ABSTRACT

CHARACTERISTICS OF A CELL-FREE PROTEIN SYNTHESIZING SYSTEM ISOLATED FROM LACTATING BOVINE MAMMARY GLANDS

by Donald C. Beitz

The molecular mechanisms of milk protein synthesis were studied in a cell-free system using defined components. Freshly obtained mammary tissue was immediately frozen, pulverized, thawed, and extracted with buffer. The microsome and enzyme fractions were then separated by differential centrifugation. The complete incubation mixture for amino acid incorporation was composed of microsomes, soluble RNA, aminoacyl-sRNA synthetases, ATP and an ATP generating system, amino acids, reducing agent, buffer and salts. Protein synthesis was measured as the incorporation of C^{14} leucine into TCA precipitable protein. This system incorporated 500 cpm per mg of microsomes per minute or 50 μμmoles of leucine over a 40 minute period. Incorporation in the controls was negligible.

This incorporation of C^{14} leucine was completely dependent upon microsomes and ATP and only partially dependent upon the aminoacyl-sRNA synthetase and sRNA. Incorporation was not affected by deoxyribonuclease, chloramphenicol, poly A, poly U, growth hormone, cortisone, prolactin, estrogen, or insulin but was inhibited by ribonuclease, puromycin, cycloheximide, sodium fluoride, and sodium deoxycholate.

Chromatography of the incubated complete system on

Sephadex showed that radioactivity was associated with the microsomes and with a protein fraction of about 32,000 molecular weight. Isotope dilution tests indicated a net synthesis of specific milk proteins--α_s-casein, β-lacto-globulin, β-casein, α-lactalbumin, and κ-casein. Radio-autography of precipitin bands of an immunodiffusion test of a complete system incubated for 0 and 40 minutes showed that the latter sample contained labeled α_s-casein. This system is then potentially useful in studying control of milk protein synthesis by the lactating dairy cow.

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A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Departments of Biochemistry and Dairy Science

1967

ACKNOWLEDGMENTS

The author wishes to express his sincere appreciation to Professors W. A. Wood and J. W. Thomas for their guidance, encouragement, and understanding throughout the course of this work. He is also grateful to Dr. R. S. Emery for his enthusiasm and counsel. His thanks go to Professor A. J. Morris, and H. A. Tucker for serving on his guidance committee and to Mrs. Douglas Randall's help in the preparation of this manuscript. The many discussions and assistance of his fellow graduate students, especially Harvey Mohrenweiser, shall always be remembered. The author is especially grateful to his wife, Judy, for typing the rough drafts of this manuscript and to both his wife and son, David, for their encouragement and love throughout the course of this work. The support of the Ralston Purina Company and the National Institutes of Health is greatly appreciated.

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INTRODUCTION

Much of our present information concerning protein synthesis has come from the use of a crude cell-free system, developed by Zamecnik, Hoagland, and their associates, which permitted the study of incorporation of labeled amino acids into protein. Such cell-free preparations were obtained initially from rat liver, but have since been derived from other mammalian tissues, microorganisms, and plants. The general features of preparations from all these sources have proven to be essentially similar.

I have chosen to adapt this method of study to the protein synthesizing system of the bovine mammary gland. The gland produces a large amount of milk proteins and, hence, should be a very suitable system for the study of many aspects of protein synthesis. Not only are large quantities of protein continuously formed, but they are also excreted from the cells and thus complications introduced by subsequent catabolism largely are absent.

Many workers have studied the overall aspects of milk protein synthesis by arterio-venous differences, injection of labeled amino acids, and perfusion of isolated mammary glands with labeled amino acids. These techniques have proven that the mammary gland utilizes absorbed blood amino acids which account for most of the milk protein synthesis.

Yet, the mechanism of this synthesis and its control are unclear because of lack of biochemical studies.

Therefore, an attempt was made to develop a cell-free system which would synthesize milk proteins from amino acids. The attainment of net protein synthesis and identification of the synthesized product were ultimate goals. A cell-free system synthesizing a known protein would permit study of the mechanism of milk protein synthesis and permit elucidation of the factors which might limit protein synthesis *in vitro*. Application of this knowledge to the lactating cow could give a clearer understanding of the mechanism of control for the quantity and type of milk protein synthesized.[1]

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[1] A preliminary account of this research was presented at the annual meeting of the American Dairy Science Association in June, 1967, and an abstract appears in the Journal of Dairy Science 50, 999, 1967.

REVIEW OF LITERATURE

Studies on protein-synthesizing systems from various organisms have now provided overwhelming evidence that proteins are synthesized by a similar mechanism in all organisms. The mechanism whereby the different organisms orderly direct the synthesis of peptide bonds from free amino acids is presented in the following discussion of the model system for protein synthesis.

Model for Protein Synthesis

The individual amino acids are activated in the presence of adenosine triphosphate (ATP), magnesium ions, and specific enzymes (aminoacyl-sRNA synthetases) to form aminoacyl adenylates and inorganic pyrophosphate (P-P). This reaction constitutes the initial reaction involved in protein synthesis.

The aminoacyl-sRNA synthetases which have been partially purified from many organisms show specificity for individual amino acids (1, 2, 3). For example, aspartyl- and asparaginyl-specific aminoacyl-sRNA synthetases isolated from Lactobacillus arabinosus have been separated by fractionation (4). Amino acid specificity of the synthetase enzymes was also demonstrated by Herve' and Chapeville (5) who chemically converted sRNA-bound cysteine to sRNA-bound alanine and

showed that both cysteinyl- and alanyl-sRNA synthetases were unable to "recognize" the complex in which alanine was attached to soluble ribonucleic acid (sRNA).

These synthetases catalyze the linkage of the carboxyl group of the amino acid to the phosphate group of the adenylate moiety as an anhydride bond. This activated amino acid is bound to the enzyme.

$$\text{Amino Acid} + \text{Adenine-ribose-P-P-P} \xrightarrow[\text{Synthetase}]{\text{Aminoacyl-sRNA}} \text{Enzyme (adenine-ribose-P-amino acid)} + \text{P-P}$$

For example, aminoacyl-sRNA synthetase-bound threonyl, and valyl adenylate complexes have been isolated (6, 7).

The aminoacyl group of the enzyme-bound complex is transferred to amino acid-specific sRNA's where the amino acid is attached as an ester linkage to the ribose portion of the terminal adenosine residue. This reaction may be illustrated as follows:

$$\text{AMP-amino acid-enzyme} + \text{sRNA} \longrightarrow \text{amino acid-sRNA} + \text{AMP}$$

The end of the sRNA molecule where the amino acid is attached consists of a cytidylate-cytidylate-adenylate sequence (1) while guanylic acid is the predominant 5' terminal nucleotide. The complete nucleotide sequences for tyrosine (8), and alanine sRNA's (9) have been determined.

Evidence that several different sRNA's can accept a particular amino acid has been presented by showing that 1) five leucine-specific sRNA's can be separated from extracts of *E. coli* (10), and 2) two valyl-sRNA's have been isolated

from yeast (11). In general, however, the purified sRNA's are specific to only one amino acid (2).

Studies with combinations of sRNA and aminoacyl-sRNA synthetases from different organisms indicate that species differences exist between the enzymes and sRNA's. The sRNA from different species which accept the same amino acid have different structures (1). The specificity of binding amino acids to sRNA is maintained when synthetases from different species are used with a particular sRNA, but all of the multiple sRNA's which are specific for single amino acids may not become esterified with that amino acid by heterologous enzymes. For example, two serine sRNA fractions from E. coli are esterified by E. coli enzymes, but only one is charged to the extent of 60 per cent by yeast enzymes (1). More recent studies with homologous and heterologous combinations of preparations to synthesize aminoacyl-sRNA's suggest that the amino acid specificity between sRNA and aminoacyl-sRNA synthetases is maintained among different organisms such as E. coli, Neurospora crassa, rat liver, and yeast (12, 13, 14).

The aminoacyl-sRNA's react with messenger RNA (mRNA) bound to ribosomes (polyribosomes) at specific positions determined by the nucleotide sequence of the mRNA. This binding process was first demonstrated by the finding of a polyuridylic-dependent binding of phenylalanyl-sRNA to reticulocyte ribosomes (15). The noncovalent binding reaction is non-enzymatic and requires magnesium ions and a

monovalent cation--either potassium or ammonium (16). If guanosine triphosphate (GTP), transfer enzymes, and glutathione are added, the bound phenylalanine is incorporated into protein. In contrast to the binding process described above with E. coli, Arlinghaus and coworkers (17) have reported that the binding of aminoacyl-sRNA to reticulocyte ribosomes, prior to peptide bond formation, requires a transfer enzyme as well as GTP.

Two enzyme fractions were required for polypeptide synthesis (18, 19). No function has been assigned to these fractions, although one appears to be involved in the hydrolysis of GTP as the peptide bond is synthesized in the E. coli system (20) and the reticulocyte system (17). These aminoacyl transferring enzyme preparations show considerable species specificity with respect to ribosomes but not with respect to aminoacyl-sRNA (14, 21).

The aminoacyl-sRNA's are specifically aligned in relation to the growing peptide chain, that is, the carboxyl terminal amino acid is linked to sRNA. Nucleophilic attack by the α-amino group of the incoming aminoacyl-sRNA on the carboxyl carbon of the peptidyl-sRNA results in the formation of a new peptide bond and the release of the previously attached sRNA. The polypeptide chain containing an additional amino acid residue is linked through the new amino acid to its corresponding sRNA. Polyribosome-bound aminoacyl-sRNA and peptidyl-sRNA are, therefore, intermediates in protein synthesis.

Chapeville et. al. (22) have presented evidence that template recognition shows no specificity with respect to the aminoacyl moiety of sRNA. These workers prepared aminoacyl-sRNA compounds in which the amino acid group was modified after incorporation into sRNA and demonstrated that the incorporation of the modified amino acid was dependent on the nature of the sRNA, not on the nature of the amino acid in the hybrid. Hence, the sRNA moiety gives rise to specificity of binding of the aminoacyl-sRNA.

Wettstein and Noll (23) have found that each rat liver ribosome bound two, and at most, three sRNA molecules. Aminoacyl-sRNA and peptidyl-sRNA were tightly bound, and uncharged sRNA could be removed by washing. Thus, the authors concluded that three distinct types of binding sites exist on ribosomes--namely decoding, condensing, and exit sites. Thus, during peptide bond synthesis a particular sRNA passes through each of the binding sites.

When an amino acid is incorporated, the messenger and the ribosome move one coding unit in relation to each other. A new nucleotide sequence in messenger RNA is, therefore, placed in position to base-pair properly with the next aminoacyl-sRNA. As this process is repeated, the polypeptide chain grows from its N-terminal to its C-terminal residue by the ordered sequential addition of the amino acids of aminoacyl-sRNA's. This ordered sequential addition of amino acids is determined by the nucleotide sequence of the mRNA whose sequence of ribonucleotides was determined by complementation

of the deoxyribonucleotide sequence of DNA (1).

As peptide bond formation is proceeding, the ribosome moves far enough along the messenger molecule to allow a new ribosome to attach. A second identical peptide chain can now be initiated and synthesized in the path of the second ribosome in the same way. Consequently, several identical peptide chains can be synthesized simultaneously on a polyribosome complex. Eventually, at the end of the mRNA, the ribosome, the nascent completed polypeptide chain, and the terminal, esterified sRNA are released from the polyribosome (1).

Inhibitors of Protein Synthesis

Many antibiotics, enzymes, and inorganic compounds inhibit protein synthesis both *in vivo* and *in vitro*. Inhibitors which have specific sites of action have aided greatly in the elucidation of the preceding model of protein synthesis. The following discussion will only include those inhibitors which were used in the study reported herein.

Chloramphenicol--First shown to inhibit protein synthesis in *Staphlococcus aureus* by Gale and Folkes (24), chloramphenicol has been shown to inhibit protein synthesis both in intact cells and in cell-free systems by a number of different methods and in a wide variety of organisms (25). Chloramphenicol inhibition in microbial systems occurs at a stage subsequent to the attachment of amino acids to sRNA

and at a site related to the ribosomal assembly of amino acids on mRNA (26). It neither affects the activation of amino acids (27) nor the transfer of amino acids to sRNA (28), but it inhibits the transfer of activated amino acids from sRNA to ribosomes (29) and interferes with polymerization of amino acids when synthetic templates are added to E. coli ribosomes (30). Rendi and Ochoa (31) have shown that this inhibitor does not directly affect RNA synthesis, but interferes with the attachment of mRNA to ribosomes.

Although chloramphenicol readily inhibits protein synthesis in microbial systems, protein synthesis in mammalian systems is markedly resistant. For example, complete inhibition of protein synthesis in E. coli cell-free systems can be obtained with 1.5×10^{-4} M chloramphenicol (30), but in mammalian cell-free systems comparatively little inhibition was obtained with $5\text{-}10 \times 10^{-3}$ M chloramphenicol (32). Also, comparative in vivo experiments of bacterial and mammalian cells showed that much greater concentrations of the inhibitor are required for the latter.

Experiments utilizing reticulocyte template RNA as a stimulant of protein synthesis in the reticulocyte cell-free system resulted in a marked increase in the incorporation of C^{14} L-leucine into trichloroacetic acid (TCA) precipitable material. Addition of chloramphenicol inhibited this protein synthesis induced by added template RNA but had comparative little inhibitory effect on the unstimulated protein synthesis system (33).

The differences of response of bacterial and mammalian protein synthesizing systems to chloramphenicol must be related to differences in template RNA turnover and stability since mRNA of mammalian cells is more stable, remaining attached to ribosomes for longer times (26). Indirect evidence suggests that chloramphenicol interferes with the function of mRNA; that is, inhibits the attachment of mRNA to ribosomes or possibly directly inactivating mRNA (31, 33, 34).

Puromycin--Experiments by Yarmolinsky and de la Habe (35) firmly established that puromycin inhibited the *in vitro* synthesis of protein while the suppression of protein synthesis by puromycin *in vivo* was first demonstrated by Gorski *et al.* (36). Yarmolinsky and de la Habe (35) further showed that the action of puromycin in a cell-free system occurred in the transfer of the amino acids and that esterification of the acids to sRNA was unaffected. Specifically, the antibiotic was demonstrated to exert its effect at a level involving the sRNA-ribosomal complex, where it could substitute for aminoacyl-sRNA and become attached by its amino group to the incomplete polypeptide chain (37). The carboxyl group of puromycin is not available for additional peptide bond formation; therefore, the polypeptide chain could not grow in length. Relatively short chains, each carrying a puromycin molecule, are then released (38).

This direct effect of puromycin on the ribosome resulting in the release of protein was first reported by Morris

and Schweet (39, 40) in their studies with leucine incorporation by rabbit reticulocytes. The discharge was non-enzymatic and occurred at 10^{-3} M puromycin and was considered to result from the displacement of incomplete globin chains and earlier intermediates from the ribosome. Thus, soluble protein could be freed from ribosomes in the absence of incorporation and without ribosomal breakdown.

Cell-free protein synthesizing systems derived from many organisms are inhibited by the same mechanism as previously described. Tissiéres and Watson (41) reported that 8×10^{-5} M puromycin inhibited protein synthesis with the E. coli cell-free system. Florini (42) reported that cell-free preparations from rat skeletal muscle did not synthesize protein in the presence of puromycin. Other workers reported that rat liver (43), thymus cell nuclei (44), and rat liver mitochondria (45) cell-free protein synthesizing systems are inhibited by this antibiotic.

Cycloheximide--Protein synthesis in many organisms, excluding bacteria, is also inhibited by cycloheximide (46-50). Two groups of investigators have reported that it does not inhibit the formation of aminoacyl-sRNA but involves the transfer of amino acids from aminoacyl-sRNA to nascent polypeptide chains (47, 51, 52). Wettstein et. al. (53) have shown that cycloheximide inhibits the breakdown of polyribosomes and have suggested that the readout mechanism from mRNA is involved. Recent studies with a reticulocyte cell-

free system by Lin et. al. (54) indicated that cycloheximide inhibits the initiation of new chains on ribosomes. Further, studies on the rate of amino acid incorporation indicate that cycloheximide also decreased the rate of incorporation into nascent polypeptide chains that were initiated in intact cell and remain attached to ribosomes during their isolation.

Sodium Fluoride--Marks et. al. (55) and Ravel et. al. (56) have shown that sodium fluoride inhibits protein synthesis in a manner quite similar to that of cycloheximide. They found a decrease in polysomes and an increase in monomeric ribosomes in rabbit reticulocytes incubated in the presence of sodium fluoride. This dissociation was accompanied by a loss of nascent polypeptide chains from the ribosomes. On the other hand, this inhibitor caused little or no detectable dissociation of polyribosomes in the complete cell-free system. Washing out the sodium fluoride from intact reticulocytes permitted protein synthesis to become reestablished (48, 57). Lin et. al. (54) demonstrated that sodium flouride blocks the initiation of new peptide chains on ribosomes in a reticulocyte cell-free system, suggesting that this inhibitor directly or indirectly interfered with the reattachment of monomeric ribosomes on messenger RNA. Sodium fluoride had no apparent effect on the rate of amino acid incorporation into previously initiated nascent chains.

Ribonuclease--Many investigators have found that ribonuclease is a potent inhibitor of the cell-free protein

synthesizing systems isolated from many different organisms (58, 59, 60, 61). Incorporation of amino acids into TCA-precipitable protein was inhibited to the extent of 90% by as little as 0.1 μg of pancreatic ribonuclease in the cell-free system derived from reticulocytes (58). Possibly sRNA, ribosomal, and mRNA may have been affected; but preincubation of the purified ribosomes with ribonuclease demonstrated that the effect could have been at the ribosomal level. Later experiments with E. coli and rat liver ribosomes indicated that this nuclease caused a quantitative degradation of polyribosomes to the monomeric form of ribosomes (62, 63). Because polyribosomes are the major synthetic form of ribosomes (2), degradation of the mRNA of the polyribosome caused inhibition of protein synthesis.

Other Inhibitors--Other compounds that inhibit protein synthesis in vitro are nucleocidin (64), gougerotin (65), blasticidin S (66), amicetin (67), tetracyclines (68), and neomycin B (68). Since these substances were not used in this research, the mechanism of their inhibition of protein synthesis will not be discussed.

Effect of Hormones on Protein Synthesis

Most tissues in higher animals are to some degree dependent on hormones for their normal growth and development. Knox et. al. (69), who demonstrated that synthesis of certain enzymes was increased in animal cells by the administration

of corresponding substrates, observed that the steroid hormone, cortisone, induced the synthesis of adaptive enzymes. These findings provided investigators an explanation for the selective regulation of enzyme synthesis by individual hormones. Prior to these experiments, the most prevalent view of hormone action involved a type of hormone-enzyme interaction resulting in enzyme regulation (70).

Tata et. al. (71), in attempting to describe the mechanism of thyroxine action, found that it increased the rate of in vivo RNA synthesis in the nucleus and protein synthesis in the cytoplasm. The stimulation of nuclear RNA synthesis occurred prior to any noted change in the amino acid incorporation activity of ribosomes in the cytoplasm. Similar sequential stimulation of RNA synthesis followed by that of protein synthesis have been recorded for other hormones as well; i.e., growth hormone acting on liver (72) and muscle (73) and testosterone on prostate gland (74).

The observations that hormones affect a change in the rate of protein synthesis in vivo prompted Korner (75), Mueller (76), and Williams-Ashman et. al. (74) to study the regulation of protein synthesis in liver and accessory sexual tissues by growth hormone, estrogens, and testosterone, respectively. These workers showed that the addition of hormones involved in growth and development to cell-free protein synthesizing systems prepared from their respective target organs had no effect on their protein synthesizing capacity. On the other hand, the respective hormones, when

administered *in vivo*, markedly stimulated protein synthesis in the target organ. This stimulatory effect was largely localized in the ribosomes of the target cells.

Karlson (77) proposed that hormones act as inducers, which, by combining with appropriate repressors, control mRNA synthesis and thereby regulate enzyme synthesis. Tata (71) proposed two other modes of action: 1) hormones might regulate the rate of transfer of mRNA from the site of synthesis into the cytoplasm, and 2) hormones could regulate the type and rate of protein synthesis at the level of translation of mRNA by ribosomes.

Hormones such as prolactin, cortisone, estrogen, progesterone, insulin, and growth hormone regulate the synthesis of milk by mammary tissue. Insulin, hydrocortisone, and prolactin stimulate synergistically the synthesis of casein-like phosphoproteins and whey proteins in organ cultures of mouse mammary glands. Stimulation was not observed or was minimal when one or more of these hormones were omitted from the culture medium (78, 79). No study has been reported concerning the effect of adding hormones to a cell-free protein synthesizing system obtained from this tissue.

Milk Protein Synthesis

Although the lactating mammary gland is particularly suitable for the study of protein biosynthesis because of its high rate of synthesis and ease with which its product can be

characterized, only a few studies have been conducted on the different reaction steps leading to milk proteins. Most of the reported studies have involved arterio-venous (A-V) differences measurement of amino acid concentration across the mammary gland or perfusion studies designed to delineate precursor-product possibilities. Only a few reports on protein syntehsis in cell-free preparations have appeared.

In the absence of specific information it could be postulated that the proteins of milk are formed from either amino acids, peptides, or complete proteins of blood. For many years a debate existed among the first investigators of milk protein synthesis concerning the site of synthesis. To settle this question, investigators utilized the techniques of studying the changes in the level of the possible milk protein precursors in the arterial and venous blood supply systems of the mammary gland.

Arterio-Venous Difference Studies--The first attempt to study the precursors of milk proteins by this technique was conducted by Cary (80). Because the concentration of free amino nitrogen was 16-34% lower in the plasma collected from the mammary vein than the concentration in jugular plasma and only 3-5% lower for dry cows, he concluded that the A-V differences were in the order of magnitude to be expected if all the milk proteins originate completely from the free amino acids of blood.

Later Blackwood (81) repeated Cary's work using

improved methods of measuring amino nitrogen and arrived at the same conclusion. Calculations of the total weight of amino nitrogen absorbed by the mammary gland versus the amount secreted in the milk led Graham (82) to conclude that blood amino acids could not be the sole source of milk protein. Similar experiments conducted by Shaw and Peterson (83) indicated that the blood amino acids could not account for more than 35% of the milk proteins while Nikitin (84) arrived at a value of 45%. Since A-V difference studies pose certain limitations on quantitative conclusions, the findings from these early studies are limited, but indicate that free amino acids are absorbed from the blood by the mammary gland in significant amounts (85).

More recently there has been a clear demonstration of uptake of specific amono acids by lactating glands. Borchaert (86) showed that the concentrations of the ten essential amino acids in the blood decreased markedly during a perfusion study. In further studies, absorption of glutamine, glutamic acid, threonine, and serine as measured by A-V differences of lactating cows were shown to provide a significant portion of those same amino acids in casein. Work by Verbeke and Peters (87) showed that lactating bovine glands were capable of absorbing enough arginine, glutamine, isoleucine, leucine, lysine, valine, threonine, and histidine to provide all the respective amino acid residues in milk protein. Taurine, urea, and α-amino-butyric acid were not absorbed. According to this report, absorption could not account for all of the

aspartic acid, asparagine, glutamic acid, serine, and proline in the milk proteins. Other workers (88) have also shown that the uptakes of the ten essential amino acids and glutamic acid by lactating goat glands were approximately equal to the corresponding output figures. They found that the uptake of serine was consistently less than the output and that the uptake of the other non-essential amino acids were inconsistent.

Studies on Injection of Labeled Precursors--Tracer experiments have been utilized to more accurately study the compounds of the blood which are used by the mammary gland as precursors of milk proteins. Campbell and Work (89) reported that injected radioactive valine and lysine were incorporated into the whey proteins and casein, but the portion of valine and lysine of milk protein that may have originated from plasma proteins could not be estimated. In a later experiment these same workers concluded that 90% of the lysine and serine of milk protein came directly from free amino acids in the blood. Furthermore, Askonas (90) also concluded that at least 90% of the valine and lysine residues of crystalline β-lactoglobulin and casein came from a single pool of free amino acids in the mammary gland. Barry (91, 92) concluded that in the lactating goat at least 70% of the lysine, tyrosine, and glutamic acid and glutamine residues of casein were absorbed from the corresponding free amino acids of the plasma. For asparagine and proline, these percentages amounted to 50%.

The possibility that a major fraction of the plasma proteins could be hydrolyzed to a limited extent within the gland and contribute to the pool was not excluded in the above experiments. When plasma proteins labeled with C^{14} glycine were injected into lactating rabbits by Campbell and Work (89), not more than 10% of the glycine or serine of the milk proteins came from these plasma proteins. In a later experiment Askonas and coworkers (93) compared the maximum specific radioactivities of milk and plasma proteins and found that casein and the whey proteins were over fifty times more radioactive than the plasma proteins. Clearly a significant part of the amino acids of milk proteins did not come from any major fraction of the plasma proteins.

The origin of the milk protein amino acids which are not absorbed from the bloodstream in sufficient amounts has been the concern of many research investigators. Volatile fatty acids (e.g. acetate and propionate) were incorporated into aspartic and glutamic acids of casein by the isolated, perfused cow's udder (94). Black and Kleiber (95) concluded from infusion experiments that the carbon of volatile fatty acids, glucose, and of fructose was utilized in the synthesis of glutamic acid, aspartic acid, serine, and alanine. Moreover, acetate and fructose were incorporated, respectively, into proline and glycine. Wood et. al. (96) calculated from their experiments in which glucose C^{14} was supplied to the mammary gland by injection into the pudic artery, that

approximately 25% of the serine in casein was synthesized in the udder.

In conclusion, reported experiments indicate that the udder obtains the amino acids for milk protein synthesis by two mechanisms: 1) absorption of blood amino acids and 2) by synthesis of amino acids from absorbed precursors.

Campbell and Work (89) and Askonas et. al. (93), working with rabbits and goats, found that the total casein isolated from milk taken within a few hours after intravenous administration of radioactive amino acids contained higher levels of radioactivity than the total whey proteins. Part of the difference was accounted for by a component of whey proteins--immune globulins--which apparently entered the gland as intact protein. Furthermore, Larson and Gillespie (97) demonstrated that α-casein, β-casein, α-lactalbumin, and β-lactoglobulin were synthesized in the mammary gland from a common free amino acid pool and that the immune globulins, milk serum albumin, and γ-casein entered the milk preformed from the blood stream. McCarthy (98) verified portions of the above findings by showing that α-lactalbumin and whole casein were derived from a common pool of free amino acids in the udder. Available chemical and immunological evidence indicated that milk serum albumin was identical to albumin of the blood (97, 99).

In Vitro Studies--Initial in vitro studies of amino acid incorporation into proteins by Hoagland and his coworkers (100) clearly showed that certain small (15 mμ), dense cyto-

plasmic particles were essential for synthesis and, indeed, that the labeled amino acids became attached to the particles, later termed ribosomes. In preparations from mammalian tissues, amino acid-incorporating activity was associated with microsomes which consist of free ribosomes and ribosomes bound to the endoplasmic reticulum. Apparently, the mammary gland contains similar structures since electron micrographs of thin sections of mouse mammary glands have shown that the endoplasmic reticulum of the secretory cell is rather similar to that of the secretory cells in the pancreas (101). The "rough membranes," consisting of lipoprotein with associated electron dense nucleoprotein particles, were particularly prominant. However, areas of smooth membranes, Golgi zones, and secretory granules were also present. All these portions of the cytoplasm probably contribute to the microsome fraction isolated from the mammary gland.

Bailie and Morton (102) were the first investigators to attempt to isolate microsomes from the mammary gland of dairy cows. In this case mammary gland microsome fractions were isolated, not to study protein synthesis, but to compare various enzymatic activities such as alkaline phosphatase with the corresponding activity of microsomes found in milk.

Brew and Campbell (103) determined the RNA to protein ratio of microsome fraction from guinea pig mammary glands to be 0.35-0.38. Since the ratio obtained from liver microsomal preparations is usually 0.2 (104), this high ratio suggested that the microsome fraction was rich in ribosomes which were

not attached to membranes. Sucrose density gradient centrifugation of such preparations indicated that a substantial peak of free ribosomes was present--the peak area being greater than the corresponding fraction from liver (103).

Fraser and Gutfreund (105) first detected aminoacyl-sRNA synthetase activity in mammary gland tissue isolated from guinea pigs, cows, and rats. Homogenates from all three sources catalyzed the formation of hydroxamates from a complete mixture of amino acids; however, the rate of hydroxamate formation was only 20% above that with tryptophan alone. The same results were obtained using a particle-free supernatant of guinea pig mammary gland as a source of the synthetases. Barely significant activity was found for most of the amino acids tested, but tyrosine gave more stimulation over the endogenous level of activity than did most amino acids. Compared to the crude homogenate, the pH 5 enzymes[2] retained full activity for coupling leucine and glycine to soluble RNA but would not catalyze an exchange of ATP and radioactively labeled inorganic pyrophosphate. This lack of exchange was unexpected since amino acids are activated by the following reaction:

$$\text{Amino acid} + \text{ATP} \xrightleftharpoons{\text{Enzymes}} \text{aminoacyl-AMP} + \text{pyrophosphate}$$

Conversely, Bucovaz and Davis (106) found that five of the nineteen common amino acids tested (L-tyrosine, L-tryptophan, L-isoleucine, L-cysteine, and L-leucine) markedly stimulated the ATP and labeled pyrophosphate exchange reaction in

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[2]The pH 5 enzymes are equivalent to the precipitate obtained after adjusting the 105,000 x g supernatant to pH 5.

an aparticulate fraction of rat mammary glands. Purification of this soluble fraction by Norit treatment not only lowered the endogenous exchange rate but also resulted in the observation that fifteen of the nineteen tested amino acids stimulated the exchange reaction. Using the aparticulate fraction, L-leucine and L-glutamic acid significantly increased the rate of exchange. Passage of the aparticulate fraction through Sephadex G-25 resulted in a 4-fold increase in the exchange rate due to tyrosine addition over the rate obtained by Norit treatment. Based on these studies, it is not known whether the mammary gland contains aminoacyl-sRNA synthetases specific for each amino acid as has been found for other organisms.

Bucovaz and Davis (106) also showed that in rat mammary tissue the specific activity of the leucine and glutamic acid-activating enzymes increased in the early stages of lactation and decreased toward the latter stages of lactation. These data suggested that as the demand for protein biosynthesis by the mammary gland increased, there was a concomitant increase in the ability of the intact gland to activate amino acids.

Fraser et. al. (107) studied the esterification of activated amino acids to sRNA in mammary tissue preparations by measuring the amount of C^{14}-labeled amino acids that were incorporated into the pH 5 enzyme fraction. Under their experimental conditions, significant quantities of amino acids became esterified to the sRNA contained in the pH 5 enzymes. When rat liver sRNA was added to the guinea pig mammary gland

pH 5 fraction, the amount of glycine coupled to sRNA was proportional to the total sRNA concentration. Inspection of the graphs of amino acid concentration versus a measure of bound amino acid residues indicated a biphasic process. First, there was a rapid increase of amino acid-sRNA formation with increasing concentration of amino acids up to about 20 mM followed by a slower continuous increase in amino acid-sRNA formation which was proportional to amino acid concentrations. At high concentrations, amino acids combined to the extent of one residue per sRNA molecule. Further incubation of the total pH 5 fraction with ATP and glycine or leucine did not significantly diminish the reaction of sRNA with valine which suggests sRNA specificity. These investigators found that this reaction may be reversed by enzymatic removal of the amino-acyl-AMP (the precursor of amino acid-sRNA). Only the L isomer of the amino acid was esterified to the sRNA. Gutfreund (108) concluded that the acylation of amino acids to the sRNA and not the formation of the aminoacyl-AMP was the rate limiting step in amino acid-sRNA formation.

Fraser and Gutfreund (105) reported the first demonstration that subcellular fractions of mammary tissue possessed the ability to incorporate amino acids into TCA-precipitable material. They incubated a homogenate of lactating guinea pig mammary gland with radioactive amino acids and fractionated the resultant incubation mixture. Most of the radioactivity was associated with the mitochondrial

rather than the microsomal fraction. Because addition of ribonuclease inhibited synthesis in the mitochondrial fraction, incorporation by this fraction was considered to be due to contamination by microsomes. Later Turba and Hilpert (109) demonstrated that the microsomal fraction isolated from lactating guinea pig mammary glands incorporated amino acids into protein by an ATP-dependent mechanism.

More recently Brew and Campbell (103) incubated slices of lactating guinea pig mammary gland with radioactive amino acids and they, too, found that the mitochondrial fraction was the most active. However, the mitochondrial and microsomal fractions were equally active when incubated with labeled amino acids, pH 5 enzyme, and an energy generating system. The combined mitochondrial and microsomal fractions incorporated labeled amino acids into α-lactalbumin. This incorporation depended on the presence of energy and resulted in synthesis of complete molecules of α-lactalbumin from radioactive amino acids based on usual peptide analyses of the hydrolyzed product.

MATERIALS

Mammary Tissue Source

The mammary tissue utilized in this study came from lactating cows and was obtained from Van Alstine's slaughter house in Okemos, Michigan. All cows were completely milked prior to slaughtering. Since the cows usually available at this source were generally below average in production, special arrangements were made to obtain normal mammary tissue of normal cows from the dairy herd of Michigan State University. Higher rates of amino acid incorporation were obtained with this source presumably because better control of milk production levels, udder health, and freedom of milk in the tissue preparations was possible.

Chemicals

The chemicals used in this study and their sources are listed in the following paragraphs. Amino acids-A grade, adenosine triphosphate (ATP)-disodium salt, guanosine triphosphate (GTP)-trisodium salt, protamine sulfate, bovine growth hormone (1.0 USP units/mg), recrystallized bovine pancreatic insulin (23.4 IU/mg), bovine lactogenic hormone or prolactin (grade B, 20 IU/mg), ammonium salt of polyuridylic acid, and potassium penicillin G were obtained from Calbiochem, Los

Angeles. Trisodium salt of phosphoenol pyruvic acid, crystalline pyruvate kinase, tris (hydroxymethyl) aminomethane (Tris-HCl buffer), cycloheximide, crystalline bovine serum albumin, and sodium deoxycholate were preparations of the Sigma Chemical Company. Cortisone acetate, 2 X crystallized deoxyribonuclease, and puromycin dihydrochloride were obtained from Nutritional Biochemicals and reduced glutathione and polyvinyl sulfate, potassium salt, were purchased from General Biochemicals. Crude collagenase (125-200 units/mg), and electrophoretically purified horseradish peroxidase were obtained from Worthington Biochemical Corporation. Polyadenylic acid, potassium salt, was purchased from Miles Chemical Company and crystalline L-chloramphenicol was obtained from Parke, Davis and Company. Bentonite (325 mesh) was purchased from E. H. Sargent Company.

L-Leucine-U-C^{14} (200-250 μc/μmole), sodium pyrophosphate-P^{32} were obtained from New England Nuclear Corporation while L-lysine-U-C^{14} (1.17 μc/μmole) and L-phenylalanine-U-C^{14} (1.3 μc/μmole) were purchased from Volk Radiochemical Company. 2,5-Diphenyloxazole (PPO) and 1.4-di [2-(5-phenyloxazolyl)] -benzene (POPOP) were preparations of the Packard Instrument Co. Inc. Sephadex G-75 was obtained from Pharmacia, Limited. Fresh milk was supplied by the Michigan State University dairy herd.

Dr. J. R. Brunner of the Department of Food Science at Michigan State University donated antibodies against whole casein, proteose-peptones, α_s-casein and κ-casein. The proteins were isolated and purified by methods devised by the

following investigators: whole casein, McKenzie and Wake (110), α_s-casein, Thompson and Kiddy (111), κ-casein, Swaisgood *et. al.* (112), and proteose-peptones by Brunner and Thompson (113). Antibodies against these purified proteins were prepared according to the method of Kabat and Mayer (114). Ten mg of each of these proteins were suspended in complete Freund's adjuvant which was purchased from Difco Laboratories and then injected intramuscularly into rabbits. Ten mg of each of the milk proteins suspended in incomplete Freund's adjuvant (minus the antibody production stimulant) were injected at two week intervals twice more into the same respective rabbits. At the end of the six week exposure period, the rabbits were each bled by heart puncture and the blood sera containing the antibody were collected.

Equipment

Some of the homogenizations of mammary tissue were performed in a Virtis "45" high speed homogenizer and in a French high pressure tissue press. All radioactivities were determined in a series 3000 Packard TriCarb scintillation counter. The Spinco Model L and the Model B-60 International Preparative ultracentrifuge were used for sucrose density gradient centrifugation and microsome isolation. Sedimentation velocity was determined with a Model E Spinco ultracentrifuge equipped with sclieren optics. A Gilford record-

ing spectrometer was used for measuring enzyme activities by spectrophotometric methods.

METHODS

Preparation of Single-Cell Suspensions

Single-cell suspensions of mammary tissue were prepared according to the method of Eoner et. al. (115). One gram of the washed mammary tissue was suspended in 3 ml of Hanks' basic salt solution (see Appendix) which contained 3 mg of collagenase per ml. This suspension was incubated for 1 hour at 37°C with constant stirring. The tissue was 1) recovered by centrifuging the suspension for 10 minutes at 5,000 x g, 2) resuspended in the same volume of collagenase solution as used in the previous mixture, 3) incubated for one hour at 37°C 4) again recovered by centrifugation, and 5) washed twice with the salt solution by centrifugation. This process resulted in a suspension of intact mammary cells, secretory and otherwise, which was devoid of the normal connective tissue.

Preparation of Subcellular Fractions

For the preparation of the subcellular fractions, the mammary tissue was homogenized by the following procedure. The relatively milk-free tissue was cut with scissors into small pieces and washed with a Medium A salt solution (see Appendix Table 5) and frozen with Dry Ice as soon as possible.

After transporting the tissue to the laboratory, it was further frozen with liquid nitrogen and then powdered with a mortar and pestle in a cold room.

The procedure for preparation of the microsomes and the aminoacyl-sRNA synthetases (pH 5 enzyme) is presented in Figure 1. The suspension of thawed, homogenized tissue was suspended in 2 to 3 volumes of Medium A and centrifuged at 10,000 x g for 10 minutes at 4°C to remove mitochondria, nuclei, and cell debris. The supernatant solution was then centrifuged for 90 minutes at 105,000 x g at 4°C in a SW 30 rotor for the Spinco Model L centrifuge. The aparticulate supernatant solution was utilized for the preparation of the aminoacyl-sRNA synthetases and the pellet was used as the microsomal fraction.

Preparation of the Microsomal Fraction--The procedures for microsome preparation were adapted from those utilized by Korner (60) who isolated a similar fraction from rat liver. The pellet was suspended with the aid of a Broeck homogenizer in one-third the original volume of Medium A. This opalescent solution was again centrifuged at 105,000 x g. The washed microsomes were resuspended in Medium A to a final concentration of 8 to 12 mg of ribosomes per ml based on the extinction coefficient of 11.3 absorbancy units at 260 mμ per mg ribosomes per ml (116). This solution was subsequently used for studying amino acid incorporation by the cell-free system. The ribosomes, i.e., particles not bound to the endoplasmic

PREPARATION OF MICROSOMES AND pH 5 ENZYME

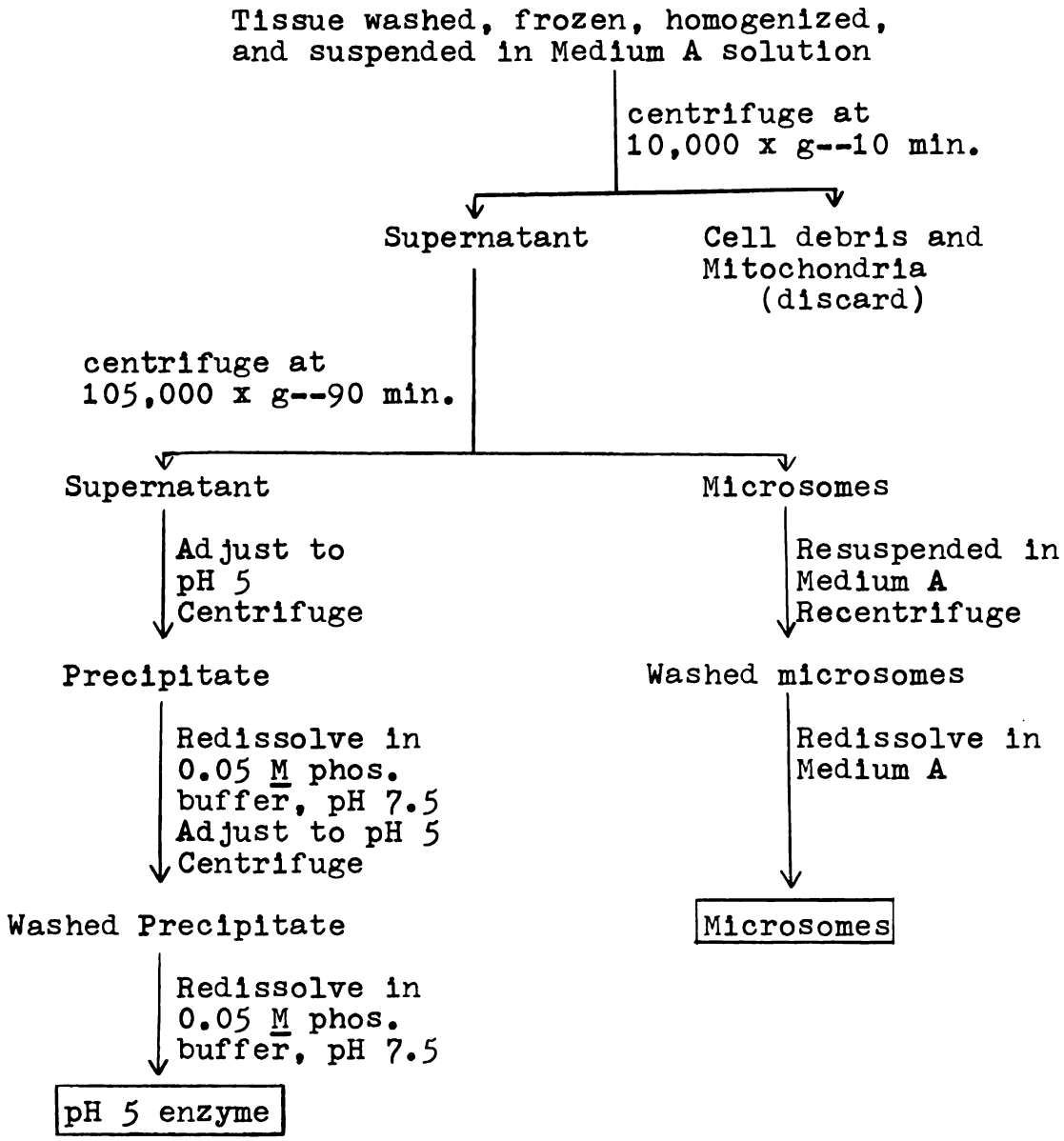

Figure 1. Preparation of microsomes and pH 5 enzyme from mammary tissue.

reticulum, were prepared from this microsome solution by the addition of a 10% solution of sodium deoxycholate to a final concentration of 0.25%. The ribosomes were reisolated by centrifuging the solution at 105,000 x g for 90 minutes at 4°C. This procedure usually resulted in the isolation of about 250 mg of microsomes per 100 g of tissue.

Preparation of pH 5 Enzyme--Aminoacyl-sRNA synthetases were isolated by acid precipitation of pH 5 from the aparticulate fraction (supernatant obtained after the 105,000 x g centrifugation) according to the method of Hoagland et. al. (117, see Figure 2). This fraction was held at 0°C and was adjusted to pH 5.0 by the addition of 1 N acetic acid. After gently stirring the solution for 10 minutes, the mixture was centrifuged for 10 minutes at 30,000 x g. The pellet was dissolved in a volume of 0.05 M phosphate buffer (pH 7.5) equal to that of the original solution. The solution was again adjusted to pH 5.0 with 1 N acetic acid and centrifuged as before. The resulting pellet was dissolved in phosphate buffer to give a final concentration of 4 to 8 mg of protein per ml. This solution--the pH 5 enzyme fraction--was subsequently utilized in the amino acid incorporation experiments. When it became necessary to remove the sRNA which contaminated this preparation, neutralized protamine sulfate was added to a final concentration of 1.0 mg per ml of the aparticulate fraction. The precipitate was then removed by centrifugation at 30,000 x g for 10 minutes at 4°C. The pH

5 enzyme was prepared from this solution as previously described. Usually 500 mg of pH 5 enzyme was isolated from each 100 g of tissue.

Preparation of the AS_{70} Enzyme--Aminoacyl-sRNA synthetases were also isolated from the aparticulate solution by ammonium sulfate fractionation according to the procedure of Allen and Schweet (58). Powdered ammonium sulfate was added slowly to the aparticulate supernatant solution to 40% saturation. After gently stirring for 1 hour, the mixture was centrifuged, the precipitate discarded, and the supernatant solution was adjusted to 70% of saturation with solid ammonium sulfate. After standing for 2 hours with occasional stirring, the precipitate was collected by centrifugation at 20,000 x g for 15 minutes and the supernatant was discarded. The precipitate, termed the AS_{70} enzyme, was suspended in 0.05 M phosphate buffer at pH 7.5 and utilized for amino acid incorporation studies.

Isolation of sRNA from Mammary Tissue--The procedure for precipitation of the sRNA (see Figure 2) was based on the work of Rosenbaum and Brown (118). Mammary tissue frozen at -20°C was sliced into small pieces and homogenized in the Virtis "45" homogenizer for 1 minute at full speed. The tissue was suspended in one volume of redistilled, water-saturated phenol, one volume of 0.14 M NaCl-0.01 M phosphate buffer at pH 7.0, and 0.02 volume of 1% EDTA at 0°C. The homogenate was centrifuged at 5,000 x g and separated into

PREPARATION OF sRNA

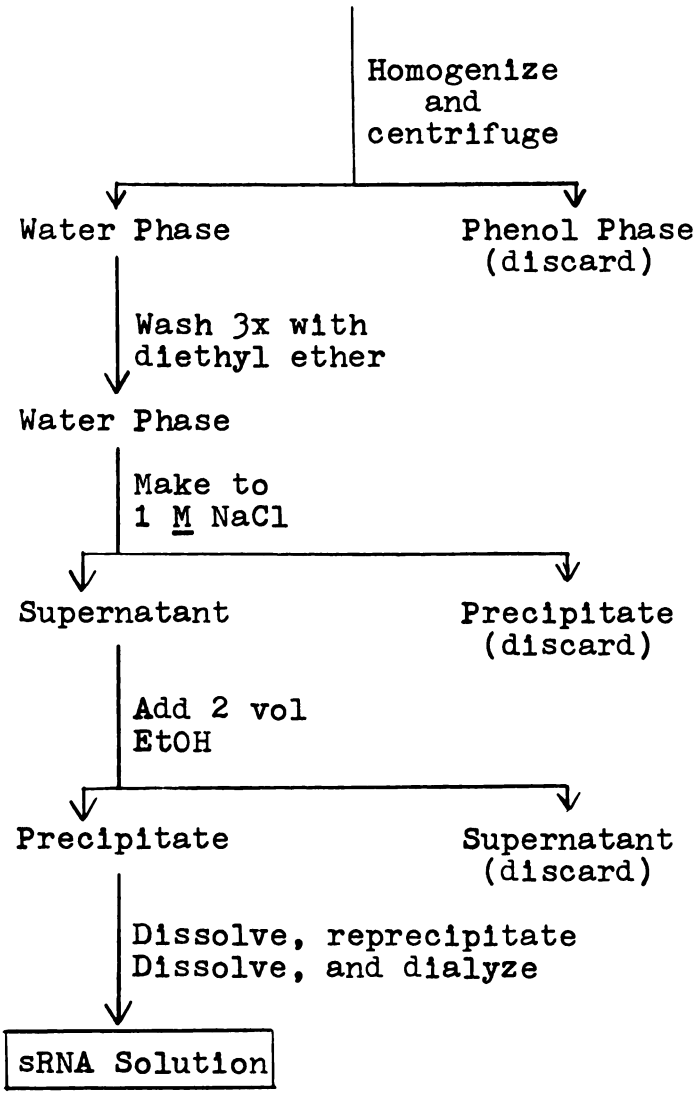

Figure 2. Isolation of sRNA from mammary tissue.

two phases. After the phenol was removed from the water phase by ether extraction, sodium chloride was added to a concentration of 1 M. The solution was kept at 4°C overnight. The white flocculent precipitate was removed by centrifuging the solution for 10 minutes at 20,000 x g. Two volumes of absolute ethanol were added to the supernatant solution to precipitate the sRNA. The precipitate was collected by centrifugation, redissolved, reprecipitated with ethanol, and dialyzed against several changes of 0.01 M phosphate buffer, pH 7.4. This solution containing approximately 1 mg of sRNA per ml was subsequently utilized in amino acid incorporation experiments. The concentration was determined by the relationship: 1 mg per ml equals 24 absorbance units [260 mμ and 1 cm light path (119)]. Usually 25 mg of purified sRNA was prepared from each 100 g of mammary tissue.

Analysis of Ribosomes by Sucrose Density Gradient Centrifugation

Analysis of the degree of aggregation of ribosomes was performed by sucrose density gradient centrifugation. One-tenth ml of a ribosome and polyribosome solution containing ten absorbancy units (260 mμ) per ml was layered on a linear 10 to 34% sucrose gradients (4.8 ml total) prepared in an apparatus similar to that described by Martin and Ames (120). The gradient solution contained, in addition to the sucrose, 0.005 M $MgCl_2$, 0.025 M KCl, and 0.01 M

Tris-HCl buffer, pH 7.4. The gradient was centrifuged for 90 minutes at 39,000 rpm in a Spinco SW swinging bucket rotor and allowed to stop without braking.

After centrifugation, the ribosomal sedimentation pattern was obtained by adapting the Gilford recording spectrometer as shown in Figure 3. When the gradient had been placed in the holder, toluene containing β-carotene as an indicator was pumped by means of a Sage pump into the void space above the gradient and into the outlet tube. When the air was completely expelled, this part of the system was then closed. The gradient tube was punctured at the bottom by the adjustable needle which was connected via teflon tubing to the flow cell. This tubing, the flow cell, and the outlet tubing were previously filled with 34% sucrose. After baseline adjustment on the recorder and opening the outlet valve, the toluene was pumped into the gradient tube at a rate of 5.0 ml per hour. The recorder was adjusted to 1.0 absorbancy unit full scale and the chart speed was 0.1 inch per minute. The special Gilford flow cell with a pin hole cross section had a 1 cm light path. Teflon tubing having an internal diameter of 0.053 cm was used and the spectrometer wavelength was set at the 260 mμ.

Two experiments involving sucrose density gradient centrifugation were performed with the type SB-283 rotor for the International Preparative Ultracentrifuge, Model B-60. The rotor was operated at 40,000 rpm for 1 hour. All procedures were identical to that previously described except

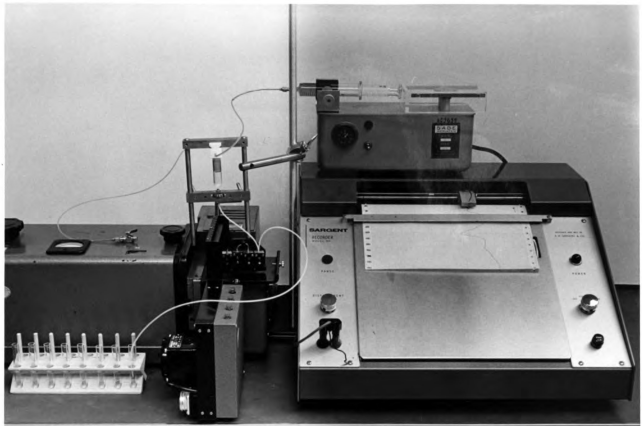

Figure 3. The apparatus for obtaining ribosomal patterns from sucrose density gradients. The procedures for performing the analysis are given in the text of the Methods section.

that approximately 10 absorbance units (260 mμ) of ribosomes and polyribosomes were layered onto the 12 ml sucrose gradient and the flow rate through the flow cell was 10 ml per hour.

Electron Microphotographs--The ribosome sample used for electron microscopy consisted of a solution from the polyribosome fraction of a sucrose gradient. Droplets of these ribosome samples were placed on Formvar-coated copper grids. The excess fluid was removed by touching filter paper to the edge of the grid and extensively rinsing each grid with solutions of decreasing concentrations of sucrose, but each solution contained 0.025 M KCl, 0.005 M $MgCl_2$, and 0.01 M Tris-HCl buffer, pH 7.5. The polyribosomes on the copper grids were shadowed in a Kenny shadow caster with oxidized tungsten, and then photographed in a RCA, type EMU 2A, electron microscope.

Aminoacyl-sRNA Synthetase Assays

Two general methods were used to assay aminoacyl-sRNA synthetase activity in various fractions isolated from mammary gland tissue. They were 1) the radioactive pyrophosphate exchange with ATP by the method of Bucovaz and Davis (106) and 2) aminoacyl hydroxamate formation according to the procedure of Berg (121).

The reaction mixture used for the measurement of aminoacyl-sRNA synthetase activity by ATP-PP^{32} exchange

contained 100 μmoles of Tris-HCl buffer, pH 7.5, 5 μmoles of ATP, 5 μmoles of sodium pyrophosphate containing approximately 100,000 cpm, 5 μmoles of $MgCl_2$, 0.5 ml of the 0.02 M amino acid mixture whose composition was similar to that of whole casein, 50 μmoles of KF, and 1 mg of enzyme, all in a total volume of 1.0 ml.

After the incubation, two ml of trichloroacetic acid (10% w/v) was added to stop the reaction. Two hundred mg of acid-washed Norit A were added per ml of the supernatant. The Norit A was removed by centrifugation at 2,000 x g and washed with 4 ml of 0.1 M sodium acetate (pH 4.5). The Norit A was next washed 1) with 0.05 M sodium acetate-0.1 M sodium pyrophosphate solution (pH 4.5), 2) three times with a 0.1 M sodium acetate, and 3) finally with distilled water. The washed Norit A was then suspended in 5 ml of 1 N HCl heated at 100°C for 15 minutes and then centrifuged. An aliquot of the supernatant solution was suspended in Kinard's solution for scintillation counting (see Appendix). The inorganic phosphate content was measured by the method of Fiske and SubbaRow (122).

Aminoacyl-sRNA synthetase activity in various preparations was also measured by aminoacyl hydroxamate formation. The assay mixture contained 10 μmoles of ATP, 0.7 ml of the 0.02 M amino acid mixture whose composition was comparable to that of whole casein, 10 μmoles of $MgCl_2$, 20 μmoles of Tris-HCl buffer, pH 7.4, the enzyme fraction and 1000 μmoles of neutralized hydroxylamine in a total volume of 3 ml. The

control contained no amino acids. The mixture was incubated for 30 minutes and then the reactions were stopped by adding 1.4 ml of 100% trichloracetic acid (w/v). After 0.6 ml of 2 M $FeCl_3$ was added, the mixture was centrifuged at 2,000 x g and the absorbancy of the supernatant at 520 mμ was determined. These results were converted to the amount of hydroxamate formed by comparison to a standard curve prepared with succinic anhydride.

Properties of Mammary Gland sRNA

The homogeneity and sedimentation coefficient of sRNA was determined by sucrose density gradient centrifugation according to the method described by Martin and Ames (120). Linear gradients of 5 to 20% sucrose solutions containing 0.005 M $MgCl_2$, 0.025 M KCl, and 0.01 M Tris-HCl buffer at pH 7.4 were centrifuged 15 hours in an SW 39 Spinco swinging bucket rotor at 4°C and then fractionated by collecting 6 drop fractions. Peroxidase and cytochrome c were utilized as markers of known sedimentation coefficients and they were assayed according to the method developed by Worthington Biochemical Corporation and by measuring absorbance at 415 mμ, respectively.

Esterification of amino acids to the purified sRNA was studied by the method of Fraser et. al. (107). The incubation mixture contained 0.15 mg sRNA, 15 μmoles of $MgCl_2$, 300 μmoles of Tris-HCl buffer, pH 7.4, 0.1 ml of 0.02 M amino acid mixture whose composition was comparable to that of whole

casein minus glutamate, 0.02 μc of L-glutamate-U-C^{14} (1.5 μc/μmole), and 11 to 15 mg of the enzyme fraction in a volume of 3.0 ml. This mixture minus sRNA served as the control. The systems were incubated for 30 minutes at 37°C and the reaction was stopped by acid precipitation at pH 5.0. The precipitate was 1) collected by centrifugation at 30,000 x g for 10 minutes, 2) dissolved in 10 ml of 0.01 M phosphate buffer, pH 7.0, and 3) extracted twice with an equal volume of water-saturated phenol. Two volumes of absolute ethanol were combined with the aqueous phase. The precipitate was collected by centrifugation and the radioactivity was determined by dissolving an aliquot in Kinard's counting fluid (see Appendix Table 4).

Ribonuclease Activity Determination

Ribonuclease activity of the various fractions of the mammary tissue was measured by the method of Kalnitsky et. al. (123). The incubation mixture contained 10 mg of yeast RNA, the enzyme fraction to be tested, 10 μmoles of $MgCl_2$, in a volume of 2.0 ml of 0.01 M phosphate buffer, pH 7.5. The solution was incubated for 30 minutes at 37°C. The reaction was stopped by the addition of 1.0 ml of a solution of 0.75 g of uranyl acetate dissolved in 25% perchloric acid. After standing for 15 minutes, the precipitate was collected by centrifugation at 2,000 x g and the absorbance at 260 mμ was determined on the supernatant.

Unincubated samples served as controls for each enzyme preparation.

Isolation of DNA from Mammary Tissue

Mammary gland DNA was prepared according to the method of Chargaff as follows (124): Two hundred grams of fresh mammary tissue was suspended in 400 ml of 0.1 M NaCl-0.05 M sodium citrate, pH 7, and homogenized at 4°C for 30 seconds in a Waring Blender. All subsequent operations were also performed at 0 to 4°C. The sediment obtained from centrifuging the homogenate at 2,000 x g was washed twice with the above buffer. The washed sediment was combined with 1,250 ml of a sodium chloride solution (10% w/v) and gently stirred overnight. After a second similar extraction, the combined supernatants were injected into two volumes of 95% ethanol. The nucleohistone precipitate was spooled out, washed successively with 70% and 80% ethanol, and then dissolved in 2,500 ml of a 10% sodium chloride solution. The proteins were removed by extracting this solution eight times with chloroform-amyl alchohol (3:1). After injecting the deproteinized solution into 2 volumes of 95% ethanol, the precipitate was spooled out and washed with 70%, 80%, and 100% ethanol in succession. The final yield was 450 mg of air-dried DNA.

Protein and Ribosome Determinations

Protein concentrations in the various preparations were determined by the method of Warburg and Christian (125). Ribosome and polyribosome content of various solutions was determined by the method of Tso' and Vinograd (116). They found that 1 mg of ribosomes per ml have an absorbance of 11.3 at 260 mμ and 1 cm light path.

Assay of Amino Acid Incorporation

The complete system for measuring C^{14} leucine incorporation was composed of the components (each adjusted to pH 7.4) listed in Table 1. Unless specified, the complete system was incubated for 40 minutes at 37°C and contained the pH 5 enzyme as a source of aminoacyl-sRNA synthetases. The incubation period was considered to have been initiated when the final component, i.e., the labeled amino acid, was added to the incubation mixture. Six mg of bovine serum albumin was added to the mixture immediately before stopping the reactions by adding 10 ml of 5% trichloracetic acid (TCA). The proteins in the zero time sample was precipitated immediately after the addition of the labeled amino acid. The TCA-precipitated proteins of each incubation mixture were washed and counted according to the method of Casjens and Morris (65). Briefly, this procedure involved 1) washing the precipitated proteins twice with 5% TCA, 2) dissolving the precipitates in 0.5 ml of 1 N sodium hydroxide, 3)

TABLE 1

Components of the complete amino acid incorporating system

The amounts of the components in this table were utilized in each ml of the complete incubation mixture. The specific amounts of microsomes, enzyme, and labeled amino acid are quoted with each experiment. The composition of the amino acid mixture was comparable to the amino acid composition of whole casein as analyzed by Jenness and Patton (126).

Component	Amount per ml
Microsomes	0.3-0.6 mg
pH 5 enzyme or AS_{70} enzyme	1 to 2 mg
sRNA	50 µg
ATP	1.0 µmole
GTP	0.25 µmole
Phosphoenolpyruvate	5.0 µmole
Pyruvate kinase	40 µg
KCl	50 µmoles
$MgCl_2$	5.0 µmoles
Glutathione (reduced)	20 µmoles
Amino acids minus leucine (see Appendix for composition)	1.0 µmole
C^{14} leucine (200 to 250 µc/µmole)	1.0 to 1.5 µc
Tris-HCl buffer, pH 7.4	50 µmoles

reprecipitating the proteins with TCA, 4) washing the precipitates again with 5% TCA, and 5) dehydrating the precipitates with washes of acetone containing 0.1 N HCl, of acetone-HCl and ether (4:1), and finally ether alone. The dried samples were transferred to a scintillation vial and dissolved in 0.5 ml of 1 N sodium hydroxide. Thixotropic counting solution (see Appendix Table 1) was mixed with the dissolved sample for the determination of radioactivity.

Identification of the Possible Synthesized Proteins(s)

Gel Filtration Studies of Radioactive Product--Column chromatography on Sephadex G-75 was utilized to assess the approximate molecular weight and homogeneity of the proteins that may have been synthesized in the cell-free system. The Sephadex gel was packed in a column of 1 x 70 cm dimensions and equilibrated with 0.05 M phosphate buffer, pH 7.5. Two and one-half ml of the 40 minute incubated complete system containing peroxidase and cytochrome c as standard proteins of known molecular weights were applied to the column. One ml of the 0 time sample containing the same markers was also analyzed by the same procedures. The 0.05 M phosphate buffer, pH 7.5, was allowed to flow through the column at a rate of approximately 3 ml per hour. One ml fractions were collected. Peroxidase activity and cytochrome c in the collected fractions were assayed by the methods previously described. Radioactivity was determined by combining 0.25 ml of each fraction with 0.5 ml of 1 N sodium hydroxide and suspending

the resulting solution in the thixotropic counting solution (see Appendix Table 1). Determination of the molecular weights of the unknown proteins was based on the method described by Andrews (127).

Isotope Dilution Studies--Partial identification of the synthesized protein in the incubated complete system was also performed by isotope dilution studies. Incubation mixtures which were incubated for 0 and 40 minutes were combined with fresh skimmed milk. Precipitation of the caseins and subsequent isolation of individual casein fractions--α_s, β, and γ,--were performed according to the method of McKenzie and Wake (110) as shown in schematic form in Figure 4. Isolation of α-lactalbumin and β-lactoglobulin was conducted according to the method of Aschaffenburg and Drewry (128) as shown in Figure 5. Aliquots of each protein was then dissolved in sodium hydroxide and the resulting solution was suspended in the thixotropic counting solution for radioactivity determinations.

Immunodiffusion Study--Antigen-antibody reactions utilizing the technique of immunodiffusion according to the method of Ouchterlony (114) were performed to more adequately define the protein that was synthesized in the cell-free system. One percent ion-free agar dissolved in 0.15 M NaCl and 0.15 M phosphate buffer (pH 7.2) was poured into a 2 mm thick layer in a petri dish and allowed to solidify. The antigen (0.05 ml) was placed in the center well while the

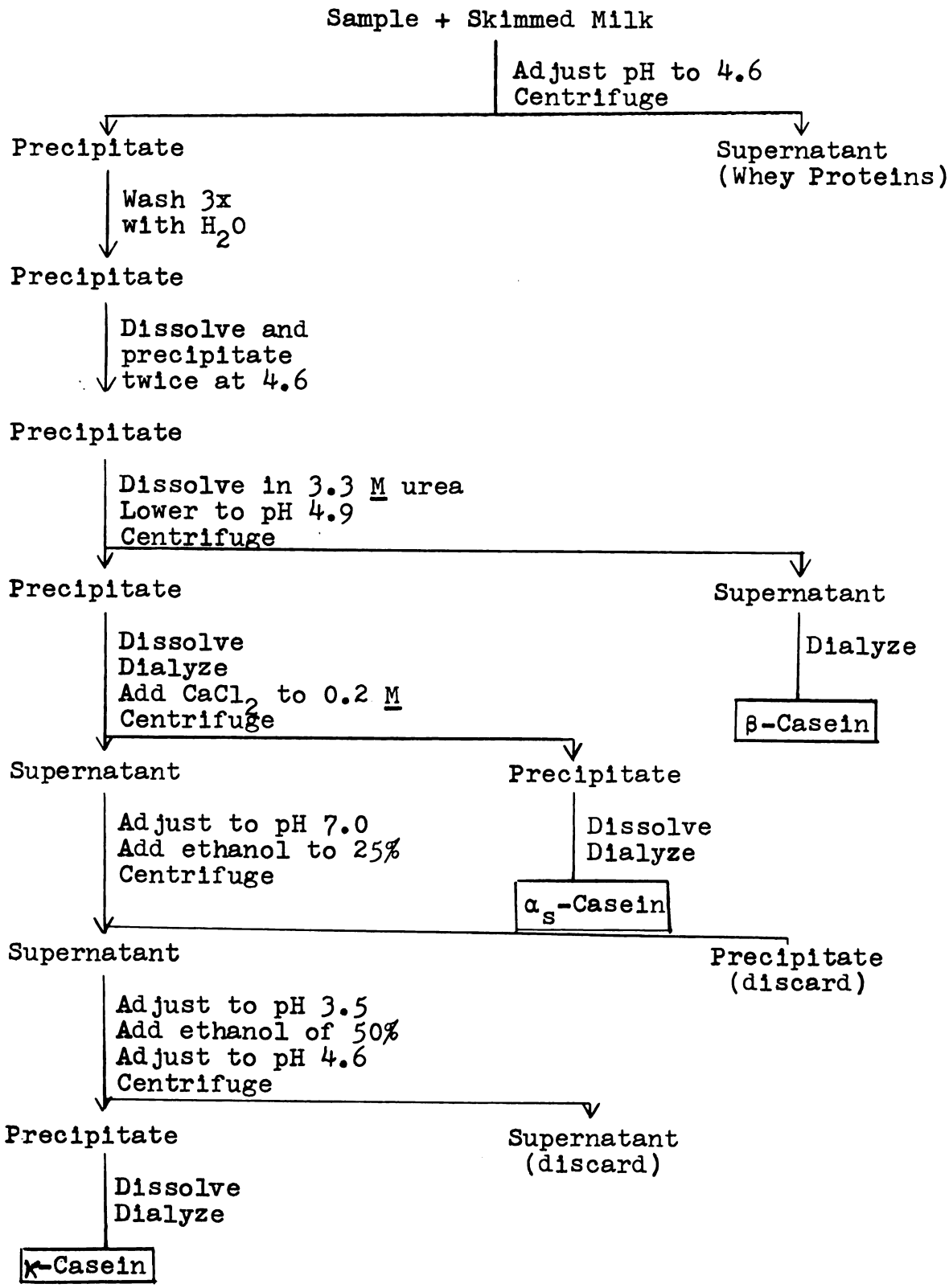

Figure 4. Isolation procedures for α_s-casein, β-casein, and κ-casein from skimmed milk.

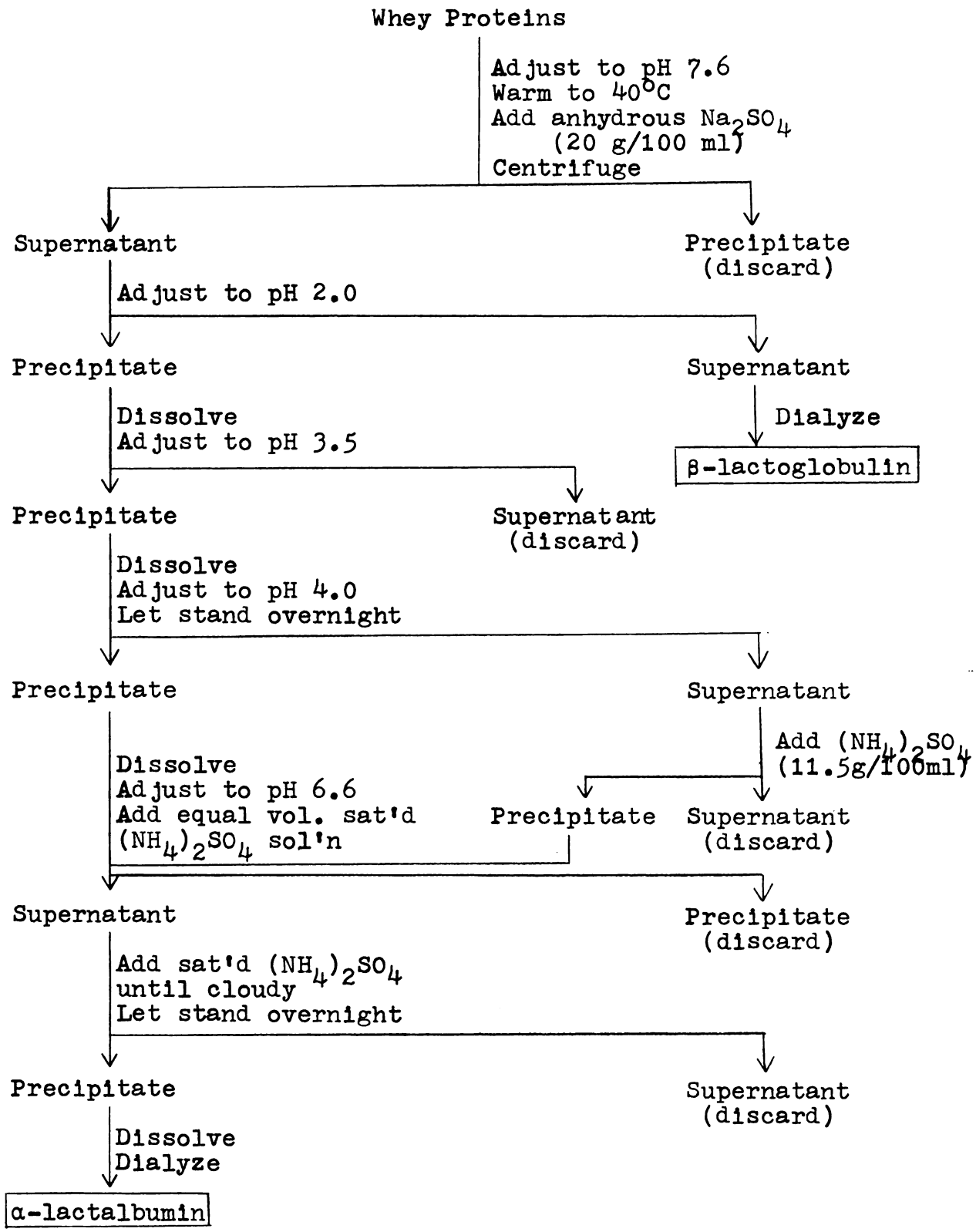

Figure 5. Isolation procedures for α-lactalbumin and β-lactoglobulin from the whey fraction of skimmed milk.

antibodies against the four proteins (α_s-casein, whole casein, κ-casein, and proteose-peptone fraction) were placed in wells equidistant (10 mm) from the center well. The petri dish was placed in a room temperature, water vapor-saturated chamber. Immunodiffusion was permitted to proceed for three days. The unprecipitated protein was then leached from the agar by repeatedly placing 0.15 M NaCl-0.15 M phosphate buffer at pH 7.2 over the agar for a period of 12 hours. After air-drying the agar containing the precipitin bands, the position of these bands was recorded. Next the agar sheet was placed in contact with Kodak No Screen Medical X-Ray film for 30 days. The exposed X-ray film was developed by normal photographic methods.

RESULTS

PART I

CHARACTERIZATION OF COMPONENTS

The aim of this work was to develop a cell-free system for synthesis of specific milk proteins utilizing components of bovine origin isolated from lactating mammary glands. Synthesis of specific proteins would be an essential property of any system that might later be used to study the control of synthesis of milk proteins.

Many characteristics of this system have been studied and their presentation is organized in the following manner. First, each of the purified cellular components which was utilized in the complete protein synthesizing system was partially characterized. These include microsomes, sRNA, and the aminoacyl-sRNA synthetase preparations. Subsequent studies utilizing these components for amino acid incorporation will then be discussed. This latter section includes a description of experiments designed to determine 1) the dependence of the complete process on each of the components of the complete system, 2) inhibition of amino acid incorporation by known protein synthesis inhibitors, 3) possible stimulation by polynucleotides and hormones, and 4) type of

protein synthesized by the complete system.[3]

Characteristics of the Ribosome Fraction

Preparation of the Ribosomal Fraction--The presence of polyribosomes, i.e., aggregates of ribosomes held together by strands of RNA, has been demonstrated in many different cell types in various species (1, 2, 3). Polyribosomes have been shown to be required in cell-free systems that are capable of synthesizing amino acids (129). These fragile structures are extremely sensitive to ribonuclease and mechanical forces. Since polyribosomes are required for cell-free protein synthesis, procedures for their preparation from lactating bovine mammary tissue were investigated. In order to determine which method of preparation yielded a higher percentage of polyribosomes, mammary tissue was disrupted by

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[3]Note: Throughout this thesis, except in some Figure titles, the following terminology will be used:

Ribosomes--Particles consisting of the single or monomeric ribosome.

Polyribosomes--Particles consisting of more than one ribosome.

Ribosomal fraction--A solution which contains both ribosomes and polyribosomes and contains sodium deoxycholate (0.25% w/v) to solubulize the endoplasmic reticulum.

Microsomes--The particles in the microsomal fraction and consisting of free ribosomes, free polyribosomes and membrane-bound ribosomes and polyribosomes.

As a matter of convenience, in certain titles of Figures, the word "ribosomes" refers to both ribosomes and polyribosomes as defined above.

the Virtis "45" tissue homogenizer, the French press, and by breaking frozen tissue with a mortar and pestle.

After the initial homogenization of the tissue by each of the techniques, the microsomes were isolated as described in the Methods section. Sodium deoxycholate was added to the microsome suspensions to a final concentration of 0.25% (w/v) to solubulize the endoplasmic reticulum. An aliquot of the solution from each of the three tissue preparations was subjected to sucrose density gradient centrifugation and the extent of aggregation of the ribosomes was determined by scanning the gradient at 260 mμ (see Methods).

Patterns of the individual ribosomal preparations (Figures 6, 7, and 8) show a sharply defined peak of single ribosomes. Ribosomes in this fraction were determined to be monomer units by comparison of this mammary ribosome pattern with similar density gradient patterns of reticulocyte ribosomes where the state of aggregation has been thoroughly investigated (129). The absorbing material to the left of this large ribosome peak represents a series of polyribosome components. Figures 6, 7, and 8 demonstrate that the homogenization of the tissue in the frozen state produced a microsome fraction which contained a higher proportion of polyribosomes than was found in the microsome fractions isolated after the tissue had been homogenized with the Virtis "45" or the French press. Since polyribosomes are necessary for protein synthesis, it is important to obtain a preparation with the highest proportion of

Figure 6. The sedimentation behavior of ribosomes and polyribosomes prepared by homogenizing frozen tissue. The ribosomal fraction containing 0.25% DOC (w/v) was isolated from tissue which had been frozen in the liquid nitrogen and disrupted with the aid of a mortar and pestle (see Methods). Seven-tenths of an absorbance unit (260 mμ) of ribosomes and polyribosomes dissolved in 0.1 ml of Medium A (see Appendix Table 5) was layered onto a linear sucrose gradient of 10 to 34% sucrose containing 10 mM Tris-HCl buffer (pH 7.4), 25 mM KCl, and 5 mM $MgCl_2$ (final volume 4.8 ml). The gradient was centrifuged 1.5 hour at 39,000 rpm in a SW 39 rotor at 4°C. The contents of the centrifuge tube was then analyzed optically for ribosomal and polyribosomal content (see Methods).

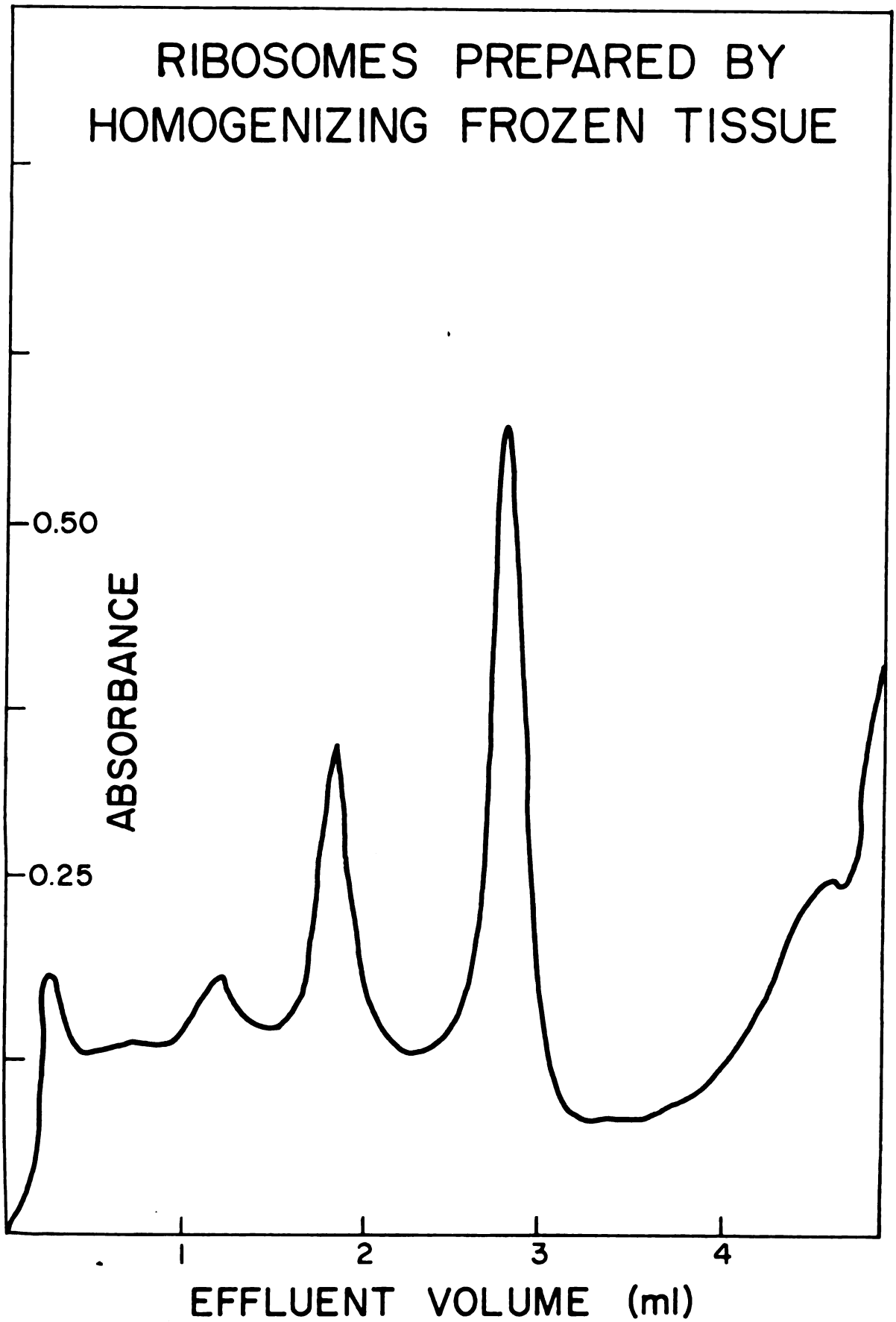

RIBOSOMES PREPARED BY
HOMOGENIZING FROZEN TISSUE
0.50
0.25
ABSORBANCE
1
2
3
4
EFFLUENT VOLUME (ml)

Figure 7. The sedimentation behavior of ribosomes and polyribosomes prepared with a Virtis "45" homogenizer. The ribosomal fractions containing 0.25% DOC (w/v) were prepared from tissue which had been disrupted with the Virtis "45" homogenizer by methods described in the Methods section. Eight-tenths of an absorbance unit of ribosomes and polyribosomes were analyzed for ribosomal and polyribosomal content as described in Figure 6.

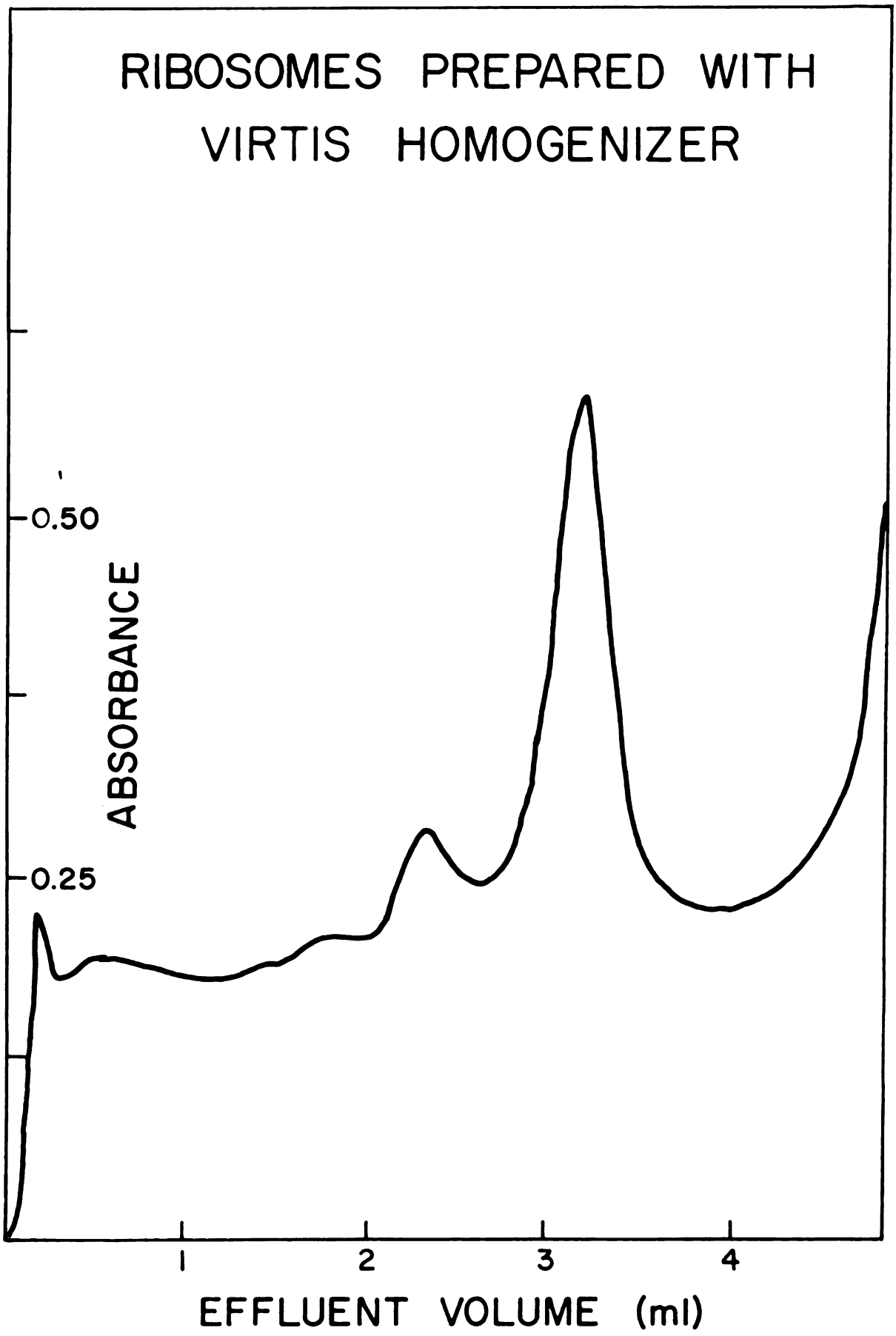
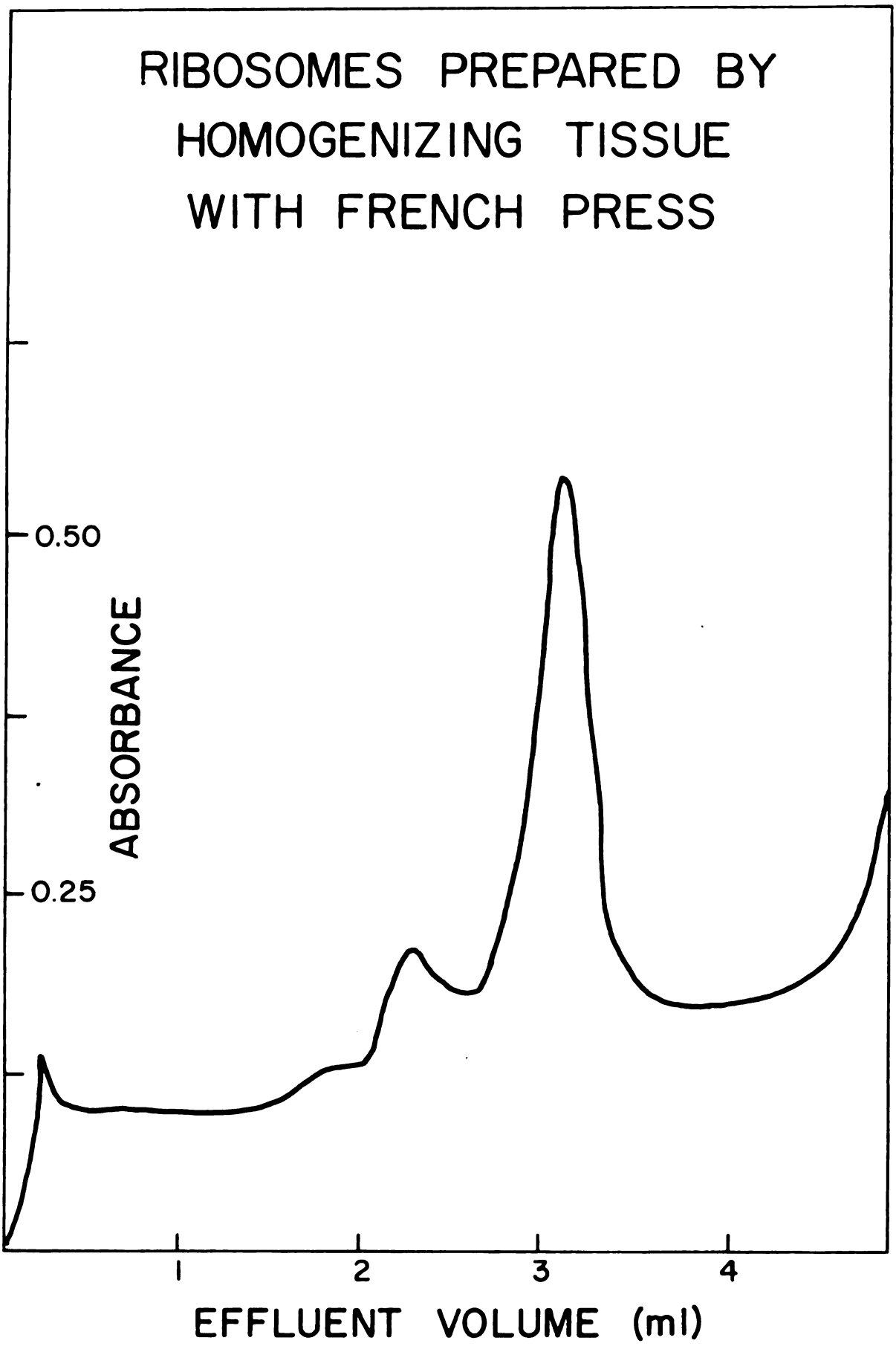

RIBOSOMES PREPARED WITH
VIRTIS HOMOGENIZER
0.50
0.25
ABSORBANCE
1
2
3
4
EFFLUENT VOLUME (ml)

Figure 8. The sedimentation behavior of ribosomes and polyribosomes prepared by homogenizing tissue with the French press. The ribosomal fraction containing 0.25% DOC (w/v) was isolated from tissue which had been disrupted by utilizing the Aminco-French pressure cell at 12,000 to 16,000 psi (see Methods). Eight-tenths of an absorbance unit of ribosomes and polyribosomes were analyzed for ribosomal and polyribosomal content as described in Figure 6.


RIBOSOMES PREPARED BY
HOMOGENIZING TISSUE
WITH FRENCH PRESS
0.50
0.25
ABSORBANCE
1
2
3
4
EFFLUENT VOLUME (ml)

polyribosomes. Therefore, the procedure of homogenizing frozen tissue became the method of choice for all the future studies.

Effect of Additives on Polyribosome Yield--Electron micrographs have shown that in the liver and pancreas, ribosomes are primarily attached to an intracellular lipoprotein membrane, the endoplasmic reticulum, and a small proportion apparently is free in the cytoplasm (130). Palade and Siekevitz (131) demonstrated that pancreatic ribosomes are released from the endoplasmic reticulum while maintaining their structural integrity by treating the microsomal fraction with sodium deoxycholate (DOC). Burka (132) has recently demonstrated that the free ribosomes of the reticulocyte ribosomal fraction are broken down by deoxycholate. Insignificant breakdown of ribosomes and polyribosomes was observed when DOC was added to rat liver microsomes at levels varying from 0.3% to 1.2% (w/v); however, concentrations higher than 1.66% destroyed the ribosomal particles (63). Because sodium deoxycholate releases the bound ribosomes from the endoplasmic reticulum, this anionic detergent was used to ascertain whether mammary gland ribosomes existed as bound and/or free, and whether this treatment altered the sedimentation behavior of the various aggregates of ribosomes.

Sodium deoxycholate (DOC) was combined with an aliquot of a microsome solution to a final concentration of 0.25% (w/v). Equal absorbancy units at 260 mμ of the DOC-treated and the non-treated solutions were each subjected to sucrose

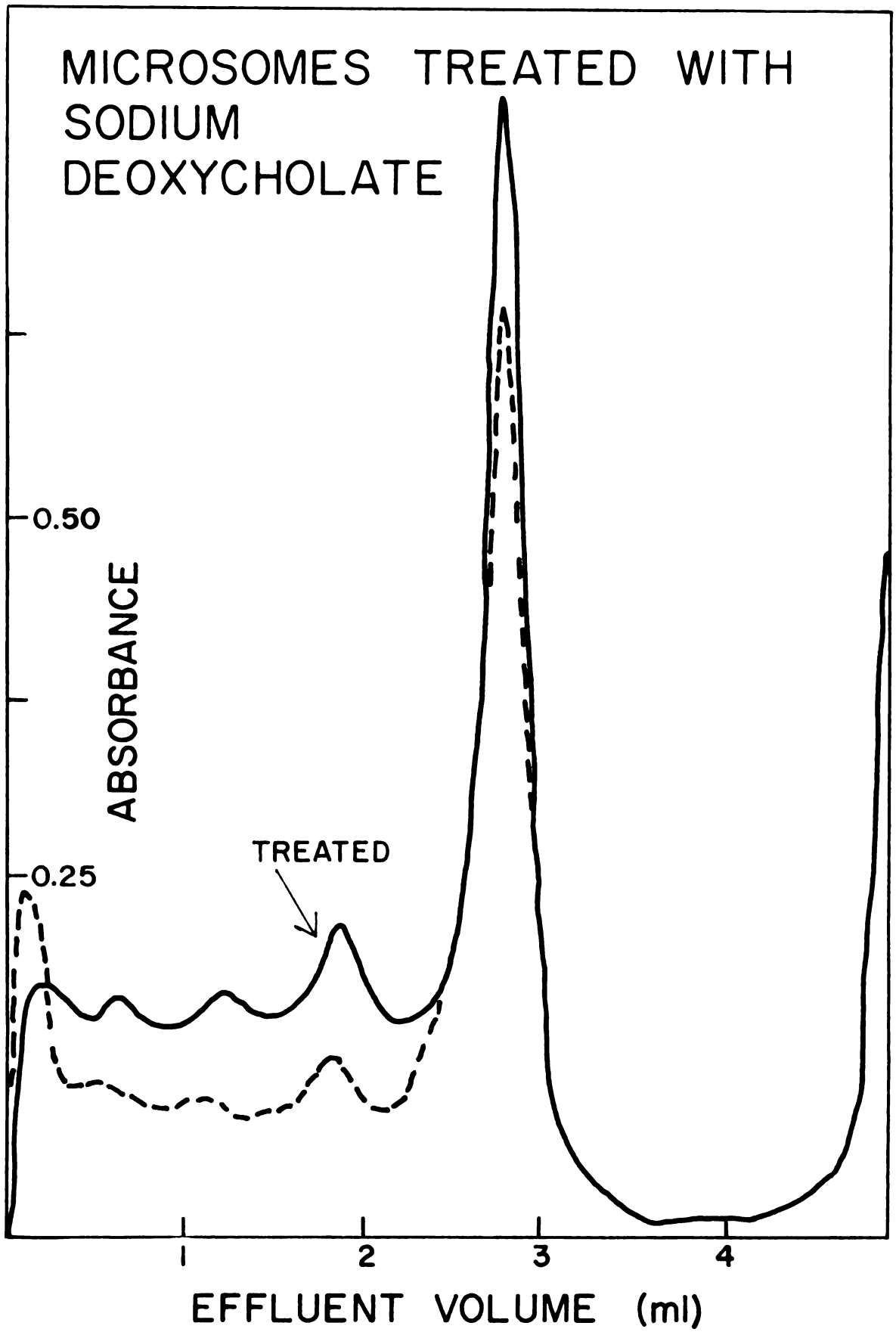
density gradient centrifugation and the pattern of ribosome sedimentation was determined as previously described (see Methods).

The results of this experiment are illustrated in Figure 9. The ribosomal pattern of the microsome preparation showed that unbound ribosomes and polyribosomes were present since not all the 260 mμ absorbing material sedimented to the bottom of the centrifuge tube. Treatment of the microsome preparation with deoxycholate resulted in a substantial increase in the amount of each of the ribosomal peaks, thereby illustrating a release of ribosomes from the endoplasmic reticulum. Also, the DOC treatment did not interfere with the ribosomal structures or the binding of messenger RNA to the ribosomes since the polyribosomal peak heights were also substantially increased.

The proportion of polyribosomes in a given preparation of microsomes is dependent on the activity of ribonuclease in the solution. Because polyribosomes are necessary for protein synthesis, it is desirable to reduce the action of ribonuclease to a minimum. Ribonuclease has been shown to bind to bentonite and thereby become inactivated (133). With this fact in mind, it was decided to determine if treatment of the crude homogenate of mammary tissue with bentonite would increase the yield of polyribosomes.

Bentonite, previously washed and sized by the method of Fraenkel-Conrat and Singer (133), was added at a concentration of 10 mg per ml to the crude homogenate. The

Figure 9. The sedimentation behavior of microsomes before and after treatment with sodium deoxycholate. These preparations were isolated from a crude homogenate which had been prepared by powdering the frozen mammary tissue (see Methods). The microsome fraction was clarified by adding DOC to a final concentration of 0.25% (w/v) to prepare the ribosomal fraction (endoplasmic reticulum solubulized). One absorbance unit (260 mμ) of both preparations were layered onto a linear sucrose gradient which was subsequently centrifuged and analyzed for ribosomal and polyribosomal content as described in Figure 6.


MICROSOMES TREATED WITH SODIUM DEOXYCHOLATE
0.50
0.25
ABSORBANCE
TREATED
1
2
3
4
EFFLUENT VOLUME (ml)

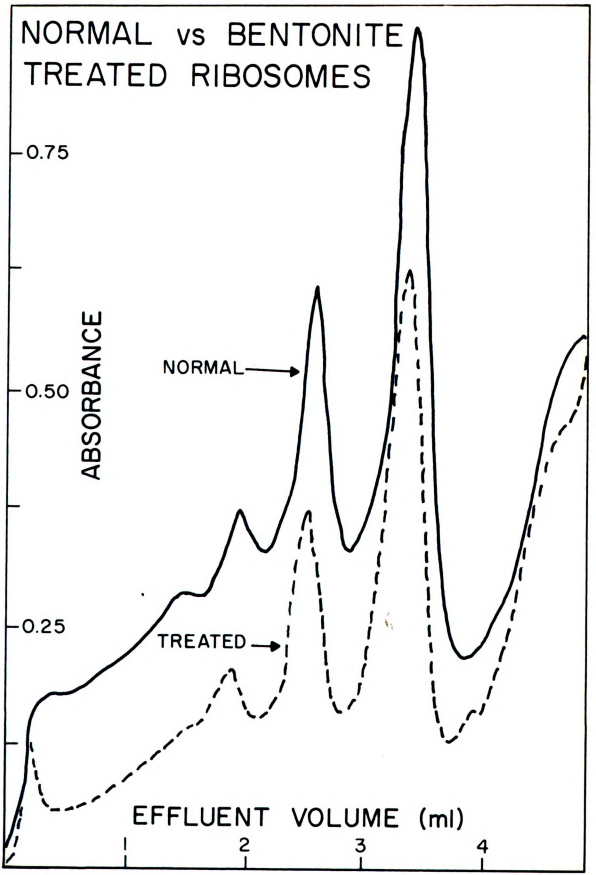
microsomes were isolated as usual and the endoplasmic reticulum was solubulized with 0.25% (w/v) of DOC. Analysis by sucrose density gradient centrifugation was conducted as described in the Methods section.

Sucrose density gradient patterns of normal and bentonite-treated ribosomes are shown in Figure 10. Ribosomal patterns of the two preparations were equivalent with respect to the proportion of polyribosomes, illustrating that bentonite treatment was not advantageous. Bentonite-treated ribosomes and polyribosomes equivalent to 1.10 absorbancy units at 260 mμ were centrifuged while 0.7 absorbancy units of normal ribosomes and polyribosomes were centrifuged to obtain ribosomal patterns. Yet, the pattern of the normal ribosomes and polyribosomes showed the most 260 mμ absorbing material. This suggests that binding of a portion of the ribosomes to bentonite had occurred and consequently these were not scanned by the usual gradient analysis. Tester and Dure (134) have explained a similar loss of ribosomes as the formation of a ribosome-bentonite complex formed by salt binding of negatively charged ribosomes to the negatively charged surface of bentonite through magnesium ions.

In summary, bentonite did not increase the proportion of polyribosomes in a given microsome preparation, and therefore, this procedure was not utilized in subsequent studies.

In addition to bentonite, polyvinyl sulfate also has been shown to inactivate ribonuclease (135). Therefore, it was logical to examine whether this inhibitor, if added to

Figure 10. The sedimentation behavior of normal and bentonite-treated ribosomes and polyribosomes. The normal ribosomal fraction was isolated from a crude homogenate which had been prepared by powdering the frozen mammary tissue (see Methods). The bentonite-treated ribosomes and polyribosomes were isolated from a similar crude homogenate containing washed bentonite at a concentration of 6 μg per 10 ml of protein. Five-tenths absorbance unit (260 mμ) of the normal ribosomes and polyribosomes (0.1 ml) and 0.42 absorbance unit of the same preparation treated with bentonite (0.1 ml) were layered onto linear sucrose gradients. These gradients were subsequently centrifuged and analyzed for ribosomal and polyribosomal content as described in Figure 6.


NORMAL vs BENTONITE
TREATED RIBOSOMES
0.75
0.50
0.25
ABSORBANCE
NORMAL
TREATED
EFFLUENT VOLUME (ml)
1
2
3
4

the crude homogenate, would increase the proportion of polyribosomes in the ultimate microsome fraction.

Polyvinyl sulfate was added to the crude homogenate at a 0.01 M concentration. The ribosomes and polyribosomes were isolated and analyzed by the usual sucrose density gradient centrifugation (see Methods). The results of the experiment are presented in Figure 11. Comparison of polyvinyl sulfate-treated ribosomes and polyribosomes with the control illustrates that a partial destruction of the ribonucleoprotein structure of the ribosomes had occurred. Each ribosomal peak in the treated sample was drastically reduced in area and a ribosomal subunit smaller in size than the monomer ribosome was produced. Consequently, this ribonuclease inhibitor was not utilized in further future experiments.

Since Dupanol might decrease ribonuclease activity and thus produce a higher proportion of polyribosomes in the microsome fraction, this possibility was next investigated. Microsomes were prepared as usual (see Methods). Dupanol was added at a concentration of 1% (w/v) to an aliquot of the microsome solution. Sucrose density gradient centrifugation of the normal DOC-treated microsomes and the Dupanol-treated microsomes was conducted as usual.

Results of this experiment are presented in Figure 12. Since no ribosomal peaks were observed in the treated sample, it was concluded that Dupanol completely destroyed the physical structure of the ribosomes. Hall and Doty (136) have

Figure 11. The sedimentation behavior of normal and polyvinyl sulfate-treated ribosomes and polyribosomes. The normal ribosomal fraction was isolated from a crude homogenate as described in Figure 10. The polyvinyl sulfate-treated ribosomes and polyribosomes were isolated from a similar crude homogenate which contained polyvinyl sulfate at a concentration of 0.01 M. Five-tenths absorbance unit (260 mμ) of the normal ribosomal fraction in 0.1 ml of Medium A (see Appendix Table 5) and 1.1 absorbance unit of the polyvinyl sulfate-treated ribosomes and polyribosomes dissolved in 0.1 ml of Medium A were layered onto linear sucrose gradients which were subsequently centrifuged and analyzed for ribosomal and polyribosomal content as in Figure 6.

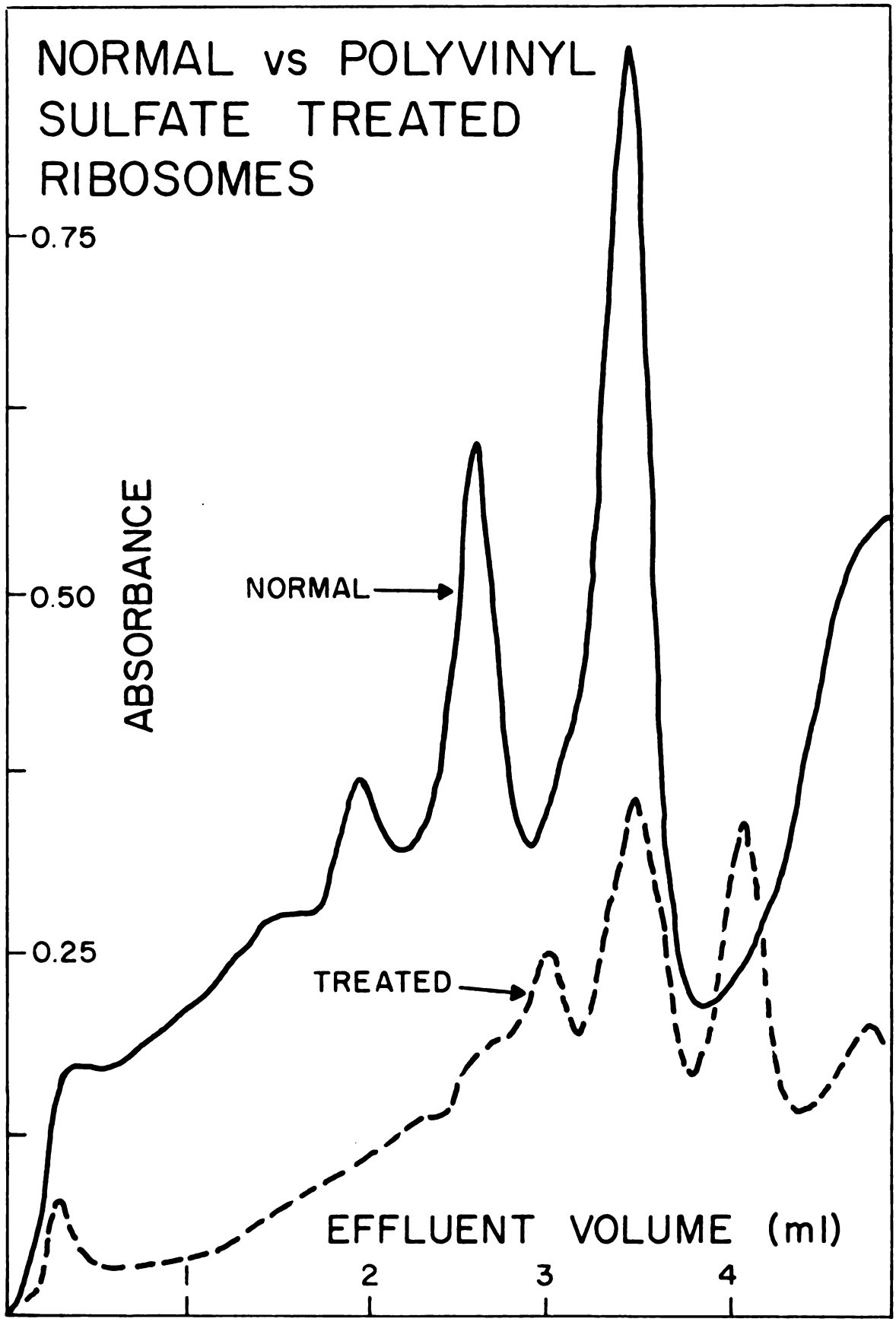
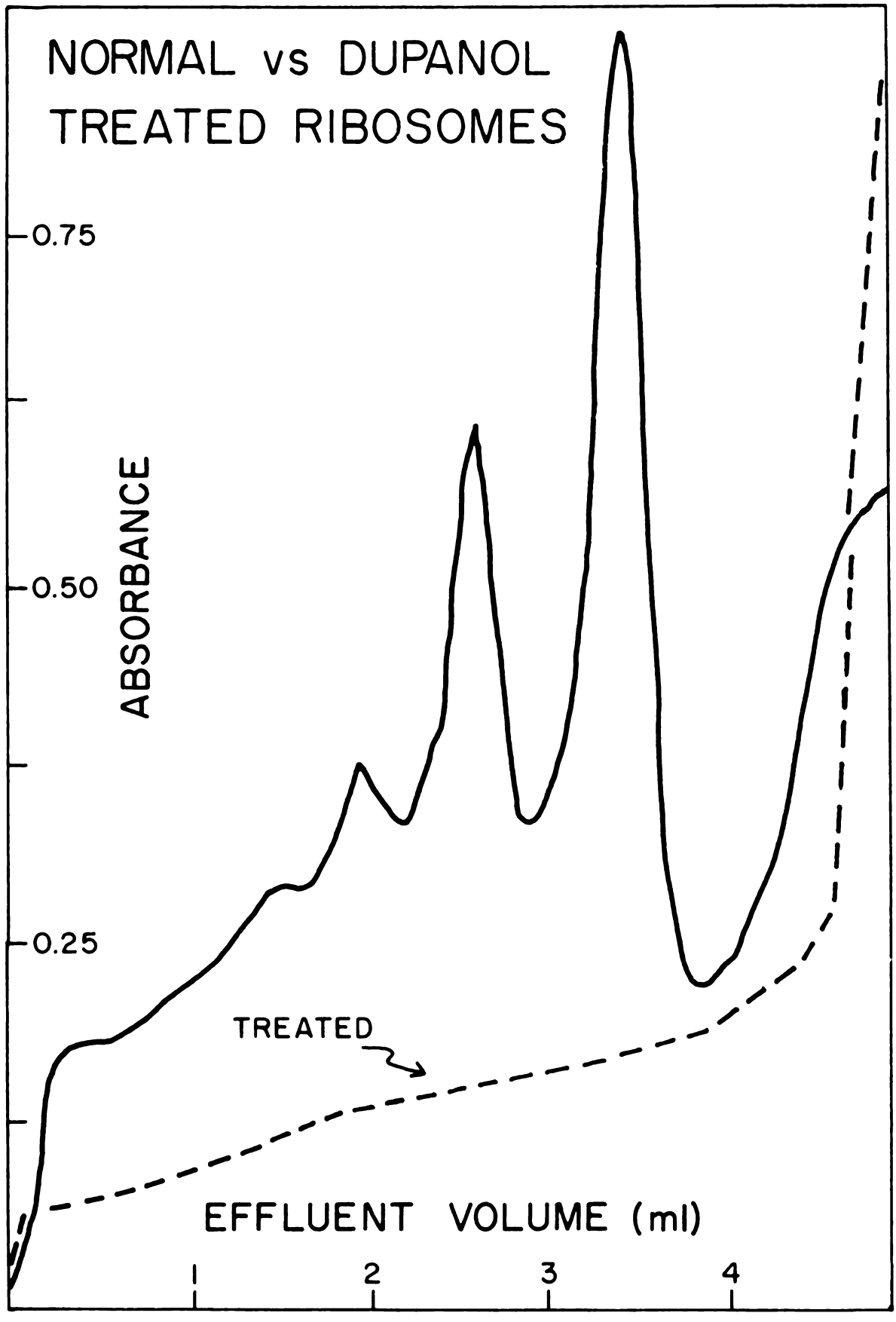

NORMAL vs POLYVINYL SULFATE TREATED RIBOSOMES
0.75
0.50
0.25
ABSORBANCE
NORMAL
TREATED
EFFLUENT VOLUME (ml)
2
3
4

Figure 12. Comparison of the sedimentation behavior of normal and Dupanol-treated ribosomes and polyribosomes. The normal ribosomal fraction was isolated from a crude homogenate (same as Figure 10) which had been prepared by powdering the frozen mammary tissue (see Methods). The Dupanol-treated ribosomes and polyribosomes were isolated from a similar crude homogenate by treating the microsome fraction with Dupanol (1% w/v). Five-tenths absorbance unit (260 mμ) of the Dupanol-treated microsomes and 0.5 absorbance unit of the normal ribosomes dissolved in 0.1 ml of Medium A were layered onto linear sucrose gradients and subsequently centrifuged and analyzed for ribosomal and polyribosomal content as described in Figure 6.


NORMAL vs DUPANOL
TREATED RIBOSOMES
0.75
0.50
0.25
ABSORBANCE
TREATED
EFFLUENT VOLUME (ml)
1
2
3
4

shown that this anionic detergent readily attacked liver ribosomes by dissociating them into an RNA fraction and a protein-detergent complex fraction. Quite obviously, this procedure could not be of use in future experiments.

Effect of Incubating Microsomes with Ribonuclease--Polyribosomes have been shown to be degraded to monomeric forms by the action of ribonuclease (58, 63). With this in mind, a study of the effect of ribonuclease on mammary gland ribosomes and polyribosomes was undertaken. One ml solutions of Medium A (see Appendix Table 5) containing 0.82 mg of microsomes and 0.01 μg of crystalline pancreatic ribonuclease were incubated at 37°C for various times. In another study, 30 times more ribonuclease was added to the incubation medium. In both experiments the samples were cooled rapidly and subjected to the usual sucrose density gradient analysis.

Data of this experiment is presented in Table 2. There was a time-dependent degradation of the polyribosome peaks with a concomitant increase in the peak representing the monomeric form of ribosomes. Incubation of the microsomes with the higher level of ribonuclease (1 μg RNase per 2.7 mg of microsomes) resulted in extensive ribosomal degradation. After 5 minutes of incubation, all the ribosomes were destroyed.

These results demonstrate that polyribosomes and even ribosomes are unstable in the presence of ribonuclease at

TABLE 2

Incubation of microsomes with ribonuclease

The reaction mixture contained 0.82 mg of microsomes and 0.01 µg of ribonuclease per one ml of Medium A (see Appendix Table 5). The column on the extreme right show results which refer to a reaction mixture containing 0.3 µg of ribonuclease and 0.82 mg of microsomes per ml. After incubation at 37°C, aliquots were subjected to sucrose density gradient centrifugation (see section on Methods) and the areas under each peak was calculated and compared to the total area. Ribosome size was calculated from the sedimentation rate in the sucrose gradient according to Martin and Ames (120) and using the reticulocyte monomeric ribosome (78 S, 129) as a standard.

Ribosome Size	Time of Incubation (min.)				
	0[1]	1[1]	5[1]	10[1]	5[2]
~ 80 S	74.4%[3]	78.8	92.4	91.2	Trace
~120 S	22.2	19.0	7.6	7.8	None
~190 S	3.3	2.3	Trace	None	None

[1] 1 µg of RNase added per 82 mg of ribosomes and polyribosomes

[2] 1 µg of RNase added per 2.7 mg of ribosomes and polyribosomes

[3] Value represents percent of total area under ribosomal peaks

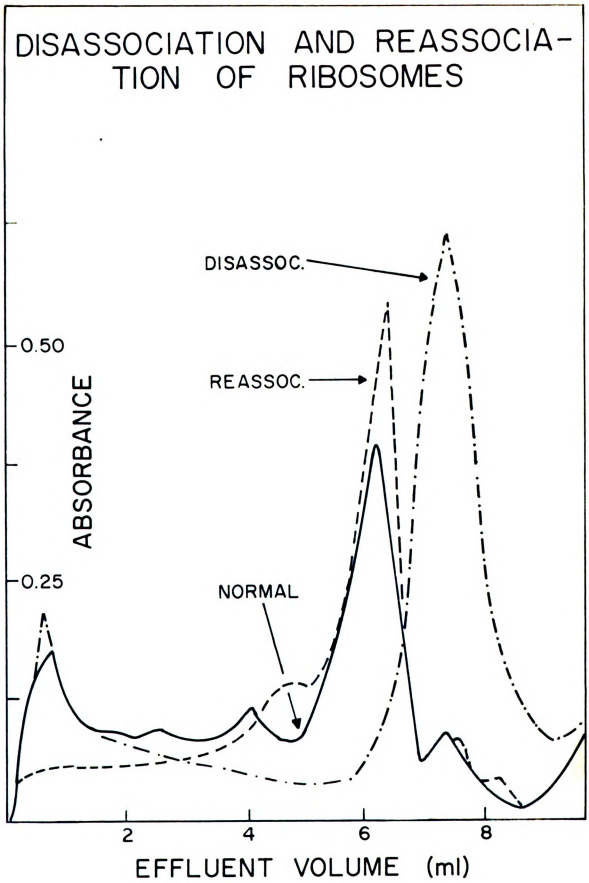
37°C. Also the dissociation of polyribosomes to ribosomes illustrates that the former are held together by RNA.

Dependency of Polyribosomal Character Upon Magnesium Ions--Previous workers (63) have demonstrated that magnesium ion concentration of at least 1 mmolar was necessary for stability of rat liver polyribosomes. Magnesium concentration of 0.1 mM or less resulted in a considerable decrease in the amount of polyribosomes as analyzed by sucrose gradient density centrifugation. The purpose of the following experiment was to determine whether mammary gland polyribosomes possess a similar dependence on magnesium ion concentrations.

An aliquot of a microsome pellet was suspended in a volume of 0.5 M KCl equal to the volume of the original microsome solution and recentrifuged at 105,000 x g for 90 minutes. The resultant microsomes were suspended at a final concentration of about 1 mg per ml in Medium A which contained no magnesium chloride. In order to determine the effect of adding magnesium back to these "shocked" microsomes, magnesium chloride was added to a final concentration of 8 mM. These three preparations of microsomes containing the three levels of magnesium chloride--0, 4, and 8 mM--were subjected to sucrose density gradient centrifugation.

The results of this study are presented in Figure 13. In the absence of magnesium ion, ribosomes in all states of aggregation were changed to a slower moving component which

Figure 13. The sedimentation behavior of dissociated and reassociated microsomes. The normal microsomes were isolated by the procedure described by the Methods section. The dissociated microsomes were prepared by suspending the microsomes in 0.5 M KCl, reisolating the dissociated microsomes by centrifugation at 105,000 x g for 90 minutes and suspending them in Medium A (see Appendix Table 5) minus $MgCl_2$. The microsomes were reassociated by adjusting an aliquot of this latter preparation to 8 mM $MgCl_2$. Twelve and six-tenths absorbance units (260 mμ) of the normal microsomes (0.1 ml), 9.5 units of dissociated microsomes (0.1 ml) and 9.5 units of reassociated microsomes (0.1 ml) were layered on linear sucrose gradients of 10 to 34% sucrose containing 10 mM Tris-HCl buffer (pH 7.4), 25 mM KCl, and 5 mM $MgCl_2$ (final volume 12.0 ml). The gradients were centrifuged 1 hour at 40,000 rpm in the Model SB-283 rotor at 4°C in the Model B-60 International Preparative Ultracentrifuge. The contents of the centrifuge tubes were then analyzed for ribosomal and polyribosomal content (see section on Methods). Note--the disassociated ribosome peak may actually be "swelled" ribosomes as described in the text.


DISASSOCIATION AND REASSOCIATION OF RIBOSOMES
DISASSOC.
REASSOC.
NORMAL
0.50
0.25
ABSORBANCE
2
4
6
8
EFFLUENT VOLUME (ml)

was equivalent to a 60 S particle. These particles may be a subunit of an 80 S ribosome or may represent a particle of the same molecular weight as the 80 S particle but having an altered (swollen) conformation. This possibility has previously been suggested for reticulocyte ribosomes subjected to similar treatment (145). This approximate sedimentation coefficient is based on 1) the calculation that the major peak in the normal preparation is an 80 S particle with monomeric reticulocyte ribosome of 78 S used as standard (129), 2) the method of calculation of sedimentation coefficients from known values as reported by Martin and Ames (120). When magnesium chloride was added to these "shocked" microsomes at a concentration of 8 mM, the ribosomes and polyribosomes reappeared in the gradient pattern and the peak representing the slower moving component decreased in height.

Thus, magnesium ion is necessary to preserve the integrity of the ribosomes and the polyribosomes. Removing magnesium ions from the solution containing both ribosomes and polyribosomes results in ribosomal dissociation or "swelling". However, these ribosomal "subunits" may be reassociated (altered) in such a way as to form ribosomes and even polyribosomes by bringing the magnesium ion concentration back to 8 mM

Sedimentation Coefficient Determination--In order to substantiate the calculation of sedimentation coefficients

from the sucrose density gradient data, two preparations of ribosomes suspended in Medium A and containing 0 and 4 mM magnesium chloride were subjected to sedimentation velocity measurements in the analytical ultracentrifuge. The solution of ribosomes and polyribosomes containing magnesium chloride also contained DOC at a concentration of 0.25% (w/v) to solubulize the endoplasmic reticulum. The sedimentation patterns are presented in Figure 14 and the data for the calculations are presented in Table 3.

The sedimentation coefficients of these two samples of ribosomes were similar to that calculated from the sucrose gradient data when utilizing the monomeric reticulocyte ribosomes as a 78 S standard (129). The monomeric ribosomes were found by this experiment to possess a sedimentation coefficient, corrected to 20°C and water as the solvent, of 79 while the ribosomes in the solution containing no magnesium possesses an S^{w}_{20} of 54. The value of 79 for normal mammary gland ribosomes compares well with the S^{w}_{20} of 75 reported for pancreatic ribosomes (137) and 83 reported for rat liver ribosomes (138).

Electron Microscopy of Polyribosomal Preparation--Warner et. al. (129) have verified the existence of polymers of ribosomes in reticulocyte preparations by electron microscopy. A similar test was conducted with the mammary gland ribosomes in order to obtain more information concerning the integrity of the preparations.

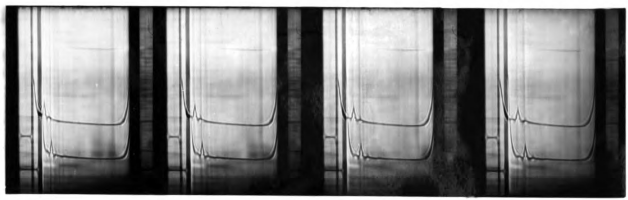


Figure 14. Sedimentation velocity of mammary gland ribosomes. Normal ribosomes and polyribosomes were dissolved in Medium A (see Appendix Table 5) containing 0.25% deoxycholate at a concentration of 2.5 mg per ml. This preparation is represented by the lower sedimentation pattern in the Figure. The "shocked" microsomes (microsomes which had been suspended in 0.5 M KCl were dissolved in Medium A containing no $MgCl_2$ at a concentration of 2.5 mg per ml and are represented by the upper sedimentation pattern. Six-tenths ml of these solutions were centrifuged at 42,040 rpm in Spinco Model E analytical ultracentrifuge. The photographs of the schlieren patterns were taken at 4, 6, 8, and 10 minutes after the centrifuge had reached maximum velocity. The velocity of movement of each peak was calculated and the sedimentation coefficient (not corrected to zero concentration) was calculated from the following differential equation: $S = dr/dt/w^2r$. The camera lens magnification factor was 21.53 and the inner reference was 5.72 cm from the center of rotation while the outer reference was 7.30 cm.

TABLE 3

Sedimentation coefficients of mammary gland ribosomes

The normally prepared ribosomes and polyribosomes were dissolved at a concentration of 2.5 mg per ml in Medium A (see Appendix Table 5) containing 0.25% deoxycholate and "shocked" ribosomes (microsomes suspended in 0.5 M KCl solution) were dissolved in Medium A containing no $MgCl_2$ at a concentration of 2.5 mg/ml. Six-tenths ml of these solutions were centrifuged at 42,040 rpm in a Spinco Model E analytical ultracentrifuge and the sedimentation velocity was monitored by photographing the schieren optics pattern at time intervals. Viscosity of the solutions was determined with the Ostwald viscometer in order to correct the calculated S valve to 20°C and water as a solvent.

Sample	Viscosity of Solution at 4°C	Sedimentation Coefficient	
		Uncorrected S	Corrected to Water and 20°C (S^{W}_{20})
Normal Ribosomes	3.51	22.6	79.2
Shocked Ribosomes	3.47	16.1	56.0

For this study, ribosomes were prepared and centrifuged in a sucrose gradient as previously described (see section on Methods). That fraction of the gradient which contained material sedimenting faster than the ribosomes was isolated, and aliquots of this solution were analyzed by electron microscopy as previously discussed (see section on Methods).

As shown in Figure 15, particles were indeed observed in the polyribosome fraction. They are also of the correct dimensions (200-250 A in diameter, 129) to be ribosomes. Some particles appear to exist as ribosomes while many are seen in various states of aggregation. Some polyribosomes appear to be linearly arranged as if attached to a strand of messenger RNA.

Properties of Mammary Gland sRNA

Determination of Homogeneity and Sedimentation Coefficient--In addition to the ribosomal fraction, sRNA is required in cell-free protein synthesizing systems. Activated amino acids become esterified to the sRNA and are thereby transferred to the ribosomal complex. In order to determine the specific requirement of sRNA in a mammary gland cell-free system, the RNA must be purified to remove other RNA's present in mammary tissue.

Mammary gland sRNA was purified according to method of Rosenbaum and Brown (118) and assayed for purity by sucrose density gradient centrifugation. Horse radish

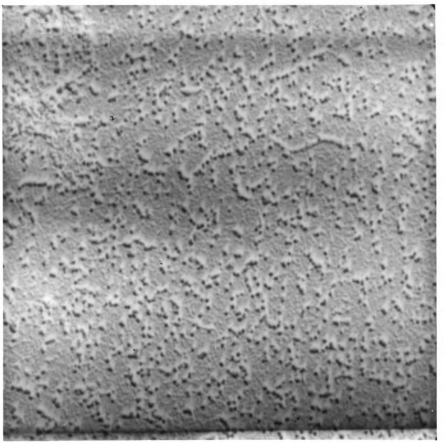


Figure 15. Electron micrograph of mammary gland polyribosome preparations. One absorbance unit (260 mμ) of normally prepared ribosomes and polyribosomes were layered onto a linear sucrose gradient (10 to 34%) containing 10 mM Tris-HCl buffer (pH 7.4), 25 mM KCl, and 5 mM $MgCl_2$ (total volume, 4.8 ml). The gradient was centrifuged at 39,000 rpm for 1.5 hours and the polyribosome fraction was isolated and used for the electron microscopic analysis. Droplets of this polyribosome sample was placed on Formvar coated copper grids and washed several times (see section on Methods). The grids were then shadowed with tungsten oxide and photographed in an RCA, type EMU 2A, electron microscope. Magnification of the photograph is approximately 45,000.

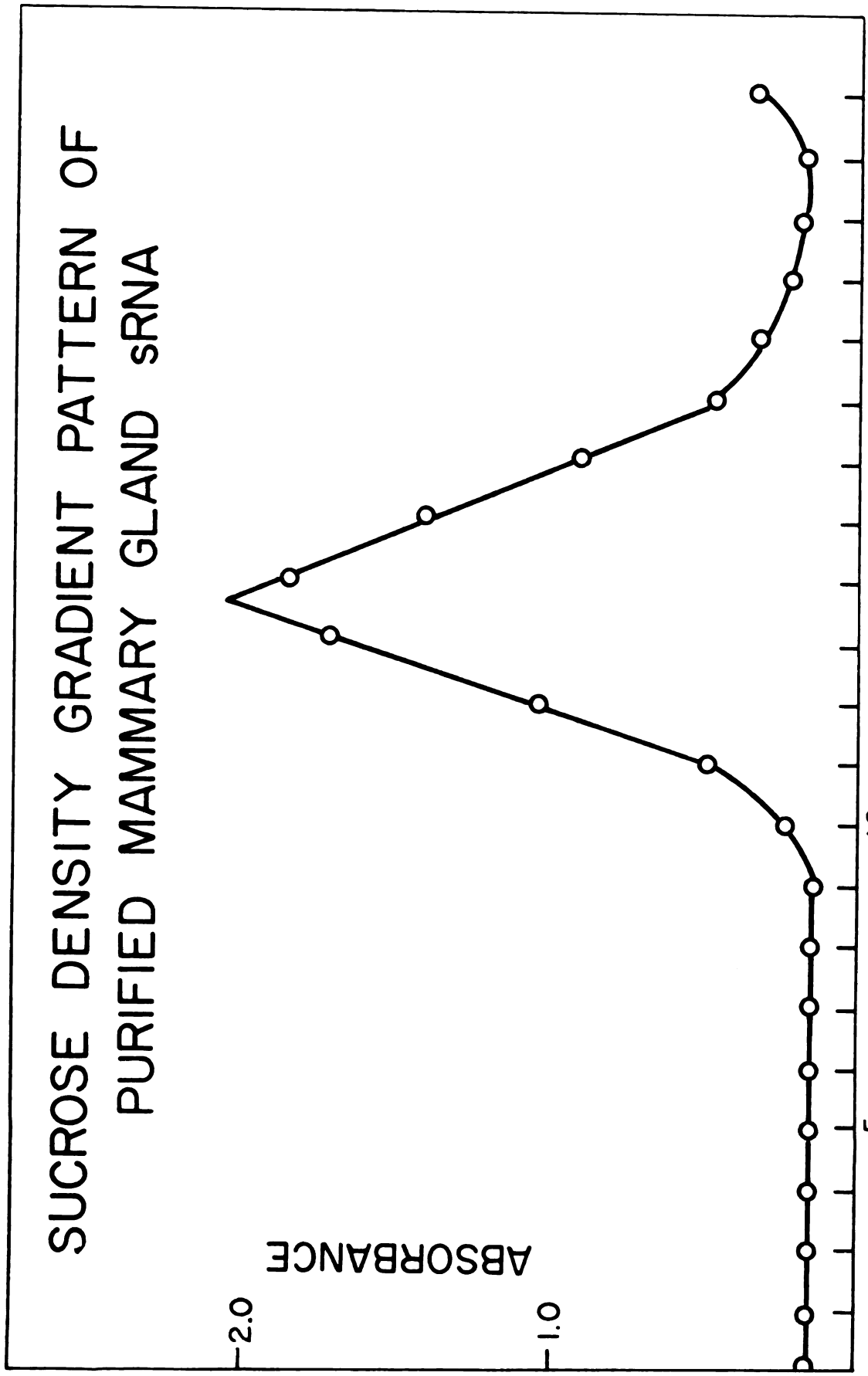
peroxidase was used as a marker of known sedimentation coefficient.

Results of this study are illustrated in Figure 16 and indicate that the sRNA preparation was homogeneous and free of other RNA's such as ribosomal RNA. Calculation of the sedimentation coefficient by the method of Martin and Ames (120) gave an S^{w}_{20} value of 4.1.

Esterification of Amino Acids to sRNA--If the isolated mammary gland sRNA is to function as an intermediate in protein synthesis in a manner similar to that found in other cell-free systems (2), enzymatic esterification of amino acids to the sRNA must occur. This property was tested by the following experiment.

The incubation mixture of 3.0 ml volume contained 0.15 mg of sRNA, 15 μmoles of ATP, 15 μmoles of $MgCl_2$, 300 μmoles of Tris-HCl buffer, pH 7.4, a total of 2 μmoles of an amino acid mixture minus glutamate whose composition was equal to that of whole casein (see Appendix Table 2), and 0.02 μc of L-glutamate-U-C^{14} possessing a specific activity of 9.5 μc per μmole. The esterification was catalyzed either by aminoacyl-sRNA synthetases precipitated at pH 5 (pH 5 enzyme) or by the same enzymes isolated by ammonium sulfate fractionation (AS_{70} enzyme). Isolation procedures of both enzyme fractions were described in the Methods section. This incubation mixture minus the addition of sRNA served as a control. The system was incubated for 30 minutes at 37°C and then the sRNA was reisolated to determine

Figure 16. Sucrose density gradient pattern of purified mammary gland sRNA. The sRNA was isolated and purified from mammary tissue according to the method of Rosenbaum and Brown (118). Nine absorbance units (260 mμ) of sRNA dissolved in 0.1 ml of 0.01 M phosphate buffer (pH 7.0) was layered onto a linear sucrose gradient of 5 to 20% sucrose containing 25 mM KCl, 10 mM Tris-HCl buffer (pH 7.0), and 5 mM $MgCl_2$ (final volume 4.8 ml). The sRNA solution also contained 5 μg horseradish peroxidase as a marker of known sedimentation coefficient. The gradient was centrifuged 15 hours at 39,000 rpm in a SW 39 rotor at 4°C. The contents of the centrifuge tube were then divided into 22 fractions containing 6 drops per fraction. The sRNA content of each fraction was determined from the absorbance at 260 mμ (119). Peroxidase activity in the various fractions was determined as described under Methods.


SUCROSE DENSITY GRADIENT PATTERN OF
PURIFIED MAMMARY GLAND sRNA
ABSORBANCE
2.0
1.0
5
10
15
20
FRACTION NUMBER

the radioactivity as also described in the Methods section. The results of this experiment are presented in Table 4.

Both the AS_{70} and the pH 5 enzyme catalyzed the esterification of glutamate and the isolated sRNA. Under the conditions used, the AS_{70} enzyme catalyzed the esterification of 0.1% of the added glutamate while the pH 5 enzyme catalyzed the esterification of 0.17% at the end of the 30 minute incubation.

These data indicate that the purified mammary gland sRNA can be charged with amino acids and thus may serve as an intermediate in milk protein synthesis. It has been assumed that, in addition to glutamyl-sRNA, sRNA's specific for every other amino acid found in proteins are present in the preparation.

Enzymatic Activities of Various Fractions

Aminoacyl-sRNA Synthetase Activity--Before amino acids can be incorporated into proteins, activation by the formation of the aminoacyl-AMP compound must occur. This activated amino acid must then react with sRNA to form an aminoacyl-sRNA. Both reactions have been shown to be catalyzed by the aminoacyl-sRNA synthetases. Aminoacyl-sRNA had previously been shown to be synthesized by the mammary gland preparations. The next study was designed to demonstrate the initial reaction in aminoacyl-sRNA synthesis; namely, the activation of amino acids.

TABLE 4

Esterification of L-glutamate-U-C^{14} to mammary gland sRNA

The 3.0 ml incubation mixture contained 0.15 mg of sRNA, 15 µmoles of ATP, 15 µmoles of $MgCl_2$, 300 µmoles of Tris-HCl buffer, pH 7.4, 2 µmoles of mixed amino acids minus glutamate (see Appendix Table 2 for composition), 0.02 µc of L-glutamate-U-C^{14} (9.5 µc/µmole) and the enzyme fraction. The preparation of the enzyme fractions were described in the Methods section. The reaction mixture was incubated for 30 minutes at 37°C and the sRNA isolated and assayed for radioactivity (see Methods for isolation procedures).

Preparation	mg of enzyme	mg sRNA[1]	net dpm[2]	% C^{14} glutamate esterified to sRNA
AS_{70} enzyme	15.0	0.15	567	0.104%
pH 5 enzyme	11.0	0.15	901	0.166%

[1] Based on 24 absorbance units (260 mµ for 1 mg sRNA/ml (119)

[2] Corrected for the control containing no added sRNA

Partial purification of aminoacyl-sRNA synthetases by acid precipitation was studied by measuring the extent of hydroxamate formation of amino acid which was catalyzed by the various fractions. The complete incubation mixture contained 10 μmoles of ATP, 0.7 ml of the 0.02 M amino acid mixture whose composition was comparable to that of whole casein, 10 μmoles of $MgCl_2$, 20 μmoles of Tris-HCl buffer, pH 7.4, the enzyme fraction, and 1000 μmoles of neutralized hydroxylamine in a total volume of 3 ml. After incubation for 30 minutes, the amount of amino acid hydroxamates formed was measured as described by Berg (121).

Results of this study are presented in Table 5. The specific activity of the pH 5 precipitate was nearly 20 times that of the crude homogenate. Insignificant activity remained in the pH 5 supernatant indicating that enzymes which activate all the amino acids were precipitated at pH 5.

Aminoacyl-sRNA synthetase activity was also measured in the pH 5 and AS_{70} enzyme fractions by determining the rate of exchange of inorganic pyrophosphate into ATP according to the method of Bucovaz and Davis (106). The one ml incubation mixture contained 100 μmoles of Tris-HCl buffer, pH 7.4, 5 μmoles of ATP, 5 μmoles of sodium pyrophosphate containing 100,000 cpm, 5 μmoles of $MgCl_2$, 0.5 ml of the 0.02 M amino acid mixture whose composition was similar to that of whole casein, 50 μmoles KF, and 1 mg of the enzyme fraction. The mixture was incubated for 30 minutes. After the reaction was stopped by the addition of trichloroacetic

TABLE 5

Purification of aminoacyl-sRNA synthetases based upon hydroxamate formation

The fractions which were assayed for aminoacyl-sRNA synthetase activity were isolated from mammary tissue as described in the section on Methods. The incubation mixture contained 10 μmoles of ATP, 0.7 ml of the 0.02 M amino acid mixture (see Appendix Table 2 for composition), 10 μmoles of $MgCl_2$, 20 μmoles of Tris-HCl buffer, pH 7.4, 1000 μmoles of neutralized hydroxylamine, and aliquots of the various mammary tissue fractions in a final volume of 3.0 ml. The measurement of the aminoacyl-hydroxamates formed was described in the Methods section.

Fraction	Control[1]	Complete	Protein (mg)	Specific Activity[2]
Crude Homogenate	3.70[3]	4.20	77.0	.0144
15,000 x g Supernatant	2.06	3.50	34.0	.042
105,000 x g Supernatant	2.70	3.50	12.0	.033
pH 5 Supernatant	1.52	1.72	10.5	.0192
pH 5 Precipitate	1.60	4.32	10.4	.260

[1] Incubation mixture minus amino acids

[2] Specific activity units: μmoles/mg protein/hour

[3] All values are expressed as μmoles hydroxamates formed per hour

acid, Norit A was added to separate the ATP from the reaction mixture. After several washed of the Norit A, the specific radioactivity of the bound ATP was determined by procedures described in the Methods section.

The activities of the pH 5 enzyme and the AS_{70} enzyme are presented in Table 6. Although both enzymes catalyzed the exchange reaction, the AS_{70} enzyme possessed 3 times higher specific activity than the pH 5 enzyme. Therefore, these results suggest that the AS_{70} enzyme would be the preparation of choice, but results in the next study give reasons for utilizing the pH 5 enzyme in future acid incorporation studies.

Ribonuclease Activity--Many investigators have shown that ribonuclease is a potent inhibitor of protein synthesis in cell-free systems (58-61). Accordingly, to obtain maximal amino acid incorporation by cell-free systems, components must possess minimal ribonuclease activity.

The subsequent study was conducted to ascertain the ribonuclease activity of the different preparations from the mammary gland. Both the pH 5 and the AS_{70} enzymes were prepared by the usual procedures (see section on Methods) and assayed for ribonuclease activity according to the method of Kalnitsky (123) by measuring the release of acid-soluble nucleotides from the RNA substrate.

The ribonuclease activity of many fractions of mammary tissue is shown in Table 7. Concerning the pH 5 enzyme

TABLE 6

Aminoacyl-sRNA synthetase activity as measured by radioactive pyrophosphate exchange into ATP

The isolation procedures for the preparation of these enzyme fractions were described in the Methods section. The incubation mixture contained 100 µmoles of Tris-HCl buffer, pH 7.4, 5 µmoles of ATP, 5 µmoles of sodium pyrophosphate containing 100,000 cpm, 5 µmoles of $MgCl_2$, 0.5 ml of the amino acid mixture (see Appendix Table 2), 50 µmoles of KF, and 1 mg of the enzyme preparation. Both enzyme preparations were dissolved in 0.05 M phosphate buffer, pH 7.4. The reaction mixture was incubated 30 minutes and the amount of radioactivity exchanged into the ATP was measured (see Methods for procedure).

Enzyme	Control[1]	Complete	Protein (mg)	Specific Activity[2]
pH 5 enzyme	1.21[3]	3.64[3]	1.0	2.43
AS_{70} enzyme	0.358[3]	8.24[3]	1.0	7.89

[1] Control contained no added amino acids

[2] Specific activity units = µmoles/mg protein/hour

[3] Values are expressed as µmoles pyrophosphate exchanged per hour

TABLE 7

Ribonuclease activities of various fractions

The procedures for preparing fractions which were assayed for ribonuclease activity and the procedure for assaying ribonuclease activity are described in the Methods section. The rate is expressed as the amount of acid soluble nucleotides released from RNA per unit of time.

	Preparation	Specific Activity (units[1]/mg proteins)
	Crude Homogenate	0.033
	15,000 x g Supernatant	0.072
	105,000 x g Supernatant	0.076
I.	pH 5 enzyme Supernatant[2]	0.40
	pH 5 enzyme Precipitate[2]	0.070
	pH 5 enzyme Supernatant[3]	0.330
	pH 5 enzyme Precipitate[3]	0.045
II.	40% Ammonium Sulphate Precipitate	0.065
	70% Ammonium Sulfate Supernatant	0.077
	70% Ammonium Sulfate Precipitate (AS_{70} enzyme)	0.460

[1] Unit = amount of acid-soluble nucleotides that give an increase in absorbancy of 1 unit per minute at 260 mμ (1 cm light path)

[2] Results of first pH 5 precipitation

[3] Results of reprecipitation of the first pH 5 precipitate

preparation, most of the ribonuclease was in the pH 5 enzyme supernatant. A reprecipatation of pH 5 enzyme further reduced the ribonuclease activity in the pH 5 enzyme precipitate. In contrast, most of the ribonuclease remained with the AS_{70} enzyme rather than being removed by fractionation.

PART II

IN VITRO PROTEIN SYNTHESIS

C^{14} Leucine Incorporation by a Crude Homogenate

The previous discussion dealt with the cellular components which comprise a cell-free system that synthesizes protein. The second part of this thesis will relate to experiments concerning amino acid incorporation by a complete protein synthesizing system composed of the previously characterized cellular components plus other non-cellular constituents. However, before studying amino acid incorporation by the complete system composed of defined components, incorporation was first verified in crude preparations of mammary tissue; e.g. crude homogenates and single-cell suspensions.

To study amino acid incorporation in a crude homogenate, fresh mammary tissue was chopped into small pieces and washed three times with Hanks' basic salt solution (see Appendix Table 3). The tissue was again extensively macerated with scissors and suspended in Hanks' solution at a concentration of 0.5 g per ml. Three-tenths of a gram of tissue was incubated with 0.35 mg of penicillin G, and 2.0 μc of L-leucine-U-C^{14} (251 μc/μmole) in a final volume of 2 ml. Aliquots were taken after incubating the mixture at 37°C for 0, 1, and

2 hours. The reactions were stopped by adding 5% trichloroacetic acid. The protein precipitates were washed twice with 5% trichloroacetic acid, dissolved with 1 N sodium hydroxide, reprecipitated with 5% trichloroacetic acid, dehydrated with acetone and diethyl ether and assayed for radioactivity by the method of Casjens and Morris (65).

This particular system incorporated 16.9, 144.12, and 162.6 μμmoles of the added leucine per gram of tissue for 0, 1, and 2 hours respectively (Table 8). Because the effective specific activity of the L-leucine-U-C^{14} was significantly reduced by 1) the intracellular pool of free leucine and 2) the quantity of non-synthetic connective tissue present, the observed amount of incorporation represents only a part of the total amount of protein synthesis actually occurring. But these results do show that mammary gland tissue which has been isolated from the animal, homogenized, and incubated at 37°C can synthesize protein.

C^{14} Leucine Incorporation by Single-Cell Suspensions

Ebner et. al. (115) reported that a single cell suspension of mammary tissue was capable of synthesizing protein. Therefore, it was decided to measure the amount of C^{14} leucine incorporated into trichloroacetic acid-precipitated material and, in addition, to compare its level of incorporation to that found above with the crude homogenate.

One gram of collagenase-isolated cells (see section on Methods) were suspended in 4.0 ml of Hanks' solution

TABLE 8

C^{14} leucine incorporation by a crude homogenate of mammary tissue

Each incubation mixture contained 0.3 g of homogenized tissue, 0.35 mg of penicillin G, and 2.0 µc of L-leucine-U-C^{14} (251 µc/µmole) in a final volume of 2 ml. The mixture was incubated for the various times at 37°C with intermittent shaking. Amino acid incorporation was assayed according to the method of Casjens and Morris (65) as described in the Methods section. The cpm incorporated were corrected for quenching. The efficiency of counting the external C^{14} benzoic acid standard was 82%.

Incubation Time (hours)	cpm Incorporated	µµmoles incorporated per g tissue
0	2,863	16.9
1	19,532	144.2
2	22,057	162.6

(see Appendix Table 3) which also contained 40 μc of L-leucine-U-C^{14} (0.9 μc/μmole) and 0.4 ml of the 0.2 M amino acid mixture minus leucine (see Appendix Table 2). One ml aliquots were taken after 0, 1, 10 and 60 minutes of incubation at 37°C. The reaction was stopped by the addition of trichloroacetic acid (5% w/v) and the radioactivity associated with the protein was determined as described in the previous experiment.

The results of this study are presented in Table 9. The level of incorporation at 60 minutes was about seven times the 0 time control. The 60 minute level of incorporation of leucine (corrected for 0 time values) in the single cell suspension was 40 times greater than the amount of leucine incorporation by the crude homogenate. The amount of synthesis per gram of tissue for the crude homogenate was reduced an unknown amount by the presence of nonsynthetic connective tissue. Yet, both crude systems did show significant leucine incorporation and suggested that partial purification and recombination of the correct cellular components would perform similarly.

Amino Acid Incorporation by the Cell-Free System

The study of milk protein synthesis was then extended to a cell-free system utilizing the components which were defined in the first sections of the thesis. A discussion of many characteristics of C^{14} leucine incorporation by the cell-free system such as time, microsome, energy, and

TABLE 9

C^{14} leucine incorporation by isolated mammary gland cells

The incubation mixture contained 1 g of collagenase-isolated cells (see Methods for preparation procedures), 4 ml of Hanks' basic salt solution as described in Appendix Table 3, and 40 μc of L-leucine-U-C^{14} (0.98 μc/μmole). One ml aliquots were removed at time intervals from the incubation mixture and assayed for amino acid incorporation. Efficiency of counting an external standard was 72%.

Time (min.)	cpm/g cells	μμmoles leucine incorporated / g cells
0	1565	1097
1	1955	1372
10	3250	2280
60	9940	6970

magnesium dependence will be presented. This discussion will be completed by the partial identification of the proteins that were synthesized by this cell-free system.

Amino acid incorporation was studied in the complete system composed of the following components dissolved in 1 ml volume: Microsomes (0.3 to 0.6 mg), sRNA (50 μg), pH 5 enzyme (1 to 2 mg), ATP (1 μmole), GTP (0.25 μmole), phosphoenol pyruvate (5 μmoles), pyruvate kinase (40 μg), KCl (50 μmoles), $MgCl_2$ (5 μmoles), reduced glutathione (20 μmoles), amino acid mixture minus leucine whose composition was comparable to that of whole casein (1.0 μmole total), Tris-HCl buffer, pH 7.4, (50 μmoles), and L-leucine-U-C^{14} (1 to 1.5 μc, specific activity 200 to 250 μc/μmole). This complete system was incubated at 37°C for 40 minutes. The reaction was stopped by the addition of 6 mg of carrier protein (bovine serum albumin) and 10 ml of trichloroacetic acid (5% w/v). The precipitated protein was washed twice more with trichloroacetic acid, dissolved in 1 N sodium hydroxide, reprecipitated and washed once again with trichloroacetic acid, dehydrated with acetone-HCl and ether, and assayed for radioactivity according to the method of Casjens and Morris (65). Unless specified, this will be the composition of the complete system which will be utilized in later experiments. Once such variation was the inclusion of the AS_{70} enzyme instead of the pH 5 enzyme in certain experiments.

C^{14} Leucine Incorporation vs Time of Incubation--The time dependence of C^{14} leucine incorporation was determined by stopping the reactions at 0, 5, 10, 20, 40, and 60 minutes by the addition of trichloroacetic acid (5% w/v) and measuring the radioactivity that was associated with the washed precipitate (Figure 17). Incorporation into trichloroacetic acid precipitable material was linear for the first twenty minutes and was nearly completed after 40 minutes. However, when the complete system was incubated overnight, incorporation reached a very high level probably due to bacterial growth. Microbial growth appeared to contribute insignificantly to the observed incorporation in the short term experiments since the addition of 35 µg of penicillin G did not reduce the incorporation of leucine. The amount of radioactivity at 40 minutes was usually one hundred to five hundred times that at 0 time. When the AS_{70} enzyme was utilized instead of the pH 5 enzyme, incorporation increased linearly with time for 10 minutes and reached a maximum at 40 minutes (Figure 18).

The reason for the cessation of incorporation is not known since the addition of more components after incorporation had ceased was not performed. However, the failure may have resulted from the lack of integrity of the ribosomes and polyribosomes. In order to test this hypothesis, the following experiment was conducted. The complete system was incubated for 0, 10, and 40 minutes at 37°C. After incubation aliquots of the mixtures were subjected to the usual

Figure 17. C^{14} leucine incorporation vs time using pH 5 enzyme. The standard assay mixture contained microsomes (0.64 mg), pH 5 enzyme (2.2 mg), L-leucine-U-C^{14} (1.0 μc/μmole) and the usual amounts of the other components. Amino acid incorporation was determined by the usual methods (see section on Methods).

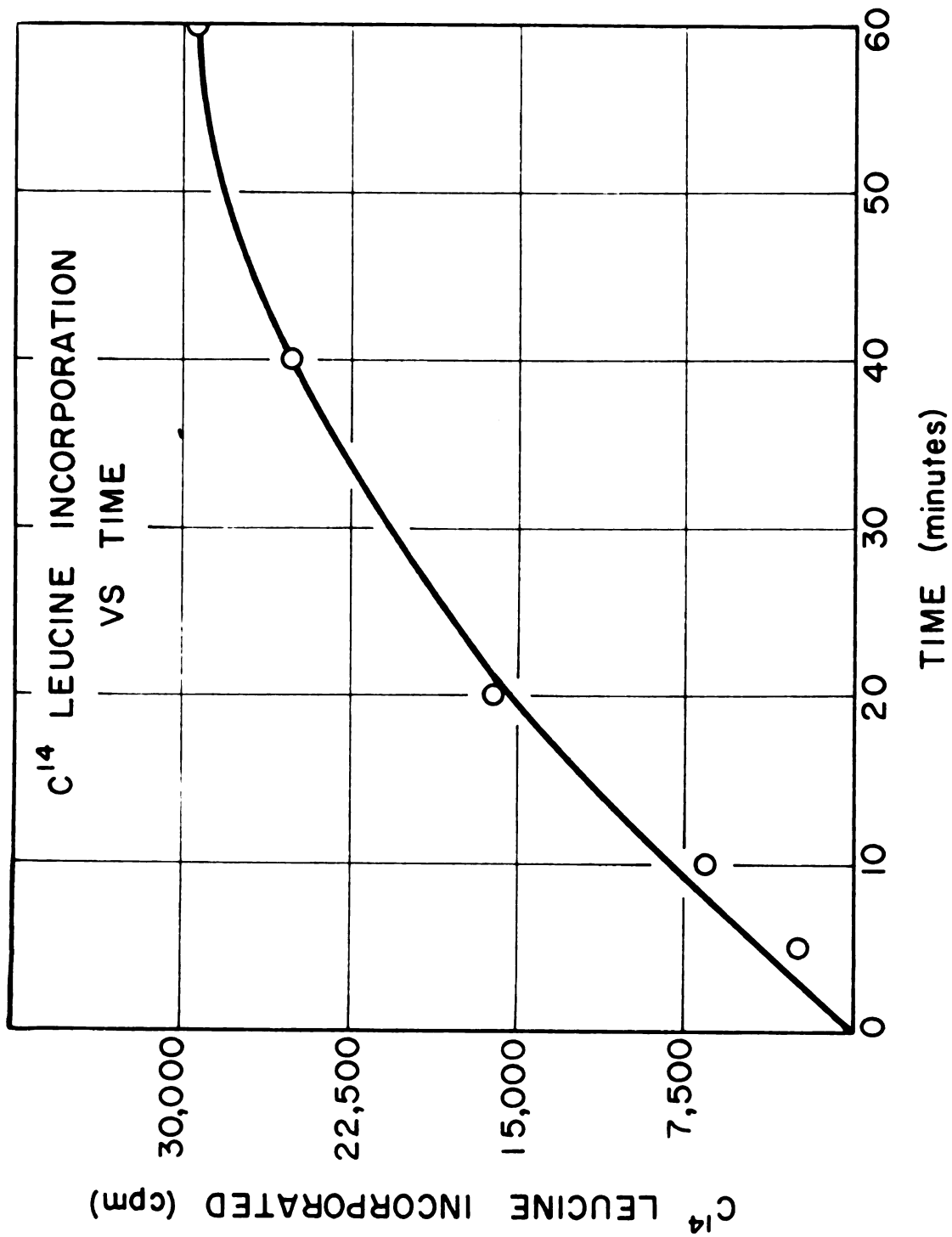
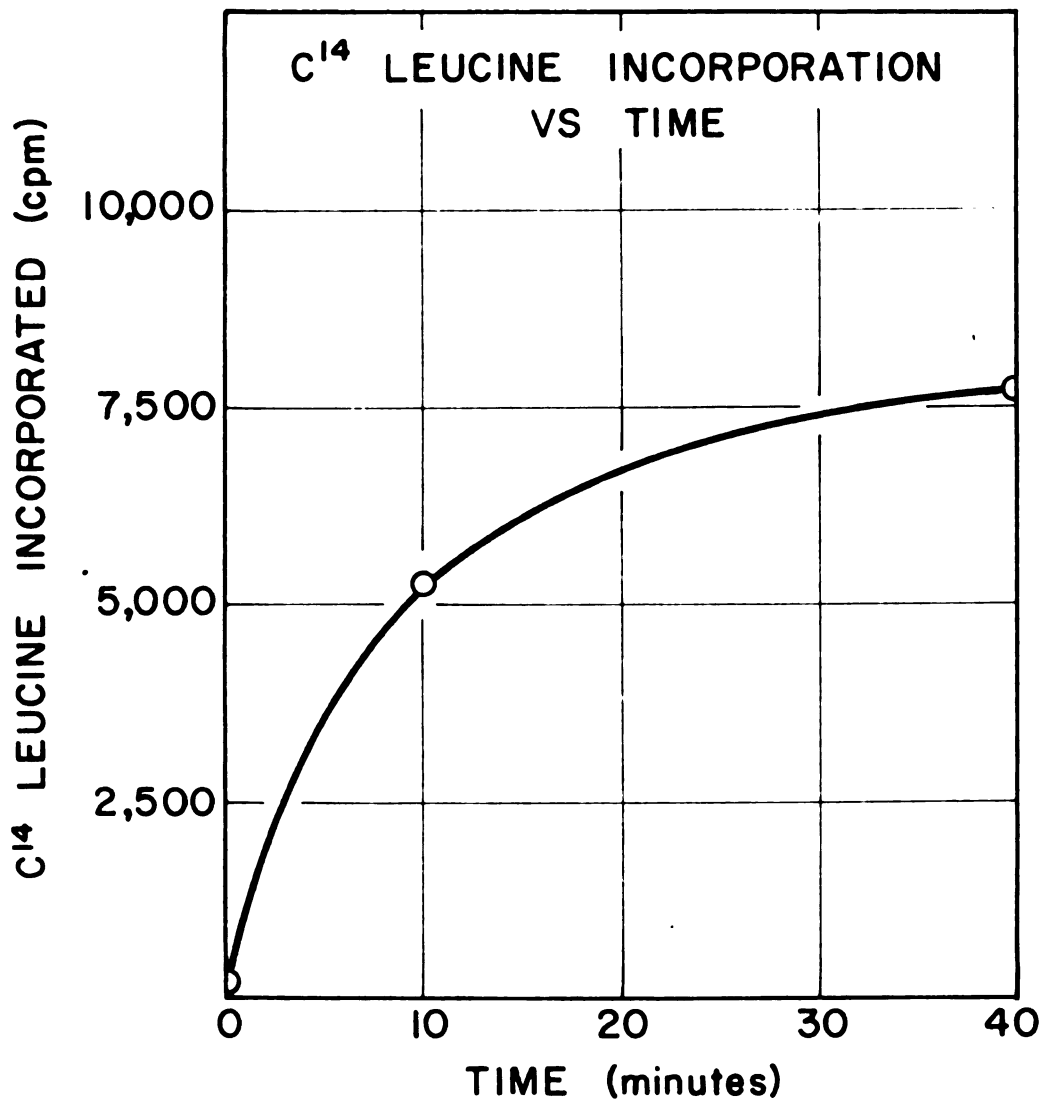

C14 LEUCINE INCORPORATION VS TIME
C14 LEUCINE INCORPORATED (cpm)
30,000
22,500
15,000
7,500
0
10
20
30
40
50
60
TIME (minutes)

Figure 18. C^{14} leucine incorporation vs time using AS_{70} fraction. The standard assay mixture contained microsomes (1.4 mg), AS_{70} enzyme (1.7 mg), and L-leucine-U-C^{14} (1.5 µc, 210 µc/µmole), and the usual amounts of the other components. Amino acid incorporation was determined by the usual procedures (see section on Methods).

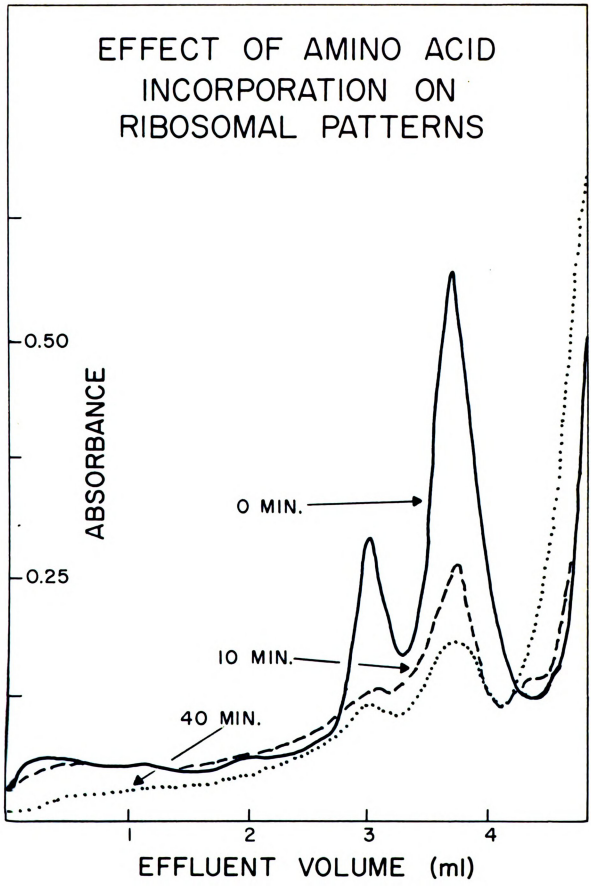

C14 LEUCINE INCORPORATION
VS TIME
C14 LEUCINE INCORPORATED (cpm)
10,000
7,500
5,000
2,500
0
10
20
30
40
TIME (minutes)

sucrose density gradient centrifugation for analysis of the ribosomal components (Figure 19). The 0, 10, and 40 minute sample had incorporated 158, 6,770, and 19,183 cpm, respectively, when assayed by the usual method. The results demonstrate a time-dependent degradation of the ribosomes associated with each peak of the ribosomal pattern. Although it is impossible to decide whether this degradation was a direct result of protein synthesis, as described by Noll et. al. (138), or the result of ribonuclease activity, the ribosomes and polyribosomes were unstable under these conditions.

This observed instability suggests that incorporation ceases after 40 minutes of incubation because the amount of active polyribosomes and ribosomes present in the assay mixture were insufficient to sustain incorporation. These results agree with those of Korner (60), who postulated that amino acid incorporation reached a maximum level after one hour because liver microsomes were unstable to incubation of the complete system at 37°C. Allen and Schweet (58), Sing et. al. (139), and Talal (61) also reported that amino acid incorporation reached a maximum after 1 hour of incubation in the reticulocyte, thyroid, and spleen cell-free protein synthesizing systems, respectively. However, no specific causes for this phenomenon was observed or proposed.

Incorporation of C^{14} Leucine into Mammary Gland Microsomes--Studies in mammalian reticulocyte, liver, and

Figure 19. Effect of amino acid incorporation on the sedimentation behavior of microsomes. The standard assay mixture contained microsomes (0.4 mg), pH 5 enzyme (1.2 mg), L-leucine-U-C^{14} (1.25 μc, 210 μc/μmole), and the usual amounts of the other constituents. The mixtures were incubated for 0, 10, and 40 minutes and then equal aliquots of each sample (0.1 ml) were layered onto a linear sucrose gradient of 10 to 34% sucrose containing 25 mM KCl, 5 mM $MgCl_2$, and 10 mM Tris-HCl buffer (pH 7.5). The gradients were centrifuged for 1.5 hours at 39,000 rpm in a SW 39 rotor at 4°C. The contents of the centrifuge tubes were then analyzed for ribosomal and polyribosomal content (see section on Methods).


EFFECT OF AMINO ACID
INCORPORATION ON
RIBOSOMAL PATTERNS
0.50
0.25
ABSORBANCE
0 MIN.
10 MIN.
40 MIN.
1
2
3
4
EFFLUENT VOLUME (ml)

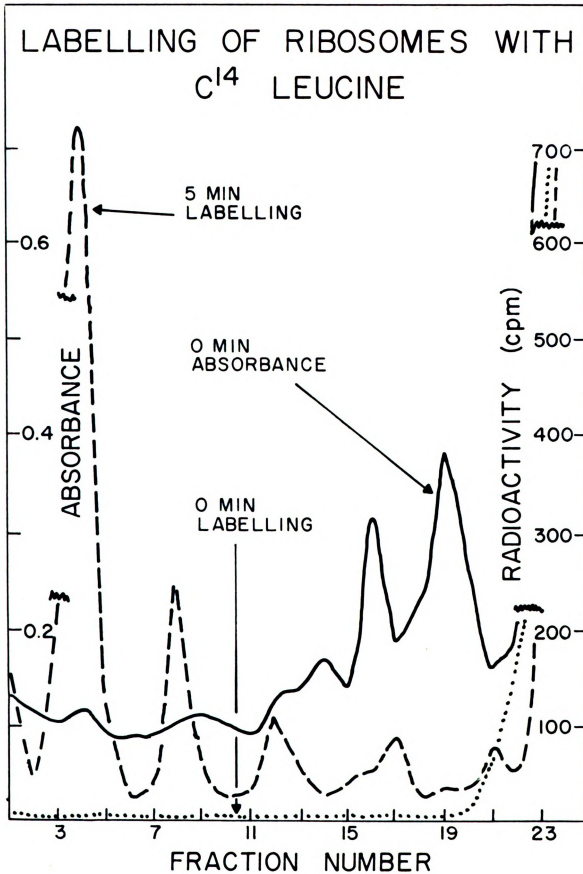
bacterial cell-free protein synthesizing systems indicated that protein synthesis was performed by aggregates of ribosomes complexed with messenger RNA (129, 140, 141). Similarly the function of polyribosomes in the incorporation of C^{14} leucine was investigated by means of the next two experiments. The complete system was incubated for 0 and 5 minutes. Aliquots of the mixtures were then subjected to the usual sucrose density gradient centrifugation; equal volume fractions were collected and the radioactivity in each was determined.

The 0 time ribosomal pattern and the amount of radioactivity in each fraction of the gradients which contained an aliquot of the 0 and 5 minute incubation mixture are presented in Figure 20. No C^{14} leucine was bound to the ribosomes or polyribosomes in the non-incubated system. After incubation for 5 minutes, C^{14} leucine became attached to the polyribosomes. In this test insignificant radioactivity was observed in the fractions containing the monomeric ribosomes. The 0 time and 5 minute samples had incorporated 87 and 7,361 cpm, respectively, when assayed by the usual methods.

These data suggest that the mammary gland polyribosomes rather than ribosomes are most active in protein synthesis. Also, these data emphasize the importance of obtaining microsome preparations for amino acid incorporation studies which contain the highest possible proportion of polyribosomes.

The next experiment was also designed to study the

Figure 20. Labeling of ribosomes and polyribosomes with C^{14} leucine. The standard assay mixture contained microsomes (0.4 mg), pH 5 enzyme (0.8 mg), and L-leucine-U-C^{14} (0.7 μc, 225 μc/μmole), and the usual amounts of the other constituents. Three-tenths ml of the samples incubated for 0 and 5 minutes were layered onto a linear sucrose gradient of 10 to 34% sucrose containing 10 mM Tris-HCl buffer (pH 7.5), 25 mM KCl, and 5 mM $MgCl_2$ (final volume 12.0 ml). The gradients were centrifuged at 40,000 rpm for 1 hour and 45 minutes at 4°C in a Model SB-283 rotor with a B-60 International Preparative Ultracentrifuge. Fractions were collected (16 drops) and the absorbance (260 mμ) and radioactivity were determined in each fraction.


LABELLING OF RIBOSOMES WITH
C14 LEUCINE
5 MIN
LABELLING
0 MIN
ABSORBANCE
0 MIN
LABELLING
ABSORBANCE
RADIOACTIVITY (cpm)
0.6
0.4
0.2
700
600
500
400
300
200
100
3
7
11
15
19
23
FRACTION NUMBER

function of polyribosomes in amino acid incorporation. Microsomes were layered onto a usual sucrose gradient which was subsequently centrifuged and divided into ten equal fractions. An aliquot of each fraction was utilized as the source of microsomes in the complete protein synthesizing system. Incorporation was assayed by the method previously described. The results of this experiment are presented in Table 10. The polyribosomes were 2-3 times more efficient in C^{14} leucine incorporation than the ribosomes whose peak was located between fraction 6 and 7.

Henshaw et. al. (141) obtained similar results with a rat liver cell-free system. The rat liver polyribosomes were 2.5 times more efficient in synthesizing protein than ribosomes. When poly U was added to their ribosome preparation, phenylalanine incorporation by the ribosomes equalled that of the polyribosomes; this suggests that messenger RNA limited synthesis by the ribosomes. The behavior of mammary gland ribosomes with respect to synthetic messenger RNA is unknown.

The Effect of the Amount of Microsomes on C^{14} Leucine Incorporation--Many investigators (1, 2, 3) have determined the dependence of amino acid incorporation upon the amount of ribosomal fraction added to the cell-free system. For example, Allen and Schweet (58) observed a linear increase in incorporation as the reticulocyte ribosome and polyribosome concentration was increased to 8 mg per ml. The purpose

TABLE 10

Amino acid incorporation by gradient-fractionated microsomes

One and nine-tenths mg of normally prepared microsomes (0.3 ml) were subjected to sucrose gradient centrifugation in the Model B-60 International Ultracentrifuge as described in the Methods section. Fractions containing 1.33 ml were collected by puncturing the bottom of the centrifuge tube. (Fraction #1 is nearest the bottom). Three-tenths ml of each fraction was utilized in the standard assay mixture containing 1.2 mg of pH 5 enzyme and 1 μc of L-leucine-U-C^{14} (251 μc/μmole) for measuring amino acid incorporation. This mixture was incubated 40 minutes and the amount of amino acid incorporation was measured by the usual procedures. These assays of amino acid incorporation were conducted in duplicate. Seventy-five percent of the microsomes layered onto the gradient were recovered in the fractions collected.

Fraction	cpm Incorporated[1]	mg Ribosome (x 10^2)[2]	cpm/mg
1	32	2.32	1378
2	60	3.56	1087
3	80	3.40	2350
4	84	3.37	2490
5	94	4.34	2168
6	72	6.57	1095
7	58	6.10	952
8	11	2.20	500
9	8	1.46	546
10	0	--	0
Control	20	--	--

[1] Average of two determinations and corrected for the control which contained no ribosomes.

[2] Based on 11.3 absorbancy units (260 mμ) for 1.0 mg ribosomes per ml (116).

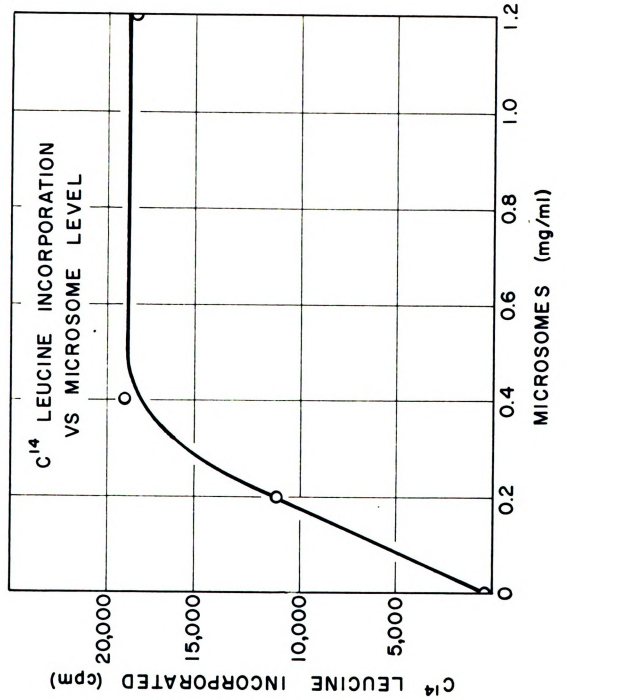
of the following experiment was to determine the dependency of C^{14} leucine incorporation on the microsome concentration in the mammary gland cell-free system. The microsome concentration was varied from 0 to 1.2 mg per ml while the concentration of the other components in the system was held constant. The mixtures were incubated for 40 minutes and the amount of incorporation determined by the usual method.

In the standard assay system, C^{14} leucine was incorporated at a level which was linearly dependent on the microsome concentration up to about 0.5 mg per ml. No incorporation occurred when the microsomes were omitted from the system. As Figure 21 indicates, incorporation was not significantly stimulated by further addition of microsomes suggesting that incorporation was limited by an insufficient amount of some other component in the system.

This preparation of microsomes contained bound ribonuclease (0.23 units/mg microsomes) whose activity may have been partly responsible for the non-linearity of incorporation with respect to microsome concentration. Elson (142) studied this phenomenon in relation to E. coli. protein biosynthesis and suggested that ribosomal-bound ribonuclease may play a role in controlling the synthesis of proteins in the cell-free system.

Dependence of Incorporation on the Source of Aminoacyl-sRNA Synthetases--Aminoacyl-sRNA synthetases catalyze the activation of amino acids and the subsequent esterification

Figure 21. C^{14} leucine incorporation vs microsome level. The standard assay mixture contained pH 5 enzyme (1.2 mg), L-leucine-U-C^{14} (1.25 µc, 210 µc/µmole), varying levels of microsomes, and the usual amounts of the other components. Amino acid incorporation was measured by the usual procedures (see section on Methods).


C14 LEUCINE INCORPORATED (cpm)
20,000
15,000
10,000
5,000
0
0.2
0.4
0.6
0.8
1.0
1.2
MICROSOMES (mg/ml)
C14 LEUCINE INCORPORATION
VS MICROSOME LEVEL

of the activated amino acids to sRNA. Both the pH 5 enzyme and the AS_{70} enzyme which were isolated from the mammary gland were shown in the first part of this thesis to possess these activities. The purpose of the next three experiments was to determine the amount of C^{14} leucine incorporated by the complete systems which contained either the 105,000 x g supernatant or aparticulate fraction (see section on Methods), pH 5 enzyme, or the AS_{70} enzyme as a source of aminoacyl-sRNA synthetases.

First, the aparticulate fraction was tested as a source of aminoacyl-sRNA synthetase activity. This system which contained the aparticulate fraction in addition to the other usual constituents was incubated 40 minutes. The amount of C^{14} leucine incorporated was then determined as usual. When 2.96 mg of the aparticulate fraction protein was added to the system, 2656 cpm were incorporated whereas only 842 cpm were incorporated when it was omitted from the system. Thus, nearly a four-fold stimulation in incorporation was observed by adding this preparation. However, the aparticulate fraction was not as efficient in stimulating protein synthesis as the pH 5 enzyme purified from this same preparation (see section on Methods for procedures). For example, 3,797 cpm were incorporated into protein by the system containing only 1.45 mg of protein as the pH 5 enzyme. This was to be expected since the specific activity of ribonuclease in the aparticulate fraction was nearly twice that contained in the pH 5 enzyme.

The dependence of amino acid incorporation on the

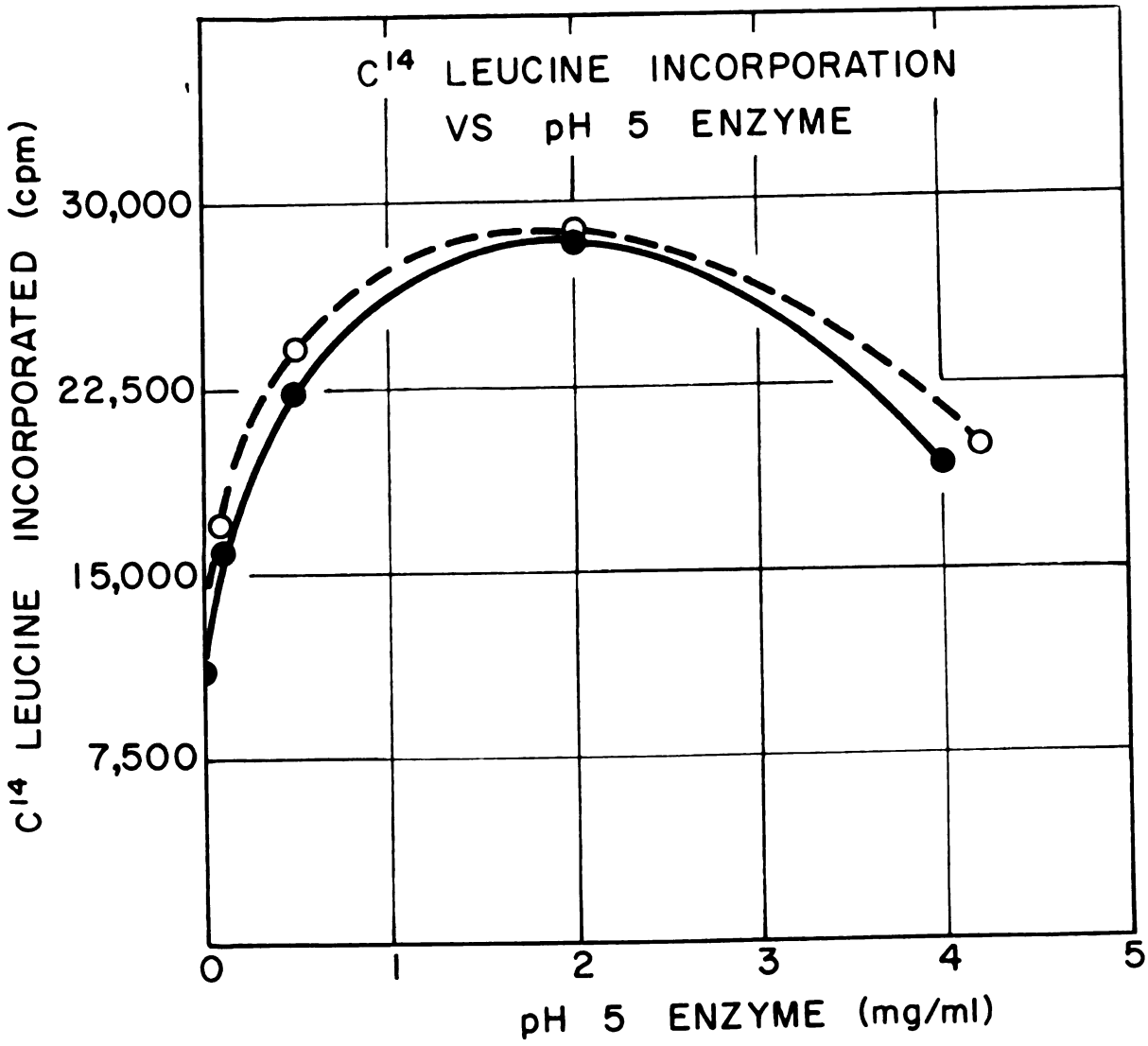
level of pH 5 enzyme was also tested. The normal pH 5 enzyme was purified by adjusting the aparticulate fraction (105,000 x g supernatant of the crude homogenate) to pH 5 and isolating the precipitate as described in the Methods section. The pH 5 enzyme was also isolated from an aliquot of the same aparticulate fraction which had previously been treated with neutralized protamine sulfate (1 mg per ml) to remove the sRNA which normally precipitates with the pH 5 enzyme. Varying levels of these enzyme preparations were added to the complete systems and the amount of C^{14} leucine incorporation after a 40 minute incubation was determined.

The results with both enzyme preparations were similar. With lower amounts of the pH 5 enzyme, amino acid incorporation was roughly proportional to enzyme concentration (Figure 22). At higher concentrations, the level of incorporation decreased. Incorporation occurring in the absence of pH 5 enzyme can be attributed to the aminoacyl-sRNA synthetases already present, probably bound as a complex with the microsomes. This decrease at higher levels of pH 5 enzyme may be due to the residual ribonuclease contamination in the enzyme preparations.

In short, these pH 5 enzymes were only partially required for maximum leucine incorporation. Removal of the sRNA from the pH 5 enzyme by protamine sulfate treatment caused no change in the amount of incorporation.

The AS_{70} enzyme rather than the pH 5 enzyme was utilized in the complete cell-free protein synthesizing system

Figure 22. C^{14} leucine incorporation vs pH 5 enzyme. The standard assay mixture contained microsomes (0.64 mg), L-leucine-U-C^{14} (1.0 µc, 254 µc/µmole), varying levels of pH 5 enzyme, and the usual amount of the other constituents. The solid line on the graph refers to data obtained with normally prepared pH 5 enzyme. The dashed line refers to data obtained with pH 5 enzyme prepared from cell sap which had been treated with protamine sulfate (1 mg/ml) (see section on Methods). Amino acid incorporation was determined by the usual procedures.

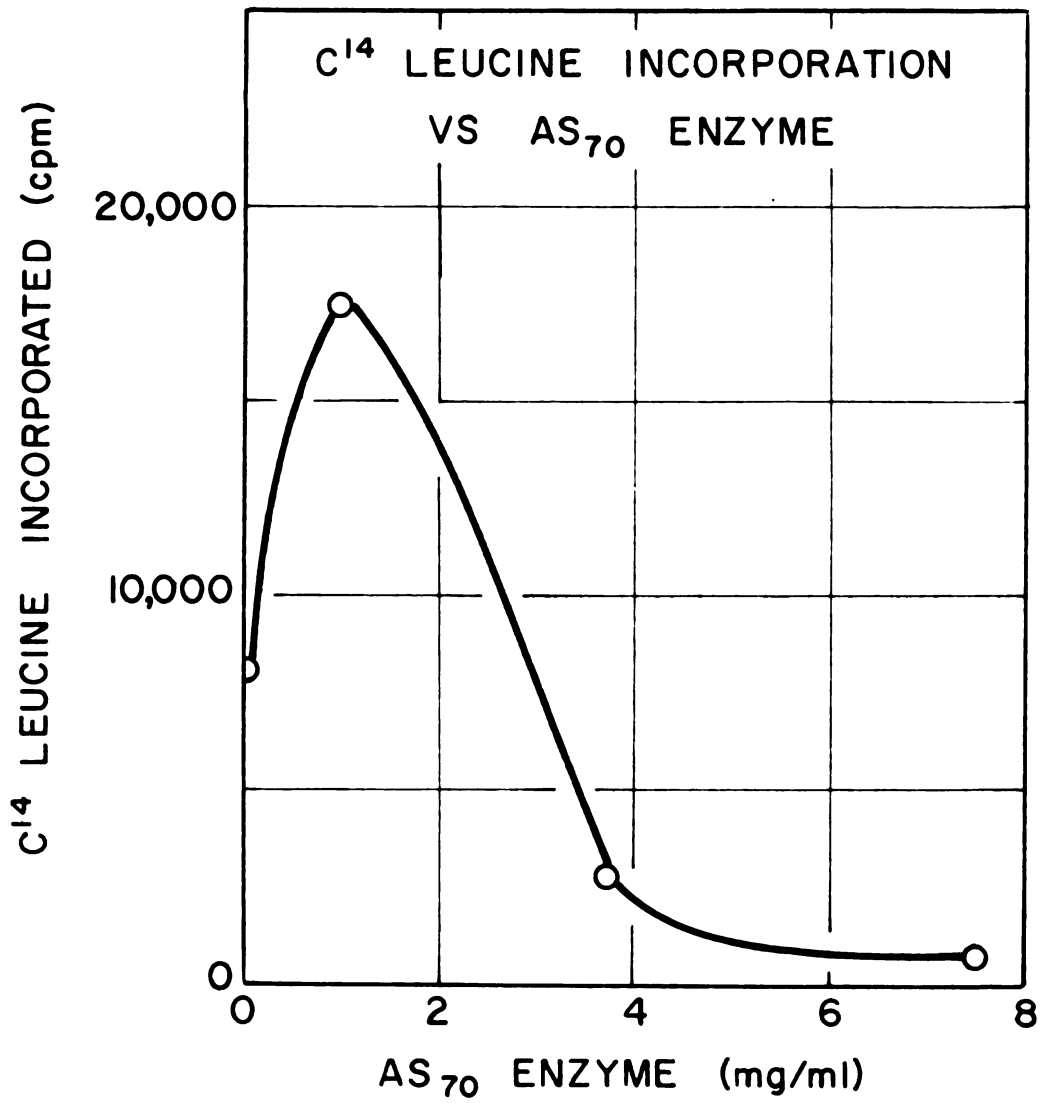

C14 LEUCINE INCORPORATION
VS pH 5 ENZYME
C14 LEUCINE INCORPORATED (cpm)
30,000
22,500
15,000
7,500
0
1
2
3
4
5
pH 5 ENZYME (mg/ml)

of reticulocytes (58). In this case, much lower levels of amino acid incorporation occurred when the pH 5 enzyme was used since most of the aminoacyl-sRNA synthetase activity was not precipitated at pH 5. Results from an earlier study indicated that the AS_{70} enzyme isolated from mammary tissue possesses three times the aminoacyl-sRNA synthetase activity of the pH 5 enzyme. Consequently, various levels of this AS_{70} enzyme was tested with the cell-free system instead of the pH 5 enzyme and the amount of amino acid incorporation was measured.

Incorporation of C^{14} leucine was greatly affected by the level of the AS_{70} enzyme added to the complete amino acid incorporating system (see Figure 23). The level of incorporation was maximal at approximately one mg per ml and decreased at higher concentrations of enzyme. This unusual dependence of incorporation on the AS_{70} enzyme concentration was probably caused by the high levels of ribonuclease activity in that enzyme preparation.

Dependence of C^{14} Leucine Incorporation on sRNA--Aminoacyl-sRNA has been shown to be an intermediate in protein synthesis (2); thus, sRNA must be present in a cell-free system which is capable of amino acid incorporation. The following experiment was conducted to determine the degree of dependence of incorporation on the addition of sRNA purified from the mammary gland by the method of Rosenbaum and Brown (118) (see section on Methods).

Figure 23. C^{14} leucine incorporation vs AS_{70} enzyme. The standard assay mixture contained microsomes (0.4 mg), L-leucine-U-C^{14} (1.25 µc, 210 µc/µmole), varying levels of AS_{70} enzyme and the usual amounts of the other constituents. Amino acid incorporation was determined by the usual procedures (see section on Methods).


C14 LEUCINE INCORPORATION
VS AS70 ENZYME
C14 LEUCINE INCORPORATED (cpm)
20,000
10,000
0
0
2
4
6
8
AS70 ENZYME (mg/ml)

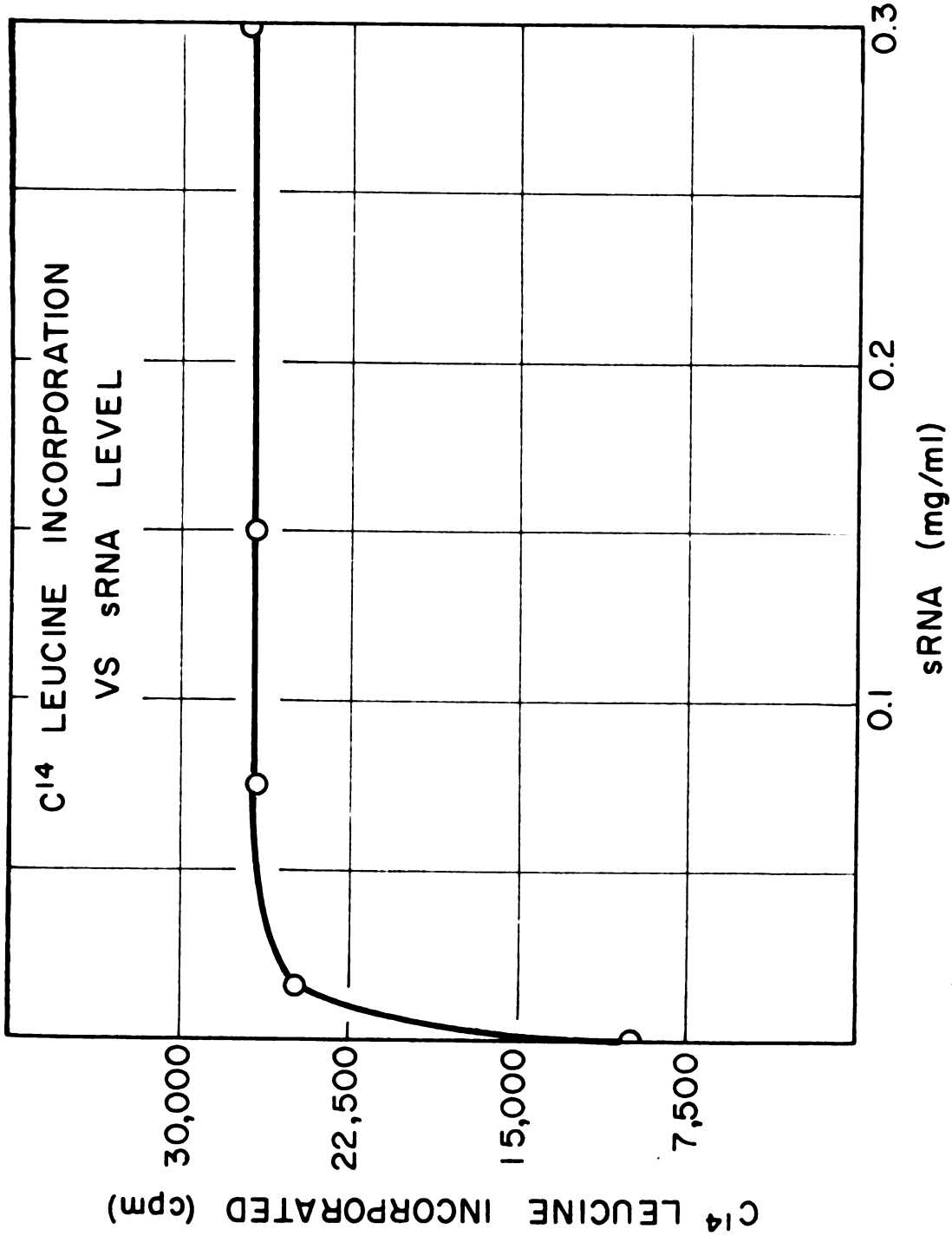
Purified sRNA was added at different levels to the complete system and radioactivity in the precipitated protein was determined. The pH 5 enzyme was isolated from an aparticulate fraction which was previously treated with neutralized protamine sulfate (1 mg/ml). Normal pH 5 enzyme (protamine sulfate step omitted) was used for comparative purposes.

The dependence of incorporation on sRNA addition is shown in Figure 24. Omission of the sRNA resulted in a 65% decrease when compared with the maximal level of incorporation. Incorporation reached its maximal level at approximately 50 μg per ml and was not altered by further additions. The presence of sRNA in the normal pH 5 enzyme was verified since omission of sRNA in the complete system containing this enzyme preparation resulted in only a 23% decrease in incorporation when compared to the maximum level of incorporation.

In summary, addition of sRNA to the cell-free system was necessary to obtain maximal incorporation. The normal pH 5 enzyme contained sRNA but the amount was inadequate to support maximal incorporation.

Stability of Microsomes and pH 5 Enzymes to Storage--Allen and Schweet (58) found that reticulocyte ribosomes stored at -20°C for 3 and 6 weeks retained 75% and 70% of their original activity, respectively. Although stored in the presence of reduced glutathione, 50% of the aminoacyl-sRNA synthetase activity in the reticulocyte AS_{70} enzyme was

Figure 24. C^{14} leucine incorporation vs sRNA level. The standard assay mixture contained microsomes (0.64 mg), pH 5 enzyme (2.1 mg) which was isolated from protamine sulfate-treated aparticulate fraction (see section on Methods), L-leucine-U-C^{14} (1.0 μc, 254 μc/μmole), varying levels of purified mammary gland sRNA, and the usual amounts of the other components. Amino acid incorporation after 40 minutes incubation was determined by the usual procedures.


C14 LEUCINE INCORPORATION
VS sRNA LEVEL
C14 LEUCINE INCORPORATED (cpm)
30,000
22,500
15,000
7,500
0.1
0.2
0.3
sRNA (mg/ml)

lost due to storage for 10 days at -20°C. In order to determine the stability of the mammary gland microsomes and pH 5 enzymes, the subsequent study was conducted.

Amino acid incorporation by a complete system composed of freshly prepared microsomes and pH 5 enzyme containing 0.001 M reduced glutathione was determined. After storing these components at -20°C for one month, they were added to a complete system and the amount of incorporation was again measured. Another test system containing one month old microsomes and freshly prepared pH 5 enzyme was also assayed for C^{14} leucine incorporation.

The system containing freshly prepared microsomes and pH 5 enzyme incorporated 19,183 cpm while the system containing freshly prepared pH 5 enzyme and one month old microsomes incorporated 16,438 cpm. The microsomes had retained 86% of their capacity to incorporate amino acids after one month's storage. However, the system which contained both pH 5 enzyme and microsomes that had been stored for one month at -20°C, incorporated only 5,681 cpm. Because the previous experiment suggests that the microsomes are stable in storage, this 71% decrease in incorporation must have been caused by a decrease in aminoacyl-sRNA synthetase activity in the one month old pH 5 enzyme. Apparently microsomes retain full activity even after one month of storage at -20°C while the pH 5 enzyme loses activity due to storage.

Dependence of Amino Acid Incorporation on ATP--Besides

the components which were isolated from tissue, ATP and a system for its continuous generation are additional constituents required for cell-free protein synthesis (2). The reticulocyte system (58) synthesized no protein in the absence of ATP and its generating system while incorporation was decreased 60% when only the ATP generating system was omitted from the complete amino acid incorporating system. The dependence of ATP and its continuous generation on amino acid incorporation in the mammary gland cell-free system was tested by omitting the ATP generating system or both the generating system and ATP from the system.

When standard assay conditions were employed, no C^{14} leucine was incorporated if ATP and the generating system were omitted (Table 11). Only 33% of the maximal level of incorporation occurred when ATP was added to the system but its generating system of phosphoenol pyruvate (PEP) and pyruvate kinase was omitted.

In conclusion, this experiment demonstrated that this cell-free system requires ATP in order to incorporate amino acids into proteins. These results are in agreement with those of Turba and Hilpert (109) and also of Brew and Campbell (103) who demonstrated an ATP dependent amino acid incorporation in a mammary gland cell-free system.

Dependence of Incorporation on Magnesium Ion Concentration--Investigators have also shown that magnesium ions are necessary for amino acid incorporation in cell-free systems

TABLE 11

Dependence of ATP for amino acid incorporation

The standard assay mixture contained microsomes (0.4 mg), pH 5 enzyme (1.2 mg), L-leucine-U-C^{14} (1.25 μc, 210 μc/μmole), and the usual amounts of the other components except for the test systems. The results are expressed as cpm incorporated per ml reaction mixture as measured by the usual procedures (see Methods section).

System	cpm
Complete System	19,183
minus PEP and pyruvate kinase	6,206
minus ATP, PEP, and pyruvate kinase	396
Complete System, 0 Time	158

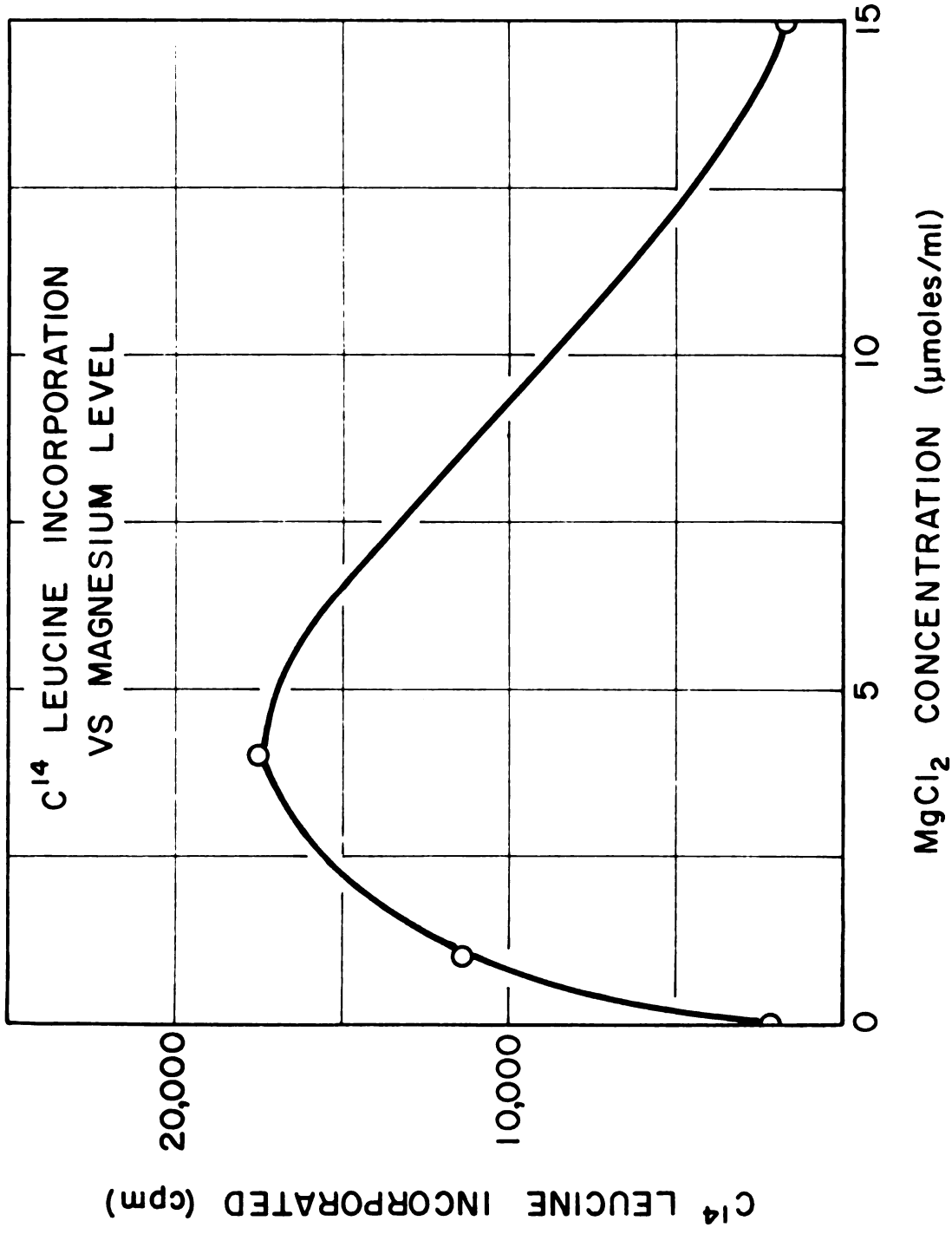
(58, 60, 61). Maximum incorporation occurred at a concentration of 5 μmoles of magnesium ion per ml while considerably less incorporation was observed at lesser or greater concentrations. Because of the magnesium dependence found in other systems, two experiments were performed to test the level of amino acid incorporation as a function of magnesium chloride concentrations.

In the first experiment, the concentration of magnesium chloride in the otherwise standard assay system was varied from 0 to 15 mmolar. Actually, the lowest concentration of magnesium in the complete system was 0.5 mmolar since the microsome preparation contained 5 μmoles of magnesium chloride per ml. The mixtures were incubated for 40 minutes and assayed for incorporation by the usual methods.

As illustrated in Figure 25, C^{14} leucine incorporation by this mammary gland cell-free system was optimal at 4 to 5 mmolar magnesium chloride. When no supplemental magnesium chloride was added to the complete system, incorporation decreased from the maximum by about 85%. Furthermore, a concentration of 15 mmolar magnesium chloride inhibited incorporation by about 85%. Therefore, to obtain maximal incorporation in a particular system, it was important to adjust the concentration of magnesium ions at 4 to 5 mmolar.

In the second experiment, microsomes, which had been washed with 0.5 M KCl and suspended in a medium A containing no magnesium ions, were incubated in complete systems containing 0 to 8 mmolar magnesium chloride. After 40 minutes

Figure 25. C^{14} leucine incorporation vs magnesium level. The standard assay mixture contained microsomes (1.4 mg), pH 5 enzyme (4.4 mg), L-leucine-U-C^{14} (1.5 µc, 210 µc/µmole), varying levels of $MgCl_2$, and normal amounts of the other constituents. Amino acid incorporation for the 40 minute incubation was determined by the usual procedures (see section on Methods).


C14 LEUCINE INCORPORATION VS MAGNESIUM LEVEL
C14 LEUCINE INCORPORATED (cpm)
20,000
10,000
0
5
10
15
MgCl2 CONCENTRATION (µmoles/ml)

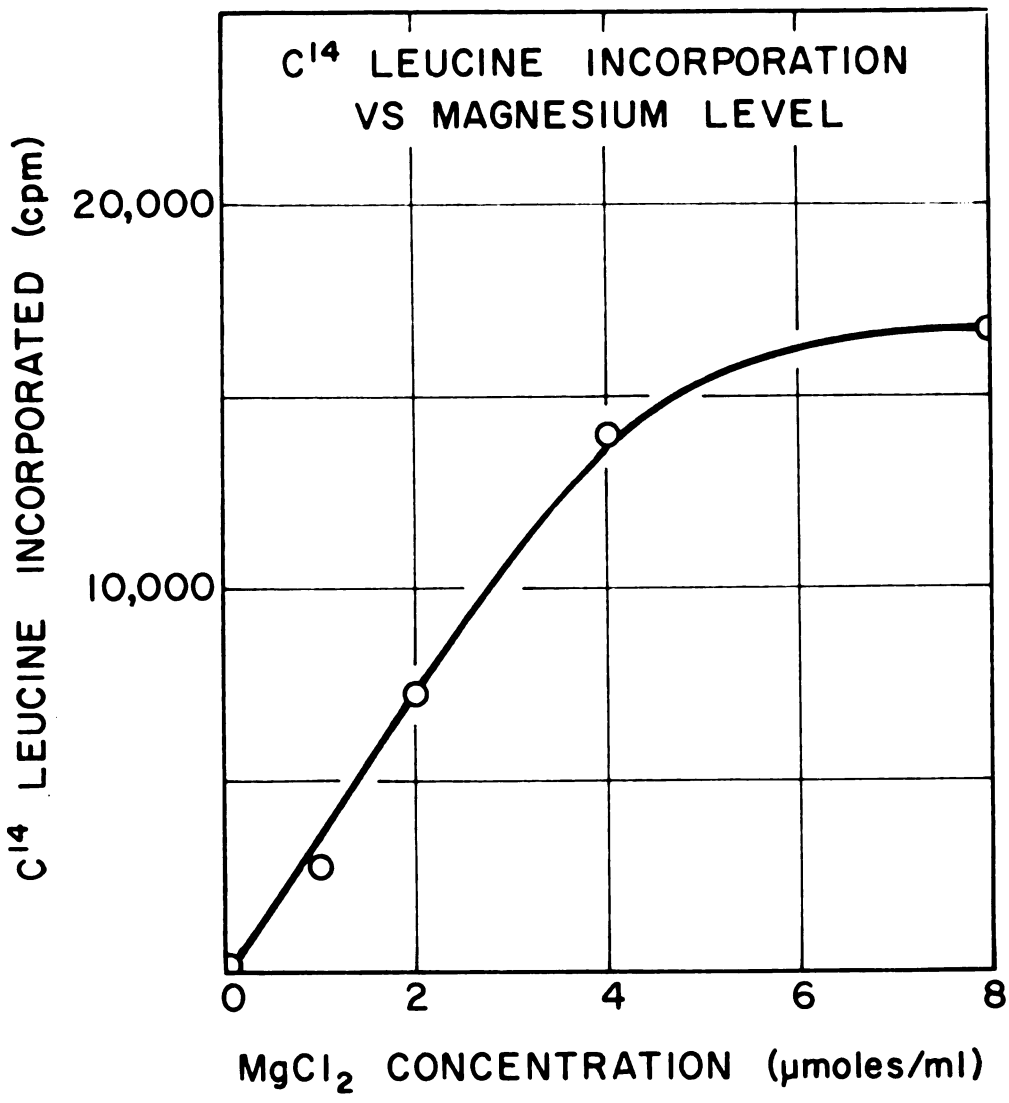
of incubation, the amount of C^{14} leucine incorporation was assayed as usual.

Figure 26 demonstrates that leucine was not incorporated in the complete system without added magnesium chloride. Incorporation increased linearly as the magnesium chloride concentration was raised to 4 mmolar. No significant increase in leucine incorporation occurred when the magnesium ion concentration was greater than 4 mmolar.

The previous sucrose density gradient centrifugation data obtained with the same 0.5 M KCl-treated ribosomes indicated that the predominant particle was a subunit of the normal ribosome or a "swelled" ribosome as previously described. The addition of magnesium chloride resulted in recombination of the subunits or in an alteration of the ribosome conformation to form normal-sized ribosomes and polyribosomes. Correlation of these results with the above incorporation studies suggest that the "shocked" microsomes are incapable of synthesis in a complete system. As magnesium ion concentration in the complete system was increased, normal ribosomes and polyribosomes were reformed and incorporation occurred. When the magnesium ion concentration in the complete system was 4 mmolar, the KCl-treated microsomes incorporated leucine 74% as well as the normal microsomes from which the treated microsomes were derived; that is, 29, 850 cpm incorporated per mg of treated microsomes compared to 40,600 cpm per mg of normal microsomes.

In summary, the maximum C^{14} leucine incorporation occurred at 4 to 5 mmolar magnesium chloride concentration.

Figure 26. C^{14} leucine incorporation vs magnesium level using "shocked" microsomes. The standard assay mixture contained "shocked" microsomes (0.5 mg) as described by Miller et. al. (145), pH 5 enzyme (2.2 mg), L-leucine-U-C^{14} (1.0 µc, 254 µc/µmole), varying levels of $MgCl_2$, and the usual amounts of the other constituents. Amino acid incorporation for the 40 minute incubation was determined by the usual procedures (see section on Methods).


C14 LEUCINE INCORPORATION
VS MAGNESIUM LEVEL
C14 LEUCINE INCORPORATED (cpm)
20,000
10,000
0
2
4
6
8
MgCl2 CONCENTRATION (μmoles/ml)

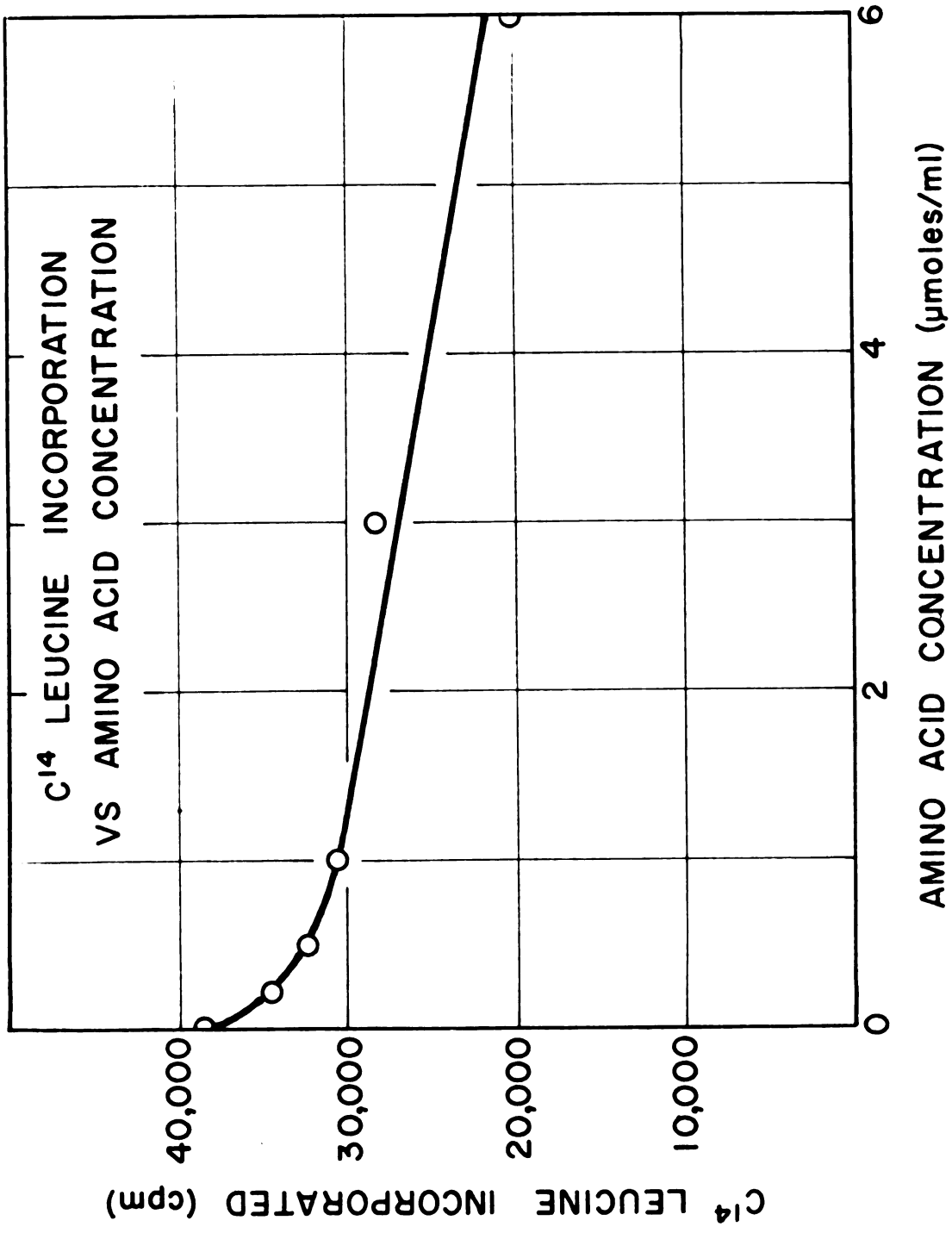
Magnesium chloride functions to maintain the integrity of the ribosomes and polyribosomes in the incubation mixture and this is necessary in order to realize amino acid incorporation.

Dependence of Incorporation on Amino Acids--Allen and Schweet (58) reported that the reticulocyte cell-free system incorporated 50% less C^{14} leucine into protein when the amino acid mixture was omitted. In contrast, Turba and Hilpert (109) and Singh et. al. (139) demonstrated that the mammary gland and thyroid gland systems incorporated less labeled amino acid into protein when an amino acid mixture was included in the complete system. No explanations for this discrepancy were reported. It is possible, however, that these results are a function of the level of free amino acids contaminating the cellular fractions involved. The effect of the amount of the amino acid mixture present in the complete amino acid incorporating system on amino acid incorporation was investigated in the present study.

A number of concentrations of amino acids minus leucine were added to complete systems. The samples were incubated for 40 minutes and the level of incorporation was determined.

As the concentration of the amino acid mixture was increased, the quantity of C^{14} leucine which became incorporated in protein decreased (Figure 27). Because this observation suggested dilution of the specific activity of the labeled leucine with unlabeled leucine, an amino acid analysis of the mixture was performed. The results showed that the

Figure 27. The effect of amino acid concentration on C^{14} leucine incorporation. The standard assay mixture contained microsomes (1.4 mg), pH 5 enzyme (4.4 mg), L-leucine-U-C^{14} (1.5 µc, 210 µc/µmole), varying levels of the amino acid mixture minus leucine (for composition, see Appendix Table 2), and the usual amounts of the other constituents. Amino acid incorporation for the 40 minute incubation was determined by the usual procedures (see section on Methods).


C14 LEUCINE INCORPORATION
VS AMINO ACID CONCENTRATION
C14 LEUCINE INCORPORATED (cpm)
40,000
30,000
20,000
10,000
0
2
4
6
AMINO ACID CONCENTRATION (μmoles/ml)

amino acid mixture which supposedly was leucine-free contained trace amounts of leucine. Nevertheless, to assure that an individual non-labeled amino acid would not limit incorporation of a C^{14}-containing amino acid, all assays were conducted with 0.05 ml of the 0.02 M amino acid mixture minus the labeled amino acid under study.

The question of dilution of the specific activity of the labeled leucine was investigated by adding non-radioactive leucine to the standard assay system. The addition of 1 μmole of unlabeled leucine to the complete system decreased incorporation by 96%. The addition of 1 μmole of leucine lowered the specific activity of the C^{14} leucine by 250 fold. Higher levels of leucine permitted only trace amounts in C^{14} leucine incorporation. Thus, it is likely that diminished C^{14} leucine incorporation with increasing amino acid mixture results from the trace amounts of leucine present in the other amino acid preparations.

Comparison of the Incorporation of Different Labeled Amino Acids--The purpose of the following experiment was to determine the rate of incorporation of different amino acids. Separate complete systems which lacked the amino acid under study were incubated in the presence of either L-glutamate U-C^{14}, L-lysine U-C^{14}, L-leucine U-C^{14}, or L-phenylalanine U-C^{14} for 40 minutes. The level of incorporation of each labeled amino acid was determined as before.

Table 12 illustrates that a higher percentage of the

TABLE 12

Amino acid incorporation vs type of labeled amino acid

The complete system contained the standard assay constituents including microsomes (0.4 mg) and pH 5 enzyme (1.2 mg). The amino acid mixture minus the amino acid under study was added to each complete incubation system. The conditions of incubation and assay of incorporation was described in the section on Methods. The results are expressed as counts incorporated per ml and as the percent of the added labeled amino acid that was incorporated into the trichloracetic acid precipitable material. The efficiency of counting was 77%.

Amino Acid	Specific Activity (μc/μmole)	μc of Amino acid added	net cpm Incorporated	% Amino Acid Incorporated
L-Glutamate-U-C^{14}	15.20	1.0	328	0.0194
L-Lysine-U-C^{14}	1.17	2.0	727	0.0214
L-Leucine-U-C^{14}	210.00	1.25	19,025	0.903
L-Phenylalanine-U-C^{14}	1.30	4.0	1,764	0.0261

added C^{14} leucine was incorporated than of the other three amino acids. Specifically, nine-tenths percent of the C^{14} leucine was incorporated while only 0.026% of the C^{14} phenylalanine and 0.02% of the glutamate and lysine were incorporated into protein. Based on the amino acid composition of whole casein, the amount of incorporation of the different amino acids was expected to be as follows (in order of decreasing amounts): glutamate, leucine, lysine, and phenylalanine. This deviation from the expected may be due to 1) the limiting amount of aminoacyl-sRNA synthetase activity for a particular amino acid, 2) a limiting quantity of an sRNA specific for a particular amino acid, 3) different rates of utilization of the amino acids for processes other than protein synthesis, and 4) synthesis of higher-than-normal amounts of non-casein proteins. Since the highest possible amount of incorporation was desired, C^{14} leucine was the labeled amino acid utilized in most experiments reported in this thesis.

Inhibition of C^{14} Leucine Incorporation--Understanding the mechanism of protein synthesis has been increased by studying inhibition of synthesis by certain substances. In order to compare the mechanism of milk protein synthesis in a cell-free system with that observed in systems derived from other organisms, the effect of many known inhibitors of protein synthesis was examined.

Puromycin inhibits protein synthesis in many mammalian

systems such as those derived from reticulocytes (58), spleen (61), liver (60), and the thyroid gland (139). The effect of puromycin on the mammary gland system was determined by adding 1 μmole of puromycin to complete amino acid incorporating systems containing either the pH 5 enzyme or the AS_{70} enzyme as a source of aminoacyl-sRNA synthetases. C^{14} leucine incorporation was determined as usual. The results of this experiment are presented in Table 13. Incorporation by the systems containing either enzyme preparation was inhibited 95% by the addition of 1 μmole of puromycin per ml.

Based on the accepted mechanism of puromycin inhibition, these data suggest that milk proteins were indeed synthesized through an aminoacyl-sRNA intermediate.

Chloramphenicol inhibits protein synthesis by bacterial cell-free systems but does not affect protein synthesis in similar systems derived from mammalian tissues (2). The effect of this antibiotic on inhibition of leucine incorporation was tested at a concentration of 50 μg per ml. The level of incorporation was determined as usual after the 40 minute incubation of the standard assay mixture. Chloramphenicol addition exerted no effect on incorporation by the system (Table 14). The standard complete system incorporated 30,838 cpm of C^{14} leucine while 31,506 cpm were incorporated by the mixture containing chloramphenicol. In another test, levels of chloramphenicol ranging from 0 to 200 μg per ml of incubation mixture did not alter the level of incorporation.

TABLE 13

Inhibition of C^{14} leucine incorporation by puromycin

Standard assay systems containing microsomes (0.4 mg), pH 5 enzyme (1.2 mg) or AS_{70} enzyme (3.6 mg), L-leucine-U-C^{14} (1.25 µc, 210 µc/µmole), and the usual amounts of the other constituents were tested for incorporation by the usual methods. Puromycin was added at a concentration of 10^{-3} M to the test systems. The results are expressed as cpm incorporated per ml of reaction mixture.

System	cpm Incorporated
pH 5 Enzyme	
Complete system	19,183
+ Puromycin	1,956
0 Time	158
AS_{70} Enzyme	
Complete system	2,806
+ Puromycin	389
0 Time	137

TABLE 14

Effect of L-chloramphenicol addition on C^{14} leucine incorporation

The complete system contained the usual constituents including microsomes (1.5 mg), pH 5 enzyme (2.2 mg), and L-leucine-U-C^{14} (1.5 μc, 210 μc/μmole). Chloramphenicol was added at a concentration of 50 μg per ml of incubation mixture and the incorporation was measured by the usual methods. Results are expressed as cpm incorporated per ml of incubation mixture.

System	cpm Incorporated
Complete System	30,838
+ Chloramphenicol	31,506
0 Time	220

Several workers (31, 33, 34) have suggested that chloramphenicol inhibits protein synthesis *in vitro* either by inhibiting the attachment of messenger RNA (mRNA) to ribosomes or by directly inactivating the mRNA. These mechanisms of action suggest two explanations for the absence of chloramphenicol inhibition of amino acid incorporation in the mammary gland cell-free system. First, the amino acid incorporation was not dependent on the *in vitro* attachment of an RNA to the ribosomes after the completion of a protein molecule. Secondly, the mammary gland mRNA was not deactivated by the chloramphenicol. Without further study, either explanation appears possible.

According to previous studies (2), low levels of ribonuclease inhibit protein synthesis in cell-free systems derived from many organisms. Protein synthesis was inhibited to the extent of 90% by as little as 0.1 μg of pancreatic ribonuclease in the reticulocyte cell-free system (68). Experiments with *E. coli* and rat liver ribosomes indicated that the nuclease destroyed the intact messenger RNA causing a degradation of polyribosomes to monomeric ribosomes (2, 63). Analogous studies of ribonuclease inhibition were performed with the mammary gland system.

Pancreatic ribonuclease was added at a concentration of 1 μg per ml to a complete system containing either the AS_{70} enzyme or the pH 5 enzyme. In a second experiment the level of ribonuclease was varied from 0 to 10 μg per ml of the reaction mixture. Inhibition of incorporation was

determined by incubating the system for 40 minutes and measuring the radioactivity associated with the washed trichloroacetic acid precipitate.

When either the pH 5 enzyme or the AS_{70} enzyme was utilized as a source of aminoacyl-sRNA synthetase activity, no C^{14} leucine was incorporated into protein when only 1 μg of ribonuclease was added to the complete system (Table 15). In the experiment where the concentration of ribonuclease was varied, as little as 0.1 μg of the inhibitor resulted in 90% inhibition of incorporation whereas the higher levels completely abolished incorporation (Figure 28). Since two types of RNA, messenger RNA and transfer RNA, are presumed to be required, this level of inhibition was to be expected.

Deoxyribonuclease should inhibit protein synthesis if DNA is a required component of the cell-free system. However, protein synthesis in most of the reported cell-free systems involves only translation of preformed RNA and not the transcription of DNA to form RNA. Therefore, the effect of deoxyribonuclease addition to the standard assay system was investigated.

Fifty μgrams of deoxyribonuclease was added to the standard assay mixture containing either the pH 5 enzyme or the AS_{70} enzyme. This mixture was incubated for 40 minutes and assayed for C^{14} leucine incorporation by the usual methods.

The addition of deoxyribonuclease did not alter the rate of synthesis by the complete system (Table 16). The result of this study demonstrates that incorporation in

TABLE 15

Inhibition of C^{14} leucine incorporation by ribonuclease

The complete system contained the usual constituents including microsomes (0.4 mg), either pH 5 enzyme (1.2 mg) or AS_{70} enzyme (3.6 mg), and L-leucine-U-C^{14} (1.25 µc, 210 µc/µmole). Crystalline pancreatic ribonuclease was added at concentrations of 1 µg per ml. Results are expressed as cpm incorporated per ml of the reaction mixture.

System	cpm Incorporated
pH 5 Enzyme	
Complete System	19,183
+ Ribonuclease	316
0 Time	158
AS_{70} Enzyme	
Complete System	2,806
+ Ribonuclease	222
0 Time	137

Figure 23. C^{14} leucine incorporation vs ribonuclease level. The standard assay mixture contained microsomes (0.7 mg), pH 5 enzyme (2.6 mg), L-leucine-U-C^{14} (1.0 µc, 251 µc/µmole), and the usual amounts of the other constituents. Aliquots of a pancreatic ribonuclease solution containing 100 µg/ml were added at varying levels to the complete systems which were incubated 40 minutes. Amino acid incorporation was determined by the usual procedures (see section on Methods).

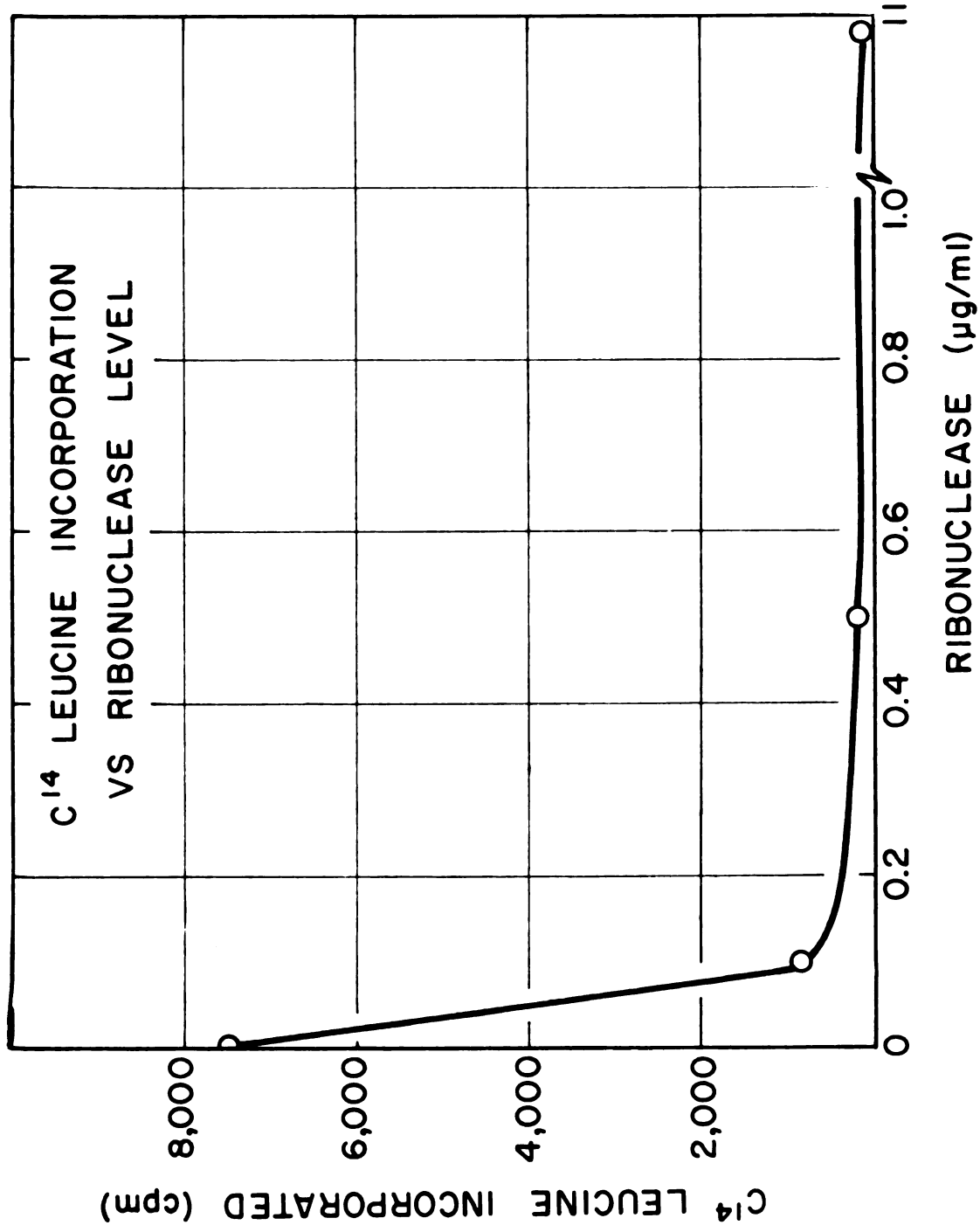

C14 LEUCINE INCORPORATION
VS RIBONUCLEASE LEVEL
C14 LEUCINE INCORPORATED (cpm)
8,000
6,000
4,000
2,000
0
0.2
0.4
0.6
0.8
1.0
11
RIBONUCLEASE (µg/ml)

TABLE 16

Effect of deoxyribonuclease addition to the complete system

The complete system containing the pH 5 enzyme (4.4 mg) was composed of the standard assay constituents including microsomes (1.4 mg) and L-leucine-U-C^{14} (1.5 µc, 210 µc/µmole). The complete system containing the AS_{70} enzyme (3.6 mg) included microsomes (0.4 mg), L-leucine-U-C^{14} (1.25 µc, 210 µc/µmole), and the usual amounts of the other constituents. Crystalline deoxyribonuclease, dissolved in 0.05 M phosphate buffer (pH 7.0), was added to the test systems at a concentration of 50 µg per ml of incubation mixture. Incorporation was assayed as described in the Methods section and the results are expressed as cpm incorporated per ml of the complete systems.

System	cpm Incorporated
pH 5 Enzyme	
Complete System	30,838
+ Deoxyribonuclease	29,726
0 Time	158
AS_{70} Enzyme	
Complete System	2,806
+ Deoxyribonuclease	2,825
0 Time	137

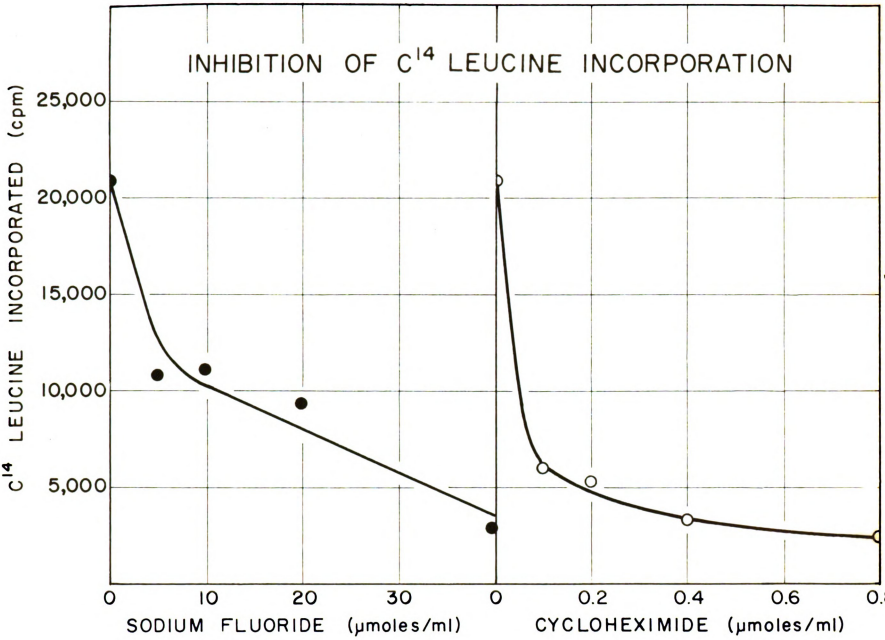
this system is a result of translation of preformed RNA rather than both transcription of DNA to form RNA and then translation of the RNA to synthesize protein.

Sodium fluoride and cycloheximide inhibited protein synthesis in the reticulocyte cell-free system (54). Studies by Lin et. al. (54) illustrated that cycloheximide slows, but does not completely block, the extension of existing nascent peptide chains while sodium fluoride exerted no effect on this process. Both compounds inhibited the in vitro initiation of polypeptide chains on the ribosomes. The effect of these inhibitors on C^{14} leucine incorporation was investigated in the mammary gland cell-free system.

Complete systems were incubated for 40 minutes in the presence of varying levels of sodium fluoride (0 to 40 mmolar) and cycloheximide (0 to 0.8 mmolar). Amino acid incorporation was determined as usual.

The data in Figure 27 indicates that C^{14} leucine incorporation was partially inhibited by either compound. Increasing the concentration of cycloheximide beyond 0.4 mmolar did not significantly increase the degree of inhibition. According to the accepted mechanism of action of these inhibitors, this residual level of incorporation reflects only that incorporation obtained by the continuation of existing polypeptide chains, amounting to about 10% of the total incorporation as in the uninhibited system. Similar results were obtained when sodium fluoride was used as an inhibitor except

Figure 29. Inhibition of C^{14} leucine incorporation by cycloheximide and sodium fluoride. The standard assay mixture contained the usual complete system constituents including microsomes (0.4 mg), and pH 5 enzyme (0.8 mg), and L-leucine-U-C^{14} (0.70 µc, 225 µc/µmole). Amino acid incorporation after 40 minutes of incubation in the presence of the varying levels of both inhibitors was determined as usual (see section on Methods).


INHIBITION OF C14 LEUCINE INCORPORATION
C14 LEUCINE INCORPORATED (cpm)
25,000
20,000
15,000
10,000
5,000
0
10
20
30
SODIUM FLUORIDE (μmoles/ml)
0
0.2
0.4
0.6
0.8
CYCLOHEXIMIDE (μmoles/ml)

that one hundred times higher concentrations were required to produce inhibition equal to that of cycloheximide. This reduction in incorporation caused by these two compounds with their known modes of action indicates that incorporation consists of both elongation and initiation of new chains in the mammary gland system. Inhibition by both compounds was of the same magnitude as found by Lin et. al. (54) in the reticulocyte cell-free system.

In conclusion, these inhibition studies suggest that incorporation by this system is a result of 1) the continuation of existing polypeptide chains, and 2) the initiation and elongation of new polypeptide chains.

Investigators have utilized the technique of adding low levels of sodium deoxycholate (DOC) to solutions of microsomes in order to solubulize the endoplasmic reticulum. However, Korner (60) utilizing the rat-liver cell-free system, noted a 79% decrease in incorporation when the system contained 0.1% DOC. A level of 0.5% caused 97% inhibition. Complete removal of DOC from the ribosome and polyribosome preparations is difficult, so the level of inhibition by this detergent was examined by the following experiment.

The complete system was adjusted to 0.1% with respect to DOC concentration and the incorporation was then measured as usual. When one mg of DOC was added to the complete system (0.1%), 6,614 cpm of C^{14} leucine were incorporated into protein. However, if the detergent was omitted, 19,183 cpm were incorporated, thus the incorporation in the DOC-treated

system was reduced by 66%. Because of this inhibition, microsomes were not pretreated with DOC in any of the amino acid incorporation experiments.

Effect of Polynucleotides on C^{14} Leucine Incorporation--There has been a number of studies concerned with the interaction between ribosomes and polyribonucleotides aimed at elucidation of the role of this complex in protein synthesis. Polyadenylic acid (poly A) and polyuridylic acid (poly U) have been shown to stimulate lysine and phenylalanine incorporation, respectively (59). For example, Henshaw et. al. (141) observed a 3-fold increase in phenylalanine incorporation in the rat liver system by the addition of 500 μg of poly U per ml while Florini and Breuer (143) found a 5-fold increase by adding 200 μg of poly U per ml to their rat muscle system. In order to investigate the possibility that various artificial messengers would stimulate amino acid incorporation by the mammary gland cell-free system, denatured DNA, poly A, and poly U additions were examined.

DNA was isolated from mammary tissue by following the procedures of Chargaff (124) as described in the section on Methods. This DNA was then denatured to single strands by maintaining the temperature of the solution at 90°C until the 260 mμ absorbance reached a maximum. At this time the solution was cooled rapidly to prevent reassociation of the DNA strands. One and one-half mg of this DNA preparation

was added to a complete system and the incorporation of C^{14} leucine was measured.

Addition of the DNA preparation to the standard assay mixture resulted in a 36% increase in leucine incorporation when compared to the system containing no DNA (Table 17). This stimulation of incorporation was minimal when compared to the 5-fold increase in phenylalanine incorporation caused by the addition of poly U to a muscle cell-free system (143). The results obtained from the mammary gland cell-free system indicate that mammary gland microsomes were unable to utilize the strands of DNA as messages for protein synthesis.

Possible stimulation of incorporation was examined also by adding poly A (70 μg per ml) or poly U (20 μg per ml) to the system. Two μc of L-lysine-U-C^{14} (1.7 μc per μmole) were added to a system containing poly A and 4 μc of L-phenylalanine-U-C^{14} (1.30 μc per μmole) were added to the system containing poly U. Incorporation of the amino acids was determined as usual.

Results of this study are illustrated in Table 17. No stimulatory effect was observed by adding the synthetic polynucleotides. Under the assay conditions for amino acid incorporation either the mammary gland ribosomes could not bind to the polynucleotides or the genetic message was not translated even though ribosomal binding had occurred. Without further study under different assay conditions neither possibility can be verified. Miller et. al. (145) in a reticulocyte system have postulated that an initiation factor is necessary

TABLE 17

Effect of addition of single-stranded DNA, poly A, and poly U to the complete system

The complete systems contained the usual constituents including pH 5 enzyme (1.4 mg) and microsomes (1.1 mg). The DNA, poly A, and the poly U were dissolved in 0.05 M phosphate buffer (pH 7.0) and were added at concentrations of 150 µg, 70 µg, and 20 µg per ml of the incubation mixture, respectively. The following labeled amino acids were added to the respective systems: single-stranded DNA system-- L-leucine-U-C^{14} (1.0 µc, 254 µc/µmole), the poly A system-- DL-lysine-4,5-H^3 (5.0 µc/µmole), and the poly U system-- L-phenylalanine-U-C^{14} (4.0 µc/µmole). The amount of incorporation was assayed by procedures described in the section on Methods. Results are expressed as cpm incorporated per ml of incubation mixture.

System	cpm Incorporated
Complete System	3,378
+ DNA (single stranded)	4,641
Complete System	17,469
+ Poly A	16,452
Complete System	1,611
+ Poly U	1,197

for amino acid incorporation to be stimulated by poly A or poly U additions. Since this mammary system did not require an initiation factor (next section), the lack of this component did not prevent a possible stimulation of amino acid incorporation by poly A and poly U addition. The results also suggest a structural peculiarity of the mammary gland ribosomes contained in the microsome fraction. According to Redman (144) proteins which are synthesized on ribosomes attached to the membranes of the endoplasmic reticulum are transferred into its cisternae. Possibly, the microsomes which are present in the mammary gland cell-free system possess this structural arrangement. Perhaps this feature restricts the binding of the ribosomes to the synthetic messages added to the system.

Conceivably, the leucine concentration in the standard assay system limited amino acid incorporation; therefore, a stimulation of incorporation by the synthetic polynucleotides was impossible. On the other hand, a limitation of C^{14} leucine incorporation by insufficient leucine was unlikely because of the following reasons: 1) the microsomes may have contained free amino acids within their cisternae, 2) the sRNA probably had leucine already esterified to it, and 3) a high level of C^{14} leucine incorporation was observed when no amino acids were added to the complete amino acid incorporation system.

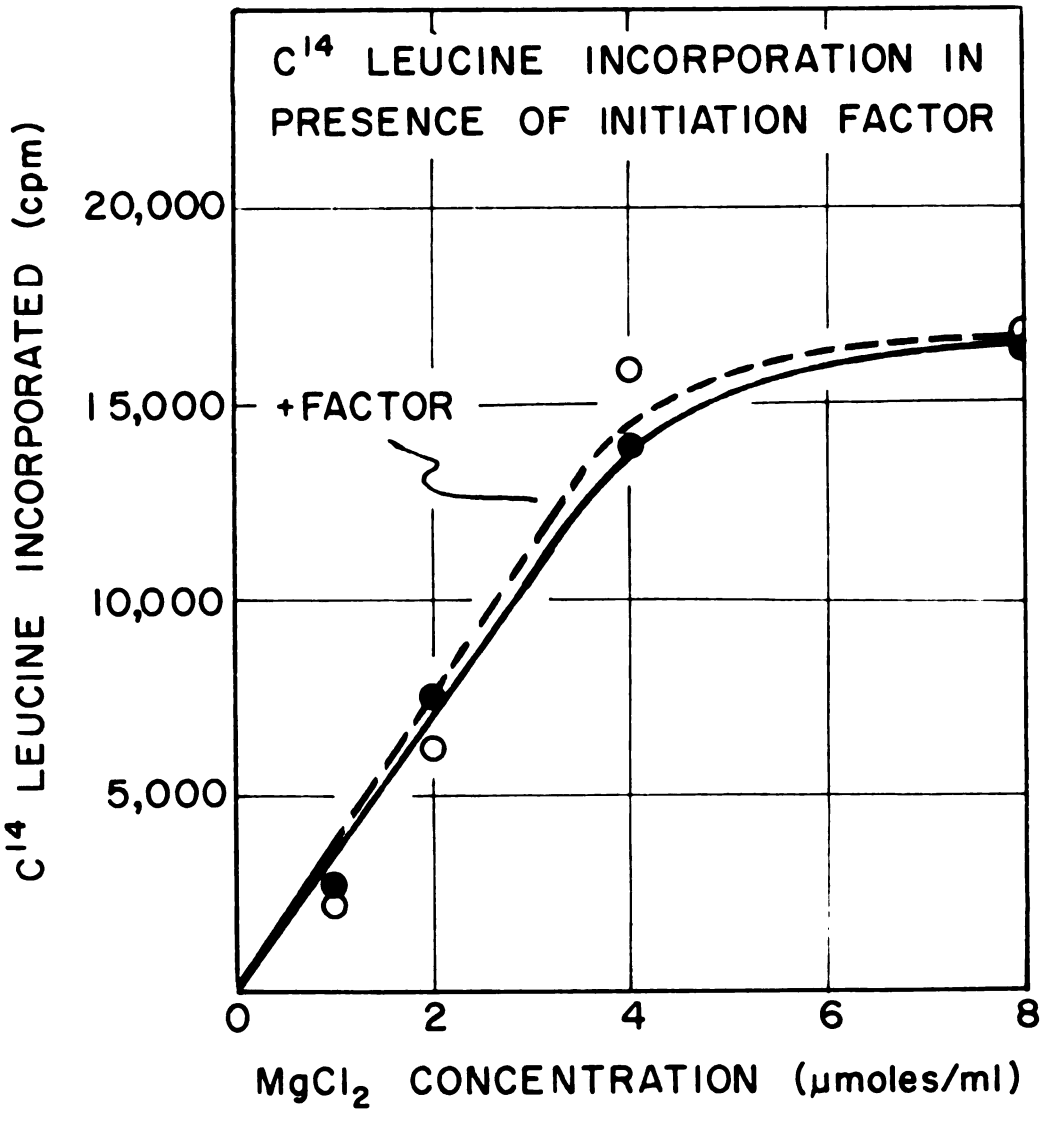
Effect of Initiation Factor on C^{14} Leucine Incorporation--Miller et. al. (145) have isolated an unidentified

factor from reticulocyte ribosomes which is necessary for polypeptide chain initiation in the cell-free synthesis of hemoglobin. This initiation factor (IF) was solubulized when the ribosomes were washed with a 0.5 M KCl solution. In the following study an attempt was made to demonstrate the role of a similar factor bound to mammary gland ribosomes.

Since Miller et. al. (145) noted that the initiation factor altered the dependence of the reticulocyte complete system on magnesium ion concentration, the following experiment was conducted with the mammary gland cell-free system. The microsomes which were suspended in 0.5 M KCl were added to the standard incubation mixtures which contained varying levels of magnesium chloride (0 to 8 mmolar). An aliquot of the fraction, presumably containing the initiation factor which was prepared by the procedure of Miller et. al. (145) as described in the Methods section, was added to the test sample prior to the incubation. The samples were incubated 40 minutes and the C^{14} leucine incorporation was assayed as usual.

The results of this study are presented in Figure 30. No differences in leucine incorporation at the various magnesium chloride concentrations were observed due to the addition of the fraction which presumably contained the initiation factor. Therefore, if active initiation factor was indeed present in the fraction obtained from the microsones, it exerted no effect on the dependence of incorporation on magnesium concentration.

Figure 30. C^{14} leucine incorporation in the presence of initiation factor. The complete incubation mixture contained the following: 0.47 mg of 0.5 M KCl-treated microsomes, 2.2 mg of pH 5 enzyme, 1 μc of L-leucine-U-C^{14} (254 μc/μmole), varying levels of $MgCl_2$, and the usual amounts of the other components. The presumed initiation factor equivalent to 0.4 mg of protein was added at the beginning of incubation. Amino acid incorporation after the 40 minute incubation was assayed by the usual techniques (see section on Methods).


C14 LEUCINE INCORPORATION IN
PRESENCE OF INITIATION FACTOR
C14 LEUCINE INCORPORATED (cpm)
20,000
15,000
10,000
5,000
+FACTOR
0
2
4
6
8
MgCl2 CONCENTRATION (μmoles/ml)

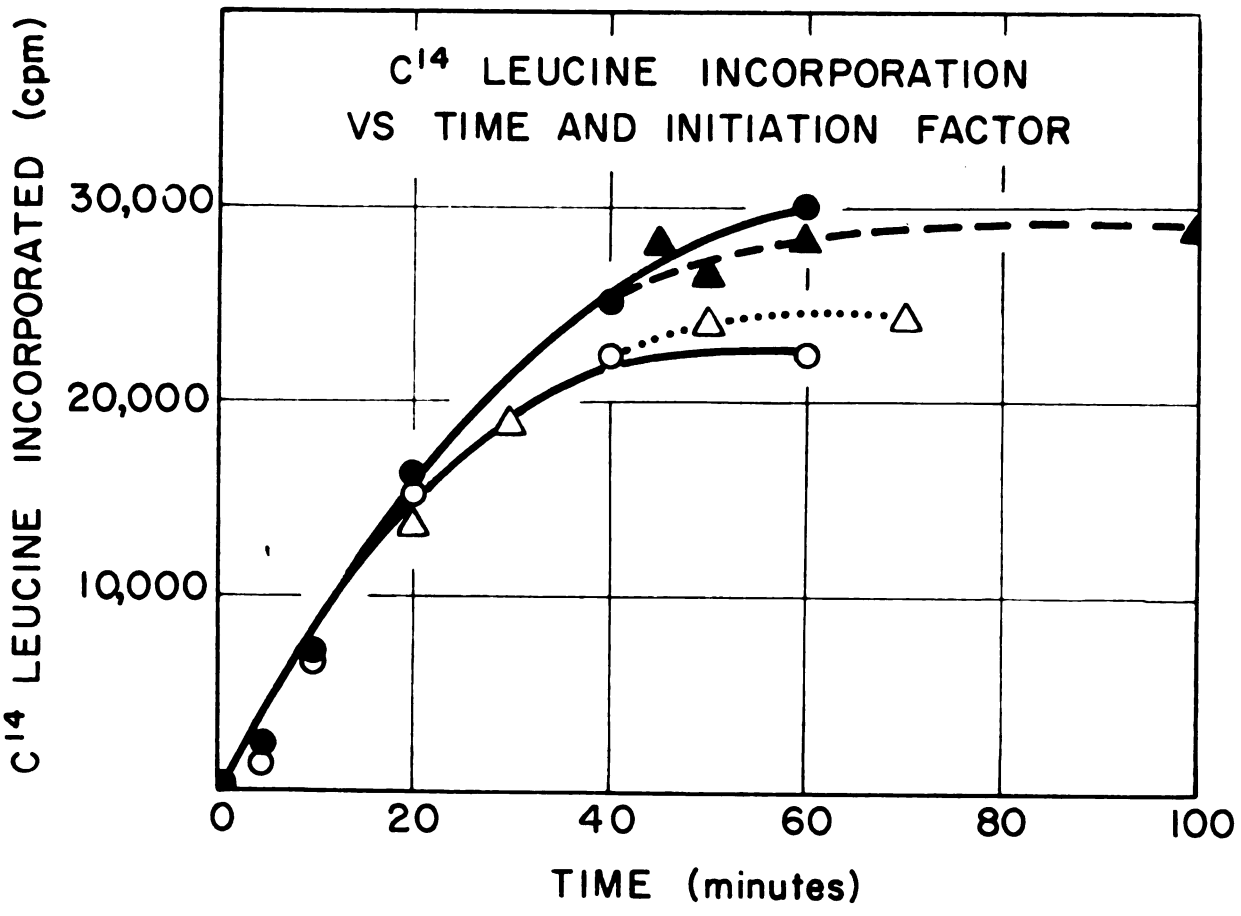
The hypothesis that amino acid incorporation approaches a maximum at approximately 40 minutes due to a deficiency of active initiation factor was also tested. A normal time course experiment was conducted to serve as a control by measuring the incorporation of leucine at various time intervals. In duplicate incubation mixtures, initiation factor was added at 0, 10, and 40 minutes after the incubation was started. In this experiment normal microsomes were utilized and the protein synthesis was assayed by the incorporation of C^{14} leucine.

The results of this experiment are presented in Figure 31. The initiation factor did not alter the level of leucine incorporation when it was added at 0 time, 10 minutes, or 40 minutes after the incubation. From these results, it may be concluded that the mammary gland microsomes either required no initiation factor or that it was not removed from the mammary gland microsomes by the methods described for their preparation.

Effect of Lactogenic Hormones on C^{14} Leucine Incorporation--Turkington et. al. (78, 79) have recently found that prolactin, insulin, and cortisone act synergistically to stimulate casein and whey protein synthesis in cultures of mammary tissue. But the effect of these hormones on cell-free preparations has not been investigated.

To examine the effect of certain hormones on amino acid incorporation, the following experiment was performed.

Figure 31. The effect of initiation factor on the time course of C^{14} leucine incorporation. The complete incubation mixture contained: 0.90 mg of normal microsomes, 2.2 mg of pH 5 enzyme, 1 μc of L-leucine-U-C^{14} (254 μc/μmole) and the usual amounts of the other components. The presumed initiation factor equivalent to 0.4 mg of protein was added at the beginning of incubation (0———0), 10 minutes after the initiation of incubation (• • •) and 40 minutes after the beginning of incubation (———). Amino acid incorporation was assayed by the usual tenchiques (see section on Methods).


C14 LEUCINE INCORPORATION
VS TIME AND INITIATION FACTOR
C14 LEUCINE INCORPORATED (cpm)
30,000
20,000
10,000
0
20
40
60
80
100
TIME (minutes)

Estrogen, prolactin, cortisone, growth hormone, and insulin were each suspended in a 0.01 M phosphate buffer, pH 7.0, at a concentration of 10 mg per ml. Various amounts of these solutions were then added to the standard assay system and the amount of C^{14} leucine incorporation was determined by the usual procedures.

The results of this study are illustrated in Table 18. Addition of growth hormone, prolactin, insulin, or cortisone did not alter the amount of leucine incorporated. Various combinations of cortisone, insulin, and prolactin also exerted no effect. These results agree with the previously published observations that growth hormone caused no stimulation in the amino acid incorporation by the rat liver system (72) and that estrogen and progesterone exerted no effect on protein synthesis in cell-free systems derived from accessory sexual tissues. The results of Florini (73) and Tata (71) obtained by studying protein synthesis in cell-free muscle and liver systems suggest that a hormone effect would be observed only if RNA was being synthesized in the system. As previously discussed in the experiment where poly U and poly A were added to the standard assay system, possibly the leucine concentration limited incorporation so as not to permit a stimulation by the lactogenic hormones.

To summarize, various hormones which act on the mammary gland in vivo were assayed for their effect on cell-free amino acid incorporation. No effect was observed for

TABLE 18

Effect of hormone additions on C^{14} leucine incorporation

The standard assay mixture contained microsomes (0.7 mg), pH 5 enzyme (2.6 mg), L-leucine-U-C^{14} (1.0 µc, 251 µc/µmole), and the usual amounts of the other constituents. The various hormones were suspended in 0.01 M phosphate buffer, (pH 7.0) at concentrations of 10 mg per ml. The hormones were added individually or in combinations at concentrations of 50 or 100 µg of each hormone per ml of incubation mixture. Activity of the hormones were as follows: insulin, 23.4 IU/mg; growth hormone, 1.0 USP unit/mg; and prolactin, 20 IU/mg. Amino acid incorporation was assayed by the usual procedures (see section on Methods).

Radioactivity (cpm) / Hormone(s) added	Amount of each hormone added		
	0	50 µg	200 µg
0 Time	38		
Control, 40 minutes	7,562		
Growth Hormone		7,384	7,237
Cortisone		7,475	7,267
Insulin		7,169	7,866
Prolactin		8,971	7,469
Cortisone and Insulin		--	8,033
Cortisone and Prolactin		--	8,556
Insulin and Prolactin		--	7,941
Cortisone, Insulin, and Prolactin		7,940	8,966

individual hormones or various combinations of the hormones. The *in vivo* effect of mammary gland sensitive hormones must then be at a level prior to the translation of RNA for protein synthesis.

Characterization of the Synthesized Protein

The standard assay of protein synthesis used in the present study measures the amount of labeled amino acid which is incorporated into trichloroacetic acid-precipitable material. This is only an approximate estimate since amino acid incorporation may occur with either polypeptide chain initiation, further elongation of existing polypeptide chains, or perhaps with random side chain reactions. Therefore, it was necessary to verify whether the labeled products synthesized by the mammary gland cell-free system were, in fact, milk proteins.

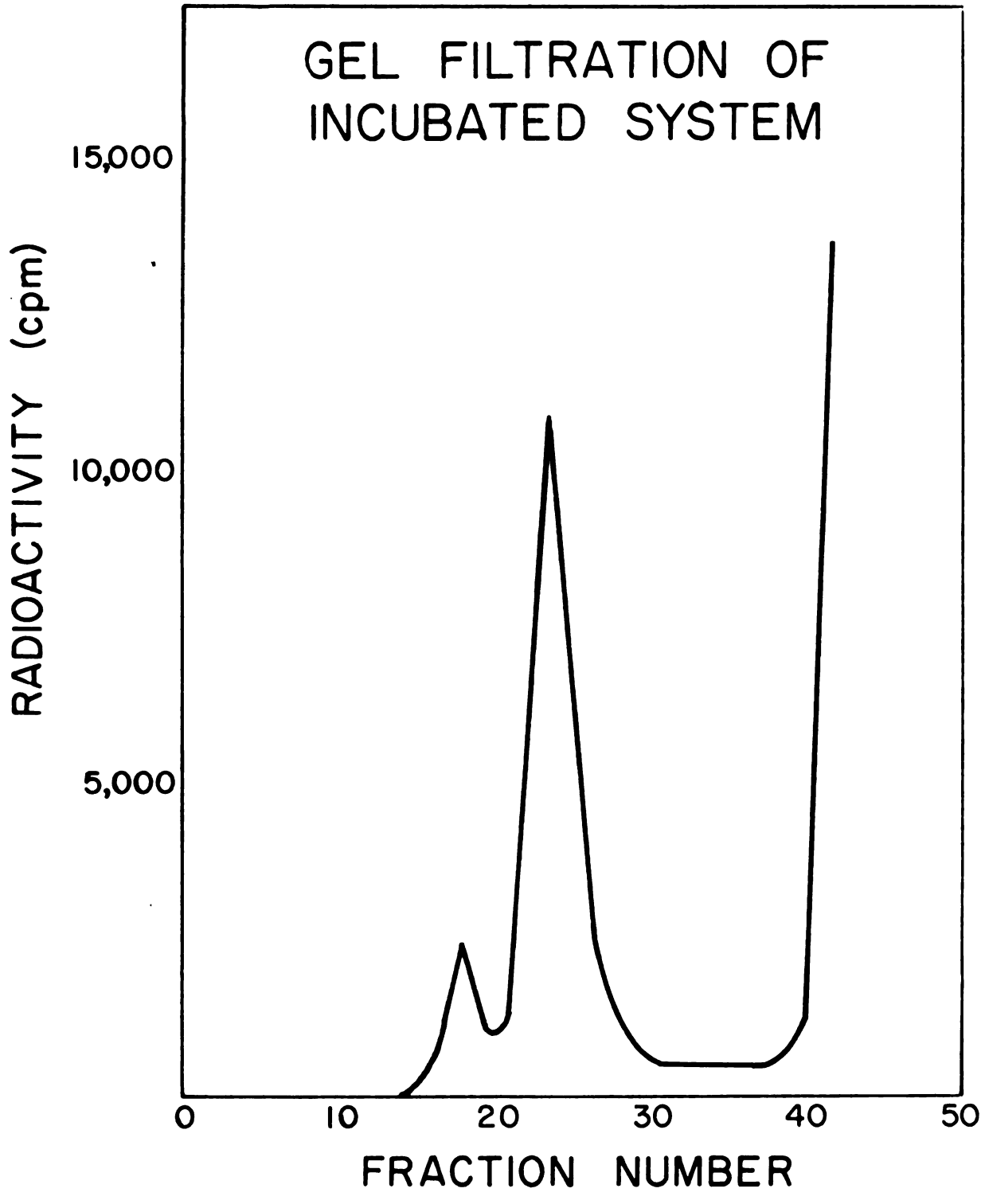
Gel Filtration Studies--According to the recent review by Thompson *et. al.* (146) on the physical properties of milk proteins, those proteins which are synthesized by the mammary gland possess molecular weights in the range of 16,000 to 36,000. If the mammary gland cell-free system synthesized authentic milk proteins, the incorporated radioactivity would be associated with proteins with molecular weights in this region. This possibility was investigated by the following gel filtration study with Sephadex G-75.

After incubating the standard assay mixture for 0 and

40 minutes, the reaction was stopped by rapid freezing. The 0 time and the 40 minute sample incorporated 57 cpm and 18,458 cpm, respectively, when measured by the usual procedures. Aliquots of these incubated preparations together with horse radish peroxidase and cytochrome c were layered onto the Sephadex G-75 columns and fractionated according to the conditions described in the section on Methods. The amount of radioactivity in each fraction was determined by combining an aliquot with 1 N sodium hydroxide (0.5 ml) and suspending the resultant solution in a thixotropic counting fluid as described by Casjens and Morris (65).

The results of these gel filtration studies are presented in Figure 32. Two major radioactive peaks in addition to the peak of free C^{14} leucine were found for the sample incubated for 40 minutes. The first radioactive material to be eluted corresponded to material eluted with the void volume of the column and presumably consisted of labeled peptides which remain attached to the microsomes. The next radioactive material to be eluted represented protein which had an average molecular weight of 32,000. This calculation was based on the method reported by Andrews (127) in which horse radish peroxidase and cytochrome c were used as protein markers of known molecular weights. None of these peaks were present when a zero time sample was chromatographed in the same manner. Thus, the above radioactive peaks represent proteins that were synthesized in the cell-free system. In addition to demonstrating *in vitro* protein

Figure 32. Behavior of the incorporated leucine. Standard assay systems contained microsomes (1.0 mg) pH 5 enzyme (0.8 mg), L-leucine-U-C^{14} (0.8 μc, 251 μc/μmole), and the usual amounts of the other constituents. Systems incubated for 0 and 40 minutes were subjected to gel filtration studies by placing 1 ml of the 0 time incubation mixture and 2.5 ml of the 40 minute mixture on Sephadex G-75 column. The specific details of this experiment are presented in the Methods section. The radioactivity in fractions 10 to 40 for the 0 time sample coincided with the base line.


GEL FILTRATION OF
INCUBATED SYSTEM
RADIOACTIVITY (cpm)
15,000
10,000
5,000
0
10
20
30
40
50
FRACTION NUMBER

synthesis, these results show that synthesis was confined to a limited molecular weight range because most of the radioactivity appeared in a well-defined area of the column chromatograph. This suggests that the radioactive proteins could be milk proteins since most of the proteins synthesized by the bovine mammary gland possess molecular weights in the 16 to 36,000 range.

Isotope Dilution Tests of the Incubated Standard Assay System--Previous examination of the incubated assay system by column chromatography illustrated that radioactively labeled proteins were synthesized by the in vitro system. Further identification of the synthesized proteins was conducted by isotope dilution tests. Of course, results derived from this experiment are based on the assumption that the synthesized product can be isolated by the same procedures as used for known milk proteins. This experiment was conducted as follows.

At zero time and at 40 minutes of incubation, 1.0 and 10.8 ml aliquots, respectively, were withdrawn and each added to 100 ml of skimmed milk. The specific milk proteins were then isolated from these solutions by the procedures described in the Methods section. The 0 time sample contained 220 cpm precipitated by the trichloroacetic acid method while the 40 minute incubated sample contained 102,751 cpm in the acid precipitate.

The β-casein, κ-casein, α-lactalbumin, β-lactoglobulin,

and α_s-casein fractions were recovered by the purification procedure and extensive dialysis (see section on Methods) and each contained radioactivity (Table 19). The amount of radioactivity in each of the respective milk protein fractions was significantly increased over the 0 time value. The radioactivity in each protein fraction was roughly proportional to the amount of each protein usually present in skimmed milk (146). When the results were corrected to equilize the volumes of the original samples, the radioactivity in each of the protein fractions isolated from the 40 minute incubated sample was significantly increased over the 0 time control protein fractions.

Based on this study, the complete system incubated for 40 minutes synthesized α_s-casein, β-casein, κ-casein, α-lactalbumin, and β-lactoglobulin.

A second isotope dilution test was conducted by Dr. B. L. Larson of the University of Illinois Dairy Science Department. Standard assay systems of 10 ml each, instead of the usual 1 ml, were incubated for 0, 5, and 40 minutes. A 1 ml aliquot of each system was assayed for C^{14} leucine incorporation by the usual methods. Four and one-half ml of the 0 and 5 minute incubated samples and 5 ml of the 40 minute incubated sample were frozen at the end of the incubation period and sent to Dr. Larson while in the frozen state. Each sample was thawed and combined with 16 ml of fresh skimmed milk by Dr. Larson who subsequently isolated, purified, and determined the radioactivity of the β-casein

TABLE 19

Identification of synthesized protein by isotope dilution

The standard assay mixtures contained microsomes (0.7 mg), pH 5 enzyme (2.6 mg), L-leucine-U-C^{14} (1.0 μc, 251 μc/μmole), and the usual amounts of the other constituents. The mixtures were incubated for 0 and 40 minutes. One ml of the 0 time sample and 10.8 ml of the 40 minute sample were each combined with 100 ml of fresh skimmed milk. Specific milk proteins were then isolated from each mixture according to the procedures described in the Methods section.

Protein Fraction	Total Radioactivity		Increase per ml* (cpm)	% of Total
	0 min (cpm)	40 min (cpm)		
β-Casein	248	38,610	3,327	35.0
α_s-Casein	1,064	57,582	4,276	45.0
χ-Casein	105	14,500	1,247	13.2
α-Lactalbumin	357	7,259	317	3.2
β-Lactoglobulin	576	9,875	339	3.5

*Expressed as the net increase in cpm per ml of incubation mixture.

and β-lactoglobulin. The β-casein and β-lactoglobulin were purified according to the methods of Aschaffenburg (147) and Aschaffenburg and Drewry (128), respectively. The isolated proteins were checked for purity by electrophoresis on cellulose acetate strips.

The results, illustrated in Table 20, again show that both β-casein and β-lactoglobulin were radioactive and, therefore, were synthesized by the cell-free system. As expected, the synthesis of these proteins was time-dependent. The amount of dpm present in the trichloroacetic acid (TCA) precipitates which corresponds to actual milk protein synthesis may be estimated. In doing this one must assume that the same ratio exists in vitro as in vivo between the relative amounts of milk proteins synthesized. The assumption was also made that β-lactoglobulin comprises about 10% and β-casein about 25% of the total milk proteins synthesized in the mammary gland. Further, an assumption was made that 17% and 31% of the leucine present in the total milk proteins normally synthesized in vivo is represented by β-lactoglobulin and β-casein, respectively. These results are also presented in Table 20 and indicate that about 25% of the dpm found in the TCA precipitable protein are due to the synthesis of milk proteins with the remaining counts due to other reasons including the synthesis of other proteins perhaps both by the components from the secretory cell as well as from the other cells present in mammary tissue. Perhaps the in vitro synthesis of these two milk proteins (β-casein and

TABLE 20

Identification of synthesized protein by isotope dilution as conducted by Dr. B. L. Larson

The standard assay mixture contained microsomes (0.4 mg), pH 5 enzyme (0.8 mg), L-leucine-U-C^{14} (0.7 µc, 225 µc/µmole) and the usual amounts of the other components. Four and one-half ml of the 0 and 5 minute and 5 ml of the 40 minute incubation mixtures were each combined with 16 ml of fresh skimmed milk. β-casein and β-lactoglobulin were isolated and purified from these mixtures by the method of Aschaffenburg (147) and of Aschaffenburg and Drewry (128), respectively. Aliquots of the incubation mixtures were also assayed for amino acid incorporation by the usual procedures (see section on Methods).

Time (min)	Corrected dpm in TCA precipitable protein per ml[1]	dpm per ml of reaction mixture due to:		Estimated dpm per ml in reaction mixture due to total milk protein synthesized.[2]	
		β-lact	β-casein	β-lact	β-casein
0	0	0	0	0	0
5	7,274	254	670	1,495	2,160
40	21,699	670	2,540	3,950	8,200

[1] Corrected for 0 time control, 87 dpm/ml.

[2] For example, 1) 254 x 100/17 = 1495 dpm/ml where 17 equals the percent of leucine in the total synthesized milk proteins contributed by β-lactoblobulin.
2) 670 x 100/31 = 2160 dpm/ml where 31 equals the percent of leucine in the total synthesized milk proteins contributed by β-casein.

β-lactoglobulin) is not a good index from which to extrapolate the synthesis of the other milk proteins.

In summary, the results indicate that milk proteins have been synthesized by the mammary gland cell-free system. However, the results are complicated by the presence of additional counts in the TCA precipitated protein.

Identification Studies by Immunodiffusion--The technique of immunodiffusion has been utilized by investigators to identify the products of protein synthesis in cell-free systems. For example, Stenzel and Rubin (148), who studied protein synthesis in a rabbit spleen system, identified one of the synthesized proteins as γ-globulin by using a combination of immunodiffusion and radioautography. Because this technique permitted identification of minute quantities of labeled protein, it was utilized to identify the milk proteins which were synthesized in the mammary gland cell-free system.

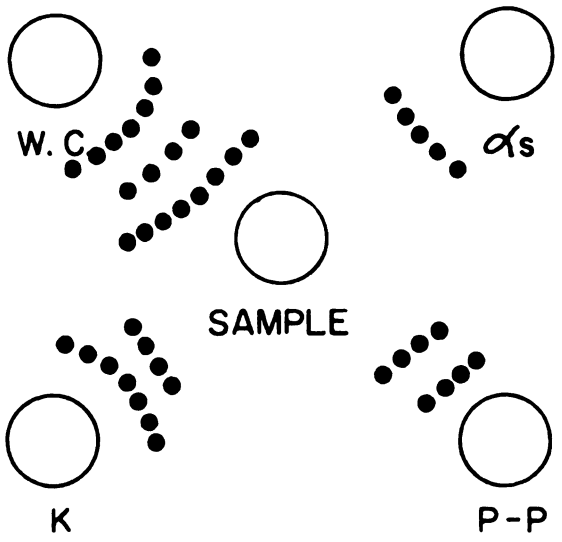
An aliquot (0.05 ml) of a complete system at 0 time and after 40 minutes of incubation were utilized for the immunodiffusion studies as described in the Methods section. These samples had been stored at -20°C for 6 months. The 0 time and 40 minute sample had incorporated 220 cpm and 19,183 cpm per ml, respectively, into protein as assayed by the usual trichloroacetic acid precipitation procedure. The samples were placed in the center well of an agar plate and the antibodies against α_s-casein, κ-casein, proteose-peptone,

and whole casein were placed in wells equidistant from the center. The immunodiffusion was allowed to proceed for two days. The unprecipitated proteins were then leached from the agar gels, and the gels air-dried. Then the agar films were exposed to x-ray film for visualization of the areas of radioactivity.

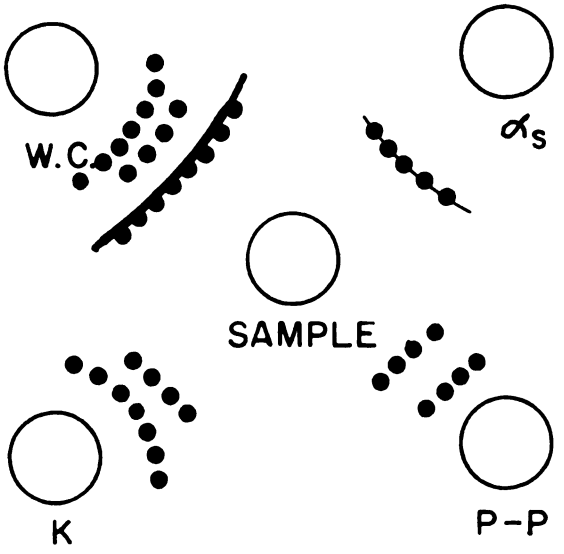
Precipitin bands were observed in the agar gels for both test samples and each authentic antibody. However, after radioautography, a radioactive precipitin band was observed only between the sample incubated for 40 minutes and both the whole casein and the α_s-casein antibodies (see Figure 33). The location of the band between the whole casein antibody and the 40 minute sample corresponded exactly with the position of the radioactive band between the 40 minute sample and the α_s-casein antibody. In addition, these radioactive bands coincided with the respective precipitin bands observed prior to the drying of the gel. No radioactive bands were observed for the κ-casein and proteose-peptone antibodies probably because of inadequate sensitivity. Furthermore, no radioactive bands were formed in the 0 time control sample. Therefore, the synthesis of milk proteins by the mammary gland cell-free system was again verified. Specifically, this particular study demonstrated a synthesis of α_s-casein.

Figure 33. Radioautographs of immunodiffusion studies of incubated complete systems. Standard assay mixtures contained microsomes (0.4 mg), pH 5 enzyme (1.2 mg), L-leucine-U-C^{14} (1.25 μc, 210 μc/μmole), and the usual amounts of the other constituents. Five hundredths ml of each antibody were placed in the outer wells. The immodiffusion and radioautography were conducted as described in the section on Methods. The following abbreviations were used: w.c., whole casein; α_s, α_s-casein; κ, κ-casein; and P-P, proteose-peptones. The precipitin bands are indicated by the solid circles (● ● ●) while the solid circles connected with a line (●—●—●) represent radioactive precipitin bands.

RADIOAUTOGRAPHS OF IMMUNODIFFUSION STUDIES OF INCUBATED COMPLETE SYSTEM



0 MIN



40 MIN

DISCUSSION

The object of this research was to obtain a cell-free system from bovine mammary tissue which would synthesize at least one of the well-defined milk proteins. The steps in attaining this goal were 1) preparation of well-defined components such as ribosomes and polyribosomes, soluble RNA, and aminoacyl-sRNA synthetases, 2) assembly of the synthesizing system, 3) study of amino acid incorporation into protein, 4) attainment of net protein synthesis, 5) testing the effect of the lactogenic hormones on the total system, and 6) partial identification of the protein synthesized. The discussion which follows pertains to some aspects of each of these steps.

The complete amino acid incorporating system includes the partially purified mammary gland cellular components (microsomes, sRNA, and aminoacyl-sRNA synthetases), amino acids, ATP and its generating reactions, certain salts, a reducing reagent and a buffer. Achievement of any C^{14} leucine incorporation necessitated inclusion of the microsomal fraction and the energy source, ATP, in the incubation mixture. Partial dependence of incorporation was observed for the addition of the aminoacyl-sRNA synthetase or the purified sRNA. Probably this phenomenon was caused by both of the components being incompletely removed from the isolated

microsomes. The pH 5 enzyme also contained sRNA since the treatment of this enzyme fraction with protamine sulfate resulted in a 2-fold increase in the dependence of amino acid incorporation upon the addition of sRNA. Possibly, further purification of the microsome fraction may lead to a demonstration of complete dependence of sRNA and amino-acyl-sRNA synthetases in the cell-free system.

Polyribosomes are composed of ribosomes, messenger RNA, and polypeptide chains undergoing active synthesis. The properties established for these ribosomal aggregates include 1) sedimentation in a sucrose density gradient ultracentrifugation as a structure considerably heavier than ribosomes, 2) dissociation into ribosomes and/or smaller subunits after either ribonuclease treatment of suspension in a solution containing no magnesium ions, 3) ability to cosediment with radioactive polypeptides while active in synthesis, and 4) ability to support amino acid incorporation in a cell-free system. Based upon these criteria and others the presence of polyribosomes in the bovine mammary gland has been demonstrated. To fulfill these criteria, the following observations were noted: Electron microscopy demonstrated a presence of linear assays of ribosomal particles in the polyribosomal solution. Since the mammary gland is rich in ribonuclease, it is therefore possible that these aggregates already represent partial breakdown products formed by the hydrolytic action of mammary gland ribonuclease of messenger RNA. Pulse incubation of the

complete amino acid incorporation system and subsequent analysis of radioactivity of various polyribosomal aggregates indicated that the large aggregates bind more radioactivity and hence were more active in protein synthesis than the ribosomes. Utilization of microsomes which were fractionated by sucrose density gradient centrifugation as the ribosomal component on the complete amino acid incorporating system demonstrated that the polyribosomes incorporated at least 3 times more leucine than the ribosomes when expressed as the amount of C^{14} leucine incorporated per unit weight of polyribosome or ribosome.

Another experiment which demonstrated the dependence of amino acid incorporation on normal-sized ribosomes and polyribosomes is as follows. Sucrose density gradient centrifugation and sedimentation velocity runs of microsomes dissolved in 0.5 M KCl suggest a dissociation of the polyribosomes and ribosomes to ribosomal subunits. Addition of magnesium chloride to these dissociated ribosomes causes a recombination of these subunits to form a nearly-normal complement of ribosomes and polyribosomes. When the dissociated ribosomes are utilized as the ribosomal component in the standard amino acid incorporating mixture, no incorporation is observed at zero magnesium chloride concentration. As the concentration of magnesium chloride was increased, incorporation increases to a maximum at 5 mM magnesium chloride which also is the approximate concentration at which these ribosomal subunits recombine to form a near-normal complement of

ribosomes and polyribosomes. The potency of the ribonuclease as an inhibitor of protein synthesis also suggests that incorporation is a function of polyribosome integrity.

Another very interesting point which has come from the detailed study of the requirements for C^{14} leucine incorporation has been the narrow optimal ranges for most of the components. For example, pH 5 enzymes treated with protamine sulfate, and the AS_{70} enzyme must be added at optimal concentrations. Lower incorporation occurred at higher as well as lower concentrations of enzyme. The decrease in incorporation at the lower concentration of enzyme in the standard assay system was related to the dependence of incorporation on this enzyme while the decrease at higher concentration apparently resulted from the higher amounts of ribonuclease which contaminated the enzyme preparations. The amount of leucine incorporation increased when the sRNA or the microsomes were present in higher concentrations in the standard assay system. However, amounts higher than a certain level of either component caused no change in the amount of incorporation.

Concerning stability of the components, the pH 5 enzyme was unstable when stored at $-20^{\circ}C$ for one month while the microsomes were fully active after similar storage for one month. Practically speaking, testing an additive such as glycerol to maintain the pH 5 enzyme activity during storage deserves further attention. The microsomes used in the described experiments were not freed of the endoplasmic

reticulum by sodium deoxycholate treatment as this anionic detergent was found to inhibit leucine incorporation.

The mechanism of the condensation of amino acids to peptide chains for the formation of protein molecules has to fulfill two tasks. First, it has to couple the endergonic peptide-bond formation with an exergonic reaction to make the overall reaction thermodynamically possible. Secondly, it has to include a step or series of steps which determine the specific sequence in which the amino acids condense. Before milk proteins are synthesized, amino acids must be first activated. Aminoacyl-sRNA synthetase activity was present in both the pH 5 enzyme and the AS_{70} enzyme which are partially required for C^{14} leucine incorporation. Presumably, the activation reaction gave rise to the specific requirement of ATP and its continuous generation in the cell-free system. The second reaction prior to peptide bond formation, that is, the esterification of this activated amino acid to sRNA, also occurs in the cell-free system as indicated by the formation of glutamyl-sRNA by either the pH 5 enzyme or the AS_{70} enzyme. The dependence of sRNA for maximum incorporation and inhibition by puromycin also lends support to an aminoacyl-sRNA intermediate which has been suggested by other workers (107). At this point it is unknown whether there are aminoacyl-sRNA synthetases and sRNA's in these mammary tissue preparations which are specific for each amino acid.

Amino acid incorporation by this system appears not

to involve transcription but only the translation of performed RNA. The absence of inhibition by deoxyribonuclease and the absence of an effect of lactogenic hormones indicate the absence of the transcription process in the mammary gland cell-free system.

Based on the mechanism of action of chloramphenicol inhibition in bacterial systems (26), reattachment of ribosomes to the message is not involved in the incorporation process. Addition of synthetic polynucleotides (poly A and poly U) also did not alter the amount of amino acids incorporated. Perhaps this absence of binding or of translation was a function of the conditions that were used. Further study of the effect of additions of poly A and poly U is needed to clearly understand this lack of stimulation since in other mammalian systems amino acid incorporation is stimulated 3-fold (141). Perhaps a dissociation of the microsomes prior to the addition of either poly A or poly U would facilitate binding and translation of the message.

Although the mechanism of inhibition of cycloheximide and sodium fluoride, part of their inhibitory effect involves the prevention of initiation of new peptide chains. From these postulates and the results of the present experiment, one can conclude that amino acid incorporation involved the initiation of new peptide chains as well as the elongation of pre-existing chains.

One of the unsolved problems in this system revolves around the question of why incorporation ceased after a short

period. Unfortunately, additions of any components after incorporation had reached a maximum were not performed. The time-dependent loss of polyribosomes and ribosomes in the standard assay incubation mixture suggests that a decrease of ribosomal integrity causes cessation of incorporation. Korner likewise postulated a similar mechanism for the time dependence of incorporation in the cell-free system derived from rat liver (60). Miller et. al. (145) suggested that an initiation factor which is bound to the reticulocyte ribosome was responsible for causing the initiation of synthesis of new polypeptide chains of hemoglobin in a cell-free system. The loss of activity of this factor after a time in the incubation mixture caused incorporation to cease. However, this study indicated that the mammary gland possesses no initiation factor as do reticulocytes or that the isolation procedure did not remove the initiation factor from the microsome. Therefore, it could be neither established nor disproven that an active initiation factor limits in vitro milk protein synthesis.

A puzzling problem concerns the efficiency of incorporation of different amino acids. One would expect that the amino acid which exists in largest amounts in milk protein would be incorporated to the greatest extent by the cell-free system. Based on the amino acid composition of casein, the predominant protein fraction of milk protein, suggests the following order of amino acids (expressed in decreasing amounts) to be incorporated by the standard assay system:

glutamate, leucine, lysine, and phenylalanine. However, leucine was incorporated to the greatest extent followed by glutamate, phenylalanine, and lysine. Further work on this problem is needed to decide whether glutamate enters into many side reactions or whether a limited amount of a specific sRNA or aminoacyl-sRNA synthetase for any of the amino acids causes the unexpected result.

The value of such a protein synthesizing system for future use is greatly increased when synthesis specific milk proteins occurs. Column chromatography studies demonstrate that the system does synthesize authentic proteins. Isotope dilution studies indicated that α_s-casein, χ-casein, β-casein, β-lactoglobulin, and α-lactalbumin were indeed synthesized in the cell-free system. These proteins constitute the bulk of protein normally secreted into milk. Results of this type of analysis are a function of the similarity in the behavior during purification of the synthesized protein to the secreted milk proteins contained in the skimmed milk. Immunodiffusion in conjunction with radioautography suggest that at least α_s-casein is synthesized. Further studies to identify all the milk proteins by this method are desirable. Also, identification of the synthesized protein(s) by other techniques such as polyacrylamide gel electrophoresis, immunoelectrophoresis, and fingerprinting are needed. In general, these studies indicate that the synthesis of milk proteins conforms to the mechanism of protein synthesis found in other biological systems. Incubation of the mixture of partially defined

components in the standard assay system resulted in incorporation of C^{14} into trichloroacetic acid-precipitable material many fold higher than the control. This incorporation of leucine resulted in the synthesis of authentic milk proteins.

SUMMARY

Cell-free milk protein synthesis as measured by C^{14} leucine incorporation has been accomplished using partially defined components from bovine lactating mammary tissue. With this system, a net incorporation of C^{14} leucine into trichloroacetic acid (TCA) precipitable protein and into specific milk proteins could be demonstrated. This synthesis was dependent on the presence of ribosomes and energy and was partially dependent on the addition of sRNA and aminoacyl-sRNA synthetase preparations. The incorporation was linear with respect to time for 10 to 20 minutes and reached a maximum in approximately 40 minutes.

Polyribosomes incorporated more C^{14} leucine into TCA precipitable protein than did ribosomes. Magnesium ion concentration affected the level of incorporation by altering the proportion of polyribosomes present in the system; i.e., concentrations less than 4 mM caused both a dissociation of polyribosomes and a decrease in C^{14} leucine incorporation.

Inhibitor studies indicate that the mammary gland synthesizes protein by a mechanism similar to that established for other mammalian systems, that is, the incorporation is very sensitive to ribonuclease, puromycin, cycloheximide, and sodium fluoride but insensitive to chloramphenicol. The incorporation system was insensitive to

deoxyribonuclease and to hormones which greatly affect mammary gland physiology. This indicates that only translation of the preformed genetic code takes place in this system and that DNA-dependent RNA synthesis utilizing microsomes in the complete system, incorporation was not stimulated by the addition of polynucleotides. This suggests the existence of unique structural characteristics of the microsomes. Preliminary studies indicated that the initiation factor found on reticulocyte ribosomes was either absent from the mammary gland microsomes or not removed by the procedures which were used. The C^{14} leucine incorporated was judged to reside largely in the milk protein by gel filtration chromatography, isotope dilution tests, and by immunodiffusion and radioautography. Column chromatography demonstrated that the size of the synthesized proteins was in the 32,000 molecular weight range. Isotope dilution studies illustrated that α-lactalbumin, β-lactoglobulin, κ, α_s, and β-caseins were synthesized. Radioautography of immunodiffusion studies demonstrated a synthesis of α_s-casein by the system.

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APPENDIX

TABLE 1

Composition of the Thixotropic Counting Fluid (65)

PPO	7 g
POPOP	150 mg
Naphthalene	50 g
Toluene	200 ml
p-Dioxane	800 ml
100% Ethanol	30 ml
Thixotropic Gel Powder (Cab-O-Sil)	36 g

TABLE 2

Composition of Amino Acid Mixture (126)

Amino Acid	mg/100 ml
Glycine	19
DL-Alanine	35
DL-Valine	72
L-Leucine	103
L-Isoleucine	76
L-Proline	116
DL-Phenylalanine	55
L-Cysteine	5
DL-Methionine	31
DL-Tryptophan	12
L-Arginine Hydrochloride	40
L-Histidine Hydrochloride	32
L-Lysine Hydrochloride	82
DL-Aspartic Acid	72
L-Glutamic Acid	220
DL-Serine	59
DL-Threonine	45
L-Tyrosine	61
L-Glutamine	110
L-Asparagine	36

TABLE 3

Hanks' Basic Salt Solution (150)

	mg/100 ml
Glucose	120
NaCl	680
KCl	40
$CaCl_2$	20
$MgCl_2 \cdot 6H_2O$	20
$NaH_2PO_4 \cdot 2H_2O$	15
$NaHCO_3$	220

TABLE 4

Composition of Kinard's Counting Fluid (149)

p-Dioxane	385 ml
Xylene	385 ml
100% Ethanol	230 ml
PPO	5.0 g
POPOP	50 mg
Naphthalene	80 mg

TABLE 5

Composition of Medium A (151)

Component	Molarity	g per liter
Sucrose	0.35	119.8
$KHCO_3$	0.035	3.50
$MgCl_2 \cdot 6H_2O$	0.005	1.01
KCl	0.025	1.86
Glutathione (reduced)	0.001	0.35

Adjust final solution to pH 7.4 with HCl