HEMOGLOBIN SYNTHESIS
IN THE MATURING RABBIT RETICULOCYTE

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This thesis is affectionately dedicated
to my wife, and my mother and father.

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Abstract

A method is described for fractionating rabbit reticulocytes by buoyant density centrifugation in an albumin (BSA) gradient. The progressively lighter cells contained more ribosomes and were more active in protein synthesis than the denser cells.

Cytological, physical, and biochemical properties of the fractions showed that the procedure separates the cells according to their degree of physiological maturity. Fractions were examined for their protein synthesizing activity and ribosomal distribution by sucrose gradient analysis. With increasing cell maturation the percent of ribonucleoprotein present as polysomes declined slightly, the pentamer persisted as the major ribosomal aggregate, and the specific activity of the polysomes decreased. The loss of hemoglobin synthesis during reticulocyte maturation is closely correlated with the loss of total ribosomal material and with an increasing percentage of inactive polysomes.

A fraction of the most immature reticulocytes was labeled with H^3-leucine and transfused into a normal rabbit for *in vivo* maturation. BSA-gradient analysis of blood samples taken at various time intervals confirmed that the position of the cells in a BSA gradient is a function of their age.

A reticulocyte cell-free amino acid incorporating system is described which is capable, after an initial rapid incorporation, of a linear rate of protein synthesis for at least two hours. This system was used to further investigate the differences between reticulocyte fractions from a BSA gradient. Crossed incubations of ribosomes and supernatants showed that not only the ribosomes become less active but that supernatant factors limit amino acid incorporation in the more mature cells. The nature of the supernatant effect is discussed.

Preliminary cell-free studies using polysomes and 80S ribosomes, isolated from a sucrose gradient, showed that 80S ribosomes are inactive by themselves. However, in the presence of polysomes they participate in amino acid incorporation. The results indicate that the rate of protein synthesis is a function of the concentrations of both "monosomes" and polysomes.

TABLE OF CONTENTS

PART		PAGE
	INTRODUCTION	1
	Formation and Maturation of Erythroid Cells	3
	Cytological changes during maturation	3
	Biochemical changes during erythroblast development	4
	Maturation of Reticulocytes	8
	Cytological changes	8
	Biochemical changes	9
	Basis for the Present Studies	12
I.	PROTEIN SYNTHESIS IN RETICULOCYTE FRACTIONS SEPARATED BY ALBUMIN DENSITY CENTRIFUGATION	16
	Materials and Methods	17
	1. Reticulocyte preparation	17
	2. Whole cell incubation	18
	3. BSA gradients	18
	4. Cell lysis, and hemoglobin and ribosome preparation	20
	5. Cell counts and staining	21
	Results	22
	1. Preliminary experiments in fractionating reticulocytes	22
	2. Neutral density separation of reticulocytes	22

PART PAGE

3. Large scale cell fractionation using 25 ml BSA gradients 28

Discussion 33

II. POLYRIBOSOMES AND THE LOSS OF HEMOGLOBIN SYNTHESIS IN THE MATURING RETICULOCYTE 35

Introduction 35

Materials and Methods 36

1. Reticulocyte preparation 36
2. Albumin gradients 36
3. Cytological methods 37
4. Incubations, lysis, and ribosome preparation 40
5. Sucrose gradients 40
6. Analysis of sucrose gradients and other measurements 41
7. Determination of intracellular leucine pool 41

Results 42

Cytological and physical properties of the reticulocyte fractions 42

A. Density 42

B. Staining characteristics 43

C. Cell size distributions 46

D. Amino acid incorporation and ribosome content 52

PART | PAGE

Loss of polyribosomes during reticulocyte maturation 54

A. Sucrose gradient analysis and amino acid incorporation 56

B. Sedimentation coefficients 61

Determination of leucine pool in young and old reticulocytes 64

1. Leucine pool in the whole cell preparation 64

2. Leucine pool in young and old reticulocytes 68

Discussion 72

III. *IN VIVO* MATURATION OF IMMATURE RETICULOCYTES TRANSFUSED INTO A NORMAL RABBIT 77

Materials and Methods 77

Results and Discussion 80

IV. RETICULOCYTE CELL-FREE AMINO ACID INCORPORATING SYSTEM AND ITS USE IN INVESTIGATING THE LOSS OF PROTEIN SYNTHESIZING ACTIVITY IN THE MATURING RETICULOCYTE 88

Introduction 88

Materials and Methods 89

1. Preparation of subcellular fractions 89

PART	PAGE

2. Cell-free incubations 91
3. Determination of radioactivity 91
4. TCA-precipitation on membrane filters 92
5. Activity of 80S ribosomes in cell-free system 93
6. Materials 94

Results 95

Properties of the cell-free amino acid incorporating system 95

A. Time course 95
B. pH optimum 97
C. Amino acid incorporation in the presence of 105000 g supernatant, pH-5 supernatant, and pH 5 fraction 98
D. Effect of supernatant concentration on incorporation 104
E. Effect of reagent mix and amino acid concentrations 106

Amino acid incorporation in cell-free systems from BSA-fractionated reticulocytes 107

A. Cell-free experiments with reticulocyte fractions separated by neutral density centrifugation 110

PART | PAGE

B. Cell-free incorporation in reticulocyte fractions from a BSA density gradient 114

Stimulation of cell-free amino acid incorporation by 80S ribosomes 118

Discussion 122

REFERENCES 128

INTRODUCTION

During the past decade research in hemoglobin biosynthesis has greatly contributed to our understanding of the biochemical mechanisms involved in the ordering and assembly of amino acids into protein molecules. The impetus for such studies has been largely due to the development of the reticulocyte whole cell (1) and cell-free (2) amino acid incorporating systems and to the great ease with which these systems may be utilized. Reticulocytes are immature red blood cells intermediate between the nucleated marrow erythroblasts and the mature circulating erythrocytes. They are easily induced in large numbers in laboratory animals by phenylhydrazine injections, and they can be conveniently obtained, washed, and incubated. Reticulocytes provide an extremely simplified cell system for studying the biochemical events of protein synthesis. While they have lost their nuclei and thus their ability to make RNA (3) they still have all the cellular machinery for synthesizing protein. Their ribosomes exist free in the cytoplasm (7) mainly as polyribosomes (3, 4, 5), and may be liberated from the cells with a minimum of mechanical manipulation.

The unique feature of the reticulocyte system is that it makes predominantly one protein, hemoglobin (2, 7, 8), which is well characterized and easily isolable. Since the hemoglobin messenger is quite stable (3) and the structure of hemoglobin is known, this system offers the possibility of ultimately relating the messenger RNA base sequence to the hemoglobin amino acid sequence. As the reticulocyte matures,

its ability to synthesize hemoglobin is lost (1, 9). Thus in this system one may study the controlling factors involved in the termination of synthesis of a specific protein during cell maturation.

Reticulocytes are so called because of the appearance of a basophilic reticulum observable on staining with a supravital dye (10). It was almost 100 years ago, in 1865, that Erb first observed them in hemolyzed blood cell preparations (11). He hoted that these granular red blood cells existed in only sparse numbers in healthy individuals, and in much higher amounts during conditions of extensive blood regeneration. Erb correctly maintained that these cells were young, transitional forms in the development of mature erythrocytes. Erlich is accredited with the demonstration of intact reticulocytes in blood (12) and showed their presence in both normal and anemic blood by staining with methylene blue. He speculated that reticulocytes were either degenerate forms or were the result of an increased protoplasmic development.

Much of the research which followed centered mainly on the cell morphology. The dispute whether reticulocytes were degenerating or newly formed cells was settled around the turn of the century when several researchers confirmed Erbs earlier supposition that they are indeed immature cells resulting from increased blood cell proliferation (13, 14, 15). The ensuing research on reticulocytes during the early part of this century concerned their role in erythropoiesis and blood cell maturation, and their physical properties, structure and biochemistry (10). However, it was not until 1942 that their significance in the synthesis of hemoglobin was first demon-

strated by Reimann (16).

FORMATION AND MATURATION OF THE ERYTHROID CELLS

Before considering the course of hemoglobin synthesis in the maturing reticulocyte, it is perhaps relevant to examine how these cells are formed and how they develop their extreme specialization, hemoglobinization. The red blood cells arise in the bone marrow, as was first shown by Neumann in 1868, through a series of nucleated precursors (18). It is generally believed that the erythroid cells are derived from primitive cells called stem cells. The stem cells receive a stimulus (possibly erythropoietin) (20-23) and differentiate into the erythrocyte series composed of the proerythroblast, basophilic, polychromatic, orthochromatic erythroblasts, reticulocyte, and erythrocyte (24). Through this progression of increasingly differentiated cell stages, various cytological and biochemical changes take place. These are climaxed by loss of the nucleus and birth of the reticulocyte. One of the prime aspects of this differentiation is the onset of hemoglobinization.

Cytological Changes During Maturation

Erythroid cell maturation is accompanied by a progressive reduction of cell volume, nuclear volume, loss of nucleoli, and eventually expulsion of the nucleus (19, 25, 26). During this time the chromatin becomes denser, while the mitochondria, endoplasmic reticula, Golgi apparatus, centrioles, and ferritin granules decrease

in size or abundance and eventually disappear (26, 27). The ribosomal concentration, as observed by electron-microscopy, initially increases slightly and subsequently diminishes as the hemoglobin density rises in the polychromatic stage (26). The cytoplasmic basophilia, shown by Dustin (28) and Brachet (29) to be ribonucleoprotein, follows the same course of events (19, 30).

Biochemical Changes During Erythroblast Development

DNA - Pronormoblasts are diploid (31) and each successive division is accompanied by a decrease in chromosomal material (32). Accordingly, the DNA is lost progressively and most rapidly at the polychromatic stage (33). Erythroblast DNA content, as determined by microspectrophotometry, shows a steady decline from a relative value of 19.8 in the pronormoblast to 11.3 in the orthochromatic erythroblast nuclei (34, 35).

DNA synthesis in human erythroblasts has been studied extensively *in vitro* by Lajtha (36, 37, 38) and *in vivo* by Bond, et al. using radioautography with H^3-thymidine (39). Borsook and Weil have recently examined *in vitro* H^3-thymidine incorporation into normal and anemic rabbit erythroblasts (40, 41). All agree that the rate of DNA synthesis per cell declines from the proerythroblast to the polychromatic erythroblast. However, the rate per cell volume varies little since the cell and nuclear volumes approximately halve at each mitosis (39). The most significant conclusion reached by all groups was that DNA synthesis stops during the polychromatic erythroblast stage;

orthochromatic erythroblasts are incapable of making DNA (36-41).

RNA - From microspectrophotometric studies, Thorell, in 1947, showed that the quantity of RNA per cell decreases continuously from the stem cells to the reticulocyte where normally only traces remain (43-45). This has been recently confirmed by Ackerman (47) and by Grasso, et al. (48). In the normal animal the major loss of RNA occurs in the orthochromatic stage, whereas in the severely anemic animal the main loss takes place in the reticulocytes (42, 45). The RNA concentration increases somewhat in the basophilic erythroblasts and then also declines (48). Electron micrographs have clearly shown this change to be reflected in the cytoplasmic density of ribosomes, with a large portion of the decrease taking place in the orthochromatic erythroblasts and reticulocytes (26).

The rate of RNA synthesis as evidenced by autoradiographic studies is greatest in the earliest erythroblasts and also declines with maturation (40, 42, 48, 52). This occurs in parallel with decrease in rate of protein synthesis (52). Essentially all the RNA synthesis in erythroblasts is DNA-dependent (41) and therefore stops when the nucleus is lost (40, 48). In the normal rabbit marrow this occurs at the orthochromatic erythroblast, whereas in severely anemic rabbits this takes place usually at the polychromatic stage (40, 49).

Protein and Hemoglobin Synthesis - Thorell provided some of the first quantitative data on the macromolecular events of erythrocytic maturation (43, 44). He observed that hemoglobin absorbancy

appeared relatively late in maturation, starting from only traces in the basophilic stage, increasing rapidly in the polychromatic, and leveling off through the orthochromatic and reticulocyte stages. He also noted that the main synthesis of hemoglobin ensued after the major portion of the RNA had disappeared (43, 44). However, in hemorrhagic anemia of rabbits the pattern was modified so that the RNA persisted through somewhat later maturation stages while the appearance of hemoglobin was slightly retarded (45). The whole picture shifted toward the reticulocyte stage in phenylhydrazine anemic chickens so that the major portion of hemoglobin synthesis was observed to take place in the circulating blood (46).

In accord with Thorell's observations, the cytochemical techniques of Ackerman and microspectrophotometric methods of Grasso et al have recently confirmed that also in the fetal rabbit liver significant amounts of hemoglobin do not appear until the polychromatic erythroblast (47, 48). In addition, the total cytoplasmic protein was found to decrease progressively from the hemocytoblast to polychromatic erythroblast due to cell volume reduction and then to increase sharply to the reticulocyte stage with the onset of hemoglobin synthesis (48).

Autoradiographic studies of C^{14}-glycine incorporation into rat bone marrow cells _in vitro_ (50) and _in vivo_ (51) have indicated that protein synthesis is greatest in the youngest cells and declines with maturation. Similar findings have been reported by Pileri et al for human marrow cells _in vitro_. In addition they noted a parallel relationship between RNA and protein synthesis during erythroblast devel-

opment (52).

Borsook and co-workers (40, 49) have carried out extensive autoradiographic studies with normal and anemic rabbit marrow cells which had incorporated H^3-leucine in vitro. They observed that protein synthesizing activity per cell was greatest in the proerythroblasts and decreased to the orthochromatic cells. However, the rate of synthesis per unit cell volume changed little due to the cell volume reduction at each division. The reticulocytes in the normal rabbit were less active than the orthochromatic cells, while in the anemic animals their activity was between that of the orthochromatic and polychromatic erythroblasts. This was presumably due to their origin from earlier erythroblasts in the severely anemic animal (40). Borsook and Weil have concluded that non-heme proteins are the main proteins synthesized in the pro- and basophilic erythroblasts, while hemoglobin synthesis becomes predominant from the polychromatic erythroblasts onward. Normally, 95 percent of the hemoglobin synthesis is concluded at the orthochromatic stage while in phenylhdrazine anemia about 25 percent of the synthesis occurs in the reticulocytes (42).

It thus appears that the normal course of events leading to the synthesis of hemoglobin in erythroblasts is as follows: DNA synthesis ceases in the polychromatic erythroblasts while RNA synthesis begins to decline. At this same time, or slightly earlier, synthesis of hemoglobin is "turned on" and accelerates rapidly. With the total loss of DNA at the orthochromatic stage (or earlier) the reticulocyte is formed and RNA synthesis stops. During the maturation of the reticulo-

cyte the hemoglobin synthesizing apparatus begins its decline in quantity and activity.

MATURATION OF RETICULOCYTES

There is considerable evidence that the erythroblast loses its nucleus by extrusion (19, 48, 53, 54); in so doing it becomes a reticulocyte. This normally occurs at the orthochromatic erythroblast (18, 19, 40). However, there is an abundance of data which indicates that this may take place one or two stages earlier, particularly under erythropoietic stress (20, 22, 25, 55-58). Direct experimental evidence presented by Borsook and co-workers has shown that in phenylhydrazine anemia of rabbits that the majority of reticulocytes arise directly from the polychromatic erythroblasts, and possibly from earlier stages (40, 42, 49). On the other hand, it seems that when development is unhurried, as in the normal animal, orthochromatic erythroblasts may become erythrocytes directly (25).

Cytological Changes

Reticulocyte maturation is most conveniently defined by the loss of stainable reticulum (10, 18). Cells having a large amount of reticulum are younger than those having a small amount (60). Heilmeyer used this as the bases of classifying reticulocytes according to degree of maturity (59).

Reticulocytes are generally considered to be larger and less dense than erythrocytes (10, 18, 61, 62). Their increase in density upon maturation can be accounted for by an increase in hemoglobin

concentration, plus a decrease in the cellular contents of water and lipids (10, 57, 75). Especially large or macrocytic reticulocytes are a frequent occurrence in severe anemia (25, 33, 57). Since there is little if any reduction in volume during maturation (10), the large reticulocytes apparently mature to larger than normal erythrocytes (57).

Electronmicrographs have revealed that reticulocytes contain a moderate amount of ribosome clusters, a few mitochondria and traces of endoplasmic reticulum (26, 63-65). These all disappear upon maturation to erythrocytes (26, 63-65).

Biochemical Changes

As the reticulocyte matures to an erythrocyte, dramatic biochemical changes take place (10, 66, 67). The most significant of these is the loss of RNA (43, 61, 68-70) and the loss of the ability to synthesize protein or specifically hemoglobin (1, 9, 66). With the disappearance of the mitochondria, all the mitochondrial enzyme systems disappear including the Kreb's cycle, oxidative phosphorylation, electron transport and heme synthesis (66, 67, 75). Glycolysis and the pentosephosphate pathway persist in mature erythrocytes but diminish with cell age (75).

Reticulocytes, being anucleate, contain very little or no DNA (40, 61, 70) and are therefore unable to synthesize RNA (71-73). However, they are capable of renewing the terminal nucleotides of SRNA, as was shown recently by Warner and Rich (74). The loss of RNA has long been observed in *in vitro* maturations by the gradual disappear-

ance of stainable reticulum (10, 60, 76). By treatment with RNase, Dustin (28) and Brachet (29) confirmed that this reticulum, or cytoplasmic basophilia, is largely RNA. Subsequently it was proven that the reticulum consists of ribosomes, endoplasmic reticulum, and possibly mitochondria precipitated by the supravital dye (61, 77, 78). Further evidence for the loss of RNA was the close correlation observed between the percentage of reticulocytes and their content of chemically determined RNA (61, 70, 79) or ribosomal RNA (69). Disappearance of cytoplasmic RNA and accumulation of free nucleotides has been demonstrated during *in vitro* maturation of reticulocytes. These processes seemed to be energy dependent as they were inhibited by dinitrophenol (80, 81). In more detailed experiments, Bertles and Beck have shown that *in vitro* maturation is accompanied by a conversion of cellular RNA into extracellular purines and pyrimidines (68).

It has been known for quite some time that reticulocyte maturation is accompanied by the complete loss of protein synthesis (19, 65, 66). This was first indicated by Reiman who demonstrated that reticulocytes, but not erythrocytes, are capable of hemoglobin synthesis. By incubating reticulocyte rich blood from patients with pernicious anemia, he observed a *net increase* in hemoglobin content *in vitro* (16). He presented evidence which indicated that

> "contrary to general opinion, the main seat of hemoglobin production is not the nucleus of the red cell, but the reticular substance. Hemoglobin formation still is going on after the expulsion of the nucleus as long as there remains reticular substance. . . . The reticular substance must be considered as constituting special cell organ which is endowed with the property of providing hemoglobin for the red cell." (17).

After the introduction of isotopic tracers, the knowledge of hemoglobin synthesis rapidly progressed. In 1948, Altman and co-workers, using C^{14}-glycine, studied the *in vivo* formation of hemoglobin in rats. Of interest was their finding that incorporation was much greater in the reticulocyte-rich blood of rats made anemic by phenylhydrazine (89). With the development of a rabbit reticulocyte whole cell amino acid incorporating system by Borsook and co-workers (1), one of the major tools for studying protein and specifically hemoglobin biosynthesis was established. Erythrocytes were found to be inactive, whereas amino acid incorporation increased with the percentage of reticulocytes (1). Further evidence for the loss of hemoglobin synthesis during maturation was evidenced by the close correlation between the degree of reticulocytosis and rate of hemoglobin synthesis (70, 79). In addition, Allfrey and Mirsky observed that the maturation of chicken reticulocytes is accompanied by a decline in rate of heme and globin synthesis (90). Finally the autoradiographic studies by Gavosto and Rechenman demonstrated directly that the rate of protein synthesis decreased according to the degree of reticulocyte maturation (9).

From the foregoing discussion it can be seen that reticulocytes provide a model system for studying some of the mechanisms regulating mammalian cell differentiation and maturation. With the loss of the nucleus, various metabolic processes begin to decline. Destroyed RNA cannot be replaced and the protein synthesizing machinery degen-

erates. Thus, reticulocytes offer an opportunity for examining the macromolecular events effecting the last stage of erythroid cell differentiation, the loss of the hemoglobin synthesizing capacity.

BASIS FOR THE PRESENT STUDIES

The present work was initiated in an effort to find an answer to what appeared to be a major discrepancy in hemoglobin biosynthesis studies. In general these studies have indicated that a large fraction of the reticulocyte ribosomes are inactive. For instance, in both whole cell and cell-free experiments, a ribosomal labeling corresponding to approximately only two leucine residues per ribosome has generally been observed (82, 83). Assuming that each ribosome is making one hemoglobin polypeptide having an average of 17 leucines per peptide (84, 85) one would expect those ribosomes active in hemoglobin synthesis to contain in the steady state an average of one-half a hemoglobin peptide, the equivalent of 8.5 leucine residues. From these considerations it would appear that only one-quarter of the ribosomes are active in protein synthesis. Schweet (86) and Dintzis et al (87, 7) have made similar calculations. In addition the ribosomal dissociation studies of Lamfrom and Glowacki provided more direct evidence for only a small fraction of the total ribosomes being active in the cell-free system (88).

It was thought that at least one cause of this problem might lie in the heterogeniety of the cell populations used. The red cells isolated from rabbits with phenylhydrazine anemia represent a mixed

population. About 10 to 20% of them are mature erythrocytes, many of which are damaged by the phenylhydrazine. One would expect these to be devoid of ribosomes. The remainder of the erythrocytic cells are of a mixture ranging from the most immature to the most mature reticulocytes. One would expect these cells, therefore, to contain varying amounts of ribosomes. In addition, varying activities of the ribosomes might also be expected. Autoradiographic analysis using H^3-leucine incorporation has indicated that this might be the case. A large fraction (60%) of such cells were inactive, although they stained as reticulocytes and therefore presumably contained ribosomes. Some cells were more active than others (49).

These observations therefore prompted experiments designed to separate the active from the inactive cells. It was hoped that such studies might provide not only more active whole cell and cell free systems but might also give some insight to the degradative processes affecting protein synthesis in the maturing reticulocyte.

Part I describes a method of fractionating reticulocytes by centrifugation in a density gradient of bovine serum albumin. Examination of the amino acid incorporation and cellular contents of ribosomes and hemoglobin in each fraction indicated that a fractionation of the cells according to their protein synthesizing capacity had been achieved.

In order to correlate these findings with the degree of cell maturation in Part II the cytological and physical properties of the cell fractions were investigated and compared with their biochemical properties. These studies showed that the albumin gradient fractionates

the reticulocytes according to their degree of physiological maturity. With this established, the fate of the ribosomes and polyribosomes and their activities during cell maturation was studied by means of sucrose gradient analysis.

Part III confirms that the position of the cells in an albumin gradient is a function of their age. This was accomplished by following, with albumin gradient analyses, the *in vivo* maturation of a band of young reticulocytes transfused into a normal rabbit.

The properties of a reticulocyte cell-free amino acid incorporating system is described in Part IV. This system was used to examine the differences in the ribosomal and soluble components of the young and old reticulocytes.

In these studies amino acid incorporation into the ribosomes and into the total trichloroacetic acid precipitable material in the 105000 g supernatant was used as a measure of protein synthesis. Referring to this either as protein or hemoglobin synthesis is warranted by the following considerations: a) Incorporation into isolated hemoglobin was essentially the same as incorporation into the total TCA precipitate (7). b) Hemoglobin constituted nearly all of the TCA-precipitable material (8). c) Incorporation into hemoglobin accounted for over 90% of the protein produced (6). d) Labeled glycine, histidine, leucine, or lysine incorporation into the total TCA-precipitate was substantially the same as into isolated hemoglobin (7). e) The amino acids were incorporated largely into hemoglobin peptides isolated by

fingerprinting (87, 91). That amino acid incorporation in the reticulocyte cell-free system corresponds to a limited synthesis of hemoglobin has been justified recently by Schweet, et al. (90).

I. PROTEIN SYNTHESIS IN RETICULOCYTE FRACTIONS SEPARATED BY ALBUMIN GRADIENT CENTRIFUGATION

INTRODUCTION

It has long been known that reticulocytes are less dense than erythrocytes (60) and that young reticulocytes are lighter than older ones (62). This fact has been used by many authors to achieve partial separation of various blood cell types. The earlier methods consisted simply of centrifugation of the suspended blood cells and separation of the packed cell mass into layers (62, 93-95). This gave only a partial separation with the top layers enriched in reticulocytes. The packed cells are not only difficult to fractionate by this method, but cell density distributions are hindered by the lack of mobility in the cell mass. Neutral density separations in concentrated albumin solutions have been used by several authors to obtain reticulocyte-rich fractions (96, 97). This method yields only two cell fractions, one lighter and one denser than the suspending medium. An improved modification of this procedure, using a step gradient of albumin or gum acacia solutions, increased the number of fractions obtainable from one tube (98-100). Crude albumin gradients were first used by Graham et al to achieve good fractionation of white cells (101). These gradients were prepared by briefly stirring a step gradient of albumin solutions to obliterate the interfaces. This type of gradient is obviously quite irregular and difficult to reproduce.

A far superior procedure employing bovine serum albumin gradients for the fractionation of erythrocytes by buoyant density has recently been described by Leif and Vinograd (102). During the early stages of this work, the author, in collaboration with R. Leif, found this method also applicable to the separation of reticulocytes. In the present studies a modification of this procedure has been used to fractionate anemic rabbit reticulocytes by buoyant density centrifugation.

The experiments reported in this section investigated the amino acid incorporation of the fractionated cells and their ribosomes and hemoglobin contents. These studies revealed that lighter cells in the albumin gradient exhibit a higher capacity for protein synthesis, and contain a greater quantity of ribosomes and hemoglobin per cell than the denser cells. In addition the ribosomes of the less dense cells showed a greater amino acid incorporating activity than those of the denser cells.

MATERIALS AND METHODS

1. Reticulocyte Preparation - Reticulocytosis was induced in rabbits by the method of Lingrel and Borsook (82). After 7 days of subcutaneous injections of phenylhydrazine, blood containing 80-90% reticulocytes was obtained by cardiac puncture, heparinized, and immediately chilled on ice. All further operations were performed at $4^{o}C$ unless otherwise indicated. The blood was filtered through 3 layers of gauze to remove any clots and debris, and centrifuged at

4000 g for 10 minutes. The plasma was decanted and the cells were resuspended and washed in 4 volumes of NKM (0.13 M NaCl, 0.005 M KCl, and 0.0074 M $MgCl_2$).

2. <u>Whole Cell Incubation</u> - The reagent mixture used was that described by Lingrel and Borsook (82) with the exception that the solutions were prepared exclusively in NKM. To 10.0 ml of packed cells were added 16.5 ml of reagent mixture, 1.0 ml of anemic plasma, 1.1 ml of 0.27 m M $Fe(NH_4)_2(SO_4)_2$ in NKM. This mixture was preincubated for 10 minutes at 37°C in a Dubnoff shaker, and 1.5 ml of 0.01 M L-Leucine-1-C^{14} (Isotopes Specialties Co.) in NKM, specific activity of 3.6 - 7.6 x 10^5 cpm/μ mole, was added. Incubation with the radioactive amino acid was continued usually for 30 minutes with shaking. The reaction was terminated by chilling on ice. The cells were sedimented at 4000 g for 10 minutes and washed two times in NKM.

3. <u>BSA Gradients</u> - Albumin solutions and gradients were prepared by a modification of the method of Leif and Vinograd (102). Bovine albumin, Fraction V, (BSA), from Armour Pharmaceutical Company, was dissolved in NKM-Bicarbonate (NKM containing an equimolar amount of $NaHCO_3$ in place of NaCl) to give a 33-1/3% (w/w) stock solution of BSA. The resulting solution had a pH of 7.2 and density of 1.100 $g \cdot cm^{-3}$ at 25°C. For the gradients dilute solutions of 21.7% and 25.6% (w/w), densities of 1.065 and 1.077 $g \cdot cm^{-3}$ (25°), were prepared by diluting the stock solution with NKM-Bicarbonate.*

* These will be referred to simply as 21 and 25% BSA solutions.

The preliminary experiments in fractionating reticulocytes utilized 4.5 ml gradients of 21 - 25% BSA prepared with an apparatus of the type described by Kuff, Hogeboom, and Dalton (103). This consists of two syringes, one containing the light and the other the dense solution, driven at varying rates by twin programmed cams. The cams may be so designed so as the rates of flow from the two syringes, through a mixing chamber, provide an effluent varying linearly in increasing concentration of the solute. In the preliminary experiments 0.2 ml of cells, prelabeled with C^{14}-leucine, were layered over each 4.5 ml BSA gradient prepared in this manner. These were centrifuged in the Spinco SW-39 rotor at 4°C for 1 hour at 33, 000 g. The bottoms of the tubes were pierced with a needle and fractions were dripped through a steel capillary inserted to a point just above the pellet. Cell fractions, including the pelleted cells, were washed with NKM and analyzed for radioactivity and hemoglobin as described in the next section. Preliminary cell fractionation using this method gave promising results. However, due to improperly machined cams, the apparatus used was found to be unsuitable for the production of linear gradients (102), and was also subject to jamming by the sticky BSA solutions.

These problems were aleviated by use of the glass twin tube device, described by Bock and Ling (104), popularly utilized for sucrose gradients (105). When run slowly, so that equal fluid level is maintained in both chambers, this device produced linear BSA gradients. A plot of refractive index vs. fraction number (25 fractions) gave a linear relationship with no discontinuities except at the ends of the gradient.

Linear 25 ml gradients of 21 - 25% serum albumin were prepared by this method. 6 ml of a cell suspension, containing 5 ml of packed cells from the preceding incubation plus 1 ml NKM were layered over each BSA gradient and centrifuged in a Spinco SW 25.1 rotor at 19000 g for 90 minutes at 4°C. Fractions (generally 8 or 9) were collected dropwise as already described with the bottom pelleted cells being fraction number 1. Cell fractions were washed free of albumin with NKM, aliquots were removed for staining and cell counting, and the remainder of the fractions were centrifuged down at 2000 g for 15 minutes.

In some of the earlier experiments neutral density separation was used to obtain larger yields of cells and ribosomes. A 5 ml of 1 suspension of 2 volumes of reticulocytes plus 1 volume of NKM were layered over 25 ml of 25% BSA. These were centrifuged for 1 hour at 40000 g in the Spinco SW 25.1 rotor. Top cells were removed with a Pasteur pipette and both top and bottom cell fractions washed as previously described.

4. Cell Lysis, and Hemoglo n and Ribosome Preparation - Packed cells from the albumin gradients were lysed essentially by the method of Schweet, Lamfrom, and Allen (2). To 1 volume of packed cells were added 4 volumes of 5×10^{-3} M $MgCl_2$ with swirling for 30 seconds. Isotonicity was immediately restored with 1 volume of 1.5 M sucrose containing 0.15 M KCl. This procedure disrupts only the red cells and leaves the white cells intact (3, 82). Stroma was removed by centrifugation at 12000 g for 10 minutes. The 12000 g supernatant

(S_{12}) was removed and centrifuged for 3 hours at 105000 g to sediment the ribosomes. Free C^{14}-leucine was removed from the 105000 g supernatants (S_{105}) by dialyzing for 36-42 hours against 3 changes, 200-300 volumes each, of a buffer composed of 0.025 M NaH_2PO_4, 0.021 M Na_2HPO_4, 0.010 M NaCN, saturated with CO. Hemoglobin concentration in each sample was determined from the absorbance at 522 mμ, the isobestic point of ferrihemoglobin cyanide and carbonmonoxy hemoglobin (106, 107). Ribosomal pellets were washed twice by homogenizing in 11 ml of Solution M (0.1 M KCl, 0.002 M $MgCl_2$, 0.0075 M Tris., pH 7.5 at 25°C) and centrifuging at 105000 g for 2 hours. Ribosomal pellets were homogenized in 5-6 ml of Solution M and large particulate matter was removed by centrifuging for 10 minutes at 12000 g. Quantity of ribosomes was determined from the absorbance at 260 mμ (108). Radioactivity in the hemoglobin and ribosomes was determined on liquid-plated samples with a Nuclear Chicago gas-flow counter.

5. Cell Counts and Staining - Cells were counted in either of two ways: (a) Hemocytometer - duplicate counts of at least 1000 cells were made on appropriately diluted aliquots in NKM. (b) Coulter Electronic Cell Counter, Model A - duplicate cell dilutions were made in NKM and several count determinations made on each sample (109) Reticulocytes were stained with Brecher's New Methylene Blue (110) and classified according to stage of maturation by the system of Heilmeyer (111).

RESULTS - PART II

1. Preliminary Experiments in Fractionating Reticulocytes - In the early fractionation experiments, reticulocytes previously incubated with C^{14}-leucine were centrifuged in 21 to 25% albumin gradients prepared by the twin-cammed device described by Kuff, et al. (103). The results of such a cell fractionation are given in Table 1. A fractionation according to amino acid incorporating activity was achieved, as can be seen from the increasing specific activity of the hemoglobin from the bottom to the top of the gradient. The two top fractions, which contained only 18% of the total hemoglobin in the cell population, accounted for 48% of the total incorporation.

From a similar albumin gradient, approximately 50 fractions were collected by dripping and each analyzed for hemoglobin content and radioactivity. These results are shown in Figure 1. Here it can be seen that the distribution of radioactivity is displaced toward the less dense fractions with respect to the cell distribution, indicated by the absorbance at 522 mμ. As a result the specific activity of hemoglobin profile is of a negligible value in the densest cell fractions and increases progressively through the gradient to the upper fractions. Thus the lightest cells exhibit the greatest amino acid incorporating ability.

2. Neutral Density Separation of Reticulocytes - These first fractionation experiments prompted further quests to determine if the differing degrees of amino acid incorporation observed in the BSA separated cell fractions are reflected in the quantity and activity of

TABLE 1

DISTRIBUTION OF HEMOGLOBIN AND RADIOACTIVITY

IN BSA-FRACTIONATED RETICULOCYTES

	BSA Fraction No.					
	1 (Bottom)	2	3	4	5	6 (Top)
1. Total mg. Hemoglobin	185.0	11.9	48.4	73.5	51.3	20.7
2. Total cpm x 10^{-3}	6.94	3.24	26.3	65.5	62.3	32.6
3. cpm/mg Hb	37.5	272	544	897	1212	1575
4. % of Total Hb	47.4	3.0	12.4	18.8	13.1	5.3
5. % of Total cpm	3.5	1.6	13.4	33.2	31.6	16.5

After a 1 hour incubation with C^{14}-leucine, 0.2 ml of packed cells were fractionated on a 4.5 ml gradient of 21-25% BSA. Cell fractions were lysed and lysates analyzed for hemoglobin and total TCA-precipitable counts. Lines 4 and 5 represent percent of total hemoglobin and counts found in the gradient. Cell fractionation, incubation, and analyses were as described in Methods.

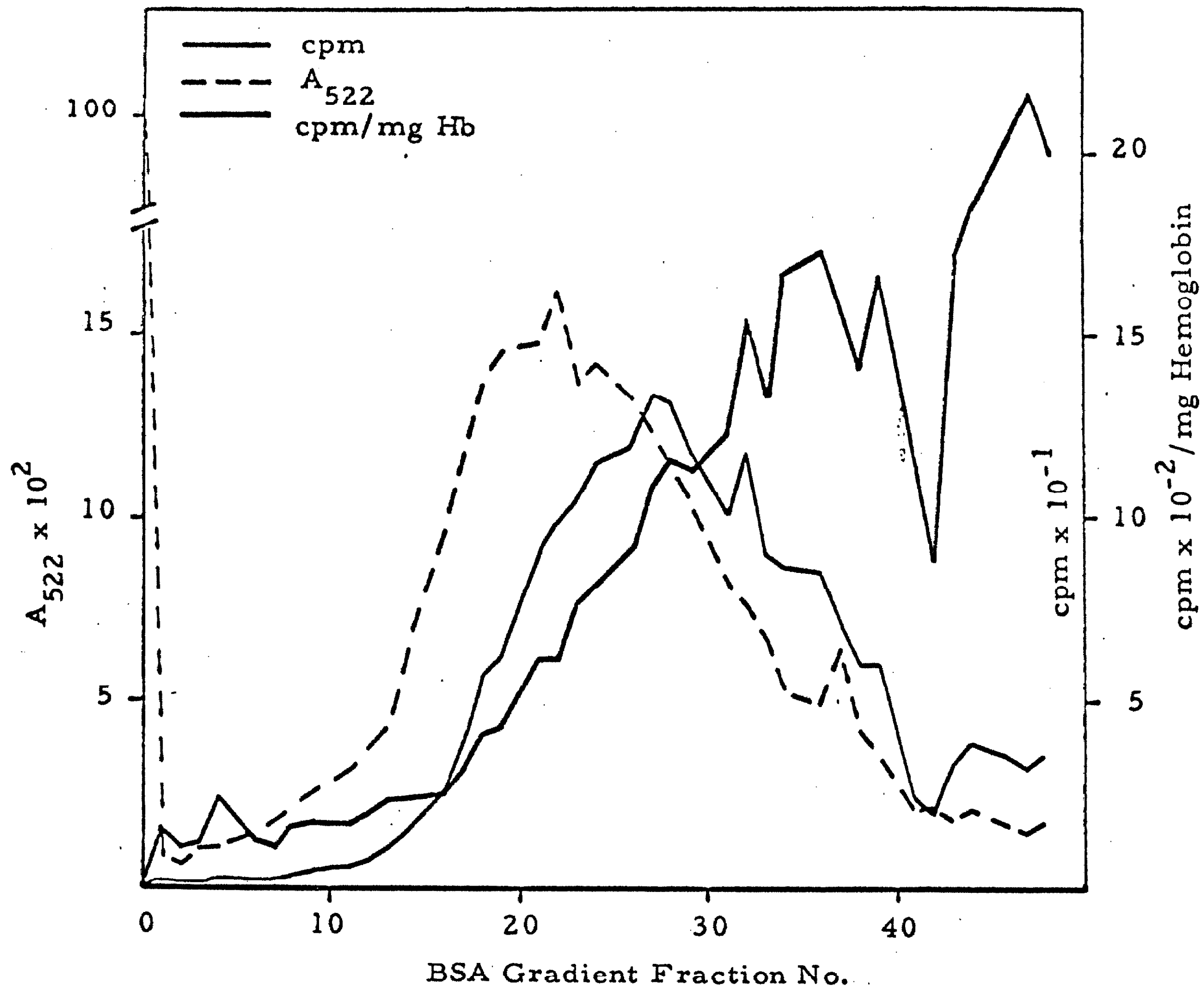


Fig. 1. Distribution of C^{14}-Leucine Labeled Reticulocytes in a BSA Gradient. Reticulocytes were incubated for 30 minutes with C^{14}-leucine. 0.2 ml of cells were centrifuged on a 4.5 ml, 21-25% BSA gradient and 48 fractions were collected by dripping. Cell fractions were lysed and assayed for hemoglobin (A_{522}) and redioactivity. Experimental details are described in Methods. Cell distribution is represented by the hemoglobin absorption of 522 mμ. Fractions are numbered from the densest to lightest.

the ribosomes. In order to obtain large enough quantities of fractionated cells from which the ribosomes could be easily isolated, reticulocytes were separated by neutral density centrifugation in 25% BSA. This technique of course yielded only two cell fractions, one lighter and the other heavier than the suspending medium. Cell counts were made on each fraction in order to calculate the amount of ribosomes and hemoglobin per cell. The results of two such experiments are shown in Tables 2 and 3. The experiment in Table 2 gave a very small top cell fraction caused by the high salt content of the Sigma BSA used in this particular experiment. Fluctuations in cell distribution may also arise from variations in the percent reticulocytes and the degree of maturation of each batch of cells. However, this experiment (Table 2) shows the striking result that a very small group of very light reticulocytes, representing only 0.5% of the total cell population in hemoglobin content, exhibited 49% of the total amino acid incorporating activity. Also noteworthy is that the specific activity of the ribosomes as well as the hemoglobin synthesized per ribosome is greatest in the light cells.

In a similar but separate 25% BSA neutral density separation, Table 3, an enumeration of the cells in each fraction permitted the calculation of the quantity of ribosomes and hemoglobin per cell. More nearly isotonic conditions were maintained in this experiment by the use of low-salt Armour BSA, so that a larger percentage of cells appeared in the light fraction. These light cells, amounting to 45% of the total population, contained virtually all of the ribosomes.

TABLE 2

NEUTRAL DENSITY SEPARATION OF RETICULOCYTES

Amino Acid Incorporation into Hemoglobin and Ribosomes

	Light Cells	Dense Cells
Total mg Hemoglobin	8.84	1635
% of Total Hemoglobin	0.5	99.5
Total Incorporation into Hb (cpm)	15,000	15,890
% of Total Incorporation	48.6	51.4
Total mg Ribosomes	0.588	34.5
Incorporation into Ribosomes (cpm)	76	1620
Specific Activity of Ribosomes (cpm/mg)	129	46.9
Hb Incorporation (cpm)/Ribosome (mg)	25,500	460

Cells were incubated 30 minutes with C^{14}-leucine and separated into 2 fractions by neutral density centrifugation. Lysates were analyzed for hemoglobin, ribosomes, and radioactivity. Percent values represent percent of the total found in the light plus dense cell fractions. Experimental conditions and procedures were as described in Methods.

TABLE 3

NEUTRAL DENSITY SEPARATION OF RETICULOCYTES

Hemoglobin and Ribosomes per Cell

	Light Cells	Dense Cells
Total No. Cells x 10^{-10}	1.27	1.56
% of Total Cells	45	55
Total mg Ribosomes	14.63	0.05
% of Total Ribosomes	99.7	0.3
pg Ribosomes/Cell	1.15	0.00342
Total mg Hemoglobin	317	178
% of Total Hemoglobin	64	36
pg Hemoglobin/Cell	24.9	11.4
No. Ribosomes x 10^{-3}/Cell	173.0	0.515

Incubation and fractionation conditions were as in Table 2, but in a separate experiment. Cells were counted by hemocytometer. Number of ribosomes were calculated using molecular weight of 4×10^6 (Dinztis, Borsook and Vinograd 6). Percent values are percent of the total found in light plus dense cells. Experimental conditions and analyses were as described in Methods.

They also possessed twice as much hemoglobin per cell and 300 times as many ribosomes per cell as the heavy cells. From these experiments it became apparent that the less dense reticulocytes not only have the higher amino acid incorporating capacity but also the greater quantity of ribosomes and hemoglobin per cell.

3. Large Scale Cell Fractionation Using 25 ml BSA Gradients - With these encouraging findings, a more exhaustive study of the properties of reticulocyte fractions from the albumin gradient was undertaken. It was found that at least 5 ml of packed cells could be fractionated on a 25 ml gradient without appreciable loss of resolution. This permitted a greater number of cell fractions to be taken from one batch of reticulocytes for the isolation of their ribosomes. Reticulocytes were incubated with C^{14}-leucine and then centrifuged in an albumin gradient as described in Methods. Six to nine fractions were collected from each gradient analyzed for their hemoglobin and ribosome contents, and amino acid incorporation. Tables 4a and 4b present a summary of the data from three such experiments. The three experiments are not directly comparable in all ways since in each cells from a different rabbit and C^{14}-leucine of different specific activity were used, and the number of cell fractions taken varied. However, several general conclusions may be drawn from these data. In Table 4a it can be seen that the hemoglobin per cell is lowest in the bottom fraction and generally increases toward the middle fractions to a value up to 2.5 times that observed in fraction 1. In two of the experiments this quantity declines in the upper fractions. These dif-

TABLE 4A

Hemoglobin, Ribosomes and Protein Synthesizing Activity per Cell in Fractions Separated by BSA Gradients

	Exp. No.	BSA Gradient Fraction No. 1 (Bottom)	2	3	4	5	6	7	8	9
pg Hb/Cell	1	16.0	22.6	28.2	20.0	28.9	38.6			
	2	18.1	27.1	34.7	35.6	48.6	41.6	28.0	27.1	28.8
	3	*	27.2	33.0	37.5	32.5	28.4	30.3	21.7	
pg Rib/Cell	1	0.0191	0.728	1.72	1.38	1.55	1.64			
	2	0	0.596	1.49	1.56	2.04	2.07	2.38	1.77	2.63
	3	*	0.508	0.926	1.45	1.54	1.63	1.04	0.75	
Hb syn./cell (cpm Hb/cell)+	1	0.062	4.37	12.4	11.9	11.9	32.0			
	2	4.37	25.1	171	348	685	748	711	788	740
	3	*	8.0	22.4	37.9	56.5	71.4	61.3	61.3	

Cell counts were made by hemocytometer in Exp. 1 and 3, and by Coulter Counter in Exp. 2. Data continues on Table 4B.

* Sample lost

\+ Total cpm in hemoglobin per cell

TABLE 4B

AMINO ACID INCORPORATING ACTIVITY OF RIBOSOMES IN CELL FRACTIONS SEPARATED BY BSA GRADIENTS

	Exp. No.	BSA Gradient Fraction No. 1 (Bottom)	2	3	4	5	6	7	8	9
Hb Syn/Rib.	1	3.29	5.89	7.18	8.58	7.61	19.54			
(cpm Hb/μg Rib)+	2	**	41.9	116	222	336	362	300	446	281
	3	*	15.8	24.2	26.2	36.7	43.8	59.1	81.4	
Tot. cpm Hb / Tot. cpm Rib.	1	208	306	282	288	388	418			
	2	**	495	984	1230	1330	1240	678	931	593
	3	*	608	755	649	538	492	618	750	
Specific Activity	1	1.41	1.71	2.27	2.66	1.75	4.15			
of Ribosomes	2	**	7.60	10.4	16.1	22.4	26.0	39.3	42.4	42.2
(cpm/OD_{260})	3	*	2.31	2.85	3.60	6.08	7.95	8.68	9.66	

Reticulocytes were incubated with C^{14}-leucine and fractionated in 25 ml gradients of 21-25% BSA in three separate experiments. Each fraction was analyzed for number of cells, quantity of ribosomes and hemoglobin, and amino acid incorporation. Specific activity of C^{14}-leucine used and number of fractions taken varied between experiments. For experimental details see Methods. * Sample lost. + Total cpm in hemoglobin per μg ribosome in each fraction. ** No ribosomes in this sample.

ferences are likely caused by variations in the severity of the anemia. This spread of cell hemoglobin content is similar to that observed by Ambs in humans of 15 to 48 pg Hb/cell (113). The values in the bottom fractions agree with the hemoglobin content of normal rabbit erythrocytes, reported by Fichsel, et al. to be 20.1 pg/cell (112) and determined by this author to be 17.9. The hemoglobin content of the upper fractions corresponds to values for reticulocytes reported by other authors. Ambs has given the average figure of 37.1 pg Hb/cell for a population of 60% reticulocytes (113) and Weicker, et al. have reported that reticulocytes contain approximately twice the quantity of hemoglobin found in the erythrocyte at 2/3 the concentration (114). Brecher and Stohlman have shown that anemic rat reticulocytes contain about 1-1/2 times as much hemoglobin as erythrocytes (57).

The quantity of ribosomes per cell (Table 4a), as determined from the U. V. absorption of material sedimenting at 105000 g and cell counts, is seen to be negligible in the heavy, bottom cell fractions, and to increase to a maximum of about 2.6 pg/cell in the top, light cells. Thus, as was indicated in the neutral density separations, the lighter cells contain, in general, not only more hemoglobin but also more ribosomes.

The hemoglobin synthesis per cell increases sharply from the bottom to the middle gradient fractions and levels to an approximately constant value in the upper cell fractions.

Since it is known that the ribosomes are the site of protein synthesis, one would expect a close relationship between the rate of

hemoglobin synthesis and the amount of ribosomes. As seen from Table 4b the hemoglobin synthesis per ribosome (C^{14}-leucine incorporated into hemoglobin per mg ribosome) shows a drastic decrease from the lightest to the densest cells. The ribosomes in the lighter, more active cells are making hemoglobin at a rate approximately 6 times higher than those in the heavy, least active cells. Thus, in progressing from the most active to the least active cell fractions, the rate of hemoglobin synthesis falls off much faster than the decrease in ribosomes.

More closely following the amino acid incorporation into soluble protein is the incorporation into the ribosomes, as judged by the ratio of cpm Hb to cpm ribosomes (Table 4b). With the exception of Exp. 2 this ratio exhibited (in general) no large variations over the entire range of reticulocyte fractions. Table 4b also shows that the specific activity of the ribosomes decreases from the lightest to the heaviest cells. This occurs in parallel with the decline in rate of protein synthesis per ribosome.

Examination of the cells from each fraction stained with New Methylene Blue indicated that the lightest fractions were composed of the youngest reticulocytes while the densest fractions were the most mature cells. This was later studied in more detail and will be described in Part II.

DISCUSSION

The foregoing results have shown that an albumin density gradient can be used to fractionate reticulocytes from a phenylhydrazine anemic rabbit according to their amino acid incorporating activity. The cells are distributed in the gradient from the most active ones at the top to the least active ones at the bottom. The dense, pelleted cells were virtually devoid of ribosomes and inactive in protein synthesis. In progressing from the densest to lightest cell fractions the number of ribosomes per cell increased. However, even more striking was the corresponding rise in the hemoglobin synthesizing activity per cell and per ribosome.

The lighter cells were more active in protein synthesis, not only because they contained more ribosomes, but, as evidenced by the quantity of hemoglobin synthesized per unit of ribosomes, also because their ribosomes were making protein at a faster rate. There are two possible explanations for this. Either (1) there is a greater percentage of inactive ribosomes in the denser cells or (2) the ribosomes in the less active cells are synthesizing protein at a slower rate due to limiting concentrations of one or more non-ribosomal components required for protein synthesis. In the succeeding portions of this thesis evidence will be presented that both of these factors play a role.

In these experiments, the examination of New Methylene Blue-stained smears from each fraction gave the encouraging indications that the fractionation also separated the cells by their degree of

maturity. The lightest cells stained as young reticulocytes while the heaviest cells appeared to be largely mature erythrocytes. The cytology of the BSA cell fractions was examined in more detail in succeeding experiments and will be discussed further in the next section.

In accord with the staining characteristics, the bottom, pelleted cells were found to have a hemoglobin content comparable to that of normal erythrocytes. The lighter cells, however, contained up to 2.5 times this amount of hemoglobin in agreement with values reported for reticulocytes by other authors (57, 113, 114). The reason for the greater hemoglobin content has been explained by the observations of Borsook, et al. (42, 49) and Brecher and Stohlman (57) which showed that reticulocytes in severe anemia are abnormally large because they arise from more immature erythroblasts by skipping one or more mitoses. This will be discussed more extensively in Part II.

II. POLYRIBOSOMES AND THE LOSS OF HEMOGLOBIN SYNTHESIS IN THE MATURING RETICULOCYTE

INTRODUCTION

In the previous section it was demonstrated that rabbit reticulocytes may be fractionated in terms of their protein synthesizing activity by buoyant density centrifugation in a bovine serum albumin gradient. The lighter cell fractions appeared to be composed of the more immature reticulocytes. Therefore it seemed that this fractionation procedure offered a means of studying the prime feature of reticulocyte maturation, the loss of the hemoglobin synthesizing apparatus.

A detailed examination of the cytological and physical properties of the cell fractions was undertaken and is described in this section. In agreement with the previous biochemical findings, this study confirmed that the albumin gradient indeed separates the reticulocytes according to their degree of physiological maturity.

During the course of these studies several laboratories established that the site of protein synthesis in the reticulocyte is a multiribosomal aggregate called the polyribosome or polysome (4-6). This has been subsequently confirmed also for liver cells (123, 124), HeLa cells (125), bacteria (126, 127), and slime molds (128). In view of the previous findings of differing ribosomal activities in the cell fractions (Part I), the discovery of polysomes prompted an investigation of the activity and fate of these structures in relation to the loss of hemo-

globin synthesis in the maturing reticulocyte. These experiments have confirmed that the decline in protein synthesis is directly associated with the disappearance of both polyribosomes and single 80S ribosomes. In addition, with increasing cell maturation there occurs a progressive decrease in the activity of the polysomes.

The experiments in this section were performed in collaboration with Miss E. R. Glowacki.

MATERIALS AND METHODS

1. Reticulocyte preparation was carried out as described in Part I, except that cells were washed in New NKM (0.153 M MaCl, 0.005 M KCl, 0.005 M $MgCl_2$) (117). This saline solution was found to be superior to the "old" NKM in preventing cell lysis (117). All operations were carried out at 4^oC unless otherwise indicated.

2. Albumin gradients were prepared essentially as described in Part I with the exception that New NKM Bicarbonate (New NKM containing an equimolar amount of $NaHCO_3$ in place of NaCl) was used here. Since the buoyant density of the cells increases with the salt concentration (102), denser BSA solutions were required here to achieve a cell banding comparable to that obtained in the earlier experiments. Therefore, linear albumin gradients of 21.7 to 30.1% (w/w), densities 1.065 - 1.090 g/cm^3 at 24^oC, were prepared using the twin-tubed gradient-making device of Bock and Ling (104) described earlier. Four ml of a suspension of cells diluted 1:1 with New NKM (hereafter called simply "NKM") were layered onto each of three 25 ml

gradients, and centrifuged in a Spinco 25.1 rotor at 19,000 g for 90 minutes at 5°C. The appearance of the gradients after centrifugation is shown in Plate 1. Approximately 20 preliminary fractions were collected from the gradient by the method of Leif and Vinograd (102), using a Technicon Proportional Pump, through a glass capillary inserted to a point just above the erythrocyte-rich pellet. All fractions, including the pelleted cells, were diluted at least 4-fold with NKM and centrifuged to free them from albumin. Adjacent fractions were pooled so as to give six final cell fractions of roughly equal volume. Initial cell distributions in the BSA gradients and the manner of fraction pooling are indicated in Figure 2. The six pooled cell fractions were then resuspended in 12 ml NKM; aliquots were removed for staining and cell counting, and the cells were recentrifuged. These six fractions of packed cells were used for the whole cell incutations with C^{14}-leucine.

3. Cytological Methods - Cells were stained with Brecher's new methylene blue (110) and counterstained with Wright's stain. Cell counts and size distributions were determined on a Coulter Electronic Particle Counter, Model A, after dilution into a modified Eagle's saline solution described by Brecher et al (118). Volume distributions were compared to those of normal human and normal rabbit erythrocytes in the same medium. Photomicrographs of the stained cells were taken by the author with a Zeiss Photomicroscope through the courtesy of Dr. E. B. Lewis.

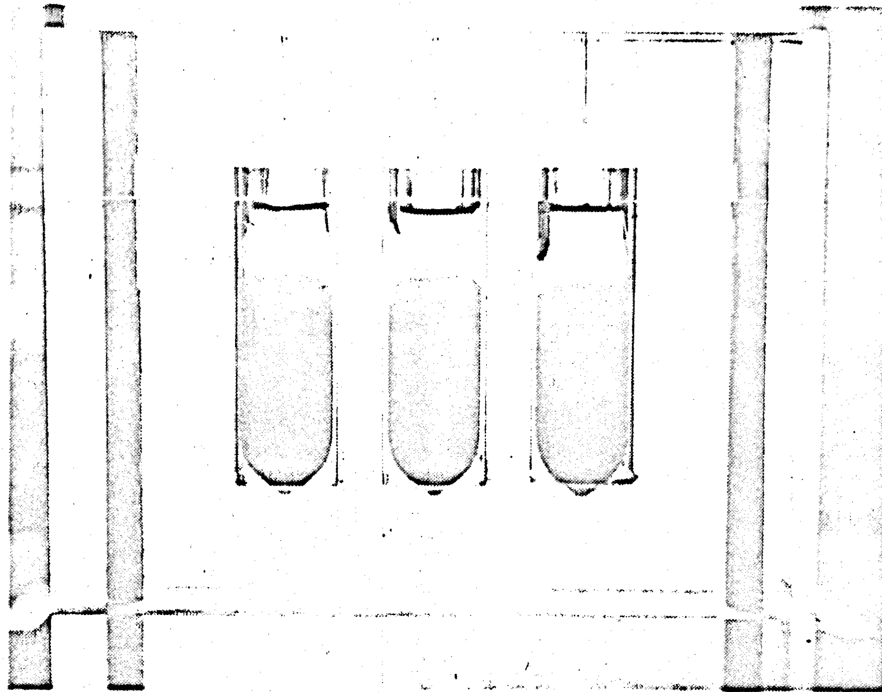


Plate 1.

Albumin gradients showing the distribution of cells after centrifugation. Contents of tube on the right were inadvertently mixed. Erythrocyte-rich pellet is at the bottom of the tubes; reticulocytes are distributed throughout gradient with a dense band in the top third. Lucite block was used for holding gradient tubes while pumping fractions.

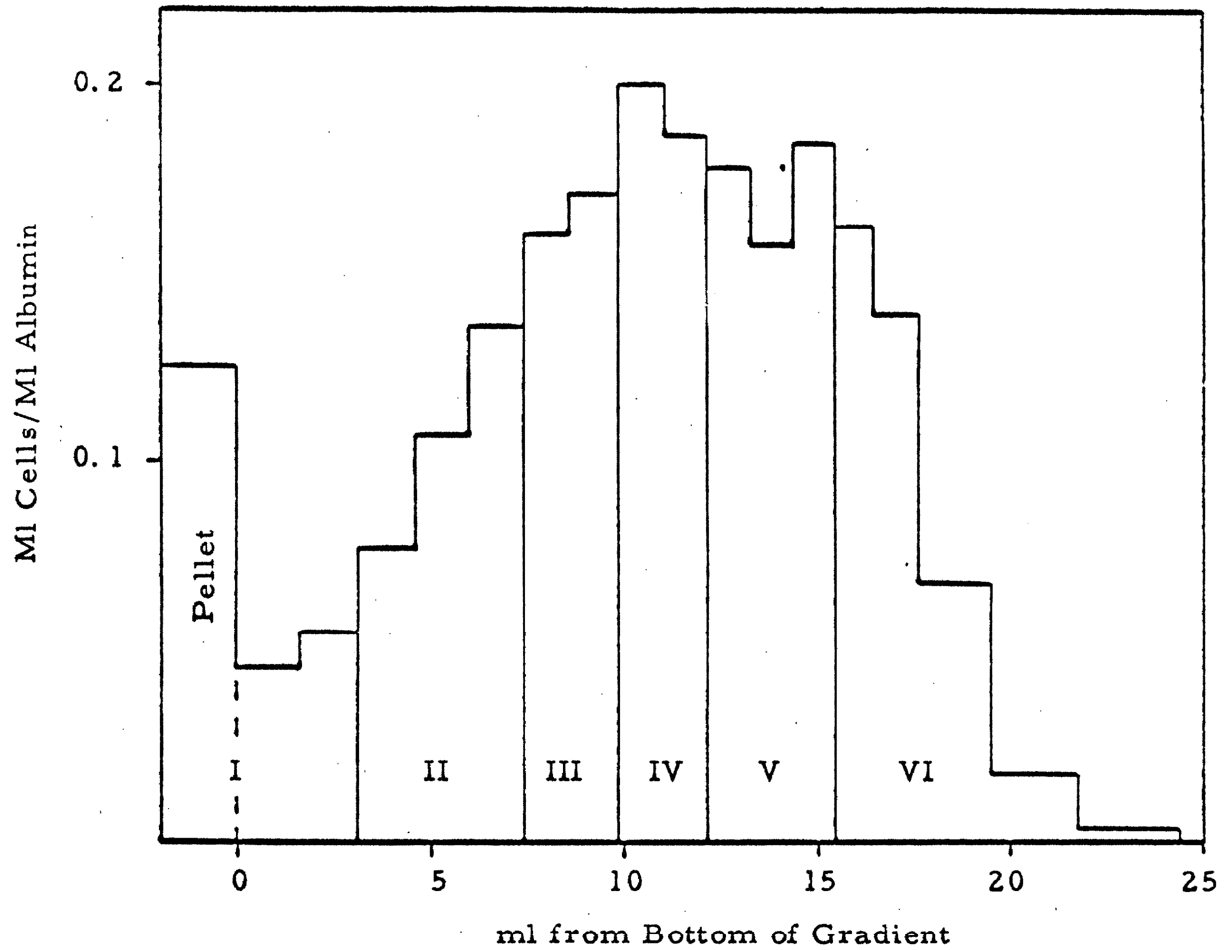


Fig. 2. Cell distribution and fractionation of a 25 ml BSA gradient. Roman numerals designate the pooled cell fractions, with I the densest and VI the lightest.

4. Incubations, lysis, and ribosome preparations were carried out as described in Part I, but with the following modifications. New NKM was used throughout in place of NKM. Preincubation of the cells was for 20 minutes and the incubation with C^{14}-leucine was usually for 10 minutes. Lysis was performed by the addition of two volumes of 0.0015 M $MgCl_2$ in 0.001 M Tris, pH 7.5 at 25°C, with mixing for 30 seconds; isotonicity was restored with 0.6 volumes of the sucrose-KCl solution.

Sedimenting the ribosomes directly out of the clarified lysate onto the sucrose gradients proved to be unsuitable (see Results). Therefore a modification of the procedure of Rueckert, Zillig, and Doerfler (119) was adopted to obtain ribosomes relatively free of contaminating radioactive hemoglobin. Clarified lysates (S_{12}) were layered over 2.5 ml of 30% (w/v) sucrose in solution P (0.08 M KCl, 0.0015 M $MgCl_2$, 0.01 Tris, pH 7.5 at 25°C) and centrifuged 4 hours in a Spinco SW-39 rotor at 39,000 rpm. Ribosomal supernatants were removed, with Pasteur pipette, for determining the amino acid incorporation into hemoglobin. The ribosomal pellets plus the bottom 0.5 ml of sucrose solution were gently resuspended in 1.5 ml solution P.

5. Sucrose Gradients - Approximately 10^7 plaque-forming units of bacteriophage ØX174 were added to each ribosomal suspension as a sedimentation marker and these suspensions were layered onto linear 25 ml sucrose gradients (15-30% w/v in solution P) prepared by the method of Britten and Roberts (105). Gradients were centrifuged 130 minutes at 24,800 rpm in a Spinco 25.1 rotor at 5°C decelerated

without brake. One ml fractions were collected from three gradients simultaneously using a Technicon Proportional Pump (102) and stored at -70°C until analysis. Mallinkrodt Analytical Grade sucrose was used.

6. Analysis of Sucrose Gradients and Other Measurements - Samples from the sucrose gradients were analyzed for RNP (ribonucleoprotein) by absorbance at 260 mμ (108) using either a Beckman DK2 Recording Spectrophotometer or a Beckman DU Spectrophotometer. Samples were assayed for incorporated C^{14}-leucine by precipitation in hot 5% TCA using 0.5 mg casein per sample as carrier and collection of precipitate on Schleicher and Schuell, type B-6 membrane filters for gas-flow counting. (Details of this method are described in Part II.) Bacteriophage ØX174 were assayed, by E. Glowacki, using standard plaque-assay procedures (138). ØX174 and its host bacterium *E. coli C* were kindly supplied by M. Yarus. Ribosomal supernatants were analyzed for hemoglobin, by absorbance at 522 mμ (106) (Methods, Part I), and for C^{14}-leucine incorporation by hot TCA precipitation and counting on membrane filters (Methods, Part IV).

7. Determination of the Intracellular Leucine Pool - Cell separations, incubations, lysis, ribosome purification, and radioactivity determinations were performed essentially as outlined in the previous paragraphs. Reticulocytes, washed once in NKM, were preincubated for 20 minutes in a series of incubation mixtures each containing 1 ml packed cells. To each were then added from 0. 1 to 10. 0 μ moles of C^{14}-leucine (1.25 μc/μ mole) and incubation was continued

for 10 minutes. Cells were lysed and ribosomes were purified by two 5 hour centrifugations at 120,000 g through 30% sucrose in solution P. Total C^{14}-leucine incorporation and specific activity of both ribosomes and soluble protein were determined. Graphic determination of the leucine pool is described under Results.

RESULTS

Cytological and Physical Properties of the Reticulocyte Fractions from the BSA Gradient

In order to correlate the amino acid incorporating activities and hemoglobin contents of the fractionated reticulocytes with their degree of maturation, cell fractions from a BSA gradient were examined for their staining properties, volume distributions, and approximate densities (estimated from their position on the gradient).

A. Density - As has been shown by Leif and Vinograd (102), cells are distributed in the gradient on the basis of their buoyant density. Since in these experiments a density gradient from 1.065 to 1.090 g cm^{-3} (24°C) was used, the pelleted cells must have a density greater than 1.090 g cm^{-3}. The densities of the rest of the cells then range from 1.090 g cm^{-3} at the bottom to 1.065 g cm^{-3} at the top of the gradient.

Brecher and Stohlman (57) have indicated that the density of reticulocytes is largely determined by their hemoglobin concentration. However, in the previous section it was shown that the lighter cells contained more hemoglobin per cell. Weicker et al (114) and Goto

(120) have pointed out that while reticulocytes may contain more hemoglobin than erythrocytes, the hemoglobin is present in 2/3 - 3/4 the normal concentration. Thus, as will be confirmed later, the lighter reticulocytes are larger cells.

B. Staining Characteristics - When reticulocytes are stained with new methylene blue a deep blue reticulum appears in the cytoplasm (10). This reticulum has been shown to consist of precipitated RNA (28, 61, 77) and ribonucleoprotein (77, 78). It has been generally accepted that the most immature reticulocytes have the largest amount of reticulum and that this progressively decreases as the reticulocyte matures to the erythrocyte (10, 18). In "old" reticulocytes only a few scattered granules or threads may be found (60). Accordingly, Heilmeyer (59) placed these cells into four classes based on their content of stainable reticulum (see Plate 2). Class I reticulocytes are those which have just lost their nucleus and contain a dense deeply-staining reticulum. Classes II and III are progressively more mature and Class IV, with sparse stainable material, is the most mature form immediately preceding the erythrocyte. However, this classification is somewhat arbitrary since actually the classes are not distinct but form a continuum from the most immature reticulocytes to the mature erythrocytes.

At least 1000 cells in each fraction were counted and classified according to Heilmeyer. These results are shown in Figure 3. As there is no clear-cut distinction between the various classes this determination is quite subjective. However, it does present a general

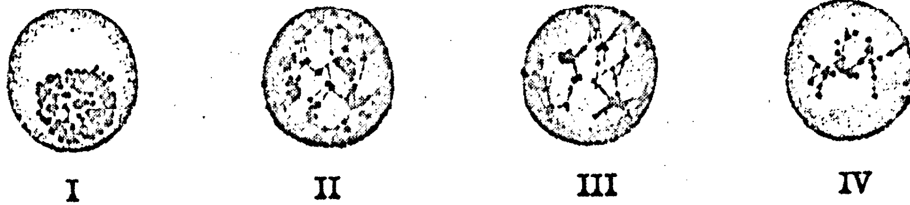


Plate 2.

Reticulocytes at different stages of maturation arranged according to the method of Heilmeyer. I represents the most immature and IV the most mature reticulocyte. (From Heilmeyer and Begemann, 121).

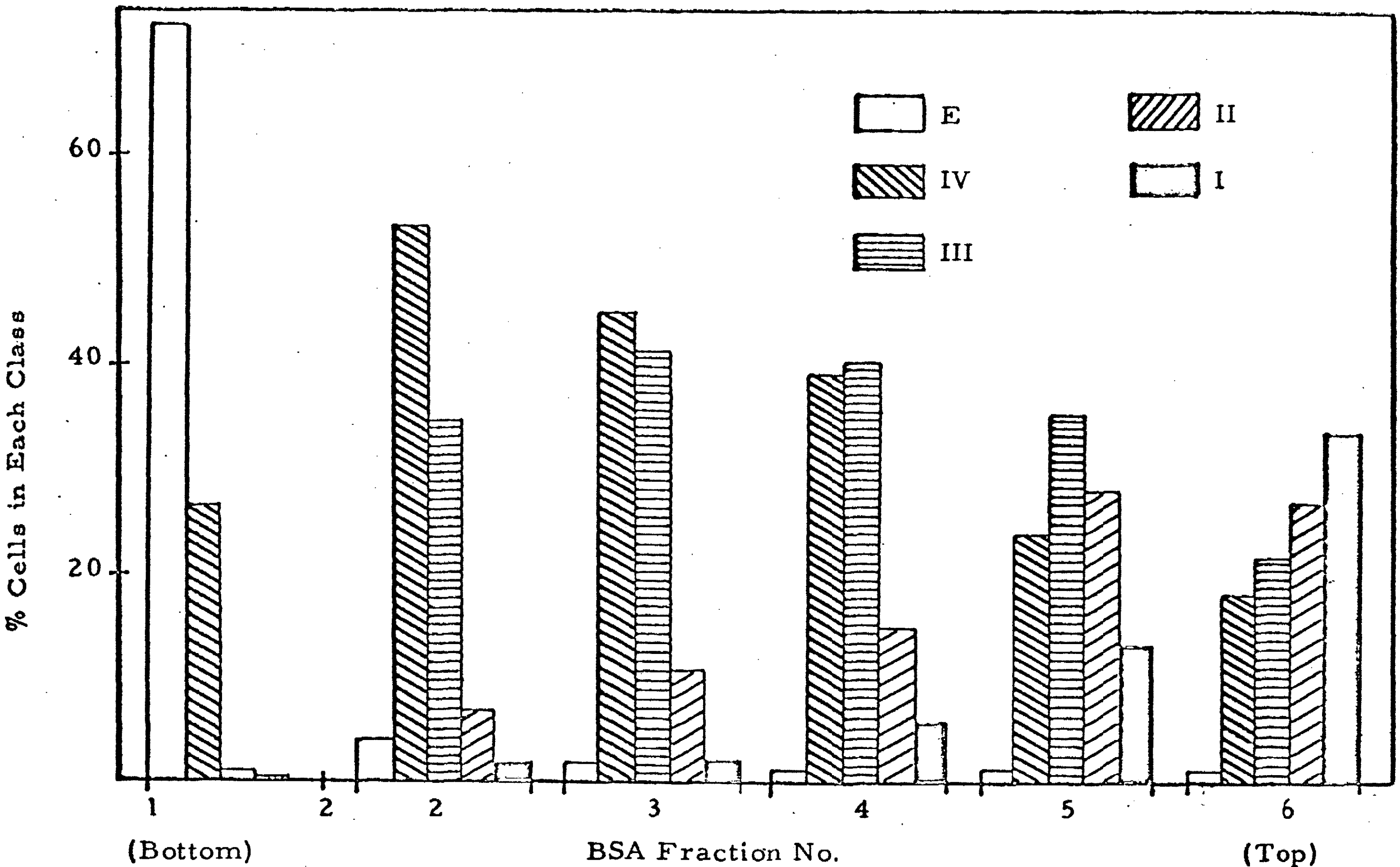


Figure 3. Distribution of Heilmeyer Classes of Reticulocytes in the BSA Cell Fraction. E represents erythrocytes, IV the oldest, and I the youngest reticulocytes.

picture of the cell distribution in the various fractions. Fraction 1, the pelleted cells, is seen to contain primarily erythrocytes (many with Heinz bodies caused by the phenylhydrazine) plus a smaller percentage of the most mature reticulocytes (Class IV). Fraction 2 contains the highest percentage of Class IV reticulocytes, while fractions 3 and 4 consist primarily of Class IV and Class III, the intermediate reticulocytes. Cell fraction 5 has fewer of the more mature forms and an increased percentage of Classes II and I. The top fraction, 6, shows the proportion of the most immature reticulocytes, Class I, with deeply stained reticulum, plus lesser amounts of the more mature cells. Photomicrographs of typical cell fractions are presented in Plate 3 and for comparison a picture of the unfractionated cells is shown in Plate 4.

C. Cell Size Distributions - Relative cell volume distributions of three of the reticulocyte fractions from a 21-30% BSA gradient, as determined on a Coulter Counter, Model A, by the method of Brecher et al (118), are shown in Figure 4. The values of the abscissa represent threshold voltage settings, with the voltage "window" width at each position being 4 voltage units from the lower to upper threshold voltage setting. The electrical pulse heights, detected by the counter at each window width, are proportional to cell volume (118). Therefore, the threshold voltage "window" settings represent relative cell volumes. An estimate of the true cell volumes was extrapolated from the size distributions of normal rabbit and human erythrocytes which have mean cell volumes of 61 μ^3 and 87 μ^3 respectively (122).

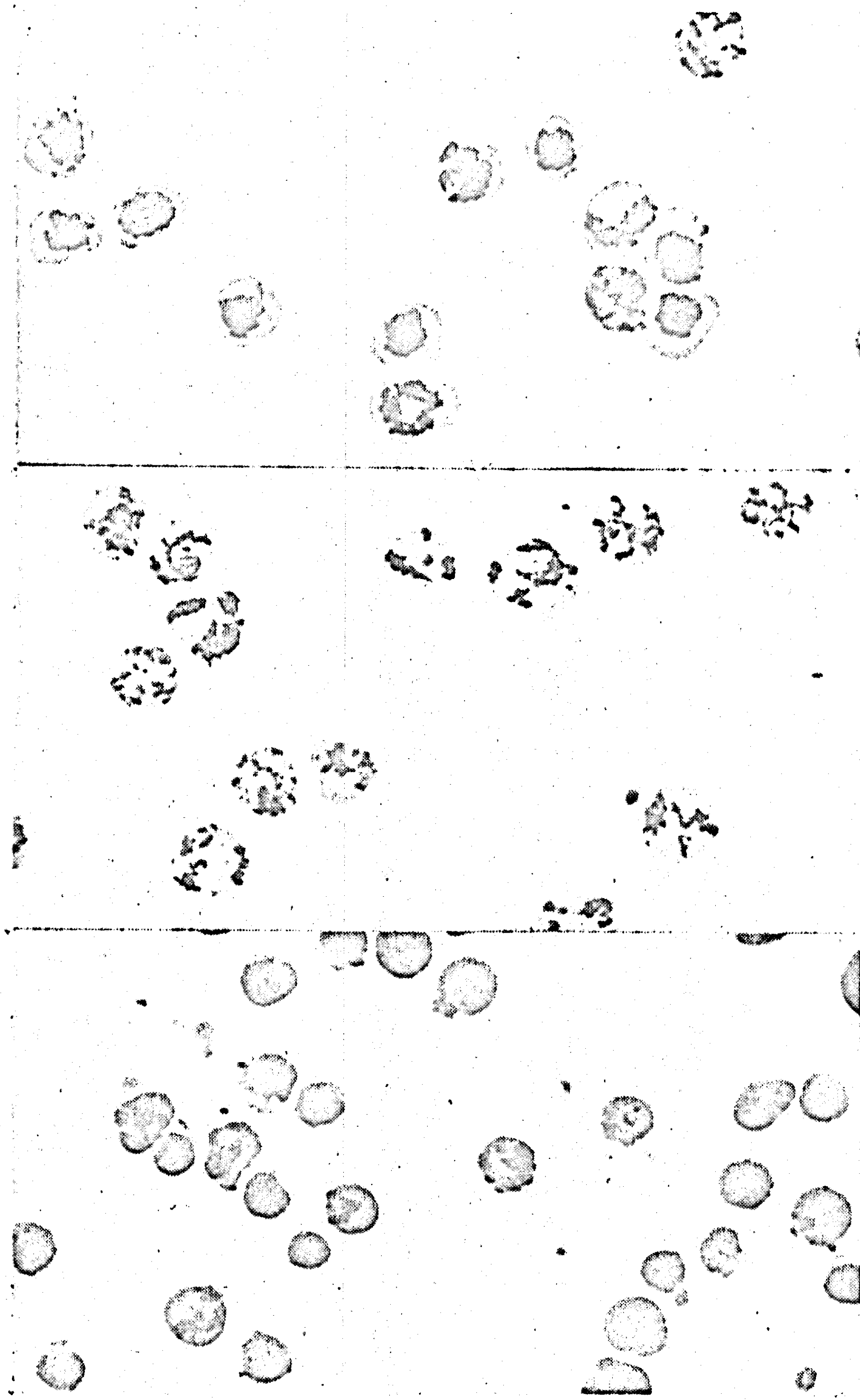


Plate 3.

Photomicrographs of reticulocyte fractions from a BSA gradient. Cells stained as described in Methods. Bottom: Fraction 1; Middle: Fraction 3; Top: Fraction 6.

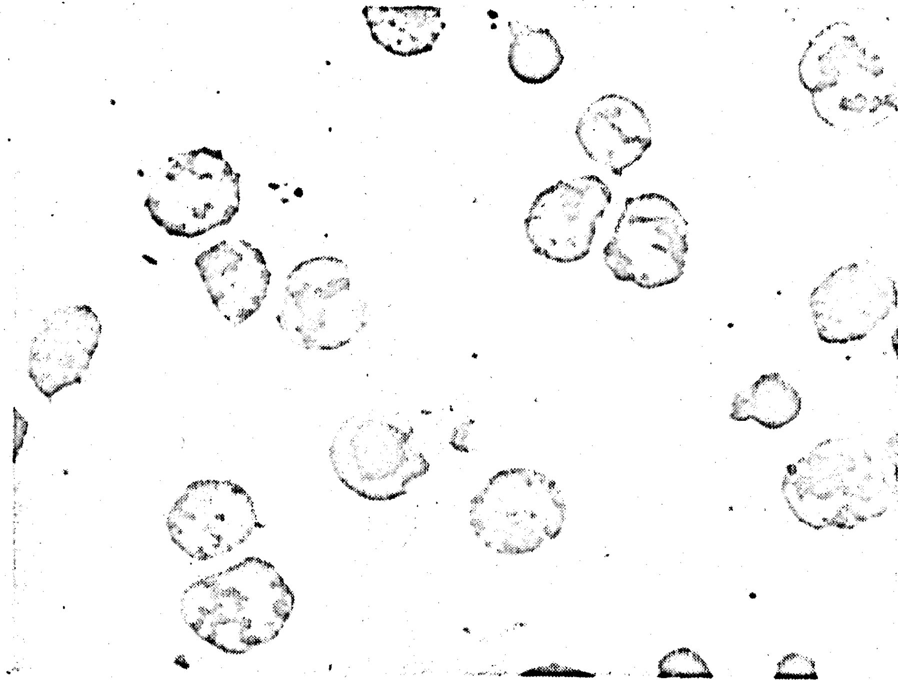


Plate 4.

Photomicrograph of unfractionated blood cells from a phenylhydrazine anemic rabbit.

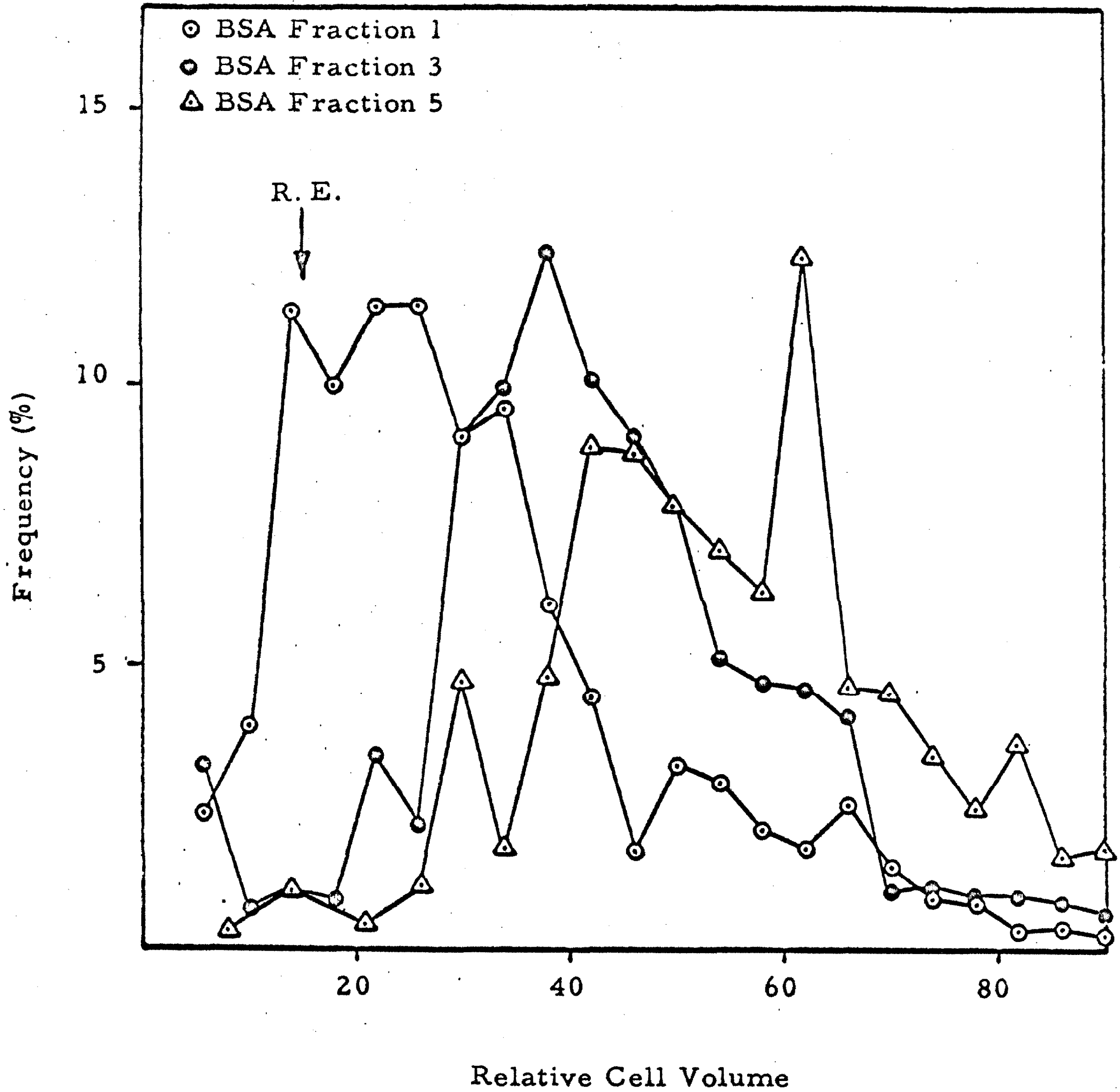


Fig. 4. Cell volume distributions of fractions from the bottom, middle, and top of a BSA gradient as determined on a Coulter Counter, Model A. R. E. - position of normal rabbit erythrocyte, 61 μ^3.

From Figure 4 it is seen that in general the cell volumes increase and distributions broaden from cell fraction 1 to fraction 6. The cells of fraction 1 are mainly of 1 to 2 times the normal rabbit erythrocyte volume. Fractions 2 through 4, banding at intermediate densities, were larger and had peak values about 2-1/2 times that of the normal erythrocyte and secondary shoulders reaching up to 4 times the erythrocyte volume. The lightest cell fractions, 5 and 6, averaged still larger, and ranged mainly from 2-1/2 to 4 times the volume of the normal rabbit erythrocyte.

Table 5 lists the cell volumes determined from size-distribution data. The relative mean cell volumes as well as the most probable cell volumes (peak frequency values) increased from Fraction 1 to Fraction 6. The ratio of relative cell volumes at major frequency peaks to that of the normal erythrocyte are also listed. Values underlined represent the predominant frequency maxima seen in Figure 3.

It has long been recognized that reticulocytes are larger than erythrocytes (62, 129, 130). Particularly large reticulocytes are produced in phenylhydrazine anemia with reported values ranging from 2 to 3.5 times the volume of the normal erythrocyte (57, 114, 130, 131, 132). Cell volume distributions reported here indicate a majority of cells in this size range, and, in addition, are in good agreement with the reticulocyte size distributions observed by Brecher and Stohlman in phenylhydrazine anemic rats (57). Borsook and co-workers (40, 49, 133) have shown that in severe anemia large reticulocytes are formed directly from younger erythroblasts before the orthochromatic stage. According to Borsook et al (49) the size of such a reticulocyte is that

TABLE 5

Size of Cells in the BSA Fractions

BSA Cell Fraction	Relative[a] Mean Cell Volume	Relative[a] Most Probable Cell Volume	Ratio of Size[b] of Major Cell Peaks to Size of Normal Erythrocyte	Most[c] Probable Volume μ^3
1 (Bottom)	32.2	24	1.0, 1.5, 2.0	92
2	43.7	34	2.2, 3.5	131
3	43.1	38	2.5, 3.2, 4.0	146
4	49.4	38	2.5, 3.3, 4.2	146
5	56.2	62(42)[d]	2.0, 2.75, 4.0	239
6 (Top)	53.4	42	2.0, 2.75, 4.0	161

a. Relative cell volumes represent threshold voltage settings on Coulter Counter, Model A.

b. Determined from position of cell peaks (Fig. 3) to position of normal rabbit erythrocyte.

c. Calculated from known distribution positions and volumes of normal rabbit and human erythrocytes (Fig. 3).

d. Figure in parentheses represents the major (but not highest) cell peak in fraction 5.

The tabulated cell volumes for fraction 5 appear greater than those of fraction 6 due to a higher secondary distribution peak in fraction 5 (see Fig. 4). Actually the size distribution profiles of 5 and 6 were quite similar as evidenced by the major peaks in column 4.

of its parent cell minus its nucleus. Since the earlier marrow cells have not only a larger cell volume (40, 43) but a larger cytoplasmic volume (48, 134), the larger, lighter reticulocytes have most likely arisen from the more immature erythroblasts.

D. Amino Acid Incorporation and Ribosome Content - In experiments described in Part I it was found that the protein synthesizing activity increased progressively toward the lighter cell fractions. Similar results were obtained under the present experimental conditions as shown in Table 6. The three experiments are not directly comparable because in each cells from a different rabbit were used. However, within any one given experiment both the leucine incorporation and the amount of ribosomal material per cell increase steadily from the bottom (Fraction 1) to the top (Fraction 6) of the gradient. The cells in the lightest fractions are as much as 200 times as active in protein synthesis and contain up to 40 times as many ribosomes as those in the heaviest fractions.

It is well documented that the amino acid incorporation as well as the amount of RNA is greatest in the youngest reticulocytes and decreases with maturation (10, 66-70). These data are consistant with an increasing degree of cell maturation from the top to the bottom of the albumin gradient.

The cytological and physical properties of the reticulocyte fractions, in agreement with their protein synthesizing activities, demonstrate that the albumin density gradient fractionates the cells according to their degree of physiological maturity. The strongest

TABLE 6

Leucine Incorporation and Ribosomes per Cell

	Expt. Number	Time of Incubation Min.	BSA Gradient Cell Fraction					
			1	2	3	4	5	6
pg ribosomes per cell*	1		0.112	0.620	1.03	1.71	2.59	4.64
	2		0.089	0.753	0.899	1.10	1.56	1.67
	3		0.117	0.540	0.759	0.953	1.45	2.63
μ moles C^{14}-leucine incorporated per 10^{12} cells+	1	5	0.071	1.44	2.96	5.44	8.57	15.1
	2	10	0.850	27.8	41.3	68.7	104	136
	3	20	3.60	23.2	37.4	56.0	99.7	216

* Calculated from total ribosomal material from sucrose gradient analyses and total number of cells in each fraction.

\+ Based on total TCA-precipitable radioactivity in the cell lysates.

evidence for this was provided by cell staining characteristics with additional support provided by studies of cell density, size distribution, amino acid incorporation, and ribosomal activities and contents. The lighter cells are more immature not only in the sense of the Heilmeyer progression of reticulocytes but by virtue of the fact that they are produced directly from the more immature bone marrow cells.

The Loss of Polyribosomes During Reticulocyte Maturation

The initial studies on the cell fractions (Part I) showed a loss of ribosomes and ribosomal activities paralleling the decrease in protein synthesis. This section deals with the fate of the polyribosomes in relation to the loss of protein synthesizing capacity during reticulocyte maturation.

In the initial work on this problem, the cells were first incubated with C^{14}-leucine and then fractionated on albumin gradients. They were washed with NKM containing non-radioactive leucine to reduce contamination by free C^{14}-leucine. Clarified lysates from the cell fractions were centrifuged directly on sucrose gradients. This gave the results shown in Figure 5. Although the usual incorporation was observed into soluble protein, the polyribosomes were virtually "cold", containing on the average only 0.07 μ moles leucine per μ mole ribosome. The theoretical value should be 8.5 (see pg. 61) (84, 85). It was suspected that the cold leucine wash might be chasing out the nascent counts in the polyribosomes. However, even without this washing the polysomal incorporation was only somewhat improved. Apparently the polysomal label was still partially chasing during the BSA

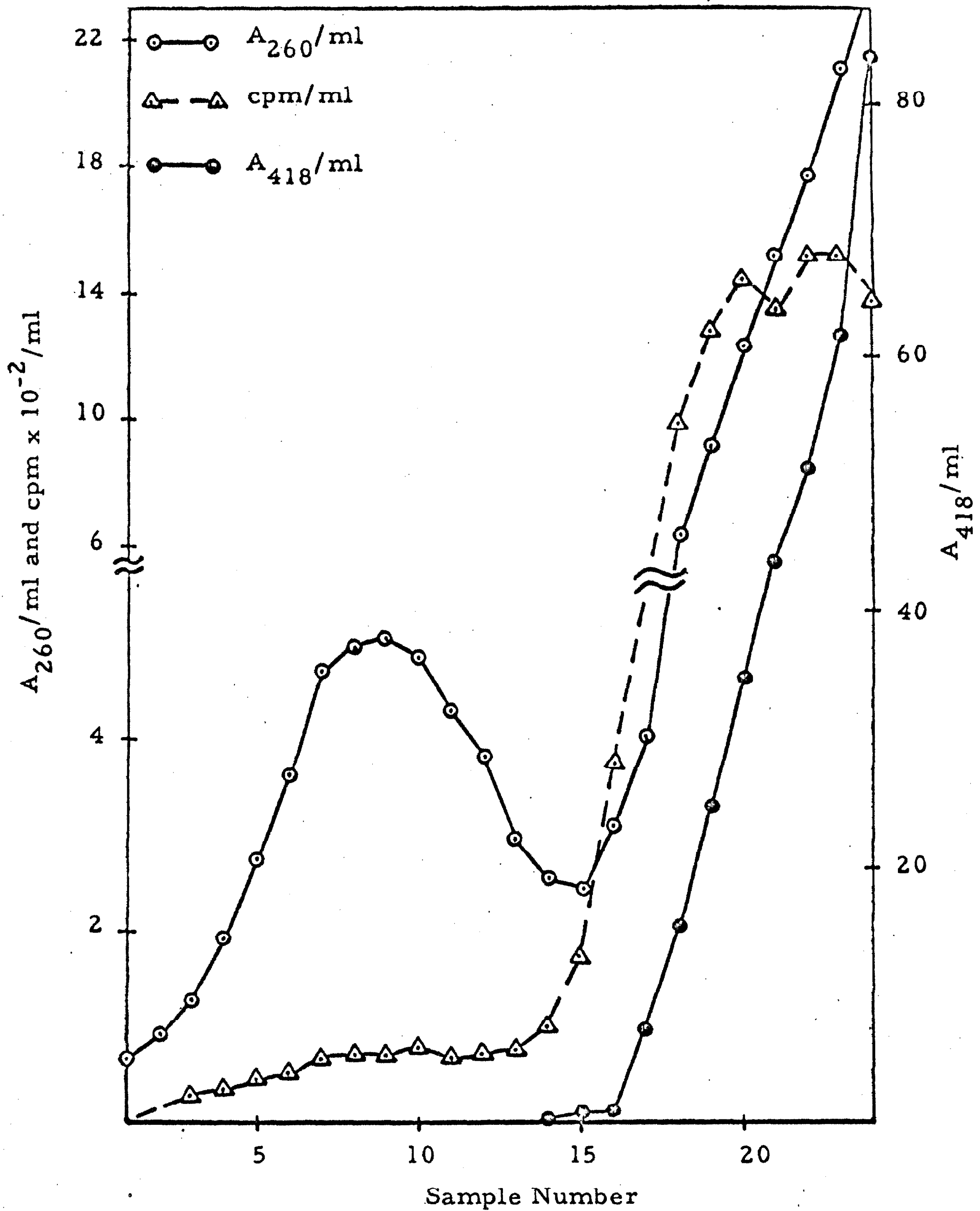


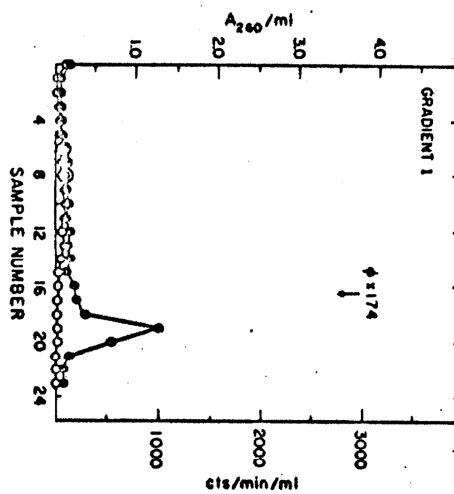
Fig. 5. Sucrose gradient analysis of reticulocyte lysate. 2.0 ml lysate, from 0.75 ml cells incubated with C^{14}-leucine, were centrifuged on a 15-30% sucrose gradient. Procedures described in Methods. Gradient is numbered from densest to lightest sample.

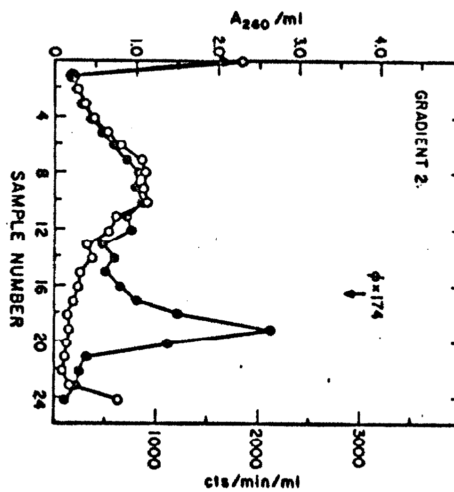
treatment (see Discussion). Therefore in succeeding experiments the cells were first fractionated on BSA gradients and then the individual fractions incubated separately with C^{14}-leucine in the whole cell system. It was found that this procedure retained the full activity of the cells and labeling of the polysomes.

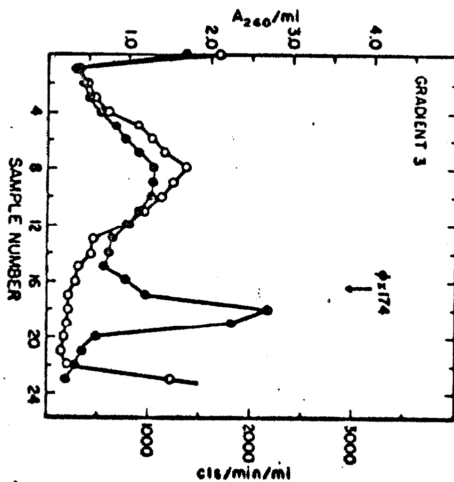
An additional problem arose from the centrifugation of clarified cell lysate on a sucrose gradient (Fig. 5). Only the polyribosomal optical density peak (sample 9) is discernable. The 80S ribosome peak is obscured by the absorbancy of protein carried down through the gradient as is witnessed by the hemoglobin absorption curve (A_{418}). In addition, this radioactive protein obscured the true counts in the 80S region. This problem was alleviated in further experiments by first purifying the ribosomes by a 4 hour centrifugation through 30% sucrose (see Methods). Ribosomes so treated gave the results which follow.

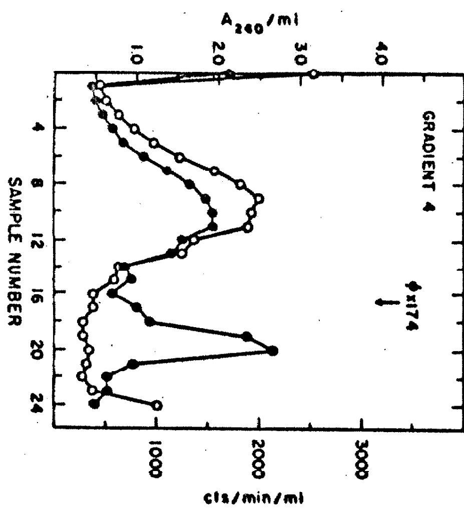
A. Sucrose Gradient Analysis and Amino Acid Incorporation - Sucrose gradients of the washed ribosomal material from each of the six cell fractions are shown in Figure 6. Each gradient contained the ribonucleoprotein from 2.0 ml lysate, corresponding to 0.75 ml packed reticulocytes. The optical density and radioactivity profiles show a marked decrease from fraction 6 to fraction 1. Two major absorbancy peaks can be seen, a narrow one of single 80S ribosomes (samples 18-20) and a broader peak of polyribosomes (samples 6-12). Most of the TCA precipitable counts are found in the polyribosomal region and coincide closely with the optical density curve. The very low levels of radioactivity in the 80S region indicate that negligible

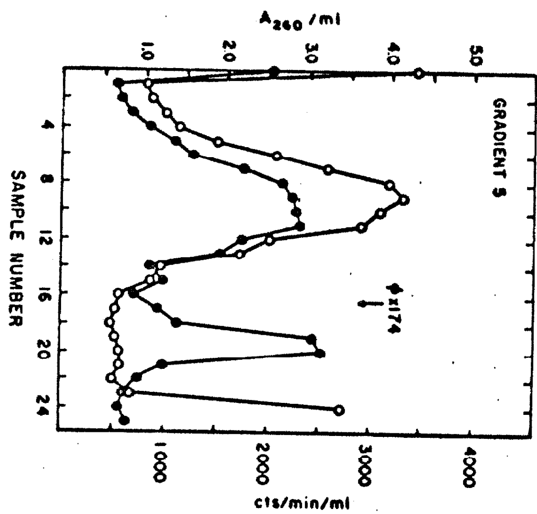
Figure 6. Sucrose gradients of ribosomal material from the six cell fractions of the BSA gradients. Each fraction was incubated 10 min. with C^{14}-leucine under conditions described under Methods. Gradients are numbered from the densest to the lightest BSA fraction. All data are normalized to 2. 0 ml. of cell lysate (corresponding to approx. 0. 75 ml. packed cells). ØX174 is included for sedimentation marker. (●) A_{260}/ml. ; (O) cts /min/ml.

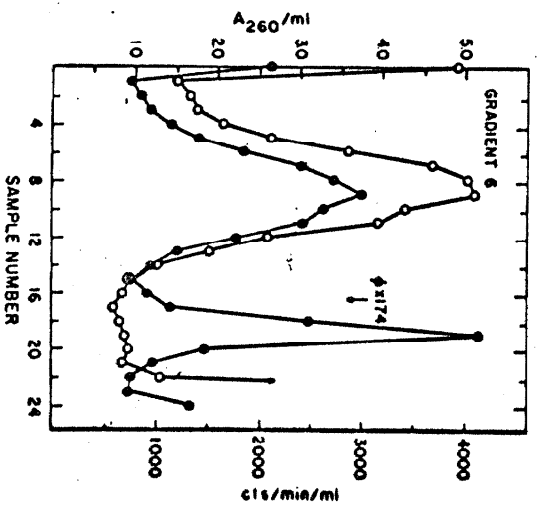

GRADIENT 1
φx174
SAMPLE NUMBER
A_{260}/ml
cts/min/ml


GRADIENT 2.
φx174
SAMPLE NUMBER
A_{260}/ml
cts/min/ml


GRADIENT 3
φx174
SAMPLE NUMBER
A_{260}/ml
cts/min/ml


GRADIENT 4
φx174
SAMPLE NUMBER
A_{260}/ml
cts/min/ml


GRADIENT 5
φx174
SAMPLE NUMBER
A_{260}/ml
cts/min/ml


GRADIENT 6
φx174
SAMPLE NUMBER
A_{260}/ml
cts/min/ml

polysome degradation to single ribosomes occurred during the isolation procedure. Most of these counts are probably due to contaminating radioactive hemoglobin from the supernatant. The maximum leucine incorporation appears in the same position on each gradient, indicating that ribosomal aggregates of similar size must be responsible for the bulk of protein synthesis in all classes of reticulocytes.

The specific activities of the ribosomal material from the sucrose gradients are plotted in Figure 7. This illustrates again the negligible incorporation into 80S ribosomes. It is of interest to note that the polyribosomes from the most active reticulocytes (Gradient 6) have a higher specific activity than those from the least active (Gradient 1). This difference in specific activity is not due to failure of the polysomes from the less active cells to reach saturation labeling during the incubation period, for the same labeling differences were observed for both 5 and 20 minute incubations. As will be shown later, the apparent decrease in the ability of polyribosomes to incorporate amino acids probably results from an increasing number of inactive polyribosomes in the more-mature cells. Evidence for this in *in vitro* maturation has been reported by Marks, Rifkind, and Danon (135).

A summary of data from the six sucrose gradients and their respective ribosomal supernatant fractions is presented in Table 7. The protein synthesizing activity of the cells, represented by the total counts in hemoglobin (line 1), shows a large decrease from the youngest cells (Fraction 6) to the oldest (Fraction 1). A corresponding but less severe decrease is seen in the total ribosomal and polysomal material (lines 3 and 4). As a result the amount of protein synthesized

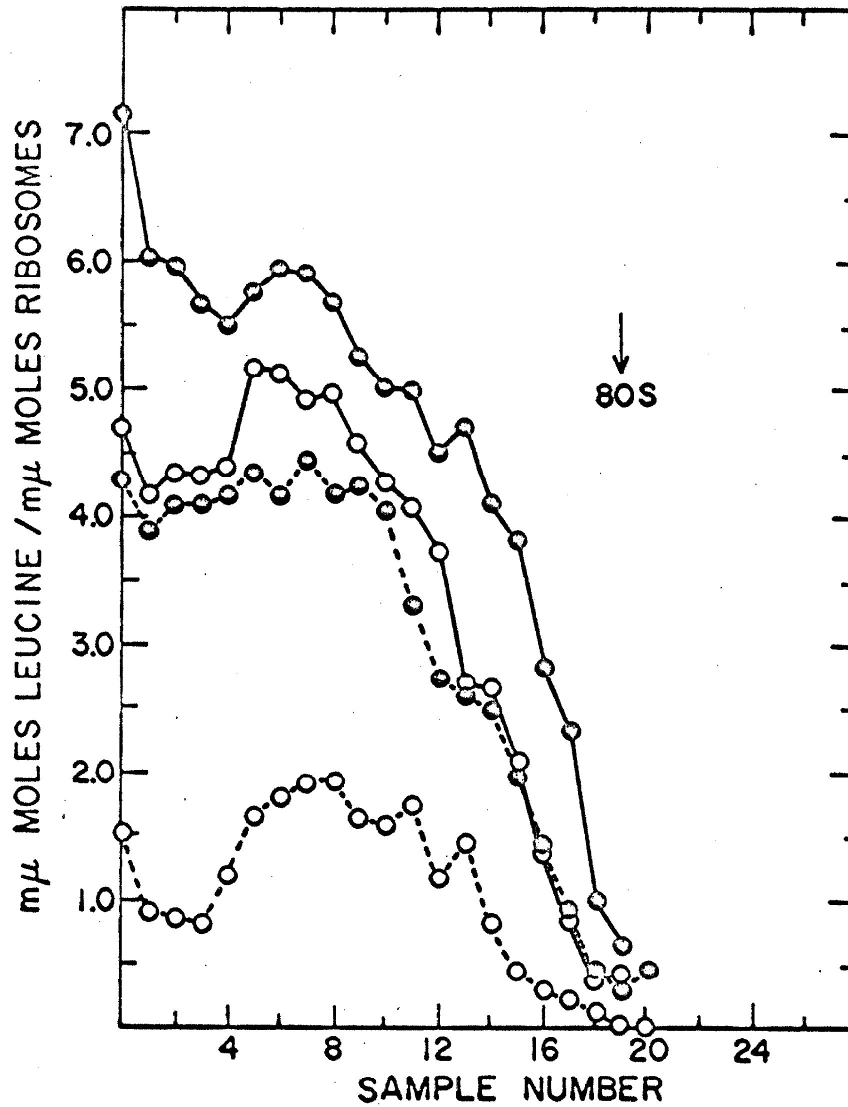


Figure 7. Specific activity of the polyribosomes. O -- O Gradient 1, ◉ -- ◉ Gradient 2, O—O Gradient 3, ◉—◉ Gradient 6. Gradient 4 (not shown) falls between 3 and 6; gradient 5 is superimposable with 6.

TABLE 7

Amino Acid Incorporation and Ribosomal Content of Reticulocyte Fractions

	BSA Gradient Cell Fraction					
	1 (Bottom)	2	3	4	5	6 (Top)
1. Total cts hemoglobin x 10^{-4}	3.98	63.4	88.0	153	227	325
2. Cts/min/mg hemoglobin x 10^{-3}	0.377	9.19	10.8	18.5	36.8	46.5
3. Total ribosomes (A_{260})	5.09	20.6	23.3	30.0	41.1	48.5
4. Total polyribosomes (A_{260})	1.74	11.8	13.4	19.8	28.5	32.8
5. $\frac{\text{Total cts protein}}{\text{Total polyribosomes } (A_{260})} \times 10^{-4}$	2.28	5.36	6.59	7.71	7.94	9.91
6. Percent ribosomes as polyribosomes	34.2	56.6	57.3	66.1	69.4	67.5
7. Total cts polyribosomes x 10^{-2}	5.20	94.7	124	200	315	374
8. Cts/min/A_{260} polyribosomes	1.42	3.77	4.41	4.78	5.19	5.34

Data based on the results of the 10 minute amino acid incorporation and 2 ml of lysate (Fig. 1).

Total cts protein are the total TCA-precipitable counts in the ribosomal supernatants.

Total ribosomes represent total A_{260} found on the sucrose gradients.

Total polyribosomes include sucrose gradient samples 1 through 14 plus pelleted polyribosomes.

per polyribosome (A_{260}) (line 5) is greatest in the most immature cells. The total incorporation is not directly proportional to either the total ribosomal or polyribosomal material. On the other hand, the incorporation into hemoglobin (line 1) varies proportionally with the incorporation into polysomes (line 7). The percentage of ribosomes found as polyribosomes decreases comparatively little with increasing state of maturation of the reticulocytes. This is in contrast to the large shift to single ribosomes observed during both in vitro (135) and simulated in vivo (136) maturation by Marks and co-workers.

When one plots the amount of incorporation into protein vs. the amount of ribosomal material or the amount of polyribosomal material (Fig. 8), one finds a linear relationship extrapolating to finite values of the abscissa. This is again indicative of some inactive ribosomes and polyribosomes. However, the most precise measure of protein synthesizing ability is the amount of radioactivity associated with the polyribosomes (Fig. 9). In all of these experiments it has been found that the total amino acid incorporation is directly proportional to the total counts on polyribosomes. Assuming that all the functionally active ribosomes are synthesizing hemoglobin peptides under steady-state conditions this would imply that the rate of protein synthesis is proportional to the number of active ribosomes in polyribosomes.

B. Sedimentation Coefficients - Sedimentation coefficients of the various ribosomal components in these experiments were determined by Ellen R. Glowacki (137). Band centrifugation by the method of Vinograd, et al. (138) showed five clearly separable components with

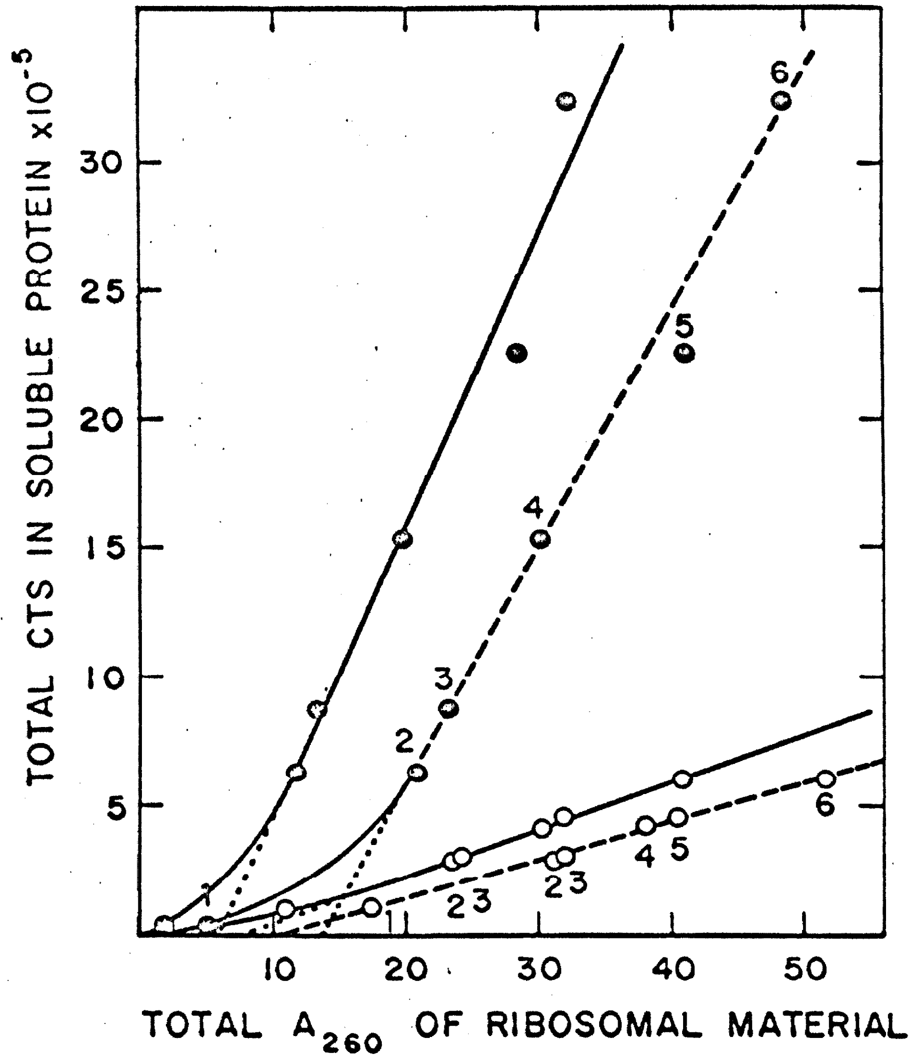


Figure 8 Incorporation of C^{14}-leucine into soluble protein as a function of the total ribosomal material (broken lines) and polyribosomal material (solid lines). (O) 5 min. and (⊙) 10 min. incubation with C^{14}-leucine. Numbers correspond to BSA gradient cell fractions.

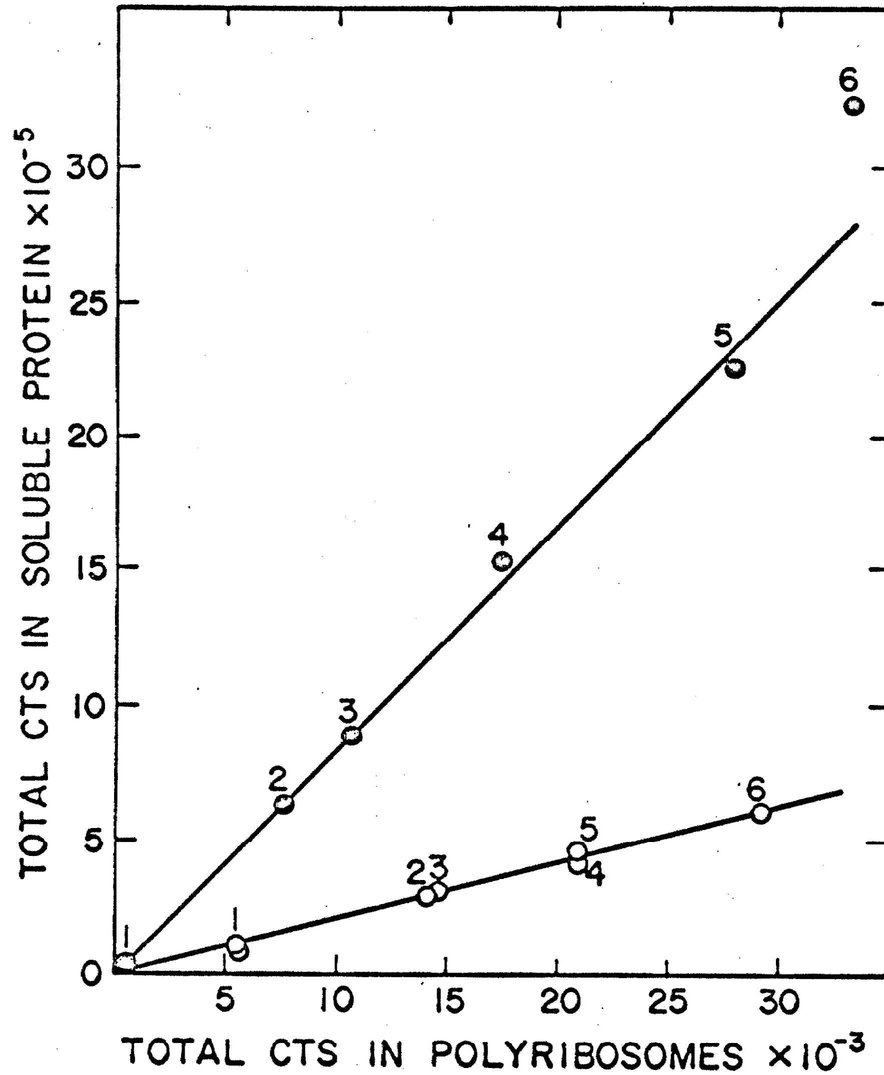


Figure 9. Incorporation of C^{14}-leucine into soluble protein as a function of total incorporation into polyribosomes. (O) 5 min. and (⊙) 10 min. incubation with C^{14}-leucine. Numbers correspond to BSA gradient cell fractions.

$S_{20,w}$'s of 80, 130, 153, 190 and 220. Approximate S-values of 86, 123, 181, 205 and 238 were obtained from the sucrose gradients by the Martin and Ames method (139). From Gierer's equation for S-value vs. number of ribosomes in the polysome, these values correspond to a regular series from the monomer (80S) to the pentamer 220S). The main polysomal peak in the sucrose gradients is a ribosome pentamer.

Determination of the Leucine Pool in Young and Old Reticulocytes

Examination of the amino acid incorporation into reticulocyte polysomes revealed that theoretical labeling was not achieved. This was based upon the assumption that a ribosome active in hemoglobin synthesis should contain, on the average, 1/2 a hemoglobin peptide or the equivalent of 8.5 leucine residues (84, 85). The polysomes of the most active cells had a specific activity of 6 leucines per ribosome. A possible explanation for this was that the cells contained a significant intracellular leucine pool, of greater magnitude in the denser cells, which was diluting the specific activity of the added C^{14}-leucine. This hypothesis was tested in the following experiments.

1. Leucine Pool in the Whole Cell Preparation - Unfractionated reticulocytes were incubated in a series of reaction mixtures containing varying amounts of added radioactive amino acid (see Methods). A plot of the total C^{14} incorporation into soluble protein (I_o) vs. the amount of C^{14}-leucine added (H) is depicted in Figure 10. Here it can be seen that the incorporation normally observed (using 1.5 μ moles

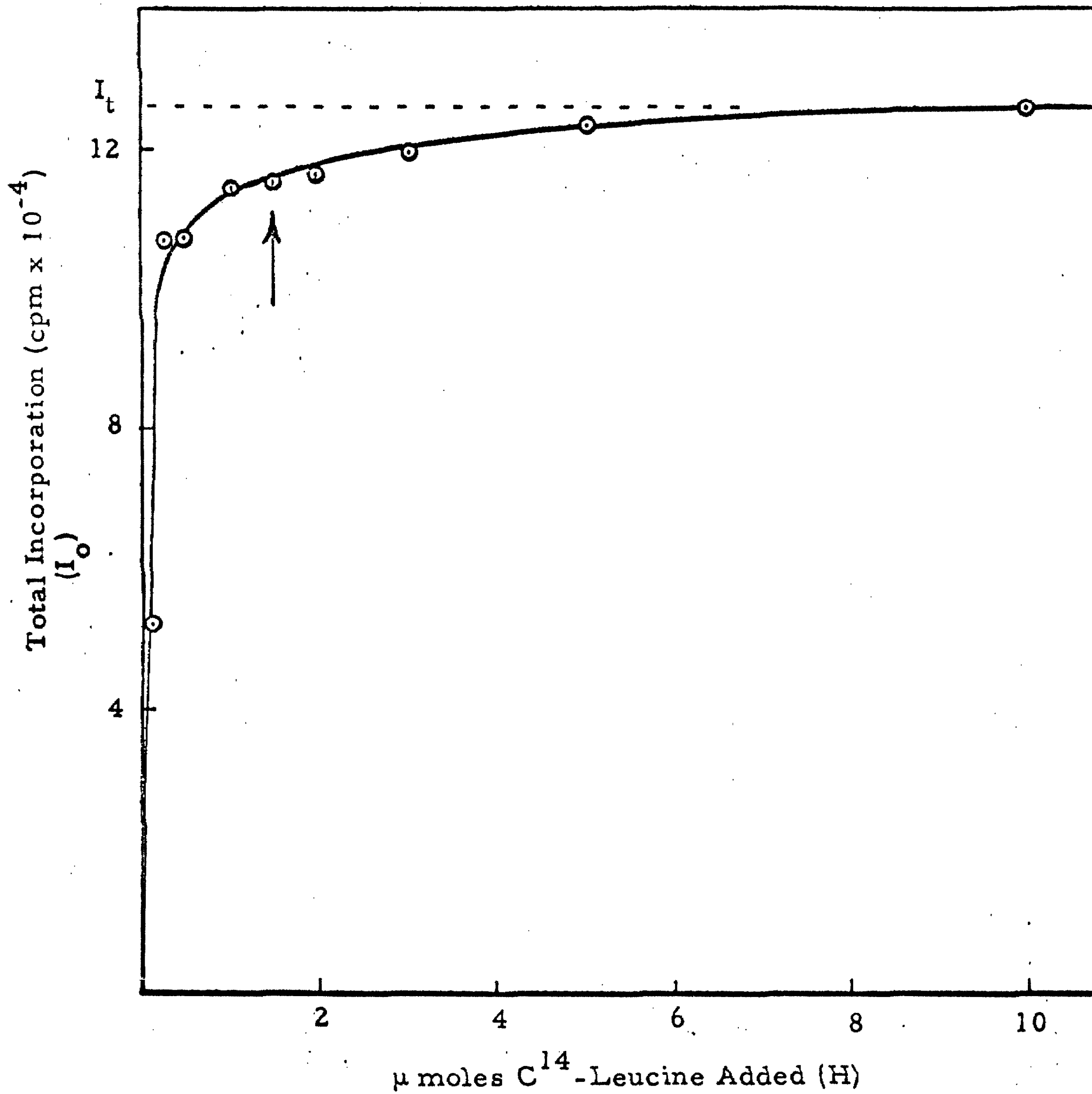


Figure 10. Total Incorporation vs. Amount of C^{14}-leucine Added. Ordinate values represent the total TCA-precipitable counts in soluble protein of 1 ml cells incubated 10 min. Arrow indicates incorporation at the concentration of C^{14}-leucine normally used (1.5 μ moles/ml cells).

C^{14}-leucine per ml cells) is about 92% of the maximum incorporation attainable (I_t) .

Let us assume that the added external C^{14}-leucine, H (μ moles), is in rapid equilibrium with the intracellular "cold" leucine pool, P (μ moles). Then $I_o = I_t \cdot \frac{H}{H+P}$, where I_o is the observed incorporation in cpm and I_t is the C^{14}-leucine incorporation in the absence of a leucine pool (see Fig. 10). Inverting this equation and multiplying both sides by H we obtain

$$\frac{H}{I_o} = \frac{H}{I_t} + \frac{P}{I_t} \quad .$$

If we now plot H/I_o vs. H , the slope of the resulting line will be $1/I_t$ and the x-intercept will be -P (Fig. 11). From the slope of this line I_t is calculated to be 1.24×10^5 cpm (in contrast to a value of 1.15×10^5 cpm observed using the usual amount of C^{14}-leucine). The leucine pool size, from the x-intercept, is found to be 0.1 μ mole for 1 ml packed cells.

As an added check a similar plot was performed using the specific activity of the ribosomes. Since the ribosomal counts are turning over rapidly they would be expected to reflect more precisely the changes in the isotope concentration. Moreover, the specific activity would not be as subject to concentration errors as would the total incorporation. Such a plot is shown in Figure 12. From the slope, the specific activity in the absence of a leucine pool (I_t) is calculated as 70.9 cpm/OD_{260}; the specific activity of the ribosomes at the C^{14}-leucine level normally used was 66.1 cpm/OD_{260}.

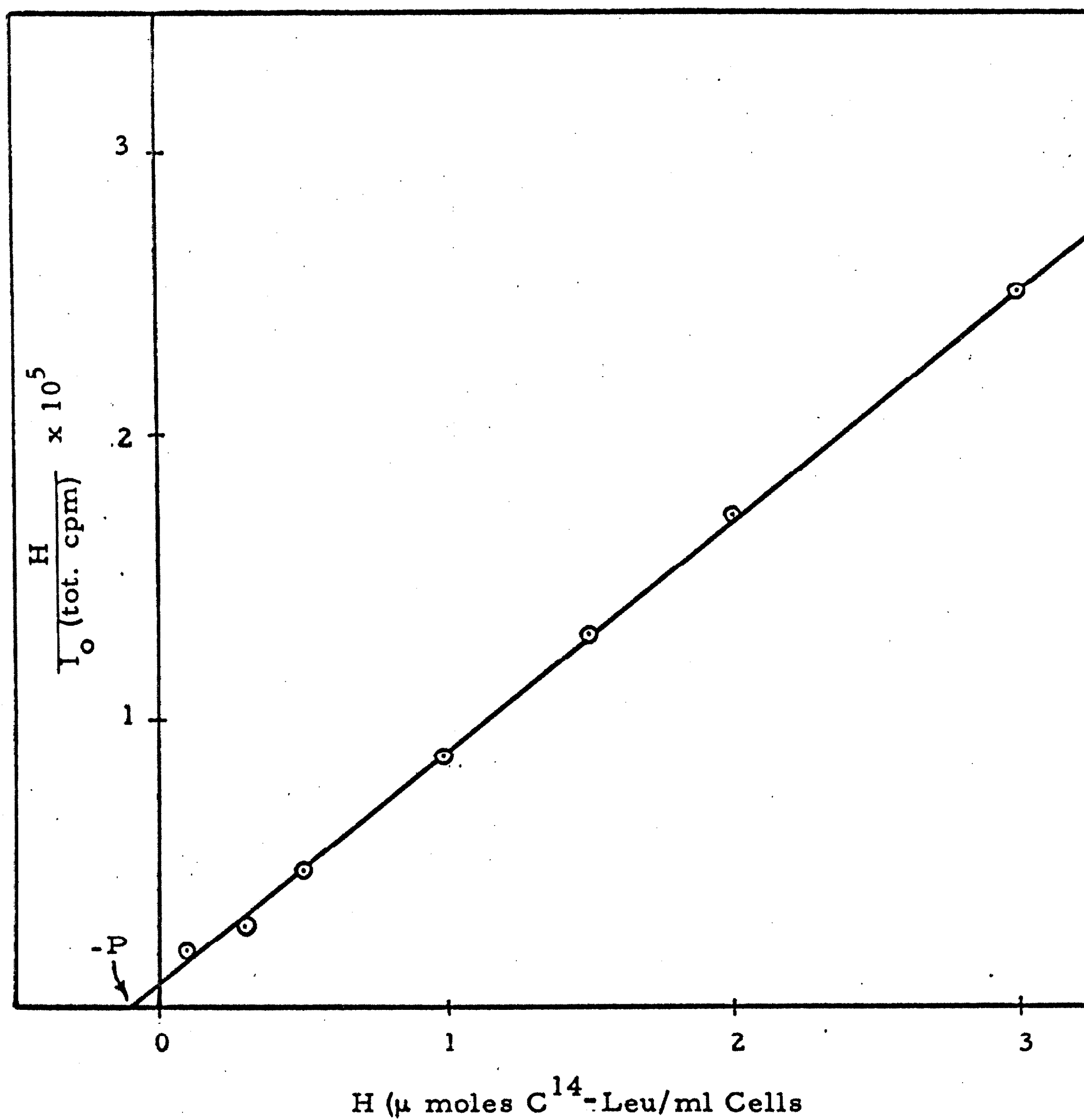


Fig. 11. Plot of H/I_o vs H for total incorporation into soluble protein. The slope, $1/I_t = 8.06 \times 10^{-6}$ cpm^{-1} ; this gave a true incorporation I_t of 1.24×10^5 cpm. The x-intercept (-P) = -0.1 μ mole.

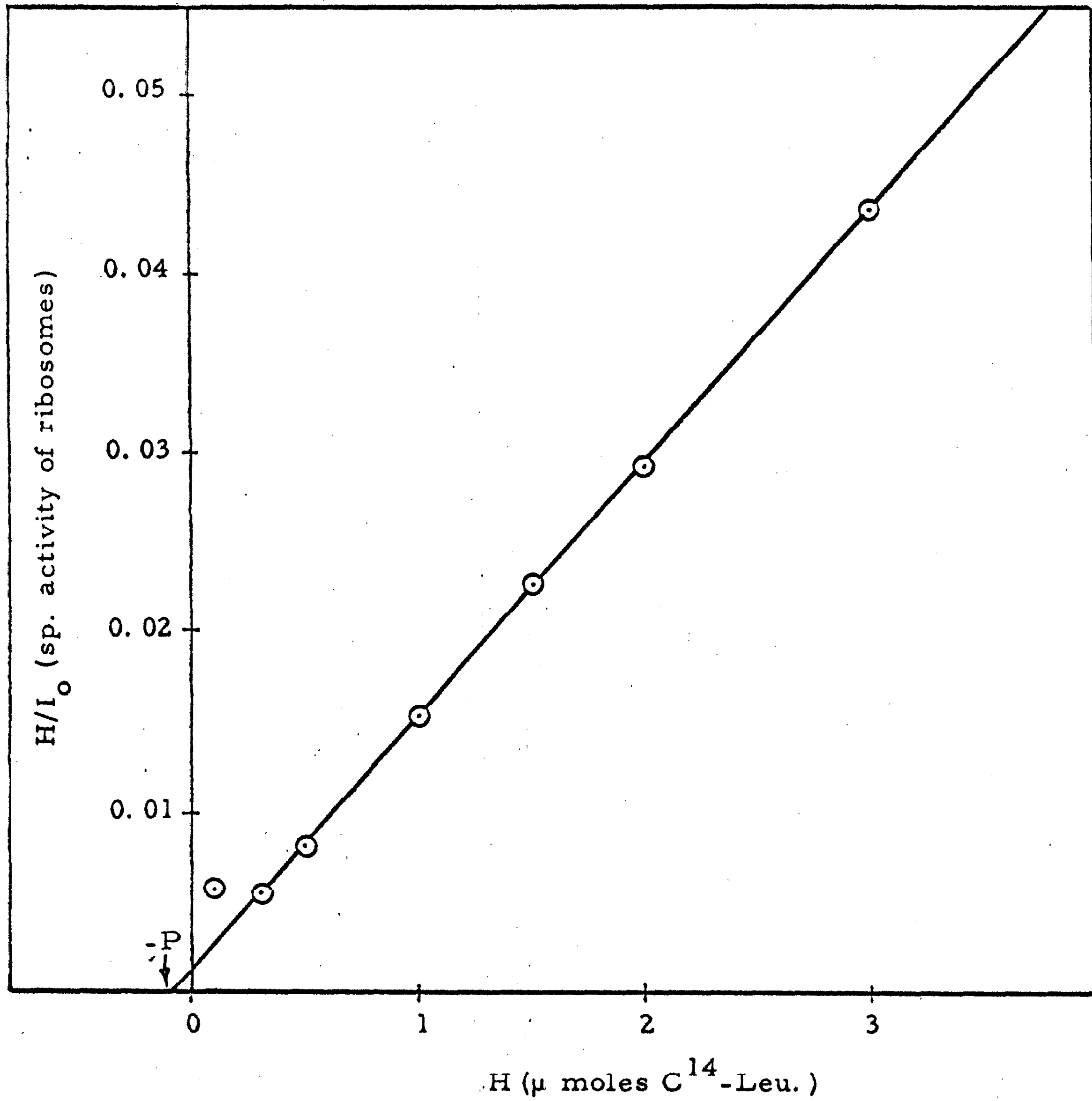


Fig. 12. Plot of H/I_o vs H for specific activity of the ribosomes. The slope, $1/I_t$ = 0.0141 $OD_{260} \cdot cpm^{-1}$; I_t = 70.9 $cpm \cdot OD_{260}^{-1}$. -P = 0.1 μ mole.

The apparent leucine pool is again found from the intercept at the abscissa to be 0. 1 μ mole per 1 ml packed cells. This figure is in accord with the reticulocyte leucine concentration of approximately 10^{-4} M (determined by Borsook, unpublished); this would be essentially 0.1 μ mole/ml cells.

2. Leucine Pool in Young and Old Reticulocytes - In order to determine if the lower ribosomal incorporation observed in the older, heavier cells is due to a larger free leucine pool, 8 ml of cells were fractionated using two 25 ml BSA gradients. The top 15% and the bottom 15% of the cells (not including the pellet) were isolated and washed for incubation in an isotope dilution experiment as before.

A series of 10 minute incubations, using 0. 2 ml cells in each reaction tube with 0. 1 - 0. 6 μ moles of C^{14}-leucine were performed on aliquots of the top and bottom cell fractions. Total incorporation was determined on the 12000 g supernatants from the cell lysates by the method described darlier. The plots of H (μ moles C^{14}-leucine added) vs. H/I_o for the top and bottom cells are shown in Figures 13 and 14. The values of P determined from these graphs are 0.12 μ moles/ml of top cells and 0.10 μ moles/ml of bottom cells. These figures represent the apparent intracellular leucine pools which are in equilibrium with the added C^{14}-leucine in washed reticulocytes. It should be noted here that the added anemic plasma does not contribute significantly to the leucine concentration of the reaction mixutre. Borsook, et al. (1) have shown the leucine concentration to be 0.0305 μ moles/ml plasma; this is only about 0.2% of the amount of added

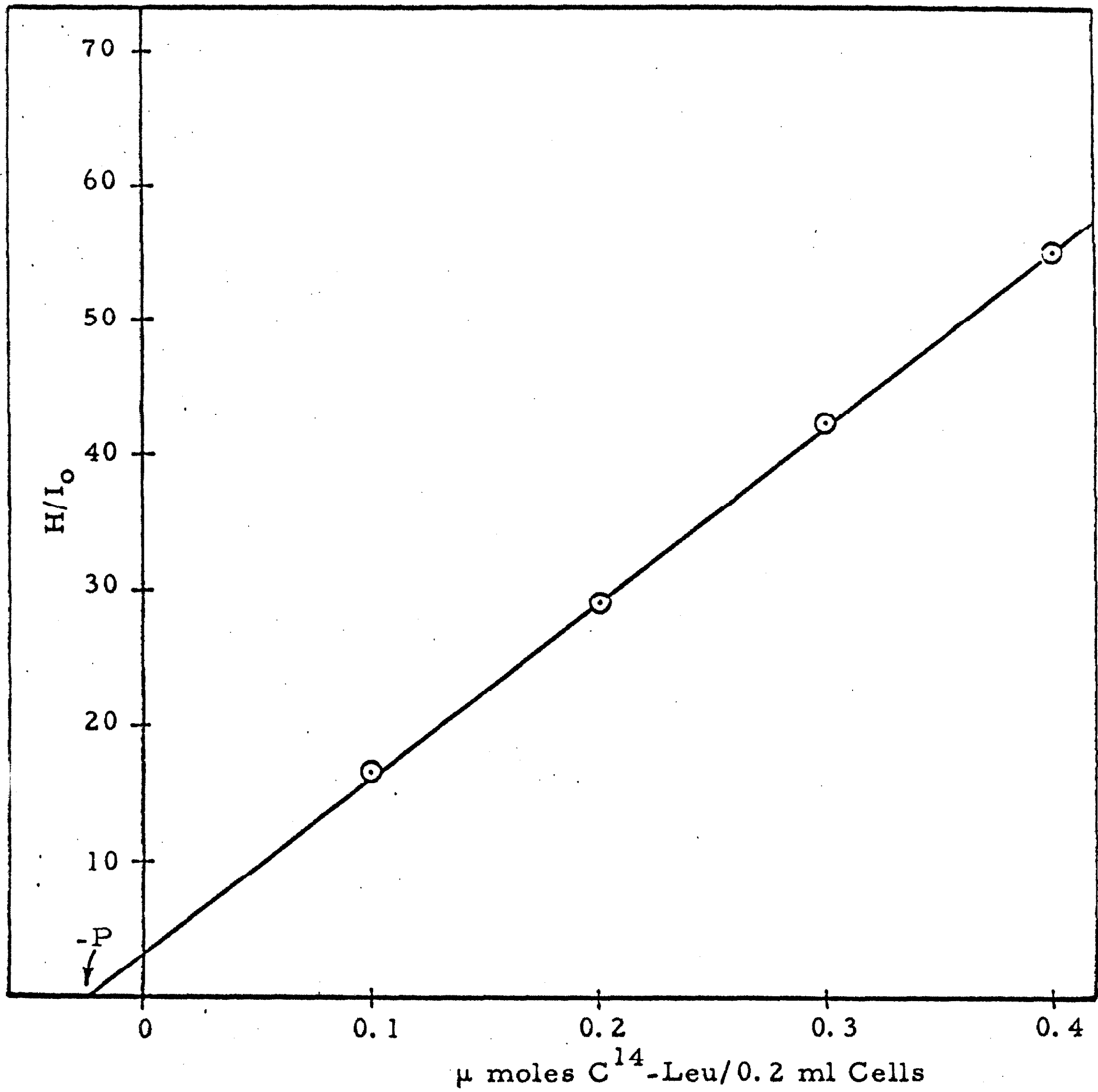


Fig. 13. Plot of H/I_o vs. H for the lightest reticulocytes. I_o represents total TCA-precipitable counts in the 12000 g supernatant. -P = 0.024 μ moles for 0.2 ml cells.

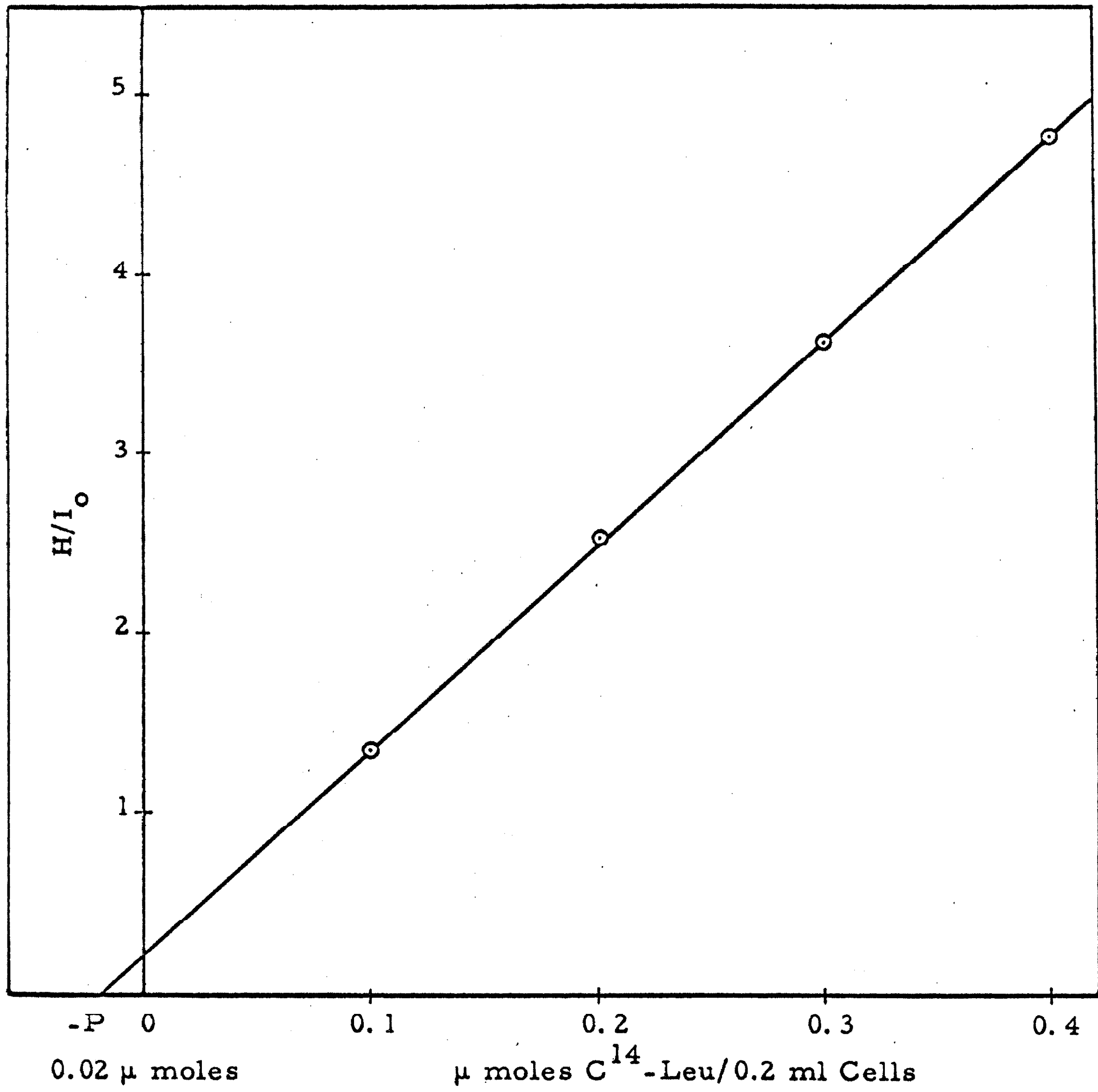


Fig. 14. Plot of H/I_o vs. H for the densest cells. I_o same as Fig. 13. -P = 0.020 μ moles for 0.2 ml cells.

C^{14}-leucine.

Since 1.5 μ moles of C^{14}-leucine are normally added to the incubation of 1 ml packed cells, the specific activity of polyribosomes observed in the previous experiments is low by only 7-1/2% in the top, light cells and 6-1/2% in the bottom, dense cells. Therefore both the lower than theoretical labeling of the polysomes from the most active cells as well as the decreased specific activity of the ribosomes from the less active, denser cells, cannot be explained by isotope dilution from the intracellular leucine pool.

DISCUSSION

In the present investigation it was found that albumin density-gradient centrifugation fractionates rabbit reticulocytes according to their degree of maturity. This conclusion is based on concordant data from cytological characteristics, cell volume distributions, ribosomal contents, and amino acid incorporation, and it has been corroborated further by *in vivo* maturation experiments (Part III).

This fractionation has made possible a study of the role and fate of the polyribosome in relation to the loss of hemoglobin synthesis in the maturing reticulocyte. Although other authors have examined the disappearance of reticulocyte polysomes during *in vitro* (135) and *in vivo* (136) maturation, this system has permitted the study of differences in the protein synthesizing machinery within a given cell population of varying stages of maturation without the use of prolonged incubations.

One of the main features brought out by sucrose-gradient analyses is that increasing cell maturation is accompanied by a nearly parallel decrease in both polyribosomes and ribosomes. In contrast to the observations of Marks and co-workers (135) on reticulocytes maturing *in vitro*, a progressive shift to smaller polysomal aggregates and monomer ribosomes was not observed in these experiments. With the exception of the pelleted cells, which contained only traces of ribosomes, there was little reduction in the percent polysomes with increasing cell maturation. Moreover, the main polysomal peak in all cell fractions occurred in approximately the same position in

the sucrose gradients. It thus appears that the integrity of the polyribosomes is maintained up to the very last stages of maturation. These conclusions have been corrobroated by the electron micrograph studies of Mathias et al (144) which have revealed that even in cells containing few ribosomes (and presumably nearly mature) the bulk of the ribosomes occur as "tetra-" and "pentosomes".

These experiments have not shed much light on the dynamic mechanisms involved in the ribosomal disappearance. However the process of polysomal breakdown found in the cell-free system by Goodman and Rich (140) and Hardesty et al (141a) does not appear to be operative in the whole cells. Since only extremely low levels of ribonuclease activity have been found both in the ribosomes (144, 152) and in the ribosomal supernatant (154), and since the polysomes of even the most mature reticulocytes are predominately tetra- and pentamers, it seems highly improbable that the indiscriminate action of a ribonuclease is responsible for this phase of maturation.

Evidently a balance is maintained within the cell between the concentration of polysomes and "monosomes" thereby keeping the polysomes intact for hemoglobin synthesis up to the most mature stages. It may very well be that the small percentage increase in single ribosomes in the older cells is the result of the equilibrium between ribosomes and messenger RNA predicted from the scheme of ribosomal attachment and release from polysomes observed in the cell-free system (140, 141a, b). From these considerations and from the cell-free experiments to be presented in Part IV it seems that the

rate of protein synthesis depends on the concentration of free ribosomes as well as polyribosomes.

One of the more interesting findings was that the specific activity of the polyribosomes decreased from the youngest to the oldest cells. The maximum labeling observed in the most active reticulocytes was about six molecules of leucine per ribosome. This is somewhat below the theoretical labeling of 8.5 leucines residues per ribosome, assuming an average of one-half a hemoglobin peptide per ribosome. In progressing to the least active cells this labeling was further reduced to approximately two leucine residues per ribosome at the pentamer polysome peak. This observation was somewhat surprising since, assuming that the majority of polysomes are making hemoglobin, one would expect a similar specific activity in the polysomes of all the fractions. There are several possible explanations for this decreased labeling: a) Failure of the polysomes to reach saturation labeling in the more mature cells. Since the incorporation into ribosomes is known to plateau within five minutes (82) and the same incorporation differences were observed for 5, 10, and 20 minute incubations, this explanation has been ruled out. b) Partial chase-effect caused by C^{12} -leucine coming from the breakdown of non-heme proteins during the handling and washing of the cells. Evidence for this was presented on page 54, and is supported by the observation that protein synthesis procedes at O°C (142). It is known that non-heme proteins but not hemoglobin turn over in reticulocytes (40) and that stroma proteins are degraded during reticulocyte maturation and

re-utilized for hemoglobin synthesis (67). Since this effect would be greater in the younger reticulocytes which contain more stroma (67) it could account for the submaximal labeling in the younger cells but not for the even lower specific activity of polysomes in the older cells. c) A significant and greater intracellular leucine pool in the more mature fractions. This has been discounted by showing the leucine pool to be approximately the same and negligible in both the light and heavy cells. d) Non-specific aggregation of 80S ribosomes. This seems unlikely since it would affect the ribosomes from all cell fractions. The low Mg^{++} high K^{+} concentrations used do not favor aggregation, and treatment of the ribosomes with RNase under these conditions obliterates essentially all the polyribosomes (154).

It therefore appears that the observed specific activities represent the true state of polyribosomes. Both the lower than theoretical labeling in the lightest cells and the decreased polysomal counts in the more mature cells can be best explained by the presence of a fraction of inactive polyribosomes which increases with cell maturation. Additional support for this is a) the extent of protein synthesis in each fraction is directly proportional to the total counts on the polysomes and not to the total quantity of polysomes, b) the amount of protein synthesized per polysome (A_{260}) decreases with increasing degree of maturation, and c) the plot of total incorporation into hemoglobin vs. total polyribosomal A_{260} extrapolated in all experiments to a finite amount of ribosomal material at zero incorporation into hemoglobin. A similar decrease in specific activity of polysomes has also been noted in <u>in vitro</u> maturation studies (135) and reticulo-

cytes from patients with thalassemia major (143). However, Rich et al (156) have reported preliminary findings that the polysomes in all classes of reticulocytes are equally active. The reason for this difference cannot be ascertained until more of his data is available.

Since the reticulocytes are in the process of losing their ribosomal material, the lowered incorporation into polysomes can be best explained by the presence of impaired or inactive polysomal aggregates which still sediment as intact structures. This could be brought about by damaged ribosomes, SRNA, and/or messenger RNA blocking the normal transcription mechanism. The presence of either damaged SRNA molecules or SRNA attachment sites on the ribosomes might cause a premature release of the incomplete peptide chains and give rise to incompletely labeled polysomes. This would be compatible with Philipps' finding peptidyl-SRNA in the reticulocyte ribosomal supernatant (155).

The data presented have shown that the loss of protein synthesizing activity in the maturing reticulocyte involves a loss of total ribosomal material, both monomers and polyribosomes, and a progressive decrease in the activity of the polysomes for protein synthesis. This is most likely due to an increasing percentage of inactive or damaged polyribosomes.

III. *IN VIVO* MATURATION OF IMMATURE RETICULOCYTES TRANSFUSED INTO A NORMAL RABBIT

INTRODUCTION

To further substantiate the conclusion that the albumin density gradient fractionates the reticulocytes according to their degree of physiological maturity, an *in vivo* maturation experiment was performed. A narrow band of the youngest, lightest reticulocytes were first recovered from the cells of an anemic rabbit by means of the albumin gradient technique (experimental details are described in Methods, Part II). These young reticulocytes were first allowed to incorporate H^3 -leucine and were then injected intravenously into a normal rabbit. At successive time intervals thereafter, blood samples were drawn and the cells were fractionated on a BSA gradient. The gradient samples were analyzed for tritium-labeled protein and hemoglobin in order to follow the banding position of labeled cells with respect to the *in vivo* maturation time.

MATERIAL AND METHODS

Reticulocyte fractionation by buoyant density centrifugation in gradients and *in vitro* incubations were performed as previously described (Part II, Methods). 1.75 ml of the most immature reticulocytes were isolated from 9.7 ml of red blood cells from a phenylhydrazine anemic rabbit using three 25 ml BSA gradients and pooling the fractions. Cell distribution in the albumin density gradients and

the reticulocyte fraction used in the experiment are illustrated in Fig. 15. The isolated cells were incubated in the complete reaction mixture with 2 mc of L-leucine-4,5-H^3, 3.37 mc/μmole (Nuclear Chicago), for 1 hr. at 37°C. The H^3-labeled cells were washed free of the reagent mixture by centrifugation in NKM, mixed with 3.2 ml rabbit serum containing 5 mg/ml sodium citrate, and injected into the marginal ear vein of a normal rabbit. Blood samples of 10 ml each were taken from the ear of the recipient after 1 hr and at various time intervals thereafter for 15 days, as indicated in Fig. 15. Each sample was centrifuged for 15 min at 1000g in a 15 ml graduated centrifuge tube and blood and packed-cell volumes were recorded. The cells were washed in and suspended in 1/2 vol. of NKM. Five to six ml of this cell suspension were fractionated on a 25 ml BSA gradient and collected in 1 ml fractions. Cells from each fraction were washed with NKM and lysed with 0.8-1.6 ml of lysing solution (Methods, Part II). Stroma pellets were washed with 0.2-0.4 ml 0.1 M KCl, 0.1 M tris, pH 7.5 and the washings added to the stroma-free lysate.

Lysates were assayed for relative hemoglobin content by the oxyhemoglobin method (Wintrobe, 1961), measuring the absorbancy at 542 mμ. Absorbancy of very dilute samples was measured at 415 mμ and converted to A_{542} unites by multiplying by 0.116.

Radioactive protein was determined by treating 0.1 - 0.05 ml of lysate with 5 ml of 1% HCl in acetone to remove the heme. The protein precipitate was centrifuged, washed with acetone, dried free of acetone, and dissolved in 3.0 ml of H_2O. One-tenth ml aliquots

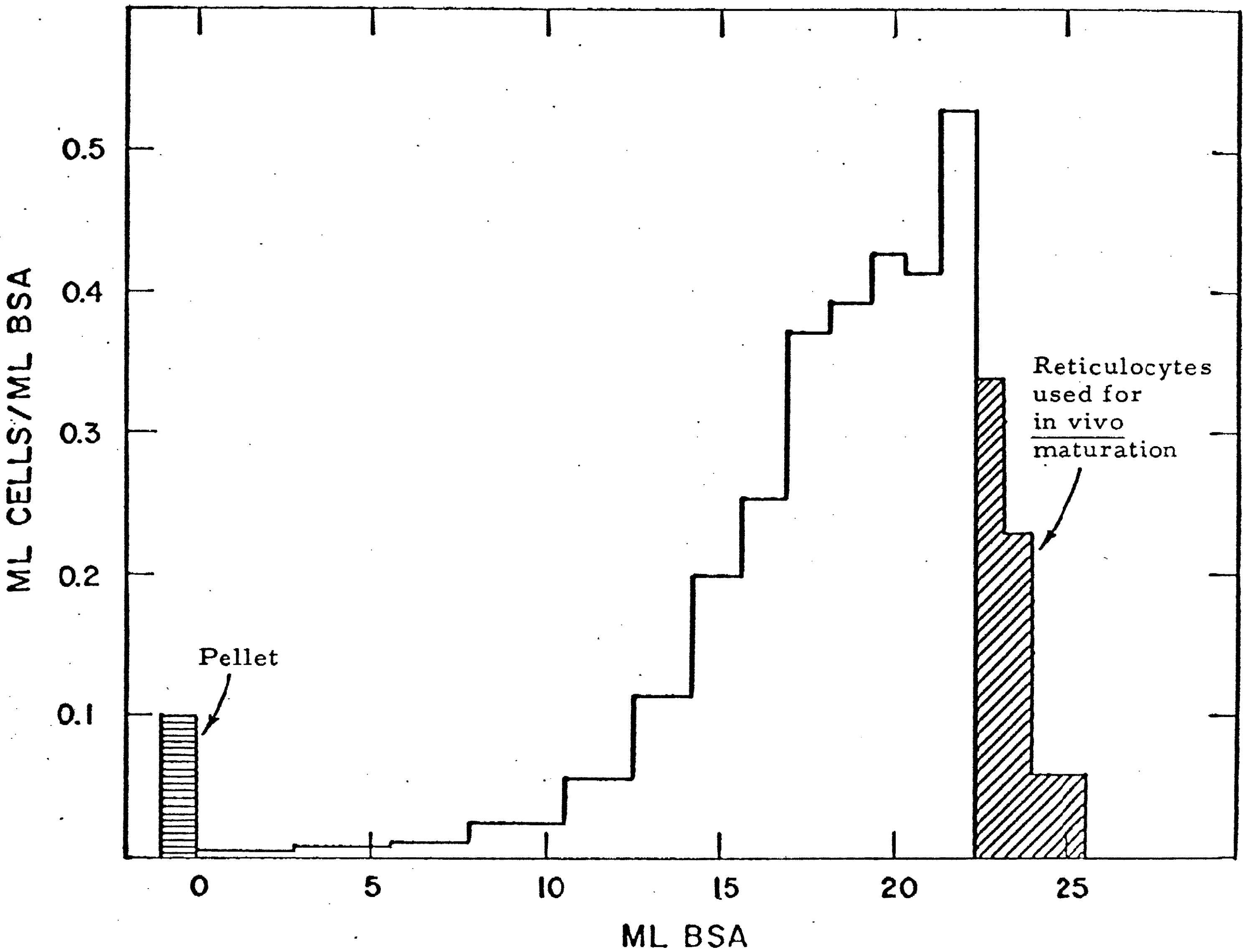


Figure 15. Distribution of reticulocytes in the albumin density gradient.

of this final protein solution were added to 10 ml of Bray's solution (145) for liquid scintillation counting.

RESULTS AND DISCUSSION

The centrifuged BSA gradient containing the first cell sample taken from the normal recipient is shown in Plate 5. The normal erythrocytes are seen distributed in the bottom third of the tube. The injected tritium-labeled reticulocytes initially banded at the position of the leucocyte band (visible in the top third of the gradient tube).

The distribution of radioactivity in the various blood samples analyzed on BSA gradients is shown in Fig. 16. The time in hours, when each sample was taken after the initial injection of labeled cells, is indicated for each curve. In the first sample, taken 1 hr after injection, the labeled cells rebanded at almost the same position as on the original gradient from which they were isolated. In blood samples taken during the first two days following the injection, the tritium-labeled cells banded in successively lower (denser) positions in the gradient as they matured to erythrocytes. During this time the position of the peak of radioactivity on the gradients moved linearly with time toward more dense fractions (Fig. 17). A similar but slower increase in density with cell age has been observed in normal rabbit erythrocytes by Leif and Vinograd (102) using buoyant density centrifugation. . The present results confirm the earlier results based on cytological and biochemical evidence (Part II) that the position of the cells in the gradient is a function of their age.

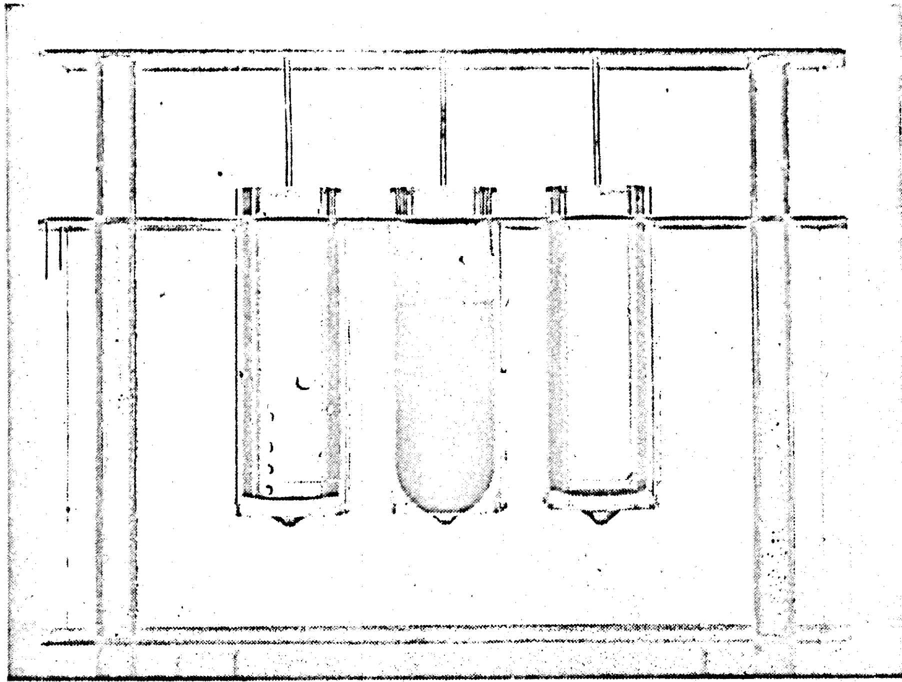


Plate 5.

BSA gradient of the first blood cell sample from the *in vivo* maturation experiment. Normal erythrocytes of recipient are seen in lower third of the tube. Transfused young reticulocytes are banded in top third of the tube.

Figure 16. Distribution of Radioactivity in the Blood Cell Samples as Analyzed on BSA Gradients. Time, in days, after injection of H^3-labeled reticulocytes is indicated for each curve. Gradient samples are numbered from the densest (1.090 g cm^{-3}, 24° C) to the lightest (1.065 g cm^{-3}, 24° C). Cross-hatched area represents the position of the initial band of young reticulocytes as isolated from a phenylhydrazine anemic rabbit.

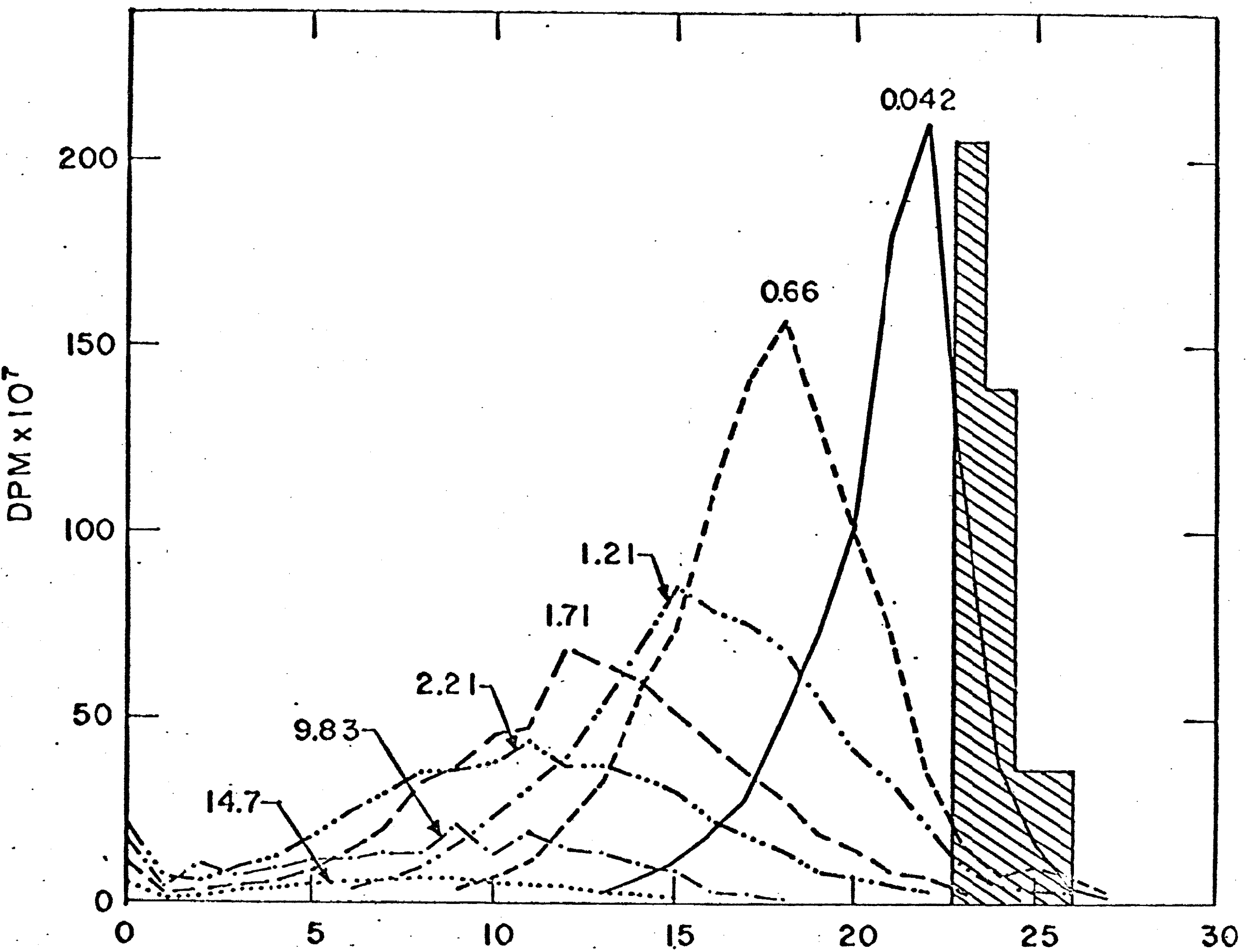


Figure 16. Distribution of Radioactivity in the Blood Cell Samples as Analyzed on BSA Gradients.

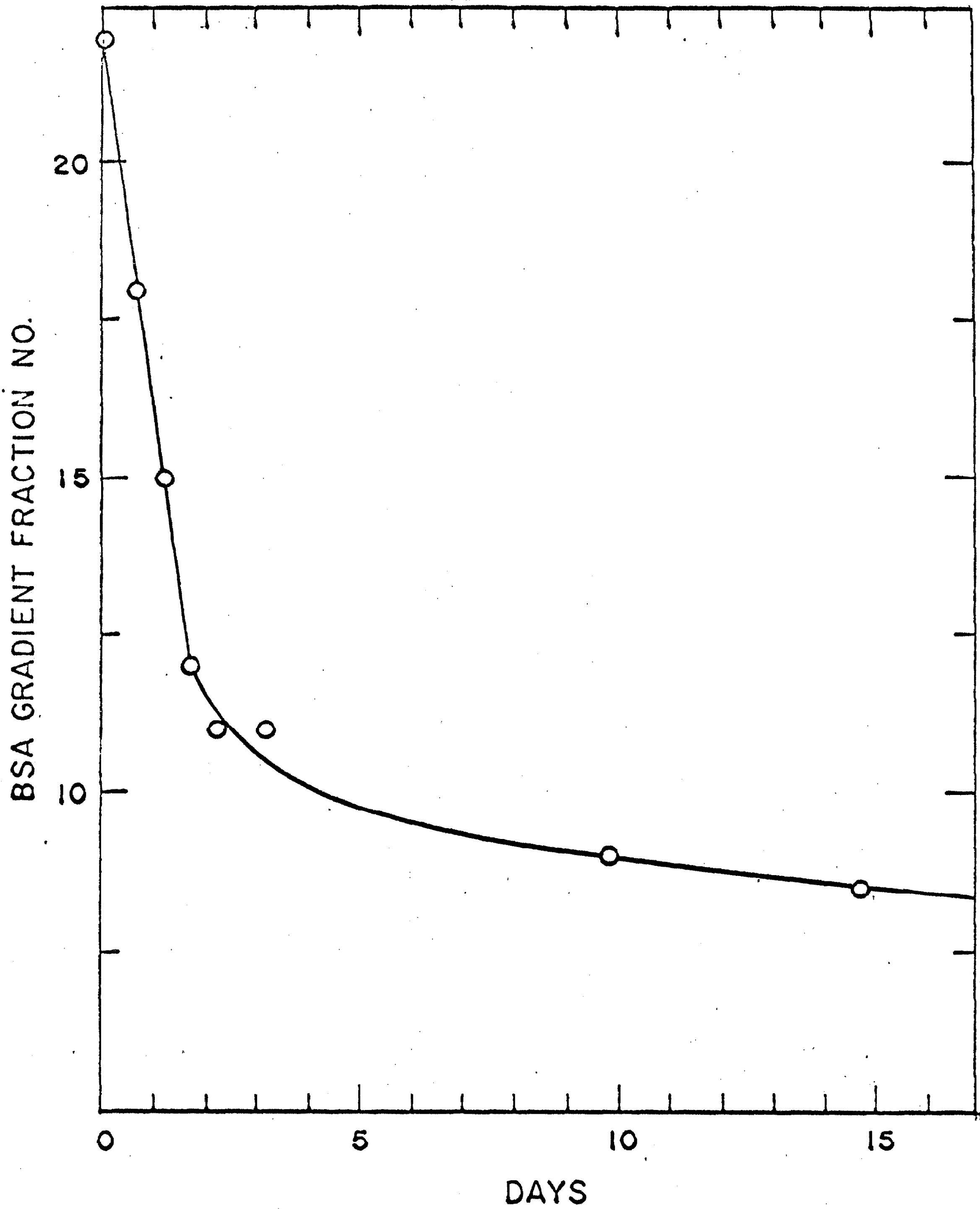


Figure 17. Position of Peak of Radioactive Cells as Analyzed on BSA Gradients, as a Function of Time After Transfusion.

After the first two days, the banding position of the tritiated cells rapidly levels off about two-thirds of the way down the gradient at fraction 9 (Fig. 17). This final banding position corresponds in density to that of the lightest normal erythrocytes (see Plate 5). It has previously shown that the least dense cell fractions consist of the most immature, macrocytic reticulocytes (Part II). The present findings show that such cells mature to erythrocytes which are less dense than the majority of normal erythrocytes. This maturation, in terms of buoyant density changes, takes place in about 48 hours. Since the injected reticulocytes band well above the normal erythrocytes during the first day, this method offers the possibility of studying the biochemical events of maturation in vivo. Such studies have been precluded in anemic animals due to the constant influx of new reticulocytes from the bone marrow.

A graph of the percent survival of the labeled reticulocytes versus time (Fig. 18) shows two distinct cell populations. One population, approximately 55% of the injected cells, had a life span of about 2 days. The remaining 45% of the cells disappeared from the circulation by 17 days. A similar survival curve of sickle cells transfused into normal humans has been reported by Singer and Fisher (146). This is evidence for the extremely short lifetime of the red blood cells which arise in response to a hemolytic crisis. Previous reports, based on in vivo C^{14}-glycine incorporation (147) and Fe^{59} incorporation (148) have indicated considerably longer half-lives, of about 19 days, for such reticulocytes. However, the present findings are not

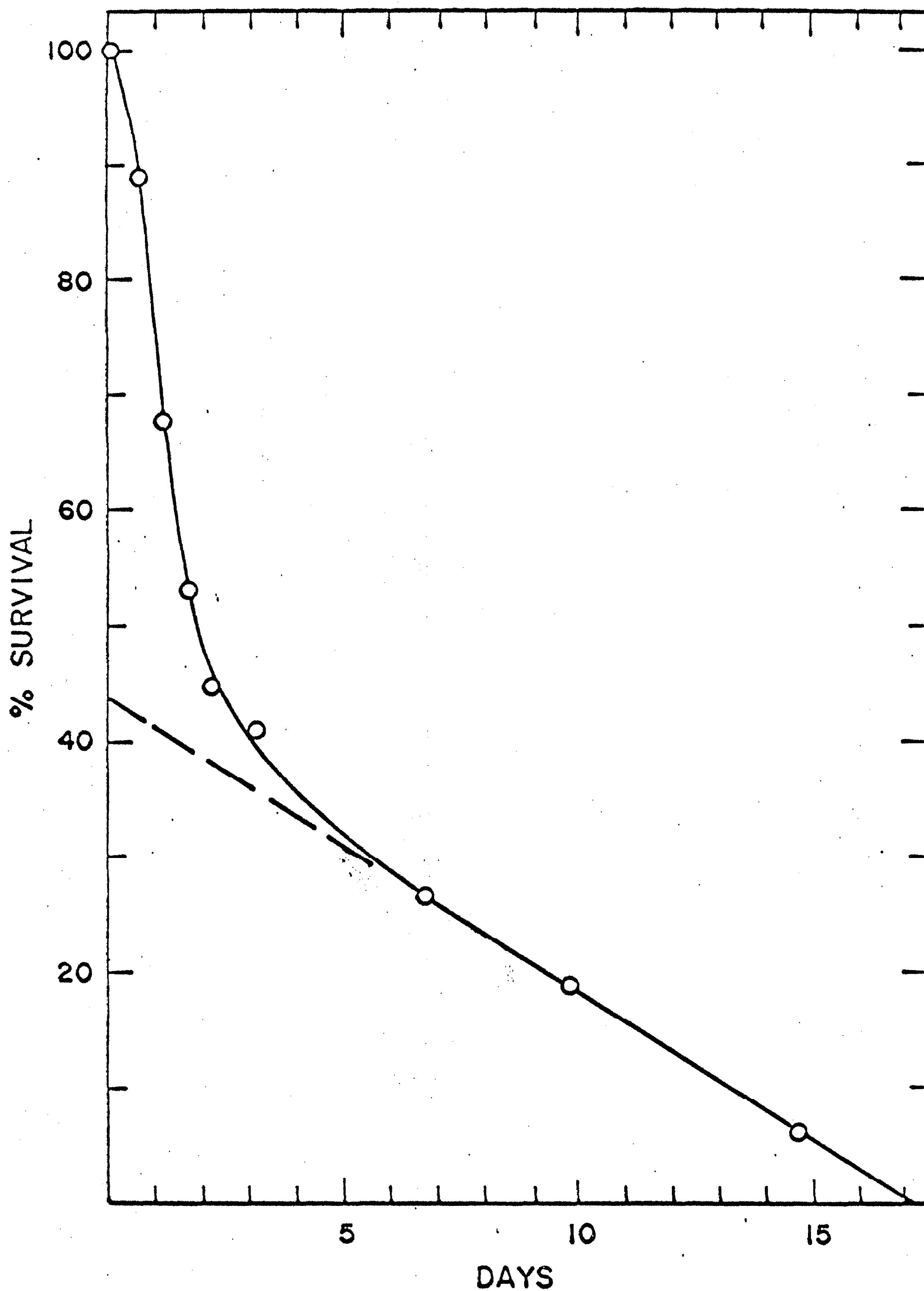


Figure 18. Percent Survival of Tritium-Labeled Reticulocytes as a Function of Time after Transfusion. Values represent percent of initial radioactivity injected, corrected for cell samples removed.

only consistent with the results of *in vivo* C^{14}-valine labeling of reticulocytes (Borsook, Lingrel, Scaro, and Millette, 49), but are in excellent agreement with the survival time of C^{14}-glycine labeled reticulocytes, transfused into normal recipients (Mazur and Carleton, 149). Such extremely short life spans are not unexpected since survival times as short as 4 days have also been observed in several hemolytic anemias of humans (150, 151, 152).

These experiments were performed with the collaboration of Ellen R. Glowacki.

IV. RETICULOCYTE CELL-FREE AMINO ACID INCORPORATING SYSTEM AND ITS USE IN INVESTIGATING THE LOSS OF PROTEIN SYNTHESIZING ACTIVITY IN THE MATURING RETICULOCYTE

INTRODUCTION

In the early observations on the amino acid incorporation into ribosomes and soluble protein in reticulocyte fractions (Part I) it was found that with increasing maturation the capacity for protein synthesis declined faster than the decrease in ribosomes. Thus, the rate of hemoglobin synthesis per ribosome in the youngest cells was as much as 6 times higher than that in the oldest. These observations were correlated with the decreasing specific activity of the ribosome. It seemed most likely that an increasing percentage of inactive ribosomes was to blame for the diminishing activity of the ribosomes during maturation. The sucrose gradient analyses of the ribosomal material in each fraction (Part II) showed only a small shift to the inactive 80S ribosomes in the more mature cells. This was insufficient to account for the observed decrease in activity. A look at the polyribosomal amino acid incorporating activities in the various fractions further strengthened and extended the original hypothesis to include an increasing fraction of inactive polysomes. These studies, however, did not exclude the

possibility of a decline of some essential soluble factor(s) during cell maturation.

It was therefore hoped that the cell-free system would offer a different approach toward solving these problems. However, the cell-free system is not completely analogous to the whole cell system. Its rate of amino acid incorporation is usually only a few percent of the whole cell rate and it usually ceases by about 30 min. Nevertheless it presented the possibility of testing separately the ribosomes and supernatant factors from the albumin gradient fractionated cells in order to pinpoint the source of this declining activity.

With this in mind, the first part of these studies is devoted to developing a satisfactory cell-free system and examining its properties and limitations. The system was then used to investigate the differences between the most active and least active reticulocytes from a BSA gradient, so as to complement the observations from whole cell studies.

MATERIALS AND METHODS

1. Preparation of Subcellular Fractions - Reticulocytes were prepared, washed, and lysed as described in Part I. All operations besides the incubations were performed at 0° to 4° C. The lysate was centrifuged at 12000 g for 10 minutes to remove the cell debris and the supernatant (S_{12}) was further centrifuged at

10500 g for 2 hours to sediment the ribosomes. The supernatant (S_{105}) was either used directly or further fractionated by pH5 precipitation (108).

The pH5 supernatant (5-Sup) was prepared by adjusting the pH of S_{105} to 5.15 at 0°C with 1N acetic acid. After standing 10 to 15 minutes on ice it was centrifuged 15 minutes at 12000 g. The supernatant was carefully decanted, sufficient glutathione was added to make 0.001M, and the pH readjusted to 8.1 at 0°C with a saturated Tris base solution. The 5-Sup was frozen at -70°C until further use.

The pH5 fraction (5-Enz) was prepared by dissolving the pH5 precipitate in solution F (0.025 M KCl, 0.004 M $MgCl_2$, 0.03 M Tris., pH 7.5 at 25°C), adjusting the pH to 4.1 with 1N acetic acid, and centrifuging 15 minutes at 12000 g. The precipitate was again dissolved in solution F and centrifuged for 2-1/2 hours at 105000 g to remove any remaining ribosomes. The resulting supernatant (5-Enz) was frozen at -70°C until use.

Ribosomes were washed twice by homogenizing in solution F with a loose-fitting Potter-Elvehjem homogenizer and centrifuging for 2 hours at 105000 g. Denatured ribosomal material was removed prior to the second ultracentrifugation by spinning 10 minutes at 12000 g. Ribosomal pellets were frozen under 0.2 ml solution F at -70°C until use.

In some instances 5-Sup was dialyzed, to remove endogenous amino acids, against 2 changes of 20 volumes of Dialysis Medium -1, DM-1, (10^{-4} M EDTA, 10^{-3} M GSH, 10^{-2} M Tris., pH 7.5 at 25°C). This was followed by a 7 hour dialysis against 2 changes, 20 volumes each, of solution F containing 10^{-3} M GSH.

2. Cell-Free Incubations - Incubation conditions were essentially as described by Lamfrom (108) except that all reagents were prepared in solution F. The basic reaction mixture contained 2.7 ml (unless otherwise indicated) 1.5 - 2.5 mg ribosomes, 1.0 - 2.0 ml supernatant (S_{105} or 5-Sup), 0.09 ml of the complete amino acid mixture of Lingrel and Borsook (82) minus leucine, 340 μg creatine kinase, 0.45 μ mole GTP, 1.8 μ moles ATP, 36 μ moles creatine phosphate, 26 μ moles glutathione, 67.5 μ moles KCl, 9.45 μ moles $MgCl_2$, 21-51 μ moles Tris. (pH 7.5 at 25°C) and 250-500 μ moles sucrose (depending on the volume of supernatant used), and 0.19 μ mole L-leucine-1-C^{14} (2.5 - 5.6 x 10^6 cpm/μ mole). Incubations were performed at 37°C for various times as stated in the text.

3. Determination of Radioactivity - Incubations were terminated by chilling in ice-water and the ribosomes removed by precipitation at pH 5.1 with either N/3 HOAc or 3 ml of 0.2 M NaOAc buffer, pH 5.0, containing 0.01 M $MgCl_2$. After centrifugation at 12000 g for 10 minutes the supernatant was decanted and the

ribosomal pellets rinsed twice with about 0.5 ml of the pH 5 buffer. The ribosomal pellets were suspended in 0.5 ml solution F, and dissolved by the addition of 0.5 ml of 0.1N NaOH. After 5 to 10 minutes 1.0 ml of supernatant (S_{105}) was added to each ribosome sample as carrier protein, and both ribosomes and ribosomal supernatants were precipitated with 7% TCA, washed, freed of heme, and dried according to Lamfrom[108]. The dried protein samples were dissolved in 10 ml of 1N NH_4OH and duplicate 0.5 ml aliquots plated on aluminum sand-blasted planchets and oven-dried. Radioactivity was determined with a Nuclear Chicago gas flow counter.

4. TCA-Precipitation on Membrane Filters - In the later experiments, radioactive TCA-precipitates were plated and counted on membrane filters. To a solution containing 0.2 - 0.8 mg protein was added sufficient 20% TCA containing 0.05 M leucine to give final concentrations of 5% TCA and 0.2 to 0.75 mg protein per ml. After mixing and standing for 1 hour the mixtures were heated at 90° C for 30 minutes to hydrolyze the amino acyl-SRNA. The tubes were chilled, and the precipitates were dispersed using a Vortex Mixer and collected with suction on membrane filters. Tubes which contained the precipitates were rinsed once with approximately 1 ml of 5% TCA containing 0.05M leucine, twice with 5% TCA, and the precipitates were rinsed three times on the filters with 5 ml of 5% TCA. When partially dry the filters

were attached to aluminum planchets with rubber cement and allowed to dry completely.

Ribosomes were precipitated and plated in a similar manner using a 2.5% casein solution as carrier to bring the final amount of precipitated protein to approximately 5 mg. With this method, pH 5-precipitated ribosomes were first dissolved in 1 ml of 0.05 N NaOH, made up of 5 - 10 ml with H_20, and appropriate aliquots were taken and quickly precipitated with TCA. Washings and platings on membrane filters were as described for the soluble protein.

5. Activity of 80S Ribosomes in Cell-free System - Ribosomes were obtained by layering 7.5 ml of clarified lysate (S_{12}) over 4.5 ml of 30% (w/v) sucrose in solution P in Spinco No. 40 tubes and centrifuging for 4 hours at 105,000 g. The upper 5.5 ml of supernatant (S_{105}) from each tube was saved for the incubations and the rest was discarded. Ribosomal pellets were rinsed twice with solution P and resuspended in solution P to yield a solution of 76 OD_{260}/ml. Over each of three 25 ml sucrose gradients were layered 2 ml of the ribosomal solution. Gradients were prepared, centrifuged, and fractionated as in Part II, Methods. Twenty-five 3 ml pooled fractions were collected; fractions #2 through 11 were combined as the polysomal fraction and #16 through 21 as the 80S ribosome fraction. Polysomes and robosomes were recovered from the sucrose solution by a 4 hour centrifugation at 105,000 g and

resuspended in a small volume of solution F.

Each incubation tube contained 0.5 ml of S_{105} plus the complete reagent mixture listed in par. 2. Specific activity of C^{14}-leucine was 12.5 mc/m mole. One series of tubes contained 0.64 mg polysomes and 0 - 0.74 mg 80S ribosomes in each. Another series contained only the varying amounts of 80S ribosomes. Incubation was for 90 minutes at 37°C. Amino acid incorporation into pH 5-precipitated ribosomes and soluble protein was determined by TCA precipitation on membrane filters (par. 4).

6. Materials - Tris and EDTA (ethylenediaminetetracetic acid) were obtained from Sigma Chemical Company, and L-Leucine-1-C^{14}, at a specific activity of 12 to 14 mc per m mole, was purchased from Isotopes Specialties Company (now Volk Radiochemical Company). Non-radioactive amino acids and glutathione came from Nutritional Biochemicals Corporation and California Corporation for Biochemical Research. ATP and GTP were obtained as sodium salts, from Nutritional Biochemicals Corporation or Pabst Laboratories. Creatine phosphate, disodium salt, A grade, was procurd from California Corporation for Biochemical Research and crystalline creatine kinase from C. F. Boehringer and Sons. Membrane Filters were type B-6 from Schleicher and Schuell.

RESULTS

Properties of the Cell-Free Amino Acid Incorporating System

The goal of these studies was to develop a good cell-free amino acid incorporating system which could be used to investigate the nature of the loss of the protein synthetic apparatus in the maturing reticulocyte. Therefore experiments were undertaken to improve the cell-free incorporating activity and to define the limitations of the system with respect to various parameters as time, pH, enzyme concentration, and reagent concentrations.

A. Time Course - The time course of amino acid incorporation into ribosomes and soluble protein is shown in Figure 19 for two different 105000 g supernatant (S_{105}) concentrations. After an initial fast rise during the first 30 minutes, the incorporation into soluble protein continued to increase linearly for at least 2 hours. This is in strong contrast to the cell-free incorporation, reported by Allen and Schweet (158), Goodman and Rich (140), and others (162), which usually ceases in 30 minutes. The incorporation into ribosomes shown here reaches a plateau corresponding to 1.9 μmoles leucine per μmole ribosome. This level is maintained throughout most of the time course and is comparable to the ribosomal labeling that has been observed in the reticulocyte whole-cell incorporations by this author and Lingrel and Borsook (82). The fact that the specific activity of the ribosomes does not decrease appreciably during prolonged incubations (at least between 15 and 110 minutes) is indicative of negligible

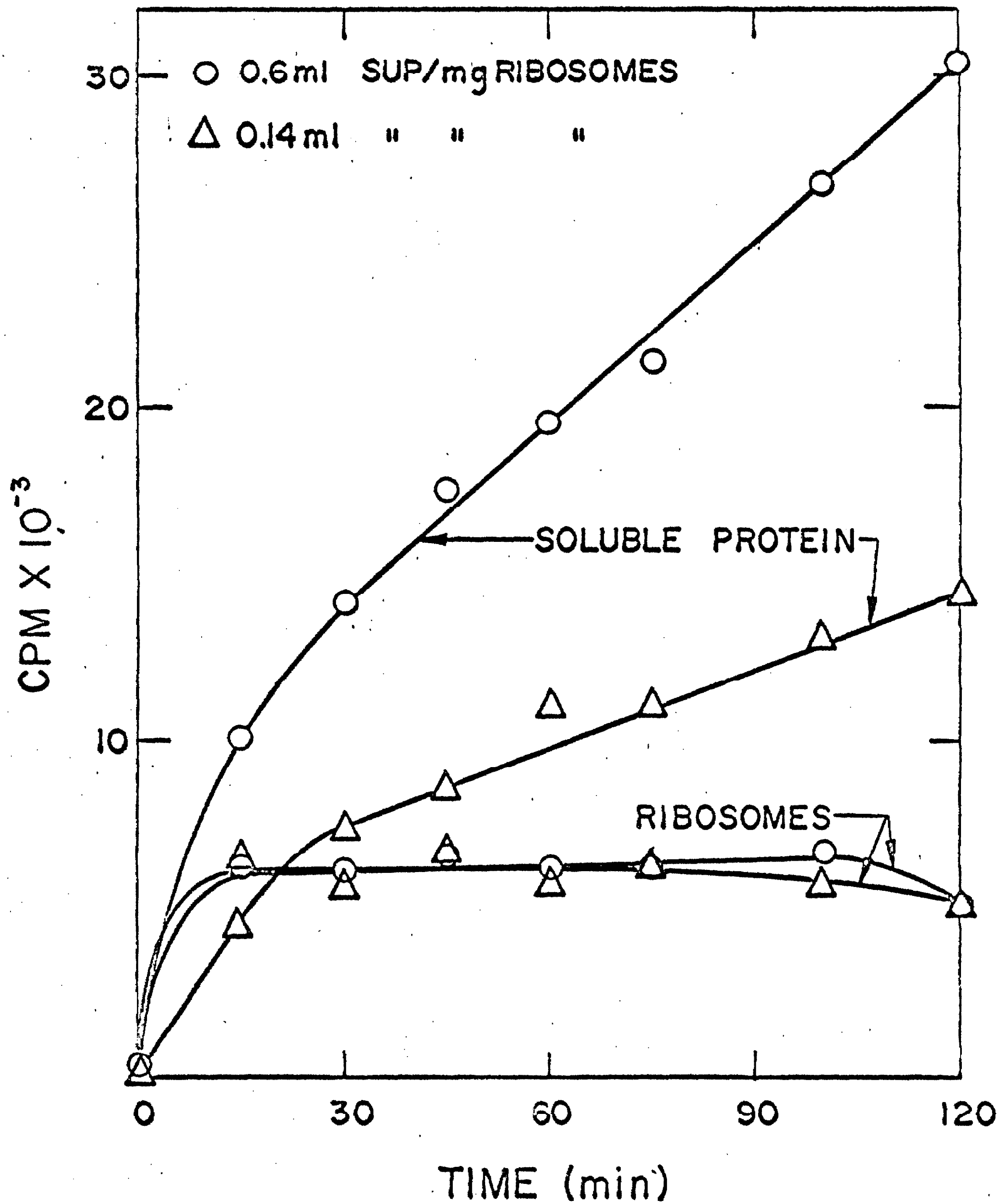


Figure 19. Time course of amino acid incorporation into ribosomes and soluble protein using two different amounts of supernatant. Each point corresponds to a 3 ml incubation containing 3.3 mg ribosomes, 0.48 or 2.03 ml S_{105}, and the complete reagent mixture containing C^{14}-leucine at 4.3 x 10^6 cpm/μ mole (see Methods).

ribosomal breakdown occurring during this time. (Additional data to support this is presented in a later section.) This finding is contrary to recent reports by Hardesty, et al. (141) and Lamfrom and Knopf (162) which indicate a large decrease in ribosomal radioactivity associated with polysomal breakdown during similar cell-free incubations.

Most cell-free amino acid incorporating systems have suffered from the failure of the ribosomes to "turn over", i.e., to initiate and release newly synthesized peptide chains (86). The failure of ribosome reattachment to polysomes has recently been implicated as a cause of this. However, it does not appear to be a serious problem in the system reported here. The fact that the 120 minute soluble protein incorporation is five times greater than the ribosomal incorporation suggests that the ribosomal counts have turned over 5 times in the system with higher supernatant concentration. A comparable release of radioactive protein in the reticulocyte cell-free system has recently been reported by Hardesty, et al. (159) using once sedimented (1x-) ribosomes. These authors showed released counts to be related to the de novo synthesis of complete peptide chains. The amount of soluble TCA-precipitable counts (Fig. 19) is seen to be strongly influenced by the amount of supernatant used. The effect of varying supernatant concentrations is examined later in more detail.

B. pH Optimum - The effect of pH on the amino acid incorproation was tested for a 100 minute incubation using Tris buffers of varying pH. The pH of the mixtures was measured at 2°C before and

after incubation. From these values the average pH during incubation was determined. The total TCA-precipitable counts were measured and are plotted against the pH of the reaction mixtures in Figure 20. The pH curve shows a plateau between pH 7.9 and 9.8, with midpoint at pH 8.1 at 2°C. This corresponds to a value 7.25 at 25°C.

C. Amino Acid Incorporation in the Presence of 105000 g Supernatant, pH-5 Supernatant, and pH-5 Fraction - These experiments were designed to test the system's requirements for the various supernatants (S_{105} and 5-Sup) and the pH-5 fraction (5-Enz). First the stimulatory effect of these three components was tested in a basic system containing an initial low amount of S_{105} (Table 8). The complete system consisted of 3 mg ribosomes, 0.3 ml S_{105}, plus the usual reagent mixture. Total C^{14}-leucine incorporation was determined in the ribosomes and soluble protein fractions.

The addition of the 5-Enz to the active system is seen to cause a decrease in the soluble protein incorporation while increasing the ribosomal counts (line 3). These differences, though not great, are reproducible (observed in at least four experiments). Addition of pH 5-supernatant (5-Sup) gives an increase in total incorporation equivalent to that observed with added S_{105}, but also favors a higher release of soluble counts (lines 2 and 4). When 5-Enz and 5-Sup are added to the system lacking the initial amount of S_{105} it is seen that the 5-Sup works equally as well as the equivalent amount of S_{105} (lines 5 vs. line 2). However, again the 5-Enz is seen to reduce the soluble amino acid incorporation while at the same time increasing the ribosomal counts

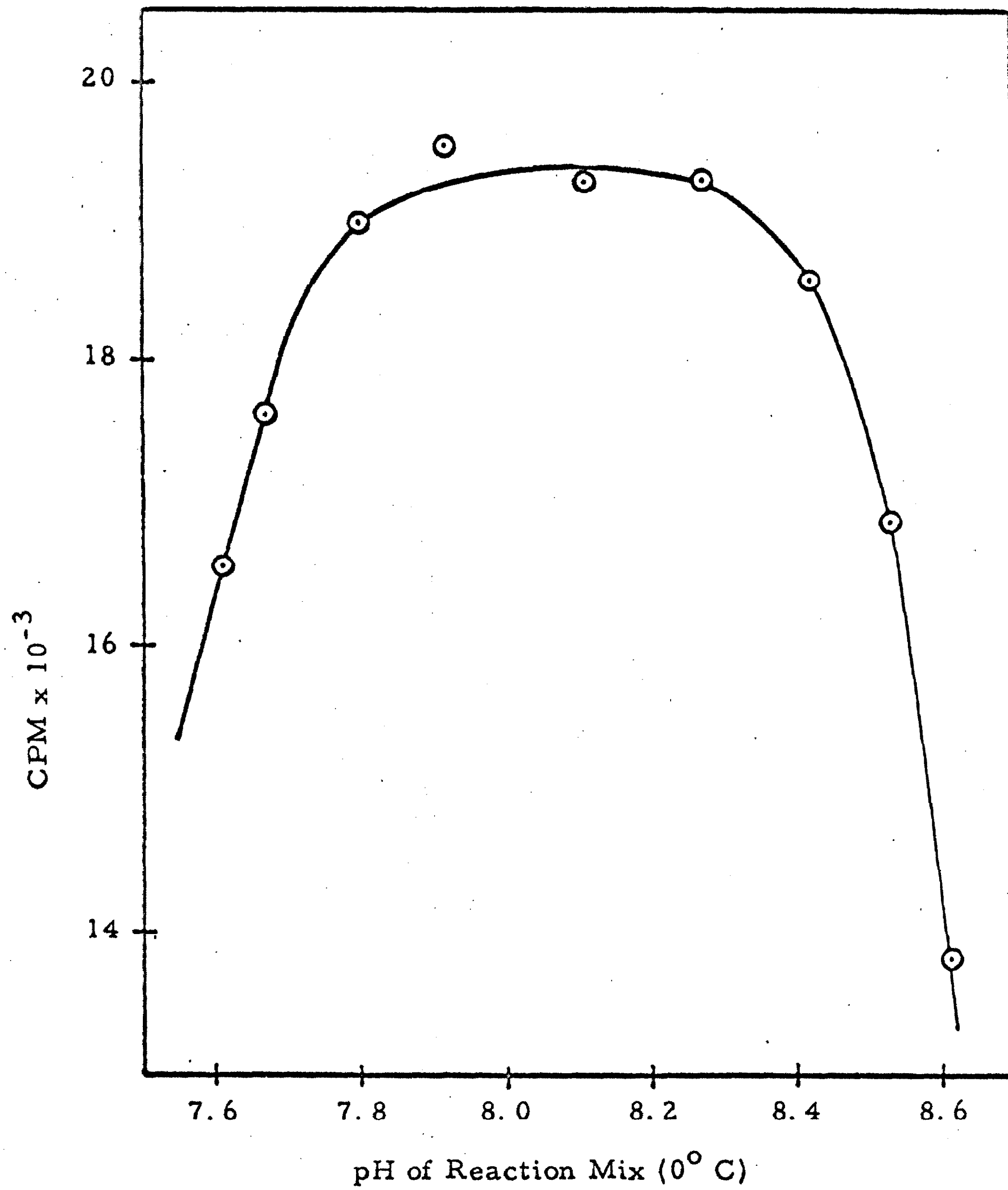


Figure 20. Effect of pH on amino acid incorporation. Assay conditions were as described in the text.

TABLE 8

EFFECT OF SUPERNATANT FRACTIONS ON AMINO ACID INCORPORATION IN THE COMPLETE CELL-FREE SYSTEM

System	cpm		
	Soluble Protein	Ribosomes	Total
1. Complete	6420	3770	10190
2. " + 0.7 ml S_{105}	8610	3990	12600
3. " + 5-Enz	5540	4160	9700
4. " + 0.7 ml 5-Sup	9580	3250	12830
5. " $-S_{105}$ + 1.0 ml 5-Sup	8980	3450	12430
6. " $-S_{105}$ + 1.0 ml 5-Sup + 5-Enz	8230	4130	12360

The complete system contained the complete reagent mix, 3 mg ribosomes, and 0.3 ml S_{105} (8 mg protein) in a total volume of 2.7 ml. Additions were S_{105} and 5-Sup both containing 27 mg protein / ml, and 5-Enz containing 0.56 mg protein. Incubation was for 90 min. at 37° C.

(line 5 vs. line 6).

The results of a similar experiment are portrayed in Figure 21. To once sedimented, unwashed ribosomes (1x Rib) were added 105000 g supernatant (S_{105}), pH 5-supernatant (5-Sup), or pH 5-enzyme (5-Enz) under the usual reaction conditions with C^{14}-leucine. Samples were removed at the times indicated and assayed for C^{14}-incorporation into soluble protein and ribosomes. It can be seen that the incorporation into the soluble protein was comparable using either S_{105} or 5-Sup. However, the incorporation into ribosomes using 5-Sup is lower than that observed in the presence of S^{105} as was indicated in the previous table. This difference in the ribosomal incorporation in the presence of the two supernatants resides in the activity of the ribosomes observed in the presence of 5-Enz. Since the amount of 5-Enz used was equivalent to the amount initially present in the S_{105}, the ribosomal incorporation using S_{105} is roughly the sum of that observed using 5-Sup and 5-Enz alone. This difference is probably due to the presence of contaminating ribosomes both in the S_{105} and 5-Enz preparations (108). In contrast to the reticulocyte cell-free system of Kruh, et al. (160), which consists of ribosomes and "pH 5-enzymes" as the sole cellular components, the system reported here shows negligible incorporation into the soluble protein when only ribosomes and 5-Enz are used.

Another experiment using twice washed ribosomes with the various supernatant fractions is shown in Table 9. In contrast to the previous experiments, the 5-Enz preparation was centrifuged 2 hours

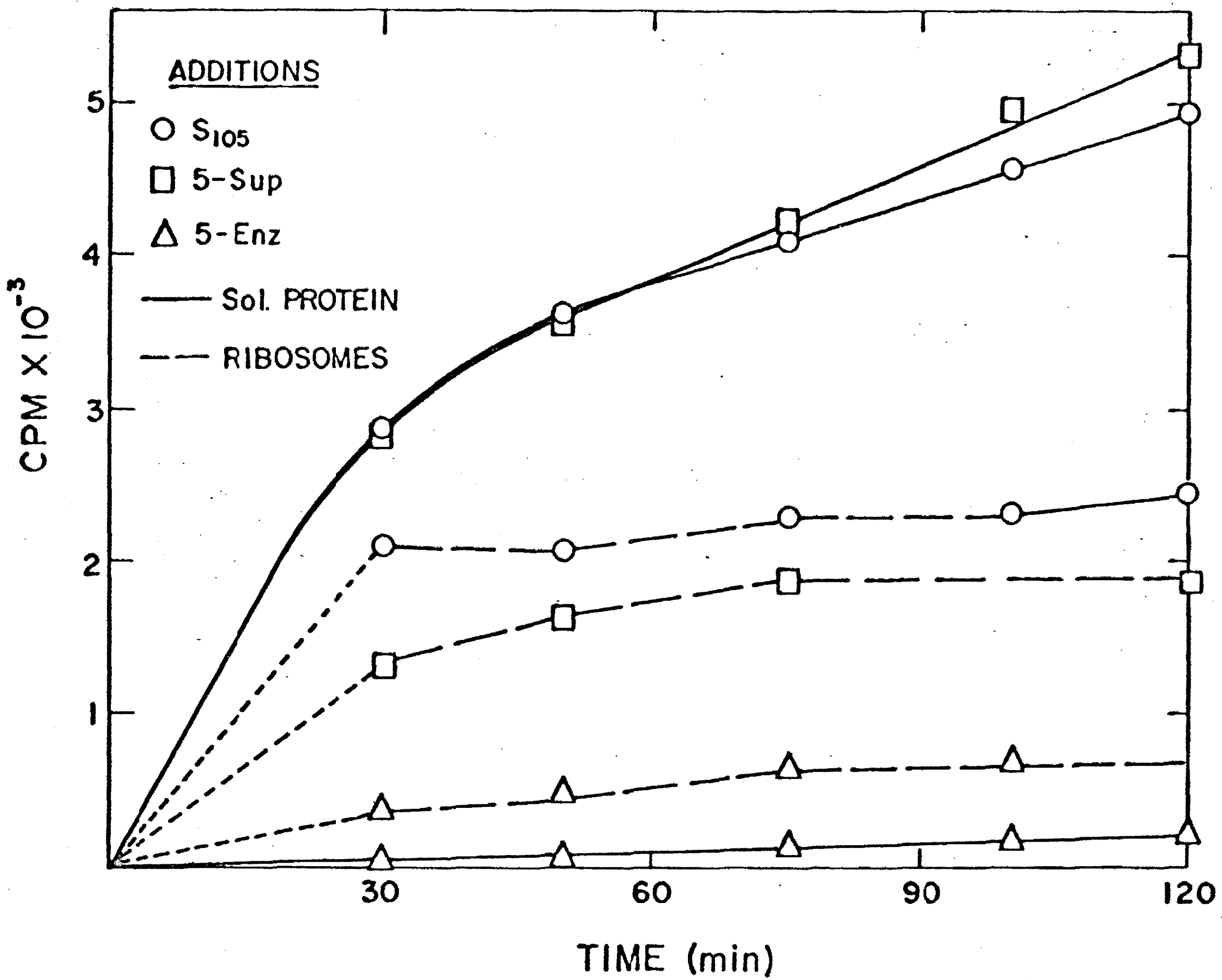


Figure 21. Time course of amino acid incorporation in the presence of various supernatant fractions. Each point corresponds to a 2 ml incubation containing 2.2 mg 1x ribosomes, 1.1 ml supernatant (S_{105} or 5-Sup) or the equivalent amount of 5-Enz. Incubation and assay conditions were as previously noted.

TABLE 9

EFFECT OF VARIOUS SUPERNATANT FRACTIONS ON CELL-FREE AMINO ACID INCORPORATION

	Additions				cpm		
						Sol.	
	Rib.	S_{105}	5-Sup	5-Enz.	Rib.	Protein	Total
1.	+	+			4820	6550	11570
2.	+		+		3210	5290	8500
3.	+			+	409	211	620
4.	+		+	+	4270	4980	9250
5.		+			666	1890	2556
6.			+		36	28	64
7.				+	38	82	120
8.			+	+	38	56	94

Each reaction mixture contained in a final volume of 2.7 ml the complete reagent mixture and the following additions as indicated: twice-washed ribosomes (Rib), 3 mg; 105000 g. supernatant (S_{105}) and pH 5-supernatant (5-sup), 1.5 ml containing 45 mg protein; pH 5-enzyme (5-Enz), 1.97 mg protein. Incubation was for 100 min. at 37° C.

at 105000 g to remove contaminating ribosomes. Their presence in the S_{105} is indicated by the significant incorporation in the absence of ribosomes (line 5), whereas 5-Sup and 5-Enz are ribosome-free as witnessed by their very low incorporation under the same conditions (lines 6, 7, 8). When the results of line 1 are corrected for this residual ribosome incorporation (line 5) it is again apparent that total incorporation in the presence of 5-Sup is equivalent to that seen with S_{105}, whereas the release of soluble counts is favored by the 5-Sup. Only a slight enhancement of total activity is achieved by the addition of 5-Enz to 5-Sup (line 4), The main effect of 5-Enz still seems to be the shifting of the distribution of incorporation toward the ribosomes and reducing soluble counts.

From these studies it was concluded that the pH 5-fraction is not required for this system; only ribosomes and pH 5-supernatant are necessary for optimal amino acid incorporation. As was shown in Figure 21, the rate of incorporation into soluble protein using 5-Sup is the same as, if not slightly higher, than that of the complete system with 105000 g supernatant.

D. Effect of Supernatant Concentration on Incorporation - In order to determine the limitations of the pH 5-supernatant on both the ribosomal and soluble protein labeling, a series of cell-free incubations were run with varying 5-Sup concentrations. The results of a 15 minute incubation are shown in Figure 22. At low supernatant concentrations the ribosomes are not fully labeled and soluble counts are low. At about 0.55 ml 5-Sup per mg ribosome the ribosomal labeling

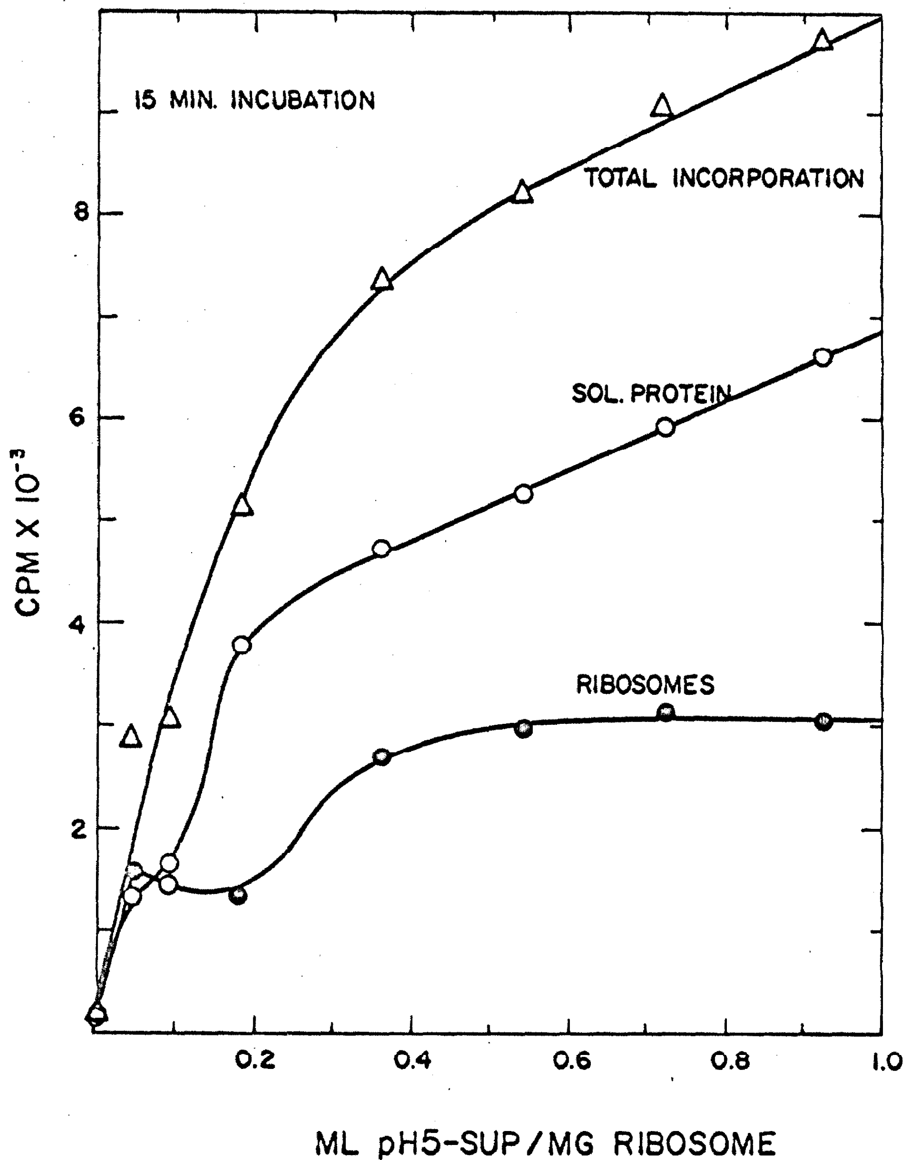


Figure 22. Effect of supernatant concentration cell-free amino acid incorporation. Each incubation contained 2.22 mg ribosomes, C^{14}-leucine at 5.58 x 10^6 cpm/µmole. Incubation and assay conditions were as previously described.

reaches a maximum. No maximum is achieved in the soluble protein incorporation, at least up to 0.82 ml supernatant per mg ribosome. Above 0.2 ml 5-Sup the soluble counts increase linearly with supernatant concentration. Apparently under the conditions of these incubations incorporation into soluble protein is limited by supernatant factors. Similar labeling patterns were obtained also for 50 and 110 minute incubations.

It should be noted that the incorporation in the absence of pH 5-supernatant is essentially zero (Fig. 22). The total incorporation at zero supernatant concentration was 236 cpm, or 2.5% of the counts observed at the maximum supernatant level used. Thus the 2x washed ribosomes must be virtually free of at least one or more supernatant factors essential for protein synthesis.

E. Effect of Reagent Mix and Amino Acid Concentrations - Since the greatest incorporating activities in these studies were obtained using supernatant concentrations much higher than used previously by other authors (2, 88, 108, 161), the optimum concentrations of amino acids and reagent mix were sought under the current incubation conditions. To accurately determine the optimal conditions, the endogenous small molecules were removed from the 5-Sup by dialysis against DM-1 buffer. KCl, $MgCl_2$, Tris, and sucrose were restored to their original concentrations in the incubation mixtures. Only 8% of the total incorporating activity was lost after 9 hours of dialysis. The standard reagent mixture, lacking the amino acids, was prepared in 2-fold concentration. Its composition was 2.24 mM GTP, 9.0 mM

ATP, 130 mM GSH, 180 mM creatine phosphate, and creatine kinase, 1.7 mg ml in solution F. This was added in varying amounts to an incubation mixture consisting of amino acids plus C^{14}-leucine, ribosomes, and dialyzed 5-Sup (D 5-Sup). The incorporation into ribosomes and supernatant was determined after a 30 minute incubation (Fig. 23). The optimum incorporation occurred at 0.2 ml, corresponding to the reagent concentrations normally used. Incorporation was essentially zero in the absence of these compounts. The rapid drop in activity with higher reagent concentrations is believed to be due to inhibition by high levels of ATP (158).

The same conditions were used for the amino acid concentration curve (Fig. 24), but the reagent mixture was kept constant and the amino acid mixture (containing the usual concentration of C^{14}-leucine) was varied. Saturation level in both ribosomes and supernatant was reached at about 0.1 ml amino acid mixture per 2.7 ml reaction volume. This represents approximately the concentration usually used.

Amino Acid Incorporation in Cell-Free Systems from BSA-Fractionated Reticulocytes

From the whole cell amino acid incorporation studies using reticulocytes fractionated in albumin gradients (Parts I and II) it was shown that there occurs a progressive loss of protein synthesizing capacity during reticulocyte maturation. By comparing the incorporation into polysomes, ribosomes, and soluble protein it was determined that this loss was accompanied by a progressive disappearance of total

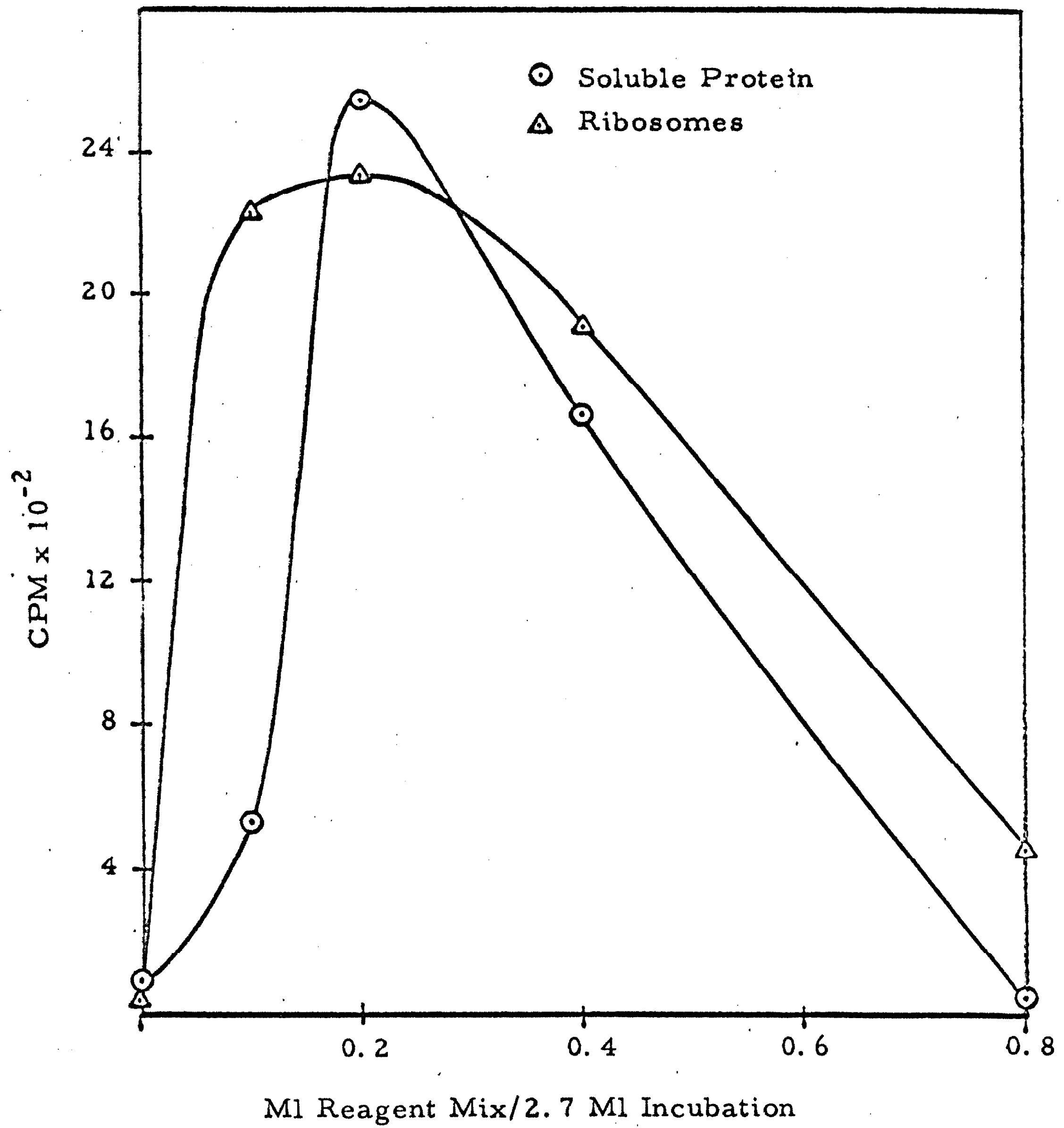


Figure 23. Effect of Reagent Concentrations on Amino Acid Incorporation. Reagent mix consisted of ATP, GTP, creatine phosphate, creatine kinase, and glutathione in the proportions described in Methods. Incubation and assay conditions were as previously noted.

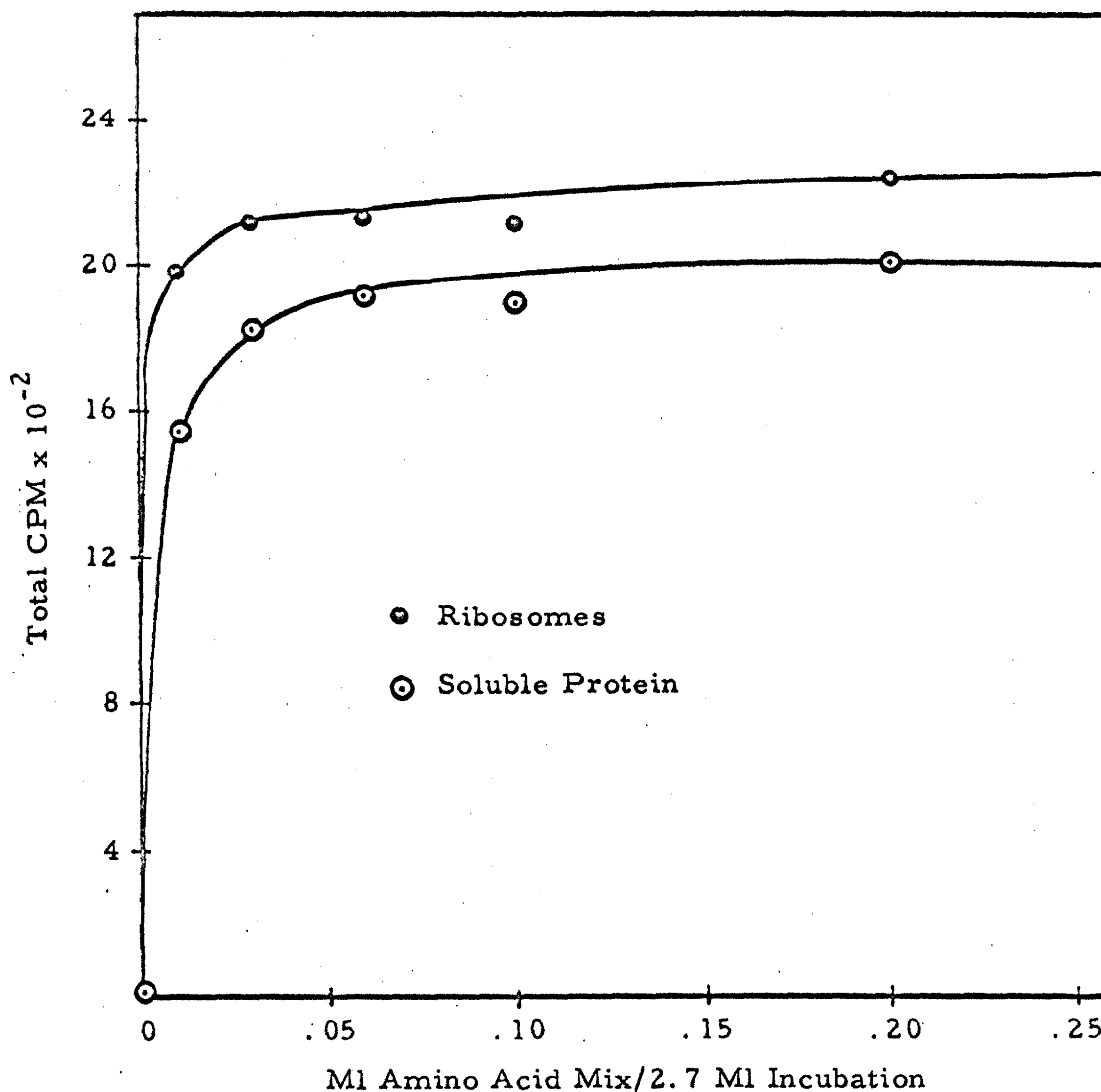


Figure 24. Effect of Concentration of Amino Acid Mixture on C^{14}-Leucine Incorporation. The complete amino acid mix including C^{14}-leucine were added in the proportions given in Methods. 0.1 ml represents amount normally used. Incubation and assay conditions were as indicated in Methods.

ribosomal material (80S and polysomes). Furthermore, it was shown that the decreasing protein synthetic activity was directly related to the amount of nascent protein on the polyribosomes. Evidence was presented which indicated that the observed decrease in amino acid incorporating activity of the polysomes is due to an increasing percentage of inactive ribosomes or polysomes with increasing cell maturation. The cause of this inactivation was not apparent. Although the active ribosomes sedimented predominately as pentamer polysomes in the more mature reticulocytes, their incorporating activity was low.

In order to further elucidate the nature of this inactivation process a series of cell-free studies were carried out with ribosomes and supernatants from the various reticulocyte fractions. By using supernatant and ribosomes from young and old reticulocytes in cross experiments it was hoped that the cause of the ribosomal inactivation and the influence of supernatant factors on this process could be determined.

A. <u>Cell-Free Experiments with Reticulocyte Fractions Separated by Neutral Density Centrifugation</u> - Neutral density separation of reticulocytes was essentially as described in Methods, Part I, but using a 21% albumin solution. 18 ml of cell suspension (3 vol. cells : 1 vol. NKM) were centrifuged in 3 SW-25 tubes. The cells of one tube were recombined and washed as a BSA-treated control (C). The remaining two tubes were separated into top (T) and bottom (B) cells representing 45% and 55%, respectively, of the original cell population. As a non-BSA treated control, an additional aliquot of

cells (W) was held on ice during the cell fractionation. From the two fractions (T and B) and control cells (C and W) were prepared 1x-ribosomes and 105000 g supernatants. These components were incubated in duplicate with C^{14}-leucine in the complete cell-free reaction medium using equal quantities of ribosomes and supernatants. After one hour the amino acid incorporation into pH 5-precipitated ribosomes and soluble protein was determined. Table 10 shows the effect of centrifugation in BSA on the cell-free amino acid incorporating activity. Prior BSA-treatment resulted in only a small loss of activity; the total soluble protein counts were reduced by 12% and the total ribosomal counts by only 5%. The main experimental results are summarized in Table 11. The findings of experiments 2 and 3 agree with the previous observations in the whole cell experiments in that the system from the light cells (T) incorporated more amino acids into protein than that from the dense cells (B). However, in contradiction to the whole cell studies, the ribosomes from both T and B cells show the same specific activities. The reason for this is not obvious, but may be due to the fact that in these incubations the ribosomes from both T and B cells are at the same concentrations. It was shown in whole cells that the ribosomal concentrations are much less in the dense cells than in the light ones. The earlier cell-free data (see Results, Section D) demonstrated that the ratio of supernatant to ribosomes does indeed influence the degree of labeling on the ribosomes.

The complete top-cell system (Exp. 2) exhibits both a higher total incorporation per mg ribosome and a greater turnover of ribosomal counts (ratio of soluble counts to ribosome counts)than either

Effect of BSA Treatment on the Cell-free Amino Acid Incorporation

Pretreatment of the cells	Soluble Protein		Ribosomes	
	cpm/ mg	Total cpm	cpm/ mg	Total cpm
Centrifugation in BSA	352	12, 300	130	3670
None	355	14, 000	144	3850

TABLE 11

AMINO ACID INCORPORATION IN CELL-FREE SYSTEMS FROM RETICULOCYTES SEPARATED BY NEUTRAL DENSITY CENTRIFUGATION.

	Components		Soluble Protein	Ribosomes		Total Incor-
Exp. No.	Ribosomes	S_{105}	Tot. cpm	cpm/mg	Tot. cpm	poration
1	C	C	12,300	130	3670	15,970
2	T	T	14,800	127	3480	18,280
3	B	B	11,900	129	3570	15,470
4	T	B	12,200	130	3610	15,810
5	B	T	14,900	112*	3140*	18,040

*Results from only one incubation.

	Soluble cts /	Relative Incorporation		
Exp. No.	Ribosome cts	Ribosomes	Sol. Protein	Total
1	3.35	105	83.0	87.5
2	4.25	100	100	100
3	3.34	103	80.5	84.5
4	3.38	104	82.5	86.5
5	4.74	90	101	99.0

Each incubation contained 2.5 mg ribosomes, 1.5 ml of S_{105}, 0.18 μmole L-Leucine-1-C^{14} (3.6 x 10^5 cpm/μmole), plus the complete reagent mixture in a total volume of 2.7 ml. Incubation was for 1 hr. at 37° C. Relative incorporation represents data normalized to a value of 100 for the data in Exp. 2.

the complete B system (Exp. 3) or the BSA-treated control cells (Exp. 1).

From the cross-experiments (4 and 5) it is demonstrated that this difference in activity resides in the supernatant. Thus the B ribosomes (from dense cells) show an activity equal to the complete light cell system (T, T) in the presence of T-supernatant (Exp. 5). Likewise the T ribosomal activity is reduced to that of the B system when incubated with B supernatant (Exp. 4). It is of interest to note that the control cell system (Exp. 1) has an amino acid incorporating activity at the level of only the bottom, dense cell system.

The observed differences are not great inasmuch as the cells were separated into only two fractions. However, the results are significant since determinations from duplicate incubations agreed within $\pm$ 2% and similar results were found in following experiments. It seems most likely that the observed differences in supernatant activities can be best explained by a critical concentration of one or more essential factors in both the control and bottom supernatants. However, the presence of an inhibitor substance in both the whole cell (control) and the dense cell (B) supernatants cannot be ruled out by this data.

B. Cell-Free Incorporation in Reticulocyte Fractions from a BSA Density Gradient - Approximately 12 ml of packed reticulocytes were separated into 7 fractions of varying densities by means of 3, 21-30%, 25 ml BSA gradients (Methods, Part II). The seven fractions, roughly equal in volume, were numbered P, 1 to 6, from the densest pelleted cells, P, to the lightest, 6, (see Fig. 2). As a control, 2 ml

of unfractionated, packed cells were held on ice during the gradient separation. Cells were lysed and 8 ml of clarified lysate from each fraction were layered over 5 ml of 30% sucrose in solution G (0.08 M KCl, 0.005 M $MgCl_2$, 0.005 M Tris, pH 7.5 at 25°C) in Spinco No. 40 tubes; ribosomes were recovered by centrifuging 4 hours at 105000 g. The upper 85% of the supernatants were removed from the sucrose layer for the S_{105} fraction of the incubations. The remaining sucrose solutions were decanted, the ribosomes were rinsed free of sucrose and supernatant with solution F, and all fractions were frozen until use. Ribosomes were dissolved in solution F, and equal quantities of ribosomes and 105000 g supernatant were incubated with C^{14}-leucine in Spinco No. 40 tubes under the conditions described in Table 12. At the end of the incubation the tubes were quickly chilled on ice, 5 ml of cold solution F was added, and 4.5 ml of 30% sucrose in solution P were layered under the reaction mixtures. Ribosomes were sedimented through the sucrose solution by centrifugation at 105000 g for 4 hours. Incorporation of C^{14}-leucine into ribosomes and soluble protein was determined on aliquots precipitated with hot TCA and collected on membrane filters. The results are presented in Table 12. Once again the lowest amino acid incorporation is observed in the systems derived from the heaviest cells (line 1 and 2). Since equal quantities of ribosomes were used in each experiment, the amount of protein synthesized per ribosome is lowest in these fractions. As in the previous cell-free experiment and unlike the whole cell experiments (Part II), the ribosomal incorporation (specific activity of the ribosomes) remains constant throughout the series. In contrast to the

TABLE 12

Amino Acid Incorporation in Cell-free Systems

from Reticulocytes Fractionated in a BSA Gradient

Exp. No.	* Ribos.	* Sup.	cpm sol. Protein	Ribosomes		Total Incorporation	
				cpm/ OD_{260}	Tot cpm	cpm	Relative
1	P	P	3360	170	1520	4880	37.4
2	1	1	4293	184	1775	6068	46.6
3	2	2	10120	167	1740	11860	91.1
4	3	3	11700	175	1590	13290	102.0
5	4	4	9770	173	1755	11525	88.5
6	5	5	10710	164	1465	12180	93.5
7	6	6	11510	167	1515	13030	100.0
8	6	1	8960	171	1535	10500	80.6
9	1	6	8930	154	1565	10500	80.6
10	C	C	5340	186	1610	6950	53.4

Incubations were in duplicate for 65 min at 37° C with 1.11 mg ribosomes, 0.75 ml Sup (S_{105}), 0.27 μmoles L - leucine-1-C^{14} (12.5^{mc}/m mole), and the complete reagent mixture in a total volume of 1.97 ml. Relative total incorporation represents data normalized to a value of 100 for Exp. No. 9.

* Figures correspond to the BSA gradient cell fraction from which the ribosomes and supernatants were obtained. P represents pelleted cell fraction and C, unfractionated control cells.

gradient of activities seen in the whole cell BSA fractions, the amino acid incorporation in the systems from fraction 2 through 6 remains fairly constant.

The incorporation into the control cell system (C), composed of ribosomes and supernatant from the unfractionated cell population, is much lower than would be expected, judging from the values observed in the systems from fractionated cells. One can calculate a theoretical value for incorporation into the control cell-free system from the weighted averages of fractions P through 6 (based on the ml of cells in each fraction). The calculated value is 10, 908 cpm total incorporation; the observed figure is 6950 cpm (Exp. 10).

The crossed cell-free incubations of cell fractions 1 and 6 (lines 8 and 9) indicate that the activity of the fraction 1 system may be boosted to 81% of the fraction 6 system by using supernatant from the lightest (# 6) cells. Conversely, the addition of # 1 supernatant to # 6 ribosomes decreases their incorporation by 20%

A comparison of # 1 and # 6 ribosomes incubated with # 6 supernatant (lines 7 and 9) agrees with the whole cell experiments in that the ribosomes from dense cells were less active than those from the light cells. The lower activity of the supernatant from the more mature cells (fraction 1) can be seen from a comparison of experiments 7 and 8, and 2 and 9. These results suggest, as in the previous experiment, that either suboptimal concentration(s) of an essential factor(s) or the presence of an inhibitor limit the cell-free amino acid incorporation in the presence of supernatant from the more mature reticulocytes.

Stimulation of Cell-free Amino Acid Incorporation by 80S Ribosomes

It was observed (Part II) that even the most mature reticulocytes contained polysomes, and that the proportion of single ribosomes to polysomes showed a relatively small increase from the youngest to oldest cells. The studies of Goodman & Rich (140) and Hardesty et al (141, 141a) provided strong evidence for a cyclic mechanism of interaction between 80S ribosomes and polysomes during the course of protein synthesis. According to the model a ribosome becomes attached to one end of a polysome and then gradually moves along the messenger RNA strand while the peptide chain being synthesized increases in length. At the end of the messenger the ribosomes detach and release their polypeptide chain (140). This suggested that in the whole cell studies (Part II), the ratio of polysomes to "monosomes" could be a reflection of a steady-state between them, and that the rate of protein synthesis is a function of the concentrations of both 80S ribosomes and polysomes. This possibility could be tested in the present cell-free protein synthesizing system.

Reticulocyte ribosomes were centrifuged through 30% sucrose and fractionated by sucrose-density gradient centrifugation as described in Methods. In a series of cell-free incubations each containing a constant amount of polysomes, the effect of varying amounts of added "monosomes" was tested in a 90 min. amino acid incorporation. In a similar series of incubations without polysomes, the activity of 80S ribosomes alone was determined (Fig. 25).

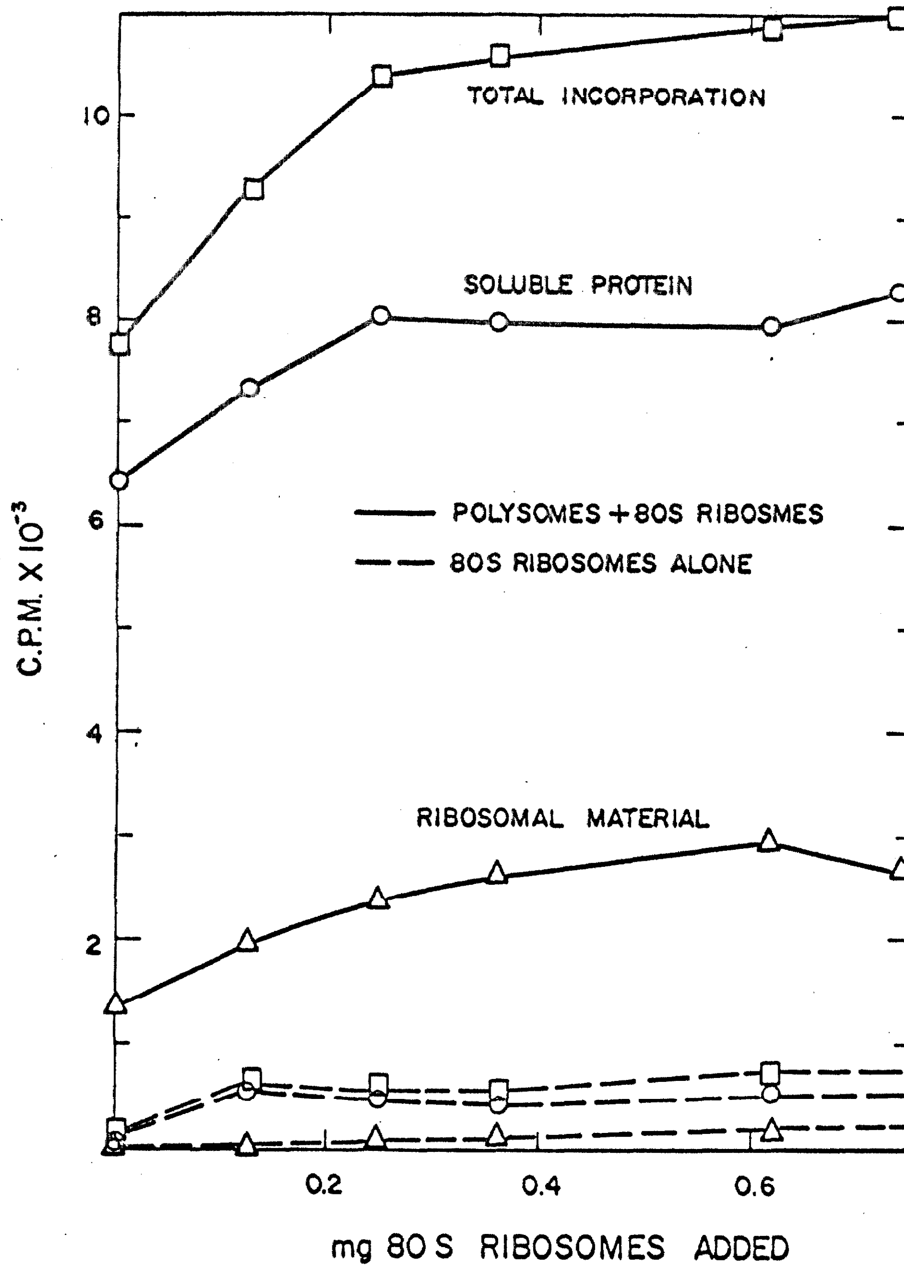


Figure 25. Activity of 80S Ribosomes in a Cell-Free System. Each 1.0 ml incubation contained 0.5 ml S_{105}, the complete cell-free reagent mixture listed in Methods, and 0.067 μ moles C^{14}-leucine (12.5 μc/μmole). (——) contained 0.64 mg polyribosomes plus 0-0.74 mg 80S ribosomes. (----) contained 0-0.74 mg 80S ribosomes only. After a 90 min. incubation at 37°C, amino acid incorporation into ribosomal material (Δ) and soluble protein (O) was determined. (□) represents total incorporation.

Contrary to the recent report of Lamfrom and Knopf (162) the 80S ribosomes as isolated here are virtually inactive in protein synthesis. They not only exhibited less than 10% of the activity (per mg ribosome) found in the polysomes, but increasing amounts of monosomes alone gave no stimulation of protein synthesis (bottom Fig. 25). However, the addition of 80S ribosomes to the polysome cell-free system raised the total incorporation up to 41%. There was a nearly linear increase in the total amino acid incorporation up to a level of 0. 25 mg 80S ribosomes per 0. 64 mg polysomes (an average of two ribosomes per polysome) Fig. 25). At the same time the incorporation per mg ribosome as well as the specific activity of the total ribosomal material remained relatively constant (Table 13). Higher levels of added monosomes failed to stimulate the system as evidenced by the plateau in soluble protein counts (Fig. 25) and the decrease in the incorporating activity per ribosome (Table 13).

These results indicate that 80S ribosomes are capable in the presence of polysomes of participating in protein synthesis. In agreement with the scheme proposed by Goodman and Rich (140) the rate of protein synthesis is a function of the concentrations of both single ribosomes and polysomes.

TABLE 13

Ribosomal Amino Acid Incorporation in a Cell-free System with Increasing Amounts of 80S Ribosomes and Constant Amount of Polysomes.

mg 80S Rib.	mg Poly-somes	Tot. mg Rib.	Tot. Incorp. (cpm)	Tot. Incorp. x 10^{-4} per mg Rib.	Spec. acty. Rib. (cpm/mg)
0	0.64	0.64	7776	1.21	2100
0.124	0.64	0.76	9287	1.21	2560
0.248	0.64	0.89	10410	1.17	2670
0.362	0.64	1.00	10590	1.06	2600
0.620	0.64	1.26	10890	0.86	2340
0.744	0.64	1.38	10980	0.79	1940

DISCUSSION

One of the most significant features of the present cell-free system is its ability to incorporate amino acids at a linear rate (after an initial rapid increase) for at least two hours. This is in sharp contrast to the usual reticulocyte systems which cease incorporating within 30 minutes (156, 158, 162). The reason for the success of this system is speculative at this time. Its composition is essentially the same as that used by Lamfrom (108) with the main exception that higher levels of supernatant (5 to 10 times as much per mg ribosome) were used. As was seen in Figures 19 and 22 the higher levels of supernatant have increased ribosomal labeling and greatly promoted the incorporation into soluble protein.

A possible explanation for the activity of the system might lie in the energy generating enzyme used, creatine kinase. Glowacki (154) has examined the cell-free components for ribonuclease activity assayed by the loss of bacteriophage MS-2 RNA infectivity in the protoplast system. Only very low levels were detectable in ribosomes and supernatant. However some creatine kinase samples (Sigma) contained high RNase activity, while the enzyme used in these studies (Boehringer) contained only neligible nuclease activity.

Further evidence for the very low RNase activity in this system is the constant level of ribosomal labeling which is maintained for almost two hours (Fig. 19). This is in contrast to other reticulocyte cell-free systems in which the ribosomes exhibit not only lower labeling but also a decreasing specific activity with time (141, 156, 162).

It now seems that a general characteristic of cell-free systems is the breakdown of polysomes during the course of incubation (140, 141a, 156, 162, 168). Generally this involves a progressive shift to smaller polysomal aggregates to single 80S ribosomes. At the same time the incorporation into ribosomes simultaneously decreases and shifts toward lighter ribosomal components (141a, 156). In extreme cases this proceeds rapidly to nearly quantitative conversion to single ribosomes and the total loss of all ribosomal radioactivity within 25 minutes (162). From the studies of Hardesty et al (141, 141a) and Goodman and Rich (140) it is clear that a major factor in the polysomal breakdown in reticulocytes is the limiting or failure of ribosomal reattachment. After a 65 minute incubation of the present system, although about 70% of the polysomes shifted to monosomes, the major portion of the ribosomal counts were still in the polysome region of a sucrose gradient (preliminary results).

From these considerations and observations it appears that the properties of the present system may be explained by the following working hypothesis: During the initial 30 minutes of incubation there is a shift in the monosome to polysome ratio to a new steady-state resulting in the reduction of the rate of incorporation observed in Figure 19. This shift is largely the result of dilution of the cytoplasmic components, although partial inactivation of a ribosomal attachment mechanism could play a part. A lower percentage of active polysomes than originally present remains and continues making protein at a linear rate (Fig. 19). The validity of this hypothesis could

be tested by time course studies of the ribosomal distributions and labeling by sucrose gradient analysis.

The incorporating activity generally observed in the cell-free system was only 1 to 2% that of the whole cell system. That the ribosomes were diluted about 7-fold and the supernatant 10 to 15-fold over their concentrations in the intact cell, could account for a large part of the reduced activity. By using higher supernatant concentrations (which increased both ribosomal and supernatant incorporation, (Fig. 22) and ribosome concentrations a greater steady-state concentration of polysomes might possibly be favored and result in a higher incorporating activity per ribosome.

It was shown that 80S ribosomes are capable of participating in protein synthesis in the cell-free system. That a greater stimulation was not achieved may be due to several factors. The polysomes provide a substantial rate of protein synthesis by themselves and most likely contribute markedly to the monosome pool. On the other hand, above the observed saturation level of approximately two monosomes per polysome, supernatant factors or enzymes required for ribosomal attachment may become rate-limiting. From the present data it cannot be ascertained if ribosome attachment proceeds throughout the incubation or declines and stops. The linear second phase of incorporation and the high incorporation into soluble protein would indicate that reattachment continues, provided the mechanism suggested by Goodman and Rich (140) is correct. Further research will be needed to prove this. Nevertheless, the system presented offers the

possibility of investigating the kinetics and mechanism of ribosome-messenger RNA interaction in the cell-free system. The obtaining of "clean" 80S ribosomes presents the opportunity of studying the interaction of these ribosomes with exogenous messenger RNA molecules in the synthesis of specific proteins other than hemoglobin.

The cell-free experiments with the BSA gradient cell fractions differed from the whole cell studies (Part II) in that the ribosomes from all fractions showed the same specific activities (Tables 11 and 12). Although the cell-free system cannot necessarily be equated to the whole cell system, the uniform ribosomal labeling might be the result of using equal quantities of ribosomes and supernant in all the cell-free experiments. In the intact cell it was shown (Part II) that a wide range of ribosomal concentrations exist.

The specific activity of the ribosomes in the cell-free studies, unlike the whole cell, was not directly related to the amino acid incorporating activity. Although the reason for this is not apparent, it would indicate that there was a similar percentage of functioning ribosomes in all incubations. An explanation of this difference must also await further investigations of the ribosomal distributions and their specific activities in the cell-free systems of each BSA cell fraction.

In spite of the equal labeling of ribosomes from all cell fractions, the amino acid incorporating activity of ribosomes from light and heavy cells in the presence of the same supernatant was

greatest in the lightest cells. This is in agreement with the previous whole cell findings. However, due to the use of constant amounts of ribosomes and supernatants in the cell-free studies, the differences between fractions were less striking and the incorporation from fraction 2 through 6 remained fairly constant.

Also brought out by these studies was the limitation of incorporation by the supernatant from the older cells. Thus as the reticulocytes mature there is not only a loss of ribosomal material and a decrease in ribosomal activity, but a loss of supernatants ability to support protein synthesis. A similar situation has been found in the comparison of normal and regenerating rat liver by Hoagland (165). Unfortunately these studies have not progressed to the point of identifying the source of the lowered supernatant activity in the older cells. It can be speculated that one or more components essential for protein synthesis, such as activating enzymes, transfer enzymes, or SRNA become limiting in the more mature cells. These factors might also become defective through degradative processes occurring during cell maturation. Hoagland and Askonas (169) have found that part of the enhanced activity of soluble fractions from regenerating liver can be accounted for by a fraction having properties resembling messenger RNA. Although this possibility should not be overlooked it seems that messenger RNA does not exist in the reticulocyte soluble fraction (164, 166) but only in the ribosomal fraction (73, 165, 166). Another cause of the reduced supernatant activity in the older cells could be the presence of an inhibitor. This was indicated by the lower than

expected activity in the control cells (Tables 11 and 12). Such a hypothesis is strengthened by the report by Hoagland et al (167) of an inhibitor, isolable from normal rat liver microsomes, which acts antagonistically to GTP. He proposes that the inhibitor functions by a negative control of protein synthesis. Since it was shown earlier (Part II) that the polysomes from the more mature cells had a lower protein synthesizing capacity as well as a lower specific activity, an inhibitor would provide an attractive explanation for both the whole cell and cell-free observations and be of interest from the aspect of a control mechanism.

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