# TRANSLATION OF M-RNA AND SYNTHESIS OF BASIC PROTEINS EARLY DURING RAT LIVER REGENERATION



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# TRANSLATION OF mRNA AND SYNTHESIS OF BASIC PROTEINS EARLY DURING RAT LIVER REGENERATION

by

© Nancy Louise Kariel, B.S.

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of the requirements for the degree of
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## Abstract

The regenerating rat liver, a model for rapid, controlled growth in a mammalian system, was used to investigate molecular events occurring during a transition in growth-rate. This investigation focused on mRNA translation during the early phase of liver regeneration following partial hepatectomy.

The study included examination of: 1) the translation of polysomal (polyribosomal) mRNA; 2) the possible presence of a polysome-associated factor for stimulating or inhibiting initiation frequency or translation rate; and 3) the synthesis of basic proteins (primarily histones and some ribosomal proteins) during the first 10 h of regeneration. Intact polysomes were isolated, an *in vitro* translation assay for use with rat liver polysomes (instead of purified mRNA) as template was developed, and a criterion for operationally defining basic proteins was determined. Polysome-associated mRNA, isolated by centrifugation through a discontinuous sucrose gradient, was translated in a wheat germ *in vitro* translation system; and the translation products were fractionated on a carboxymethyl-cellulose column to quantitate basic proteins.

It was shown that: 1) the $A_{260}$ polysomes/g liver, an estimate of the quantity of mRNA/g liver, is maximal between 7 and 8 h after partial hepatectomy; 2) an active, polysome-associated factor which influences initiation frequency or translation rate is undetectable;

and 3) the peak rate of basic protein synthesis does not occur within the first 10 h postoperatively and thus does not coincide with the peak of polysome content. The peak rate of basic protein synthesis appeared to be much less than that reported in uncontrolled, rapidly growing Ehrlich ascites tumor cells. The results are compatible with the findings of others concerning a) DNA transcription; b) mRNA synthesis, transport, and translation; c) ribosomal protein and histone synthesis early during rat liver regeneration; and d) the absence of a detectable, endogenous, initiation frequency or translation rate-stimulating or inhibiting factor.

A review of the literature on DNA template activity and on ribosomal proteins and histones and their synthesis following partial hepatectomy in the rat is also included.

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## List of Abbreviations

| Abbreviation | Meaning |
|---|---|
| BSA | bovine serum albumin |
| CM-cellulose | carboxymethyl-cellulose |
| DTT | DL-dithiothreitol |
| EDTA | ethylenediaminetetraacetic acid |
| HEPES | N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid |
| SDS | sodium dodecyl sulfate |
| TCA | trichloroacetic acid |
| TEMED | N, N, N', N'-tetraethylmethylethyldiamine |
| tris | tris(hydroxymethyl)aminomethane |

# 1 INTRODUCTION

## 1.1 Overview

Studies of the sequence of intracellular events which occur in normal, controlled growth may aid in understanding that process and in contrasting it with intracellular events present in abnormal, uncontrolled growth, as in some tumor cells. The mammalian liver has the unusual property of being able to undergo rapid, well regulated compensatory growth, also called regeneration. Although the liver must continue to perform many functions for the rest of the body while increasing its cell number, neoplastic cells have no apparent external "obligations". Many proteins absent or present in low concentration in quiescent cells are required for cell replication.

This thesis will focus on the two major families of basic proteins in somatic cells, histones and most ribosomal proteins. Histones are associated with DNA and are important in its conformation and transcription. Ribosomal proteins are essential for mRNA translation and thus protein synthesis. Synthesis of both types of protein is obligatory for cell replication.

Protein synthesis may be monitored at the following steps: RNA transcription from DNA, processing of the transcript (HnRNA to mRNA), transport of mRNA to the cytoplasm, association of mRNA with ribosomes, translation of mRNA, posttranslational modification of

protein, and presence of translated protein at its site of action. The literature on the synthesis of ribosomal proteins and histones early during liver regeneration has been reviewed with respect to these stages of gene expression.

The current research was designed to examine: 1) the amount of mRNA present per g liver, 2) the apparent translatability of this mRNA, and 3) the active synthesis of basic proteins in the liver as a function of time during the first 10 h following partial hepatectomy in rats. To perform this work, it was necessary to isolate intact polysomes (polyribosomes), optimize conditions of a wheat germ *in vitro* translation assay, and establish a criterion for differentiating basic and nonbasic proteins.

## 1.2 Reasons for studying regenerating liver

Partial hepatectomy induced liver regeneration is a useful, easily available model for studying mammalian regulatory mechanisms of differentiation, mitosis, and growth at the organ, cellular, and macromolecular levels (Bucher and Malt 1971). This model is probably the best adult, mammalian example of compensatory growth (Colbert *et al.* 1977). This growth is precisely regulated and ceases after restoration of the liver mass (Bucher and Malt 1971). The peak rate of cell doubling in regenerating liver, nearly once per 24 h, is comparable to that observed in many embryonic and cultured cells.

Understanding the controlled, stimulated growth, including the regulatory mechanism(s) and the dramatic shift in the synchronized biological function of the cell, may be useful for understanding a

number of features of the cellular growth and the "developmental growth" observed as the entire animal grows; the "additive growth" present during pregnancy, lactation, hyperphagia, and exposure to some drugs (Bucher 1963; Bucher and Malt 1971); tissue growth during injury repair (Morley 1976); differentiation present during embryogenesis; and the lack of cellular differentiation which results in the aberrant growth of neoplastic transformation (Van Lancker 1969; Becker 1974; Morley 1976; LaBrecque 1979). Aspects of regeneration include: the mechanisms of initiating and terminating cell proliferation; the sequence of macromolecular biosynthesis and the sources of bioenergy during hypertrophy and hyperplasia; the mechanism of ultrastructural construction and multiplication of subcellular organelles including ribosomes, nuclei, endoplasmic reticulum, and mitochondria in the new cells; the mechanism of histological organization in the hyperplastic lobe, i. e. the relationship between vasculature, reticular endothelial cells, fibroblasts, connective tissues, and hepatic cells; and the effect of liver regeneration on hepatic function (Van Lancker 1969).

Information about two fundamental forms of reaction to injury, hypertrophy and hyperplasia, can also be ascertained by studying the liver after partial hepatectomy. During liver regeneration, the individual cells hypertrophy prior to undergoing cellular division (Van Lancker 1969). Following partial hepatectomy, nearly all the normally quiescent hepatocytes enter the cell cycle relatively synchronously, and the accompanying extensive changes

in cellular metabolism tend to remain synchronous during the first 36 h (Colbert et al. 1977; Lewan et al. 1977; Krieg et al. 1979). Thus, the cells' well regulated transition from the resting $G_0$ into the $G_1$, S, $G_2$, and M phases of the cell cycle and the regulation of these transitions, including changes in gene expression, may be studied (Tsukada and Lieberman 1964; Colbert et al. 1977; Lewan et al. 1977; Krieg et al. 1979).

Examining the sequence of synthesis of specific proteins and nucleic acids required for mitosis may aid in understanding the mechanisms which regulate DNA replication, DNA dependent RNA transcription, mRNA translation, other aspects of nucleic acid metabolism, and the consequent control of the processes of growth and differentiation (Orlova 1971; Smal'ko and Platonov 1977b). Thus, liver growth after partial hepatectomy is a useful model for determining whether regeneration initiates a massive activation of repressed genes or only an increase in the expression of pre-existing mRNA (Fausto et al. 1976; Smal'ko and Platonov 1977b). In addition, the orderedness of cell processes following partial hepatectomy provides a good experimental system for studying the effects of antitumor agents, other foreign substances, and irradiation on enzyme activity and metabolism; RNA polymerase and gene activation; DNA polymerase, replication, and mitosis; and polyploidization (Lewan et al. 1977).

## 1.3 Description of the liver

### 1.3.1 Function

The liver is a complex organ; it performs a variety of functions and is composed of several cell types. The liver's gross structure, tissue organization, and cellular constitution and the cytological and cytochemical features of its different cell types reflect the organ's diverse, specialized functions (Lane 1974). It is the body's largest visceral organ, forming about 3 to 5 per cent of the body weight (Leevy 1978; Andrews 1979). The liver is a soft, flexible, reddish brown organ which changes shape and size according to the amount of blood present (Andrews 1979) and the space available in the peritoneal cavity (Elias and Sherrick 1969). In mammals, the liver is attached by means of ligaments to the dome of the diaphragm, and it is protected by the ribs from mechanical damage (Andrews 1979). Approximately 25 per cent of the cardiac output passes through the liver each minute, allowing it to carry out effectively its many duties affecting the rest of the body (Lane 1974).

The liver functions both as a ductless gland, producing many blood proteins and lipids, and also as a ducted gland, secreting bile into the intestinal lumen (Lane 1974). Among the liver's many other functions are: storage of carbohydrates, some elements, and a few vitamins; metabolism of carbohydrates, fats, and protein; monitoring and control of the plasma concentrations of small molecules involved in metabolism; regulation of blood volume; synthesis of certain blood clotting factors; destruction of old red blood cells by phagocytosis and concommitant conservation of

hemoglobin; detoxification or removal by filtration of materials of a range of dimensions and various origins in the blood stream, including some foreign substances, drugs, and hormones; the formation of bile; and the production of heat as a by-product (Lane 1974; Sherlock 1977; Andrews 1979). The liver's secretory, metabolic, and phagocytic responsibilities tend to be independent under nonpathologic circumstances (Lane 1974).

Although the liver is not an important protein store, it is active in protein metabolism; indeed almost all plasma proteins have an hepatic origin. The liver is also capable of converting protein into carbohydrate or fat and is the only tissue capable of deaminating amino acids (Andrews 1979).

### 1.3.2 Structure

#### 1.3.2.1 Gross structure

In rats, the liver is a single organ; however, anatomists divide it into four lobes, primarily on the basis of blood supply (Elias and Sherrick 1969; Andrews 1979). There are two principal lobes, the right and left or caudate. The former is approximately six times the size of the latter (Elias and Sherrick 1969; Sherlock 1977). The liver is structurally and functionally further composed of lobules approximately 1 mm in diameter. Each lobule is centered around a small branch of the hepatic vein and is bounded by portal canals (Elias and Sherrick 1969).

#### 1.3.2.2 Cell types

Histologically, the liver is a continuous mass of parenchymal cells tunneled both by capillaries lined with littoral cells, which

transport predominately venous blood from the gastrointestinal canal to the heart, and by a system of secretory channels, which empty into the intestine. Morphologically, however, the liver is an indivisible continuum (Elias and Sherrick 1969).

The mammalian liver is composed of several cell types, differing in number, size, and function (Lewan et al. 1977). Hepatic parenchymal cells, hepatocytes, are the liver cells best-studied. Although they represent only 60 per cent of the liver cells by number (Bucher 1963; Lane 1974; Lewan et al. 1977; Jungermann and Sasse 1978), they comprise 79.8 per cent (Jungermann and Sasse 1978) to 95 per cent (Bucher 1963) of the organ's volume. Parenchymal cells are themselves diverse, reflecting the heterogeneous expression of the hepatic genome (Lane 1974). Mammalian hepatocytes are characterized by a highly ordered structure and function (Becker 1969); a large cytoplasm containing an unusually high number of organelles (Lane 1974); a large, central, often heteroploid nucleus; and being polygonal, 0.025 to 0.035 mm in diameter, with three different, specialized cell surfaces (Lane 1974; Sherlock 1977).

Because parenchymal cells tend to be polyploid (in contrast to the other liver cells, which tend to be diploid), they contain a disproportionate fraction of the organ's total DNA (Bucher 1963). In the normal adult liver, 25 to 30 per cent of hepatocyte cells are binucleate; 70 to 80 per cent of the parenchymal nuclei are tetraploid, while 1 to 2 per cent are octaploid.

Both the subcellular structures, including the type of

endoplasmic reticulum and the size and shape of mitochondria, and the active enzymes of hepatocytes vary with time, location within the liver, and functional demands (Lane 1974; Lewan et al. 1977; Jungermann and Sasse 1978). These characteristics allow the cells to be capable of responding rapidly to changes in functional demands, exogenous stress, or injury (Lane 1974). In addition, hepatocytes have an extremely complex functional capacity (Becker 1974).

Kupffer and other littoral cells and blood vessel cells comprise the second most numerous class of hepatic cells, 35 per cent of cells and 16.6 per cent of liver volume (Lane 1974; Jungermann and Sasse 1978). In contrast to hepatocytes, they are simple; containing clear cytoplasm, few non-lysosome organelles, small mitochondria, and pinocytotic vesicles (Lane 1974). Kupffer cells are important in antibody production, blood formation, and synthesis of bilirubin from old erythrocytes (Sherlock 1977; Andrews 1979).

Bile duct cells and connective tissue each comprise 2 per cent of cells; together they form 3.6 per cent of liver volume (Jungermann and Sasse 1978). Liver endothelial cells are similar to endothelial cells of other organs (Andrews 1979). Mesenchymal, ductular, and lymphatic-vascular cells are also present in the liver (Leevy 1973). The pattern of enzyme activity and RNA labeling of hepatocytes differs from that of non-parenchymal cells (Lewan et al. 1977).

The heterogeneity of intraliver functions may be caused by the periportal to perivenous gradients in oxygen, substrate and hormone concentrations, and possibly different sympathetic and

parasympathetic innervation of the parenchymal zones (Jungermann and Sasse 1978).

## 1.4 Regenerative capacity of the liver

### 1.4.1 General aspects

In addition to being highly specialized, liver cells tend to be long-lived, some lasting for the adult life of the animal (Bucher and Malt 1971). No other mammalian organ matches the liver's latent capacity for growth (Bucher and Malt 1971; Andrews 1979). Although the basal mitotic rates are reported to range from 1 in 10 000 to 1 in 100 000, hepatocytes retain the ability to prepare for and participate in mitosis readily (Bucher 1963; Becker 1974). During regeneration, the mitotic rate increases to 3 to 5 in 100 (Bucher 1963). Such mitoses can be induced by the loss of a single cell or by the loss of up to 90 per cent of the original cell number, if the remaining liver cells retain the capacity to divide, regardless of whether loss is caused by chemicals, toxins, infection, radiation, or trauma (including surgery) (Leevy 1973; Becker 1974). Each of these stresses affects the equilibrium of normal hepatic molecular, cellular, and organ structure and functions, including the supply of essential nutrients, disposal of endogenous metabolites, liver tissue oxygenation, and development of immunologic reactivity (Leevy 1973).

Restoration of liver mass following partial hepatectomy is extremely rapid. This capability of rat liver to respond to partial hepatectomy is maintained even when the operation is performed once

a month for an entire year (Bucher 1963). In normal, adult rats the residual lobes nearly double in size by 48 h, and the original mass is restored within 7 to 10 d (Bucher and Malt 1971; Lewan et al. 1977). Via cellular hyperplasia, the liver is capable of creating sufficient new, functionally and structurally equal cells to restore its original cell number and metabolic capacity (Bucher et al. 1969; Becker 1974). Despite the considerable cell movement which occurs during the restorative enlargement of the liver, the original liver lobe structures are not restored (Bucher and Malt 1971; Lewan et al. 1977). Because the response to amputation occurs equally throughout the residual tissue, liver regeneration is not a "wound phenomenon" in the usual sense; it is, in the broader sense, a form of repair. Thus, although the normal liver's response to injury or partial hepatectomy is often called "regeneration", "compensatory hyperplasia" is a more accurate term (Becker 1974).

1.4.2 In rats following partial hepatectomy

The regenerative response of liver following partial hepatectomy has been studied in several mammalian species, including man (Lewan et al. 1977). The most detailed, systematic, experimental work has been carried out with rats, mice, and dogs (Bucher 1963). Surgically induced regeneration is generally initiated by amputation of 60 to 70 per cent of the liver, following the method of Higgins and Anderson (1931); the two main lobes, median plus left lateral, are excised. This method is used most frequently, because it is relatively simple, is more reproducible, and leads to more replicable observations from animal to animal and from experimenter to

experimenter, is well-tolerated, and has a low mortality rate (Van Lancker 1969; Bucher and Malt 1971; Lewan et al. 1977). In addition, partial hepatectomy results in the most consistent, reproducible, and best in vivo mammalian, synchronous response.

The most synchronous, acute response is observed in the rat. Synchrony is affected primarily by the proportion of liver removed (Becker 1974). Within a strain of animals, each liver lobe constitutes a consistent proportion of the liver, with a mean standard error of only ± 2.5 per cent (Bucher 1963; Bucher et al. 1969; Bucher and Malt 1971; Becker 1974). However, this proportion varies from strain to strain and species to species (Bucher 1963). The amplitude and synchrony of the regenerative response are also influenced by the amount of liver removed, the animal's age, stress, maintenance of natural circadian rhythms, health, hormonal status, nutritional status, and dietary factors (Bucher 1963; Leduc 1964; Bucher and Malt 1971; Becker 1974; Morley 1976; Lewan et al. 1977).

## 1.5 Regenerative events in rat liver following partial hepatectomy

### 1.5.1 Initiation of regeneration

The exact molecular mechanism(s) which signals the body to commence the regenerative response and regulate it is unknown, although many postulates have been proposed and examined (Becker 1974; LaBrecque 1979). It is thought that tissue growth is regulated by both activators and inhibitors of DNA synthesis or earlier steps in the biochemical cycle, or of mitosis (Morley 1976). There appears to be consensus that the signal for liver regeneration is

humoral and that its appearance is not initiated by an overloading of the liver remnant with metabolic waste, increased portal blood flow, changes in functional demand or levels of known hormones, or the loss of growth inhibitors in the liver (Morley 1976; Andrews 1979). Only a few descriptions of the many hypothesized regeneration inducing substances are presented here.

Okazaki et al. (1978) have measured the blood plasma (humoral) levels of the bile pigment biliverdin 5, 10, 20, 30, 40, 60, 120 and 180 min following partial hepatectomy or a sham operation in rats. On the basis of these results, they hypothesize that biliverdin initiates liver cell multiplication.

Although the precise origin and identity of the humoral signal factor(s) for liver regeneration are unknown, Morley (1976) has isolated a possible in vivo DNA synthesis stimulating, heat stable, polypeptide "regenerating liver serum factor" of molecular weight 17 000. It is first detectable 12 h following partial hepatectomy. The occurrence of other, regeneration induced events during the 12 h lag prior to the appearance of this regenerating liver serum factor suggests that a number of regenerative factors may be induced by partial hepatectomy, each of which is responsible for a particular phase of the regenerative cycle (Morley 1976).

LaBrecque (1979) also has isolated a liver-specific, polypeptide regenerative, DNA synthesis-and-mitosis "stimulator substance". Like Morley's (1976) regenerating liver serum factor, LaBrecque's "stimulator substance" is also humoral, being present only in the soluble cell fraction, and heat resistant. In addition, it is

inactivated by perchloric acid, is unique to actively growing liver, is ethanol precipitable, is nondialysable, and has a molecular weight of approximately 10 000. Both *in vivo* and *in vitro* (liver cell cultures) there is a 12 to 15 h lag between synthesis or administration of "stimulator substance" and the presence of an observable effect on DNA synthesis. This 12 h lag suggests that "stimulator substance" acts early in the sequence of events leading to DNA synthesis and that it does not exert a simple mass action effect. Further experiments suggest that although "stimulator substance" enhances regenerative or rapid growth, it is unlikely to be the major control factor in normal DNA synthesis.

Using mathematical interpretations of reported mitoses during liver regeneration, Bard (1978; 1979) has suggested that the theoretical model most capable of predicting the actual observed mitotic rate is comprised of a liver synthesized, mitotic inhibitor with a half life of 10.0 or 11.4 h, present in the same concentration in both the liver and the blood.

1.5.2 General events early after partial hepatectomy

Despite the fact that liver regeneration has been studied for over forty years, there is still no detailed knowledge of the sequence of events early after partial hepatectomy (Wondergen and Potter 1978). Liver injury, in this case created by partial hepatectomy, induces a relatively orderly and well-regulated series of morphological and biochemical adaptive responses in subcellular organelles as the organ prepares for and undergoes compensatory hypertrophy and hyperplasia while the remnant attempts to maintain

the whole liver's glandular and metabolic functions (Leevy 1973; Lewan et al. 1977). Although initially the liver remnant does not function as efficiently as the whole organ, the individual cells, in addition to preparing for mitosis, perform liver functions as well as or better than cells in the normal state (Becker 1969; Bucher and Malt 1971; Lewan et al. 1977).

Restoration of the liver mass is a coordinated process, in which hepatocytes, mesenchymal cells, ductular cells, and lymphatic-vascular cells are equally involved (Leevy 1973). Only the hepatocytes respond initially; the vascular and littoral cells, peritoneal mesothelium, connective tissue, and other elements lag behind by nearly a day (Bucher 1963; Leduc 1964; Bucher et al. 1969; Becker 1974). Thus, studies conducted during the first 24 h after partial hepatectomy have the advantage of dealing primarily with only hepatocyte events. Other advantages to studying events early during regeneration are the greater synchronization of events, the higher level of cellular participation, and the greater ease in examining a burst of activity than a tapering off of events (Bucher et al. 1969; Bucher and Malt 1971).

When examining liver regeneration, an attempt should be made to differentiate between the morphologic and biochemical events involved in "glandular compensation" and in the growth process of liver restoration (Bucher et al. 1969). To date, no specific morphologic change following standard partial hepatectomy has been shown to be absolutely required for mitosis (Becker 1974).

All tissue growth occurs by means of either hypertrophy, an

increase in cell size or mass, or hyperplasia, an increase in cell number; or by a combination of both. In the liver, regeneration results primarily from hyperplasia, supplemented by some hypertrophy (Morley 1976). Following partial hepatectomy the cells change from the preoperative state of high metabolic and low mitotic activity first to a state of increased metabolic activity characterized by changes in RNA metabolism and preparation for cell division, lasting 12 to 16 h and associated with hypertrophy. The cells then go to a state characterized by DNA synthesis and mitosis commencing 14 to 16 h postoperatively, associated with hyperplasia (Bucher 1963; Leduc 1964; Bucher et al. 1969; Bucher and Malt 1971; Fausto et al. 1976; Colbert et al. 1977).

Morphologic changes in the hepatocytes can be observed less than thirty min after partial hepatectomy (Bucher 1963). Within the first hour postoperatively, the remaining cells start to enlarge (Bard 1978). Soon thereafter, many morphologic changes can be observed throughout the residual lobes (Bucher 1963; Bucher and Malt 1971). Dispersion of basophilia, infiltration with lipid, and loss of glycogen and protein storage products can be observed in hepatocytes (Bucher 1963; Leduc 1964; Lewan et al. 1977); and the endoplasmic reticulum, mitochondria, lysosomes, Golgi apparatus, and microbodies begin to undergo structural and functional changes (Leevy 1973). Histologically, the ultrastructural changes closely resemble those observed after starving and refeeding (Bucher and Malt 1971). The most visible morphologic change during the first day following partial hepatectomy is the increasing paleness of the

liver remnant; the paleness is caused by an extensive temporary infiltration of neutral lipid (Bucher and Malt 1971; Becker 1974).

By 12 h postoperatively, the size of hepatocyte cells, nuclei, and nucleoli has increased perceptibly, primarily because of the accumulation of lipids and water (Bucher 1963; Lewan et al. 1977); but DNA synthesis has not commenced (Fausto et al. 1976). Approximately 60 per cent of the hepatocytes remaining after partial hepatectomy enter the mitotic cycle synchronously (Leduc 1964; Saml'ko and Platonov 1977b). Following the start of cell division, which peaks at about 24 h postoperatively (Leduc 1964; LaBrecque 1979), the number of binucleate hepatocytes decreases rapidly while the number of polyploid hepatocytes increases (Bucher 1963). This increased polyploidy remains after regeneration is complete; and although the quantity of DNA is restored, the number of hepatocyte nuclei never attains the preoperative level (Bucher 1963; Leduc 1964). These morphologic changes occur in the periphery prior to their occurrence in the center of the lobule (Bucher 1963).

Many of the peripheral hepatocytes appear to undergo more than one division, while some hepatocytes at the center of the lobule may not divide at all. Although the proliferative response appears to be greater in the hepatocytes than in the biliary, vascular and supporting elements, events in the non-parenchymal cells induced by loss of liver mass appear to be independent of cellular location within the liver. Mitosis in the littoral cells, peritoneal mesothelium, blood vessel walls, bile duct, and connective tissue is maximal late in the second day or early on the third. Connective

tissue is the last hepatic component to be restored (Bucher 1963).

1.5.3 Biochemical and molecular biological considerations

As already stated, during the regenerative process the liver must synthesize many proteins absent in quiescent cells and increase the rate of synthesis of intracellular proteins normally having low turnover rates. Among the macromolecules for which there is an increased demand in regenerating liver are the following: export proteins and their mRNAs; RNA polymerase and ribosomal proteins; molecules involved in gene activation; the purine and pyrimidine ribonucleotide and deoxyribonucleotide synthesizing enzymes; and DNA polymerase, nuclear and chromosomal proteins, and mitosis related proteins. Evidence to date suggests that these dynamic changes are ordered, well regulated, and coordinately controlled.

In the context of molecular biology, the ideal situation would be to monitor the timing of and changes in gene transcription, rate of pre-mRNA processing, transport of processed mRNA to the cytoplasm, translation of the mRNA, and presence of the protein at its site of action. Because the last of these is the easiest, it has received the most study. One difficulty encountered when attempting to correlate information from different research groups about this sequence of events in regenerating rat liver, is limited overlap of observation times or methods. Thus, it is not always possible to determine whether the different reported results are compatible.

1.5.4 Template activity

Early experiments to determine gene transcription or, more accurately, DNA template availability for transcription used one of

two methods. *In vivo* studies were made by injecting labeled isotope for varying times prior to animal sacrifice and measuring labeled nuclear RNA. *In vitro* studies were performed by observing the template activity of chromatin with *Escherichia coli* RNA polymerase. Although neither of these methods can distinguish whether the DNA available for transcription codes for chromosomal RNA, rRNA, tRNA, or HnRNA (and thus mRNA), they can both provide information about the timing of nuclear events following partial hepatectomy.

Using the *E. coli* RNA polymerase technique at 0, 4 and 10 h postoperatively, Mayfield and Bonner (1972) report a maximum increase in template activity in regenerating rat liver of 35 per cent at 10 h. Using the same method and measuring at 4, 6, 8, 10, 12, 18, 25, 26, 28, 32, 35, and 39 h after partial hepatectomy, Hwang *et al.* (1974) found a maximum increase, of approximately 45 per cent, at 6 to 8 h of liver regeneration. The next greatest increase in template activity, about 60 per cent, was observed at 35 h of regeneration. Because transcription of rRNA is much greater than transcription of mRNA, these observations are probably indicative of the former activity.

1.5.4.1 *Hybridization studies of transcription*

Hybridization experiments have attempted to elucidate the type(s) of nuclear RNA being synthesized, i. e. which genes are available for transcription; the complexity of nuclear and cytoplasmic mRNA; and the homology between RNA populations from normal and regenerating liver. It is likely that the HnRNA, including that coding for histones and ribosomal proteins, is not

transcribed from highly repetitive portions of the genome, although it may be transcribed from an intermediate repetitive portion of the genome. In addition, HnRNA is a minor nuclear species which has a relatively short nuclear life before it is processed to mRNA and transported to the cytoplasm.

Fausto and co-workers have used hybridization techniques to investigate RNA synthesis following partial hepatectomy in rats. Examining rat liver 3 and 6 h post partial hepatectomy, they found: 1) no massive derepression of repetitive genes; 2) no increased polyadenylation of nuclear RNA; 3) apparent increased transport of repetitive sequence RNA, including some poly(A) RNA, from the nucleus to the cytoplasm; and 4) that the proportion of repetitive and nonrepetitive DNA sequences represented in polysomal mRNA is unchanged during the first 6 h of regeneration (Fausto et al. 1976; Greene and Fausto 1977). These observations led them to conclude that although increased amounts of poly(A) mRNA enter the cytoplasm, the proportion of new mRNA species is small, less than 10 per cent of the total poly(A) mRNA population.

Krieg and co-workers (1979) have also used hybridization techniques to study the effect of partial hepatectomy on the content and composition of poly(A) HnRNA in 3 and 12 h regenerating rat liver. Like Fausto's group, they found no derepression of repetitive genes for poly(A) RNA early during regeneration (3 h) and that there may actually be an increase in the repressed portion of the genome. In contrast to Fausto's group, they report a loss of rare sequences in sham operated and in partially hepatectomized

animals. However, again like Fausto and co-workers, they observed an increase in the abundance of poly(A) RNA to some repetitive sequence DNA 3 h postoperatively, and the presence of about 10 per cent new poly(A) RNA species in 12 h regenerating liver. In these experiments Krieg and co-workers (1979) were unable to determine whether the observations indicate the transcription of previously untranscribed sequences or merely the addition of poly(A) to sequences already being transcribed.

Because poly(A) RNA is thought to be mRNA or an mRNA precursor, Greene and Fausto (1974) examined its characteristics in liver following partial hepatectomy. Using *in vivo* labeling times of 3, 6, and 12 h, they found that after 3 and 12 h of regeneration the proportion of poly(A) in nuclear RNA was essentially constant at a level slightly lower than that in sham operated animals. Hybridizing whole nuclear and cytoplasmic poly(A) RNA to redundant sequences at 3, 6, and 12 h postoperatively, they observed a slight but consistent reduction in the proportion of poly(A) present in total nuclear RNA during the first 12 h. This observation is consistent with the simultaneous increase in the synthesis of rRNA. However, the proportion of poly(A) present in hybridized RNA from regenerating liver cytoplasm is 2 to 4 times higher than that in sham operated animals; it reached a maximum at 3 h and declined to control levels by 12 h (Greene and Fausto 1974).

Because most mRNA sequences are probably transcribed from single or low multiplicity genes, Fausto's group examined poly(A) polysomal mRNA in greater detail (Fausto *et al.* 1977; Colbert *et al.*

1977). Hybridizing complementary DNA (cDNA) to poly(A) RNA of hepatectomized and sham operated rats, to DNA, they found that approximately 18 per cent of polysomal poly(A) mRNA in both normal and regenerating liver is homologous to the intermediate repetitive portion of the genome. Hybridization of the cDNA to its homologous mRNA showed that the sequence complexity of poly(A) polysomal RNA is quite similar in sham operated and partially hepatectomized rat livers. However, 12 h regenerating liver contains about 20 per cent more poly(A) polysomal mRNA molecules per cell than 12 h sham operated liver.

To examine gene expression during liver regeneration, Fausto and co-workers cross hybridized "sham cDNA" with poly(A) polysomal mRNA from regenerating liver and the converse, "hepatectomized cDNA" with polysomal poly(A) from sham operated liver (Fausto et al. 1977; Colbert et al. 1977). They detected that approximately 14 per cent of the polysomal poly(A) mRNA from normal liver is either absent or under-represented in regenerating liver polysomes, while about 11 per cent of the polysomal poly(A) mRNA from regenerating liver is not detectable in normal liver polysomes. Because the products of gene transcription may either be confined to the nucleus or present in the cytoplasm outside of polysomes, the detection of new mRNA sequences in polysomes of regenerating liver does not necessarily imply that these mRNAs are transcribed from genes activated by the regenerative process. It should also be recalled that these experiments have not analyzed polysomal nonpoly(A) RNA.

Tedeschi et al. (1978) further investigated the possible

transcription of new mRNA species in regenerating liver by determining the percentage of the non-repetitive genome transcribed and the complexity of nuclear RNA from normal and 12 h regenerating liver. Cross hybridizing nuclear RNA from normal liver to cDNA from poly(A) polysomal RNA from normal liver, plus the appropriate homologous hybridizations, they found that the percentage of the nonrepetitive DNA transcribed into nuclear RNA is the same in normal and 12 h regenerating liver. This observation supports their earlier conclusion that extensive genomic derepression does not occur during liver hypertrophy following partial hepatectomy (Fausto et al. 1976; Greene and Fausto 1977). However, qualitative differences between the two populations may remain undetected, as definitive conclusions would require methods capable of detecting subtle changes. The previously observed differences in poly(A) polysomal mRNA populations (Colbert et al. 1977; Fausto et al. 1977) may be explained by differential DNA transcription or by post transcriptional events such as selectivity at the nucleocytoplasmic transport level.

Using the same approach, Glazer (1977) investigated the in vivo labeling of rat liver polysomal poly(A) RNA. He injected [5-$^{3}$H]orotic acid 90 min prior to killing the animals 2, 6, 12, or 20 h after sham or partial hepatectomy operations. He observed that although the specific activity of polysomal poly(A) RNA was increased by 50 per cent at 2 h following partial hepatectomy and by 30 per cent at 6 h, by 12 h it was no longer elevated. These findings agree with his previous observation of maximal poly(A) RNA synthesis 2 h post operatively (Glazer 1976). Like most in vivo

labeling in complex organisms, where pool sizes and rates of reuse of the labeled entity are unknown, it is difficult to know whether the observations are more accurate representations of events early during the labeling period or later on.

Because increased DNA template availability, transcription, and transport of the resulting mRNA to the cytoplasm are essential preliminary stages for the increased synthesis of proteins, including the synthesis of new proteins observed in regenerating rat liver, these events should be observed early following partial hepatectomy. Reported experimental observations suggest that synthesis of poly(A) RNA is maximal 2 to 3 h postoperatively (Greene and Fausto 1974; Glazer 1977). There is also increased transport of nuclear poly(A) RNA to the cytoplasm during the first 12 h of liver regeneration (Greene and Fausto 1974; Fausto et al. 1976; Colbert et al. 1977; Fausto et al. 1977; Glazer 1977; Greene and Fausto 1977; Krieg et al. 1979), but transcription of new mRNA species is limited at this time (Fausto et al. 1976; Greene and Fausto 1977; Tedeschi et al. 1978; Krieg et al. 1979).

1.5.4.2 HnRNA, mRNA, and polysomes

To study the relative rates of HnRNA synthesis in sham operated and partially hepatectomized rats, Glazer (1974) injected [5-$^3$H]orotic acid 15 min prior to sacrifice of the animals 2, 6, 12, or 20 h postoperatively. Isolating the parenchymal from the nonparenchymal nuclei, he found that the specific radioactivity of HnRNA from both types of nuclei had already increased significantly by 2 h, and maximum incorporation occurred at 6 h in parenchymal

nuclei and at 20 h in nonparenchymal nuclei. Unfortunately, Glazer does not present 0 h data, making it impossible to determine whether the sham operated 2 h values are greater than preoperative values.

Glazer (1976) examined the characteristics of the HnRNA by injecting [5-$^{3}$H]orotic acid only 5 min before killing the animals and measuring the synthesis of poly(A) and nonpoly(A) HnRNA. He found that maximum incorporation into poly(A) HnRNA occurs at 2 h following partial hepatectomy, whereas maximal incorporation into nonpoly(A) HnRNA does not take place until 12 h postoperatively. Taken in conjunction with Greene and Fausto's (1974) observation that the proportion of poly(A) nuclear RNA remains unchanged early during liver regeneration, a net synthesis of poly(A) HnRNA at this time appears probable.

Smal'ko and Platonov (1977a) have examined rat liver polyribosomes (polysomes) at different times following partial hepatectomy. After injecting [$^{14}$C]orotic acid 1 h prior to death 3, 6, 12, or 24 h postoperatively, they report that precursor incorporation into polysomes (rRNA plus mRNA) increased sharply between 3 and 6 h and increased slightly to a maximum at 12 h of liver regeneration. Using linear 10 to 40 per cent sucrose gradients, Smal'ko and Platonov (1977a) examined changes in the polysome sedimentation profiles over time following partial hepatectomy. They noted that the proportion of monosomes decreases and the proportion of "heavy" polysomes (greater than trimers) increases during regeneration, particularly at 6 and 12 h.

Because, presumably, mRNAs being actively translated are not

necessarily associated with a maximum number of ribosomes, changes in polysome profiles need not be indicative of changes in mRNA size. Therefore, Smal'ko and Platonov (1977b) attempted to analyze the newly synthesized mRNA present in polysomes and present in the cytoplasm outside of polysomes. They injected [2-$^{14}$C]orotic acid 1 h prior to sacrifice into rats 3, 6, 12, 15, 18, 21, or 24 h following partial hepatectomy or a sham operation. Polysomal and cytoplasmic RNAs were analyzed on 5 to 20 per cent sucrose gradients. There was no significant change in the distribution of polysomal and cytoplasmic RNAs during the first 24 h postoperatively. The proportion of 6 to 15S RNA (mRNAs) bearing or lacking poly(A) sequences was noted at 6 and 21 h of liver regeneration. In both polysomal and cytoplasmic RNA populations, at 21 h there is approximately half as much poly(A) 6 to 15S RNA per total RNA as at 6 h.

The information coded for by mRNAs entering the cytoplasm at 6 and 21 h may code for different proteins. Electrophoresis of the 6 and 21 h cytoplasmic RNAs in 6 per cent polyacrylamide gels showed that both preparations have substantial amounts of 5 to 6S RNA, 6 h RNA has a low proportion of 7 to 9S RNA and a high proportion of 10 to 12S RNA, and 21 h RNA has a high proportion of 7 to 9S RNA and a low proportion of 10 to 12S RNA (Smal'ko and Platonov 1977b).

Calculations by Smal'ko and Platonov (1977b) suggest that the 5 to 6S RNA may code for regulatory proteins, the 7 to 9S for histones, and the 10 to 12S for ribosomal proteins. These observations led them to conclude that following partial hepatectomy,

there are two maxima for informational RNAs to enter the cytoplasm. At the first maximum, occurring 6 h postoperatively, there is more poly(A) RNA; and this RNA is of an appropriate size, 10 to 12S, and is present at a reasonable time to code for ribosomal proteins. At the second maximum, after 21 h of regeneration, the informational RNA contains more nonpoly(A) RNA and is of an appropriate size, 7 to 9S, and is present at a reasonable time to code for histones.

Atryzek and Fausto (1979) have investigated poly(A) nuclear, polysomal, and cytoplasmic RNA transcripts 6, 12, 24, and 48 h following partial hepatectomy. Contrary to Smal'ko and Platonov's findings (1977b), but in agreement with their own previous observation (Colbert et al. 1977), they detected no changes in the size distribution of any of the poly(A) RNA classes during liver regeneration. However, in accordance with the theory that HnRNA is processed to mRNA, they report that nuclear poly(A) RNA molecules tended to be larger than their cytoplasmic counterparts. In agreement with Smal'ko and Platonov (1977a), they noted an increase in large (greater than octomers) polysomes as early as 3 h following partial hepatectomy. The number of cytoplasmic and polysomal poly(A) RNA molecules per mg DNA increased approximately 2.2 fold by 12 h and remained elevated until 24 h postoperatively. This timing is later than that reported by Smal'ko and Platonov (1977a).

Atryzek and Fausto (1979) also reported an increase in the ratio of polysomal poly(A) RNA to cytoplasmic poly(A) at 12 h of liver regeneration. In contrast, little difference was noted in the number of nuclear poly(A) RNA molecules per mg DNA in partially

hepatectomized and sham operated animals. Like Smal'ko and Platonov (1977b) and the hybridization researchers, they suggest that the increase in mRNA during liver regeneration is a result of an increased efficiency of HnRNA transcription and conversion to cytoplasmic poly(A) RNA. The timing of poly(A) RNA synthesis reported by Glazer (1974; 1976), Smal'ko and Platonov (1977a; 1977b), and Atryzek and Fausto (1979) is also in good agreement with the observations of the hybridization studies. When analyzing this information, it should be kept in mind that there is also a population of nonpoly(A) HnRNA and mRNA.

1.5.5 Ribosomal proteins

1.5.5.1 Ribosome description

Knowledge of the pattern of ribosome biogenesis may lead to a deeper understanding of the molecular mechanism(s) used by the cell for regulating protein synthesis. Cells are capable of adapting to a variety of environmental demands and conditions, each of which requires a specific variety of proteins in relatively exact amounts. Thus, at different times during a cell's life, it requires or can support different numbers of ribosomes. Ribosomes are the smallest cellular organelles requiring a specific assembly of macromolecules and are vital constituents of all living cells. Because ribosomes, with the aid of tRNAs, amino acids, initiation factors, elongation factors, and termination factors, translate information from mRNA into protein, they play a central role in gene expression.

It is accepted that the growth rate of mammalian (and other) cells is proportional to the rate of ribosome production. The rate

of ribosome biogenesis is observed to increase during a cell's transition from a resting to a growing state, prior to the commencement of DNA synthesis, although the mechanism(s) by which the rate of mammalian ribosome biogenesis is modulated under different growth conditions has not yet been elucidated. It should be recalled that ribosomes determine the cell's protein content only indirectly; they translate the supplied mRNA, but do not determine which or how much mRNA is supplied.

Because ribosomal proteins, as ribosome components, play a prominent role in the synthesis of all proteins, their own biosynthesis is an integral part of cell growth (Warner and Gorenstein 1977). Since, in growing cells, ribosomal proteins can comprise up to 5 per cent of total cellular protein (Maden 1971), the mechanism(s) and regulation of ribosomal protein synthesis are also important.

Each ribosome is formed from two ribonucleoprotein (RNP) subunits or subparticles, a large one and a small one, which associate reversibly during protein synthesis. Each ribosomal subparticle contains an RNA core which interacts with a variety of distinct proteins, each of which demonstrates activity only when present in the assembled ribosomal subparticle or whole ribosome. The average hepatocyte has $7.6 \times 10^6$ ribosomes (Hadjiolov and Nickolaev 1976).

Ribosome biogenesis involves the synthesis of the ribosome components, rRNA and ribosomal proteins, and their interaction and assembly into functional RNP particles. The rat liver

(and nonliver) ribosome is about 50 per cent RNA and 50 per cent protein by weight. It has a sedimentation coefficient of 80S, and its subparticles have sedimentation coefficients of 60S and 40S. The 60S ribosomal subparticle, mass about $2.8 \times 10^6$ daltons, consists of one strand of 28S rRNA, mass about $1.7 \times 10^6$ daltons; one strand of 5S rRNA; one strand of 5.8S rRNA, about 150 nucleotides, associated by hydrogen bonding; and one each of 39 or 40 different proteins. The 40S ribosomal subparticle, mass about $1.5 \times 10^6$ daltons, consists of one strand of 18S rRNA, mass about $0.7 \times 10^6$ daltons, and one each of 31 different proteins (Cox 1977). Because ribosomal proteins are defined operationally as those proteins present after a high salt wash or as those proteins required for ribosome function, it is not possible to enumerate them exactly.

As a class, the approximately 70 different ribosomal proteins tend to be quite basic and generally smaller than other cellular proteins. The individual rat liver ribosomal proteins have molecular weights ranging from 8 000 to 40 000 (Cox 1977). Relatively little is known about the transcription of ribosomal protein genes and their processing and translation (Tsurugi et al. 1972; Hadjiolov and Nikolaev 1976). This lack is due primarily to the numbers of proteins involved and their lack of easily identifiable activities in their unassembled form. It is unknown whether a polycistronic RNA is processed to form monocistronic ribosomal protein mRNAs; however, reported observations agree with the hypothesis that each higher eukaryote protein, including ribosomal proteins, is translated from a monocistronic mRNA. Evidence to date indicates

that the mRNAs for ribosomal proteins, like all other reported proteins, are translated in the cell's cytoplasm (Ogata et al. 1967; Tsurugi et al. 1972; Ogata et al. 1974). In a variety of experiments Nabeshima (1975) and others found that, as expected for a nonexported protein, the mRNAs for ribosomal proteins are translated by free and loosely bound polysomes, especially the former, rather than by tightly bound polysomes (Tsurugi et al. 1972; Ogata et al. 1974; Warner 1974; Nabeshima et al. 1975). After translation, the ribosomal proteins are relatively rapidly transported to the nucleolus (Warner 1974) where with 5S rRNA and pre-rRNA, they form RNP complexes (Perry 1976).

Assembly of ribosomal subparticles probably follows the time sequence: 1) mRNAs for ribosomal proteins are translated in the cytoplasm, 2) 5S rRNA and the majority of the ribosomal proteins are transported to the nucleolus where, 3) they become associated with the primary pre-rRNAs as the latter are being transcribed (Maden 1971; Warner 1974; Hadjiolov and Nikolaev 1976; Tsurugi et al. 1972; Higashinakagawa and Miramatsu 1974), subject to inhibition by already present nucleolar pre-rRNA, 4) the 60S and 40S ribosomal subparticle precursors are formed by specific endonuclease cleavages, and 5) are transported to the cytoplasm where final assemblage occurs. The rate at which a single ribosome is formed is identical in resting and regenerating liver (Chaudhuri and Lieberman 1968b; Maden 1971).

Because the cell must be capable of responding to a variety of environmental conditions requiring varying intensities of protein

production; ribosome production, a necessity for protein synthesis, must be regulated so that the supply of ribosomes matches the demand. Coordinated control of ribosomal protein synthesis in rat liver has yet to be reported. However, Gorenstein and Warner (1976; Warner and Gorenstein 1977) have reported coordinate regulation of ribosomal protein synthesis at the transcriptional level in the yeast Saccharomyces cerevisiae.

1.5.5.2 mRNA for ribosomal proteins

As part of an ongoing project in Ogata's laboratory, Nabeshima et al. (1979) investigated whether individual ribosomal proteins can be synthesized by a wheat germ in vitro translation system using [$^3$H]leucine and poly(A) mRNA as substrate. They found that poly(A) mRNA includes mRNA for the bulk of ribosomal proteins and is translated with fidelity in the wheat germ cell-free system. In addition, the translated ribosomal proteins were present in approximately equimolar amounts. Confirmation of all ribosomal proteins was limited by purification procedures which tended to exclude some of the less basic ribosomal proteins.

Nabeshima and co-workers (1979) also examined whether the poly(A) mRNA coding for ribosomal proteins is polycistronic. They first looked for a correlation between the size of liver polysomes and the molecular weights of ribosomal proteins coded for by the polysomes. Prior to translation with free polysomes, 18 h regenerating liver postmitochondrial supernatant, and [$^3$H]leucine, free polysomes were fractionated into 3 classes: those containing 4 or fewer ribosomes, those with 5 to 14 ribosomes, and those with 15

or more ribosomes. Ribosomal proteins with molecular weights of 20 000 or less were synthesized by the first two fractions, ribosomal proteins with molecular weights of 21 000 to 40 000 were synthesized by the second fraction, and two high molecular weight ribosomal proteins (L1, 60 000 and L2, 54 000) were synthesized by the last two fractions. Assuming that the distance between ribosomes on mammalian polysomes is approximately 30 amino acid residues (90 nucleotides) and that the average molecular weight of an amino acid is 105, these results suggest that, in general, individual rat liver ribosomal proteins are synthesized by polysomes, the size of which depends on the molecular weight of the protein.

To obtain more precise information on the size of the mRNA relative to the size of the translated protein, Nabeshima et al. (1979) isolated the poly(A) mRNA from the polysomes and used centrifugation through a sucrose gradient to fractionate it into 7 size classes. These mRNAs were then translated using the wheat germ cell-free system. The extent of synthesis of each ribosomal protein by each of the 7 mRNA size classes was examined. The amount of stimulation by each of the 7 fractions was similar, except for the heaviest fraction, which was somewhat lower. Almost all ribosomal proteins were synthesized from mRNA smaller than 17S. Again, the results indicate that almost all, if not all, rat liver ribosomal proteins are synthesized by monocistronic mRNAs having lengths which are generally proportional to the molecular weights of the corresponding proteins. Similar results have been reported for the yeast Saccharomyces carlsbergensis (strain S74) (Mager and

Planta 1976) and for 36 to 68 Ehrlich ascites tumor cell ribosomal proteins encoded by poly(A) mRNA (Hackett et al. 1978). These observations are in agreement with Smal'ko and Platonov's (1977b) conclusions of the size of the mRNAs coding for ribosomal proteins.

1.5.5.3 Synthesis of ribosomal proteins following partial hepatectomy

Many investigations of ribosome biogenesis in regenerating rat liver have emphasized the rate and timing of biogenesis by monitoring the transcription and processing of rRNA, particularly 18S and 28S rRNA. Because neither ribosomal proteins nor RNA appear to be reutilized during ribosomal turnover, it is reasonable to examine 45S pre-rRNA or 18S and 28S rRNAs, a limited number of well identified, relatively easily isolated species of RNA, instead of 70 less well characterized proteins. In addition, rRNA and rRNA precursors are more abundant than mRNA and mRNA precursors, even in the nucleus. The synthesis of preribosomal 45S RNA may also be a measure of the availability of protective ribosomal proteins. Because ribosome biogenesis is a relatively complex event involving transcription of rRNA in the nucleolus, transcription of mRNA for ribosomal proteins in the nucleus, synthesis of ribosomal proteins in the cytoplasm, transport of the proteins to the nucleolus for ribosome assembly, and transport of the ribosomal subparticles to the cytoplasm, the timing of the appearance of incorporated radioactive entities into assembled cytoplasmic ribosomes may not always be a precise measure of their time of synthesis.

Using cyclohexamide to inhibit protein synthesis, Chaudhuri and

Lieberman (1968a) showed that the increase in rat liver ribosome biogenesis following partial hepatectomy is totally dependent on an increased rate of synthesis of one or more proteins. Because cycloheximide blocked all protein synthesis, but lowered ribosome production by only 65 per cent, they also concluded (probably incorrectly) that there is a large pool of particulate ribosomal protein in the liver.

Tsurugi and co-workers (1972) injected [$^3$H]leucine into control rats and partially hepatectomized animals 6, 12, 18, or 48 h postoperatively. The animals were sacrificed 1 h later. Maximal incorporation into liver ribosomal proteins was observed in the animals injected at 12 h of liver regeneration, when it was ten times that of control values. The increase in labeling was not due to a change in the size of the leucine pool of the regenerating liver.

Information concerning synthesis of individual ribosomal proteins following partial hepatectomy is far from definitive. Scheinbuks *et al.* in a 1974 report indicated that at least four ribosomal proteins appear to have altered synthesis rates in 1 d regenerating rat liver relative to 1 d sham operated rat liver. Using the nomenclature of Sherton and Wool (1972), they report that proteins S6 and L5 appear to be synthesized 50 per cent more slowly in 1 d regenerating rat liver than in sham operated rat liver, and that proteins L18 and L27 appear to be synthesized 67 per cent more slowly. However, they mention that the S6 data may not reflect its phosphorylation-dependent migration pattern, and the L18 and L27

data were variable.

Looking 0, 5, 12, 18, 28, and 96 h following partial hepatectomy in rats, Wu and co-workers (1977) reported that poly(A) mRNA for the ribosomal proteins they term S1 through S6, as measured in a wheat germ *in vitro* translation system, begins to increase immediately. The quantity of this mRNA reaches a maximum, 3 to 4 times the level of the control, 18 h postoperatively.

These diverse reports of ribosomal protein synthesis following partial hepatectomy in rats are not easily interpreted. The findings of Tsurugi *et al.* (1972) appear to be in good agreement with the reported observations of the postoperative synthesis of rat liver rRNA. Unfortunately, these latter reports are also difficult to interpret, because the timing of many of the observations has not resulted in well defined peaks of synthesis. Nucleolar RNA synthesis appears to reach a maximum between 10 and 18 h following surgery (Tsukada and Lieberman 1964; Muramatsu and Busch 1965); however, there appear to be no reports of nucleolar RNA synthesis between 10 and 18 h of liver regeneration. The former observation is in general agreement with reports that synthesis of rRNA, as measured by the presence of 18S and/or 28S rRNA in cytoplasmic ribosomes, appears to be maximum at about 12 h following partial hepatectomy (Chaudhuri *et al.* 1967; Fausto and Van Lancker 1968; Tsurugi *et al.* 1972). The increase in ribosome synthesis, as monitored by the incorporation of radioactive nucleotides into rRNA, may not be an accurate reflection of the timing of ribosomal protein synthesis.

## 1.5.6 Histones

### 1.5.6.1 Histones description

Histones comprise the second major group of basic intracellular liver proteins; they contain an average of 25 per cent basic amino acids. Histones have been defined as "basic proteins that at some time are associated with DNA in somatic cells and cells in the early stages of spermatogenesis" (De Lange and Smith 1979). Most tissues have 5 major different types of histones. In liver these histones are: histone 1 or H1, histone 2A or H2A, histone 2B or H2B, histone 3 or H3, and histone 4 or H4. Histones have been classified as arginine rich or lysine rich on the basis of the major basic amino acids present. The resulting molecular properties have been utilized in some isolation techniques.

Histone 1, the largest histone ($M_r$ about 22 000), is very lysine rich with 28.7 per cent lysine and 1.7 per cent arginine (Hnilica 1972). Histone 1 shows the greatest variation in composition from species to species. Even within a specific tissue, histone 1 tends to have several subfractions which differ in amino acid sequence at certain positions and sometimes in the O-phosphoserine content; these differences can be distinguished immunologically (De Lange and Smith 1979). Histone 2B ($M_r$ about 13 800) has been termed moderately lysine rich or lysine-serine rich; it has 16.7 per cent lysine, 6.4 per cent arginine, and 10.9 per cent serine (Hnilica 1972). Histone 2A ($M_r$ about 14 000) has been classified as slightly lysine rich, arginine-lysine rich, or intermediate; it has 12.5 per cent lysine and 9.3 per cent arginine

(Hnilica 1972).

Histone 4 ($M_r$ about 11 300) is the smallest histone and has been termed arginine rich or glycine-arginine rich; it has 9.8 per cent lysine, 13.9 per cent arginine, and 15.9 per cent glycine (Hnilica 1972). In addition, histone 4 shows the greatest conservation of sequence throughout evolution (De Lange and Smith 1979). Histone 3 ($M_r$ about 15 300) has been classified as arginine rich or alanine-arginine rich; it has 10.0 per cent lysine, 13.6 per cent arginine, and 13.5 per cent alanine. Histone 3 is the only histone containing cystine (Hnilica 1972) and has the second greatest conservation of sequence (De Lange and Smith 1979).

Histones probably contain the highest proportion of acetylated, phosphorylated, or methylated amino acids of any proteins, thiolation is also present. These amino acid modifications result in subfractions of each histone. It is thought that changes in these amino acid modifications occur in a regular manner during the cell cycle and may be involved when histones become dissociated from or associated with DNA, allowing or disallowing its replication or transcription, and in facilitating transfer of histones from the cytoplasmic site of synthesis into the nucleus (De Lange and Smith 1979). Because it has been demonstrated that not all the molecules of a specific histone fraction are completely modified at even one site, it is likely that modifications of histone amino acids have a functional role(s) (De Lange and Smith 1979). Histones are usually synthesized during the S phase of the cell cycle (De Lange and Smith 1979).

Histones are associated with DNA in an approximately one to one mass ratio. They probably have several functions, including being structural components of chromatin, DNA protective proteins, cationic neutralizers of the DNA phosphate groups, and repressors of replication and/or transcription (De Lange and Smith 1979). The best elucidated of these roles is that of being structural components allowing DNA to fold efficiently, forming nucleosomes approximately 10 nm in diameter (Kornberg 1977). Each nucleosome is comprised of about 200 base pairs, 140 of which are wrapped around an octomer composed of 2 molecules of each of histone 2A, histone 2B, histone 3, and histone 4. These units are linked by the remaining 60 base pairs. Histone 1 appears to be associated with the 30 base pairs of these linker regions closest to the histone octomer associated DNA (Kornberg 1977). Like ribosomes, nucleosomes are not static; there is some turnover of histones in quiescent cells.

There has been limited research on the synthesis of histones in regenerating rat liver. The reported investigations tend to have measured histone synthesis by monitoring the appearance of nuclear, DNA associated histone.

1.5.6.2 Synthesis of histones following partial hepatectomy

In 1962 Holbrook, Evans, and Irvin published a paper about the appearance of chromatin associated histones in the liver following removal of only one-third of the liver (rather than the more customary removal of two-thirds). They isolated three histone fractions on the basis of acid solubility and extractability in different concentrations of NaCl. [1-$^{14}$C]glycine was injected 1 h

prior to sacrifice of the rats 0, 15, 19, 22, 25.5, 26, 29.5, 31, 33, 37, or 42.5 h postoperatively. By 15 h of liver regeneration, an increase in histone synthesis was noted in all fractions. One of the fractions exhibited a sharp peak in specific activity at 19 h. The other two had broader peaks of specific activity at 19 to 22 h. The radioactive incorporation values at these times were 3.4 to 5.8 fold greater than the control values. A second, smaller, peak of synthesis was noted at 37 to 43 h of liver regeneration.

Injecting rats with L-[$^{14}$C]lysine 30 min prior to death and observing at 0, 1, 6, 10, 12, 18, and 22.5 h following partial hepatectomy, Butler and Cohn (1963) noted that between 6 and 10 h of liver regeneration, the relative specific activity of liver histones decreased to less than pre-operative values. The relative specific activity of histones then rose to above average between 12 and 22.5 h, peaking at approximately 16 h postoperatively.

Takai and co-workers (1968) labeled rat liver histones *in vivo* with [$^{3}$H]leucine, which was found to be a better histone labeler than [$^{3}$H]arginine or [$^{3}$H]lysine, for 1 h prior to sacrifice 8, 12, 16, 19, or 28 h after partial hepatectomy. No increase in histone synthesis was noted until 16 h of liver regeneration. Maximum synthesis was observed to occur at 19 h, in agreement with Holbrook *et al.* (1962).

Orlova and Rodionov (1970) separated rat liver histones on the basis of their being lysine rich (histone 1, histone 2A, and histone 2B) or arginine rich (histone 3 and histone 4). They injected [2-$^{3}$H]leucine 30 min prior to death 6, 12, 15, 18, 24, or or 28.3 h following partial hepatectomy. No increase in the

labeling of the lysine rich histones was observed during the period examined.

Orlova (1971) refined this work by using methodology more specific for isolating DNA associated histones. The rats were injected with [2-$^{14}$C]glycine 30 min before sacrifice at 12.5, 14, or 15 h postoperatively. This time a five fold increase in total histone synthesis was noted at 12 h of liver regeneration. By 15 h, when there was a 7.6 fold increase in histone synthesis, the rate of synthesis was still increasing.

In 1971, Gutierrez-Cernosek and Hnilica reported on the incorporation in rat liver of L-[$^{14}$C]lysine, injected 1 h before death, into each of the five histones. Observations, corrected for the lysine content of the different histones, at 0, 2, 4, 6, 12, 18, 24, 30, or 36 h postoperatively showed that radioactive incorporation increases gradually from the time of partial hepatectomy. The specific activity of histone 3 reached a maximum at 24 h, and the other four histones reached a maximum at 30 h of liver regeneration. They also reported that the corrected specific activity of the very lysine rich histone 1 and of the arginine rich histone 3 is approximately twice that of the slightly lysine rich histone 2A and histone 2B and of the arginine rich histone 4.

These findings are in contrast to Orlova and Rodionov (1970), who noted increased specific activity only in the arginine rich histones. It is of interest that like Holbrook et al. (1962) and Orlova and Rodionov (1970), Gutierrez-Cernosek and Hnilica (1971) observed that not all the histones become associated with DNA in a

synchronous manner. This phenomenon may be indicative of differential synthesis, transport from the cytoplasm to the nucleus, or formation of the DNA-histone ionic bonds.

Examining changes in the mass proportions of rat liver chromatin proteins 0, 1.5, 3, 8, 12, 14, 18, 20, 25, and 44 h following partial hepatectomy Garrard and Bonner (1974) observed a 7 per cent decrease in the histone:DNA mass ratio during the first 8 h of liver regeneration. Although small, this decline was statistically significant at the 95 per-cent confidence limit. The time of 8 h correlates well with the observation of Butler and Cohn (1963) that labeling of histones declined to below normal values between 6 and 10 h postoperatively.

Garrard and Bonner (1974) then investigated the possibility that the 7 per cent decrease might be due to preferential declines of some histone species. Their data suggest that the decline results from a proportional decline in all the histone species. To examine the synthesis of histones, they used *in vivo* labeling with L-[4,5-$^{3}$H]lysine between 2.5 and 4 h after partial hepatectomy, when the animals were sacrificed. They observed that the labeling of histones 2A and 2B did not change significantly in response to partial hepatectomy. In contrast, the labeling of histone 1, histone 3, and histone 4 increased by 23, 81, and 19 per cent respectively. Except for histone 1, the histones whose synthesis is affected are in agreement with Orlova and Rodionov (1970). However, the two groups report different timing of the increase in synthesis. Using several criteria, Garrard and Bonner (1974) also found that

histone 1 showed a considerable turnover during the first 12 h of liver regeneration.

Smirnova and Rodionov (1974) examined the change in incorporation of [$^{14}$C]glycine into rat liver histone fractions 1, 2A and 4, 2B, and 3 at 5.5, 9.5, 11, 12.5, 14, 15.5, 17, 19, 21, 23, 25, and 27 h following partial hepatectomy. The radioactive amino acid was injected 30 min prior to animal sacrifice. The rate of incorporation of [$^{14}$C]glycine into all the histone fractions began to increase at 12.5 h of liver regeneration, and incorporation peaks were noted at 14 to 15 h and 23 h.

Rapoport and Khasigov (1975) used long labeling times; [$^{14}$C]lysine was injected 24 h prior to partial hepatectomy. They report that differential rates of rat liver histone synthesis persist at 24, 48, and 72 h postoperatively. Rapoport and Khasigov (1975) agree with the finding of Garrard and Bonner (1974) that following partial hepatectomy, histone 1 undergoes the greatest change in rate of synthesis. The specific activity of histone 1 decreased relative to an increase in the specific activity of histones 2A, 4, 2B, and 3. Because Rapoport and Khasigov administered large amounts of unlabeled amino acids during regeneration, and the specific activity of the nonhistone proteins remained constant, it is unlikely that the observations can be accounted for by reutilization of labeled amino acids. However, it is possible that previously labeled histones become associated with DNA during regeneration.

Although there is not a precise consensus on the synthesis of

rat liver histones following partial hepatectomy, some probably reliable observations can be made. Early in the postoperative period, approximately the first 10 to 12 h, synthesis of histones which have become associated with nuclear DNA is limited (Takai et al. 1968; Orlova and Rodionov 1970; Gutierrez-Cernosek and Hnilica 1971; Smirnova and Rodionov 1974) and may in fact decrease to below control values between 6 and 10 h (Butler and Cohn 1963; Garrard and Bonner 1974). There are probably at least two peaks of histone synthesis, the first at 14 to 20 h postoperatively (Holbrook et al. 1962; Butler and Cohn 1963; Takai et al. 1968; Smirnova and Rodionov 1974) corresponding to the initiation of DNA synthesis and the second at about 24 h (Orlova and Rodionov 1970; Gutierrez-Cernosek and Hnilica 1971; Smirnova and Rodionov 1974) corresponding to maximum DNA synthesis. It is possible that, as some researchers have noted, at some or all times of liver regeneration, the various histone fractions are synthesized at different rates (Holbrook et al. 1962; Orlova and Rodionov 1970; Gutierrez-Cernosek and Hnilica 1971; Rapoport and Khasigov 1975).

There are no major contradictions between the reported findings of the transcription and mRNA studies and the ribosomal and histone research in regenerating rat liver.

## 1.6 Statement of the problem

Much of the information presented here concerning the synthesis of the two major groups of basic proteins, histones and some ribosomal proteins, in rat liver following partial hepatectomy was

unavailable in 1976 at the commencement of the investigations presented here. The aim of the research was to examine several aspects of hepatic mRNA during the first 10 h following partial hepatectomy. Three questions were posed: 1) Does the amount of mRNA being translated change as a function of time following partial hepatectomy? 2) Does the rate of translation of the mRNA being translated vary as a function of time following partial hepatectomy?, and 3) Does the proportion of basic proteins being synthesized change as a function of time following partial hepatectomy?

To examine all the mRNA being translated, it was decided to use polysomes, instead of the more commonly used purified mRNA, as substrate for an *in vitro* translation assay. This decision necessitated the characterization of the assay with polysomes as substrate. It is hoped that the results of the investigations will provide some insight into the molecular aspects of controlled, rapid, normal (non-neoplastic) growth.

# 2. MATERIALS AND METHODS

## 2.1 Reagents

Chemicals were purchased as follows.

Calbiochem: some unlabeled amino acids for trichloroacetic acid (TCA) precipitable counts, spermine

Difco Laboratories: Casamino acids

Eastman Chemical Company: acrylamide, Coomassie brilliant blue, N, N'-methylene-bis-acrylamide, N, N, N', N'-tetraethylmethyl-ethyldiamine (TEMED)

Kodak: X-Omat R XR-1 film, developer and fixer for X-Omat R XR-1 film.

New England Nuclear: radioactive amino acids, Aquasol 2, Enhance, Omnifluor, Protasol, Scintiverse

Pharmacia: Sephadex G-25 medium

Picker Nuclear: ribonuclease-free sucrose

Sigma: unlabeled amino acids for translation assays, some unlabeled amino acids for TCA precipitable counts, ATP, boyine serum albumin, bromphenol blue, creatine phosphokinase, carboxymethyl-cellulose (CM-cellulose), deoxycholate, DL-dithiothreitol (DTT), glycerol, glycine, GTP, heparin, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), lysozyme, 2-mercaptoethanol, phosphocreatine, poly U, polyvinylsulfate, puromycin, sodium dodecylsulfate (SDS), trichloroacetic acid (TCA),

tris(hydroxymethyl)aminomethane (Tris, [Trizma base]), Triton X-100 BDH Chemicals, Canlab, or Fisher Scientific Company Limited: all other chemicals

2.2 Isolation of polysomes (polyribosomes)

2.2.1 Buffer A for isolation of polysomes

10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), pH 7.4 at room temperature; 100 mM KCl, 250 mM ribonuclease-free sucrose, 5 mM $Mg(OAc)_2$, 0.1 mM ethylenediaminetetraacetic acid disodium salt ($EDTA\text{-}Na_2$) in autoclaved distilled or reverse osmosis water

2.2.2 Buffer A without sucrose

10 mM HEPES, pH 7.4 at room temperature; 100 mM KCl, 5 mM $Mg(OAc)_2$, 0.1 mM $EDTA\text{-}Na_2$ in autoclaved distilled or reverse osmosis water

2.2.3 Modified Buffer A for isolation of polysomes

Buffer A containing 200 µg/mL heparin

2.2.4 Other reagents

15% (w/v) deoxycholate

2.0 M ribonuclease-free sucrose in Buffer A

0.5 M ribonuclease-free sucrose in Buffer A

10% (w/v) Triton X-100

2.0 M ribonuclease-free sucrose in modified Buffer A

0.5 M ribonuclease-free sucrose in modified Buffer A

2.2.5 Method

To minimize ribonuclease contamination, autoclaved glassware (20 psi for 25 min) was used in all situations.

Female Sprague-Dawley rats bred and raised by the Animal Care Facility, Health Sciences Centre, Memorial University of Newfoundland and weighing 150 to 200 g were used in all experiments. Following cervical dislocation, each rat was decapitated with a guillotine. The liver was excised using autoclaved scissors and disposable plastic gloves to minimize ribonuclease contamination. It was then placed immediately in cold Buffer A.

All remaining procedures were performed at 0 to 4°C. The livers were rinsed in Buffer A to remove blood, minced with the scissors, and homoginized with 2.5 volumes of Buffer A in a precooled glass tissue grinder with a motor driven, teflon pestle (3 strokes). The homogenate was centrifuged at 20 000 g for 15 min in a Sorval centrifuge. A one-tenth volume of 15% (w/v) deoxycholate, to solubilize membranes, was added to the supernatant which was then layered on top of discontinuous sucrose gradients. The gradients were constructed as follows: bottom layer--2.0 M ribonuclease-free sucrose in Buffer A [2.25 mL or 6.5 mL], middle layer--0.5 M ribonuclease-free sucrose in Buffer A [3.00 mL or 8.75 mL], top layer--deoxycholate treated supernatant [3.5 mL or 10.0 mL]. Prior to use, the centrifuge tubes were autoclaved or thoroughly rinsed with autoclaved distilled or reverse osmosis water. A Beckman 65 rotor was used with the smaller volumes, and a Beckman 45 or 50.2 Ti rotor was used with the larger volumes. Centrifugation was at 165 000 g for 3 h at 4°C. After centrifugation, the polysomes are present as pellets at the bottom of the tubes. The overlaying lipid at the top surface; the top, ferritin-containing

sucrose layer; the membranes at the sucrose-sucrose interface; and the lower sucrose layer were removed with a Pasteur pipette. If the polysomes were not to be used immediately, the interior of the centrifuge tube was carefully rinsed with Buffer A without sucrose to remove remaining sucrose prior to storage at -70°C.

2.3 Preparation of ribosomal subparticles

2.3.1 Buffer B for solubilizing polysomes

20 mM tris(hydroxymethyl)aminomethane (Tris), pH 7.8 at room temperature; 500 mM KCl, 1.5 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 10 µg/mL polyvinylsulfate

2.3.2 Other reagents

10 mM puromycin

2.3.3 Method

Polysomes were isolated as described above. Buffer B, 0.5 mL per g liver from which the polysomes were isolated, was added to the polysome pellet. The pellet was then solubilized by gentle stirring for 1 h at 0 to 4°C. The polysome-buffer mixture was centrifuged at 27 000 g at 4°C for 10 min in a Sorvall centrifuge. A 0.01 volume of 10 mM puromycin was added to the supernatant (final concentration 0.01 mM puromycin). The mixture was kept at 0°C for 15 min, incubated at 37°C for 15 min and centrifuged at 27 000 g at 20°C for 10 min. The supernatant was kept and considered to contain intact ribosomal subparticles.

## 2.4 Preparation of whole ribosomes

### 2.4.1 Reagents

1 M $Mg(OAc)_2$

ethanol

### 2.4.2 Method

Ribosomal subparticles were prepared as described above. The supernatant was made 20 mM in $Mg^{++}$ by the addition of 0.02 volumes of 1 M $Mg(OAc)_2$. An equal volume of cold ethanol was added to the supernatant-$Mg^{++}$ mixture, and the resulting solution was kept at $0^{\circ}C$ for 15 to 60 min. Whole ribosomes, monosomes, were pelleted by centrifugation at $4^{\circ}C$ and 10 000 g for 10 min in a Sorval centrifuge.

## 2.5 Preparation of ribosomal proteins

### 2.5.1 Buffer C for separating ribosomal proteins

10 mM Tris, pH 7.7 at room temperature; 100 mM $Mg(OAc)_2$

### 2.5.2 Other reagents

glacial acetic acid (HOAc)

acetone

### 2.5.3 Method

Ribosomal proteins were prepared by isolating polysomes as described above; forming ribosomal subunits as described above, except that incubation with puromycin at $37^{\circ}C$ was for a minimum of 30 min; and preparing intact ribosomes as described above, except that incubation with $Mg(OAc)_2$ at $0^{\circ}C$ was for a minimum of 60 min. After centrifugation of the ribosomes, they were gently suspended in Buffer C to a maximum concentration of 5-6 mg/mL (50-60 $A_{260}$/mL); and

2 volumes of glacial acetic acid were then added. The solution was stirred for 60 min at 0°C followed by centrifugation at 12 000 g for 10 min in a Sorvall centrifuge to remove RNA. The supernatant was saved, and the pellet was washed with 2 pellet volumes of Buffer C:glacial acetic acid (1:2) and centrifuged at 12 000 g for 10 min in a Sorvall centrifuge. This supernatant was combined with the previous supernatant, and the ribosomal proteins were precipitated overnight at 4°C by adding 9 volumes of acetone. The ribosomal proteins were then pelleted by centrifugation at 7 500 g for 8 min in a Sorvall centrifuge.

2.6 Testing polysome integrity

2.6.1 Buffer D for solubilizing polysomes

10 mM Tris, pH 7.4 at room temperature; 50 mM KCl, 10 mM $MgSO_4$ (Parish 1972).

2.6.2 Buffer E for solubilizing polysomes

10 mM HEPES, pH 7.4 at room temperature; 100 mM KCl; 0.1 mM EDTA-$Na_2$, 5 mM 2-mercaptoethanol, 10 µg/mL polyvinylsulfate; and 1, 3 or 5 mM $Mg(OAc)_2$

2.6.3 Other reagents

15% ribonuclease-free sucrose in Buffer D

30% ribonuclease-free sucrose in Buffer D

10% ribonuclease-free sucrose in Buffer E

40% ribonuclease-free sucrose in Buffer E

2.6.4 Method

Autoclaved glassware (20 psi for 25 min) was used to minimize

exogenous ribonuclease.

Using a Beckman 65 rotor, fresh (unfrozen) polysomes were prepared as described above. After centrifugation to pellet the polysomes, the supernatant was removed using a Pasteur pipette, and the interior of each ultracentrifuge tube was carefully rinsed with Buffer D or Buffer E to remove traces of sucrose. The polysomes were then gently solubilized in 0.5 mL of the same buffer at 4°C. Absorbance at 235, 240, 260, 280, and 320 nm was measured. Ideal polysomes have $A_{240} = A_{280}$ and $A_{280}/A_{260} \geq 1.7$. Then 1, 3, or 5 corrected absorbance units [corrected absorbance units = $A_{260} - 1.51\ A_{320}$ (P. Tas, personal communication)] were layered on continuous, linear sucrose gradients, 15-30% sucrose in Buffer D or 10-40% sucrose in Buffer E, in cellulose nitrate tubes. The gradients were then centrifuged using a Beckman SW 27.1 rotor at 8,000 g for 18 to 19 h at 20°C. After centrifugation, the tubes were punctured at the bottom and the absorbance of the contents was monitored by an Isco gradient fractionator with a 280-310 nm filter and recorded by a strip chart recorder.

To confirm the identification of the polysome peaks, ribosomal subparticles and intact monosomes were prepared as described above, and absorbance at 235, 240, 260, 280, and 320 nm were measured. Three or 5 similarly corrected absorbance units of ribosomes were layered on linear 10-40% sucrose gradients in Buffer E containing 3 mM $Mg(OAc)_2$ in cellulose nitrate tubes. These gradients were centrifuged and analyzed concurrently with the polysome gradients.

## 2.7 Partial hepatectomy

Both before and after the operation, all rats were allowed to eat standard rat chow as supplied by Faculty of Medicine Animal Care Facility and to drink water *ad libitum*. Partial hepatectomies were performed according to the method of Higgins and Anderson (1931). The operations were carried out using ether as an anesthetic. A median line incision was made, and prior to excision, the left lateral and the median lobes were tied off using a slip knot made of unwaxed dental floss. The body cavity was closed by sewing with surgical silk; the skin was closed with surgical staples.

For each series of rats whose polysomes were to be examined as a function of time following partial hepatectomy, litter mates were used to minimize possible effects of heredity. The partial hepatectomies on these rats were performed between 0500 and 0730 local time. Polysomes isolated from the liver of a non-anesthetized rat or from the partially-hepatectomized liver of siblings, depending on the population of each litter, were considered to be polysomes from nonoperated, or time zero, animals. Sham operation (anesthesia, opening of the peritoneal cavity, handling of the liver, and closure of the incision) were not performed, because of limiting numbers of animals and reports by many researchers that the macromolecular effects observed after partial hepatectomy appear to be induced by the removal of a portion of the liver rather than by the operation itself (Bucher and Malt 1971).

## 2.8 Wheat germ in vitro translation assay

Because the wheat germ in vitro translation system was satisfactory, it was unnecessary to use a reticulocyte lysate system. Advantages of the former system included probable higher specific activity of the translation products, time required for characterization, and cost.

### 2.8.1 Master mix

Autoclaved glassware and micropipette tips (20 psi for 25 min) and autoclaved reverse osmosis or distilled water were used in all situations. After mixing, all solutions were stored in aliquots at -70°C.

The 16 amino acids: L-aspartic acid, L-asparagine, L-arginine, L-alanine, L-glutamic acid, L-glutamine, L-cysteine, L-isoleucine, L-histidine, L-glycine, L-phenylalanine, L-threonine, L-serine, L-proline, L-valine, and L-lysine were made 5 mM in a final volume of 100 mL of 1 mM dithiothreitol (DTT) which had been neutralized to pH 7.0 at room temperature with solid $KHCO_3$. A similar mixture without L-leucine was also prepared.

The following amino acid solutions were made in 1 mM DTT and neutralized to pH 7 with solid $KHCO_3$: 5 mL of 50 mM L-methionine, 5 mL of 50 mM L-leucine, 10 mL of 25 mM L-tryptophan, and 10 mL of 2 mM L-tyrosine. Using these solutions, 1 mL of a mix which was 0.5 mM with respect to each of 19 amino acids [L-leucine, L-lysine, or L-methionine was eliminated] or to all 20 amino acids was made.

In addition, the following stock solutions were made: 1.0 M HEPES, pH 7.6 at room temperature (pH adjusted with solid KOH);

2.0 M KOAc, 1.0 M $Mg(OAc)_2$, 1.0 M DTT, 0.2 M ATP-$Na_2$, 0.05 M GTP(Na salt), 0.05 M spermine; all, except the HEPES, were neutralized to pH 7 with solid $KHCO_3$ at room temperature; 0.5 M phosphocreatine (Na salt) neutralized to pH 7 with HCl, 5 mg/mL creatine phosphokinase in autoclaved 50% glycerol and neutralized to pH 7 with solid $KHCO_3$. Again, each solution was stored in small aliquots at -70°C.

The preceding ingredients were combined in a "master mix" in the following quantities/200 µL master mix:

| component | µL |
|---|---|
| 1.0 M HEPES, pH 7.6 | 25.0 |
| 2.0 M KOAc | 62.5 |
| 1.0 M $Mg(OAc)_2$ | 3.12 |
| 1.0 M DTT | 2.5 |
| 0.2 M ATP | 6.25 |
| 0.05 M GTP | 0.5 |
| 0.5 M phosphocreatine | 20.0 |
| 5 mg/ml creatine phosphokinase | 10.0 |
| 0.05 M spermine | 1.25 |
| 0.5 mM 19 amino acid mix | 62.5 |
| (20 amino acid mix) | (12.5) |
| water | 6.38 |
| | (56.38) |

The translation assay, in a final volume of 50 µL, contained 8 µL of the master mix, contributing 20 mM HEPES, 100 mM KOAc, 2.5 mM $Mg(OAc)_2$, 2 mM DTT, 1 mM ATP, 0.02 mM GTP, 8 mM phosphocreatine, 0.04 mg/mL creatine phosphokinase, 0.05 mM spermine, and 0.025 mM (0.005 mM) of each unlabeled amino acid.

2.8.2 Wheat germ S-30 fraction

2.8.2.1 Buffer F for grinding wheat germ

20 mM HEPES, pH 7.6 at room temperature; 100 mM KCl, 1 mM $Mg(OAc)_2$, 2 mM $CaCl_2$, 6 mM 2-mercaptoethanol

2.8.2.2 Buffer G for use with Sephadex G-25 column to obtain wheat germ S-30 fraction

20 mM HEPES, pH 7.6 at room temperature; 120 mM KCl, 5 mM $Mg(OAc)_2$, 6 mM 2-mercaptoethanol

2.8.2.3 Method

All labware was autoclaved (20 psi for 25 min) prior to use.

The wheat germ S-30 extract was prepared basically according to the method of Roberts and Paterson (1973). Equal weights, 6 g, of wheat germ and autoclaved ground glass were ground together with a cold mortar and pestle for 60 s. Then 28 mL of cold Buffer F was added slowly with swirling for 60 s. The resulting paste was centrifuged at 30 000 g at 2°C for 12 min. The supernatant (wheat germ S-30 fraction) was loaded onto a Sephadex G-25 (medium) column (2.5 x 30 cm) equilibrated at 5°C with Buffer G. The flow rate was adjusted to 1.3 to 1.4 mL/min. The column was rinsed with Buffer G, and 2 mL fractions were collected. The fractions containing the highest turbidity were dropped into liquid nitrogen; the frozen drops were stored at -70°C.

The final *in vitro* translation assay contained 15 µl ($\sim$1.1 $A_{260}$) of the above wheat germ S-30 fraction, contributing 6 mM HEPES, 36 mM KCl, 1.5 mM $Mg(OAc)_2$, and 1.8 mM 2-mercaptoethanol.

2.8.3 Preparing polysomes for translation

2.8.3.1 Buffer H for solubilizing polysomes for translation

10 mM HEPES, pH 7.6 at room temperature; 15 mM KOAc, 2 mM $Mg(OAc)_2$, 0.1 mM $EDTA\text{-}Na_2$

2.8.3.2 Modified Buffer H for solubilizing polysomes for translation

10 mM HEPES; pH 7.6 at room temperature, 15 mM KOAc, 2 mM $Mg(OAc)_2$

2.8.3.3 Method

Polysomes used for the initial characterization of the in vitro translation system were prepared as described above. Polysomes used in later experiments were prepared as described above, except that modified Buffer A, 0.5 M sucrose in modified Buffer A, 2.0 M sucrose in modified Buffer A, and 10% (w/v) Triton X-100 were used instead of Buffer A, 0.5 M sucrose in Buffer A, 2.0 M sucrose in Buffer A, and 15% (w/v) deoxycholate respectively. The rinsed polysomes were stored at -70°C prior to use. The frozen polysomes were found to retain their suitability for translation.

Initially, the polysomes for translation were gently suspended in approximately two pellet volumes of Buffer H for a maximum of 15 min. Later experiments used modified Buffer H in place of Buffer H; and the final in vitro translation assay contained 20 µL of the polysome mixture in modified Buffer H, contributing 2.7 mM HEPES, 4 mM KOAc, and 0.5 mM $Mg(OAc)_2$.

2.8.4 Wheat germ in vitro translation assay

Each 50 µL translation assay was composed of 8 µL master mix,

15 μL wheat germ S-30 fraction, 2 μL $H_2O$, 5 μL radioactive amino acid(s) (5-10 μCi), and 20 μL of polysomes. Thus, the final translation assay contained 29 mM HEPES, pH 7.6 at room temperature; 140 mM $K^+$, 3.0 mM $Cl^-$, 108 mM $OAc^-$, 4.5 mM $Mg^{++}$, 2.0 mM DTT, 1.0 mM ATP, 0.02 mM GTP, 8.0 mM phosphocreatine, 0.04 mg/mL creatine phosphokinase, 0.05 mM spermine, 0.02 mM of each unlabeled amino acid, and 1.8 mM 2-mercaptoethanol.

*In vitro* translations were performed as follows: Small borosilicate, disposable glass culture tubes (10 x 75 mm) or 1.5 mL polypropylene microfuge tubes were placed on ice, and 8 μL master mix, 15 μL wheat germ S-30 fraction, and 2 μL water were placed sequentially into each tube. This mix was then incubated at 34°C for 7 min and rapidly cooled. This incubation step was omitted after initial characterization of the *in vitro* translation assay. Next, 5 μL of radioactive amino acid, containing 6 μCi L-[$^{35}$S]methionine in the final translations, followed by 20 μL of polysomes solubilized in Buffer H or modified Buffer H were added. Translations were carried out at 25°C for 60 min.

Translations of polysomes present at different times following partial hepatectomy, to be analyzed by CM-cellulose chromatography, were performed simultaneously for each litter of rats.

## 2.9 Detection of translation

Translation was determined to have occurred on the basis of radioactivity of trichloroacetic acid (TCA) precipitable material. During translation, small glass test tubes containing 10 μL of 10 mg/mL bovine serum albumin as carrier protein, 10 μL of 10 mg/mL unlabeled

amino acid corresponding to the radioactive amino acid, and 1 mL of water were prepared and kept at 0°C. Casamino acids were used when a radioactive amino acid mix was utilized for translation. After translation, 5 or 10 µL from an assay was added to a test tube. The test tube's contents were mixed prior to the addition of 110 µL of 100% (w/v) TCA to precipitate the protein. The samples were then placed in a water bath at 90°C for 20 min. They were next cooled, and the precipitates were collected on glass fiber Reeve-Angel filters, using a Millipore vacuum filtration manifold. The test tubes were rinsed with 5% (w/v) TCA containing 1 mg/mL unlabeled amino acid, and the filters were rinsed with ethanol and lamp dried for 10 min. The filters were then placed in glass scintillation vials, and 1 mL Protosal was added to solubilize the proteins and thereby increase counting efficiency. The vials were capped with teflon-lined caps and incubated at 60°C for at least 20 min. Then 50 or 46 µL of glacial acetic acid, to optimize the pH, and 10 or 9 mL, respectively, of toluene-Omnifluor were added to each vial. Finally, the samples were counted for 10 min by a Beckman liquid scintillation counter.

## 2.10 Laemmli gels, for separating in vitro translation products

### 2.10.1 Stock solutions

#### 2.10.1.1 Solution J-I

30% acrylamide (w/v), 0.8% N, N'-methylene-bis-acrylamide (w/v)

#### 2.10.1.2 Buffer J-II (4X lower Tris)

1.5 M Tris, pH 8.8 at room temperature, 0.4% sodium dodecylsulfate (SDS) (w/v)

2.10.1.3 Buffer J-III (4X upper Tris)

0.5 M Tris, pH 6.8 at room temperature, 0.4% SDS (w/v)

2.10.1.4 Buffer J-IV (sample buffer)

2.10.1.4.1 Composition

62.5 mM Tris, pH 6.8 at room temperature, 3% electrophoresis grade SDS (w/v), 5% 2-mercaptoethanol (v/v), 10% glycerol (v/v)

2.10.1.4.2 Preparation

Buffer J-IV was prepared by mixing 12.5 mL Buffer J-III, 30 mL 10% electrophoresis grade SDS (w/v), 5 mL 2-mercaptoethanol, 10 mL glycerol, and adding water to a total volume of 100 mL.

2.10.1.5 Buffer J-V (8X Tris-glycine reservoir buffer)

200 mM Tris, 1.5 M glycine

2.10.2 Working solutions prepared immediately prior to use

2.10.2.1 Solution J-VI

7.5% ammonium persulfate (w/v)

2.10.2.2 Solution J-VII (10% acrylamide running gel)

The running gel was prepared by mixing 33.3 mL Solution J-I, 25 mL Buffer J-II, and 40.2 mL water; adding 1.5 mL Solution J-VI; and mixing well. Next 25 µL N,N,N',N'-tetraethylmethylethyldiamine (TEMED) were added, and the resulting solution was thoroughly mixed. The gels were poured immediately and overlaid with Buffer J-V:water (7:1).

2.10.2.3 Solution J-VIII (3% acrylamide stacking gel)

The stacking gel was prepared by mixing 1.0 mL Solution J-I, 2.5 mL Buffer J-III, 6.34 mL water; adding 150 µL Solution J-VI and mixing well; and adding 10 µL TEMED and mixing well. The gels were poured immediately, and a 12-sample comb was inserted.

2.10.2.4 Buffer J-IX (electrophoresis reservoir buffer)

2.10.2.4.1 Composition

25 mM Tris, 375 mM glycine, 0.1% SDS (w/v)

2.10.2.4.2 Preparation

The electrophoresis reservoir buffer was prepared by mixing 12.5 volumes Buffer J-V, 1 volume 10% SDS (w/v), and 86.5 volumes water.

2.10.3 Buffer K for solubilizing proteins

10 mM Tris, pH 7.5 at room temperature; 10 mM KCl, 1.5 mM $Mg(OAc)_2$

2.10.4 Method

One-dimensional, sodium dodecylsulfate (SDS) polyacrylamide gels, following the method of Laemmli (1970), as described above, were used to separate the *in vitro* translation products. Ten volumes of cold acetone were added to each of one or more translation assays, and the proteins were allowed to precipitate overnight at -20°C. The precipitated material was then centrifuged at 3 000 g for 5 min at 4°C in a Sorvall centrifuge, and the pellet was solubilized in a volume of Buffer J-IV equal to the total initial translation assay volume. The samples were quickly heated to 90°C for 2 min, and 5 µL of 0.1% bromphenol blue (w/v) tracking dye in water was added to each sample.

In some instances, each translation assay was placed into a small ultracentrifuge tube, and its volume was adjusted to 1 mL with Buffer K. It was then centrifuged at 145 000 g for 1.5 h at 4°C to sediment ribosomal subparticles. Ten volumes of cold acetone were

added to the supernatant, and the proteins were allowed to precipitate overnight at -20°C prior to centrifugation and subsequent steps as detailed above.

A Hoeffer gel electrophoresis apparatus with 1.5 mM thick gels was used with a 100 mm, 10% acrylamide running gel and a 7 mm, 3% acrylamide stacking gel. The electrophoresis reservoir buffer, J-IX, was placed in the upper gel chamber, and a microsyringe was used to underlay the samples. Electrophoresis was run from positive to negative at 50 V until the tracking dye entered the running gel and at 150 V until the tracking dye reached the lower edge of the gel.

Gels to be stained were placed in 0.5% Coomassie brilliant blue (w/v), 25% 2-propanol (v/v), and 10% glacial acetic acid (v/v) overnight. They were destained with 25% 2-propanol (v/v) and 10% glacial acetic acid (v/v).

Some gels for autofluorography were prepared prior to drying by successive soaking in 10% glacial acetic acid (v/v) and 3% glycerol (w/v) overnight, twice in previously used dimethylsulfoxide (DMSO) for 1 to 1.5 h, fresh DMSO for 1 to 1.5 h, 20% Omnifluor (w/v) in DMSO for 1 to 1.5 h, and 10% glacial acetic acid (v/v) and 3% glycerol (w/v) overnight, including at least one change of the last solution.

Other gels for autofluorography were prepared for drying by soaking (covered) in Enhance for 2 h, soaking in cold water for 2 h, and soaking in 10% glacial acetic acid (v/v) and 3% glycerol (w/v) for a minimum of 2 h.

The gels were dried for 2 h using a Hoeffer gel drier. The gels were then allowed to expose Kodak X-Omat R X-ray film at -70°C. The

autofluorograms were developed using Kodak developer and fixer, following Kodak directions.

Stained gels of unlabeled proteins were prepared for drying by soaking in several changes of 10% glacial acetic acid (v/v) and 3% glycerol (w/v). They were then dried for 2 h using a Hoeffer gel drier.

## 2.11 CM-cellulose chromatography

### 2.11.1 Precycling of CM-cellulose

All CM-cellulose was "pre-cycled" prior to use in order to remove undesirous accompanying ions and molecules and to charge the available binding sites correctly. The CM-cellulose powder was first suspended in 0.5 M NaOH (1 g CM-cellulose/15 mL) for 30 min. The resulting gelatenous slurry was filtered using a Buchner funnel, rinsed with 0.5 M NaOH until the effluent was colorless, and rinsed with water to a neutral pH. The CM-cellulose was next suspended in a volume of 0.5 M HCl equal to the initial volume of 0.5 M NaOH for 30 min, filtered using a Buchner funnel, and rinsed with water to a neutral pH. It was then resuspended in 0.5 M NaOH, immediately filtered again using a Buchner funnel, and rinsed with water to a neutral pH. The CM-cellulose was stored in saturated NaCl at 4°C until used.

### 2.11.2 Buffer L, CM-cellulose binding buffer

10 mM Tris, pH 7.5 at room temperature; 10 mM EDTA-$Na_2$, 1 mM DTT, 7 M deionized urea (Hackett et al. 1978). The urea was deionized by an "ultra high capacity cartridge" in a model LD3 Corning

demineralizer.

2.11.3 Buffer M, CM-cellulose elution buffer

2 M LiCl, 4 M deionized urea (Hackett et al, 1978). Urea deionization was carried out as described above for Buffer L.

2.11.4 Method

Pre-cycled CM-cellulose was pre-equilibrated with a minimum of 6 washes of Buffer L. Fines were removed at this time.

Columns were made by using 3 mL plastic, disposable syringes containing 1 mL of pre-equilibrated CM-cellulose above a circular support cut from a Kimwipe tissue. Columns were poured and equilibrated with a minimum of 10 additional column volumes of Buffer L. The proteins to be applied to the column (lysozyme, bovine serum albumin, translation assays) were dissolved in Buffer L prior to their application to the column.

The translation products to be applied to the columns were prepared immediately following translation by precipitation with 10 volumes of cold acetone at -20°C or -70°C overnight; centrifugation at 12 800 g for 30 min in an Eppindorf microfuge at room temperature or at 3 000 g for 5 min at 4°C in a Sorvall centrifuge; and washing the pellets twice with cold acetone to remove unincorporated radioactive amino acid and to enhance efficient drying of the pellet prior to its being dissolved in Buffer L.

After applying the samples, the columns were rinsed to background, $A_{280}$ or radioactivity as appropriate; and the proteins were eluted with Buffer M. Eluted radioactivity was detected by diluting the sample with water to 1 mL, in a glass scintillation vial; adding

9 mL of Scintiverse or Aquasol 2; and counting for 10 min in a Beckman liquid scintillation counter.

In early experiments, CM-cellulose binding of translated products was monitored by sampling fractions collected by a Gilson microfractionator. In later experiments, when the fraction collectors ceased to operate reliably, fractions were collected manually early after sample application and early after elution Buffer M application. All other eluant was collected *en masse* in beakers.

# 3 RESULTS

## 3.1 Establishing techniques

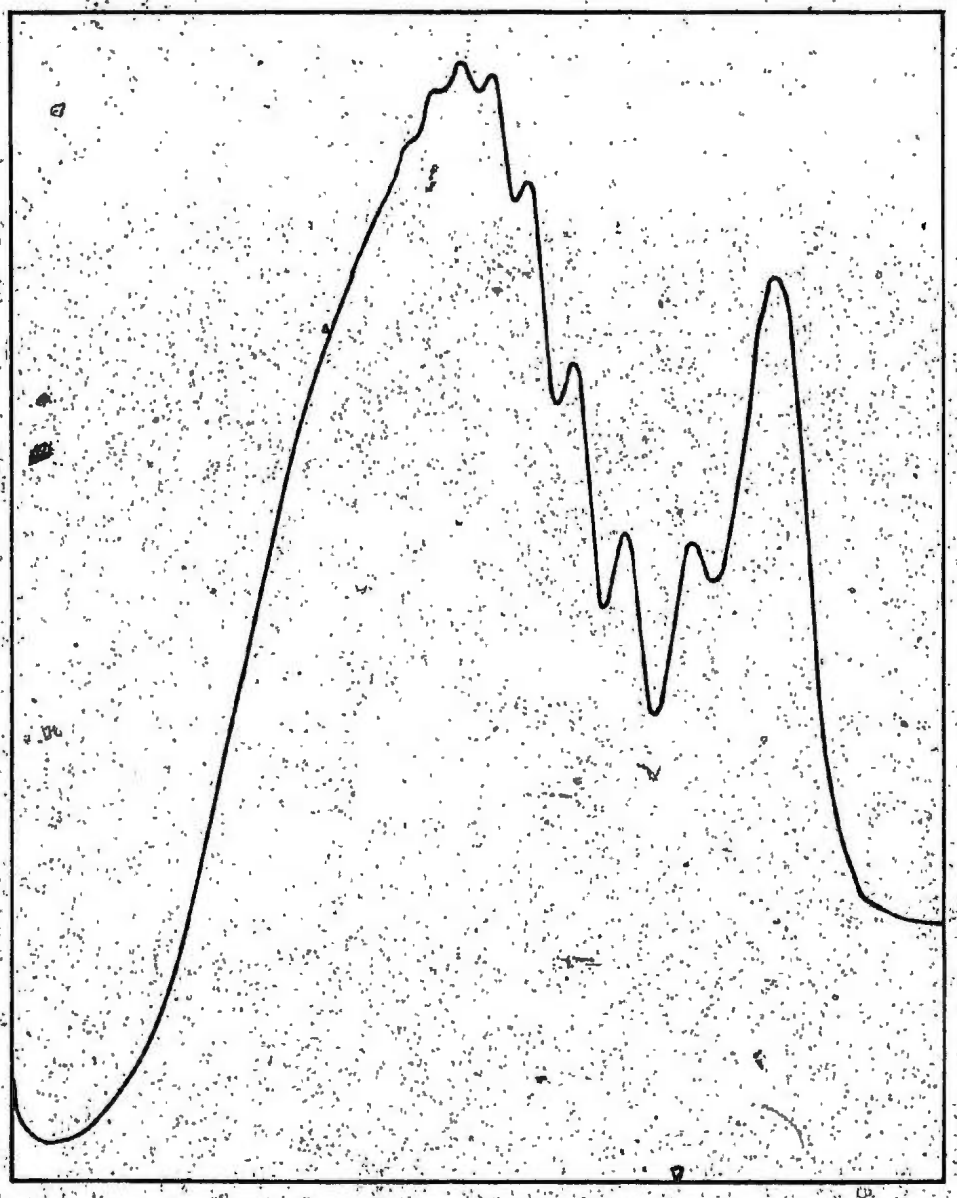
To answer the questions posed in section 1.6, techniques had to be verified or modified. Therefore, development of the methods will be discussed prior to the results obtained by using the methods.

### 3.1.1 Isolation of intact polysomes

Because subsequent experiments required intact mRNA, it was necessary to isolate intact polysomes (polyribosomes), indicating that the mRNA is undegraded. Their presence was demonstrated by using the standard technique of sucrose gradient sedimentation. Polysomes solubilized in Buffer E containing 3 mM $Mg^{++}$ and analyzed by centrifugation through a linear 10 to 40% sucrose gradient at 20°C (Figure 1) resulted in absorbance profiles showing better definition than did polysomes in similar preparations with either the same buffer containing 1 mM or 5 mM $Mg^{++}$ or polysomes solubilized in the classical Buffer D (5 mM $Mg^{++}$) (Parish 1972) prior to centrifugation through an analogous 15 to 30% sucrose gradient. To confirm identification of the monosome peak, simultaneous, analogous, independent centrifugations with ribosomal subunits and monosomes were carried out in all cases. Furthermore, the polysomes appeared more stable, based on absorption profile definition, in 3 mM $Mg^{++}$ than in the more commonly used 5 mM $Mg^{++}$. Judging from the profiles, the polysomes in 1 mM $Mg^{++}$ appeared to be more stable than those in

Figure 1. Polysome profile, normal liver polysomes. Polysomes (polyribosomes) from a normal rat liver were extracted, solubilized in 3 mM $Mg^{++}$ Buffer E, and centrifuged through a 10 to 40% linear sucrose gradient in 3 mM $Mg^{++}$ Buffer E in a Beckman SW 27.1 rotor at 8 000 g for 19 h at 20°C. Distinct peaks representing polysomes containing up to eleven ribosomes were noted. The peak at the top of the gradient (the farthest right peak) was identified as the monosome peak. This centrifugation was concurrent with that of Figure 2.

NORMAL POLYSOMES



5 mM $Mg^{++}$. That well defined polysome profiles were obtained at 20°C instead of 4°C was also unexpected. However, under these conditions (both 3 mM $Mg^{++}$ and 5 mM $Mg^{++}$, 20°C) ribosomal subunits appeared to aggregate, centrifuging slightly faster than monosomes reconstituted from subparticles.

Preliminary studies demonstrated that similar polysome profiles can be produced using 17 h-regenerating liver (Figure 2). The profile in Figure 2 was obtained concurrently with that of Figure 1; a comparison of the profiles implies that differences in the relative abundance of the various polysome size classes may result from processes occurring during liver regeneration.

Several rat livers were co-homogenized and the polysome profiles displayed on sucrose gradients to demonstrate that polysomes from different animals have similar size distributions. As anticipated (McGowan et al, 1979), a relative increase in the size of the monosome peak and a decline in polysomes ($A_{260}$) per g liver were observed when the animals had been starved, but allowed water, for 17 h prior to death the following morning (Figures 3 and 4). Although polysomes stored at -20°C or -70°C had less defined polysome absorbance profiles after centrifugation through a sucrose gradient, polysomes stored at -70°C remained suitable for cell free, in vitro translation. Polysomes stored at -20°C were not examined for this latter characteristic.

3.1.2 Characterization of the in vitro translation system

In contrast to many in vitro translation systems which use purified (protein-free) mRNA as template for protein synthesis,

Figure 2. Polysome profile, 17 h regenerating liver polysomes. Polysomes of a liver 17 h after partial hepatectomy were extracted, solubilized in 3 mM $Mg^{++}$ Buffer E, and centrifuged through a 10 to 40% sucrose gradient in 3 mM $Mg^{++}$ Buffer E in a Beckman SW 27.1 rotor at 8 000 g for 19 h at 20°C. Distinct peaks representing polysomes containing up to ten ribosomes were noted. The peak at the top of the gradient (the farthest right peak) was identified as the monosome peak. This centrifugation was concurrent with that of Figure 1.

REGENERATING POLYSOMES

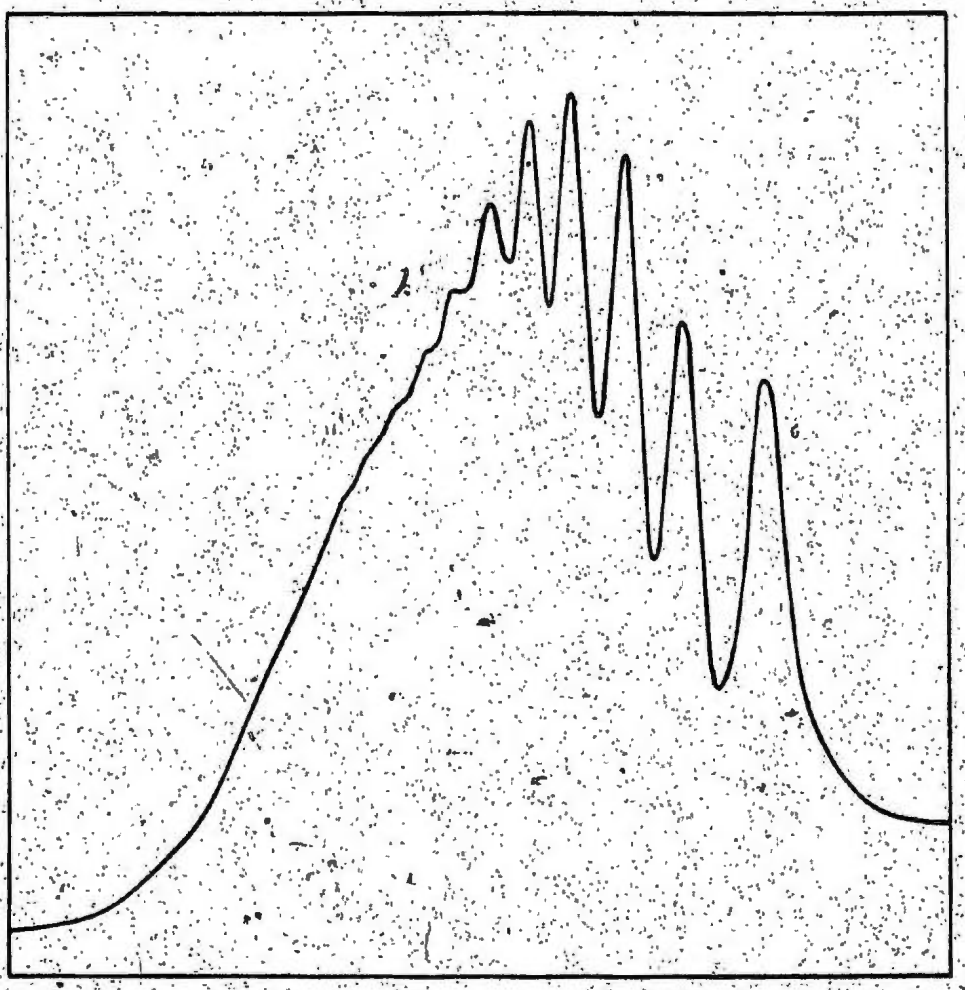


Figure 3. Polysome profile, 17 h starved, normal liver polysomes. Polysomes from a normal rat, starved for the previous 17 h, liver were extracted; solubilized in 3 mM $Mg^{++}$ Buffer E, and centrifuged through a 10 to 40% linear sucrose gradient in 3 mM $Mg^{++}$ Buffer E in a Beckman SW 27.1 rotor at 8 000 g for 19 h at 20°C. Distinct peaks were observed for polysomes containing up to only six ribosomes. The peak at the top of the gradient (the farthest right peak) was identified as the monosome peak. This centrifugation was concurrent with that of Figure 4.

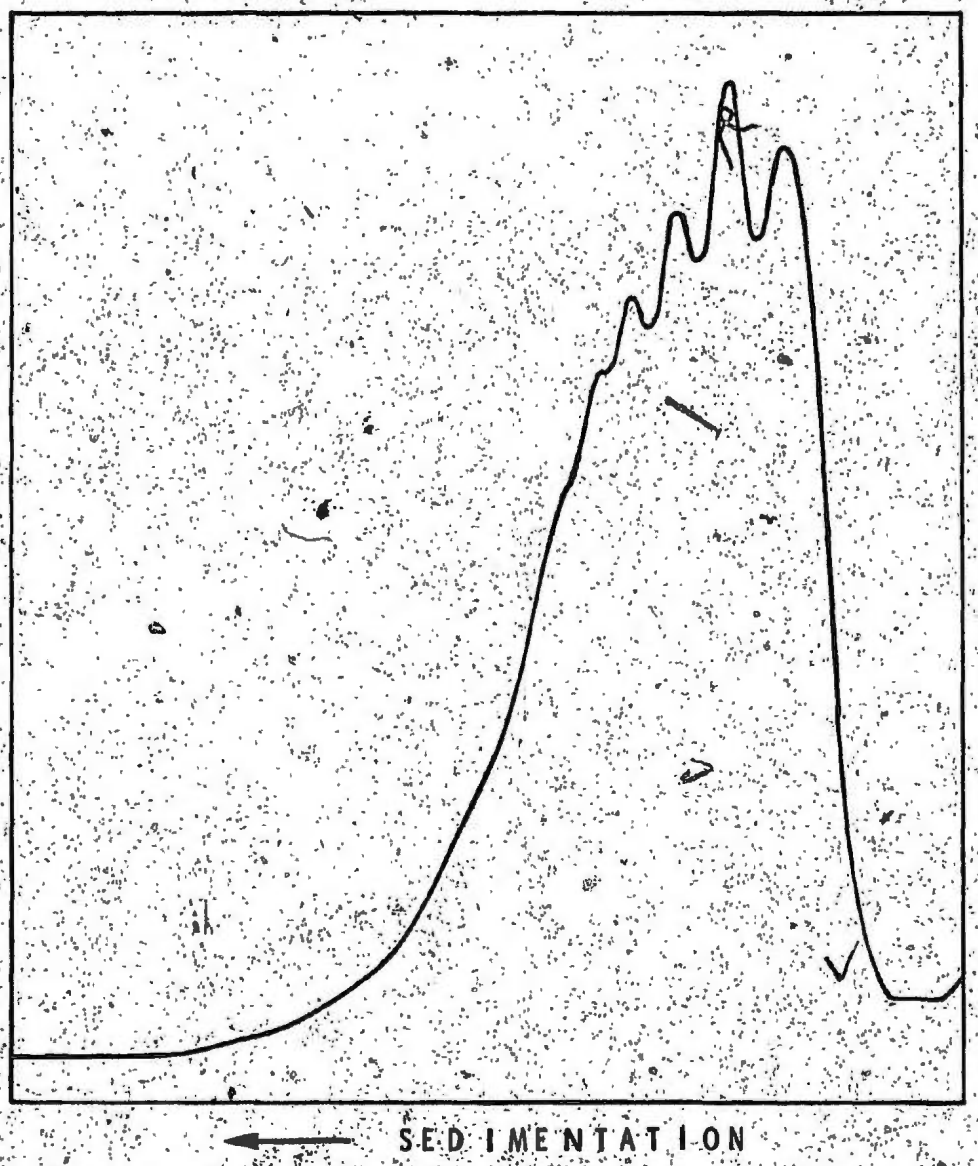
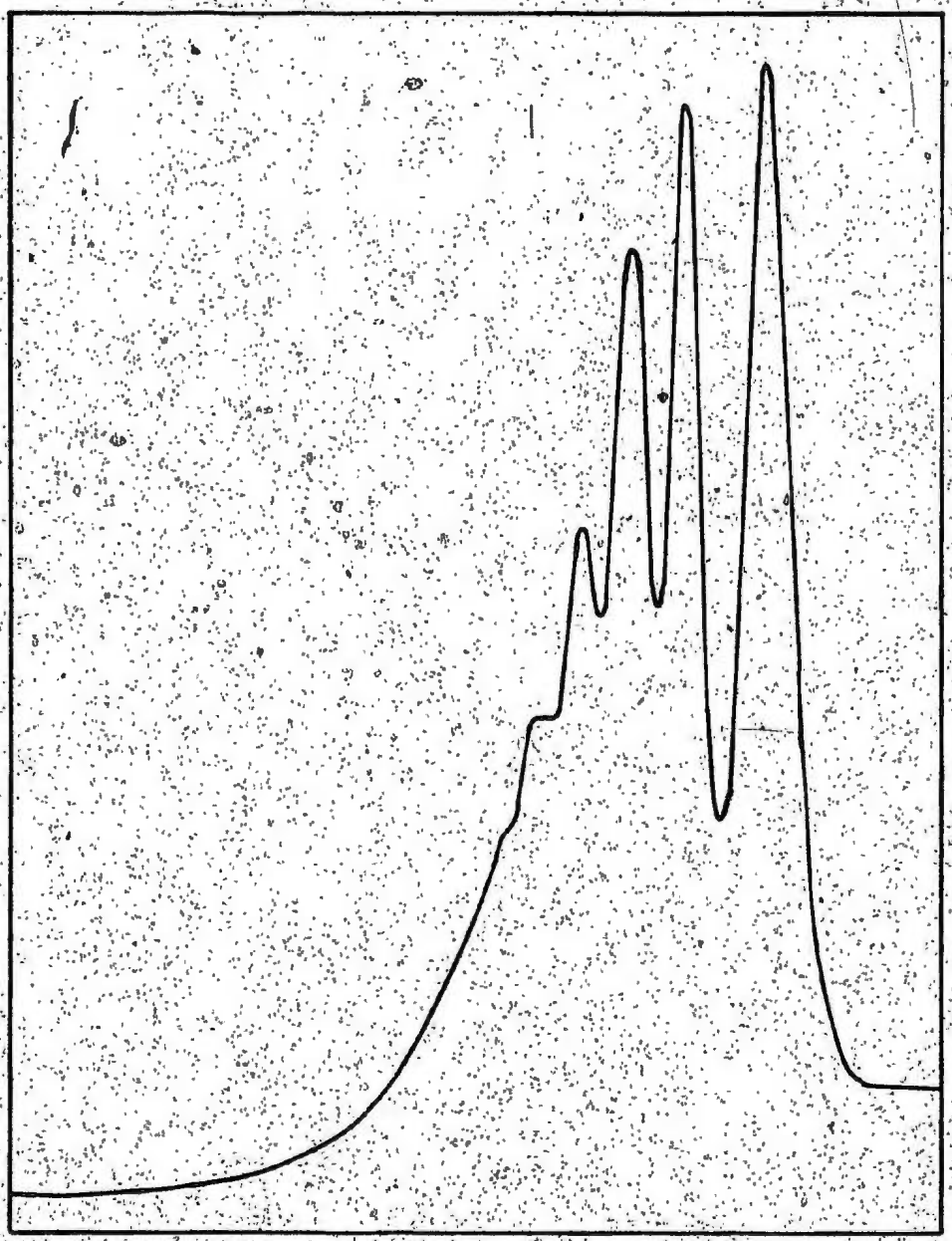

STARVED
NORMAL
POLYSOMES
A 280
SEDIMENTATION

Figure 4. Polysome profile, 17 h starved, regenerating liver polysomes. Polysomes of a liver 17 h following partial hepatectomy and feeding were extracted, solubilized in 3 mM $Mg^{++}$ Buffer E, and centrifuged through a 10 to 40% linear sucrose gradient in 3 mM $Mg^{++}$ Buffer E in a Beckman SW 27.1 rotor at 8 000 g for 19 h at 20°C. Distinct peaks were observed for polysomes containing up to only six ribosomes. The peak at the top of the gradient (the farthest right peak) was identified as the monosome peak. This centrifugation was concurrent with that of Figure 3.


STARVED REGENERATING POLYSOMES
$A_{280}$
SEDIMENTATION

these experiments utilized polysomes. Because the wheat germ based *in vitro* translation assay is sensitive to ionic strength and the buffer used, the system was optimized for use with rat liver polysomes. The determination of these translation assay parameters is detailed below.

Quench and correction of cpm to dpm were not carried out, because of the uniformity of the various samples. Furthermore, the relative increase in incorporation during translation was more important than the absolute increase.

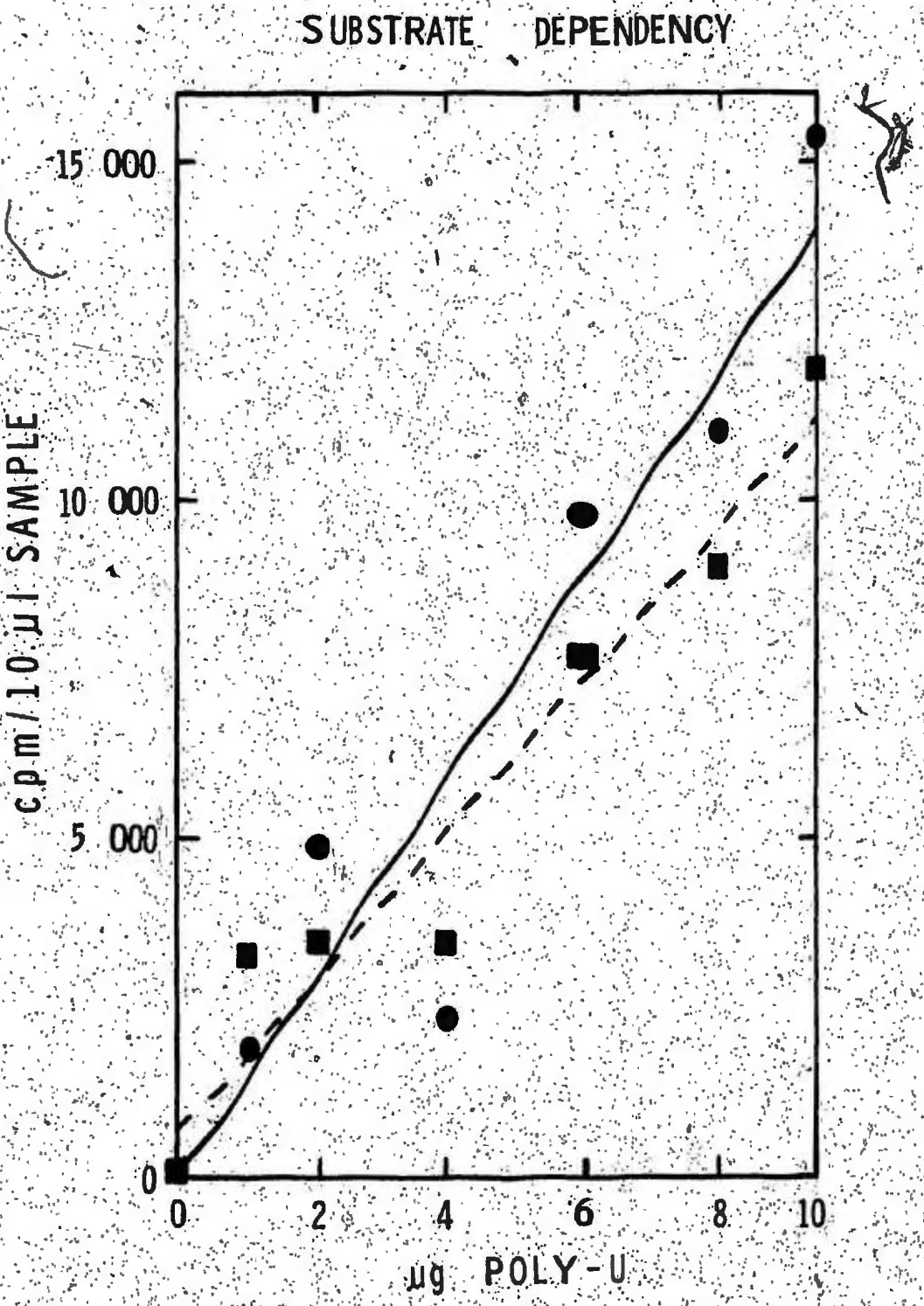
3.1.2.1 *The in vitro translation assay is functioning*

Before characterizing the *in vitro* translation assay, it was necessary to demonstrate that it was operational. The assay's ability to function was established by using poly-U as an RNA template, master mix (section 2.8.1) containing no amino acids, and [1-$^3$H]phenylalanine and measuring the trichloroacetic acid (TCA) precipitable radioactivity as a function of the quantity of poly-U in the assay. Translation was observed to be dependent on the amount of poly-U template present (Figure 5).

3.1.2.2 *Volume of wheat germ S-30 fraction*

The *in vitro* translation system was then characterized for use with rat liver polysomes. The wheat germ S-30 extract is the principal source of components required for translation, including initiation, elongation, and termination factors. It was necessary, therefore, to determine a volume of S-30 extract required for optimal translation while allowing a maximal volume of non-S-30 fraction components. A nonlimiting volume of wheat germ S-30 extract for

Figure 5. Substrate dependency of the wheat germ *in vitro* translation system. Poly-U was used as an artificial mRNA, with L-[$^3$H]phenylalanine, to test the activity of the wheat germ S-30 extract based, *in vitro* translation assay system. Each point is the average of two 10 μL samples from the same assay. Two independent experiments (●, ——; ■, - - -) were performed. The difference between the slopes of the two experiments was found not to be significantly different at the 90% confidence level.


SUBSTRATE DEPENDENCY
15 000
10 000
5 000
0
cpm/10 µl SAMPLE
0
2
4
6
8
10
µg POLY-U

varying amounts of polysomes was determined by using master mixes which compensated for the lower levels of HEPES, KOAc, and $Mg(OAc)_2$ in nonmaximal wheat germ extract volumes; these master mixes resulted in equal concentrations of HEPES, KOAc, and $Mg(OAc)_2$ in all assays. Translation, measured as incorporation of TCA precipitable radioactivity, as a function of volume of wheat germ S-30 and as a function of quantity of polysomes was examined. It appears that 15 μL of wheat germ S-30 extract is a nonlimiting volume under the conditions used (Figure 6), and that translation is polysome dependent (Figure 7).

3.1.2.3 Choice of polysome solubilizing buffer

Because it is more common to use purified mRNA than polysomes to program the *in vitro* translation assay, it was important to choose a buffer capable of solubilizing and stabilizing the polysomes prior to translation. A comparison of the translational capacity of polysomes solubilized in the chosen buffer (Buffer H) and in Buffer E, used for solubilizing the polysomes for sucrose gradient centrifugation, was made. Both buffers were found to be suitable for solubilizing polysomes for translation (Figure 8).

Specifically, a Student's *t* test applied to the linear regression coefficients indicates that the observed differences were not significant at the 90% confidence level for the choice of buffer for solubilizing the polysomes prior to translation. [For significance with 20 degrees of freedom (d. f.) at the 90% confidence limit, $t \geq 1.325$; $t_{\text{solubilizing buffer}} = 0.432$, $t_{\text{reaction vessel}} = 0.730$.]

Figure 6. Determination of a non-limiting volume of wheat germ S-30 extract. Varying volumes of wheat germ S-30 extract were used with a constant quantity of polysomes, L-$C^{14}$]lysine, and constant final concentrations of HEPES, KOAc, and $Mg(OAc)_2$. Each point is the average of a 10 µL aliquot from each of two simultaneous assays, (▲ 5 µL polysomes, ■ 10 µL polysomes, ● 15 µL polysomes). The presence of 15 µL of S-30 extract per 50 µL assay was found to be appropriate.

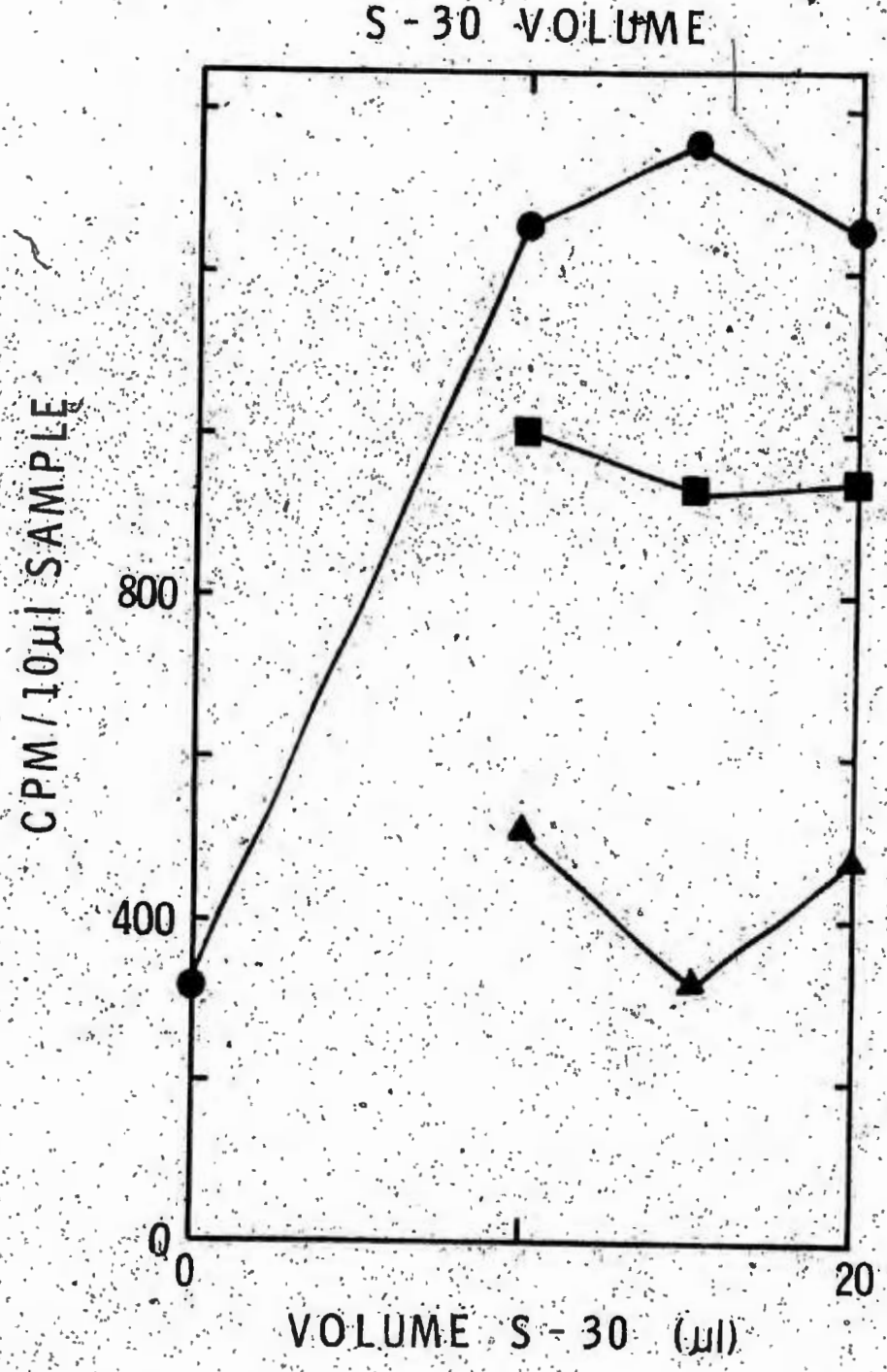

S - 30 VOLUME
CPM / 10µl SAMPLE
800
400
0
0
20
VOLUME S - 30 (µl)

Figure 7. Dependency of the wheat germ *in vitro* translation assay on polysome concentration. Fresh (unstored) polysomes were used as a substrate with L-[$^{14}$C]lysine to test the practical viability of the proposed wheat germ S-30 extract based, *in vitro* translation assay system. Each point is the average of a 10 µL aliquot from each of two simultaneous assays (▲ 10 µL S-30, ■ 15 µL S-30, ● 20 µL S-30). Lines are best fit regression lines. [These data are the same as those displayed in Figure 6.]

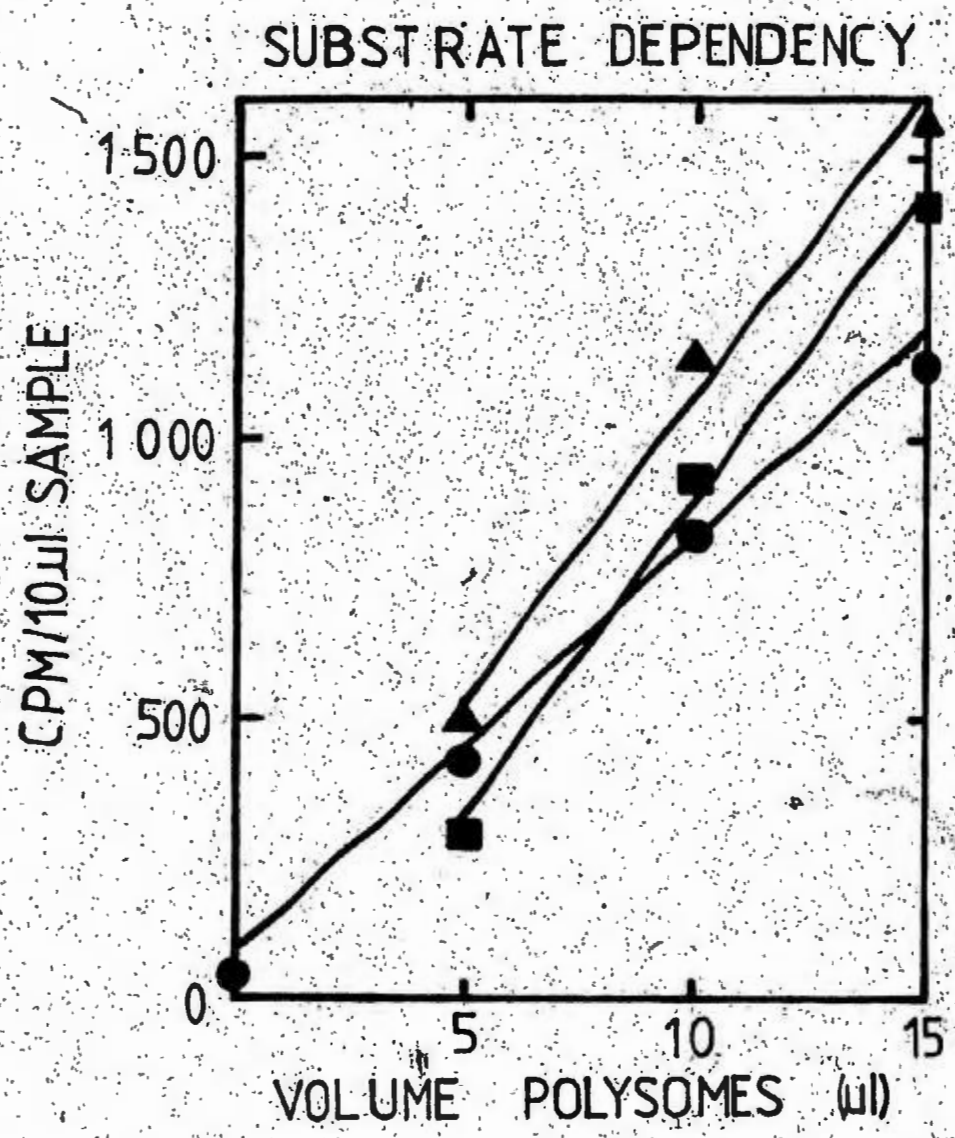
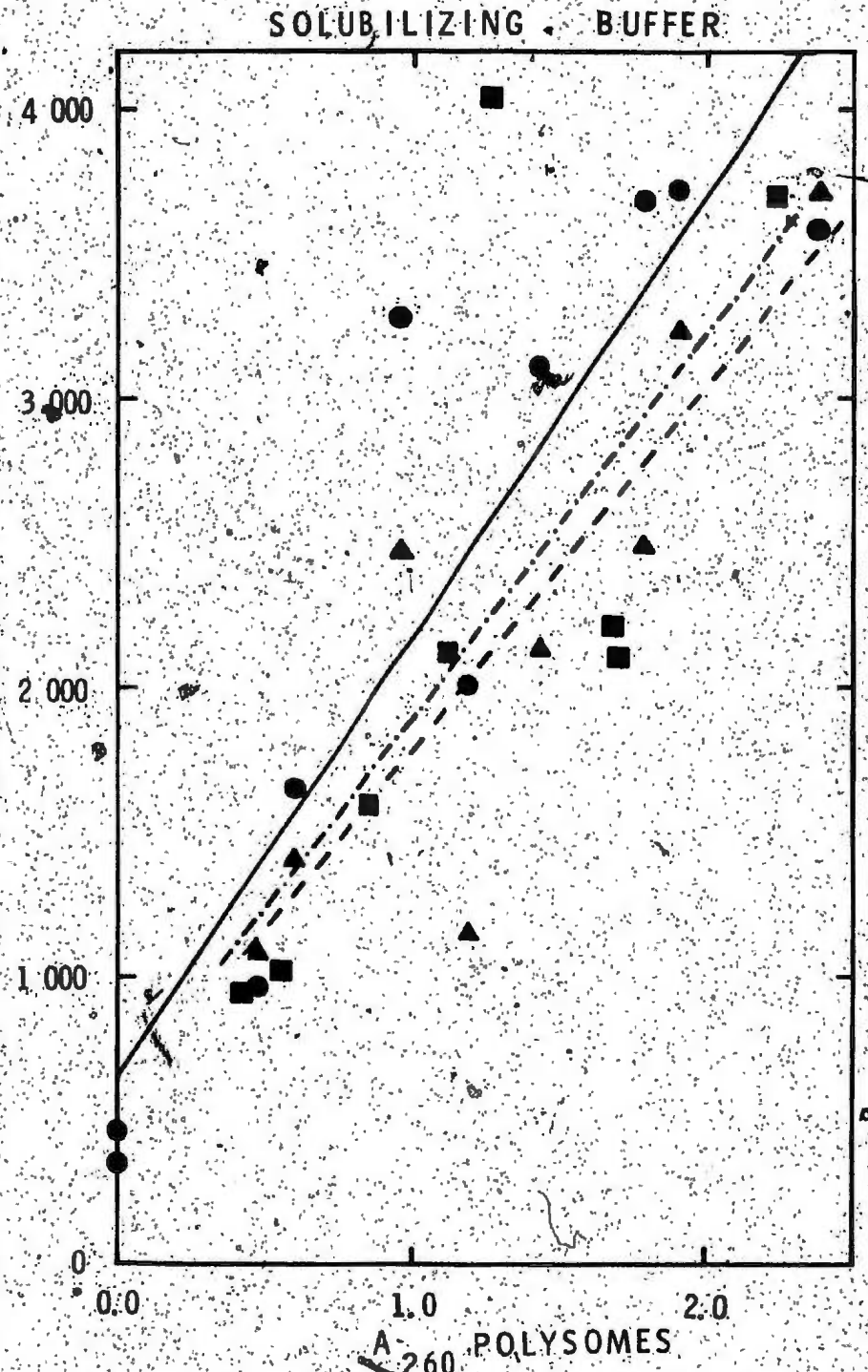

SUBSTRATE DEPENDENCY
1500
1000
500
0
CPM/10µl SAMPLE
5
10
15
VOLUME POLYSOMES (µl)

Figure 8. Choice of solubilizing buffer for in vitro translation. Buffer H and Buffer E were compared for their ability to solubilize polysomes favorably for in vitro translation. Fresh (unstored) polysomes and L-[$^3$H]leucine were used; each point is the average of two 5 µL samples from the same assay. (●—— and ▲- - -, Buffer H; ■—·—·—, Buffer E).


SOLUBILIZING BUFFER
4 000
3 000
2 000
1 000
0
CPM /.5µl SAMPLE
0.0
1.0
2.0
A 260 POLYSOMES

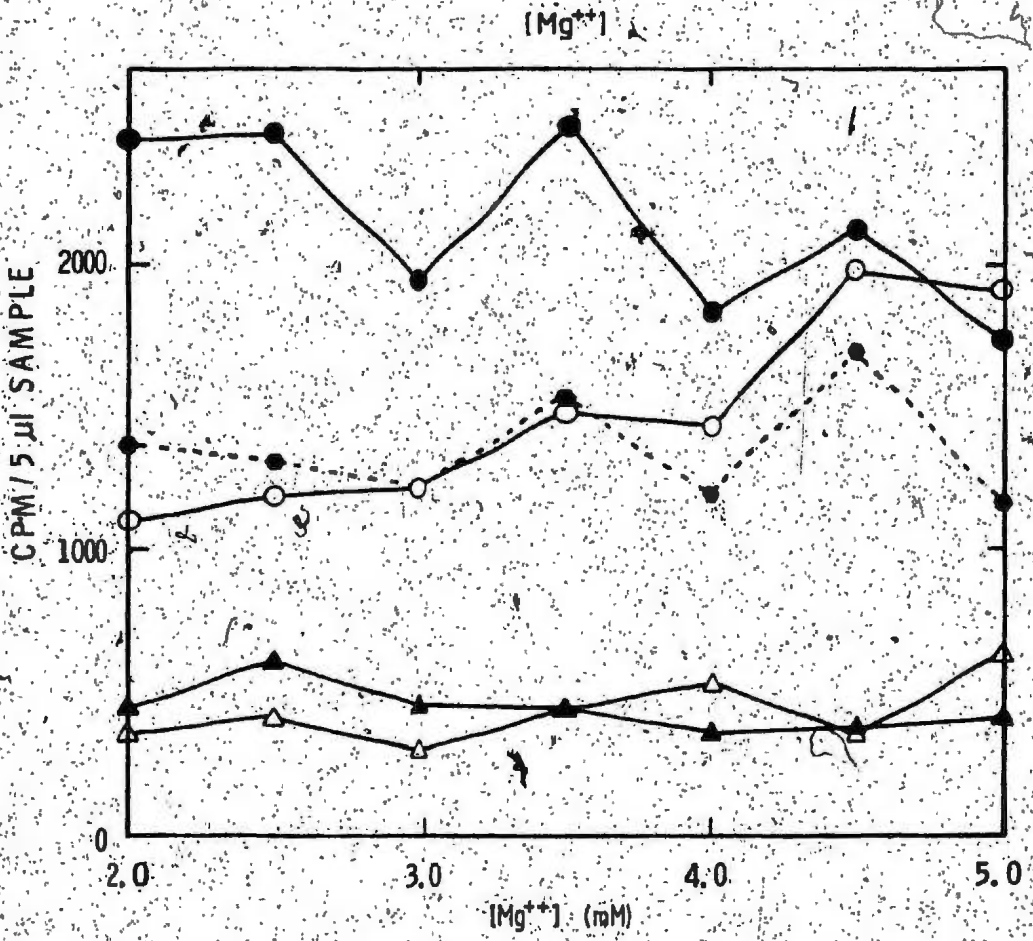
### 3.1.2.4 $Mg^{++}$ concentration

As stated in the Material and Methods section, the *in vitro* translation assays contained 4.5 mM $Mg^{++}$. Although the use of 2 mM $Mg^{++}$ (Webber 1977) to 3.5 mM $Mg^{++}$ (Zehavi-Willner 1976) has been reported, most groups use 2.5 mM or 3.0 mM $Mg^{++}$ for mRNA *in vitro* translation assays. Recalling that polysomes displayed on sucrose gradients appeared to be stable at lower than normally reported $Mg^{++}$ concentrations, it seemed appropriate to investigate the relationship between $Mg^{++}$ concentration and the *in vitro* translation of rat liver polysomes. Amino acid incorporation over the range of 2.0 mM to 5.0 mM $Mg^{++}$ was examined. The results (Figure 9) suggest that under the assay conditions used, translatability is relatively unaffected by $Mg^{++}$ concentration. Therefore, there appeared to be no good reason to change from the use of 4.5 mM $Mg^{++}$. These results also indicate that the presence of heparin during the isolation of polysomes, the lack of incubation of the wheat germ S-30 extract prior to polysome addition, and the absence of EDTA while solubilizing the polysomes do not hinder translation.

### 3.1.2.5 Time course

*In vitro* translation as a function of time was investigated to ensure that reinitiation of translation occurs and to determine an appropriate incubation time. Reinitiation of translation is a feature of a good *in vitro* translation assay and is necessary for obtaining reasonable quantities of radioactively labeled product. If the average translation rate is 10 amino acids per second, or 600 amino

Figure 9. Optimum [$Mg^{++}$] for *in vitro* translation. The concentration of $Mg^{++}$ in the translation assays was varied while keeping the concentrations of all other components constant. The radioactive amino acid was L-[$^3$H]leucine. Each point is the average of two 5 µL samples, (●, ○ —— total cpm; ▲, △ —— background cpm; ●- - - average stimulated cpm [stimulated cpm = total cpm - background cpm], (○, △ experiment using no heparin during polysome isolation, with EDTA present while solubilizing the polysomes, and pre-incubation of the wheat germ; ●, ▲ experiment using heparin during polysome isolation, no EDTA present while solubilizing the polysomes, and no pre-incubation of the wheat germ).


[Mg++]
CPM / 5 µl SAMPLE
2000
1000
0
2.0
3.0
4.0
5.0
[Mg++] (mM)

acids per minute, and if no reinitiation of protein synthesis occurs, then no significant incorporation of radioactive amino acids would occur after 5 min, a time sufficient for synthesis of a protein of $M_r$ 300 000, if the $M_r$ of the average amino acid is 100. However, it is clear from the results (Figure 10) that reinitiation of translation probably does occur, since TCA precipitable radioactivity increases for at least 60 min at 25°C. These two experiments also demonstrate, as previously noted (Figure 9), that the presence of heparin during the isolation of polysomes, the lack of incubation of the master mix-wheat germ S-30 extract-water mixture prior to the addition of the radioactive amino acids and polysomes, and the absence of EDTA while solubilizing the polysomes do not hinder the translation. In fact, the latter translation is significantly better than the translation performed with polysomes isolated in the absence of heparin, solubilized in the presence of EDTA, and translated with a pre-incubated master mix-wheat germ S-80 extract-water mixture. [For significance at the 99.9% confidence level with 34 d. f., $t \geq 3.36$. A Student's t test of the regression coefficients of the power curve regressions resulted in $t = 7.39$.]

3.1.2.6 Demonstration of full length polypeptides

In any in vitro translation system it is important to have both efficient translation of full-length polypeptides (a low level of prematurely terminating peptides) and a high level of reinitiation of translation. The latter has already been demonstrated (Figure 10). The presence of full-length polypeptides was demonstrated by using Laemmli (1970) SDS gels and autofluorography. Figure 11 is a

Figure 10. Time course of translation. Translation assays were set up and the extent of translation in a separate assay was examined every 5 min. Each point is the average of two 5 μL samples from the same assay. The lines are best fit regression lines to a power curve. (▲ experiment without heparin during polysome isolation, with EDTA present while solubilizing the polysomes, and pre-incubation of the wheat germ; ● experiment using heparin during polysome isolation, no EDTA present while solubilizing the polysomes, and no pre-incubation of the wheat germ). The radioactive amino acid was L-[$^3$H]leucine. Using Students *t* test, the two lines were found to differ significantly at the 99.9% confidence level.

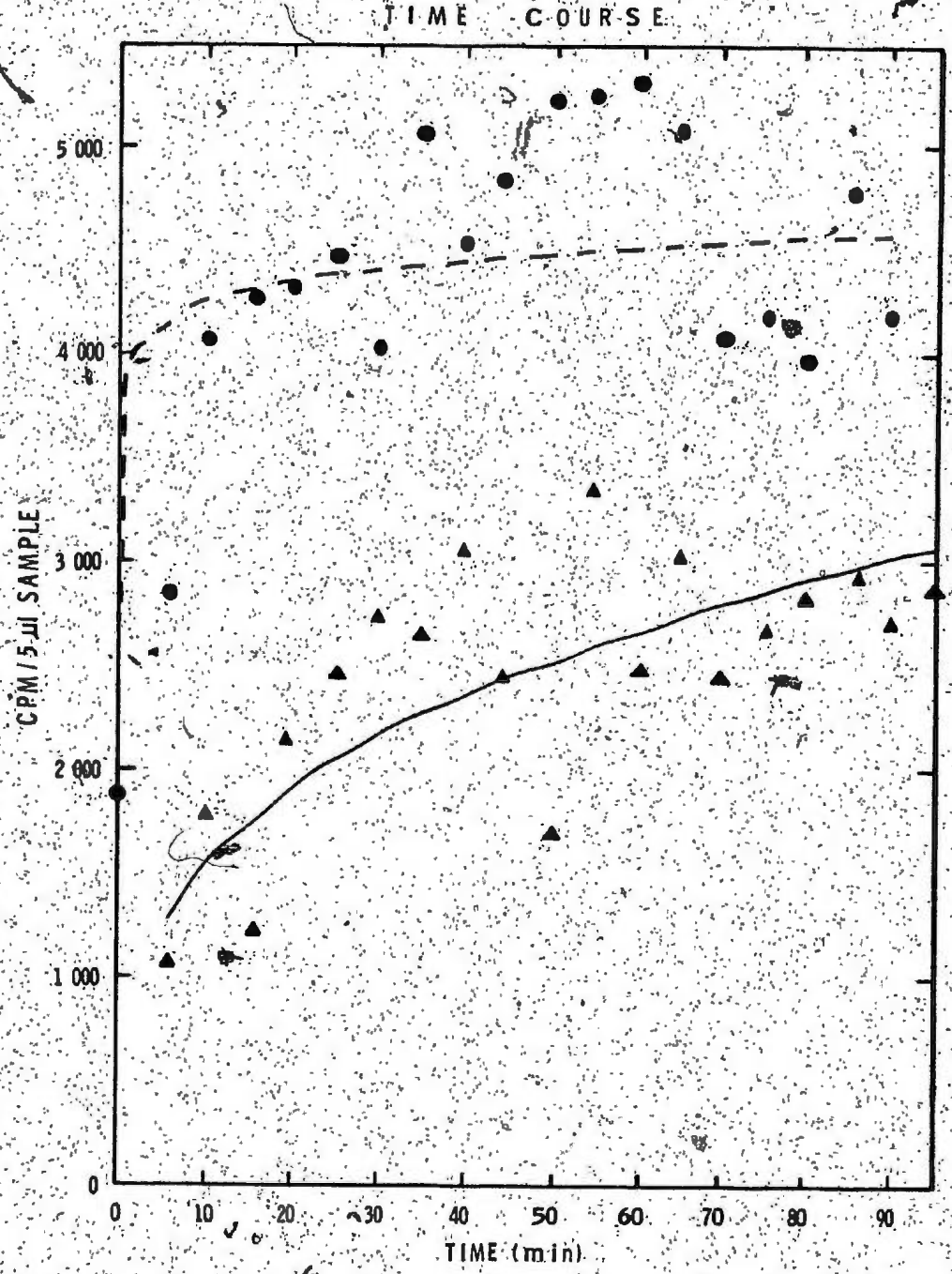
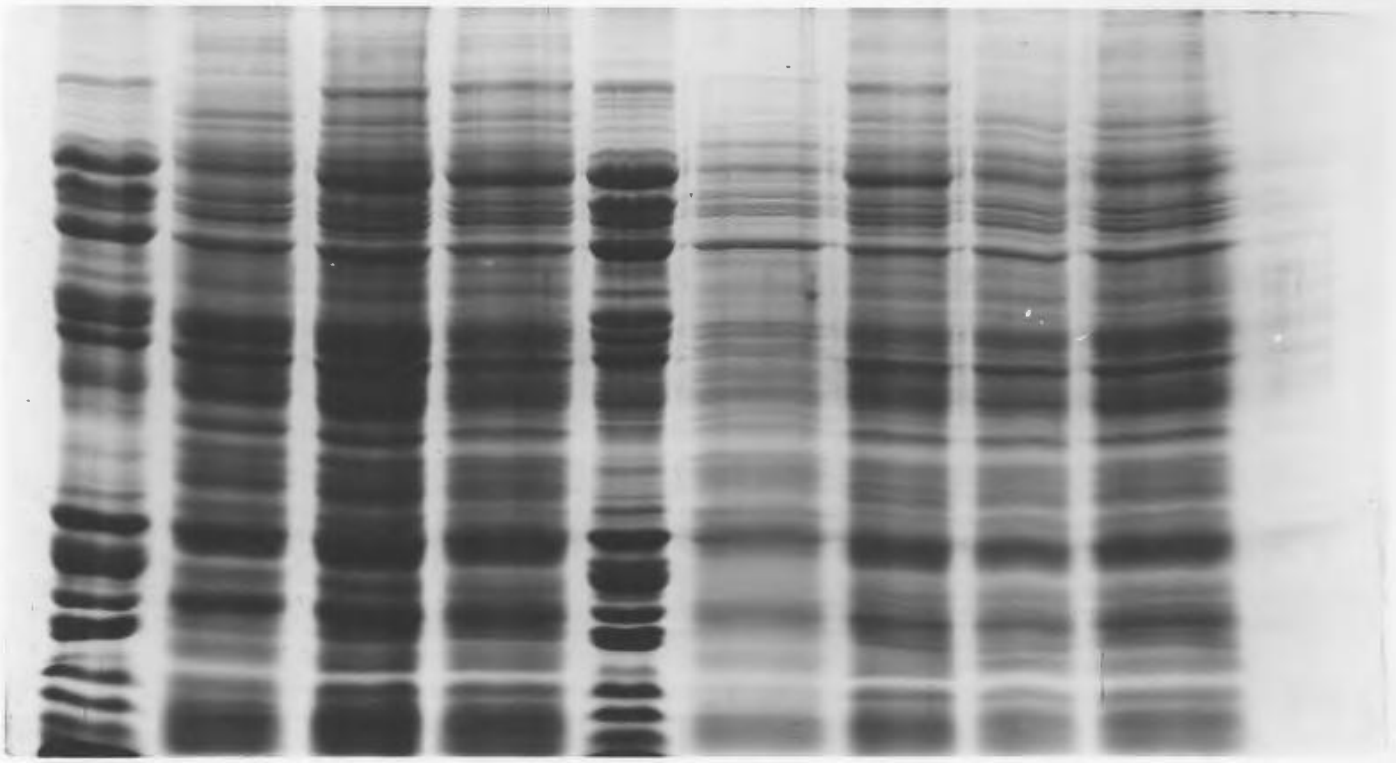

TIME COURSE
5 000
4 000
3 000
2 000
1 000
0
CPM/5µl SAMPLE
0
10
20
30
40
50
60
70
80
90
TIME (min)

Figure 11. Coomassie blue stained Laemmli SDS gel of *Escherichia coli* [$^3$H]ribosomal proteins, *in vitro* translation endogenous (wheat germ S-30 extract) proteins, and *in vitro* translation products. Numbering the slots from left to right, slots 1 and 5 have *E. coli* [$^3$H]ribosomal proteins (gift from S. Dowsett); slots 2, 8, and 9 have endogenous wheat germ (no added polysomes) translated with L-[$^3$H]amino acid mixture; slots 3 and 7 have polysomes translated with L-[$^3$H]amino acid mixture; slot 4 has polysomes translated with L-[$^{35}$S]methionine; slot 10 has L-[$^3$H]leucine; and slot 6 is from another experiment. The contents of one translation assay were applied to each of slots 2, 3, 4, 7, 8, and 9. The faint bands visible in slot 10 are probably due to spillover from slot 9.



photograph of a gel stained prior to autofluorography, showing that good separation of proteins occurs in the molecular weight range of interest (ribosomal and histone proteins); the stained "in vitro translation proteins" are primarily those present in the wheat germ S-30 fraction. Autofluorography, (Figures 12 and 13), shows that discrete polypeptides of all lengths are being synthesized, and that there is a low level of translation of wheat germ coded proteins.

3.1.2.7 Pre-incubation of wheat germ S-30 fraction

An experiment was performed to determine if incubation of the master mix-wheat germ S-30 fraction-water portion of the translation assay prior to the addition of radioactive amino acids and polysomes (template) was effective in reducing the background translation of endogenous mRNAs present in the wheat germ fraction. The results (figure 14) suggest that pre-incubation of the wheat germ S-30 fraction is not necessary, and it may in fact hinder translation of rat liver polysomes. Similar findings are shown in figures 9 and 10. In all subsequent experiments, the wheat germ portion of the assay was not incubated prior to the addition of polysomes.

3.1.2.8 Radioactivity in nascent peptides

To ensure that the majority of incorporated radioactivity was present in full length peptides, rather than in nascent peptides, an attempt was made to determine the proportion of counts present in nascent peptides and therefore not detected by the techniques used for measuring incorporated radioactivity. To release nascent peptides, the translation system could be incubated with ribonuclease $T_1$ and ribonuclease A or with puromycin prior to TCA

Figure 12. Autofluorograph of Laemmli SDS gel, demonstrating length of *in vitro* translation products. Numbering the slots from left to right, slots 1 and 5 have [$^3$H]*E. coli* ribosomal proteins (gift from S. Dowsett); slots 2, 8, and 9 have endogenous wheat germ (no added polysomes) translated with L-[$^3$H]amino acid mixture; slots 3 and 7 have polysomes translated with L-[$^3$H]amino acid mixture; slot 4 has polysomes translated with L-[$^{35}$S]methionine; slot 10 has L-[$^3$H]leucine; and slot 6 is from another experiment. The contents of one translation assay were applied to each of slots 2, 3, 4, 7, 8, and 9. The autofluorograph is of the stained gel of Figure 11.

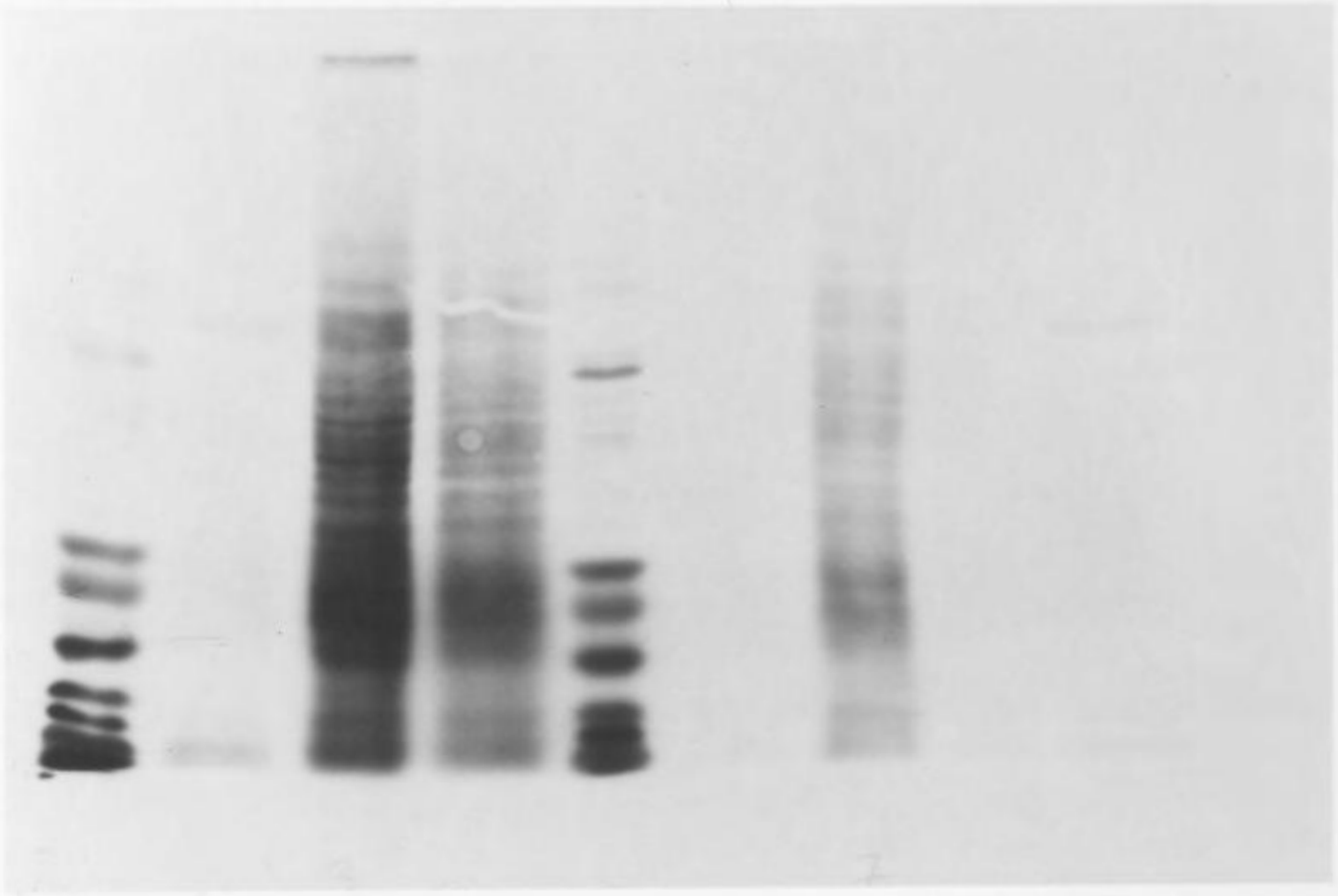


Figure 13. Autofluorograph of Laemmli SDS gel of *in vitro* translated proteins. Slot a has L-[$^3$H]leucine; slot b has *E. coli* [$^3$H]ribosomal proteins (gift from S. Dowsett); slot c has endogenous wheat germ (no added polysomes) translated with L-[$^3$H]amino acid mixture; slot d has polysomes translated with L-[$^3$H]amino acid mixture; slot e has endogenous wheat germ translated with L-[$^{35}$S]methionine; and slot f has polysomes translated with L-[$^{35}$S]methionine. The contents of one translation assay were applied to each of slots c, d, e, and f. All the slots were run simultaneously on slab gels.

# TRANSLATED PROTEINS

a b c d e f

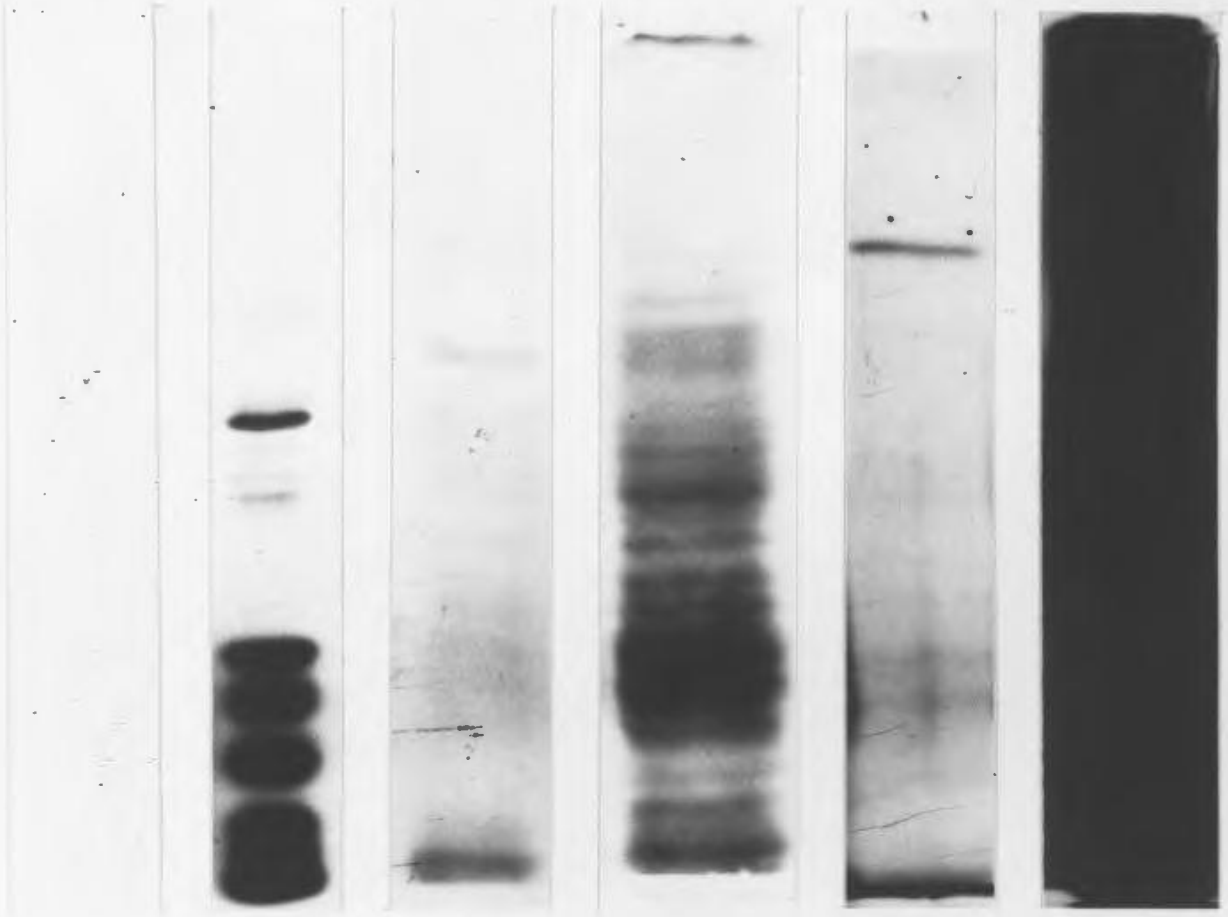
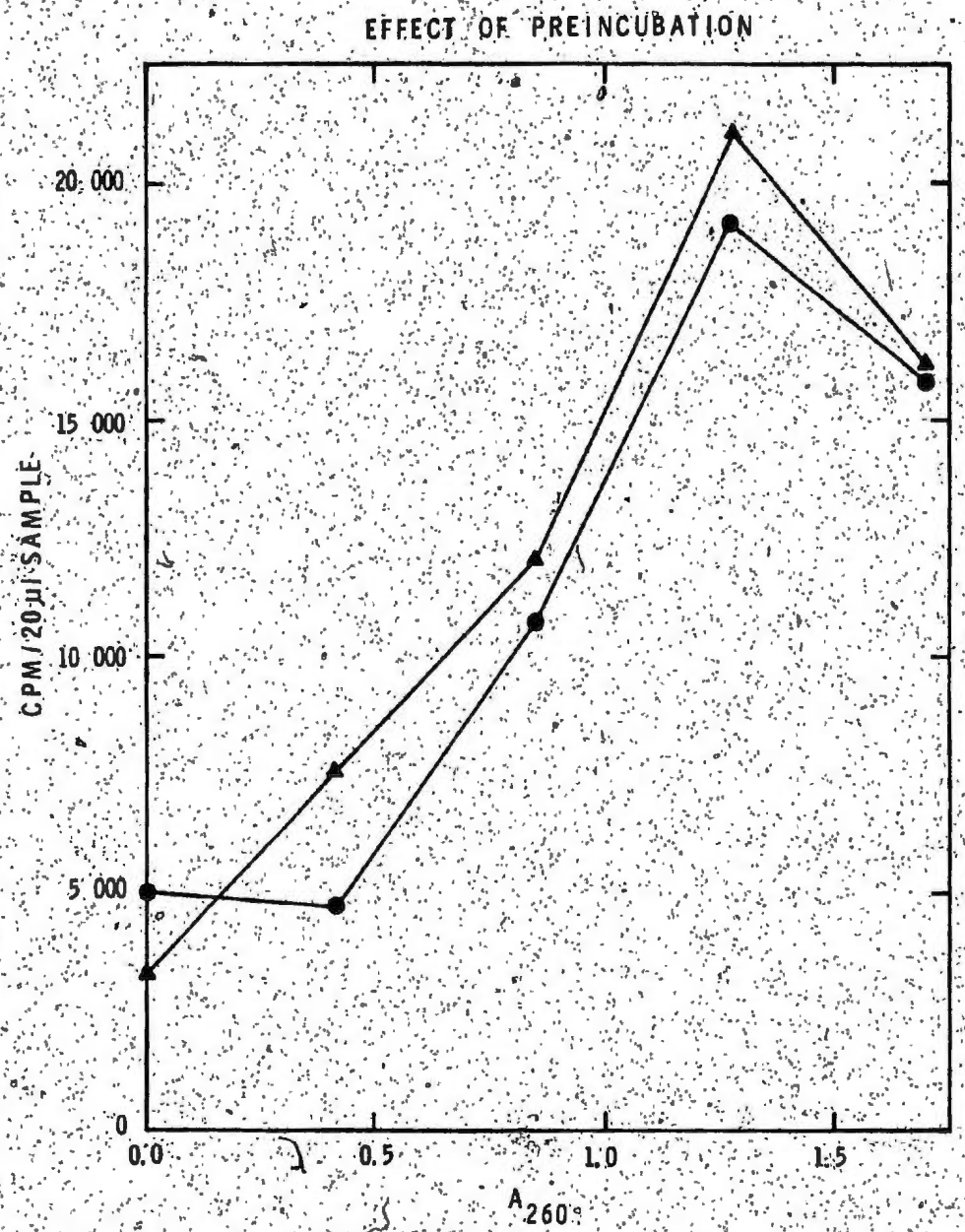


Figure 14. Effect of pre-incubation. The incorporation of L-[$^3$H]amino acid mixture by different quantities of polysomes (as measured by $A_{260}$) during *in vitro* translation with pre-incubated (●) and nonpre-incubated (▲) master mix-wheat germ S-30 fraction-water mixture prior to the addition of polysomes was compared. Each point is the average of two 20 µL aliquots from each of two simultaneously run assays.


EFFECT OF PREINCUBATION
20 000
15 000
10 000
5 000
0
CPM/20 µl SAMPLE
0.0
0.5
1.0
1.5
A260

precipitation. Two experiments using incubation with puromycin were performed. After translation, the assays were pooled. The pooled mix was split in half, and 0.01 volume of 10 mM puromycin was added to one half. The half was then incubated at 0°C for 15 min, followed by incubation at 37°C for 30 min prior to TCA precipitation. If a significant amount of radioactive amino acids were present as nascent peptides, TCA precipitable radioactivity would increase following incubation in the presence of puromycin. No such increase was observed (Table 1).

3.1.2.9 Quantity of mRNA

When translation (incorporation of radioactive amino acids) is examined as a function of mRNA present, there is an optimum quantity of mRNA for maximal translation, and that mRNA present in excess of this optimum frequently results in equal or lower levels of translation. Throughout the preliminary characterization of the *in vitro* translation system, I attempted to determine or exceed the optimum quantity of polysomes. Although polysome-Buffer H mixtures having $A_{260}$ as high as 1.6 per 20 µL were used, the system was not saturated and maximum rates of amino acid incorporation were not achieved.

3.1.3 CM-cellulose binding

Basic proteins were defined as those proteins which bind to carboxymethyl-cellulose (CM-cellulose) in 10 mM Tris, pH 7.5 at room temperature (Buffer L). The appropriateness of this definition was tested by attempting to bind lysozyme, a basic protein; bovine serum albumin (BSA), a nonbasic protein; a lysozyme-BSA mixture; and

Table 1

Radioactivity in nascent peptides

| experiment | $A_{260}$/assay | cpm in 10 μL -puromycin | cpm in 10 μL +puromycin | -/+ |
|---|---|---|---|---|
| 1 | 2.05 | 5 636 | 3 616 | 1.56 |
| 2 | 2.66 | 63 747 | 52 830 | 1.21 |

Experiment 1 used [$^3$H]amino acid mixture for translation while experiment 2 used [$^3$H]lysine. Each value in experiment 1 is the average of four 10 μL samples, while each value in experiment 2 is the average of three 10 μL samples. Both experiments had heparin present during polysome isolation, lacked EDTA in Buffer G used for solubilizing the polysomes, and had no pre-incubation of the wheat germ.

ribosomal proteins to the column using the proposed experimental conditions.

3.1.3.1 General efficacy

The binding characteristics of the CM-cellulose column were tested using lysozyme and BSA. As shown in Figure 15, CM-cellulose binds lysozyme efficiently, has a low nonspecific binding of BSA, and is capable of separating lysozyme from BSA. The column was capable of binding at least 10 $A_{280}$ (1 mL, 1 cm path length 0.2 mm slit width [Beckman model 24 spectrophotometer]) of protein, which is five times the anticipated maximum protein to be applied.

3.1.3.2 Ribosomal proteins

Because the initial purpose of the project was to examine the synthesis of ribosomal proteins, many of which are basic, the binding of ribosomal proteins to CM-cellulose under the experimental conditions was determined. A typical binding profile is shown in Figure 16. Three experiments showed that 67 $\pm$ 6% of total ribosomal proteins bind to CM-cellulose under the experimental conditions.

3.1.3.3 In vitro translation products

The ability of CM-cellulose to resolve basic and nonbasic components of *in vitro* translated polypeptides was also examined; a separation profile is shown in Figure 17, demonstrating the presence of basic and nonbasic proteins, that the two can be separated, and that the proportion of the former is much smaller than the proportion of the latter.

Figure 15. Efficacy of CM-cellulose binding. In separate experiments, ▲ lysozyme (1 mg/mL) alone, ■ bovine serum albumin (BSA) (10 mg/mL) alone, and ● a lysozyme-BSA (each 10 mg/mL) mixture were applied to CM-cellulose columns. Elution Buffer M was applied as indicated by the arrow, and 1 mL fractions were collected. The $A_{280}$ units on the left side apply to lysozyme and BSA alone while those on the right side apply to the mixture.

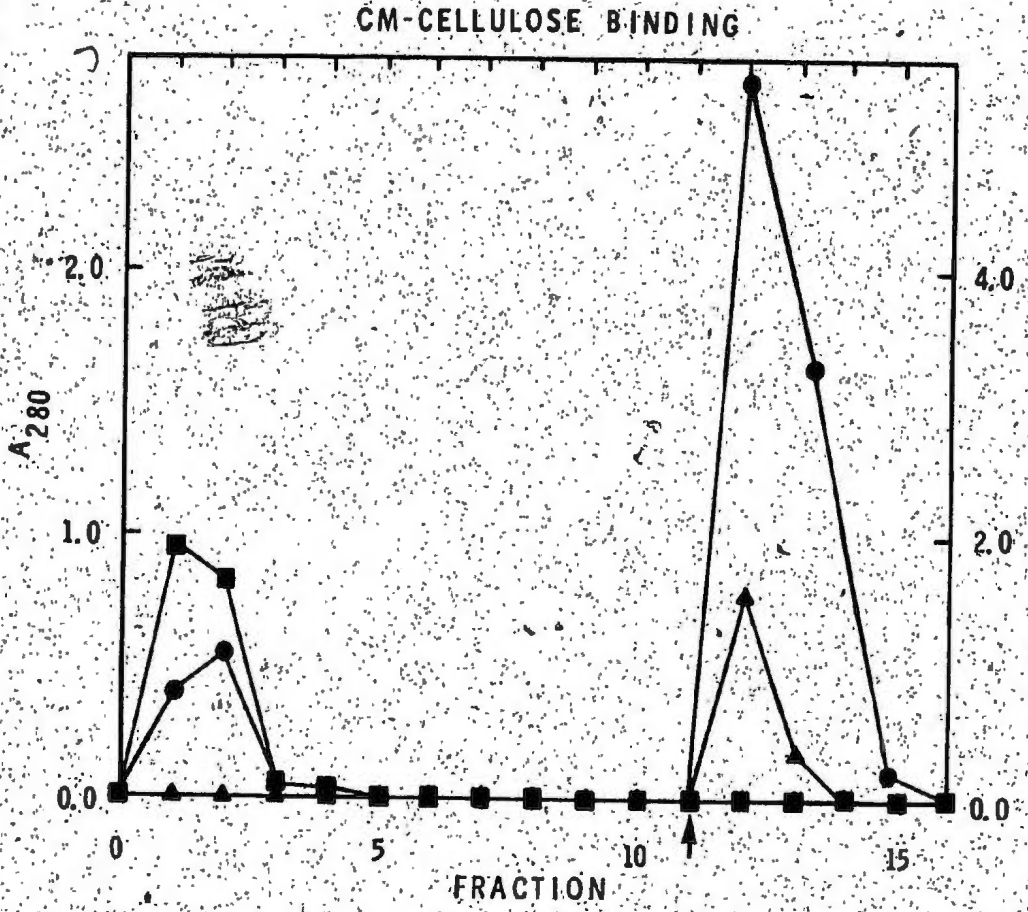

CM-CELLULOSE BINDING
2.0
1.0
0.0
$A_{280}$
4.0
2.0
0.0
0
5
10
15
FRACTION

Figure 16. Determination of CM-cellulose binding component of ribosomal proteins. Approximately 2.6 $A_{280}$ units ribosomal proteins in 1 mL binding Buffer L were applied to the column, and 1 mL fractions were collected. Elution commenced at fraction 13.

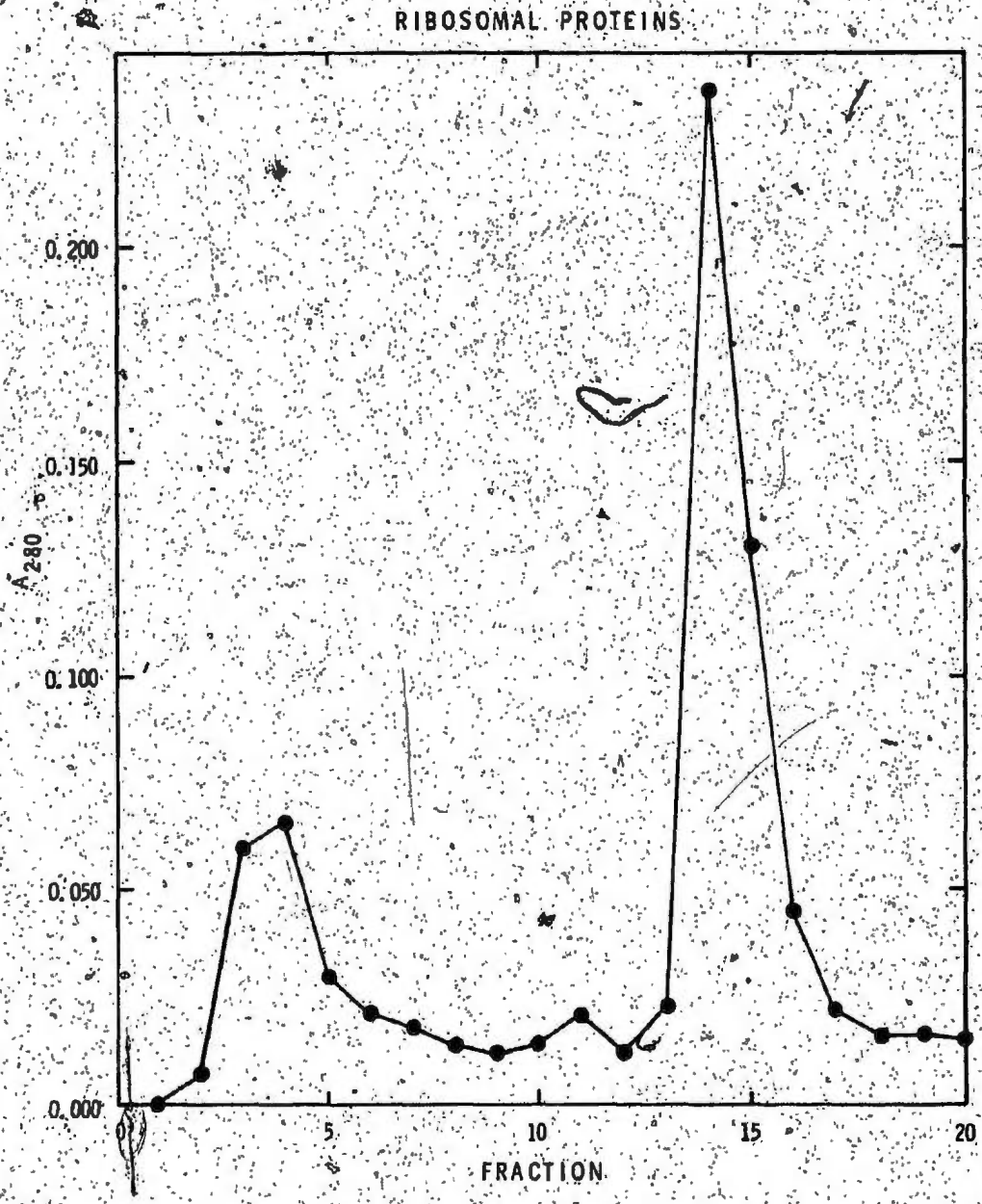
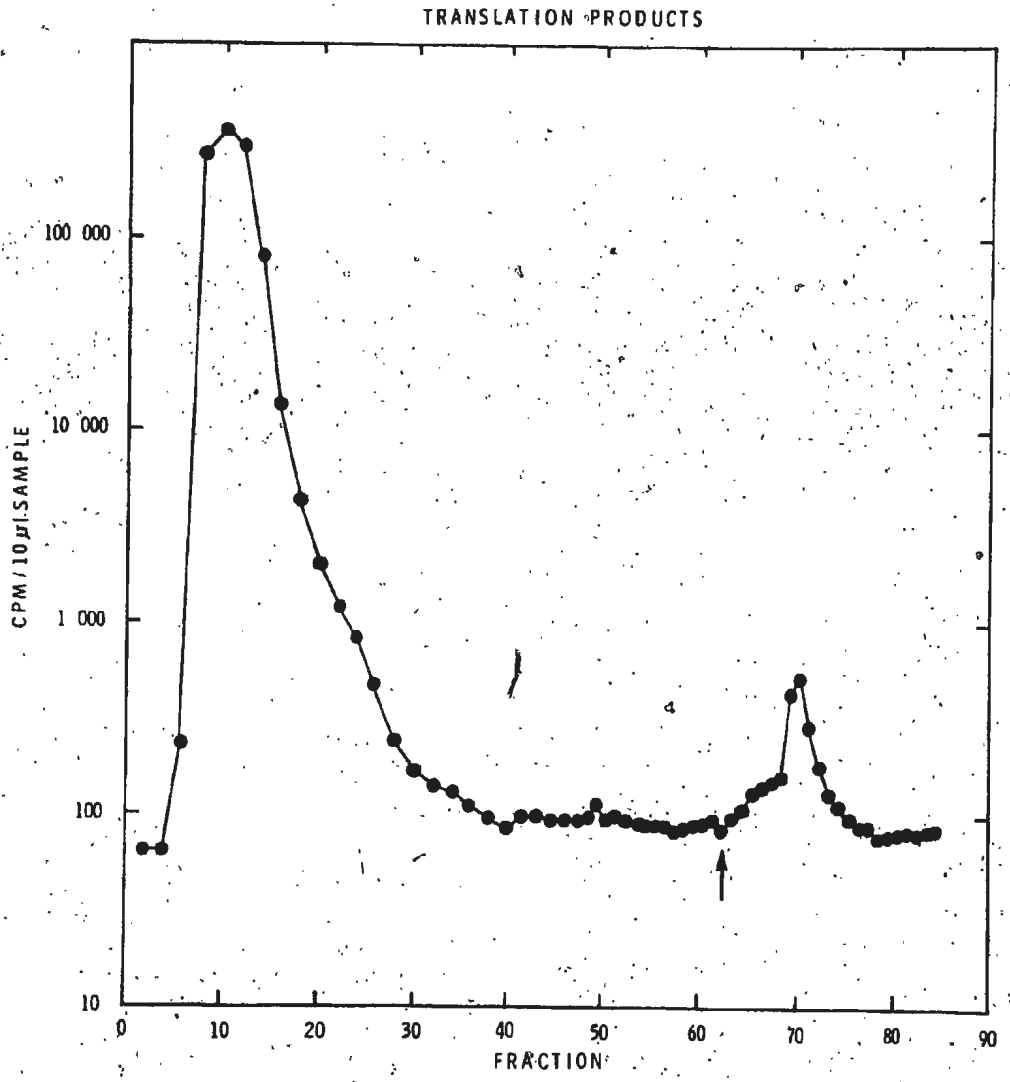

RIBOSOMAL PROTEINS
0.200
0.150
0.100
0.050
0.000
$A_{280}$
5
10
15
20
FRACTION

Figure 17. Determination of CM-cellulose ability to fractionate *in vitro* translated and labeled proteins. Translation assays, in binding Buffer L, were applied to the column and fractions were collected. Elution commenced at the arrow. This elution profile is of proteins translated by polysomes of a 6 h regenerating liver. Note the logarithmic scale.


TRANSLATION PRODUCTS
CPM / 10 µl SAMPLE
100 000
10 000
1 000
100
10
0
10
20
30
40
50
60
70
80
90
FRACTION

## 3.2 Some rat liver macromolecular changes following partial hepatectomy

Several aspects of mRNA synthesis following partial hepatectomy were examined.

### 3.2.1 Amount of mRNA present

It is well-accepted that the rate of protein synthesis in the residual liver tissue increases early after partial hepatectomy (Bucher and Malt 1971, Lewan et al. 1977). This increase may be a consequence of an increase in the rate of reinitiation of translation or of an increase in both the amount of mRNA and ribosomes available for participation in translation. The quantity of mRNA and ribosomes active in translation, measured as $A_{260}$ polysomes present per g regenerating liver, was investigated as a function of time following partial hepatectomy within each of 9 litters of rats. The unprocessed data (Appendix A) indicate that the quantity of mRNA per g regenerating liver increases early following partial hepatectomy; this increase is manifest within 2 h postoperatively and lasts more than 10 h. The unprocessed data also suggest that the quantity of mRNA per g regenerating liver reaches a plateau prior to 10 h of regeneration.

The data were analyzed by determining the coefficients of correlation of 7 regression models [linear ($y = a + bx$), parabolic ($y = a + bx + cx^2$), power ($y = ax^b$), exponential ($y = ae^{bx}$), logarithmic ($y = a + b\ln x$), hyperbolic ($y = a - b/x$), and linear (var) ($1/y = a + bx$)]. Examination of the rankings of these coefficients of correlation suggested that the data within each litter are best characterized by a power model or a parabolic model. However, visual

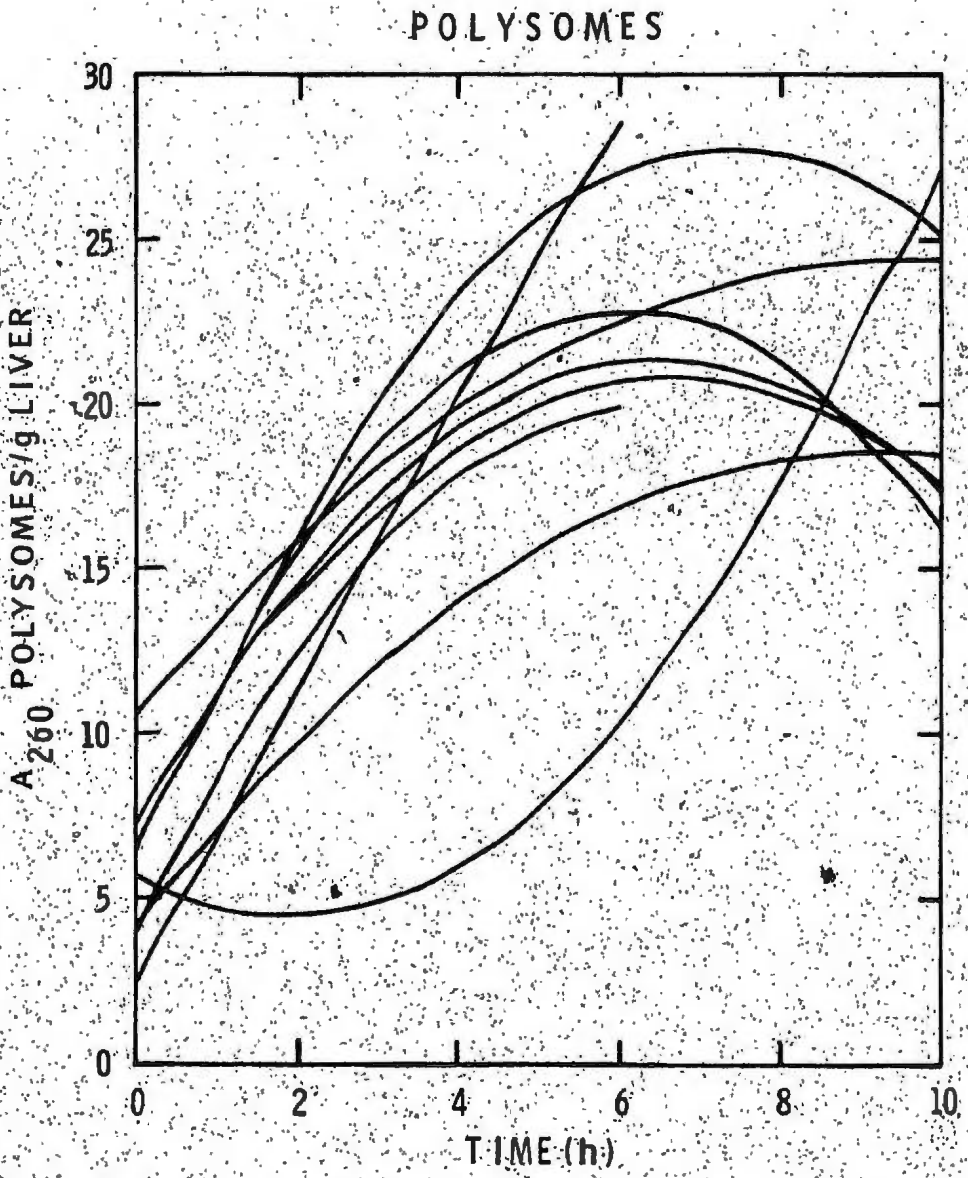
examination of the data with the power and parabolic regression lines indicated that the latter more accurately describes the situation. Biologically, this description is reassuring; because, mathematically, the parabolic model allows an increase, a peak, and a decrease in the value of the dependent variable. The parabolic regression curves from each litter are shown in Figure 18; parameters are in Appendix B. The average time of maximal $A_{260}$ polysomes/g liver was found to be 7.4 h, and a Student's t test showed that the amount of polysomes present at 10 h was still significantly greater than the pre-operative amount ($t = 11.6$, 14 d. f.).

3.2.2 Translatability of mRNA

From time to time it has been suggested, although not demonstrated, that the apparent increase in mRNA translation in rapidly growing cells may be the result of an increased initiation of translation or of an increased rate of translation relative to that present in resting cells. Preliminary observations were made to test the validity of this hypothesis with respect to polysomes in regenerating liver, by examining total radioactivity incorporated in 60 min (during *in vitro* translation) per mRNA nucleotide present ($A_{260}$ polysomes) as a function of postoperative time. More definitive conclusions would require determination of radioactivity incorporated at discrete, intermediate time points during translation.

On a litter by litter basis the unprocessed data (Appendix C), suggest a positive relationship between time following partial hepatectomy and radioactivity incorporated per mRNA nucleotide, during *in vitro* translation. Examination of the rankings of the

Figure 18. Family of regression curves of $A_{260}$ polysomes/g liver. The amount of mRNA present, measured as $A_{260}$ polysomes, as a function of time following partial hepatectomy was found to be best described by the parabola ($y = a + bx + cx^2$) regression curves shown. Each curve represents the function generated by the parabolic regression coefficients of one litter (4 or 5 animals). Parameters are detailed in Appendix B.

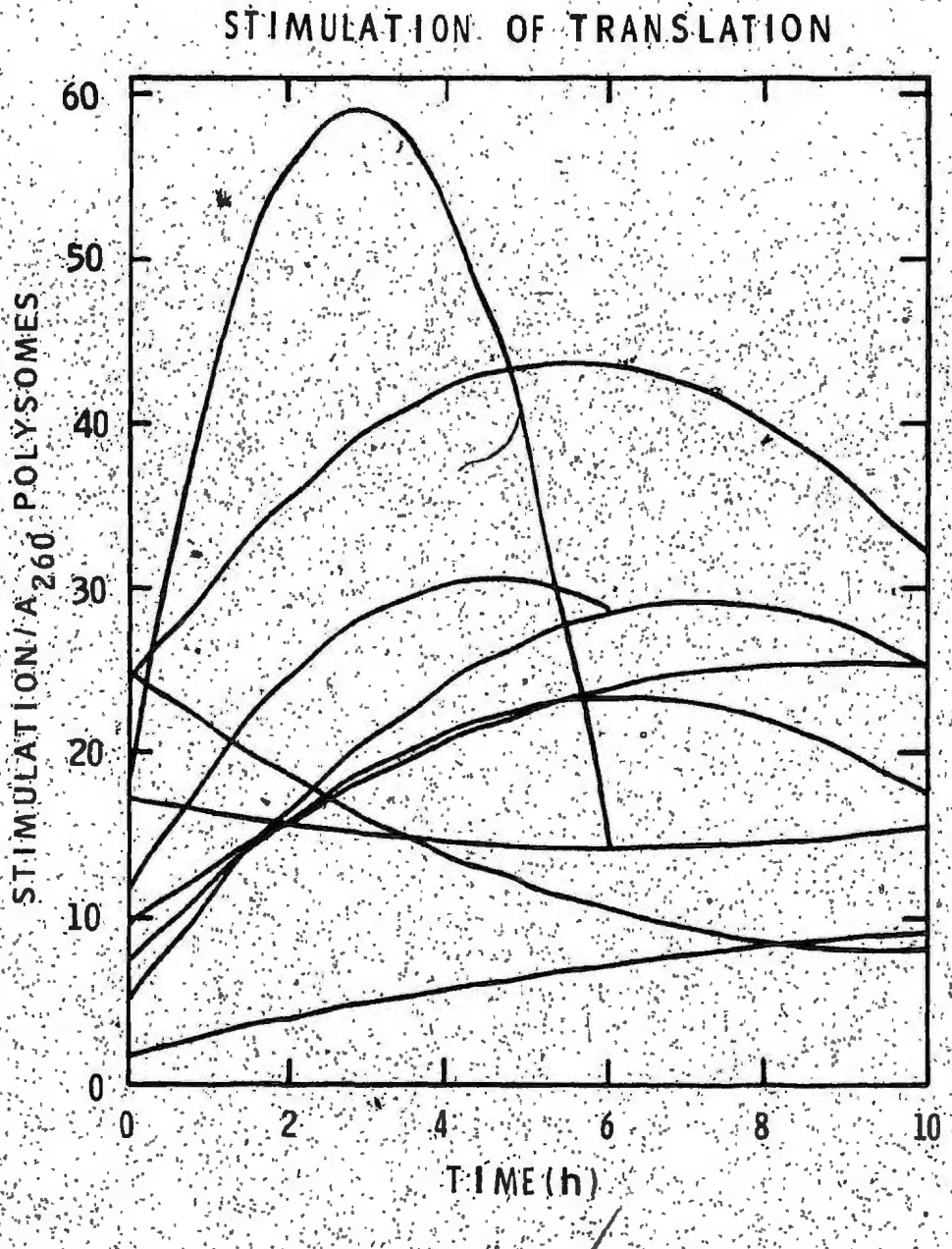

POLYSOMES
30
25
20
15
10
5
0
$A_{260}$ POLYSOMES/g LIVER
0
2
4
6
8
10
TIME (h)

coefficients of correlation suggested that this relationship follows the general model for a parabola ($y = a + bx + cx^2$). An equivalent analysis is not possible utilizing all the data, because although the specific activity of the L-[$^{35}$S]methionine used was constant within a litter, it was not identical between litters due to the short half life of $^{35}$S (87 d), the significant rates of oxidation and decomposition of L-[$^{35}$S]methionine to methionine sulfoxide and methionine sulfone, and that not all time intervals were represented within each litter. In addition, the size of the endogenous methionine pools is unknown and may be variable.

Use of the unitless measure "stimulation" will aid in overcoming the difficulties caused by variations in the specific activity of the L-[$^{35}$S]methionine used for *in vitro* translations, in ascertaining any patterns within all the data. "Stimulation" observed in an *in vitro* translation assay is the incorporation of radioactivity in the assay containing exogenous mRNA divided by the incorporation of radioactivity in the assay containing no added mRNA (Appendix D). These calculated data provide no evidence for the presence of an active translation stimulating or inhibiting factor associated with the polysomes isolated.

This observation is supported by statistical analysis. Examination of the rankings of the coefficients of correlation of "stimulation" (of translation) per $A_{260}$ polysomes as a function of postoperative time shows that again the parabolic model can best account for the observations. Figure 19 is a plot of the parabolic regression curves of the stimulation data; parameters are in

Figure 19. Family of regression curves of stimulation of translation/$A_{260}$ polysomes. On average, the amount of stimulation of translation as a function of time following partial hepatectomy was found to be best characterized by the parabola ($y = a + bx + cx^2$) regression curves shown. Each curve represents the function generated by the regression coefficients of one litter. Parameters are detailed in Appendix E.


STIMULATION OF TRANSLATION
STIMULATION/A260 POLYSOMES
60
50
40
30
20
10
0
0
2
4
6
8
10
TIME(h)

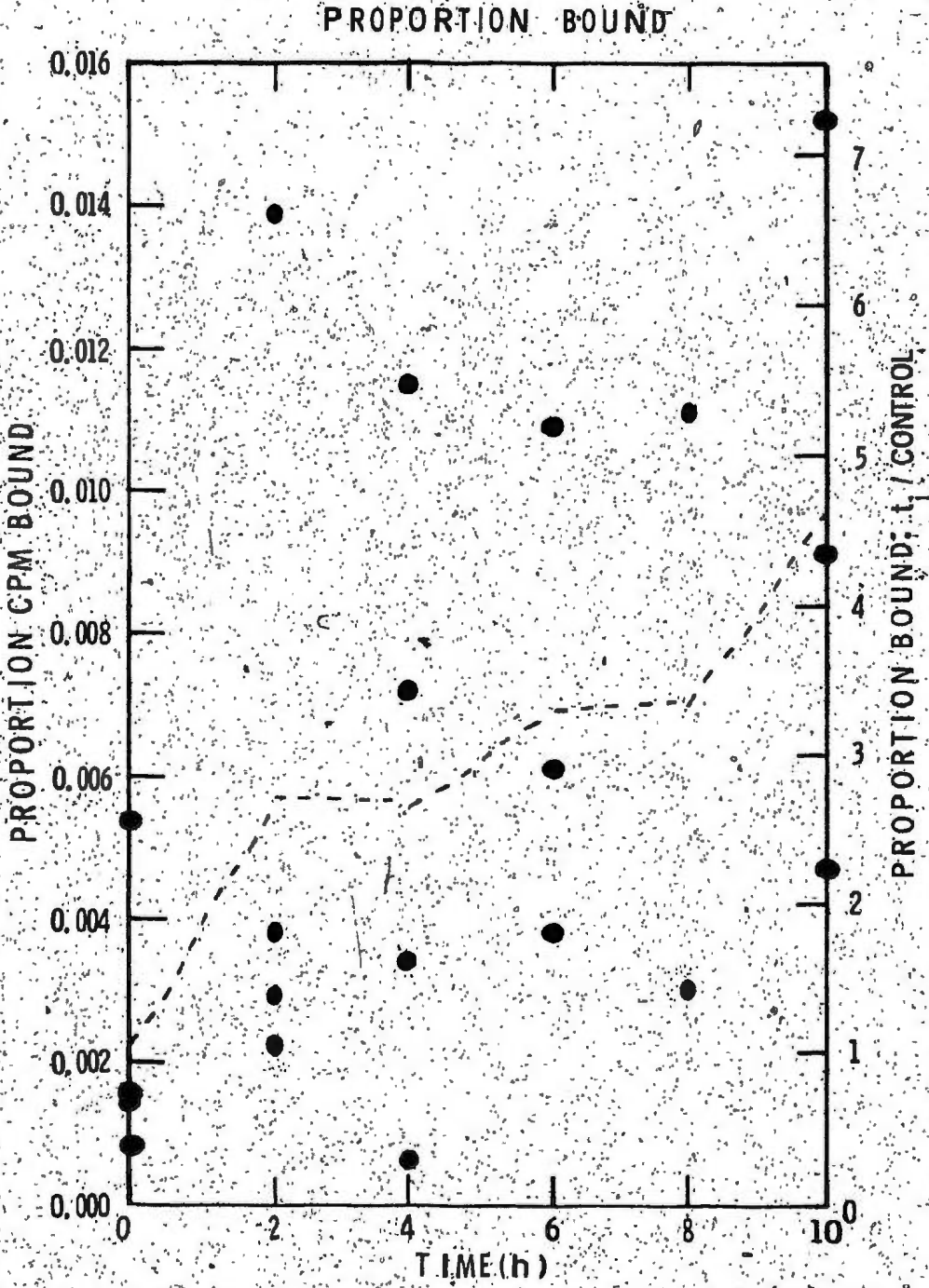
Appendix E. Dunnett's test indicates that at none of the times observed is stimulation significantly greater, at the 95% level, than the pre-operative stimulation. Thus, an analysis of stimulation/$A_{260}$ polysomes at different times following partial hepatectomy suggests that the operation does not affect the rate or frequency of translation of the mRNA present. The analysis also indicates that the existence of an active polysome associated, translation influencing factor is unlikely.

3.2.3 Synthesis of basic proteins

These studies were designed to determine if the proportion of basic (CM-cellulose binding) proteins being translated changes as a function of time following partial hepatectomy. Regression analyses of the proportion of proteins bound to CM-cellulose as a function of postoperative time (unprocessed data Appendix F) suggests that there is no good, relatively simple model of the results obtained (Figure 20). However, there is an increase in the proportion of CM-cellulose binding proteins with time following partial hepatectomy.

Use of Student's t test showed that the proportion of basic proteins translated from pre-operative polysomes does not differ significantly from that of control polysomes ($t = 0.15$, 5 d. f.), but does differ at the 90% confidence level ($t = 2.6$, 5 d. f.) from the proportion of basic proteins translated from 10 h regenerating liver polysomes. (Total confidence level = 0.9 x 0.9 = 0.81). Thus, these results suggest that there is an increase in the synthesis of basic proteins following partial hepatectomy, and that the peak of this increase does not occur prior to 10 h of regeneration.

Figure 20. Proportion of translated proteins bound to CM-cellulose. The proportion of *in vitro* translated proteins bound to CM-cellulose increases as a function of time following partial hepatectomy, as shown by the dashed line connecting the averages of the values obtained. Each point is the result of one experiment, involving one animal. The units on the left side show the actual proportion bound; the units on the right side show the proportion bound relative to the control.


PROPORTION BOUND
PROPORTION CPM BOUND
PROPORTION BOUND; t / CONTROL
TIME (h)
0.016
0.014
0.012
0.010
0.008
0.006
0.004
0.002
0.000
0
2
4
6
8
10
0
1
2
3
4
5
6
7

To determine the variability of the binding of the translated proteins to CM-cellulose and thus establish the significance of the results obtained, the following experiment was performed. A large number of polysomes from the same source (a group of normal rats) was translated in the wheat germ, cell-free system. These translation assays, containing radioactively labeled products, were combined; and one-third of the mixture was passed over each of 3 CM-cellulose columns. The proportion of CM-cellulose binding radioactivity on these columns was 0.0106, 0.0128, and 0.0167 resulting in an average of 0.0134 ± 0.0025.

Although the variation of up to 25% of the average value obtained in this limited system may be important when absolute values are small, as in this case, the coefficient of variation of these data (0.19) is of the same order of magnitude as that for the data reported by Hackett et al. (1978) (0.15). Thus, the scatter evident in Figure 20 is not attributable to poor reproducibility of the technique. Nonetheless, the overall pattern suggests that the synthesis of basic proteins increases during the first 10 h following partial hepatectomy. The observed variability of the system, as shown in Figure 20, may also reflect the influence of individual metabolic variations, circadian rhythms, reactions to stress, or other noncontrollable factors.

# 4 DISCUSSION

## 4.1 Findings

The quality of rat liver polysomes isolated, as measured by the number of distinct peaks visible by absorbance after centrifugation through a sucrose gradient, is comparable to the best published results I have seen (Ramsey and Steele 1976). As anticipated from the known increase in lipid content early during liver regeneration (Bucher and Malt 1971, Becker 1974), the livers were observed to be progressively paler/pinker during the first 10 h following partial hepatectomy, and the size of the lipid layer of the postmitochondrial supernatant increased. Because the cell-free translation system prepared from wheat germ and using polysomes instead of purified mRNA as template results in acceptable levels of stimulation (up to 250 times), its use eliminates the time and reagents required for isolating undegraded mRNA from polysomes.

When interpreting the data for the amount of mRNA present per unit weight of liver three assumptions should be kept in mind: 1) fluid loss, if any, during hypertrophy does not artifactually decrease the liver weight, i. e. there is no significant decrease in liver weight during the first 10 h of regeneration, 2) the number of mRNA nucleotides per ribosome does not change significantly during the time period examined, i. e. the number of ribosomes present in the polysome fraction accurately reflects the amount of mRNA being

translated, and 3) the volume of modified Buffer H added to the polysomes, for solubilization prior to translation, equaled twice the pellet volume, i. e. dilution factors were accurately calculated.

As stated in the Results section 3.2.1, the dependent variable being examined is "$A_{260}$ polysomes/g liver". An increase in the value of the dependent variable would occur if the liver weight decreased or if the observed $A_{260}$ polysomes increased. The former would occur if assumption 1) were in error, and the latter might occur if assumption 2) or 3) were in error. Analogously, the value of the dependent variable would decrease if the liver weight increased or if the $A_{260}$ polysomes decreased. Because hyperplasia is not observed until at least 14 h postoperatively (Leduc 1964, Bucher and Malt 1971, Fausto et al. 1976, Colbert et al. 1977), a significant change in liver weight seems unlikely. A decrease in the $A_{260}$ polysomes observed might occur if assumption 2) or 3) were incorrect. There appears to be a consensus that the total number of ribosomes involved in translation accurately reflects the amount of mRNA being translated. Nonetheless, any errors in these assumptions do not explain the difference in quantity of mRNA, as measured by $A_{260}$ polysomes, observed between pre-operative and control polysomes.

The average observed time of maximal $A_{260}$ polysomes/g regenerating rat liver is 7.4 h. This time is in good agreement with the following published observations: 1) the maximum increase in template activity observed by Hwang et al. (1974) occurs at 6 to

8 h; 2) maximum incorporation of [5-$^{3}$H]orotic acid into HnRNA of parenchymal cells occurs at 6 h postoperatively (Glazer 1974); 3) increased transport of nuclear poly(A) RNA, presumably mRNA or mRNA precursors, to the cytoplasm takes place during the first 12 h of liver regeneration (Greene and Fausto 1974, Fausto et al. 1976, Colbert et al. 1977, Fausto et al. 1977, Glazer 1977, Greene and Fausto 1977, Krieg et al. 1979); 4) Smal'ko and Platonov report maximal incorporation of [$^{14}$C]orotic acid into polysomes (rRNA plus mRNA) (Smal'ko and Platonov 1977a) and maximal appearance in the cytoplasm of informational RNA (mRNA) (Smal'ko and Platonov 1977b) between 6 and 12 h after partial hepatectomy; 5) Atryzek and Fausto (1979) report maximal cytoplasmic and polysomal poly(A) RNA and a maximal polysomal poly(A) RNA to cytoplasmic poly(A) RNA ratio between 6 and 12 h of regeneration; and 6) Colbert et al. (1977) observe that 12 h regenerating liver has approximately 20% more poly(A) mRNA molecules per cell than the liver cells of sham operated rats.

A second set of assumptions should be kept in mind when examining the unprocessed translatability of mRNA data (Appendix C): 1) the number of mRNA nucleotides per ribosome does not change significantly during the time period examined; 2) the amount of radioactivity incorporated during in vitro translation is proportional to the frequency of initiation of translation, i. e. the average rate of initiation of translation of the mRNAs of interest is equal to the average rate of initiation for all the isolated mRNAs; and 3) the rate of elongation is independent of the protein encoded by the mRNA.

Current thinking (1979) suggests that the first assumption is probably correct, i. e. mRNAs being translated may have a constant or maximal number of ribosomes associated with them; the second may be valid if the proteins whose translation is being compared have a similar stoichiometry with respect to the radioactive amino acid; and the third is valid. In these analyses, it has also been assumed that the $A_{260}$ of the polysome fraction is an accurate measure of the quantity of mRNA present.

The results relating to stimulation of translation are interesting. To date (1979), there is no evidence that some aspect of the mRNA itself may regulate the initiation frequency or the rate of translation, although a translation-influencing factor(s) may be associated with the mRNA. Thus, if there is either a polypeptide or a nonpolypeptide factor which influences the initiation frequency or the rate of translation, it would be expected to be more active or apparent in the polysome preparation used than in the standard, purified mRNA preparations. However, these data provide no direct evidence of such a factor. Further experiments and more detailed statistical analysis would be required to determine if there may be a relationship between the height and time of the peaks of stimulation of translation or if the area under these curves approaches a constant.

Perhaps the most significant finding was the size of the difference in the proportion of basic proteins synthesized in the controlled rapid growth of regenerating liver compared with that in an uncontrolled exponentially growing Ehrlich ascites tumor cell culture. The proportion of actively synthesized basic proteins

in a 10 h regenerating liver cell, 0.97%, is about four times the proportion in a normal liver cell, 0.23% (Appendix F). However, the proportion of basic proteins synthesized by Ehrlich ascites tumor cells during exponential growth, 13 ± 2% (Hackett et al. 1978) is approximately 55 times the proportion of basic proteins synthesized by quiescent rat liver cells and approximately 13 times the proportion synthesized by 10 h regenerating liver cells.

This contrast may indicate a difference in the relative abundance of specific proteins in the two cell types; such a difference could exist due to the extensive range of metabolic functions present in the liver, including production of some proteins and other substances for nonliver cells and processing of some proteins and wastes of extraliver origin; i. e. liver cells must carry out many more types of processes than do Ehrlich ascites tumor cells. In addition, ribosomal proteins comprise at most 5% of total liver protein (Maden 1971) and tend to have low turnover rates.

Because the synthesis of nuclear DNA-associated histones is limited during the first 10 to 12 h of liver regeneration (Takai et al. 1968; Orlova and Rodionov 1970; Gutierrez-Cernosek and Hnilica 1971; Smirnova and Rodionov 1974), the majority of synthesized basic proteins are likely to be ribosomal proteins. However, in an asynchronous, exponentially growing cell culture, histones are always being synthesized at an elevated level. Thus, it is not surprising that the synthesis of basic proteins in up to 10 h regenerating rat liver is less than 13%.

## 4.2 Further research

### 4.2.1 Modifications of technique

If the research reported here were to be continued, experimental design or technique might be modified in order to increase experimental accuracy and ease. These items will be discussed with respect to their order in the protocol. The methods for isolation and storage of polysomes prior to use in the *in vitro* translation assay appear to be reliable and satisfactory.

The wheat germ *in vitro* translation assay might be improved by several modifications, some of which were instituted during the course of these investigations. The preparation of the wheat germ S-30 fraction results in large amounts of some free amino acids; their presence inhibits the incorporation of the corresponding radioactive amino acids. Mezl (1981) has suggested the following procedure for minimizing the quantity of these free amino acids. A more active *in vitro* translation system with low endogenous activity is obtained when the wheat germ is ground in a 15 mM $Mg^{++}$, pH 6.3 buffer instead of the commonly used 1 mM $Mg^{++}$, pH 7.6 buffer. The generation of free amino acids during *in vitro* translation can be minimized by using only the early portion of the turbid fractions eluted from the Sephadex G-25 column and by eliminating the pre-incubation of the wheat germ S-30 fraction prior to translation (Mezl 1981). Use of this protocol may also minimize the activity of the endogenous, translation inhibiting protein characterized by Roberts and Stewart (1979). This protein acts at a polypeptide elongation step and is also associated with the wheat germ S-30

turbid fractions eluted from a Sephadex G-25 column. As reported in Results section 3.1.2.7, pre-incubation of the wheat germ S-30 fraction was found to be unnecessary for, and perhaps detrimental to, satisfactory stimulation of *in vitro* translation.

Because the wheat germ *in vitro* translation assay is relatively sensitive to ionic strength, and polysome primed assays have not been extensively characterized, it might be advisable to examine the effect of $K^+$ concentration on translation. The use of heparin during polysome isolation and the absence of EDTA while solubilizing the polysomes for translation is recommended.

No optimum quantity of polysomes for maximal stimulation of translation was observed during the characterization of the *in vitro* translation assay (section 3.1.2.9). However, it appeared that during some translations of polysome-associated mRNA from regenerating liver, such an optimum quantity had been exceeded, adversely affecting the results. This possible problem could be obviated by attempting to determine the quantity of polysomes which results in maximal incorporation of TCA precipitable radioactivity during *in vitro* translation and by using a constant quantity of polysomes, as measured by $A_{260}$, per translation assay.

CM-cellulose, as used in these investigations to define the proportion of basic proteins, has several negative attributes. When examining Ehrlich ascites tumor cell mRNA *in vitro* translation products, Hackett *et al.* (1978) report that the basic fraction, defined by binding to CM-cellulose, contained 13 $\pm$ 2% of recovered radioactivity. Because, in the present study, the average

proportion of basic radioactive polypeptides recovered from *in vitro* regenerating rat liver polysome translation products ranged only from 0.23 to 0.97% of recovered radioactivity (Appendix F), a variation of ± 2% of total radioactivity is unacceptably large. Variability in the proportion of proteins binding to or eluting from CM-cellulose was verified in a limited independent experiment, detailed in section 3.2.3; the proportion of *in vitro* translated, labeled, CM-cellulose binding polypeptides recovered was observed to be ± 25% of the average value.

CM-cellulose has the additional negative attributes of being time consuming to prepare, because of the necessity of precycling and removing fines, and of having its volume and flow rate significantly affected by the buffer's ionic strength. The necessity of defining basicity by binding to CM-cellulose may be avoided by use of another substrate or criterion. For example, CM-biogel might result in better reproducibility of binding of basic translation products, and it has neither of the mentioned negative attributes of CM-cellulose.

Alternatively, to more precisely define the actual translation products, polyacrylamide gel electrophoresis followed by autofluorography or by fractionation and solubilization of the gel and liquid scintillation counting might be preferable. However, such procedures would have to take into account a number of factors, including the possibility of some polypeptides preferentially entering or not entering the gel, preferential recoverability of proteins from the gel, and the time necessary to measure the radioactivity

in a large number of gel slices. The efficacy of any method chosen should be tested using total ribosomal proteins and histones. Any extensive characterization of the L-[$^{35}$S]methionine labeled products of *in vitro* translation should also take into account the possible effects of post translational oxidation of the labeled amino acid on physical and biochemical properties of the polypeptides.

Although biological events following partial hepatectomy tend to be regarded as being partial hepatectomy induced, by removal of liver tissue rather than the operation itself, the possible value of examining sham operated animals should not be forgotten. In addition, examination of events at intervals of not more than 2 h should be maintained. The absence in the literature of intermediate time points makes it difficult to ascertain accurately the timing of post partial hepatectomy events.

4.2.2 Questions to be investigated

The investigations reported here, like much basic research, have led to many related, but unanswered, questions. Perhaps the most obvious question is, "What are the characteristics of the basic proteins synthesized early during rat liver regeneration?" This question may be asked as a series of more easily investigated queries, including: "Are the basic proteins ribosomal proteins/histones?"; "If so, are all basic ribosomal proteins/histones present?"; "If so, are they present in stoichiometric amounts?". Each of these questions can be asked as a function of time following partial hepatectomy, leading to a more complex, yet fundamental, question, "Does the synthesis of the basic ribosomal proteins/histones

appear to be coordinately regulated at the level of translation, or are some ribosomal proteins/histones synthesized earlier/later than others?".

These questions could be answered most easily by using two dimensional polyacrylamide gel electrophoresis. However, the greatest obstacle to such an investigation appears to be technical; after elution, the CM-cellulose binding proteins are present in low concentration in a relatively large volume of high salt ( 2 M LiCl) high urea (4 M) buffer. The high salt concentration interferes with the running of most gels. In addition, there is not yet a gel system capable of unequivocally separating all mammalian ribosomal proteins. Another technical limitation to be kept in mind, if the gels are to be examined by means of liquid scintillation counting, is the half life of the radioactive moiety used relative to the time required for carrying out the experiments.

Nonetheless, there may be ways of overcoming the former obstacle. Perhaps there is an ion exchange column substrate which binds basic proteins so that they can be eluted by a change in pH instead of ionic strength. Polybuffer (Pharmacia) could be used with such a column to effect a continuous pH gradient and more precisely define the basicity of each protein fraction. If such a substrate is unavailable, there may be several ways to eliminate the salt from and reduce the volume of the eluant. The proteins could be dialyzed against a low ionic strength buffer and concentrated either in the dialysis bag by using polyethylene glycol or by lyophilization. Both these methods have the drawback that there may

be significant preferential or nonspecific binding of proteins to the dialysis membrane. Alternatively, the basic, polypeptide containing eluant could be prepared for electrophoresis by lyophilization, dissolving the lyophilizate in a minimal volume of ammonium bicarbonate buffer, desalting this mixture by passage over a Sephadex G-25 column, and lyophilizing again.

A number of related questions with respect to the rat liver mRNAs coding for basic proteins as a function of time following partial hepatectomy can be posed, including: "Do the poly(A) and nonpoly(A) or poly(A) and total polysomal mRNAs code for the same basic proteins?"; "If so, is the proportion of poly(A) and nonpoly(A) mRNA coding for a particular protein(s) constant?". All these studies would probably be of greater interest if the time course were of at least 24 h duration with a maximum interval of 2 h between time points. Obviously, such investigations should take into account the desirability, or lack thereof, of using litter mates or same sexed animals, sham operated controls, and an individual investigator's sleep requirements.

Many questions fundamental to a more complete understanding of the synthesis of ribosomal proteins/histones remain. These questions include: "Is the synthesis of ribosomal proteins/histones regulated at the level of transcription/formation or deformation of an RNP not being translated/translation?"; "What is the nature of the primary RNA transcript(s)?"; and "How is the transcript(s) processed; specifically, how\much/what type of splicing occurs prior to intracellular translation?". The regenerating rat liver may be

a desirable system to use in investigating these questions, because at specific times following partial hepatectomy the synthesis of ribosomal proteins/histones is significantly higher than in quiescent cells, resulting in a higher yield of material to be studied.

It may also be of interest to examine whether any nonbasic proteins cease or commence being actively synthesized in the liver following partial hepatectomy. Such an investigation could perhaps be undertaken using O'Farrell (1975, O'Farrell et al. 1977) two dimensional polyacrylamide gel electrophoresis techniques and autofluorography. This method is useful, because it allows simultaneous examination of many proteins.

# 5 Conclusions

Based on the results obtained from the investigation of translation of mRNA and synthesis of basic proteins in liver during the first 10 h following partial hepatectomy in the rat, the following conclusions can be drawn:

1. Polysomes (polyribosomes) may be used to program a wheat germ *in vitro* translation assay for preparative yields of radioactively labeled polypeptides. This technique eliminates the time and reagents required for preparation of protein-free mRNA while allowing examination of labeled proteins coded for by all the mRNA being translated rather than only of those proteins coded for by poly(A) mRNA.

2. Binding to CM-cellulose is not an ideal criterion for measuring the proportion of basic proteins present in a mixture of proteins when that proportion is small.

3. The value of $A_{260}$ polysomes/g liver, presumably a reflection of mRNA/g liver, reaches a maximum between 7 and 8 h following partial hepatectomy. The value of this parameter remains significantly larger than the normal (pre-operative) value, until at least 10 h postoperatively.

4. The investigations reported here support the concept that there is no polysome-associated initiation frequency or translation rate stimulating or inhibiting factor.

5. The proportion of basic proteins being synthesized in the normal liver (0.23%) or in the first 10 h of regenerating liver (up to 0.97%) is much less than that reported in uncontrolled, rapidly growing Ehrlich ascites tumor cells (13 ± 2% [Hackett et al. 1978]).

6. The peak of basic protein synthesis in regenerating rat liver occurs later than 10 h postoperatively.

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## Appendix A

$A_{260}$ polysomes/g liver

| | time after partial hepatectomy (h) | | | | | | |
|---|---|---|---|---|---|---|---|
| litter | control | 0 | 2 | 4 | 6 | 8 | 10 |
| A | | 3.91 | 12.03 | 18.33 | 19.77 | | |
| B | 9.27 | 4.99 | 5.00 | 26.90 | 26.43 | | |
| C | 9.16 | 7.11 | 20.75 | 21.48 | 20.53 | 19.36 | 27.53 |
| D | 8.84 | 5.80 | | 22.71 | 22.44 | 11.65 | 22.06 |
| E | 7.84 | 6.55 | 11.86 | 26.11 | 23.77 | 15.40 | 18.68 |
| F | 8.69 | 6.41 | 16.08 | 18.56 | 24.55 | 15.56 | 19.89 |
| G | 9.62 | 4.20 | 11.33 | 11.25 | | 21.08 | 17.11 |
| H | 7.69 | 7.46 | 2.25 | 2.67 | 15.29 | | 26.61 |
| J | 8.79 | 4.76 | 18.60 | 21.00 | 28.50 | 26.25 | 25.62 |
| average | 8.79 | 5.69 | 12.24 | 18.78 | 22.66 | 18.22 | 22.50 |

Absorbance was measured with a Beckman model 24 spectrophotometer using 1 mL sample volume, 1 cm path length, and 0.2 mm slit width.

$A_{260}$ polysomes was calculated by assuming that the volume of polysome solubilizing buffer (Buffer H) was twice the polysome pellet volume. Thus, $A_{260}$ polysomes = ($A_{260}$ polysomes/$\mu$L) x $\mu$L polysome solubilizing buffer x 1.5.

Control polysomes were isolated from a group of normal rats and stored as indicated in section 2.2.5. One centrifuge tube of the polysomes was processed concurrently with the polysomes from each experimental litter.

## Appendix B

Parameters of parabolic regression curves for

$A_{260}$ polysomes/g liver

| litter | animals | a | b | c | $r^2$ | $t_{max}$ (h) |
|---|---|---|---|---|---|---|
| A | 4 | 3.758 | 5.199 | -0.418 | 0.997 | 6.23 |
| B | 4 | 2.777 | 4.491 | -0.030 | 0.791 | 74.85 |
| C | 6 | 10.452 | 2.946 | -0.156 | 0.663 | 9.44 |
| D | 5 | 7.206 | 4.046 | -0.299 | 0.514 | 6.76 |
| E | 6 | 6.148 | 5.477 | -0.449 | 0.698 | 6.10 |
| F | 6 | 7.391 | 4.266 | -0.324 | 0.747 | 6.58 |
| G | 5 | 4.253 | 3.137 | -0.172 | 0.883 | 9.14 |
| H | 5 | 5.623 | -1.298 | 0.351 | 0.895 | |
| J | 6 | 5.750 | 5.985 | -0.406 | 0.956 | 7.37 |
| weighted average | | 6.235 | 4.446 | -0.289 | | 7.40 |

The parameters are for the equation defining a parabola, $y = a + bx + cx^2$; r = correlation coefficient.

$A_{260}$ polysomes was measured and calculated as in Appendix A.

$t_{max}$ is the time of maximal value of the parabola.

Litter H values were not used when calculating the weighted average values; in addition, the litter B value was not used when calculating the weighted average value of $t_{max}$.

## Appendix C

### cpm/$A_{260}$ polysomes

| | time after partial hepatectomy (h) | | | | | | |
|---|---|---|---|---|---|---|---|
| litter | control | 0 | 2 | 4 | 6 | 8 | 10 |
| A | | 11 320 | 23 880 | 29 013 | 28 068 | | |
| B | 14 533 | 8 650 | | 20 703 | 25 241 | | |
| C | 18 515 | 42 971 | 35 346 | 20 210 | 17 837 | 19 963 | 11 847 |
| D | 14 030 | 17 670 | | 35 508 | 39 651 | 49 858 | 42 503 |
| E | 18 371 | | 33 287 | 15 572 | 27 061 | 38 598 | |
| F | 6 654 | 10 839 | 16 148 | 15 315 | 27 422 | 31 956 | 13 705 |
| G | 20 582 | 21 501 | 46 046 | 50 612 | | 31 940 | 42 850 |
| H | 35 275 | | 44 675 | 32 769 | 58 761 | | 49 534 |
| J | 18 427 | 64 217 | 58 174 | 52 144 | 60 970 | 47 006 | 58 369 |

Each cpm value is the average of 5 µL aliquots from each of three *in vitro* translation assays, processed as in section 2.9.

$A_{260}$ polysomes were measured and calculated as in Appendix A.

Control polysomes were as in Appendix A.

Appendix D

Stimulation/$A_{260}$ polysomes

| | time after partial hepatectomy (h) | | | | | | |
|---|---|---|---|---|---|---|---|
| litter | control | 0 | 2 | 4 | 6 | 8 | 10 |
| A | | 11.7 | 24.9 | 30.2 | 29.2 | | |
| B | 14.4 | 8.5 | | 20.3 | 24.9 | | |
| C | 11.9 | 25.1 | 20.7 | 11.8 | 10.4 | 11.6 | 6,9 |
| D | 7.9 | 10.3 | | 20.3 | 22.4 | 28.3 | 24.2 |
| E | 4.5 | | 7.8 | 3.7 | 6.3 | 9.1 | |
| F | 6.2 | 9.8 | 15.2 | 14.4 | 25.6 | 29.8 | 12.8 |
| G | 18.7 | 19.5 | 41.8 | 45.9 | | 29.0 | 38.9 |
| H | 18.5 | | 23.4 | 17.2 | 30.8 | | 25.9 |
| J | 5.0 | 17.2 | 15.6 | 14.0 | 16.4 | 12.6 | 15.7 |
| average | 10.9 | 14.6 | 21.3 | 19.8 | 20.8 | 20.1 | 20.7 |

Stimulation of an *in vitro* translation assay = radioactivity incorporated in the assay containing exogenous mRNA/radioactivity incorporated in the assay containing no added mRNA.

$A_{260}$ polysomes were measured and calculated as in Appendix A.

Control polysomes were as in Appendix A.

## Appendix E

### Parameters of parabolic regression curves for stimulation/$A_{260}$ polysomes

| litter | number of animals | a | b | c | $r^2$ | $t_{max}$ (h) |
|---|---|---|---|---|---|---|
| A | 4 | 11.780 | 8.215 | -0.888 | 0.999 | 4.63 |
| B | 4 | 19.610 | 27.455 | -4.738 | 0.371 | 2.90 |
| C | 6 | 25.282 | -3.447 | 0.174 | 0.924 | |
| D | 5 | 10.014 | 3.452 | -0.191 | 0.932 | 9.04 |
| E | 5 | 2.011 | 1.114 | -0.041 | 0.531 | 13.55 |
| F | 6 | 7.457 | 5.107 | -0.411 | 0.547 | 6.22 |
| G | 5 | 24.247 | 6.963 | -0.616 | 0.453 | 5.66 |
| H | 5 | 5.094 | 6.585 | -0.450 | 0.767 | 7.31 |
| J | 6 | 17.132 | -0.858 | 0.066 | 0.386 | |
| weighted average | | 11.092 | 7.762 | -0.925 | | 7.21 |

The parameters are for the equation defining a parabola, $y = a + bx + cx^2$ ; r = correlation coefficient.

$t_{max}$ is the time of maximal value of the parabola.

Litter C and litter J values were not used when calculating the weighted average values.

## Appendix F

### Proportion of radioactivity bound to CM-cellulose

| | | time after partial hepatectomy (h) | | | | | |
|---|---|---|---|---|---|---|---|
| litter | control | 0 | 2 | 4 | 6 | 8 | 10 |
| A | | 0.0014 | 0.0022 | 0.0034 | 0.0038 | | |
| F | 0.0024 | 0.0008 | 0.0029 | 0.0072 | | 0.0030 | 0.0047 |
| G | 0.0010 | 0.0016 | 0.0038 | 0.0006 | 0.0061 | | 0.0091 |
| H | 0.0029 | 0.0054 | 0.0139 | 0.0115 | 0.0109 | 0.0111 | 0.0152 |
| average | 0.0021 | 0.0023 | 0.0057 | 0.0056 | 0.0069 | 0.0070 | 0.0097 |
| average/ control average | 1.00 | 1.09 | 2.71 | 2.67 | 3.29 | 3.33 | 4.62 |

Control data was from the control polysomes of Appendix A.