aggregates of polyribosomes result from decreased ribonuclease activity (39). This enzymic activity was examined in order to determine if alkaline ribonuclease activity represented a common factor important in determining the distribution of polyribosomes following acute inflammation, iron administration and zinc administration (Table 7). Acute inflammation resulted in a significant decrease in alkaline ribonuclease activity 18 h after treatment as well as a marked increase in the ratio of bound to free polyribosomes. At 4 h after zinc administration, there was a sizable increase in alkaline ribonuclease activity which was accompanied by a slight decrease in free polyribosome pool size. By 18 h after zinc administration, there was a slight decrease in alkaline ribonuclease activity which was accompanied by a sizable increase in both bound and free polyribosomes. Iron administration was shown to exhibit little change in alkaline ribonuclease activity in animals which exhibited a shift of polyribosomes into the bound pool. From these results, it is not evident that total alkaline ribonuclease activity can account for the changes in the distribution of polyribosomes following iron and zinc administration.

Although there does not appear to be a correlation between total alkaline ribonuclease activity and all the changes observed in the

distribution of bound and free polyribosomes, the significance of this major degradative enzyme in hepatic RNA metabolism should not be neglected. There is evidence to suggest that this endonuclease has an extrahepatic origin in the pancreas and is taken up by endocytosis in the liver (82). If this is the case, this enzymic activity may represent one of the vehicles by which hepatic RNA metabolism is modulated by extrahepatic factors during physiological states such as acute inflammation. The importance of the endogenous hepatic ribonuclease inhibitor should also be considered. Total hepatic alkaline ribonuclease activity was reported in this thesis, but these assays may not be indicative of in vivo levels of active alkaline ribonuclease if changes occur in endogenous ribonuclease during the physiological states tested. Hepatic levels of iron and zinc may influence endogenous ribonuclease inhibitor as is suggested by the observation that low levels of EDTA, which chelates iron and zinc, results in elevated inhibition of ribonuclease activity by the endogenous ribonuclease inhibitor (55).

Changes in polyribosome pools could occur in two ways. Either the metabolism of pre-existing RNA could be altered in the physiological state tested, or, an alteration in the rates of synthesis of RNA may be involved. Incorporation of orotate into RNA fractions was examined during acute inflammation to provide evidence for one of the possibilities. Work presented in this thesis suggests that acute inflammation results in increased synthesis of rRNA as early as 4 h after turpentine injection (Table 8). There also seems to be an increase in mRNA by 8 h

after acute inflammation although this may involve decreased degradation of the poly(A)-containing RNA fraction. Similar conclusions were drawn by Chandler and Neuhaus for stress induced by laparotomy with the exception that increased mRNA levels were attributed solely to enhanced synthesis (38). It may be significant that Chandler and Neuhaus studied total cytoplasmic RNA fractions using a phenol method of RNA isolation, whereas the results reported here involved use of affinity chromatography to study polyribosomal RNA fractions.

Studies attempting to quantitate mRNA levels in an in vitro translation system were largely inconclusive (Tables 9 & 10). This was due, in part, to a high endogenous translational activity of the system used, although this alone does not account for the lack of qualitative differences in the samples (Figures 17 & 18). However, if the results are taken at face value, they generally agree with the observation that bound polyribosomes contain higher levels of mRNA transcripts coding for α_1-acid glycoprotein than do free polyribosomes. The observation in these studies that higher levels of mRNA transcripts coding for albumin are found in free polyribosomes is not substantiated by the studies immunochemically identifying polyribosomes synthesizing albumin. This may reflect uncertainties due to non-specific adsorption of in vitro translation products onto immunoprecipitates.

From the work presented in this thesis, it appears that, during the three physiological states tested, changes occur in RNA metabolism that affect the distribution of RNA in polyribosomes. It seems that changes in the levels of mRNA transcripts alone cannot account for

these changes. Although total alkaline ribonuclease activity does not seem to account for all the changes observed, the importance of degradation of RNA cannot be neglected. Acute inflammation results in a shift of polyribosomes to the bound class, which are primarily responsible for the synthesis of α_1-acid glycoprotein and albumin, and seems to result from increased bound polyribosome aggregates. Acute inflammation results in increased levels of rRNA and mRNA, such as those transcripts coding for the acute phase reactant α_1-acid glycoprotein, and this may involve both synthetic and degradative aspects of RNA metabolism.

As previously mentioned, the aim of some of the work presented in this thesis was an experimental examination of some aspects of the "signal" hypothesis of Blobel and Dobberstein (15) and as such, some of the work presented here is discussed in the context of this model.

A central feature of the "signal" hypothesis is the statement that all polypeptide synthesis is initiated in the free polyribosome state. Extrapolation of this premise would lead to the conclusion that quantitative increases in the synthesis of polypeptides should result in an overall increase in the amount of free polyribosomes.

A common feature to the distribution of polyribosomes in the three physiological states tested is the reduction of the free polyribosome pool 4 h after treatment. Zweig and Grisham have reported a similar loss of free polyribosome aggregates at early times after laparotomy and partial hepatectomy (63). This decrease in free polyribosomes occurs at a time at which initiation of polypeptide synthesis should be elevated, and, argues against the premise that initiation

of protein synthesis occurs solely on free polyribosomes. This is consistent with a report that initiation of polypeptide synthesis can occur in the membrane-bound state (65).

By itself, the observed decrease in the free polyribosome pool at 4 h after treatment does not necessarily disagree with the "signal" hypothesis. The decrease in the pool size is not necessarily related specifically to initiation of hepatic mRNA transcripts known to be elevated following acute inflammation, iron administration and zinc administration. Another explanation that could be offered is an alteration during the physiological states tested, of precursor pools involved in translation. Starvation of animals prior to sacrifice may be an important factor involved in depletion of pool sizes. These decreases in free polyribosome pool sizes may reflect problems inherent in the assumption that the only biochemical consequences of these treatments are the elevation of synthesis of ferritin and metallothionein following iron and zinc administration and the elevation of acute phase reactant synthesis during inflammation. Some of the biochemical effects of increased hepatic iron and zinc may account for the alterations in the bound to free polyribosome ratios after zinc and iron administration.

Another feature of the "signal" hypothesis is the statement that bound polyribosomes synthesize secretory proteins while free polyribosomes synthesize primarily intracellular proteins. The authors of this model allow for the translation of some mRNA transcripts for secretory proteins in the free polyribosome pool due to initiation of polypeptide synthesis in the free state, or, a lack of binding sites on

the endoplasmic reticulum. The model does not allow for the synthesis of intracellular proteins on bound polyribosomes.

The induction of increased synthesis of intracellular proteins did not induce a shift of polyribosomes into the free state as might be expected from a simple interpretation of the "signal" hypothesis. This is in agreement, though, with reports of the synthesis of intracellular proteins on both bound and free polyribosomes (Table 1).

In contrast to the results obtained from iron and zinc administration, acute inflammation resulted in a shift of polyribosomes to the bound states as predicted by the "signal" hypothesis. This is accompanied by an increased translational capacity of bound polyribosomes, which have been shown to be the primary site of synthesis of the acute phase reactant α_1-acid glycoprotein. The shift of polyribosomes, though, does not seem to involve only changes in levels of mRNA transcripts. Also, it is likely that other factors governing peptide chain initiation, elongation, termination and release; rate of reading of mRNA; spacing of ribosomes; availability of tRNA, amino acids, enzymes and energy supply, are also important.

The results presented in this thesis are generally consistent with those presented by other authors. Acute inflammation results in hepatic RNA metabolism changes, which account, in part, for other biochemical changes which occur during this physiological state. It appears that experimental results concerned with the synthesis of secretory proteins are easier to interpret in the context of the

"signal" hypothesis than those concerned with the synthesis of intracellular proteins. This may be due to experimental difficulties arising from the different methodologies used to separate bound and free polyribosomes. The significance of the loosely bound polyribosomes has not been resolved, although there is reason to believe that these polyribosomes represent free polyribosomes artefactually associated with the microsome fraction (53). It may be significant that the mRNA transcripts for the intracellular protein histone have been localized exclusively on free and loosely bound polyribosomes (83).

It is evident from the results presented here that a simplistic view of the processes involved in the synthesis of secretory and intracellular proteins is not adequate in explaining biochemical and ultrastructural differences induced by different physiological states. However, it is hoped that the information obtained from these studies provides some insight into these processes and the direction that further studies on the control of acute phase reactant synthesis should take. There remains a great deal to be learned about the metabolism of intracellular proteins such as ferritin and metallothionein, and secretory proteins such as α_1-acid glycoprotein.

Suggestions for further studies

The results presented here suggest possible studies, related to this work, that go beyond the scope of this thesis.

The significance and consequences of the appearance of mono- and di-ribosomes in the bound polyribosome profile of control rats may be a topic of study which could provide insight into the biochemistry of the translation process.

If alkaline ribonuclease does have an extrahepatic origin, then there has been no explanation offered to date for the elevated activity of this enzyme during inflammation. Studies could be undertaken to explain this observation. Studies could also be performed aimed at determining the effect, if any, of transition metals on hepatic degradation of RNA.

Studies utilizing an *in vitro* translation system met with little success, although some of the problems associated with this type of experiment were identified. Two types of studies, related to those studies reported here, could be performed. Measurement of mRNA transcripts for acute phase reactants during acute inflammation could provide insight above that of previous reports. Sequence analysis of the *in vitro* translation products of an acute phase reactant, such as α_1-acid glycoprotein, would be meaningful in the context of present studies of workers in the field of polypeptide precursors.

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