DETERMINATION OF OPTIMUM AMINO ACID COMPLEMENT FOR MILK PROTEIN SYNTHESIS IN THE DISPERSED RAT MAMMARY CELL CULTURE

by

Chung Sun Park

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APPROVED:

Dr. P. T. Chandler, Chairman	Dr. R. G. Cragle
Dr. C. W. Heald	Dr. N. R. Krieg
Dr. C. E. Polan	Dr. F. W. Thye

Dr. K. E. Webb, Jr.

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Blacksburg, Virginia

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I. INTRODUCTION

Although it was recognized very early that the secretory product of the mammary gland contained significant amounts of protein, conclusive evidence, that the origin and site of synthesis of the major milk proteins was located in mammary secretory cells, was obtained only during the past two decades (59).

Recently, considerable research has been conducted concerning the uniqueness of the secretory cell for producing specific products, the proliferation of this cell differentiation and then synthesize milk, the active metabolic rate of this cell, and its dependency on hormonal influences. These efforts appear to be focused on exploring both control mechanism and pathways concerned with a specific function, i.e., milk synthesis with the biological process of cellular differentiation. However, knowledge of milk protein synthesis with respect to specific limiting amino acids and most desirable combination for the maximum milk protein synthesis has been quite limited.

Secretory cells of the mammary glands were found to survive well in cell culture and to continue the specialized activities associated with milk synthesis for some period of time (32, 42, 90). Secretory cells derived from the mammary gland of both rat and bovine species survived and proliferated well in dispersed cell culture, but within a few weeks lost the ability in a nonparallel manner to synthesize the unique constituents of milk (32,56,90). If an adequate amino acid

complement was included in the nutrient medium, a considerable amount of milk protein synthesis occurred during the first few days in culture with an initial rate comparable to *in vivo* (32,56,90).

Cells depend primarily on the culture medium to supply the necessary precursors and the most direct precursory role of amino acids involves protein synthesis. This dependency provides us with direct means to determine the essential amino acid requirements of the secretory cell for milk protein synthesis. Thus, the purposes of our study were to identify the limiting amino acids and to establish optimal combination of amino acids which would result in maximum milk protein synthesis.

II. REVIEW OF LITERATURE

General Role of Amino Acids in Lactation

Milk synthesis is a result of the integration of many diverse functions for millions of secretory cells, each under similar genetic control. It involves synthesis through many different metabolic pathways of several proteins, sugar, and fat from precursors available in blood. This whole process requires tremendous amounts of energy, resulting in the energy supply being perhaps one of the most critical factors for milk synthesis. Each epithelial cell in the udder generates its own energy for metabolism by oxidation processes with glucose and acetate as the main substrates oxidized (3). Protein synthesis requires energy as well as a complement of amino acids, and this energy is generated as a result of cellular metabolism. Thus, optimal milk protein synthesis cannot occur unless an adequate supply of energy is available.

As protein is synthesized and milk is produced by the mammary gland there are continuous demands for three major types of blood precursors; peptides, plasma proteins and free amino acids. To meet these demands, a ceaseless supply of dietary sources of nitrogen from either intact dietary proteins or microbial proteins, produced from nonprotein nitrogen sources, are required by the lactating animal for normal production of milk. It has been established that the major portions of milk protein are present as casein, β-lactaglobulin and α-lactalbumin. These proteins are synthesized within the mammary secretory cell from a common pool of free amino acids. Both essential and nonessential amino acids, of which the latter may be synthesized in mammary cells, are found in this pool (79,92).

Mitchell and Block (69) developed the concept of the first limiting amino acid by comparing the amino acid pattern in a specific protein with the amino acid pattern of egg protein which served as a reference. The first limiting amino acid was defined as the amino acid present in the smallest amount in comparison with the reference protein. Based on this concept, if the rate of milk protein synthesis is lowered due to an inadequate supply of the limiting amino acid, then increasing the quantity of the limiting amino acid should increase protein synthesis. Correction of amino acid imbalance would also stimulate the demand for other amino acids, and consequently may cause a lowering of their concentrations in the amino acid pool.

In practice, availability of certain critical (limiting) amino acids for synthesis of a specific protein by secretory cells appears to be one of the key limiting factors in the process of milk production. The α-lactalbumin, synthesized from free amino acids absorbed by the mammary cells, has been established to be one of the two protein constituents of lactose synthetase (13,33,34,35), the key enzyme for lactose synthesis. It has been suggested that lactose regulates the total volume of milk produced because it is the main contributor to osmotic pressure (94). Thus, the rate of milk protein synthesis may be the major limiting factor in total milk production. In addition to the direct precursory role in the synthesis of milk protein, amino acids play a major role in governing the rate of milk secretion.

Metabolic Events of the Lactating Mammary Gland

Secretory mammary cells from different species have an almost identical structure irrespective of the fact that the milk secreted among

species varies widely in its chemical composition (46). From various sources, Hallmann (46) concluded that all mammary cells are sharing a uniform ground plan and that only insignificant differences exist, such as the size of the casein granules elaborated by mammary cells of different species. The quantities of the characteristic components of milk, however, vary among species (79). It has been established that the epithelial cells of the mammary gland are similar to most cells in the body in that they have the same organelles and structural components. Also it appears that major metabolic functions of cellular organelles of secretory cells are not markedly different from those of other metabolically active cells (9,19).

When the relationship between products and precursors for milk synthesis are considered, it is apparent that the secreting epithelial cell must have the enzymatic complement to carry out a variety of biochemical reactions. These reactions involve the synthesis of polymer(s), energy transfer reactions, intermediary metabolism which generates key intermediates for the synthesis of macromolecules and coenzymes, and finally catabolic reactions. Davis and Bauman (19) stated that information on cellular localization of numerous enzymes known to be present in mammary tissue is limited. It has been suggested, however, that distribution of these enzymes, in general, is not different from other tissues where data are plentiful.

Larson in 1969 (56) reported that metabolic events of the mammary gland are characterized by a unique complement of synthetic pathways. Some are normal to most tissues and others are active at greatly increased levels. As with liver, the hexose monophosphate pathway becomes operative

in mammary tissue until more than half of the glucose is metabolized via this route instead of the glycolytic pathway. Appearance of some new pathways with specific enzymes unique to secretory cells occur at this time. Examples are those concerned with lactose and α-lactalbumin synthesis (33,34,56). Specific products of the lactating cell such as milk proteins also begin to appear (54,55,90). Generation of $NADPH_2$ by the pentose phosphate shunt leads to reductive synthesis of fatty acids which are characteristic of milk fat (7,8,19).

Species differences make it difficult to unify the characteristic pattern of intermediary metabolism associated with milk synthesis. Lindsay (62) observed that nonruminant species rely heavily upon glucose, derived from dietary carbohydrates, as a source of energy and carbon for tissue metabolism. On the other hand ruminants, because of the extensive fermentation process of dietary carbohydrates in the rumen, absorb little glucose from their digestive tract. Ballard et al. in 1968 (7) reported that ruminants depend almost entirely upon gluconeogenesis for the glucose needed for specific metabolic functions.

It is well established that lactose is synthesized from glucose and UDP-galactose in the presence of the enzyme lactose synthetase. This dual protein enzyme complex consists of two protein moieties, one of which is the α-lactalbumin found only in mammary tissue and the other is a nonspecific galactosyltransferase that is normally present in many tissues besides mammary gland (13,35).

The major roles that acetate plays in overall metabolism of the mammary gland are the supply of the carbon backbone for de novo synthesis of fatty acids (8,19) and generation of ATP via the TCA cycle (3).

Hardwick et al. (43), Bauman et al. (8) and Balmain et al. (6) have demonstrated that ruminant mammary tissue cannot use glucose as a carbon source for fatty acid synthesis; however, acetate is readily utilized for this purpose. Hardwick in 1966 (44) proposed that ruminant mammary tissue is lacking a certain key enzyme essential for generation of cytoplasmic acetyl-CoA from glucose.

It has been established that free amino acids, absorbed directly from blood by the mammary gland, provide most of the nitrogen required for synthesis of milk protein. The evidence for incorporation of amino acids into milk protein comes from two sources, namely, arteriovenous differences and radioactive isotope studies (79).

In summary, the major components of milk are synthesized within the mammary gland from simple metabolites extracted directly from the blood. Glucose, acetate, β-OH-butyrate, amino acids and fatty acids have been established as primary substrates for support of the synthetic activity of mammary tissue (79).

Tissue Cultures of Mammary Secretory Cells

General Method of Tissue Culture

Animal tissue culture is concerned with the study of cells, tissues and organs explanted from animals and maintained or grown in vitro. Dependent upon whether cells, tissues or organs are to be maintained or grown, two methodological approaches have been developed in the field of tissue culture. By definition (68), the term "cell culture" is used to denote the growing of cells in vitro, including the culture of single cells. In single cell cultures, cells are no longer organized

into tissues. The term of "tissue or organ culture" denotes maintenance or growth of tissues, organ primordia, or whole parts of an organ *in vitro* in a way that may allow differentiation and preservation of tissue architecture and/or function. In other words, organ culture indicates a somewhat larger slice of tissue or even an entire embryonic organ which is maintained on the surface of the medium in order to observe some of the physiological functions without outward growth. The organ culture method has been especially useful in delineation of endocrinological factors necessary for induction and maintenance of lactation, including milk protein synthesis. The review of Forsyth (37) provides a more complete description of its use.

Use of cell cultures of mammary tissue has provided a system desirable for many studies concerning mammary function and milk synthesis (55,58,90). Methods for preparation and cultivation of dispersed cell culture in connection with organ culture is found in the report by Anderson and Larson (2).

The defined medium for any of these methods is a basal salt solution to which is added various nutrients such as glucose, amino acids, vitamins, antibiotics and some blood serum. Modifications of published medium formulations are apt to be adopted, by the original authors and by others, either as improvements on media for their primary use or to adapt them for other types of culture (93). The survey compiled by Morton in 1970 (71), reviewing 23 commercially available tissue culture media and methods of preparation recommended by their authors, would provide a useful guide. All of these media have the need for some serum except for use with a few strains of long-term cultivated cells that

have been developed (93). Temin et al. (87), who have made an exhaustive survey of the literature relating to the need for serum in mammalian cells in culture, concluded that the nature of the biological effects of serum on cells in culture and of factors in serum which exert these effects is still largely unknown.

Certain cells, especially isolated single cells, grow much better in conditioned medium (medium in which cells have been grown before). This points out that most animal cells are very dependent on surrounding cells and tissue (45,58). The cells, upon being removed from the micro-environment of tissue, adapt to their new surroundings by becoming more generalized. This phenomenon is not quite so striking in organ culture. However, other changes such as differential cell death complicate the use of organ culture. Another consideration for any technique of cell dispersion or isolation is that any method of chemical, mechanical or enzymatic dissociation of cells could be selecting for a particular cell type that is best suited for survival under the conditions used (45,93). These selective pressures may also occur as a result of the choice of the type of flask or dish and even the kind of glass that is used as the culture vessel, the method of washing and cleaning, or even the purity of water.

Studies with Dispersed Mammary Cell Cultures

In spite of many problems inherent in tissue methods, a relatively simple system for study of the specific primary effect of hormones on their target organ is provided. The dependence of the mammary gland on a variety of hormones is well established and thus, it is a logical

tissue of choice. By using mammary organ cultures of midpregnancy mice, investigators (64,75,89) determined the combinations and amounts of hormones that were most effective in establishing the secretory appearance of the cells and stimulating protein synthesis. Rat tissue does not respond as well as mouse tissue, indicating that there are some undefined factors that affect differentiation of cells (20). These factors are not necessarily the same for all mammals (21,21).

Dispersed cell culture techniques, as developed by Lasfargues (60) with mouse tissue, were first applied to the bovine mammary gland around 1961 (31,32). This has provided a system for the study of factors and precursors concerned specially with milk protein synthesis. Although such cultures lose the ability to perform specific functions associated with milk synthesis as cells undergo a dedifferentiation process, significant milk synthesis continues for some time (1,32,48,56,58,91).

Some functions of both bovine and rat cells that have been studied in culture are synthesis of lactose (91), α-lactalbumin (42,88), β-lactoglobulin (42,47,57,80,90), β-casein (42,47,55,80), secretory lipids (48), and orotic acid (17). Several enzymes involved in milk synthesis were tested for activity at various stages in culture (11,32,56). The general conclusion was that specialized functions do not cease simultaneously, but that loss of function occurs in a nonparallel fashion. This indicates that it is not just cell death that is occurring.

Both bovine and rat cell cultures have a requirement for higher than normal amount of amino acids. Increasing amino acids 3-fold over that in Eagle's minimal essential medium (23) increased the cells ability to synthesize the milk proteins (78). Hormone additions stimulated for

a short period the synthesis of β-casein and β-lactoglobulin (2). However, both of these functions still disappeared eventually but their rate of loss relative to each other was changed in that the hormones had a greater effect on the maintenance of β-casein synthesis. Larson (56) reported that β-lactoglobulin synthesis was not as much affected by amino acid or actinomycin-D concentration as that of β-casein synthesis. This indicates different controlling mechanisms are operating for the different proteins. This, for example, could be related to the functional role of the final products such as the established relationship of α-lactalbumin synthesis and synthesis of lactose. Whatever the mechanism of control, there are still many unanswered questions about control mechanisms for milk formation and about the exact mechanism of milk protein synthesis. Some questions can certainly be answered by continued use of cell culture systems.

In Vitro Synthesis of Milk Protein

Studies Related to Milk Protein Synthesis

Studies conducted with a variety of systems have indicated that the overall mechanism of protein synthesis is, in general, similar in all cells for all species. Based on these results it is assumed that the general mechanisms of protein synthesis is similar in mammary secretory cells. The reader is referred to a volume edited by Lehninger (61) for an excellent reference on general mechanisms of protein synthesis.

Studies of mechanisms of protein synthesis in secretory cells from mammary glands have relied heavily upon the use of radioactive labeled amino acids, intricate physical methods to fractionate and characterize

subcellular components, and various methods to identify the synthesis of specific milk proteins. Investigations with whole animals and perfused mammary glands generally have involved administration of a labeled amino acid into the blood stream with subsequent isolation of the labeled proteins from synthesized milk. Studies utilizing cells and tissue slices with labeled amino acids as precursors for milk protein have generally involved the use of cold milk proteins as a carrier in the isolation procedure because of the small amount of milk protein synthesized. Procedures have been developed to determine synthesis of specific milk proteins. Such methods must be capable of measuring microgram quantities. Those methods have included the immunological procedures for the whey proteins (12,54) as well as chemical procedures utilizing ^{14}C or ^{3}H-labeled amino acids, with subsequent isolation and identification of the specific protein (2,42,47,57). The dispersed cell culture technique has provided a system which is useful to study precursors and factors associated with milk protein synthesis (2,42,47,55,57,78,90). By utilizing radioactive amino acids with bovine and rat mammary cell cultures, it was shown that in the first few days after establishment, significant synthesis of major milk proteins including β-lactoglobulin, α-lactalbumin, and β-casein resulted. Schingoethe et al. in 1967 (78) found that elevation of the quantity of amino acids had a stimulative effect on synthesis of β-lactoglobulin and β-casein in rat cells but only β-casein in bovine cells.

Such cultures appear to provide an environment in which significant quantities of labeled specific milk proteins may be obtained with

different labeling characteristics. By using various ^{14}C and ^{3}H-labeled amino acids as precursors, such proteins have been prepared (58). Addition of cold milk (radioactive free) as a source of protein carrier to the culture medium for the subsequent isolation process appears to be essential (42).

Uptake and Utilization of Amino Acids by Cell Culture

Amino acids play a diverse role in metabolism of cells. Their direct participation in synthesis of structural or export proteins and enzymes related to all aspects of metabolism designates them as "limiting factor." In general, the amino acid composition of culture medium has been for the most part based on quantities found in biological fluids. Alterations of the quantities were made by observing the growth or survival of different cell populations. The criteria for adequate amino acid nutrition generally has been based on growth, not function. Green and Todardo in 1967 (39) stated that proliferating cells, whose primary goals appear to be growth, may differ markedly in their amino acid requirements from a metabolic standpoint, when compared to differentiated tissues such as liver or mammary cell, whose ultimate goal is functional or the synthesis of transport products.

Dependence of cells on culture medium to supply the necessary precursors would make it possible to determine essential amino acid requirements of the mammary cell to synthesize protein. This can be done by varying the amino acid complement in the medium and measuring the net synthesis of protein. Schingoethe et al. (78), utilizing dispersed cell culture with the minimal essential medium (MEM) of Eagle, tested all 13 essential amino acids listed in MEM and found that with

the exception of tyrosine all were required by bovine secretory cells. Similar studies showed that cystine was not required by rat mammary cells. They found that glutamine was not required by the cells of both species. Amount of protein synthesis could be increased considerably by the inclusion of the total complement of nonessential amino acids in the culture medium as well as through elevated amounts of both essential and nonessential amino acids (56,57,78). In 1967 Schingoethe et al. (78) showed that raising the essential amino acids about 5-fold over the normal physiological quantities present in tissue culture medium had no effect on β-lactoglobulin synthesis in bovine cells, but nearly doubled net synthesis of β-casein. An even more marked effect was found in rat mammary cells for both proteins. These results suggested that certain amino acids may normally be limiting to the cell for synthesis of milk protein. If sufficient precursors could be made available through artificial means to the cell above that normally present, there was a possibility that the protein content of milk in vivo could be elevated by this means.

The basic concept of movement of amino acids across mammalian cell membranes and the kinetics involved have been developed primarily by Christensen and coworkers (18). Cells are capable of concentrating, intracellularly, pools of amino acids against a concentration gradient by a stereospecific mechanism requiring energy. Passive or facilitated diffusion are biologically significant, but only under certain conditions and in certain systems (85). The intricacy as well as the heterogeneity of the system at the cellular level is not unexpected because of problems of not only transporting nutrients into the cell but also being

responsible for the exodus (leakage) of metabolites from the cell. In addition to the intricate transport mechanisms themselves, the whole system is dependent on balance of nutrients (and not just amino acid) in culture medium. Moreover, this balance could conceivably determine whether a cell undergoes proliferation, retains or assumes a differentiated functional state, or is maintained as a nonfunctional entity (72). The extent of plasma membrane alteration and its effects on amino acid transport appears to be unclear. Hormonal effects on amino acid transport are variable (72).

Studies on uptake of exogenous sources of amino acids by a variety of cells in culture have revealed a similar pattern in terms of cell growth or yield. Usually glutamine, arginine, leucine and isoleucine are used in greatest concentrations followed by lysine, valine and phenylalanine (41).

Studies (41,50,65) on rates of amino acid uptake showed that glutamine was the most rapidly used, followed by lysine, leucine, and isoleucine. Comparing relative rates of uptake with composition of cellular protein, Kruse and coworkers in 1967 (50) reported that the ratio was not proportional. Isoleucine utilization rates, for example, were in excess of the percentage of the amino acid found in protein. Ling et al. (63) and Stoner (86) observed that modification of media to contain valine, isoleucine, and leucine in the same relative concentrations as those found in cellular protein resulted in a disappearance of the excessive uptake of isoleucine. This research suggested that a media to be used for a specific purpose of study may best be formulated on the basis of cellular demand. A high rate of amino acid uptake does

not necessarily imply high utilization of that specific amino acid. Each cell line appears to have a characteristic pattern which reflects subsequent utilization of amino acids for metabolic pathways. In 1972 Patterson (72) stated that on the basis of cell protein content, but not the secretory product of the cell, uptake correlated with relative proportions of amino acids in cell protein.

It is generally assumed that intracellular amino acid pools either derived from exogenous sources or by de novo synthesis may exist as single entities. However, Eagle et al. (25) have suggested that intracellular compartmentation of amino acids may exist. It is possible that certain endogenously and exogenously supplied amino acids are metabolized differently. Kuchler (52,53) showed that doubling the concentration of the essential amino acids of MEM failed to increase the rate of protein synthesis in L-strain of mouse fibroblasts but did increase intracellular essential and nonessential amino acids. Eagle (25) found that with some amino acids such as valine and lysine the degree of concentration in the intracellular pool varied within wide limits and seemed independent of exogenous quantities. With others, such as threonine, the degree of concentration increased with lower concentrations of exogenous supply.

The most direct precursory role of amino acids is that involving protein synthesis. Definite and extensive studies in this area have been done by Eagle et al. (25). The relationship of individual amino acids to the synthesis of proteins has been studied by using labeled precursors. Some results are collagen synthesis by dispersed rat bone cells (10) and milk protein synthesis by bovine mammary cells (78) which was described earlier in this review.

Cells in culture generally utilize amino acids through established metabolic pathways (67). Information concerning conversion of amino acids to metabolites and pathways leading to the synthesis of structural or secretory protein appears unclear when compared to the relatively rich information on the catabolic fate of amino acids. Specific knowledge relevant to milk protein synthesis is limited.

Jorgensen and Larson in 1968 (47) reported that cultures of bovine mammary secretory cells *in vitro* did not require a dietary source of tyrosine in order to synthesize milk proteins, if suitable phenylalanine was present. These workers demonstrated that secretory cells of lactating bovine mammary gland had significant phenylalanine hydroxylase activity which explains the apparent lack of requirement for tyrosine. Rat mammary cells, however, did require tyrosine for milk protein synthesis, supporting the common belief that in some mammals significant synthesis of tyrosine takes place only in the liver.

The metabolism of tryptophan in mammalian tissues occurs mainly by two pathways. Both pathways involve its oxidation with products of nicotinic acid or melatonin. Wurtman *et al.* (95) reported in cell culture systems the conversion of tryptophan to melatonin.

The diverse precursory role of glutamine should not be unexpected because of its apparent paradoxical requirement by cells in culture and its decomposition in culture medium (67). Decomposition by glutaminase gives rise to glutamic acid and ammonia. Kitos and Waymouth in 1966 (49) reported that glutamic acid through transamination and entry into the Kreb's cycle contributed carbon atoms to proline, alanine, lactic acid, carbon dioxide and aspartic acid. Eagle (23) noted that many

cells require glutamine under certain conditions as an essential amino acid. Because of this observation, glutamine is usually included among the essential group in the MEM. Particular attention has been given to utilization *in vitro* of glutamine, since it is well known that this amino acid can be synthesized *in vivo* from other precursors. The non-requirement for glutamine in secretory cells for both rat and bovine species was confirmed by demonstration of glutamine synthetase activity (78).

Methionine is utilized as a methyl and sulfur donor in cystine synthesis pathways. Although earlier studies (26) suggested that cystine was required by cells in culture, subsequent work by Eagle (27) showed that at sufficiently high cell populations, this requirement no longer existed. However, Foley *et al.* in 1969 (36) reported that cystine was required by diploid cell lines of human leukemic cells. In other studies, using growth as the criteria, Eagle (28) observed that the immediate precursor cystathionine was found to be an essential amino acid under stress or low cell numbers. This was due to the leakage from the cell of intermediates along the pathway of cystine biosynthesis from methionine. Schingoethe *et al.* (78) demonstrated that requirement of bovine cells for cystine was not affected by elevating methionine. However, this was not true with rat mammary cells, thus supporting a species difference.

Knowledge for lysine and histidine degradative pathways as well as incorporation into macromolecules is lacking.

Among those amino acids generally required for growth in cell culture, several are noteworthy because they do not represent dietary essentials

for the whole animal. Cystine, tyrosine, and glutamine are not included in the list of ten amino acids needed to support the growth of rats (76). Rose *et al.* in 1954 (77) found that even arginine and histidine could be omitted from the human diet if mere maintenance of nitrogen equilibrium rather than growth was used as the criteria. None of these five amino acids (cystine, tyrosine, glutamine, arginine and histidine) are dispensable in culture regardless of whether the cells used for assay are derived from human or other animal species (45).

Principal explanation for disparities that exist between amino acid requirements *in vivo* and *in vitro* lie in two directions (45). It is conceivable that modulation or dedifferentiation might lead to a systemic reduction in potential for amino acid biosynthesis within the isolated cells in culture. The other concept is that while biosynthesis of certain amino acids (for example, cystine) may proceed at rates sufficient for the needs of tissue cells within the closely arrayed populations of the intact animal, the yields are inadequate for the requirements of cells in monolayers and other isolated systems (29). Eagle (27,28,29) and Harris (45) have reviewed this subject from the standpoint of general interpretations.

III. OBJECTIVES

The purpose of this investigation was to determine the amino acid requirement for milk protein synthesis in the dispersed rat mammary cell culture system.

Specific objectives were:

1) To determine if ^{14}C-(U)-L-lysine, leucine and phenylalanine incorporation into milk proteins would result in the same quantity of measured protein synthesis.
2) To examine the effect of cell density in the dispersed cell culture on protein synthesis.
3) To investigate the effect of various amounts of the total amino acid complement on synthesis of milk protein.
4) To determine what effect the reduction of a single amino acid from the total amino acid complement in the medium has on milk protein synthesis.
5) To investigate the effect of single amino acid addition to the basic amino acid complement on milk protein synthesis.
6) To establish conditions under which the optimum combination of amino acids would result maximum milk protein synthesis.

IV. MATERIALS AND METHODS

Preparation of Dispersed Rat Mammary Cell Culture

Dispersion of Mammary Tissue

The mammary tissues, utilized throughout the experiments, were from Sprague Dawley rats four to ten days postpartum. The rats were sacrificed by ether anesthesia. Immediately after the rats ceased to respirate, four main mammary glands were removed and bathed in sterile balanced salt solution (BSS) containing antibiotics.

The cell dispersion procedure was essentially the method developed by Ebner et al. (31). With sterile scalpels and scissors the glands were cut into pieces of 5 to 7 mm^3, eliminating as much extraneous lymph node and connective tissue as possible. The tissue was transferred to the depression of a micro concavity slide (maximov) in a petri plate. Tissue was minced with a pair of scalpels to less than one mm^3 in 1 ml of a collagenase solution. The collagenase solution was 450 mg of crude collagenase[1] in 100 ml in Hank's BSS. It was sterilized by filteration after allowing to stand at room temperature for 4 to 8 h. Minced tissue was transferred into a sterile 50 ml centrifuge tube with the addition of 2 ml of extra enzyme solution for each piece of tissue chopped. One centrifuge tube provided enough cells for about 20 to 30 T-30 culture

[1]Clostridiopeptidase A, I.U.B. 3.4.4.19, 160.5 units/mg, Worthington Biochemical Corp., Box 650, Freehold, NJ 07728.

flasks. The tubes were incubated at 1 h at 37 C with continuous agitation. Following incubation, cells were centrifuged at 150 x g for 5 min., resuspended in fresh collagenase solution, and returned to the shaker. After the second hour of incubation, the cells were again spun and washed twice with BSS containing 1% bovine serum, and one additional time with BSS containing 15% serum. After three washings, an aliquot of 1 ml was removed and used for cell counts.

Enumeration of cells were made by the method of Bryant et al. (14). This method utilized crystal violet in .2 M citric acid and pH 6.7 phosphate buffer and stained only the viable nuclei. The cell aggregate was dispersed by homogenization with a power driven pestle after a 15 min. incubation at 37 C. An aliquot was take up in a white blood diluting pippette and shaken for 4 min. after which duplicate samples were counted in a hemacytometer.

After the cell count was established the appropriate amount of cells in BSS were precipitated by centrifugation and resuspended in the 15% serum in BSS followed by filtering through six layers of cheesecloth. Routinely, 1 to 2 x 10^7 cells were placed in each T-30 flask in 5 ml of nutrient medium. Cap tightened culture flasks containing both cells and nutrient medium were then incubated for 18 h at 37 C. In all studies, three flasks were set up for each observation with contents combined after incubation for subsequent analysis.

Culture Medium

The basic medium used was the minimal essential medium (MEM) of Eagle's (23) with slight modifications. Glucose was elevated by 25% (73) and 7% dialyzed bovine serum was added to MEM. Nutrients, other

than amino acid complement, including salts and vitamins were present at concentrations as described by Eagle (23). The conditioned MEM as described above is designated as "1X-MEM" hereafter. The amount and complements of amino acid present in 1X-MEM are termed as "1X." The terms "1X-MEM" and "1X" were utilized in all experiments. The 1X in the 1X-MEM were varied depending upon the objective of the specific study. Chemical composition of 1X-MEM is given in table 1.

Earle's (30) BSS, utilized for cell washing and suspension, contained 1% and 15% of dialyzed bovine serum, respectively. The antibiotics were penicillin-G at 100 u/ml, streptomycin sulfate at 100 µg/ml and nystatin at 50 u/ml. Antibiotics were added to all media and salt solutions except the 15% serum in BSS. In some studies, pH of the media was adjusted by addition of 7.5% sodium bicarbonate. Culture media, salt solutions, culture mixtures and other reagents were obtained from commercial sources.[2]

The isotope used most frequently for labeling cell cultures was ^{14}C-(U)-L-lysine. In one experiment, both ^{14}C-(U)-L-leucine and phenylalanine were utilized in the culture in addition to labeled L-lysine. Routinely .45~.50 µCi of the tracer was added to each culture flask. These labeled precursors were added to cultures for the purpose of determining <u>in vitro</u> synthesis of milk proteins. Following subsequent isolation and purification of synthesized proteins, approximately 5 to 10 mg of the protein was counted in Aquasol[3] in a liquid scitillation

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[2]Grand Island Biological Co., 3175 Staley Road, Grant Island, NY 14072, and Microbiological Associates, 4813 Bethesda Avenue, Bethesda, MD 20014.

[3]New England Nuclear, 575 Albany Street, Boston, MA 02118.

Table 1. Composition of 1X-MEM.

Components		Components	
L-Amino Acids[a]	(mg/L)	Glucose[b] (mg/L)	1,250.0
Arginine	105.0	Inorganic Salts[c]	
Histidine HCl-H_2O	41.9		
Isoleucine	52.5	Vitamins[d]	
Leucine	52.4		
Lysine HCl	73.1	Supplements	
Phenylalanine	33.0		
Threonine	47.6	Bovine Serum (ml/L)[e]	70.0
Tryptophan	10.2	Antibiotics[f]	
Valine	46.8	^{14}C-(U)-amino acids[g]	
Methionine	14.9		
Tyrosine	36.2		
Cystine	24.0		
Glutamine	292.0		

[a] The amino acid complement in Eagle's MEM and defined as "1X".

[b] Concentration shown was the adjusted value by 25% increase from what was given in MEM.

[c] Earle's BSS without glucose.

[d] The same components as defined in the MEM.

[e] The added 7% (V/V) serum to the MEM.

[f] Penicillin-G, streptomycin sulfate, and nystatin at levels of 100 u/ml, 100 µg/ml, and 50 u/ml, respectively.

[g] L-Lysine, leucine, or phenylalanine incorporated at ≈ 50 µCi/L, depending on the objective of the study.

spectrometer (Coru/matic II. Tracerlab) utilizing an external standard of Ra^{226} for quench correction.

Isolation of Synthesized Milk Proteins

At the termination of incubation (18 h) cells were removed from the surface of the flask by using a rubber-policeman and the contents of three flasks for each treatment were pooled and ground in a glass homogenizer. A volume of fresh bovine skim milk equal to the total ml of media present was added to the mixture of cells and medium. The skim milk served as a source of unlabeled protein carrier.

β-lactoglobulin (β-LG) and β-casein (β-CA) were isolated according to Groves and Larson (42), modified from Aschaffenburg (4) and Aschaffenburg and Drewery (5). The pH 2.0 precipitate was retained from β-LG procedure and served as the starting material for subsequent recovery of α-lactalbumin (α-LA) by the method of Aschaffenburg and Drewery (5). In order to have sufficient amounts of α-LA for isolation, the pH 2.0 precipitates from two groups of flasks (6 total) were pooled. In some studies, a pure source of α-LA (.834 mg/ml of the original medium used) was introduced to the precipitates to serve as an unlabeled protein carrier. Following an exhaustive dialysis for 5 days, the protein solutions were lyophilized for the final crystallization.

In order to calculate net synthesis of a milk protein, total decomposition per minute (dpm) in the protein of each treatment group as well as the specific activity (dpm/μg) of synthesized protein fractions were calculated through mathematical steps utilizing the necessary assumptions. The specific activity (cpm/mg) of the lyophilized fractions were obtained directly by weighing and counting of isolated proteins by liquid scintillation.

The weight of protein synthesized was assumed negligible compared to weight of unlabeled carrier protein introduced for isolation. Thus, it was possible to calculate the total dpm incorporated per flask into protein. The equations used for calculation of synthesized protein are:

$$P = [A/R]/\text{number of flasks, where}$$

P = protein synthesized (μg/flask), A = dpm incorporated into protein (dpm), R = specific activity of synthesized protein (dpm/μg), and

A = ((S.A.(cpm/mg) of isolated protein)*(mg of cold carrier))/(isotope counting efficiency)

R = ((dpm added as specific labeled amino acid)*(known % of amino acid in protein))/(total μg of specific amino acid pool).

Experimental

Design of Experiment

Effect of Labeled Precursor Sources

Two replications of a 2 x 3 factorial experiment were conducted. The factors were two quantities of lysine (1X and 3X) and three sources

of uniformly labeled ^{14}C-amino acids (lysine, leucine and phenylalanine). Two animals were used in each replication. The treatments used in this study are presented in table 2. Treatments were evaluated by determining the net synthesis of the major components of milk protein; β-LG, β-CA and α-LA.

Effect of Cell Density

A 3 x 2 factorial study with three cell densities of approximately 6×10^6(A), 2×10^7(B) and 6×10^7(C) cells per flask and with two amino acid complements at 1X and 3X was conducted in two replications. Each treatment group was composed of three observations by utilizing a total of nine flasks each. The treatments used for the study are given in table 3.

Investigation of Graded Quantities of Amino Acid Complement

Two replications of a 5 x 2 factorial study, consisting of five graded amounts of essential amino acid complement (1X through 5X) and two culturing environments (carbon dioxide regulated vs. conventional incubation) were conducted. Two observations were made in each replication on each of ten treatments. Three animals were used to supply the cells needed for 60 flasks in each replication. When additional pH control of the treatment media was required, sodium bicarbonate was added to adjust pH to 7.4 (table 4).

Effect of Single Amino Acid Omission

A total of ten replications were conducted in the omission studies. Eight replications (omission 1-8) consisted of 13 treatments each and

Table 2. Treatments used for study of labeled amino acid precursor sources.

Treatment	Base Medium	Amount of Lysine[a]	^{14}C-(U)-L-Amino Acids[b]
1	1X-MEM	1X	Lysine
2	1X-MEM	3X	Lysine
3	1X-MEM	1X	Leucine
4	1X-MEM	3X	Leucine
5	1X-MEM	1X	Phenylalanine
6	1X-MEM	3X	Phenylalanine

[a]To adjust in the final concentration by 3X (1.095 mg/flask), 2X (.730 mg/flask) concentration of lysine was added to the pertinent treatment group.

[b]Quantity of each labeled amino acids added to an individual flask was .438 µCi.

Table 3. Treatments used for investigation of cell density.

Treatment[a]	Base Medium	Amino Acid Complement[b]	Number of cells (#cells/flask)	^{14}C-(U)-L-Lysine (μCi/flask)
1	1X-MEM	1X	6×10^6	.448
2	1X-MEM	3X	6×10^6	.393
3	1X-MEM	1X	2×10^7	.448
4	1X-MEM	3X	2×10^7	.393
5	1X-MEM	1X	6×10^7	.448
6	1X-MEM	3X	6×10^7	.393

[a] Each treatment group consisted of 9 culture flasks.

[b] Values represent that amount of total amino acid complement over that present in the 1X-MEM.

Table 4. Treatments for the study of graded amino acid complement.

Treatment[a]	Base Medium	Amino Acid Complement[b]	Sodium Bicarbonate[c] (ml)	^{14}C-(U)-L-Lysine (μCi/flask)	Incubator
1	1X-MEM	1X	--	.438	Conventional
2	1X-MEM	2X	.20	.413	"
3	1X-MEM	3X	.30	.393	"
4	1X-MEM	4X	.40	.353	"
5	1X-MEM	5X	.50	.323	"
6	1X-MEM	1X	--	.453	CO_2-regulated
7	1X-MEM	2X	.25	.408	"
8	1X-MEM	3X	.35	.373	"
9	1X-MEM	4X	.50	.330	"
10	1X-MEM	5X	.60	.308	"

[a]Each treatment group consisted of 6 culture flasks/replication.

[b]Value represents that amount of total amino acid complement over that present in the 1X-MEM.

[c]Aliquot of sodium bicarbonate (7.5%) added to each flask for pH adjusted to 7.4.

the final two replications (omission 9-10) consisted of 15 treatments. Omission 9-10 were conducted under so-called "optimum conditions" as defined by a series of separate supplemental studies at this laboratory. The treatments for omission studies are presented in table 5. In each replication four animals were sacrificed and their mammary tissue pooled. Details on preparation of the dispersed cell cultures and on the construction of media were mentioned earlier. As shown in table 5, the number of essential amino acids in question, that were used to test the response on omission 1-8 and 9-10 were 11 and 13, respectively. Two control cultures were used; 1X-MEM (negative control) and 1X-MEM plus 2X essential amino acid addition (positive control = 3X-MEM). The single omission of an amino acid from the positive control back to the negative control quantity was repeated for each of the essential amino acids.

In all replications, ^{14}C-(U)-lysine was added to each culture flask at ≃ .45 to .50 μCi depending upon the conditions of media preparation. β-LG for all omissions and α-LA and β-CA for omission 9-10 were analyzed and these proteins were the criteria used to evaluate milk protein synthesis.

Study of Single Amino Acid Addition

The addition studies bore an exact inverse relation to that of the omission just described. Three series of the addition experiments (1, 2 and 3) were conducted. Addition 1 consisted of 15 treatments comprising two control groups (negative and positive). The amino acid complement used for Addition 1 is shown in table 6. Addition 2

Table 5. Treatments used for investigation of amino acid omission.

Amino Acids Components	Treatments[a] (mg/L)														
	1 Neg (-)	2 Pos (+)	3	4	5	6	7	8	9	10	11	12	13	14[b]	15[b]
Arg	105.	315.	-	+	+	+	+	+	+	+	+	+	+	+	+
His	42.	126.	+	-	+	+	+	+	+	+	+	+	+	+	+
Ile	53.	158.	+	+	-	+	+	+	+	+	+	+	+	+	+
Leu	52.	158.	+	+	+	-	+	+	+	+	+	+	+	+	+
Lys	73.	219.	+	+	+	+	-	+	+	+	+	+	+	+	+
Phe	33.	99.	+	+	+	+	+	-	+	+	+	+	+	+	+
Thr	48.	143.	+	+	+	+	+	+	-	+	+	+	+	+	+
Trp	10.	31.	+	+	+	+	+	+	+	-	+	+	+	+	+
Val	47.	140.	+	+	+	+	+	+	+	+	-	+	+	+	+
Met	15.	45.	+	+	+	+	+	+	+	+	+	-	+	+	+
Tyr	36.	108.	+	+	+	+	+	+	+	+	+	+	-	+	+
Cys	24.	72.	+	+	+	+	+	+	+	+	+	+	+	-	+
Gln	292.	876.	+	+	+	+	+	+	+	+	+	+	+	+	-

[a]Treatment 1 = (-); negative control (1X-MEM), Treatment 2 = (+); positive control (3X-MEM). + or - sign shown for treatment 3 through 15 corresponds to the actual value present in treatment 1 or 2.

[b]Treatments added for omission 9-10.

Table 6. Treatments used for investigation of amino acid additions 1[a], 2[b] and 3[c].

Amino Acids Components	Treatments[d] (mg/L)														
	1 (-)	2 (+)	3	4	5	6	7	8	9	10	11	12	13	14	15
Arg	105.	315.	+	-	-	-	-	-	-	-	-	-	-	-	-
His	42.	126.	-	+	-	-	-	-	-	-	-	-	-	-	-
Ile	53.	158.	-	-	+	-	-	-	-	-	-	-	-	-	-
Leu	52.	158.	-	-	-	+	-	-	-	-	-	-	-	-	-
Lys	73.(219.)	219.(657.)	-	-	-	-	+	-	-	-	-	-	-	-	-
Phe	33.	99.	-	-	-	-	-	+	-	-	-	-	-	-	-
Thr	48.	143.	-	-	-	-	-	-	+	-	-	-	-	-	-
Trp	10.	31.	-	-	-	-	-	-	-	+	-	-	-	-	-
Val	47.	140.	-	-	-	-	-	-	-	-	+	-	-	-	-
Met	15.	45.	-	-	-	-	-	-	-	-	-	+	-	-	-
Tyr	36.	108.	-	-	-	-	-	-	-	-	-	-	+	-	-
Cys	24.	72.	-	-	-	-	-	-	-	-	-	-	-	+	-
Gln	292.	876.	-	-	-	-	-	-	-	-	-	-	-	-	+

[a]Addition 1 study consisted of 15 treatments comprising two control treatments.

[b]Amino acid complement used for the Addition 2 study differed from the Addition 1 study only in terms of the amounts of lysine which are shown in parenthesis.

[c]Treatment composition for Addition 3 was identical to that for Addition 2 except the former comprised one additional treatment group which was the original negative control from Addition 1.

[d]See footnote a in table 5.

study differed from Addition 1 only in terms of the amount of lysine in the media. The concentration of lysine in the two control groups and of the lysine treatment in the Addition 2 study was increased threefold over that present in Addition 1. Treatment composition for the Addition 3 study was identical to that for the Addition 2 study except the former comprised one additional treatment group which was the original negative control. The treatments used for Addition 2 and 3 are presented in table 6. The analyzed milk proteins from the three studies were β-LG, β-CA and α-LA.

Response Surface Study

A three-dimensional central composite design described by Chandler et al. (16) and Gardiner et al. (38) was employed to assign treatments in this study. With this design the five different quantities of each of three independent variables were incorporated into 15 treatments. The three variables were lysine (X_1), arginine, methionine, and valine (X_2) and isoleucine, phenylalanine, threonine, tryptophan, and histidine (X_3). The amounts each variable were coded from -2 to 2. The combination of 15 treatments along with their coded values are given in table 7. The central point (treatment 9) of the experimental design was based on data accumulated at this laboratory. The measured net synthesis of three major components of milk protein was the criteria used to evaluate the study.

Statistical Procedure

Statistical procedures utilized, based on the characteristics of the experiment were: analysis of variance for main treatment effects on

Table 7. Variables, coded values and treatment combination used in response surface investigations.

Variable[a]	Coded value				
	-2	-1	0	1	2
	(X of MEM)				
X_1	3.0	6.0	9.0	12.0	15.0
X_2	1.5	3.0	4.5	6.0	7.5
X_3	1.0	1.5	2.0	2.5	3.0

Treatment Number[b]	X_1		X_2		X_3	
	(fold)	(code)	(fold)	(code)	(fold)	(code)
1	6	-1	3	-1	1.5	-1
2	12	1	3	-1	1.5	-1
3	6	-1	6	1	1.5	-1
4	12	1	6	1	1.5	-1
5	6	-1	3	-1	2.5	1
6	12	1	3	-1	2.5	1
7	6	-1	6	1	2.5	1
8	12	1	6	1	2.5	1
9	9	0	4.5	0	2	0
10	3	-2	4.5	0	2	0
11	15	2	6.5	0	2	0
12	9	0	1.5	-2	2	0
13	9	0	7.5	2	2	0
14	9	0	4.5	0	1	-2
15	9	0	4.5	0	3	2

[a] X_1; lysine, X_2; arginine, methionine, valine, X_3; isoleucine, phenylalanine, threonine, tryptophan. Values on each row represent multiples of amino acids relative to 1X-MEM.

[b] All treatments were balanced at 1X quantity of leucine, tyrosine, cystine and glutamine.

all six studies, orthogonal comparisons for contrasting replication means on the omission studies, Student-Newman-Keuls (S-N-K) test (83) for comparing treatment means on omission and addition studies and Dunnett's test (22) for the comparisons of treatment means with positive or negative control. Simple regression analysis for the graded quantities of amino acid complement study, multiple regression and analysis covariance procedures for the response surface study were used. Details on the statistical procedures and models were referred from Snedecor and Cochran (83) and Sokal and Rohlf (84).

V. RESULTS AND DISCUSSION

Effect of Labeled Precursor Sources

Amounts of milk protein synthesized, measured by incorporation of ^{14}C-(U)-L-lysine, phenylalanine, or leucine into protein are presented in table 8. The average net synthesis of β-LG in response to the three labeled precursor amino acids was significant ($P<.05$), with Lys = 15.9 μg, Leu = 11.1 μg and Phe = 9.5 μg/flasks. Similar trends in response of β-CA synthesis were 22.0 μg, 13.1 μg and 13.0 μg/flasks ($P>.05$). No significant differences ($P>.05$) existed among labeled amino acid sources for the measured synthesis of α-LA.

Increasing quantity of L-lysine from 1- to 3-fold in the 1X-MEM had a significant ($P<.05$) effect on the synthesis of both β-LG and β-CA. However, there was no significant ($P>.05$) effect on the α-LA synthesis. This was apparently due to the small number of observations (error df = 6). The interaction component between source of label and quantity of L-lysine was significant ($P<.01$) indicating that quantity of L-lysine added into the medium affected the synthesis, as measured by the three sources of labeled precursor amino acids, unequally.

It has been reported that synthesis as measured by ^{14}C-(U)-L-lysine and leucine in bovine mammary cell culture media was similar for β-LG and α-LA (42). However, the study did not report any statistical tests of differences in the two sources of labeled amino acids.

Each labeled amino acid is incorporated into the cell and utilized for protein synthesis in a different manner. Thus, the source of labeled amino acid precursor is an important consideration for

Table 8. Results of lysine, leucine and phenylalanine precursors on measured milk protein synthesis.

Lysine	Milk Protein	^{14}C-(U)-L of Lys	Leu	Phe
		----------(µg/flask)----------		
1X	β-LG[a]	7.8	5.8	6.7
	β-CA[b]	9.6	8.0	6.5
	α-LA[c]	2.7	4.0	3.6
3X	β-LG[a]	24.1	13.1	15.5
	β-CA[b]	34.4	18.1	19.6
	α-LA[c]	6.4	6.3	6.5

[a]Values in rows represent the mean of 4 observations from 2 replications, with standard error of the mean = 1.67 µg/flask.

[b]Values in rows represent the mean of 4 observations from 2 replications, with standard error of the mean = 2.31 µg/flask.

[c]Values in rows represent the mean of 2 observations from 2 replications, with standard error of the mean = 1.73 µg/flask.

interpreting the incorporation data with labeled amino acids. Schreiber and Schreiber (80) stated that the amount of a specific amino acid incorporated into protein was dependent on the concentration of precursor amino acid as well as the quantity of the nonradioactive amino acid present. It was reported for rat liver cells in tissue culture that ^{14}C-(U)-L-phenylalanine incorporation into protein was a function of the concentration of the labeled amino acid (81). In case of L-methionine, only part of added ^{14}C-(U)-L-methionine was actually incorporated into protein, and the remaining portion was tightly bound to protein rather than penetrating into the cells (81). Another pitfall of the interpretation for the incorporation experiments with radioactive amino acids is the reutilization of radioactively labeled amino acids. Some were incorporated into protein but are released thereafter by breakdown of protein (80).

Differences existing among labeled precursor amino acids, as well as the interaction of labeled precursors with lysine addition in the culture media, were primarily due to the high estimate of the response from the 3X L-lysine treatment groups especially for β-LG and β-CA. In deriving the value for the synthesized protein, an estimate of the specific amino acid pool size in the medium is required. In the case of lysine, this was increased from 1- to 3-fold as a result of the lysine addition. In theory if accurate adjustments had been made for the pool on the 3X lysine treatment group labeled by ^{14}C-(U)-L-lysine, the response in this group would not have been significant from the other two labeled amino acid groups. However, the bias introduced as a result of changing pool size relative to the labeled amino acid would

not be unique for lysine but would be encountered with the choice of any labeled precursor. Thus, labeled lysine seems to be as logical as any amino acid as a choice for measuring protein synthesis.

Effect of Cell Density

The response of milk protein synthesis in a 3 x 2 factorial experiment with three cell densities of approximately 6 x 10^6(A), 2 x 10^7(B), and 6 x 10^7(C) cells per flask and of two quantities of amino acid concentration at 1X and 3X are summarized in table 9. Effect of varying the cell population on the synthesis of β-LG was found to be nonsignificant (P>.05) with A = 30.79 µg, B = 37.75 µg and C = 23.05 µg/flask. The amount of net synthesis of α-LA for the three cell densities were .92 µg, 1.33 µg and 1.02 µg/flask, with no differences being significant (P>.05).

Although cell density did not significantly influence the net synthesis of β-LG and α-LA, peak response was obtained at cell numbers of 2 x 10^7/flask. This result agrees with the report by Rao et al. in 1974 (73) where an optimal cell density of 1.2 x 10^7 cells/flask was found. The generally lowered but nonsignificant amount of synthesis of milk protein observed at 6 x 10^7 cells/flask may be related to the "density-dependent inhibition" phenomenon (15,28,40,74). With low cell population, Eagle (29) and Harris (45) have reported that a number of metabolites (or intermediates in its synthesis) that can be synthesized by cells are lost to the surrounding medium in amounts which exceed the biosynthetic capacity of cells. The tendency for such depletion is due to the lack of a mutual buffering effect between cells in close proximity.

Table 9. Summary of cell density study.

Cell Density		Quantity of amino acid complement					
		1X		3X		$\bar{X}$	
		β-LG[a]	α-LA[b]	β-LG[a]	α-LA[b]	β-LA	α-LA
	(No/flask)						
A:	6×10^6	12.97	.56	48.61	1.28	30.79	.92
B:	2×10^7	15.54	.89	59.95	1.76	37.75	1.33
C:	6×10^7	15.58	.80	30.52	1.24	23.05	1.02
	$\bar{X}$	14.70	.75	46.36	1.43		

[a]Each value represents mean of 6 observations from 2 replications, with standard error of the mean = 6.75 µg/flask.

[b]Each value was consisted of mean of 2 observations from 2 replications, with standard error of the mean = .19 µg/flask.

Under these conditions, growth will fail to occur unless a compensating exogenous source is provided (29). At sufficiently high population densities, however, the medium can be "conditioned" - that is, the concentration in the medium and within the cell can be brought up to metabolically effective levels before cell death. However, when the population is increased and passes to a certain critical concentration, then so-called "density-dependent inhibition" is likely to occur. Griffiths in 1970 (40) summarized this phenomenon as inhibition of growth in crowded cultures depended more on the uptake of nutrients, which in turn was more a reflection of a reduced cell surface area, than on a nutrient depletion of the medium.

Increasing amounts of amino acid concentration from 1X to 3X resulted in a significant ($P<.05$) increase in the synthesis of β-LG (14.70 to 46.36 μg/flasks) and α-LA (.75 to 1.43 μg/flasks). No interaction ($P>.05$) between cell density and amino acid concentration was observed, indicating that the response to amino acid complement was consistent at all cell densities studied.

Results of the cell density study showed that: (1) cell number between 6×10^6 - 6×10^7 did not significantly alter the rates of the milk protein synthesis and (2) increased synthesis with 3X-MEM occurred at each cell number concentration studied.

Investigation of Graded Quantities of Amino Acid Complement

Table 10 and figure 1 summarize the results of a 5 x 2 factorial experiment consisting of graded amino acid complement (1X through 5X) and two culturing environments (CO_2-regulated vs. conventional incubators).

Table 10. Protein synthesis response to graded amounts of amino acid complement[a].

Amino Acid Complement	Incubation Environment						$\bar{X}$		
	CO_2-regulated			Conventional					
	β-LG	β-CA	α-LA	β-LG	β-CA	α-LA	β-LG[b]	β-CA[c]	α-LA
1X	20.6	38.2	1.27	32.2	28.1	.80	26.4	33.2	1.04
2X	77.6	73.9	1.97	59.6	50.6	1.54	68.6	62.2	1.75
3X	126.8	84.9	3.98	123.3	124.4	3.99	126.1	104.7	3.99
4X	129.6	154.9	3.84	142.9	106.9	3.58	136.2	130.9	3.71
5X	154.1	103.3	4.00	140.3	153.1	3.97	147.2	128.2	3.98

[a]Expressed as µg/flask.

[b]Regression equation:

$$Y = 7.9 + 30.9X, \ (r^2 = 90.5\%)$$

Where:

Y = predicted β-LG synthesis,
X = amounts of total amino acid complement, and
r^2 = coefficient of determination.

[c]Regression equation:

$$Y = 14.2 + 25.9X, \ (r^2 = 91.3\%)$$

Where:

Y = predicted β-CA synthesis, and X and r^2 are as previously defined.

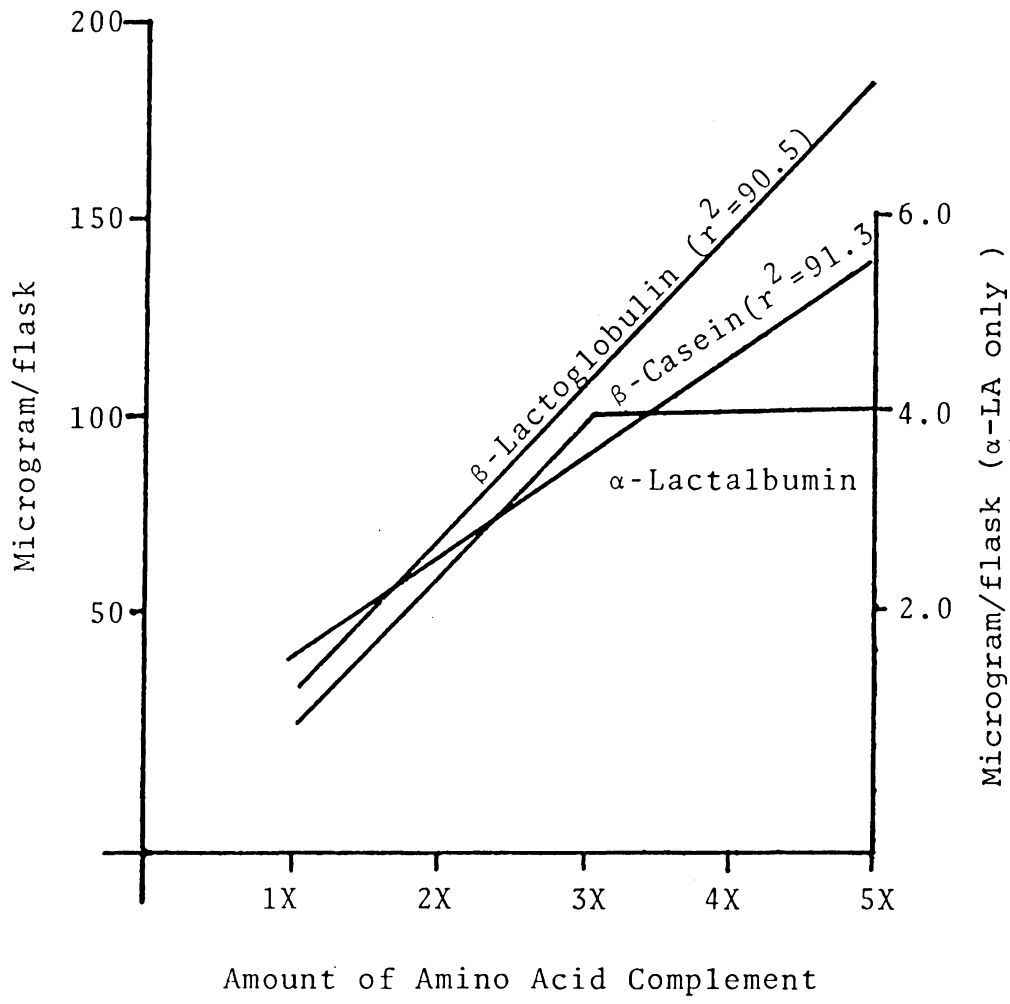

Figure 1. In vitro synthesis of milk proteins in response to graded amino acid complement from 1X through 5X.

Results showed no significant effect ($P>.05$) on culturing between CO_2 and conventional incubators from all treatment groups. Originally it was hypothesized that a CO_2-regulated incubator would bring about preferable responses for the net synthesis of milk protein by rat mammary cells in tissue culture. This assumption was based on the fact that cultures, utilized throughout our experiments, were bicarbonate-buffered media. It had been reported that such media must be maintained in a special incubator with a humidified CO_2-air atmosphere (93). McLimans (66) found that in the stoppered cell culture flask the pCO_2 (both air and media) continued to increase with a concomitant rise in carbonic acid, lactate, etc., and a drop in pH until a level of acidity was reached which terminated the cell respiration and production of CO_2. The final true equilibrium resulted after 2 to 4 days. However, in our studies the lack of a response to the CO_2-regulated incubator was likely due to termination of incubation within a 20 h period. From studies with green monkey heart cells, Searcy et al. (82) reported that a controlled environment culture system, e.g., 95% O_2-5% CO_2 for the in vitro culture of mammalian cells appeared mandatory if one was interested in the study of the morphology of the normal cell.

The nonsignificance difference observed between two systems of incubation also indicated that use of tissue culture vessels (T-30) that were closed tightly and incubated in conventional systems were as effective as incubation in CO_2-regulated systems. No interaction ($P>.05$) between graded amino acid complement and incubation system was found, indicating that the response to the system of incubation

was consistent at all graded amino acid complements studied. pH adjustment to 7.4 by titration with 7.5% sodium bicarbonate was essential for all treatment groups where amino acid complements were at or above 3X.

The response to amino acid complement of the 1X-MEM addition was linear ($P<.01$) up to 5X with regression coefficient (b) = 30.9 µg/flask and r^2 = 90.5% on β-LG synthesis. Characteristic response of β-CA synthesis was found to be quite similar to that of β-LG, with b = 25.9 µg/flask and r^2 = 91.3% (table 10 and figure 1). No improvement in α-LA synthesis resulted beyond 3X.

The linear increase in β-LG and β-CA synthesis in response to the graded elevation of total amino acid manifested that the major factor for stimulation of milk protein synthesis in the dispersed mammary cell culture system was the total amounts of amino acids available to cells. Similar studies (78), using both rat and bovine mammary cell cultures, have demonstrated that elevated quantities of essential amino acids above physiological levels stimulate the synthesis rate several fold for both β-LG and β-CA in the rat cells but only β-CA in the bovine cells. They also found that elevating glucose up to 3-fold had no effect on the synthesis of milk protein.

The increased synthesis of milk protein by increments of total amino acid complement is reasonable since 1X-MEM was developed for the mammalian cell lines to maintain or grow on solid substratum rather than for cells performing specific functions such as a biosynthesis of milk protein. The mere increase of the amino acid complement above the normal physiological range does not always result in a

corresponding rise in incorporation of substrate into protein. Mohberg and Johnson (70), using 929-L fibroblasts, showed that most amino acids in MB 752/1 were actually used in 1.5 to 2.5 times the quantities needed for protein synthesis. Much more glutamine, methionine and valine in particular were utilized than could be accounted for by increased protein synthesis. Tissue culture media may best be constructed on the basis of relative proportions of amino acids in the protein (both structural and exporting) synthesized (51,63,72).

The results clearly indicate that availability of amino acid complement to cells was a limiting factor in the rate of milk protein synthesis. However, responses obtained should not be considered as a full expression of the potentiality of amino acids directed toward protein synthesis in that many factors in the biosynthetic machinery (56) of cells are involved, including an altered in vitro environment and amino acid transport system.

Effect of Single Amino Acid Omission

Summarized results of two sets of experiments (Omission 1-8 and 9-10) for effects of a single amino acid reduction from 3X to 1X on the net synthesis of β-LG, β-CA and α-LA are in table 11.

Omission 1-8. According to the 11 treatment mean values from Omission 1-8, negative control ((-) neg) showed the lowest net synthesis of β-LG (2.7 μg/flask), whereas positive control ((+) pos) resulted in the highest response (15.3 μg/flask). Synthesis of β-LG was increased by 5-fold in going from (-) neg to (+) pos groups.

Table 11. Effect of single amino acid omission on milk protein synthesis.

Omission 1-8	Omission 9-10		
β-LG	β-LG	β-CA	α-LA
(μg/flask)			
(-) Neg (2.7)[a]	(-) Neg (4.8)[a]	(-) Neg (13.5)[ab]	(-) Neg (2.1)
Lys (3.5)[a]	Lys (5.9)[a]	Lys (19.8)[ab]	Lys (2.7)
Trp (8.3)[b]	Arg (14.1)[a]	Cys (24.2)[abc]	Met (6.6)
Val (8.4)[b]	Met (14.5)[a]	Met (26.1)[abcd]	Tyr (6.6)
Tyr (8.4)[b]	Thr (15.3)[a]	Tyr (28.4)[abcde]	Arg (6.9)
His (8.7)[b]	Val (15.9)[a]	Trp (30.8)[bcdef]	Thr (7.1)
Arg (8.8)[b]	Trp (16.9)[a]	Thr (40.1)[cdefg]	Phe (7.3)
Phe (8.8)[b]	Cys (17.0)[a]	Val (41.3)[cdefg]	His (7.7)
Ile (9.3)[b]	Phe (17.1)[a]	Arg (43.4)[defg]	Cys (8.7)
Thr (9.8)[b]	His (17.7)[ab]	His (44.8)[defg]	Val (8.9)
Leu (10.4)[b]	Leu (19.2)[ab]	Phe (47.2)[efg]	Ile (9.6)
Met (11.0)[b]	Ile (19.3)[ab]	Leu (47.4)[efg]	Trp (9.8)
(+) Pos (15.3)[c]	Tyr (20.8)[ab]	Gln (49.7)[fg]	Leu (12.6)
	(+) Pos (27.1)[b]	Ile (52.4)[g]	Gln (13.8)
	Gln (28.6)[b]	(+) Pos (55.6)[g]	(+) Pos (14.1)
CV[h] 24.22	16.30	13.77	..
SE[i] .74	1.94	3.67	..

[abcdefg]Means in same column not bearing a common superscript differ significantly (P<.05).

[h]Coefficient of variation (%).

[i]Standard error of the mean.

The 11 treatment groups other than the two controls were varied in their responses, but their values fell within range of controls.

The analysis of variance revealed that there was a significant difference ($P<.05$) among treatments. Orthogonal contrasts for the comparison among replications (Omission 1-8) revealed no significant differences ($P>.05$) for Omission 5 vs. 6 and for Omission 7 vs. 8, supporting earlier observations that the mammary cells cultured in a CO_2-regulated incubator (Omission 6 and 8) had no significant effect on the synthesis of β-LG over that observed in the conventional system (Omission 5 and 7). However, there were significant differences ($P<.05$) on Omission 5-6 vs. 7-8. This was likely due to the lower cell number (1×10^6/flask) present in Omission 5-6. According to the results in comparisons for treatment means (table 11), no significant difference ($P>.05$) existed between (-) neg and lysine treatments, but this pair was significantly ($P<.05$) different from other treatments. The (+) pos differed ($P<.05$) significantly from all treatment groups (tables 11 and 12).

Omission 9-10. Omission 9-10 studies, which consisted of two additional treatments (cystine and glutamine) were conducted under more optimal experimental conditions based on supplemental studies involving experiments with varying cell numbers (Study-2) and graded amounts of amino acid complement (Study-3). Synthesis of β-LG on (-) neg and (+) pos controls were 4.8 μg and 27.1 μg/flask, respectively (table 11). All treatment groups showed varying intermediate values of β-LG synthesis between two controls except the glutamine deleted group (28.6 μg/flask). The amount of synthesized β-CA from glutamine

Table 12. Comparisons for treatment means with the controls for omission studies.

	Omission 1-8	Omission 9-10	
	β-LG	β-LG	β-CA
		d[a]	
Control	2.63	7.90	14.88
[(+) Pos][b,*] ≠	[All groups]	[Lys],[Met],[Cys] [Arg],[Thr],[Val] [Trp],[Phe],[His]	[Lys],[Met],[Cys], [Tyr],[Trp],[Thr]
[(-) Neg][c] =	[Lys]	[Lys]	[Lys],[Met],[Cys], [Tyr]

[a] $d = t_D \sqrt{2EMS/n}$

where:

d = least significant range,

t_D = t value from the Dunnett's table, with degree of freedom (df) of error mean squares, df of protection (P) = P - 1 and $\alpha = .05$,

EMS = error mean squares and

n = number of observations grouped.

[b] Positive control.

[c] Negative control.

* $P<.05$.

treatment was 49.7 µg/flask. On the same study it was found that rate of α-LA synthesis of glutamine group (13.8 µg/flask) was second highest rank among treatments. Synthesis of β-CA on (-) neg and (+) pos were 13.5 µg and 55.6 µg/flask, respectively (table 11).

The analysis of variance for both β-LG and β-CA indicated that significant ($P<.05$) differences existed among treatments. Comparisons for treatment means (tables 11 and 12) showed that no significant difference ($P>.05$) was found to exist betwen glutamine and (+) pos groups, but this pair differed significantly ($P<.05$) from lysine, arginine, methionine, threonine, valine, tryptophan, cystine and phenylalanine groups in β-LG synthesis. Comparisons of individual treatments with controls in β-LG data revealed that (+) pos differed significantly ($P<.05$) from lysine, arginine, methionine, threonine, valine, tryptophan, cystine, phenylalanine and histidine treatments. For β-CA synthesis (+) pos differed significantly ($P<.05$) from lysine, cystine, methionine, tyrosine, tryptophan and threonine groups, whereas (-) neg did not differ significantly ($P>.05$) from lysine, cystine, methionine, and tyrosine treatments (table 12). The latter agreed with results from S-N-K test as illustrated in table 11.

When Omission 1-8 was compared to Omission 9-10, overall rate of β-LG synthesis in Omission 9-10 was much higher (33 to 146%) than synthesis on Omission 1-8. This was likely due to general improvement of techniques in this study. Over ten replications, results indicated that milk protein synthesis was lowered significantly ($P<.05$) in all cases except the glutamine treatment, when any one of the amino acids was omitted from (+) pos. In fact, the value of the glutamine treatment

exceeded (+) pos in β-LG synthesis on Omission 9-10 (table 11). This result is supported by other findings (78) stating that glutamine was not required by mammary cells of rat species. Response of (-) neg over ten replications was uniformly the lowest rate of synthesis and was not comparable to any other treatment except the lysine group. This was the only group that was found to be nonsignificant from (-) neg for synthesis of β-LG. The highest rate of synthesis for milk protein constituents on (+) pos is supported by the study of Schingoethe et al. (78) where increasing amounts of amino acid complement to 3-fold in MEM were found to be necessary for the most desirable response of milk protein biosynthesis in secretory cells of both rat and bovine species.

Single elimination of lysine from (+) pos back to (-) neg resulted in the greatest decline in protein synthesis. This result is in agreement with the study of Schingoethe et al. (78) who found that both rat and bovine mammary cells demonstrated a significant requirement for this amino acid. Both β-LG and α-LA synthesis in Omission 9-10 indicated that cystine treatment group resulted in a somewhat intermediate value of synthesis. In case of β-CA, however cystine group resulted in a lowered response next to lysine group. This response appeared to conflict with other findings (56), reporting that rat mammary cells failed to demonstrate a significant requirement for this amino acid which was affected by elevation of methionine. Eagle and Piez (27) and Eagle et al. (26) reported that cystine was an essential amino acid became a critical factor for cell growth particularly under stress or low cell number due to leakage out of the cell of intermediates on the pathway of its synthesis from methionine (29,45).

The order of magnitude of milk protein synthesis by some treatments altered, when Omission 1-8 was compared to Omission 9-10, depending upon the essential amino acid in question. Magnitudial order of synthesis (table 11) in methionine group changed from larger to lesser synthesis region in Omission 1-8 to Omission 9-10. Methionine was a far more limiting amino acid on Omission 9-10 than was the case on Omission 1-8. This shifting of order indicates that cells are reacting preferentially under better conditions where higher demand of some essential amino acids exist for cells in culture. An apparent stimulatory effect of methionine in MEM on synthesis of β-LG and β-CA for bovine mammary cell culture was reported by Larson (57). Results from both groups of omission experiments consistently revealed that glutamine and leucine were two of the least limiting amino acids in our culture system.

Conclusions for omission experiments are in table 13. The following are the order of limiting amino acids studied in rat mammary cells in tissue culture:

1) The first limiting amino acid was lysine.
2) The second limiting amino acids were methionine and cystine.
3) The intermediate group with no distinctions among them were threonine, valine, histidine, tryptophan, phenylalanine, isoleucine, arginine and tyrosine.
4) The least limiting amino acids were leucine and glutamine.

Table 13. Order of limiting amino acids for milk protein synthesis in omission studies[a].

Order	Amino Acid	Proof
1st :	Lys	By S-N-K test in Omission 1-10 and Dunnett's test in Omission 1-8 for β-LG and in Omission 9-10 for both β-LG and β-CA
2nd :	Met	By S-N-K test in Omission 9-10 for β-CA, Dunnett's test in Omission 9-10 for both β-LG and β-CA and magnitudial order of actual synthesis in Omission 9-10 for α-LA[b]
	Cys	By S-N-K test in Omission 9-10 for β-CA, Dunnett's test in Omission 9-10 for both β-LG and β-CA
3rd :	Thr, Val, His, Trp, Phe, Tyr, Ile, Arg	Intermediate group with no distinctions among them
4th :	Leu	By S-N-K in Omission 9-10 for both β-LG and β-CA and magnitudial order of actual synthesis in Omission 1-8 for β-CA and in Omission 9-10 for α-LA[b]
	Gln	By S-N-K in Omission 9-10 for β-LG, Dunnett's test in Omission 9-10 for both β-LG and β-CA and actual synthesis in Omission 9-10 for α-LA[b]

[a]Order of amino acid limitation was based upon the assumption that at least two protein constituents must be significantly ($P<.05$) altered by treatment as evaluated by either S-N-K and/or Dunnett's tests.

[b]No statistical analysis was available due to lack of observations.

Study of Single Amino Acid Addition

Response for addition experiments 1, 2 and 3, involving single amino acid addition from negative control up to quantity of positive control for each of 13 essential amino acids are presented in this section.

Addition 1. Results of Addition 1 trial are in table 14. Treatment effects were significant ($P<.05$) for β-LG and β-CA synthesis. In all three milk proteins, negative ((-) neg) and positive control ((+) pos) resulted in lowest and highest synthesis, respectively. Range of β-LG synthesis varied from 15.9 ((-) neg) to 62.4 μg/flask ((+) pos). The 13 individual additions of amino acids were varied in their responses, but values all fell within limits of the two controls. Response of β-CA and α-LA synthesis were similar in pattern to that of β-LG but relative order of rank changed (table 14). Synthesized protein for two controls were: (-) neg = 7.7 μg and (+) pos = 18.5 μg/flasks on β-CA and (-) neg = 3.8 μg and (+) pos = 13.9 μg/flasks on α-LA.

Mean comparisons for β-LG (table 14) revealed no significant difference ($P>.05$) between (+) pos and lysine treatment groups, but this pair differed significantly ($P<.05$) from remaining groups. Mean differences of β-CA appeared to be nondiscrete (table 14).

From results of Addition 1, it was difficult to identify any limiting amino acid(s) other than lysine for the protein synthesis. This was due to lack of sensitivity among mean resulting from a rather large experimental error.

Table 14. Effect of single amino acid addition on milk protein synthesis (Addition 1).

β-LG	β-CA	α-LA
----------(g/flask)----------		
(-) Neg (15.9)[a]	(-) Neg (7.7)[a]	(-) Neg (3.8)
Cys (16.1)[a]	Val (7.8)[a]	Leu (4.1)
Tyr (16.9)[a]	Arg (7.9)[a]	Val (4.2)
Thr (19.5)[a]	Tyr (8.1)[a]	Trp (4.2)
Leu (20.0)[a]	Cys (8.5)[ab]	Phe (4.3)
Trp (21.3)[a]	Phe (9.1)[ab]	Arg (4.5)
Ile (21.7)[a]	Leu (9.5)[ab]	Tyr (4.6)
Phe (22.2)[a]	Met (9.5)[ab]	Ile (4.7)
Val (22.2)[a]	His (9.7)[ab]	Cys (4.7)
Gln (23.3)[a]	Ile (9.9)[ab]	Gln (4.9)
Met (24.5)[a]	Thr (10.7)[ab]	His (5.0)
Arg (24.7)[a]	Trp (11.0)[abc]	Thr (5.3)
His (29.0)[a]	Gln (11.6)[abc]	Met (5.8)
Lys (56.1)[b]	Lys (15.6)[bc]	Lys (9.1)
(+) Pos (62.4)[b]	(+) Pos (18.5)[c]	(+) Pos (13.9)
CV[d] 18.9	18.3	..
SE[e] 3.5	1.4	..

[abc]Means in same column not bearing a common superscript differ statistically (P<.05).

[d]Coefficient of variation (%).

[e]Standard error of the mean.

Addition 2. Summary of protein synthesis data generated in Addition 2 is in table 15. Overall response obtained in Addition 2 was different from Addition 1. Upper limit for rank of responses was no longer held by (+) pos (75.3 µg/flask) but was exceeded by lysine treatment (78.0 µg/flask) for β-LG synthesis. Synthesis of β-CA on tyrosine (14.2 µg/flask) and lysine (40.8 µg/flask) treatments exceeded those on (-) neg (14.3 µg/flask) and (+) pos controls (37.0 µg/flask), respectively. Amounts of synthesis on α-LA for (-) neg and (+) pos were 5.4 µg and 16.0 µg/flask.

Synthesis of β-LG between (+) pos and lysine groups did not differ significantly ($P>.05$), but this pair differed ($P<.05$) from remaining groups. Arginine (50.2 µg/flask) was found to be different ($P<.05$) from other treatments. Data of β-CA synthesis was not significant ($P>.05$) between (+) pos and lysine groups, but this pair was different ($P<.05$) from other groups (table 15).

Mean comparisons with control groups for synthesis of β-LG revealed that synthesis of β-LG on (-) neg differed significantly ($P<.05$) from arginine, methionine, histidine, lysine and isoleucine treatments (table 16). Addition 2 study suggested that lysine was the first limiting amino acid for all three proteins with the group composed of arginine, methionine, isoleucine and histidine becoming second limiting amino acids for β-LG synthesis.

Addition 3. Addition 3 (table 17) consisted of one extra treatment (T-1) which was the negative control of Addition 1. Response to T-1 on synthesis of milk protein resulted in the lowest amount and was not comparable to any other treatment. Overall rate of protein

Table 15. Effect of single amino acid addition on milk protein synthesis (Addition 2).

β-LG	β-CA	α-LA
----------(μg/flask)----------		
(-) Neg (16.9)[a]	Tyr (14.2)[a]	(-) Neg (5.4)
Trp (18.1)[a]	(-) Neg (14.3)[a]	Gln (5.8)
Cys (18.2)[a]	Arg (14.6)[a]	Leu (6.7)
Val (19.0)[a]	Gln (15.1)[a]	Ile (6.8)
Thr (21.4)[ab]	Thr (15.6)[a]	His (7.4)
Gln (22.6)[ab]	Trp (15.6)[a]	Tyr (8.6)
Phe (23.1)[ab]	Cys (15.8)[a]	Thr (8.7)
Leu (25.8)[ab]	Met (16.7)[a]	Cys (8.9)
Tyr (28.1)[ab]	Leu (17.2)[a]	Arg (9.2)
Ile (29.8)[ab]	Val (17.2)[a]	Met (9.7)
His (33.9)[b]	His (17.2)[a]	Phe (10.4)
Met (34.9)[b]	Phe (17.8)[a]	Trp (12.7)
Arg (50.2)[c]	Ile (17.9)[a]	Val (14.2)
(+) Pos (75.3)[d]	(+) Pos (37.0)[b]	Lys (14.9)
Lys (78.0)[d]	Lys (40.8)[b]	(+) Pos (16.0)
CV[e] 11.8	9.8	..
SE[f] 2.8	3.5	..

[abcd]Means in same column not bearing a common superscript differ significantly (P<.05).

[e]Coefficient of variation (%).

[f]Standard error of the mean.

Table 16. Comparisons for treatment means with controls for addition studies.

Trial	Control	β-LG		β-CA		α-LA	
		d[a]		d[a]		d[a]	
1	[(+) Pos][b]	14.3	= [Lys]	5.4	= [Lys]	•	-
	[(-) Neg][c,*]		≠ [Lys]		≠ [Lys]		-
2	[(+) Pos][b]	11.2	= [Lys]	5.3	= [Lys]	•	-
	[(-) Neg][c,*]		≠ [Lys],[Arg],[Met],[Ile],[His]		≠ [Lys]		-
3	[(+) Pos][b]	25.9	= [Lys]	52.5	= [Lys],[Met],[Trp],[Val],[Ile],[Arg],[Tyr]	•	-
	[(-) Neg][c,*]		≠ [Lys]		≠ [Lys]		
2 + 3	[(+) Pos][b]	24.3	= [Lys]	32.7	= [Lys],[Met],[Val],[Trp],	4.6	= [None]
	[(-) Neg][c,*]		≠ [Lys]		≠ [Lys]		≠ [Lys],[Val],[Met],[Trp],

[a] $d = t_D \sqrt{2EMS/n}$ [b] Positive Control [c] Negative Control [*] P<.05

where:

d = least significant range,

t_D = t value from the Dunnett's table, with degree of freedom (df) of error mean squares, df of protection = P - 1 and $\alpha = .05$.

EMS = error mean squares and

n = number of observations grouped.

Table 17. Effect of single amino acid addition on milk protein synthesis (Addition 3).

β-LG		β-CA		α-LA	
			(μg/flask)		
	T-1 (6.4)[a]		T-1 (10.5)[a]		T-1 (2.2)
	Gln (18.4)[a]		Gln (38.6)[a]		Leu (3.3)
	Arg (20.4)[a]	(-)	Neg (43.4)[a]	(-)	Neg (3.4)
	Trp (22.1)[a]		His (52.0)[a]		Ile (3.8)
	Cys (22.6)[a]		Cys (53.0)[a]		His (4.8)
	Tyr (23.0)[a]		Thr (53.3)[a]		Gln (5.0)
	Phe (24.0)[a]		Phe (54.5)[a]		Phe (5.2)
	His (24.4)[a]		Leu (55.3)[a]		Cys (5.2)
(-)	Neg (25.4)[a]		Arg (59.1)[a]		Arg (5.9)
	Ile (28.0)[a]		Tyr (61.4)[a]		Tyr (7.7)
	Thr (30.2)[a]		Ile (62.6)[a]		Trp (8.3)
	Met (30.4)[a]		Val (64.9)[a]		Thr (8.8)
	Val (32.7)[a]		Trp (68.0)[a]		Val (9.0)
	Leu (33.0)[ab]		Met (68.5)[a]		Lys (9.7)
(+)	Pos (66.5)[ab]	(+)	Pos (109.8)[b]		Met (10.3)
	Lys (96.1)[bc]		Lys (150.2)[b]	(+)	Pos (18.3)
CV[d]	28.4		28.9		..
SE[e]	6.3		12.8		..

[abc]Means in the same column not bearing a common superscript differ significantly (P<.05).

[d]Coefficient of variation (%).

[e]Standard error of the mean.

synthesis on T-1 was much lower (24~65%) than that of (-) neg in Addition 3, confirming the inferiority of the original negative controls where lysine was present at 1X.

Response pattern of β-LG synthesis in Addition 3 was rather strange, for the order of response on (-) neg was positioned in the middle of treatments (table 17). Reason for this alternation was not clear. Lowest and highest response on β-LG synthesis resulted from glutamine group (18.4 μg/flask) and lysine treatment (96.1 μg/flask). For β-CA synthesis glutamine group was the lowest limit value (38.6 μg/flask), whereas the lysine group resulted in the highest response (150.2 μg/flask).

Mean comparisons for β-LG data revealed that there were no significant differences ($P>.05$) between lysine and (+) pos groups and among (+) pos, leucine and valine groups. Synthesis of β-CA on (+) pos did not differ ($P>.05$) from arginine, tyrosine, isoleucine, valine, tryptophan, methionine and lysine groups (table 16). Synthesis of α-LA on methionine group (10.3 μg/flask) was higher than that of lysine group (9.7 μg/flask), but significant differences between them were not obtained because of lack of observations for analysis.

Additions 2 and 3. Summary for combined Additions 2 and 3 (excluded T-1) is in table 18. Analysis of variance for combined data revealed that both replications and experiments effects were not significant ($P>.05$) for β-CA and β-LG. Treatment mean difference between lysine and (+) pos groups was not significant ($P>.05$), but this pair differed ($P<.05$) from other groups for synthesis of β-LG (table 18). Mean

Table 18. Effect of single amino acid addition on milk protein synthesis (Additions 2 and 3, combined).

β-LG	β-CA	α-LA
(μg/flask)		
Trp (20.1)[a]	Gln (26.8)[a]	(-) Neg (4.4)[a]
Cys (20.4)[a]	(-) Neg (28.8)[a]	Leu (5.0)[a]
Gln (20.5)[a]	Cys (34.2)[ab]	Ile (5.3)[ab]
(-) Neg (21.2)[a]	Thr (34.4)[ab]	Gln (5.4)[ab]
Phe (23.6)[a]	His (34.6)[ab]	His (6.1)[abc]
Tyr (25.6)[a]	Phe (36.2)[ab]	Cys (7.1)[abc]
Thr (25.8)[a]	Leu (36.3)[ab]	Arg (7.6)[abc]
Val (25.9)[a]	Arg (36.9)[ab]	Phe (7.8)[abc]
Ile (28.9)[a]	Tyr (37.9)[ab]	Tyr (8.1)[abc]
His (29.2)[a]	Ile (40.2)[ab]	Thr (8.8)[abc]
Leu (29.4)[a]	Val (41.1)[ab]	Met (10.0)[abc]
Met (32.7)[a]	Trp (41.8)[ab]	Trp (10.5)[abc]
Arg (35.3)[a]	Met (42.6)[ab]	Val (11.6)[bcd]
(+) Pos (70.9)[b]	(+) Pos (73.4)[bc]	Lys (12.3)[cd]
Lys (87.0)[b]	Lys (95.5)[c]	(+) Pos (17.2)[d]
CV[e] 38.4	40.1	19.1
SE[f] 6.4	8.6	1.4

[abcd]Means in the same column not bearing a common superscript differ significantly ($P<.05$).

[e]Coefficient of variation (%).

[f]Stand error of the mean.

comparisons for synthesis of β-CA indicated that lysine group differed significantly ($P<.05$) from other groups.

Results of Additions 1, 2 and 3 (table 19) consistently manifested that lysine was the first limiting amino acid. Even though concentration of lysine in Additions 2 and 3 was 9-fold higher than 1X-MEM (approximate to physiological level in vivo), no sign of an adverse effect was observed. The degree to which an amino acid can be concentrated by the cell did vary with environment; for example, from as much as 200-fold at an outside concentration of 0.001 mM to as little as 2-fold at an outside concentration of 10 mM (23). Next to lysine, the group of arginine, methionine, valine, tryptophan and isoleucine were determined to be the second limiting amino acids. It was reported that methionine had an apparent stimulatory effect on milk proteins β-LG and β-CA synthesis in the dispersed bovine cell culture (57). Both valine and isoleucine were limiting amino acids on synthesis of β-LG and β-CA, however, leucine, a structural analog of valine and isoleucine, was one of the least limiting amino acids.

Response Surface Study

Analysis of variance, regression coefficients for the three variables (X_1 = lysine, X_2 = methionine, arginine and valine and X_3 = isoleucine, phenylalanine, threonine, tryptophan and histidine) and actual and estimated responses for synthesis of milk protein are given in tables 20, 21 and 22.

β-LG. Overall effect of treatment was highly significant ($P<.01$). Linear component was found to be significant ($P<.01$), which was composed

Table 19. Order of limiting amino acids for milk protein synthesis in addition study[a].

Order	Amino Acid	Proof
1st :	Lys	By both S-N-K and Dunnett's tests in Additions 1, 2, 3 and 2 + 3 for β-LG, β-CA and α-LA
2nd :	Arg	By S-N-K in Addition 2 for β-LG and Dunnett's test in Addition 2 for β-LG and in Addition 3 for β-CA
	Met	By Dunnett's test in Addition 2 for β-LG, in Addition 3 for β-CA, in Addition 2 + 3 for β-CA and in Addition 2 + 3 for α-LA
	Val	By Dunnett's test in Addition 3 for β-CA, in Addition 2 + 3 for β-CA and in Addition 2 + 3 for α-LA
	Trp	By Dunnett's test in Addition 3 for β-CA, in Addition 2 + 3 for β-CA and in Addition 2 + 3 for α-LA
	Ile	By Dunnett's test in Addition 2 for β-LG and in Addition 3 for β-CA

[a]See footnote a in table 13.

Table 20. Analysis of variance of response surface study.

Source	df[a]	Mean Square			
		β-LG	β-CA	α-LA	Total[b]
Total	59(29)	....	....	....	....
Replication	1	701.3**	72.3	11.7*	1,399.2**
Treatment	14	427.2**	761.5**	28.4**	2,641.0**
Linear	3	1,724.4**	3,187.3**	123.0**	11,181.9**
X_1	1	5,136.2**	9,511.1**	367.3**	33,395.3**
X_2	1	11.5	9.4	.9	33.4
X_3	1	25.3	41.5	.9	116.9
Quadratic[c]	3	131.1**	234.3**	3.1	746.7**
Interaction	3	84.0**	47.2	.2	168.8
X_1X_2	1	18.0	80.9	.0	22.2
X_1X_3	1	231.0**	50.3	.2	483.2*
X_2X_3	1	2.9	10.3	.4	1.1
Lack of fit	5	32.4	51.0	3.6	136.5
Residual	44(14)	19.4	22.3	2.2	116.0
SE[d]		2.2	2.4	1.0	5.4
CV[e]		14.1	10.7	13.5	13.7
R-square[f]		86.5	89.4	88.8	83.8

[a]Degrees of freedom in parenthesis were for α-LA only.

[b]Total = (β-LG + β-CA + α-LA).

[c]Because of nonorthogonality, a breakdown of the quadratic component with respect to X_1, X_2 and X_3 was not possible. Quadratic components were, thus, tested collectively.

[d]Stand error of the mean.

[e]Coefficient of variation (%).

[f]Coefficient of determination (%) for the response surface model.

*P<.05. **P<.01.

Table 21. Regression coefficients describing effects of amino acids on milk protein synthesis.

Component	Regression Coefficients[a]			
	β-LG	β-CA	α-LA	Total Protein
	B ± SE[b]	B ± SE[b]	B ± SE[b]	B ± SE[b]
b	-92.73 ± 23.62**	-119.19 ± 25.36**	-10.635 ± 11.347	-217.15 ± 53.84**
b_1	11.37 ± 1.86**	15.20 ± 2.00**	1.955 ± .894*	27.54 ± 4.56**
b_2	7.49 ± 3.72*	14.36 ± 3.99**	2.132 ± 1.788	22.92 ± 9.11*
b_3	52.71 ± 12.52**	56.53 ± 13.44*	3.997 ± 6.013	111.25 ± 30.65**
b_{11}	- .31 ± .07**	- .44 ± .08**	- .055 ± .035	- .77 ± .18**
b_{22}	- 1.12 ± .29**	- 1.10 ± .32**	- .276 ± .141	- 2.36 ± .72**
b_{33}	- 9.92 ± 2.64**	- 11.80 ± 2.84**	- 1.318 ± 1.270	- 22.38 ± 6.48**
b_{12}	.17 ± .17	- .35 ± .19	.003 ± .083	- .18 ± .42
b_{13}	- 1.79 ± .52**	- .84 ± .56	.073 ± .249	- 2.59 ± 1.27*
b_{23}	.40 ± 1.04	- .76 ± 1.11	.211 ± .498	- .25 ± 2.54

[a]Regression equation:

$$Y = b_o + b_1X_1 = b_2X_2 + b_3X_3 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3$$

Where:

Y = predicted milk protein synthesis

X_1, X_2 and X_3 = actual values for the three independent variables (table 7), and

bs = multiple regression coefficients for intercept, linear, quadratic, and interaction.

[b]Standard error of regression coefficients.

*P<.05.

**P<.01.

Table 22. Actual and estimated response for milk protein synthesis.

Treatment[a]				β-LG		β-CA		α-LA		Total[b]	
No.	Combination			Act.	Est.[c]	Act.	Est.[c]	Act.	Est.[c]	Act.	Est.[c]
	(X_1)	(X_2)	(X_3)	(μg/flask)							
1	6	3	1.5	21.7	22.1	26.8	30.5	6.9	7.7	52.0	56.6
2	12	3	1.5	45.6	43.9	61.8	60.5	13.1	14.3	113.9	111.7
3	6	6	1.5	20.3	18.9	32.8	34.0	8.1	7.7	57.2	57.2
4	12	6	1.5	44.0	43.7	56.2	57.7	13.5	14.3	106.9	108.9
5	6	3	2.5	24.4	23.7	31.5	32.5	7.6	7.5	59.6	62.0
6	12	3	2.5	34.3	36.6	56.1	57.5	13.3	14.5	97.1	101.6
7	6	6	2.5	20.9	23.7	30.0	33.8	8.5	8.2	55.1	61.9
8	12	6	2.5	37.1	37.7	53.6	52.5	15.2	15.2	98.2	98.1
9	9	4.5	2	41.0	39.3	56.8	54.2	13.5	12.6	104.5	100.1
10	3	4.5	2	11.8	10.3	17.6	14.1	3.5	3.9	31.1	26.6
11	15	4.5	2	46.6	46.1	61.9	62.9	18.7	17.4	117.8	118.0
12	9	1.5	2	30.9	30.2	46.2	45.1	11.0	9.8	82.6	80.3
13	9	7.5	2	29.4	28.2	45.0	43.5	10.2	10.5	79.4	77.4
14	9	4.5	1	29.1	30.6	45.3	44.0	11.8	11.0	80.3	80.4
15	9	4.5	3	31.6	28.1	42.1	40.8	11.7	11.6	79.5	75.0

[a] Leu, Tyr, Cys and Gln were balanced at 1X to all treatment combinations. X_1: Amount of Lys (3 to 15-fold), X_2: Amount of the combination of Met, Val and Arg (1.5 to 7.5-fold) and X_3: Amount of the combination of Ile, Phe, Thr, Trp and His (1 to 3-fold).

[b] Total milk protein = (β-LG + β-CA + α-LA)

[c] Estimated value from respective fitted multiple regression model.

mainly by the X_1 variable. The collective quadratic portion was significant ($P<.01$). Significant ($P<.01$) interaction was observed only between X_1 and X_3 (table 20).

Partial regression coefficients for all linear components (b_1, b_2 and b_3), quadratic components (b_{11}, b_{22} and b_{33}) and first order interaction of b_{13} were determined to be highly significant ($P<.01$) for β-LG synthesis (table 21).

Greatest synthesis of β-LG occurred with treatment 11 (46.6 µg/flask) (table 22). Treatment 10, which comprised the lowest amount of X_1, produced the lowest response (11.8 µg/flask). Response from the central group (treatment 9) was 41.0 µg/flask. Optimum synthesis of β-LG was calculated to be 50.0 µg/flask, at $X_1 = 15.9$, $X_2 = 4.7$ and $X_3 = 1.3$.

Response to varied amounts of lysine at varying amounts of X_3, when X_2 was fixed at 4.5X, is depicted in figure 2. Syntheses ranged from 0.0 to 50.2 µg/flask. This figure indicated that effect of lysine was basically linear with maximum response resulting at 15-fold lysine addition which was the upper limit in the experiment. The highest response occurred at $X_3 = 1.5X$ and then decreased as X_3 was increased toward 3X, indicating that increasing amounts of X_3 had a decreasing effect of total synthesis of β-LG. Effects of varying X_2, with X_1 fixed at 9X and X_3 at 2X revealed that maximum response occurred at $X_2 = 4.5X$, which was the central point for this variable.

β-CA. Analysis of variance for β-CA manifested that there was a highly significant ($P<.01$) treatment effect. The X_1 and collective quadratic terms were the only significant ($P<.01$) components (table 20).

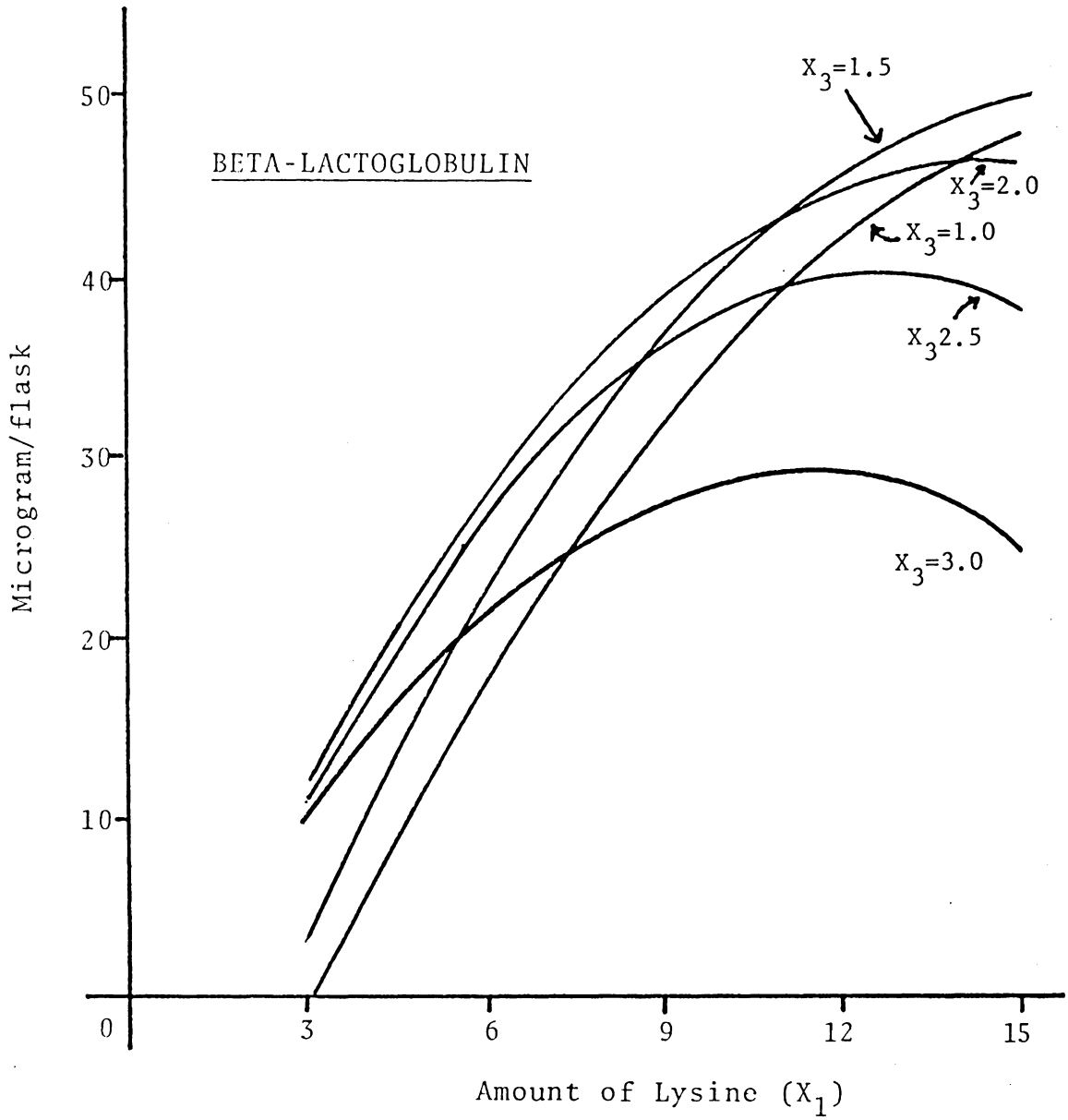

Figure 2. Response to lysine at varying amounts of X_3 when X_2 = 4.5X on the synthesis of β-LG in response surface study. (X_2 = methionine, valine and arginine, and X_3 = isoleucine, tryptophan, threonine, histidine, and phenylalanine)

Analysis of regression coefficients (table 21) indicated that there were no evidences that regression coefficients for all three constituents of the first order interaction were significantly different from zero ($P>.05$). Components other than interaction were observed to be highly significant ($P<.01$) for β-CA synthesis.

The pattern of responses of β-CA was similar to that of β-LG except in a somewhat higher amount (table 22). Calculated maximum synthesis for this protein was 64.9 µg/flask, with $X_1 = 14.2X$, $X_2 = 3.6X$ and $X_3 = 1.8X$, which resulted in the maximum being within the experimental limit. By setting X_2 at 4.5X, effects of lysine at different X_3 values on β-CA synthesis were determined and plotted (figure 3). Syntheses ranged from 0 to 64.1 µg/flask. The highest response curve obtained at $X_3 = 2X$ as with increasing the amount of X_1. When X_3 was fixed at 2X, the highest response curve occurred at $X_2 = 4.5X$.

α-LA. Only the linear component of X_1 exerted a significant ($P<.01$) effect on synthesis of α-LA (table 20). Regression analysis revealed that b_1 was a highly significant ($P<.01$) component (table 21).

Highest and lowest synthesis occurred in treatment 11 (18.7 µg/flask) and treatment 10 (3.9 µg/flask), respectively (table 22). Theoretical optimal values for the variables were $X_1 = 19.6$, $X_2 = 4.9$ and $X_3 = 2.5$, with a predicted synthesis of 18.7 µg/flask. Effect of lysine at varying X_3 on α-LA synthesis was essentially a linear response (figure 4). Syntheses ranged from 2.5 to 17.3 µg/flask.

Total protein. Overall responses for total protein (β-LG + β-CA + α-LA) were quite similar to those for β-LG in terms of significances of their components on both mean squares and regression coefficients (tables 20 and 21).

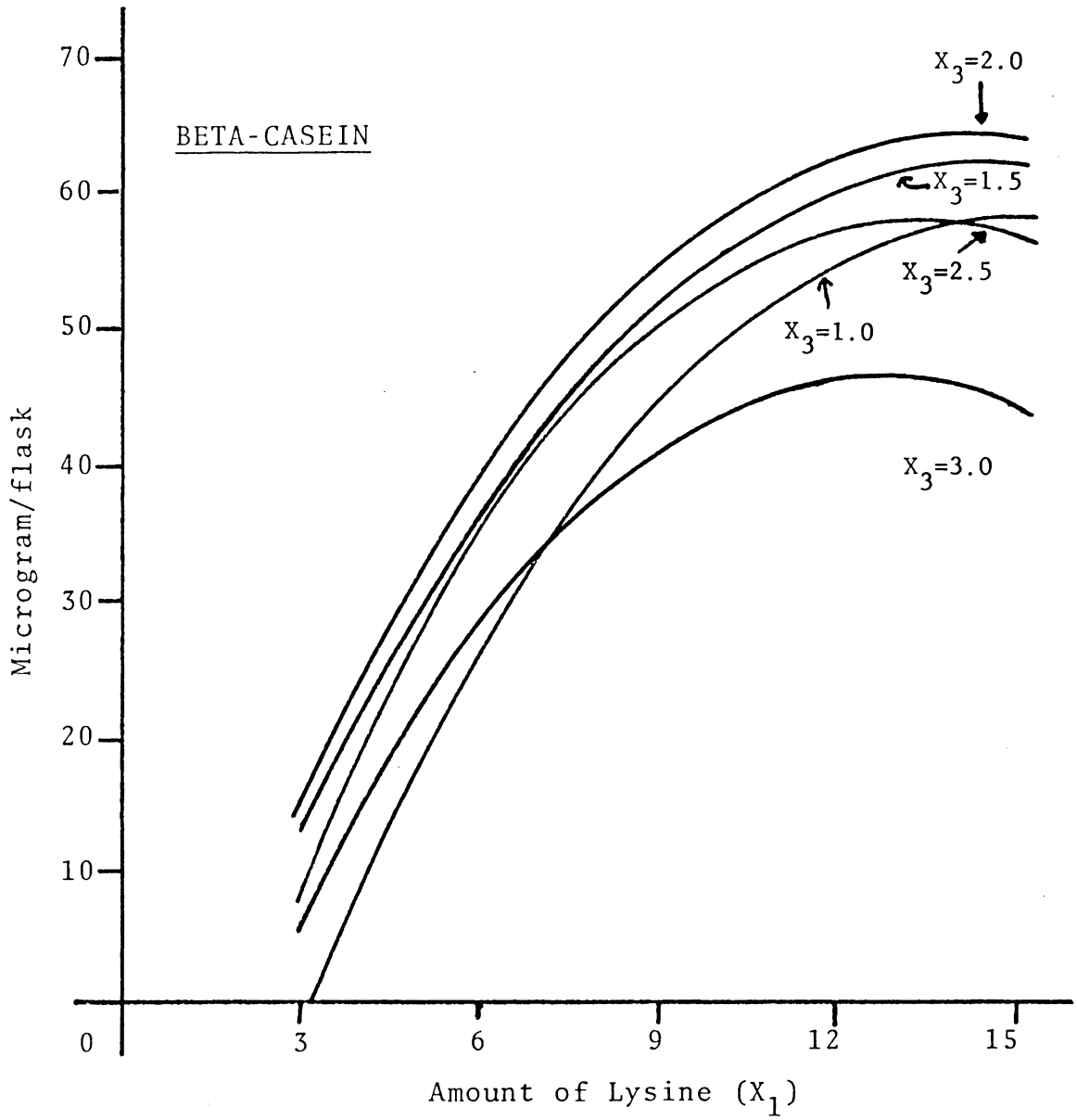

Figure 3. Response to lysine at varying amounts of X_3 when X_2 = 4.5X on the synthesis of β-CA in response surface study. (X_2 = methionine, valine and arginine, and X_3 = isoleucine, tryptophan, threonine, histidine and phenylalanine)

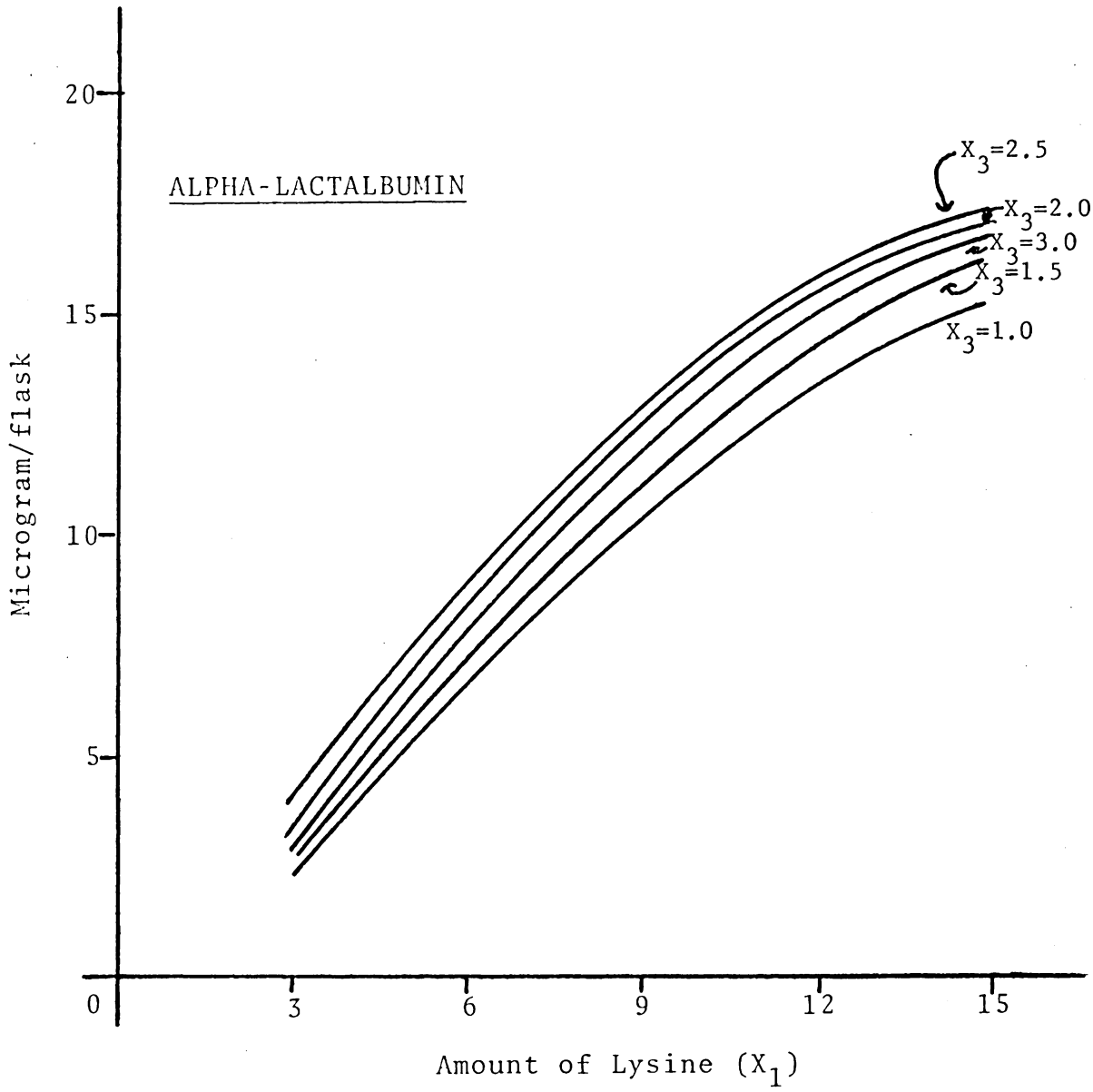

Figure 4. Response to lysine at varying amounts of X_3 when X_2 = 4.5X on the synthesis of α-LA in response surface study. (X_2 = methionine, valine and arginine, and X_3 = isoleucine, tryptophan, threonine, histidine and phenylalanine)

Optimal synthesis for total protein was 122.0 µg/flask, with X_1 = 14.6, X_2 = 4.2 and X_3 = 1.6. Similar to β-LG, effect of lysine in culture media was basically linear. Increasing amounts of X_3 from 1X to 3 X resulted in reduction of synthesis (figure 5). Synthesis ranged from 0.0 µg to 127.4 µg/flask.

The variation accounted for by regression model (R^2) ranged from 83.8 to 89.4% (table 20), indicating that the model was quite adequate in describing treatment responses.

Synthesis of milk protein was increased linearly for media containing lysine increases from 3 to 15X in all treatment combinations. Our results suggested amount of amino acids-methionine, valine and arginine for maximum milk protein synthesis was at 4.5X over that present in 1X-MEM. An interaction between lysine and the group of amino acids consisting of isoleucine, phenylalanine, threonine, tryptophan and histidine existed for β-LG and total protein synthesis. Total elevation of isoleucine, phenylalanine, threonine, tryptophan and histidine from 1X to 3X at varying amounts of lysine resulted in an overall reduction of protein synthesis when methionine, valine and arginine were fixed at 4.5X. Phenylalanine was reportedly transported into the cell via L system (18). This system was also blocked by analogues of phenylalanine. Christensen (18) stated that almost every neutral amino acid examined was inhibitory to uptake of another neutral amino acid. At the present time it is not clear if lysine had an inhibitory effect on uptake of isoleucine, phenylalanine, threonine, tryptophan and histidine or vice versa.

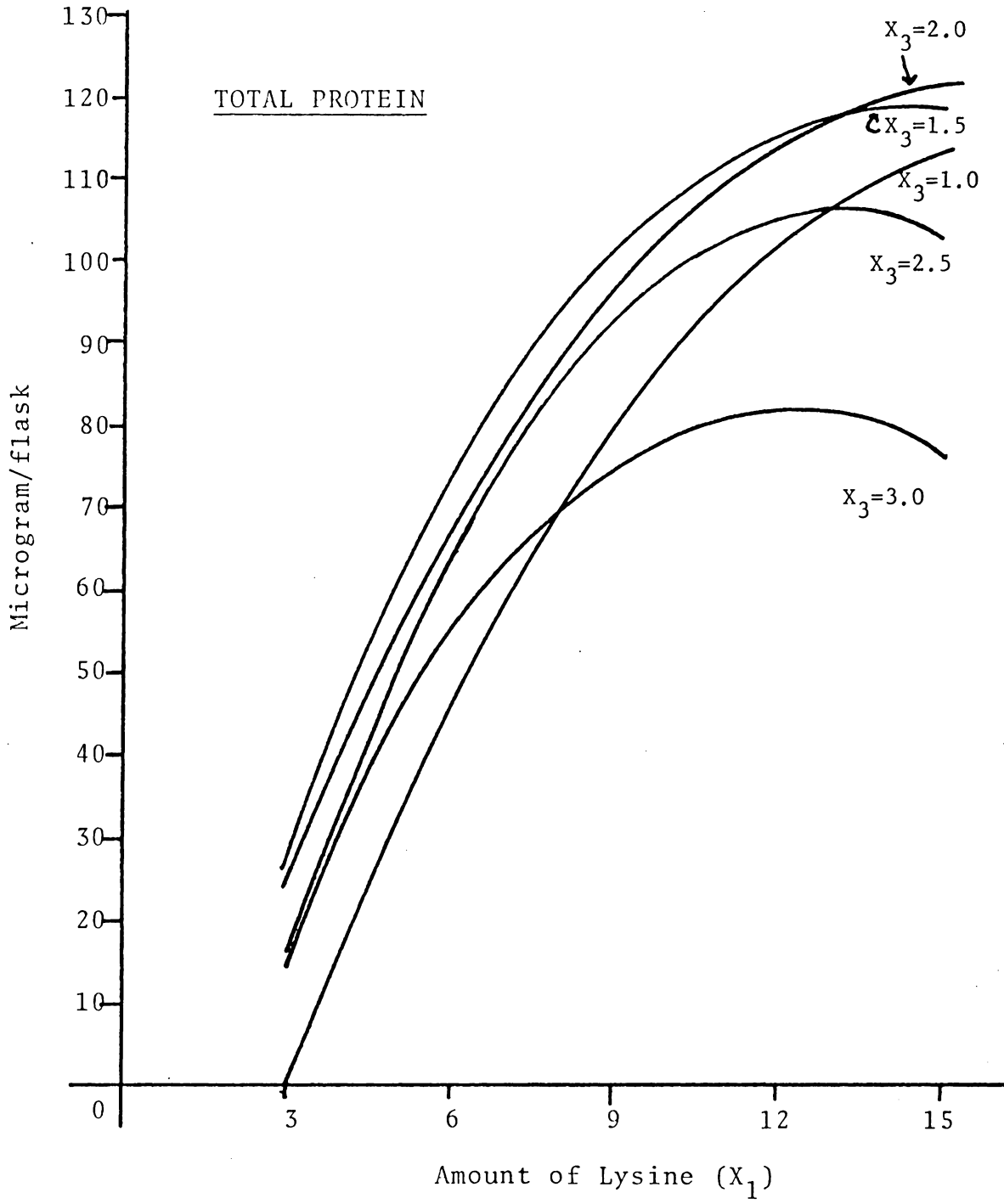

Figure 5. Response to lysine at varying amounts of X_3 when X_2 = 4.5X on the synthesis of total protein in response surface study. (X_2 = methionine, arginine and valine, X_3 = isoleucine, tryptophan, threonine, histidine and phenylalanine, and total protein = β - LG + β - CA + α - LA)

VI. SUMMARY AND CONCLUSION

In order to establish amino acid requirements for milk protein synthesis in dispersed rat mammary cell culture, three different experimental approaches were followed. These involved: 1) amino acid reduction from a positive control (3X) back to a negative control (1X) for each of the essential amino acids that were present in the 1X-MEM (Omission study) [X refers to amino acid concentration of Eagle's minimal essential medium (23)], 2) addition studies which resulted in an inverse relation to those of Omission studies, and 3) a three-dimensional central composite experiment with three independent variables (X_1 = lysine, X_2 = methionine, valine and arginine; and X_3 = isoleucine, phenylalanine, threonine, tryptophan and histidine), embracing 15 treatment combinations. In addition, supplemental studies were conducted which determined: 1) the effect of varied labeled precursor amino acids (lysine, phenylalanine and leucine) on measured synthesis of milk protein (two replications of a 2 x 3 factorial), 2) effect of varying cell density (6×10^6 to 6×10^7) on protein synthesis (two replications of a 3 x 2 factorial), and 3) response to graded amino acid complement (1X through 5X) on synthesis of milk protein (two replications of a 5 x 2 factorial).

Milk protein synthesis as measured by the incorporation of labeled lysine, leucine and phenylalanine was significantly ($P<.05$) affected by source of label for β-LG and β-CA but not for α-LA ($P>.05$). The interaction between source of label and two quantities of lysine (1X and 3X) was significant ($P<.05$).

Cell density study showed that cell numbers between 6×10^6 - 6×10^7 did not significantly ($P>.05$) alter rates of milk protein synthesis. The study also indicated that increasing amounts of amino acid concentration from 1X to 3X resulted in a significant ($P<.05$) increase in synthesis of β-LG and α-LA regardless of cell population present in treatment media.

Response of essential amino acid complement addition to 1X-MEM was linear ($P<.01$), with b = 30.9 µg/flask and r^2 = 90.5% on β-LG synthesis. Similar results were obtained for β-CA synthesis, with b = 25.9 µg/flask and r^2 = 91.3%. It was also determined that no significant effect ($P>.05$) on culturing existed between CO_2 and conventional incubators from all treatment groups. No interaction ($P>.05$) occurred between the system of incubation and graded amino acid complement.

It was observed that there were significant differences ($P<.01$) among treatments in Omission 1-8. Negative and positive controls resulted in lowest and highest syntheses. Synthesis of β-LG on lysine treatment was different significantly ($P<.05$) from other groups except from negative control. In Omission 9-10, all treatment groups showed varying intermediate values for β-LG synthesis between two controls except glutamine group. Similar results were obtained for both β-CA and α-LA with varying amounts of responses for each. Significant ($P<.01$) differences were observed among treatments on both β-CA and β-LG synthesis. Omission studies (9-10) indicated that lysine and methionine groups differed ($P<.05$) from other treatments for β-LG and β-CA.

Addition experiments 1, 2 and 3 revealed that there were significant treatment effects ($P<.01$) on synthesis of all three protein constituents. Response to negative and positive controls on synthesis of milk proteins in Addition 1 resulted in the lowest and the highest syntheses, respectively. Response of β-CA and α-LA synthesis were similar in pattern to that of β-LG but relative order of rank was altered. Addition 2 study showed that the upper limit of responses was no longer held by positive control but was exceeded by lysine treatment for β-LG synthesis. Synthesis of β-CA on tyrosine and lysine groups exceeded those on negative and positive controls, respectively. Addition 2 study also indicated that synthesis of β-LG on negative control differed significantly ($P<.05$) from lysine, arginine, methionine, histidine and isoleucine treatments. In Addition 3, the lowest and highest response on β-LG synthesis resulted from glutamine and lysine treatment. For β-CA synthesis glutamine group was the lowest limit, whereas lysine group resulted in the highest response. Synthesis of β-CA on positive control did not differ ($P>.05$) from arginine, tyrosine, isoleucine, valine, tryptophan, methionine and lysine groups.

Response surface study showed that overall treatment effect on the synthesis of β-LG was highly significant ($P<.01$). A significant ($P<.01$) interaction was observed only between the variables X_1(lysine) and X_3(isoleucine, tryptophan, threonine, histidine and phenylalanine). Partial regression coefficients for all linear components, quadratic components and first order interaction of b_{13} were highly significant ($P<.01$) for β-LG synthesis. Optimal synthesis of β-LG was calculated

to be 50.0 µg/flask, at X_1 = 15.9, X_2 = 4.7 and X_3 = 1.3. Treatment effect for β-CA was highly significant ($P<.01$). The X_1 and collective quadratic components were the only significant ($P<.01$) components. Analysis of regression coefficients indicated there was no significant ($P>.05$) differences in all three components of the first order interaction for β-CA synthesis. Calculated maximum synthesis for β-CA was 64.9 µg/flask, at X_1 = 14.2, X_2 = 3.6 and X_3 = 1.8. Only the linear component of X_1 exerted a significant ($P<.01$) effect on the synthesis of α-LA. Regression analysis revealed that b_1 was highly significant ($P<.01$). Theoretical optimal values for the variables were at X_1 = 19.6, X_2 = 4.9 and X_3 = 2.5, with a predicted synthesis of 18.7 µg/flask.

The following conclusions are made:

1) The first limiting amino acid was lysine.
2) The second limiting group of amino acids were methionine, valine and arginine.
3) The intermediary amino acids with no distinction among them were phenylalanine, threonine, tryptophan, isoleucine, cystine, and histidine.
4) The least limiting amino acids were determined to be tyrosine, leucine and glutamine.
5) The optimal combination of essential amino acids for maximum milk protein synthesis were: lysine = 15X, methionine, valine and arginine = 4.5X; and phenylalanine, threonine, tryptophan, isoleucine and histidine = 1.5X elevation over that present in the 1X-MEM, with 1X-MEM levels for leucine, tyrosine, cystine and glutamine.

On the basis of our finding, it was possible to construct an optimal essential amino acid complement for maximum milk protein synthesis (table 23) defined for *in vitro* rat mammary cells in culture. Confirmation of this proposed medium will require extensive studies where this proposed medium is compared with other accepted media used for cell culture work.

Table 23. Determined optimal amino acid complement for maximum milk protein synthesis in rat mammary cell culture.

L-amino acid	Concentration					
	Original(Eagle's)			Determined(optimum)		
	(mg/L)	(mM)	(fold)	(mg/L)	(mM)	(fold)
Arginine	105	.60	1X	473	2.70	4.5X
Histidine	42	.20	1X	63	.30	1.5X
Isoleucine	53	.40	1X	80	.60	1.5X
Leucine*	52	.40	1X	52	.40	1.0X
Lysine	73	.40	1X	1,095	6.00	15.0X
Phenylalanine	33	.20	1X	50	.30	1.5X
Threonine	48	.40	1X	72	.60	1.5X
Tryptophan	10	.05	1X	15	.08	1.5X
Valine	47	.40	1X	212	1.80	4.5X
Methionine	15	.10	1X	68	.50	4.5X
Tyrosine*	36	.20	1X	36	.20	1.0X
Cystine*	24	.10	1X	24	.10	1.0X
Glutamine*	292	2.00	1X	292	2.00	1.0X
Total		5.45			15.58	

*Unchanged.

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DETERMINATION OF OPTIMUM AMINO ACID COMPLEMENT FOR MILK PROTEIN SYNTHESIS IN THE DISPERSED RAT MAMMARY CELL CULTURE

by

Chung Sun Park

(ABSTRACT)

The amino acid requirement for milk protein synthesis was investigated in the rat mammary cells in tissue culture. Studies involved: 1) the amino acid reduction from a positive control (3X) back to a negative control (1X) for each of the essential amino acids that were present in the 1X-MEM (Omission Study) [X refers to the amino acid concentration of Eagle's minimal essential medium (MEM)], 2) the addition experiments which resulted in an inverse relation to those of the Omission studies, and 3) a three-dimensional central composite experiment with three variables (X_1 = lysine; X_2 = methionine, valine and arginine; X_3 = isoleucine, tryptophan, threonine, phenylalanine and histidine), embracing 15 treatment combinations. In addition, supplemental studies were conducted which determined: 1) the effect of varied labeled amino acid precursors (^{14}C-U-lysine, phenylalanine and leucine) on the measured synthesis of milk protein, 2) the effect of varying cell density (6×10^6 to 6×10^7) on protein synthesis, and 3) the response to graded amino acid complement (1X through 5X) on milk protein synthesis. Supplemental studies indicated that: 1) synthesis

of β-lactoglobulin and β-casein in response to the labeled amino acids were significant ($P<.05$), 2) cell numbers between 6×10^6 to 6×10^7 did not significantly ($P>.05$) alter the rates of the milk protein synthesis, and 3) the response of amino acid complement addition to the 1X-MEM was linear ($P<.01$) for β-lactoglobulin and β-casein synthesis. Data analysis from Omission and Addition experiments revealed that: 1) the first limiting amino acid was lysine, 2) the second limiting group of amino acids were methionine, valine and arginine, and 3) the least limiting amino acids were tyrosine, leucine and glutamine. The response surface study determined that the optimal combination of essential amino acids for the maximum milk protein synthesis were: 1) lysine = 15X, 2) methionine, valine and arginine = 4.5X, and 3) isoleucine, tryptophan, threonine, phenylalanine and histidine = 1.5X elevation over that present in the 1X-MEM, and 4) leucine, tyrosine, cystine and glutamine = 1X. On the basis of these findings, it was possible to construct the optimal essential amino acid complement unique to the rat mammary cells in tissue culture for the maximum milk protein synthesis.